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Original Scientific Paper

BIOFILM AND HISTOPATHOLOGICAL GRADING OF MAXILLARY SINUS MUCOSA IN PATIENTS WITH ANTROCHOANAL POLYPS

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SUMMARY – The aim of this cross-sectional study was to determine the signs of biofilm in the maxillary sinus of patients with antrochoanal polyps (ACP), and status of the mucosa on which the biofilm occurred. Mucosal samples from maxillary sinus in 40 ACP patients who underwent endoscopic sinus surgery were analyzed histopathologically and by scanning electron microscopy. Results were compared with maxillary mucosa samples of 40 patients without endoscopic and radiological signs of sinus disease. The existence of biofilm and its relation to the degree of histopathological changes according to Terrier classification of chronic mucosal inflammation of maxillary sinus were statistically analyzed. Biofilm was detected in 23 of 40 (57.5%) ACP patients; the incidence was significantly lower in the control group (2/40, 5%). Biofilm was not found in type 1 mucosa according to Terrier classification. In conclusion, biofilm showed a significant incidence in the maxillary sinus mucosa of ACP patients (57.5%). Occasionally, biofilm can be found in patients with no signs of sinus disease, but not on histologically normal mucosa. Results of this study support the theory that biofilm formation does not represent the initial stage of the inflammatory process.

Key words: *Antrochoanal polyp; Biofilm; Maxillary sinus*

Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous inflammatory disorder, a clinical syndrome characterized by persistent symptomatic inflammation of the nasal and paranasal sinus mucosa lasting for more than 12 weeks, with two phenomena present, inflammation and tissue remodeling[1-3]. CRS is most commonly divided in two major clinical phenotypes based on the presence or absence of polyps on nasal endoscopy, i.e., chronic rhinosinusitis without nasal polyps (CRSsNP) and chronic rhinosinusitis with nasal polyps (CRSwNP)[4]. Apart from these two major clinical phenotypes, antrochoanal polyp (ACP) is an insulated entity within CRS. Palfyn first reported on an ACP case in 1753, Zuckerkandl described it in 1891, and Killian distinguished ACP from CRSwNP in 1906[5-7]. ACP is a unilateral benign sinonasal lesion arising from the maxillary sinus, spreading into the nasal cavity and extending towards the choana or further to the nasopharynx. While CRSwNP always occurs bilaterally, ACP is, as a rule, a unilateral disease (only 12 cases have been described so far in the English scientific literature)[8]. It accounts for 3%-6% of all polyp cases[9].

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Bacteria exist in two biological forms, i.e., planktonic form and within the biofilm. Acute bacterial infections are caused by planktonic forms of pathogenic bacteria[10]. Biofilm occurs when the environment becomes unfavorable to bacteria, and represents primary structure of bacterial survival and proliferation[1,11]. It is estimated that 99% of all bacteria exist in the form of biofilm and only 1% in planktonic form[12]. In the biofilm form, bacteria are up to 1000 times more resistant to antibiotics typically effective against planktonic bacteria[1,13]. According to the American Center for Disease Control and Prevention, bacterial biofilms participate in the pathogenesis in up to 85% of all human bacterial infections and 65% of all nosocomial infections. Features of biofilm infections are the persistence of chronic infections; the inability to detect the microbial pathogen by standard microbiological cultivation techniques in most of the cases; the failure of antimicrobial therapy; and of the host immune system to eradicate the pathogens[10,14]. Biofilm develops in the human body, on vital or necrotic tissue, and on the inert surfaces of various biomaterials[10]. In the head and neck region, biofilm was found in periodontal diseases (dental plaque is the most famous and first described biofilm in humans, van Leeuwenhoek 1674), chronic middle ear inflammation (serous otitis and cholesteatoma), chronic tonsillitis, adenoid vegetations, cystic fibrosis, and CRS. It can also be found on artificial materials such as ventilation tubes on the eardrum, speech prostheses, endotracheal tubes, cochlear implants, central venous catheters and stents for the frontal sinus. Bacteria producing biofilm in this region are *Streptococcus pneumoniae*, *Staphylococcus aureus, Haemophilus influenzae, Pseudomonas aeruginosa*, coagulase-negative staphylococci, *Enterobacter* spp., *Proteus mirabilis*, *Hafnia* spp., and *Acinetobacter* spp.[15-20].

