Rom J Morphol Embryol 2021, 62(2):481–489 ISSN (print) 1220–0522, ISSN (online) 2066–8279 doi: 10.47162/RJME.62.2.14

ORIGINAL PAPER

RJME Romanian Journal of Morphology & Embryology http://www.rjme.ro/

Adenoidal bacterial biofilm in pediatric rhinosinusitis

RADMILA-ANCA BUGARI[1,2)], ARISTIDE SORIN BAŞCHIR[1–3)], LUMINIŢA ADELA TURCIN[1,3)], ALEXANDRU CHIOREANU[1)], CIPRIAN VALENTIN MIHALI[4,5)], ADRIAN COSMIN ILIE[6)], AFILON JOMPAN[1)], MARIA BĂLĂŞOIU[7)]

[1)]*Department of ENT, Vasile Goldiş Western University of Arad, Romania*

[2)]*Department of ENT, Dr. Turcin Clinic, Arad, Romania*

[3)]*Department of ENT, Emergency County Hospital, Arad, Romania*

[4)]*Department of Cell and Molecular Biology, Electron Microscopy, Vasile Goldiş Western University of Arad, Romania*

[5)]*Laboratory of Molecular Biology, Research and Development Station for Cattle Breeding, Arad, Romania*

[6)]*Department of Public Health and Sanitary Management, Victor Babeş University of Medicine and Pharmacy, Timişoara, Romania*

[7)]*Department of Microbiology, University of Medicine and Pharmacy of Craiova, Romania*

Abstract

The aim of the study was to observe, using scanning electron microscopy (SEM), the ratio of bacterial biofilm coverage of adenoidal tissue in children diagnosed with chronic rhinosinusitis (CR), compared to the ratio of adenoid bacterial biofilm coverage in children diagnosed with obstructive sleep apnea (OSA). We also performed histopathological and immunohistochemical tests to correlate the results with the images obtained from SEM. We estimated, using an image analysis program, the coverage ratio with bacterial biofilm on the surface of the lymphatic tissue. Adenoid vegetation extracted from children with CR had a higher percentage of bacterial biofilm coverage compared to the group diagnosed with OSA. In the nasopharynx of children with CR, the bacterial biofilm had a constant role of infection generator, and adenoidectomy was the only effective therapeutic procedure to relieve the symptoms. Allergy tests were performed in all children to establish a link between CR, OSA and allergic rhinitis.

Keywords: adenoids, bacterial biofilm, chronic rhinosinusitis, obstructive sleep apnea, scanning electron microscopy.

Introduction

The pharyngeal amygdala, a mass of lymphatic tissue located in the nasopharynx, posterior to the nasal cavity, is an important component of the Waldeyer lymphatic circle, intervening in an anti-infective defense. Adenoid hypertrophy (AH), known as adenoid vegetation, is the most common pathology that causes upper airway obstruction in children [1, 2]. In recent years, worldwide, the number of cases diagnosed and hospitalized with chronic adenoiditis in hospitals for respiratory diseases, pediatrics, otolaryngology (ear, nose, throat – ENT) has increased [3–5]. According to recent studies, the prevalence of AH is 34% in the general population, still it can vary between 42% and 70% in certain categories of children [6]. Adenoid vegetations are responsible for the onset of other diseases, such as: otitis media, acute sinusitis, obstructive sleep apnea (OSA), abnormal facial development, disorders in physical and mental development (behavioral disorders), etc. [7, 8]. In addition, lymphoid tissue hypertrophy during childhood may contribute to the development of nasopharyngeal infections, as bacterial biofilms involved in the pathogenesis of chronic adenoiditis may be present throughout the nasopharyngeal mucosa and even in the Eustachian tube [9–11]. Numerous studies consider AH being a major cause for the onset and development of acute and chronic rhinosinusitis (CR) [6, 12–14].

Currently, the pathophysiology of CR is not yet fully clarified. The role of bacteria as a trigger or an aggravating factor in CR remains debatable. However, there is evidence that bacterial biofilms are involved in severe forms of CR [15].

The presence of bacterial biofilms in AH is associated with unfavorable therapeutic results, despite adequate drug treatment (according to the antibiogram) due to the resistance of bacteria to antibiotics. The nasal mucosa and the mucosa that covers the upper respiratory tract, along with the paranasal sinuses, are generally considered a unitary system, and therefore rhinosinusitis terminology [16] is frequently used to define inflammatory processes at this level. There is a connection between upper and lower airway pathology [17]. According to specialized studies, the most common pathogenic germs in CR, isolated from the nasopharynx, are *Staphylococcus aureus* and *Streptococcus pneumoniae* [12, 18].

At the same time, allergic rhinitis is intensely associated, in specialized studies, with OSA and the onset of CR, due to the endonasal inflammatory condition secondary to the inflammatory reaction and the disturbance of rhinosinusal mucociliary clearance [19–21].

