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ARTICLE

Relevance of biofilms in pediatric tonsillar disease

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Abstract In this investigation, we study the relation between chronic inflammation of the tonsils, clinical features, and the presence of biofilms in the crypts in patients presenting with obstructive hypertrophy and recurrent upper airway pathology. Thirty-six patients who needed to undergo a tonsillectomy for obstructive reasons (aged 1 to 6 years), among which none of them had taken any antibiotics 30 days prior to surgery, were included. Samples were examined with hematoxylin-eosin and Gram staining, fluorescent microscopy, and confocal laser microscopy. The predominance of symptoms were those related to obstructive pathology rather than infection ($p<0.01$). All patients had tonsillar hypertrophy (grade III or IV), but an association with adenoids hypertrophy was detected in 66.66% of cases ($p<0.05$). 77.28% of tonsils presented biofilms in their crypts, but hypertrophy and tonsillar follicle number were not related to the presence or absence of biofilms. Here, we demonstrated that symptoms like harsh raucous sound, tonsillar and adenoids hypertrophy, apnea, and cervical adenopathies are clearly related to the presence of biofilm in tonsils. Our results allow us to propose that biofilms are involved in the pathogenesis of tonsils and adenoids hypertrophy. The prevention of biofilms formation should be focused in the early stages, attempting to restrain bacterial attachment to the respiratory mucosa.

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Introduction

Otolaryngologic diseases represent one of the most frequent problems in children. Among them, tonsillitis is one of the most common childhood pathologies and represents a real challenge because it is becoming resistant to common treatment [1, 2].

Despite the long-term and widespread use of antibiotics for this disease treatment, it is often insufficient and the infection becomes chronic, provoking hypertrophy as a manifestation of remodeling of the tonsils, as can be seen in other respiratory structures [3].

Tonsillectomy is often the choice as a consequence of obstruction of the upper airway, obstructive sleep apnea syndrome, growth delay, poor school performance, feeding difficulties, and other associated clinical features [4].

The failure of the antibiotic treatment in tonsillitis produced by susceptible organisms [5], even though it can be thought of as a consequence of antibiotic resistance [6], might be due to the presence of biofilms that can, therefore,

be considered as an etiologic factor, among others. The knowledge about biofilms existence is sustaining a new concept to explain chronic infections [2].

The adhesion of planktonic bacteria to a surface, such as the crypts of the tonsils, leads to the development of microcolonies that evolve to a biofilm community; the extracellular matrix and membrane proteins are involved in this process and the necessary energy is supplied by the bacteria [7]. The matrix provides mechanical stability for prolonged periods by hydrophobic interactions cross-linking by multivalent cations [8]. Biofilms are also a place where genetic material is easily exchanged because of the proximity of the cells maintaining a large gene pool.

Because of all of these factors, bacterial cells in biofilms are up to 500 times more resistant to antibiotics than the free cells [9]. These bacteria also resist host defense mechanisms, probably becoming a source of chronic inflammation and permanent histological changes. Free-living bacteria, on the other hand, are generally susceptible to antibiotic treatment and to host defense mechanisms.

Chole and Faddis [10] affirm that bacteria are protected by the biofilm from host defenses but continue with their metabolism and exotoxins production, leading to a chronic inflammatory response evident in respiratory mucosal changes and persistence of adenopathies.

Since the presence of biofilms may be an important factor in bacterial resistance and chronic tonsillitis [6], the present study was carried out in order to determine the connection between chronic inflammation of tonsils and the presence of biofilms in the crypts in patients presenting obstructive hypertrophy, persistent cervical adenopathy, and recurrent upper airway pathology. We also investigated a link between biofilms presence and clinical manifestations in these patients.

Materials and methods

Patients

This study was approved by the ethics committee of the Reina Fabiola Clinic, Córdoba, Argentina. Thirty-six children (18 male and 18 female) that presented to the Department of Otolaryngology with chronic inflammation of the tonsils with obstructive hypertrophy, persistent cervical adenopathy, and recurrent upper airway pathology with an age range of 1–6 years were included in the present study. None of these patients had received antibiotic therapy for at least one month prior to surgery. Only patients with five or more bouts of tonsillitis in the course of a year or three episodes per year for two years in a row were considered to have chronic tonsillitis.