Cryer *et al*. were the first to report on the existence of biofilm in CRS in patients with persistent disease, despite appropriate medical and surgical management[21]. Furthermore, when analyzing perioperative specimens, Palmer, and Sanclement *et al*. confirmed biofilm in patients with CRS[11,22]. Since then, many studies have reported on the important role of bacterial biofilms in the etiopathogenesis of CRS[1,23]. It is assumed that a healthy, normal epithelium of the upper respiratory system prevents biofilm growth, whereas the loss of cilia which occurs in CRS may favor biofilm formation[23,24]. It is considered that biofilm in CRSwNP contributes to nasal mucosa damage and increases the number of inflammatory cells with the subsequent hyperplastic process[25]. The relation between biofilm and ACP is poorly understood and so far, it has not been sufficiently investigated. The results of this study showed the existence of biofilm on the maxillary sinus mucosa in patients with ACP and contribute to better understanding the etiopathogenesis of this disease.

Patients and Methods

Forty consecutive patients (20 male and 20 female) who underwent endoscopic sinus surgery (ESS) for ACP were enrolled in this cross-sectional study. The inclusion criteria matched the diagnosis of ACP made by endoscopic examination and confirmed by computed tomography (CT). Control group consisted of 40 (20 male and 20 female) patients without any clinical, endoscopic, or CT signs of sinus disease. They underwent surgery through healthy sinuses for other pathological substrate, i.e., pituitary tumor, cerebrospinal fistula, orbital decompression, decompression of the optic nerve canal, and other lesions of the skull base. For both study groups, exclusion criteria were any sinonasal comorbidity, any conservative sinus treatment in the previous year, any previous sinus surgery, and any prior irradiation of the head and neck. All subjects whose mucosa did not appear normal during sampling were also excluded from the control group. The study was approved by the Research Ethics Committee of the Zagreb University Hospital Center. All patients were older than 18 years and each of them signed the informed consent form.

During ESS under general anesthesia, mucosal samples from the posterior wall of the maxillary sinus were taken and divided in two parts. One part was processed for histopathological analysis and the other for scanning electron microscopy (SEM), both under the code in order to guarantee patient anonymity and objectivity of the observation. The findings of histopathological analysis were grouped according to Terrier classification of chronic mucosal inflammation of the maxillary sinus, as follows: type 1, normoplastic mucosa; type 2, hyperplastic mucosa; type 3, polypous mucosa; and type 4, polypodo-polypous mucosa[26].

Samples for SEM analysis were obtained and conserved in formaldehyde solution. Before analysis, in order to remove formaldehyde residue and to dehydrate the samples at the same time, they were washed in mixtures of twice distilled water and ethanol (75/25,

50/50, and 25/75 volumes of distilled water and ethanol were used for 30 min at each volume ratio). Then, the samples were washed twice in absolute ethanol. Finally, the samples were separated from ethanol, put in a Petri dish, covered with porous cellulose paper, and dried for 24 h. The dried samples were then sliced and prepared for electron microscopy. A thermal field emission electron microscope (FE-SEM, model JSM-7000F, manufactured by JEOL Ltd. Tokyo, Japan) was used. The samples were analyzed for the existence of biofilm according to the criteria of SEM identification and the characteristic morphology of the bacterial biofilm, i.e., surface adherent bacteria organized in formations (microcolonies or macrocolonies) that can be interconnected; bacterial cells covered with extracellular matrix; two-dimensional (patches of a single cell layer) and three-dimensional (aggregates consisting of multiple cell layers) structure aggregates of the biofilm; presence of the channels and pockets free of bacteria on the surface of the extracellular matrix; formation of towers; and existence of water channels and spherical shapes in the matrix[22,27,28].

Statistical analysis was performed using Statistica 13.5 (TIBCO Inc., USA), and SPSS 23.0 (IBM, USA). For all tests, a p value lower than 5% ($p<0.05$) was considered statistically significant. Statistical difference between the groups was calculated by the use of χ^2-test for independent variables, and McNemar test for dependent variables; Spearman rank order correlation test was used for determination of correlation.

Results

Patients with ACP were significantly younger than those in the control group (mean age 26.68±7.74 *vs.* 43.48±15.22 years). Twenty-five (62.5%) ACP patients had right-sided and 15 (37.5%) left-sided polyps. This difference was borderline significant (p=0.052).