Aim

In this study, we wanted to show the presence of bacterial biofilm on the surface of adenoid tissue from children with CR and to compare the average percentage of biofilm coverage of the adenoid mucosa from these children with the average percentage of bacterial biofilm coverage of the adenoid mucosa from children diagnosed with OSA. The goal was to highlight the role of fountain of infection of the adenoids contaminated with bacterial biofilm in children with CR.

Patients, Materials and Methods

This study was conducted between January 2013 and December 2019, with the agreement of the Ethics Committee of Vasile Goldiş Western University of Arad, the Ethics Committe of the Emergency County Hospital, Arad, and the Ethics Committee of the Dr. Turcin Clinic, Arad, on a group of 107 children from Arad County, Romania, aged between four and 15 years old, hospitalized within the Department of ENT, Emergency County Hospital, Arad, and diagnosed with AH and CR or OSA.

The children were initially examined and selected by the general practitioners (GPs) in accordance with bacteriological tests of the nasal mucosa and screening forms, specially designed for the diagnosis of CR (Table 1) and OSA (Table 2).

Table 1 – *CR form*

Patient name	……
Patient age [years]	……
Hypertrophic nasal mucosa	Yes/No
Hypertrophied nasal turbinates	Yes/No
Reduction of the respiratory space	Yes/No
Purulent discharge	Present/Absent
Nasopharyngeal adenoids covering over 1/2 of the posterior nasal orifice bilateral	Present/Absent
Adenoids that block the opening orifices of the Eustachian tube bilateral	Present/Absent
Positive nasal culture	Present

CR: Chronic rhinosinusitis.

Table 2 – *OSA form*

Patient name	……
Patient age [years]	……
Nasal mucosa normal or slightly purplish appearance	Yes/No
Transparent nasal secretions	Yes/No
Nasopharyngeal adenoids covering over 1/2 of the posterior nasal orifice bilateral	Present/Absent
Adenoids that block the opening orifices of the Eustachian tube bilateral	Present/Absent
Sleep apnea	Yes/No
Negative nasal culture	Yes

OSA: Obstructive sleep apnea.

Based on the screening forms, the patients selected by the GPs were included into two groups (CR or OSA suspect) and were sent to the Clinic of ENT, Emergency County Hospital, Arad, where they underwent a complete ENT clinical consultation and an endoscopic examination. ENT endoscopy was performed with a rigid STORZ 0° endoscope with 4 mm diameter, Telecam SL II camera.

The children included in the study were tested for possible allergic reactions to respiratory allergens, using the skin test method "prick test", a minimally invasive medical procedure used to identify respiratory allergens, in this case, responsible for triggering symptoms of rhino-sinusal allergy: nasal obstruction, rhinorrhea, sneezing. All children were also clinically examined to exclude the diagnosis of asthma.

The children's parents were informed about the minors' participation in the study and gave their written consent to participate.

All patients underwent minimally invasive surgery, classical adenoidectomy under endoscopic guidance, to remove the adenoids. Of the adenoids extracted from each patient, part was sent to the Laboratory of Pathology for histopathological diagnosis and immunohistochemistry and part was collected for scanning electron microscopy (SEM) studies.

The tissue sections harvested for SEM were transported in special conditions in containers with the internal temperature maintained at 4±2ºC for at least two hours, from the Department of ENT, Emergency County Hospital, Arad, to the Laboratory of Microscopy, Vasile Goldiş Western University of Arad, where they were stored and processed according to the SEM protocol.

Fixation of adenoids for SEM was performed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for two hours, at 22°C. The samples were subsequently fixed with 1% osmium tetroxide in a PBS for one hour, at 37°C. After fixation, the samples were dehydrated using ethanol-graded series and immersed five times in a solution of hexamethyldisilane for 10 minutes and allowed to dry overnight.

The obtained specimens were mounted, and the silver sputter was covered in the preparation of the terminal. We detected the bacterial biofilm using a FEI Quanta 250 scanning electron microscope from the Laboratory of Microscopy, Vasile Goldiş Western University of Arad. On the SEM images, we measured the percentage of bacterial biofilm coverage on the surface of the adenoidal tissue, with Carnoy software, an image analysis application used in morphology to make the measurement easier, faster, and more precise.

For histopathology and immunohistochemistry studies, the resected adenoid vegetation fragments were placed in 10% neutral buffered formalin fixative solution and then embedded in paraffin, according to the usual histopathological protocol. The biological material was sectioned in the microtome, obtaining 4-µm thick sections that were stained with Hematoxylin–Eosin (HE). For the immunohistochemistry study, the microtome sections were collected on poly-L-lysine slides. The antibodies used were: anti-cluster of differentiation (CD)3 (monoclonal mouse anti-human CD3, clone F7.2.38, 1/50 dilution, Dako) to highlight T-lymphocytes; anti-CD20 (monoclonal mouse anti-human CD20cy, clone L26, 1/50 dilution, Dako) for B-lymphocytes study; anti-CD68 (monoclonal mouse anti-human CD68, clone KP1, 1/100 dilution, Dako) for macrophage study; anti-tryptase (monoclonal mouse anti-human mast cell tryptase, clone AA1, 1/100 dilution, Dako) for mast cell detection; anti-CD34 (monoclonal mouse anti-human CD34 class II, clone QBEnd-10, 1/50 dilution, Dako) for the study of microvascularization in adenoid vegetations.