A detailed clinical analysis was undertaken from each patient recording the following: age, sex, nasal obstruction and secretion characteristics, number of colds/year, presence or absence of apnea, harsh raucous sound, and cervical adenopathies.

The tonsillar hypertrophy was classified in grades as follows:

Grade I Tonsils limited to their pillar
Grade II Occupation of 25–50% of the oropharynx space
Grade III Occupation of 50–75% of the oropharynx space
Grade IV Tonsils that touch the uvula

Adenoids hypertrophy was graded as follows:

Grade 1 Adenoid obstructing 25% of the choanae
Grade 2 Adenoid obstructing 50% of the choanae
Grade 3 Adenoid obstructing 75% of the choanae
Grade 4 Adenoid obstructing 90–100% of the choanae

Tissue collection

Tonsil specimens were obtained during routine tonsillectomy. The specimens were washed with sterile saline solution and cut along the longitudinal diameter into several slices that were used for the histological studies. Twenty-two specimens were randomly selected in order to determine the bacteria number and microcolonies added to the tissue. Others were prepared for the confocal laser scanning microscopy (CLSM).

Histological studies

Tonsils were removed, washed with sterile saline solution, and cut along the longitudinal diameter into several slices, fixed in buffered (pH 7.0) 10% formaldehyde, and embedded in paraffin. The sections were stained with hematoxylin-eosin. A total of 50 slices were analyzed with a 40× objective.

Microscopy assay

The presence of biofilms in tonsils obtained by surgery was studied through Gram staining. The material was cut with a microtome and observed by an optical microscope (Carl Zeiss Axiovert, Germany). Semi-quantification of bacterial biofilms was performed according to an arbitrary scale: no micro- or macrocolonies observed (−); less than one microcolony in 50 fields observed (+1); less than one macrocolony in 50 fields observed (++); one to nine micro- or macrocolonies in 50 fields observed (+++); ten or more micro- or macrocolonies in 50 fields observed (++++).

In order to assess biofilms by fluorescence imaging microscopy (FIM), acridine orange solution (Molecular

Probes, Eugene, Oregon) 10 mg/mL for 5 min to 37ºC was used. Stained cells were observed using an Axioplant-fluorescent microscope equipped with a digital camera (DXM1200, Nikon, Japan).

Biofilms were observed by CLSM as described below. The obtained sections were then processed for double-staining. They were rinsed three times with sterile 50 mM potassium phosphate buffer (pH 7.2; no auto fluorescence detected) for 10 min and then first stained with 15 μM propidium iodide for 10 min at room temperature to detect bacterial cells in red. After being washed in phosphate-buffered saline, the sections were incubated with 50 μg/mL of fluorescein isothiocyanate (FITC) (Sigma) for 5 min at room temperature to stain the glycocalyx matrix in green. The sections were then successively washed in a combined solution of phosphate-buffered saline and demineralized water. The propidium iodide was excited at 520 nm, then, emission was monitored at 620 nm, and the FITC at 495 nm and 525 nm, respectively. The sections were examined non-destructively using a FluoView FV1000 Espectral Olympus CLSM (Olympus Latin America, Miami, FL, USA) equipped with a UPlanSApo 100×/1.40 oil UIS2 Olympus oil immersion lens. Optical sections of 0.87 μm were collected over the complete thickness of biofilms. Then, for each sample, images from three randomly selected positions were obtained and analyzed. Digital images of the CLSM optical sections were collected using an Olympus FluoView FV1000. Merged red and green images were obtained in single TIFF format and were converted to high-quality JPEG files using Adobe Photoshop version 7.0 to demonstrate the presence of biofilms.

For image analysis, three investigators (J.A.M., N.A.V., and M.G.P.) evaluated the images independently in a blinded retrospective manner. Images were analyzed for characteristic biofilm morphologic features using the following criteria: (1) the presence of bacteria, recognized by size, morphologic features, micro- and macrocolonies, and red fluorescent propidium iodide staining on CLSM images; orange fluorescent orange acridine by FIM or Gram staining by LM and (2) the presence of glycocalyx, observed on CLSM images by bright green fluorescence owing to FITC staining. A specimen was scored positive if more than one biofilm formation (based on the two criteria) were observed at an airway specimen interface or in the crypts of the specimen. Specimens that showed artifacts of cutting for CLSM and FIM were excluded. When bacteria were identified without any matrix surrounding them, they were considered to be attached individual cells or bacterial microcolonies, and the specimen was not considered to contain a biofilm.