Biofim was found in patients with ACP but also in the control group (Fig. 1). The results showed a statistically significant difference in the presence of biofilm on sinus mucosa between ACP patients and control subjects (57.5% *vs.* 5%; p<0.001) (Table 1). However, the presence of biofilm in two control subjects indicated that it may also be found in the individuals without clinical and radiological signs of rhinosinusitis (Fig. 2).

Table 2 shows distribution of ACP patients and control patients according to histopathological changes of maxillary sinus mucosa as determined by Terrier classification. In ACP patients, the most common histopathological changes were types 3 and 2, while type 1 was most common in the control group. In control group, type 1 was convincingly most

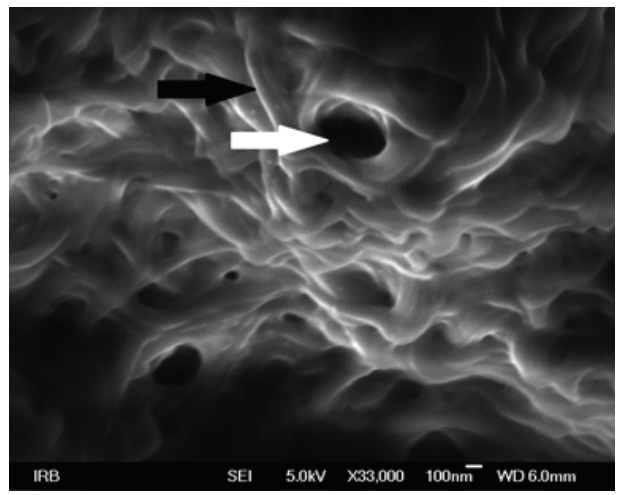

Fig. 1. Biofilm on the maxillary sinus mucosa in a patient with antrochoanal polyp: water channels (white arrow) and pila (black arrow) visible on the surface (SEM X33000).

Table 1. Presence of biofilm on sinus mucosa in ACP patients and control group

	ACP group (N=40)	Control group (N=40)	χ^a	p^a
Presence of biofilm on sinus mucosa (no/yes)	17/23 (42.5/57.5)	38/2 (95/5)	28.809	<0.001
χ^b p^b	7.875 0.051	18.375 <0.001		

ACP = antrochoanal polyp; n (%); [a]χ df=1; [b]McNemar test df=1

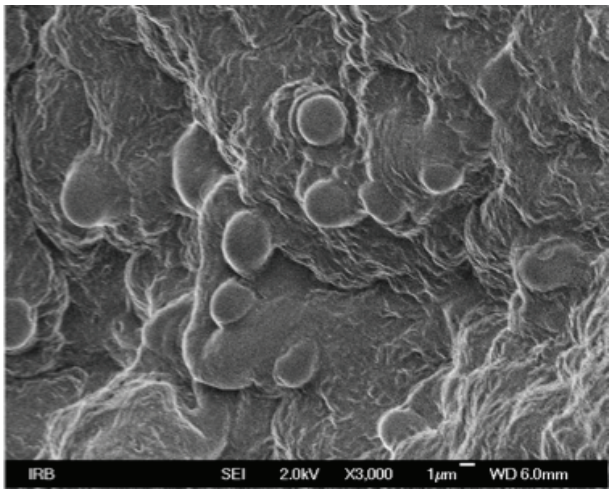

Fig. 2. Biofilm with spherical bodies that resemble cocci bacteria within the matrix in a control group patient (SEM X3000).

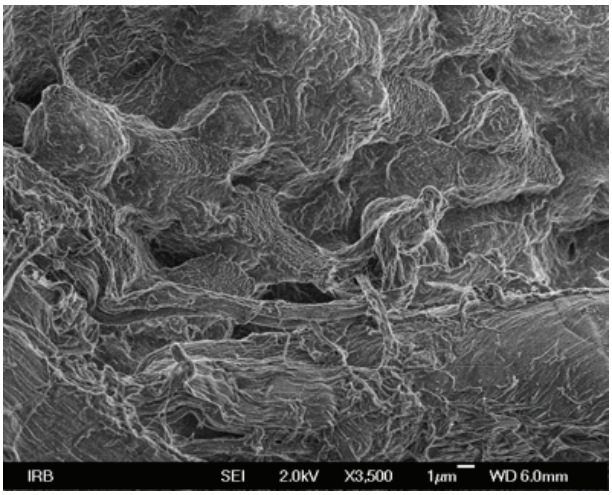

Fig. 3. Three-dimensional structure of the biofilm: channels and pockets free of bacteria on the surface of the extracellular matrix (SEM X3500).