All patients underwent nasopharyngeal clinical and video-endoscopic control three, six and 12 months after surgery.

Statistical analysis

Centralization and statistical processing of data was performed using the specialized Statistical Package for the Social Sciences (SPSS) Statistics 20 program. In the case of numerical and discrete variables (age and degree of coverage with bacterial biofilm), the following statistical procedures were used: Bravais–Pearson correlation index for the analysis of the association between variables; Student's *t*-test for comparing batches (independent samples and pairs). In the case of nominal variables, sex and the malariological test for respiratory allergens, the χ^2 (*chi*-squared) test was used. To control the age covariance, we used the analysis of covariance (ANCOVA) procedure.

Results

Clinical study

The study group consisted of 107 patients aged between four and 15 years, sent by the GPs to ENT offices, with the suspicion of chronic AH, associated with CR or OSA. The mean age was 7.24 years [standard deviation (SD) 2.61 years]. Of these, 60 (56.07%) patients were males and 47 (43.93%) females (Figure 1).

The main diagnosis criterion considered the fact that CR is a rhinosinusal infectious disease that lasts more than three months and whose treatment with oral antibiotics failed even after five weeks. Patients diagnosed with AH and CR showed the following symptoms: nasal obstruction, hyposmia or anosmia, purulent nasal discharge, anterior or posterior nasal drop syndrome, facial fullness, and facial pressure. These symptoms were accompanied by confirmation from the GP that sinus infections were repeated and with an unfavorable response to antibiotic treatment. During the video-endoscopic examination, clear macroscopic changes were found that indicate the diagnosis of CR: nasal turbinates were hypertrophic, with hypertrophied adenoid vegetations, significant obstruction of the choanal orifices and abundant mucopurulent secretions. Children with CR tested positive for intranasal bacteriological assay for *Staphylococcus aureus*.

From the initial group, 48 patients diagnosed with CR, of which 22 subjects were female and 26 male patients (Figure 2), aged between four and 15 years (mean 7.46 years; SD 2.70 years).

The two groups, OSA and CR, do not differ in terms of gender distribution (χ^2=0.129, p=0.845) nor in terms of age of the subjects [t(105)=-0.766, p=0.445].

Children diagnosed with OSA presented from a clinical point of view: nasal obstruction, oral respiration, mucous or watery nasal secretions, anterior or posterior nasal drop syndrome. Patients included in this study group did not have a history of rhinosinusitis but only confirmation of sleep disorders from their parents. In these children, ENT video-endoscopy found the presence of hypertrophied adenoid vegetations with obstruction of over 75% of the choanal orifices, bilaterally. Obstruction of the choanal orifices was correlated with a Mallampati class greater than 2.

To these data, we added parental confirmation of recurrent snoring and sleep disorders for more than 12 months and a score of at least five points on the OSA form.

Of the 107 children included in the overall group, 59 patients were diagnosed with OSA, of which 25 were girls and 34 were boys (Figure 3), aged between four and 15 years old (mean 7.07 years; SD 2.56 years). All children with OSA had negative results on nasal bacteriological exudate.

Of the total study group, 48 children had positive tests for allergy (Figure 4), of which 27 (56.25%) boys and 21 (43.75%) girls, evenly distributed between the two subgroups, 24 were part of the diagnosed group with OSA, and 24 were part of the group of children with CR (Figure 5).

Of the 48 subjects with positive allergy tests, 13 boys with OSA and 14 with CR and 11 girls with OSA and 10 with CR were identified. The ratio of boys/girls and OSA/CR in patients with positive allergy tests was relatively equal. Our study showed no statistically significant differences between the age of girls with positive allergy tests (mean 7.14 years) and that of boys with positive allergy tests (mean 7.44 years).

Of the total number of patients included in the study group, nine had recurrences, observed at postoperative controls (Figure 6).

Of the patients who had a recurrence of adenoids, there were five boys and four girls, aged between four and six years old, and five of them being part of the group initially diagnosed with OSA, and four of the group of patients diagnosed with CR (Figure 7).

All nine patients who experienced recurrences of adenoids had positive allergy tests and showed symptoms of allergic rhinitis and postoperative allergic rhino-conjunctivitis.