Statistical analysis

The obtained data were analyzed by analysis of variance (ANOVA) and multiple comparison by Fisher's test and the Chi-square test for categorical variables. The significance level was set at $p<0.05$ for all cases.

Results

Table 1 shows the most frequent symptoms presented by the children included in this study. A significantly higher percentage of patients presented obstruction manifestation (snore, nasal obstruction, tonsillar and adenoids hypertrophy, and apnea) rather than acute infection ($p<0.01$). All patients had tonsillar hypertrophy (grade III or IV), but an association with adenoids hypertrophy was detected in the 66.66% of cases ($p<0.05$).

The symptoms presented by each of the patients included in the present study were associated with the presence of biofilms in the samples obtained after surgery (36 tonsils were collected and 22 of them were randomly selected to be analyzed) and are presented in Table 2. These results demonstrate that symptoms like harsh raucous sound, tonsillar and adenoids hypertrophy, apnea, and cervical adenopathies are clearly related to the presence of biofilms in tonsils, because 81.82% of the patients who presented these symptoms had biofilms in their tonsils. It should also be noted that, out of 13 patients without cervical adenopathies, eight of them had biofilms in their tonsils.

Figure 1 shows the histopathology characteristics of the most representative tonsils obtained from our patients,

Table 1 Clinical manifestations of pediatric patients requiring tonsillectomy ($n=36$)

Clinical symptoms	%
Snore (harsh raucous sound)	94.40
Nasal obstruction	80.55
Tonsillar hypertrophy (Grade III)	75.00
Apnea	69.44
Adenoids hypertrophy (Grade IV)	58.33
Cervical adenopathies	58.33
Mucosal nasal secretion	47.22
Recurrent common cold (>5/year)	44.44
Adenoids hypertrophy (Grade III)	33.33
Purulent nasal secretion	25.66
Tonsillar hypertrophy (Grade IV)	25.00
Serous nasal secretion	16.66
Adenoids hypertrophy (Grade II)	8.33

Clinical symptoms were ordered according to the frequency observed

Table 2 Association between the most frequent clinical symptoms and the presence of biofilms in tonsils

Clinical symptoms	% of patients that presented tonsillar biofilms
Snore (harsh raucous sound)	81.82
Tonsillar hypertrophy (Grades III, IV)	81.82
Adenoids hypertrophy (Grades III, IV)	81.82
Apnea	81.25
Nasal obstruction	80.55
Cervical adenopathies	75.00
Purulent nasal secretion	66.66
Mucosal nasal secretion	50.00

where the presence of biofilms in tonsils surrounded by inflammatory infiltrates, tonsils crypts with biofilms in large colonies, and biofilms at the end of a tonsil crypt are the most remarkable findings.

These results should be related to those detailed in Table 3, which shows that 77.28% of the tonsils studied (100% clinically detected as hypertrophic) presented biofilms of different degrees and that the number of follicles were not related to the presence or absence of biofilms as demonstrated, for example, by patient 21 who presented 124 follicles and a great concentration of biofilms, and patient 29, with 501 follicles and absence of biofilms. The areas were not significantly different in patients with or without biofilms because all tonsils were hypertrophic (see Table 1).

Bacteria from biofilms were identified by Gram staining for optical microscopy, fluorescence with orange acridine for FIM, and with two fluorescence staining for CLSM. Immotile, irreversibly attached live bacteria in characteristic clusters and towers of micro- and macro-colonies were found in varying degrees of density throughout the slides. Some structures were present on the surface of the tonsils, whereas others were located in the inner part of the invaginations (Fig. 2). Specific demonstration of the presence of biofilms by CLSM was based on the fluorescence of tagged segments. Bacteria were shown by staining of the bacterial DNA and the glycopolysaccharide of the matrix (Fig. 2e–f). Bacterial cells and the surrounding glycocalyx matrix, which is indicative of bacterial biofilms formation, were present on the surface and crypts of the tonsillar tissue of our patients. Bacterial cells and nuclei of tonsillar cells stained in red, and the binding of FITC resulting in green staining indicating the presence of a bacterial glycocalyx, are also shown in Fig. 2.