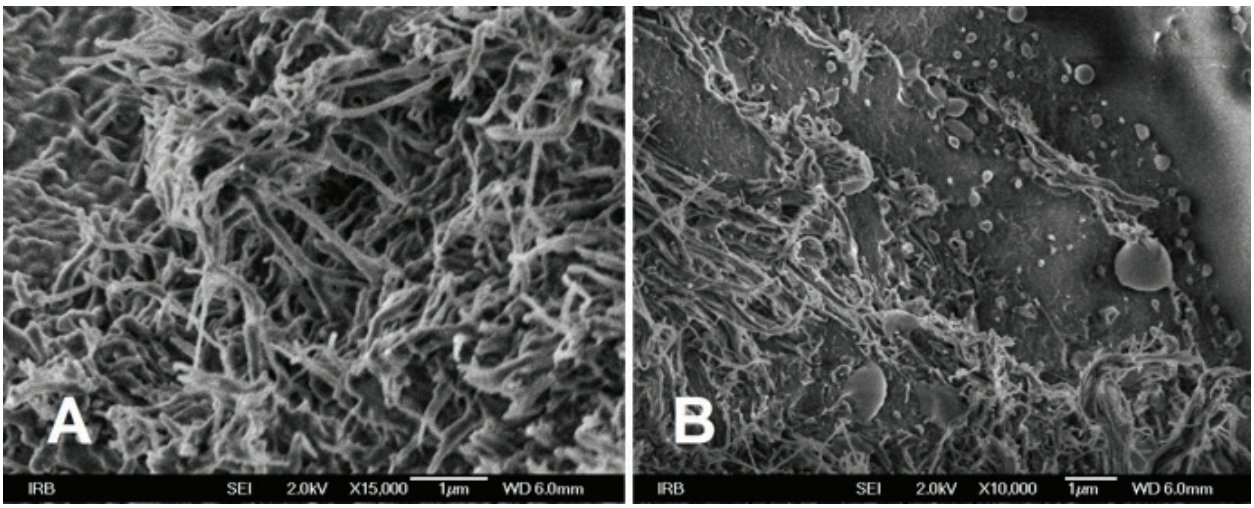

Fig. 4. Disoriented ciliary epithelium with well represented cilia without evidence of biofilm (A-SEM X10000). Destroyed ciliary epithelium, aggregates of bacteria – beginning of biofilm formation (B-SEM X15000).

common, yielding a significant difference compared to other types ($p<0.001$).

Correlation of biofilm with histopathological changes of maxillary sinus mucosa according to Terrier classification in patients with ACP is shown in Table 3. There was no statistically significant correlation between the biofilm and histopathological mucosal findings. Table 4 shows correlation of biofilm on maxillary sinus mucosa with histopathological changes in the control group. A statistically significant negative correlation was found between the biofilm on maxillary sinus mucosa and Terrier type 1 ($t(N-2)=-4.192$, $p<0.001$), and more significant positive correlation between the biofilm and Terrier type 2 ($t(N-2)=6.008$, $p<0.001$). The correlation between the biofilm and Terrier type 3 was positive but did not reach statistical significance (Fig. 3). There was no correlation of the biofilm with Terrier type 4 at all, since none of the subjects had mucosal findings of this type. Based on these results, it can be concluded that there were no

Table 2. Histopathological changes of the mucosa according to Terrier classification in patients with ACP and control subjects

Terrier classification	ACP group (N=40)	Control group (N=40)	χ^a	p^a
Type 1	2 (5)	37 (92.5)	47.478	<0.001
Type 2	13 (32.5)	2 (5)	18.356	0.028
Type 3	22 (55)	1 (2.5)	39.127	<0.001
Type 4	3 (7.5)	0 (0)	1.458	0.143
χ^b p^b	29.568 <0.001	61.651 <0.001		

ACP = antrochoanal polyp; n (%); [a]χ df=1; [b]McNemar test df=3

Table 3. Correlation of biofilm findings on maxillary sinus mucosa with histopathological changes in patients with ACP

Terrier classification		Type 1	Type 2	Type 3	Type 4
Biofilm on sinus mucosa	Spearman R t(N-2) p	-0.036 -0.215 0.831	0.057 0.350 0.728	0.036 0.219 0.827	-0.139 -0.866 0.392

ACP = antrochoanal polyp

Table 4. Correlation of biofilm findings on maxillary sinus mucosa with histopathological changes in control group

Terrier classification		Type 1	Type 2	Type 3	Type 4
Biofilm on sinus mucosa	Spearman R t(N-2) p	-0.562 -4.192 <0.001	0.698 6.008 <0.001	0.027 0.158 0.875	0.000 0.000 1.000

signs of biofilm on histologically normal mucosa; furthermore, the incidence of biofilm increased with histopathological mucosal changes (Fig. 4).