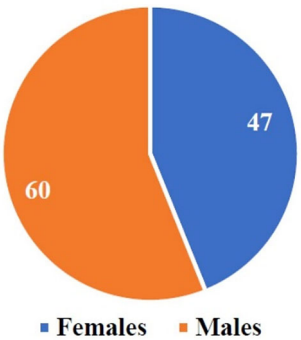

Figure 1 – ***Entire study group distribution by gender.***

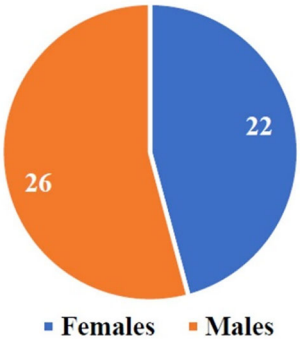

Figure 2 – ***CR group distribution by gender. CR: Chronic rhinosinusitis.***

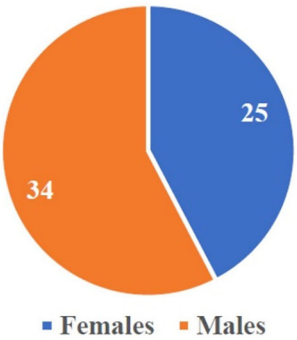

Figure 3 – ***OSA group distribution by gender. OSA: Obstructive sleep apnea.***

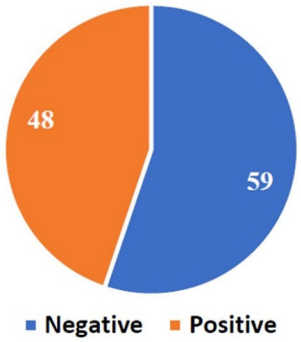

Figure 4 – ***Results of the allergy tests for respiratory allergens in the entire study group.***

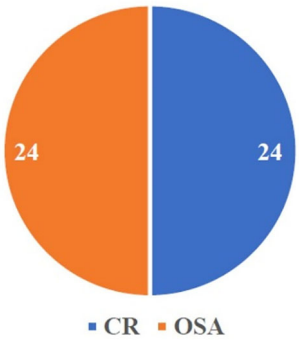

Figure 5 – ***Patients with positive allergy tests depending on the group of origin. CR: Chronic rhinosinusitis; OSA: Obstructive sleep apnea.***

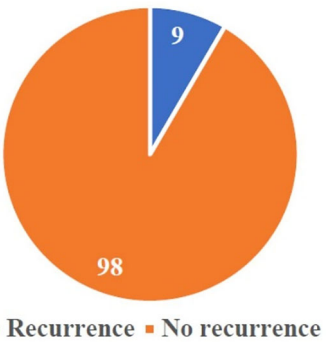

Figure 6 – ***Recurrence rate of adenoids compared to the entire study group.***

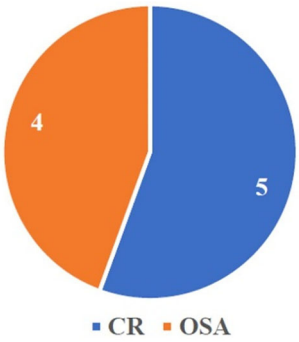

Figure 7 – ***Groups of origin of the children who presented recurrence of the adenoid vegetations.***

Histopathological and immunohistochemical studies

Histopathological and immunohistochemical studies revealed major changes in both the surface epithelium and the lymphoid tissue. The surface epithelium showed free areas, with the aspect of pseudostratified epithelium, areas of frothy metaplasia (in which the pseudostratified epithelium was replaced by Malpighian-type epithelium without keratinization similar to the epithelium in the oral cavity) and areas of epithelial erosion characterized by total disappearance. Areas of adenoid vegetation covered by the pseudostratified respiratory epithelium were strongly infiltrated with lymphocyte and macrophage cells. Also, the surface of the covering epithelium was lined with a layer of lymphocyte-like cells (Figure 8).

The lymphoid parenchyma was made up of large lymphoid follicles and associated with an abundant infiltrate with inflammatory cells located interfollicular in the adenoid stroma. The lymphoid stroma showed numerous congested blood vessels and even angiogenesis vessels.

Immunohistochemistry studies showed the presence of a cellular infiltrate rich in CD3-positive T-lymphocytes developed among lymphoid follicles, infiltrating the covering epithelium to its surface (Figure 9). B-lymphocytes were found in large numbers in lymphoid follicles but also in the interfollicular stroma. They were identified among the discontinuities of the covering epithelium and at its surface (Figure 10).

The reaction of monocyte–macrophage cells was much more intense, being identified in large numbers in the center of the lymphatic follicles, but also in the covering epithelium (Figures 11 and 12).

The study of the mast cell reaction using the anti-tryptase antibody showed that these cells had a distribution only in the follicular stroma, mainly around the blood vessels (Figure 13). This microscopic appearance demonstrates the importance of mast cells in vascular reactions in inflammatory rhinosinusal diseases.

The use of the anti-CD34 antibody allowed us to identify in the structure of adenoid vegetations a highly developed vascular network, with numerous angiogenesis vessels (Figure 14).