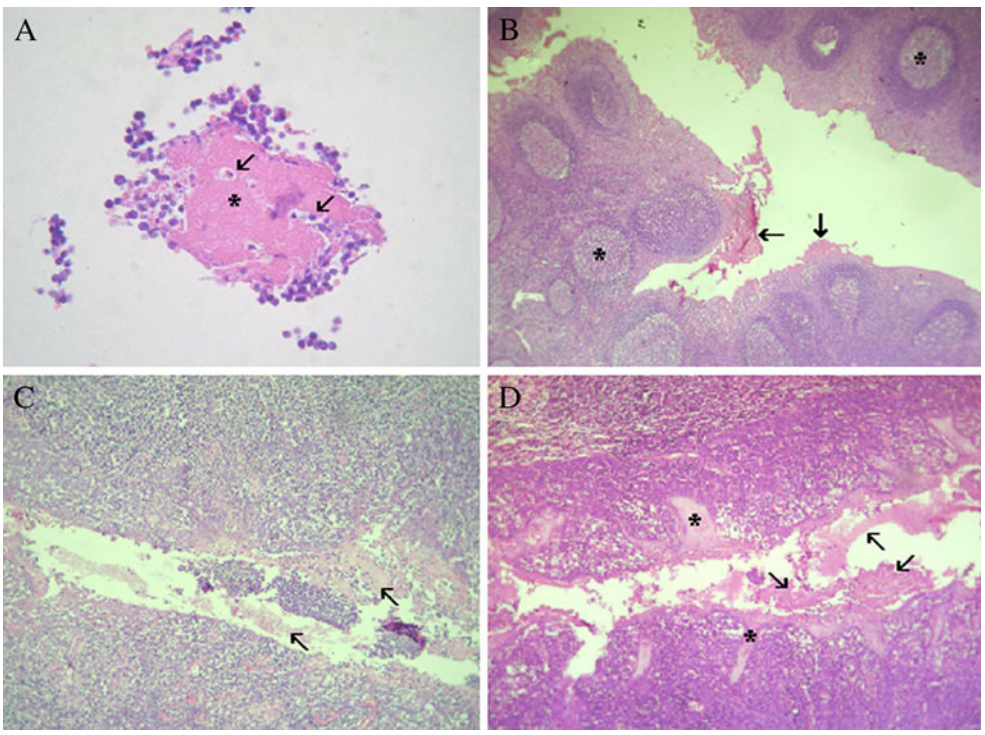

Fig. 1 **a** Biofilm in tonsil (*asterisk*) stained with hematoxylin-eosin surrounded by inflammatory infiltrate (*arrows*); 400×. **b** Panoramic view of a tonsil crypt with biofilm (*arrows*) with numerous and dilated follicles (*asterisk*) without necrosis; 32×. **c** End of a crypt with many colonies (*arrows*) and intense inflammation; 100×. **d** End of a crypt with parakeratosis (*asterisks*) and numerous biofilms (*arrows*); 150×

Table 3 Tonsillar histopathological characteristics and the presence of biofilm in the crypts (n=22)

Patient	Area (mm^2)	Number of follicles	Presence of biofilm
1	116.5±21.65	359	++++
3	110±2	208	++++
6	150	56	++++
8	178±22	187	++++
21	72,5±8.5	124	++++
mean	125.4±18	186.8±43.15	
2	97.5±14.63	444	+++
4	229.66±20.51	500	+++
7	82±11.01	118	+++
11	104.75±11.81	268	+++
12	127.75±15.38	289	+++
mean	128.33±26.38	328.8±67.88	
5	204.5±4.5	192	++
15	152.66±8.19	245	++
mean	178.58±25.92	218.5±26.5	
9	143±45.50	242	+
10	131±7.24	260	+
16	127±136.65	226	+
17	131.33±28.62	374	+
18	117.33±7.05	394	+
mean	129.9±34.13	233.2±35.17	
13	156±15.87	201	−
14	130±19.27	361	−
19	138.66±32.44	297	−
23	96.16±4.18	215	−
29	158.75±3.44	501	−
mean	135.31±11.28	315±54.78	

The data of the tonsils areas correspond to mean ± standard error and were ordered according to the concentration of biofilm detected

Discussion

Adenotonsillar disease represents a major problem in children and frequently requires surgical intervention. Chronic adenotonsillar infections and hypertrophy are frequently caused by multiple and, sometimes, resistant bacteria [11]. Many of these bacteria have the ability to form biofilms that are matrix-encased communities adapted to surface persistence. Bacterial cells attached to a suitable surface, such as tonsils for example, replicate, spread, and mature to form biofilms [9].