Discussion

Antrochoanal polyp usually occurs at a younger age, between the ages of 20 and 30, as confirmed by this study[29]. The purpose of this study was to investigate the presence of biofilm and its correlation to histopathological grading of maxillary sinus mucosa in ACP patients. Multiple studies have reported on the existence of biofilm in CRS using different detection methods. The incidence of biofilm varies from 25% to 100%[1]. In CRSsNP, it is usually about 75%[30]. The incidence of biofilm in CRSwNP is usually even higher compared to CRSsNP[31-33]. This indicates the role of biofilm in the pathogenesis of CRS, particularly in CRSwNP[2,25,30,34]. Patients with biofilm-positive CRS have a significantly higher Lund-McKay score compared to those without biofilm[35]. The presence of biofilm in CRS is associated with a higher risk

of recurrence and revision surgery[1]. Furthermore, persistent biofilms in CRSwNP patients may be responsible for surgical failure and high recurrence rates[27]. On the other hand, patients operated for ACP showed overall better symptomatic improvement compared to patients operated for CRSwNP, and the recurrence rate was lower[4,36]. In this study, the incidence of biofilm in ACP was 57.5%, and to the best of our knowledge, this is the only study that investigated the presence of biofilm exclusively in ACP patients. The lower incidence of biofilm in ACP compared to CRSwNP might be the reason for a lower ACP recurrence rate.

In the group of 40 patients with ACP, histopathological changes of the mucosa were found in 38 (95%) cases. Among them, biofilm was found in 23 (57.5%) patients. In our patients with ACP, the most common histopathological changes of the sinus mucosa were types 2 and 3 according to Terrier classification (87.5%). We believe there are two reasons that may explain this finding. First, as ACP derives from the mucosa of the maxillary sinus, its origin (sinus mucosa) should be altered histologically, as it is the case in Terrier types 2 and 3. Both of these types are characterized by edematous stroma with chronic inflammatory infiltrate. ACP has a similar histologic structure but the inflammatory infiltrate is less pronounced. Secondly, since ACP almost entirely or entirely occupies maxillary sinus cavity, it is unlikely that polyps (Terrier type 4) will develop simultaneously. That is probably the reason why type 4 was found in only three (7.5%) cases in the group of patients with ACP. There was no statistical correlation of the biofilm existence with histopathological changes of the mucosa. This can be explained by the fact that biofilm in those patients was almost always (96.88%) located on the inflammatory mucosa of the maxillary sinus (predominantly in types 2 and 3). Furthermore, Terrier type 1 describes normoplastic mucosa and was present in only two patients, as well as type 4 which was present in three patients. The incidence of biofilm did not rise with the degree of mucosal inflammation.