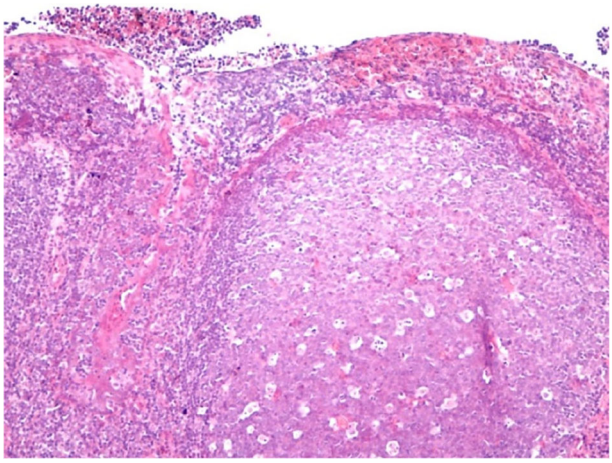

Figure 8 – ***Microscopic overview of an area of adenoid vegetation showing the absence of the covering epithelium, lymphoid follicle hypertrophy, increased lymphoid infiltrate and a lymphocytes deposit on the organ surface. Hematoxylin–Eosin (HE) staining, ×100.***

Electron microscopy study

On the SEM images from patients diagnosed with CR, we observed the characteristic morphology of the bacterial biofilm: three-dimensional (3D) structure, the glycocalyx architecture and water channels connecting the bacterial colonies. In this group of patients, the surface of the extracted lymphatic tissue was almost entirely covered with typical bacterial biofilm structure, represented as multiple connected bacterial colonies highlighted in images with blue arrows (Figure 15).

There was a direct correlation between the presence of bacterial biofilm in adenoid tissue and the presence of recurrent infectious rhinosinusal episodes. The average percentage of coverage of adenoid vegetations with bacterial biofilm in patients diagnosed with CR was 81.27% (Figure 16).

By studying the SEM images from patients diagnosed with OSA, there were observed rare 3D isolated islands with typical bacterial biofilm morphology, with no communication in between. We observed the characteristic structure of the biofilm, indicated with blue arrows, on the surface of the adenoid vegetation and the spaces without bacterial biofilm indicated by the green arrows (Figure 17).

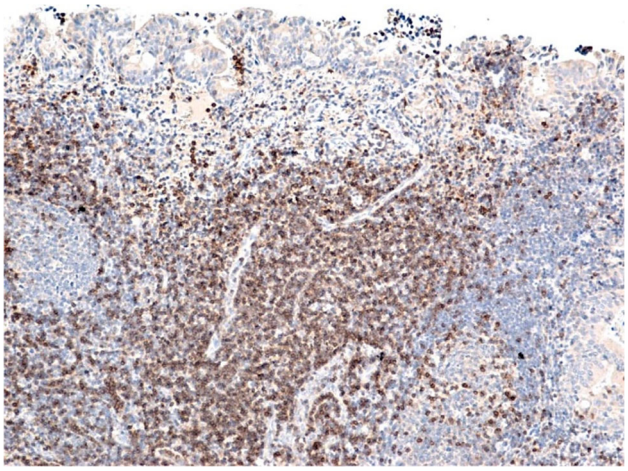

Figure 9 – ***Cellular infiltrate rich in CD3-positive T-lymphocytes arranged mainly interfollicular but also in the covering epithelium. Immunolabeling with anti-CD3 antibody, ×200. CD3: Cluster of differentiation 3.***

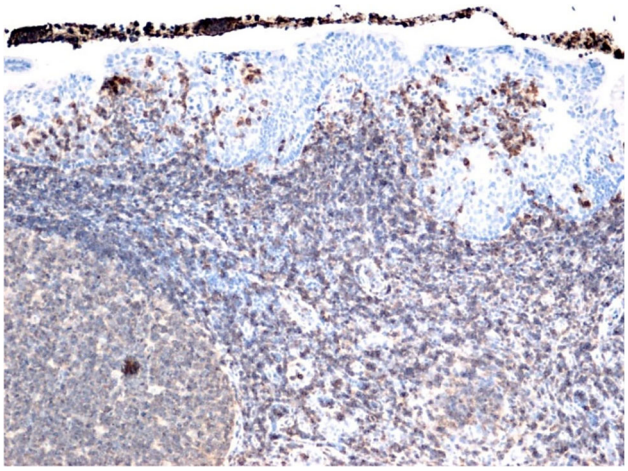

Figure 10 – ***B-lymphocytes present both in the structure of the lymphoid follicle and in the lymphoid infiltrate between the follicles. Immunolabeling with anti-CD20 antibody, ×200. CD20: Cluster of differentiation 20.***