All of these results suggest that biofilms represent a new concept in ear, nose, and throat chronic infections, and are probably involved in their pathophysiology and in the antibiotic resistance sometimes described.

This study reports the presence of biofilms in the tonsils of 77.28% of the children included in the investigation. Other authors have described the detection of biofilms in only 61% of their patients [9]. More than 77% of our patients that presented biofilms in their tonsils were children with obstruction rather than infection symptoms.

The presence of biofilms in tonsils was previously reported [8]. Al-Mazrou et al. [9] reported biofilms in 61% of 46 patients and Chole and Faddis [10] found biofilms in 15 patients with chronic tonsil disease (74%).

The persistence of tonsillar hypertrophy, which was present in 100% of the patients analyzed here, was associated with symptoms such as harsh raucous sound, tonsillar and adenoids hypertrophy, apnea, and cervical adenopathies that were clearly related to the presence of biofilms in tonsils, because 81.82% of the patients that presented these symptoms had biofilms in their tonsils. This result strongly suggests the presence of a chronic inflammatory underlying pattern, with a poor immune response [12, 13] that provoked the presence of bacterial biofilms in the crypts of 77.28% of the patients studied here and in the 81.82% of patients who presented symptoms as those described. Persistence of these symptoms must be an alert for the pediatric physician, since the presence of biofilm formation is a real possibility.

It is also interesting to analyze the histological characteristics of the tonsils with or without biofilms in their crypts. All of them were hypertrophic, as a clinical symptom, but