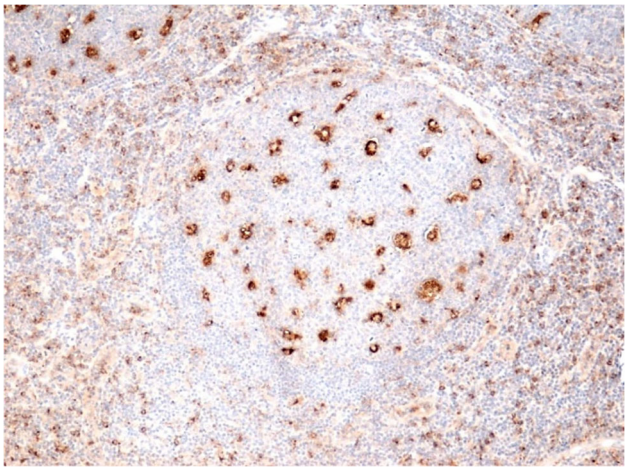

Figure 11 – ***Numerous macrophages identified in the germinal centers of hypertrophied lymphoid follicles. Immunolabeling with anti-CD68 antibody, ×100. CD68: Cluster of differentiation 68.***

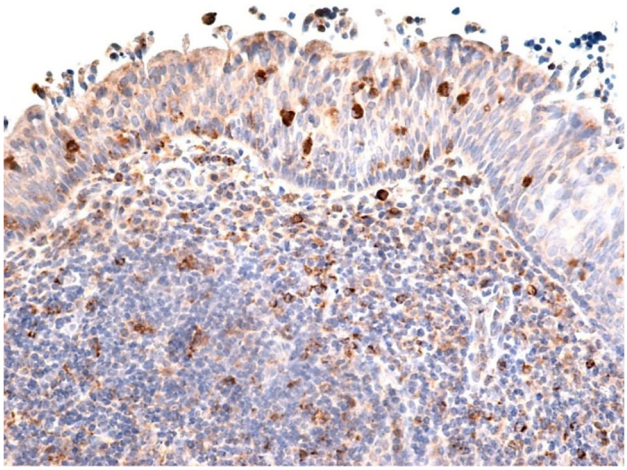

Figure 12 – ***Macrophage cells present in large numbers in the subepithelial conjunctive stroma of adenoids but also in the structure of the epithelium. Immunolabeling with anti-CD68 antibody, ×200.***

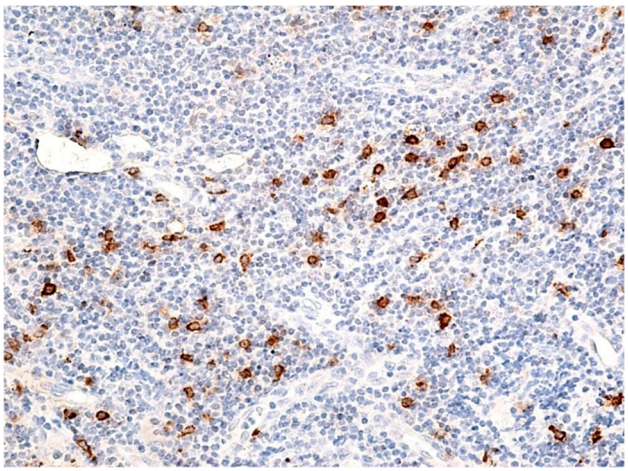

Figure 13 – ***Mast cells present in large numbers in the perivascular stroma of adenoid vegetation, mostly peri-vascular. Immunolabeling of anti-tryptase antibody, ×200.***

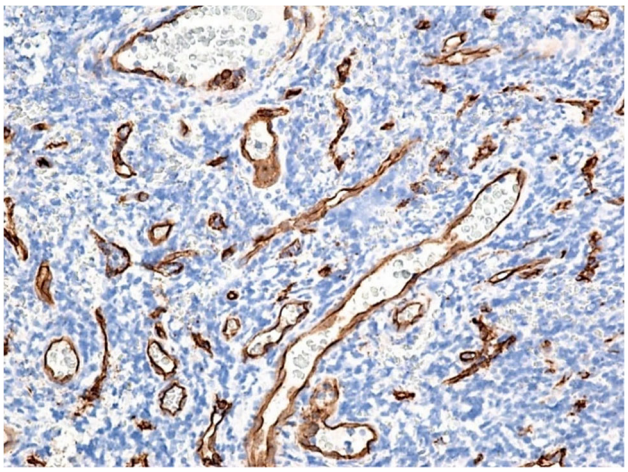

Figure 14 – ***Particularly rich vascular network, congested and with numerous angiogenesis vessels in the stroma of adenoid vegetation. Immunolabeling with anti-CD34 antibody, ×100. CD34: Cluster of differentiation 34.***

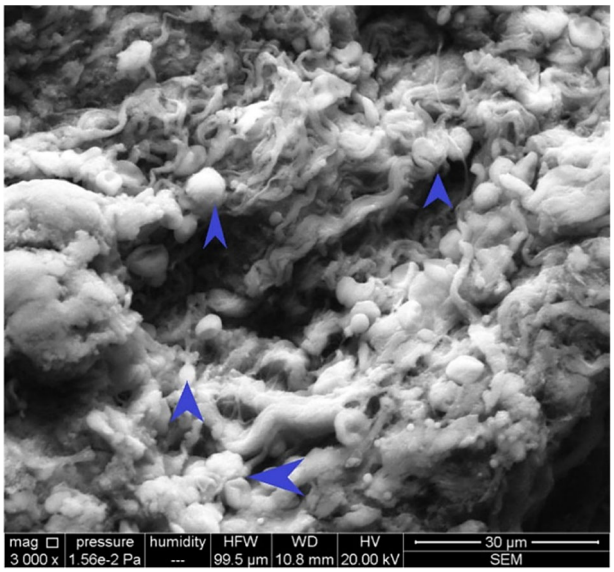

Figure 15 – ***Bacterial biofilm on the surface of adenoid lymphatic tissue from patient diagnosed with CR and AH. SEM image magnification, ×3000. AH: Adenoid hypertrophy; CR: Chronic rhinosinusitis; SEM: Scanning electron microscopy.***

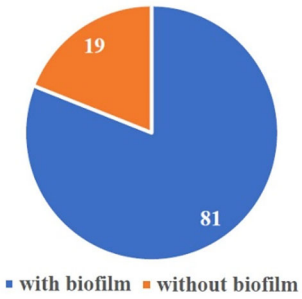

Figure 16 – ***Average percentage of biofilm coverage of the adenoid mucosa in CR children. CR: Chronic rhinosinusitis.***

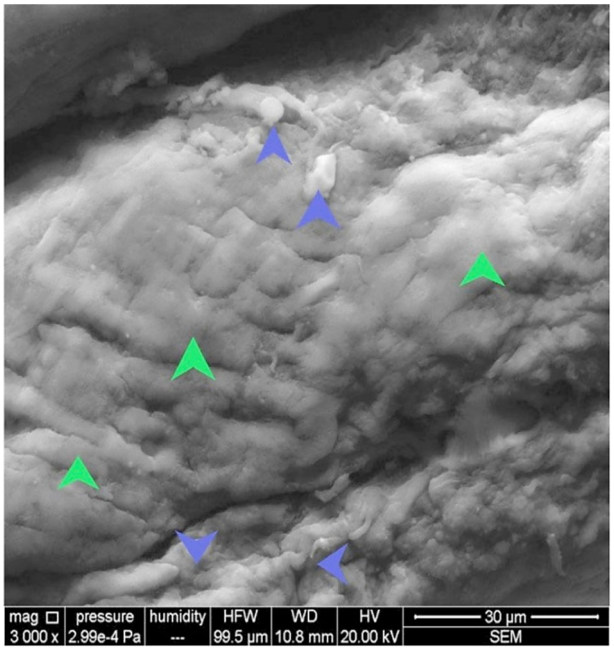

Figure 17 – ***Rare bacterial structures on the adenoidal surface. SEM image magnification, ×3000. SEM: Scanning electron microscopy.***

In the group of children diagnosed with OSA, the average percentage of bacterial biofilm coverage was only 3.87% (Figure 18), in comparison with the value we obtained in the CR group (81.27%) (Figure 19).

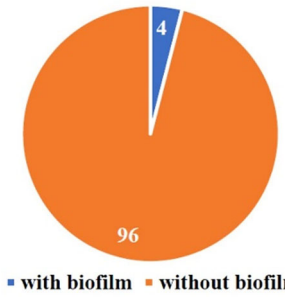

Figure 18 – ***Average percentage of biofilm coverage of the adenoidal mucosa in OSA children. OSA: Obstructive sleep apnea.***

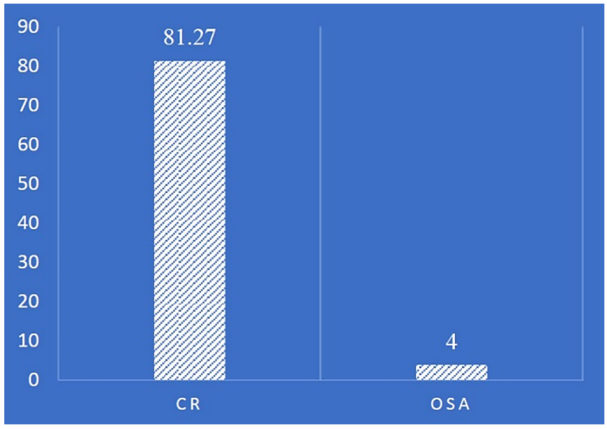

Figure 19 – ***Difference of the average percentages of adenoid coating with bacterial biofilm in the two groups. CR: Chronic rhinosinusitis; OSA: Obstructive sleep apnea.***

Compared to microscopic images from patients with CR to OSA, there was no communication between bacterial colonies.