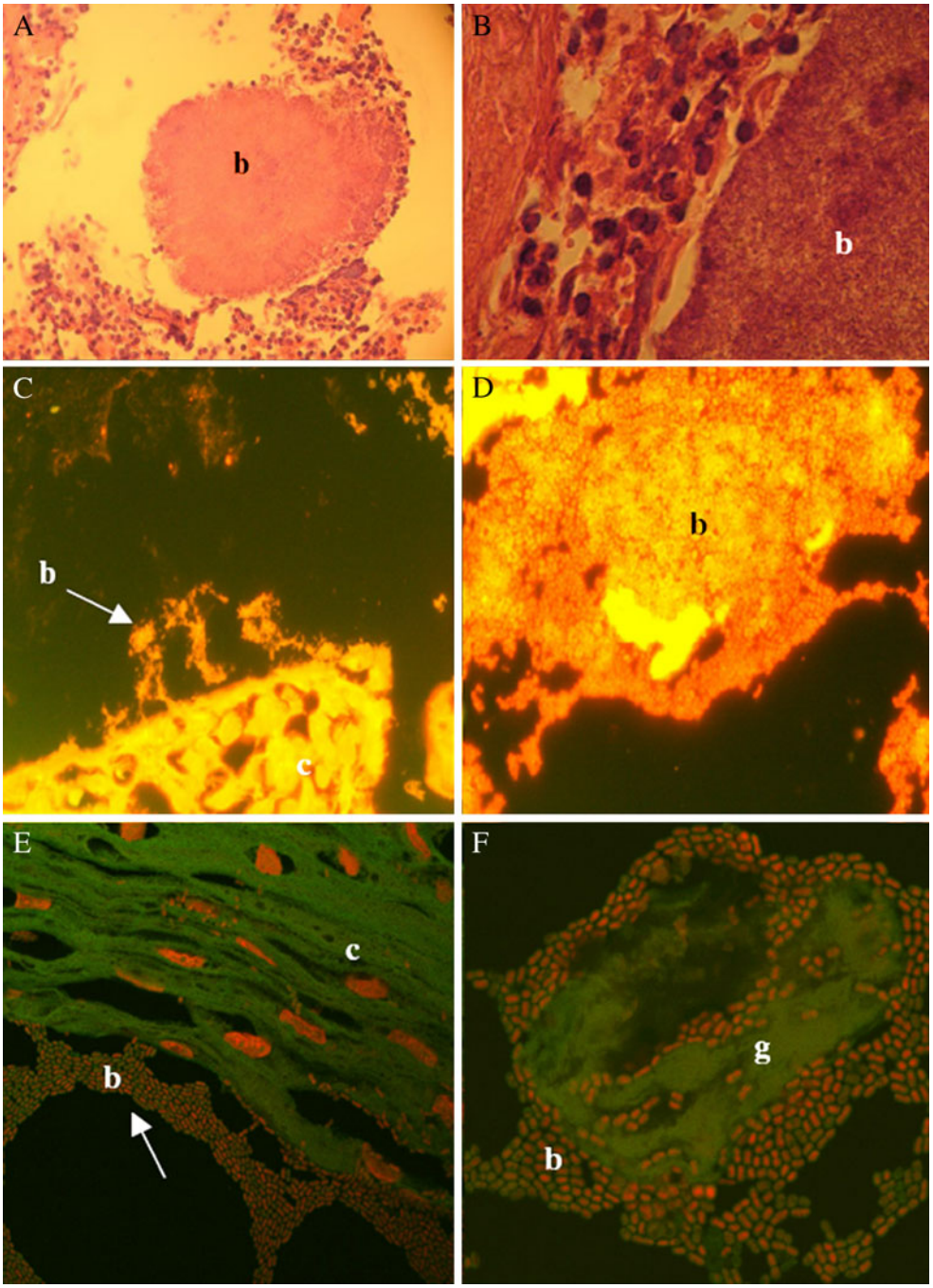

Fig. 2 Biofilms structure observed by microscopy assay. Biofilm in tonsil studied through Gram staining and observed by an optical microscope (**a**, **b**). The colony appears as a densely packed mass of bacteria. Fluorescence microscopy staining with acridine orange solution shows microcolonies (**c**) or macrocolonies encased in a glycocalyx matrix (**d**). Confocal laser scanning microscopy (CLSM) images demonstrating bacterial biofilms on human tonsils (**e**, **f**). Bacterial cells (*b*) in biofilm, as well as surrounding nuclei of tonsillar cells (*c*), stained red (propidium iodide). Green fluorescent staining (FITC) around bacteria indicates the presence of diffuse glycocalyx (*g*). (**a**, **c**, **e**: 100×; **b**, **d**: 200×; **f**: 400×)

the presence or absence of biofilms did not modify the mean area of the tonsil, as demonstrated in Table 3. Moreover, the number of tonsils follicles was not related to the presence or absence of biofilms. These results might be related to the fact that most of our patients presented obstruction symptoms rather than infection symptoms, while other authors [9, 10] explained chronic tonsillitis because of the presence of infection and of biofilms.

In chronic tonsil disease, such as the samples studied in the present paper, biofilm formation provides a mechanism for bacteria to survive, as it was clearly demonstrated in fluorescence and confocal images of tonsil tissues. Besides, as biofilms are too big to be phagocyted by macrophages, their presence is surely interfering with the normal tonsillar function [14, 15], contributing to the hypertrophy described here.

This is a novel situation that can explain the poor outcomes of most therapeutic strategies implemented by pediatricians in an effort to diminish the tonsillar size and the frequency of upper airway disease [16]. This group of patients constitutes a challenge due to their poor clinical evolution; the knowledge about the role of biofilms in this

and other infections will probably modify the treatment paradigms [17].

In the present study, we demonstrate the presence of biofilms in the crypts of 77.28% of our patients and establish a clear relation between biofilms and some clinical symptoms. As the main symptoms found in our cohort are directly associated with the hypertrophy of tonsils and adenoids, we propose that biofilms formation is directly related to the physiophatogenic mechanism of this chronic disease.

The prevention of biofilms formation should be focused in the early stages, having in mind the connection with clinical symptoms demonstrated here, trying to restrain bacterial attachment to the respiratory mucosa. Persistence of the main symptoms described here must be an alert for the pediatric physician, since the presence of biofilm formation was confirmed in most of the tonsils analyzed.

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