Discussions

The difference between the two groups, OSA and CR, was statistically significant in terms of the percentage of bacterial biofilm coverage [$t(50.62)$=-39.558, p<0.001; Cohen's d=8.04), indicating that the two distributions differ significantly, and the size of the difference is important. Children diagnosed with CR had a significantly higher percentage of bacterial biofilm coverage (81.27%) compared to the children diagnosed with OSA (3.871%). In a different study, with significantly fewer patients, Zuliani *et al.* (2006) found that the adenoids extracted from children with CR averaged 94.9% of the surface of the adenoid mucosa covered with bacterial biofilms compared to an average coverage of 1.9% in patients with OSA [12]. These differences can be generated by the disproportion of the studied population, the year of study and the geographical area.

The following idea is outlined: the nasopharyngeal bacterial biofilm is a reservoir for several species of bacteria [20–22], resistant to antibiotic therapy [22–24]. From the nasopharynx, the infectious process can migrate through

the Eustachian tube to the middle ear causing colonization of the middle ear cavity and secondary bacterial otitis media [11]. Due to the structure of biofilms (bacteria in a polymeric matrix) and its properties (quorum detection, resistance to phagocytosis and antimicrobial agents), it is resistant to host immune defense mechanisms and to antibiotics [25–27]. It was reported that various bacterial biofilms are associated with increased antibiotic resistance [28, 29]. Over time, several methods for the treatment of bacterial biofilm have been tested, such as: baby shampoo, honey, mechanical debridement, macrolides [30–33]. The role of adenoid bacterial biofilm in CR pathogenesis is also supported by the association between the two [34–37]. We consider, like other researchers [38–40], that bacterial biofilm represents the link between chronic adenoidal disease and CR.

In CR patients, there was a statistically significant tendency in older subjects to have higher percentages of bacterial biofilm coverage (r=0.342, p=0.017, d=0.12). This trend reveals the direct correlation between the colonization with the bacterial biofilm and the age of children. Similarly, in the case of the researched male population, there is a statistically significant association between age and the percentage of bacterial biofilm coverage (r=0.282, p=0.029, d=0.08), the older boys having higher percentages. In the case of the female population, age does not play a significant role.

Of the total study group, 48 children had positive allergy tests, as a percentage 44.85% of children compared to the general group, this percentage was significantly higher than the values found by consulting the specialty literature, where the general pediatric prevalence for respiratory allergens was found between 6–23% [20–22]. We found that the absolute numbers were evenly distributed between the two subgroups, 24 were part of the group diagnosed with OSA and 24 were part of the group of children with CR. Targeted studies on rhinosinusal allergens and allergic inflammation are needed to fully understand the relationship between allergy, biofilm, AH, CR, and OSA [41, 42].

The ratio of boys/girls and OSA/CR in patients with positive allergy tests was relatively equal. There were no statistically significant differences between the age of girls with positive allergy tests and that of boys with positive allergy tests. However, it seems that the presence of positive allergy tests was associated with the recurrence of the adenoids.

Observing the micromorphology of bacterial biofilm using SEM is an effective method of diagnosis despite the high costs. The bacterial biofilm located in the adenoid tissue acts as a generator of infection [43–45] in the neighboring areas, such as the middle ear [11, 46], but also in the lower respiratory tract: trachea, lungs [47, 48].

Conclusions

One challenge for future research consists in finding an efficient noninvasive therapy regarding the chronic rhinosinusal infections associated with biofilm formation. The clinical study revealed that antibiotic therapy in children with CR has an unsatisfactory result. SEM study revealed the 3D architecture of the bacterial biofilm on the adenoidal surface. Histopathological and immunohistochemical studies evidenced the intense chronic inflammatory reaction at cellular level. SEM images showed a strong correlation with histopathological results and with the clinical status of the patients. The results of this study showed that the bacterial biofilm does not influence the evolution of OSA in the studied group due to the lack of bacterial biofilm contamination of the adenoids. The bacterial biofilm covering adenoids represents a source of infection in CR patients and highly affects the quality of their life. The effective therapeutic protocol in both study groups is the surgical one. Our results indicate that bacterial biofilms have a central part in the pathogenesis of CR and that the recurrence of the adenoids could be related to the allergic status of the patients, regardless of the initial diagnostic, CR or OSA.

Conflict of interests

The authors declare that they have no conflict of interests.

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Corresponding authors

Alexandru Chioreanu, MD, PhD Student, Department of ENT, Vasile Goldiş Western University of Arad, 86 Liviu Rebreanu Street, 310414 Arad, Romania; Phone +40257–259 691, e-mail: alexandru.chioreanu@yahoo.ro

Maria Bălăşoiu, Professor, MD, PhD, Department of Microbiology, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Phone +40723–319 879, e-mail: balasoiu.maria@yahoo.com

Received: April 2, 2021

Accepted: October 28, 2021