In the control group of 40 patients, type 1 according to Terrier classification was the predominant histopathological finding. Type 1 was found in 37 (92.5%) cases, showing no evident inflammatory disease of the sinus mucosa. It represents normoplastic mucosa and this was expected. The healthy sinonasal mucosa, goblet cells that produce mucus, and ciliated cells that eliminate this mucus are the first line of defense against inhaled bacteria[24,37]. Proper mucociliary clearance is very important for normal sinus function, especially their drainage. CRS is characterized by respiratory epithelial disorders; cilia are increasingly rare or absent, resulting in decreased mucociliary clearance[24]. It can lead to the development of atypical, metaplastic, or mixed respiratory epithelium[38]. Mucociliary dysfunction is known to be present in both CRS phenotypes, as well as in CRS with biofilm[24,39,40]. Investigating biofilm and CRS on animal model, Jia *et al*. showed that the earliest changes were goblet cell hyperplasia with consequent increase in mucus production and concomitant decrease in ciliary activity[41]. Studies investigating the presence of biofilm and ciliary damage showed different degrees of epithelial destruction, ranging from partial ciliary disorder to complete absence of cilia[24,41,42]. It is still not clear whether ciliary dysfunction precedes or results from biofilm production. Out of the three cases classified as types 2 and 3 in our control group, two were positive for biofilm. This study found a statistically significant negative correlation between the biofilm on sinus mucosa in control group and Terrier type 1 findings ($t(N-2)=-4.192$, $p<0.001$). These data support the presumption that biofilm formation does not represent the initial stage of the inflammatory process leading to partial damage or destruction of the cilia. Therefore, biofilm should not appear on histologically completely normal mucosa. Furthermore, the positive correlation between biofilm and Terrier type 2 ($t(N-2)=6.008$, $p<0.001$) was found in the control group of patients. It indicates that the probability of the biofilm formation is related to histopathologically changed mucosa and it occurs on previously damaged mucosa. If cilia are in any way not functional or absent, the risk of biofilm and other risk factors for CRS are increased, and the biofilm might contribute to further epithelium damage and subsequent hyperplasia of the subepithelial layer[1,31].

The presence of biofilm of 5% (2/40) in our control group indicated that biofilm can occasionally be found in subjects without clinical, endoscopic and radiological signs of CRS. Similar results were recorded in the study by Singh *et al*., where biofilm was found in 8% (4/50) of the control group patients[43]. In our study, to reduce the incidence of 'apparently healthy' subjects in the control group, we excluded all patients whose mucosa did not seem healthy on ESS, keeping in mind that the probability of rhinosinusitis

decreases with low clinical suspicion (<50%) and negative nasal endoscopy[44]. However, three of our control group patients had hyperplastic and polypous mucosa according to Terrier classification, two of them with positive biofilm. Therefore, the absence of clinical, endoscopic and radiological signs of sinus disease need not mean histologically normal sinus mucosa. Positive biofilm findings in control groups of other biofilm studies in CRS range from 0 to almost 50%[27,45]. Such a great difference in the incidence is probably caused by different inclusion criteria, and sample size and localization. In most studies, control group was composed of individuals who underwent septoplasty with or without negative CT scan[31,32,45]. Furthermore, comorbidity can affect biofilm formation as well. Allergic rhinitis, in which mucosal edema can cause nasal and sinus obstruction, is usually mentioned in the literature; in some studies, surprisingly, it was not an exclusion criterion[46].

Conclusions

In patients with ACP, biofilm has a significant prevalence in the maxillary sinus mucosa, confirmed in 57.5% of the cases. Occasionally, biofilm can be found in patients with no signs of sinus disease, but not in normoplastic mucosa (type 1 according to Terrier classification). The results of our study support the theory that biofilm formation does not represent the initial stage of the inflammatory process.

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Sažetak

BIOFILM I HISTOPATOLOŠKA KLASIFIKACIJA SLUZNICE MAKSILARNOG SINUSA U BOLESNIKA S ANTROKOANALNIM POLIPOM

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Cilj ovog presječnog istraživanja bio je utvrditi postoje li znakovi biofilma u maksilarnom sinusu u bolesnika s antrokoanalnim polipima (ACP) i utvrditi status sluznice na kojoj se biofilm javlja. Uzorci sluznice maksilarnog sinusa 40 bolesnika s ACP-om koji su podvrgnuti endoskopskoj operaciji sinusa analizirani su patohistološki i skenirajućom elektronskom mikroskopijom. Rezultati su uspoređeni s 40 bolesnika bez endoskopskih i radioloških znakova sinusne bolesti. Analizirana je i statistički obrađena prisutnost biofilma i njegova povezanost sa stupnjem patohistoloških promjena prema Terrierovoj klasifikaciji kronične upale sluznice maksilarnog sinusa. Biofilm je pronađen u 23 od 40 bolesnika (57,5%) s ACP-om, a statistički značajno rjeđe u bolesnika kontrolne skupine (2 od 40,5%). Biofilm nije pronađen na sluznici tipa 1 prema Terrierovoj klasifikaciji. Ovo je istraživanje pokazalo značajnu incidenciju biofilma na sluznici maksilarnog sinusa u bolesnika s ACP-om (57,5%). Sporadično se biofilm može naći i u asimptomatskih bolesnika (bez kliničkih, endoskopskih i radioloških znakova sinusne bolesti), ali ne i na normoplastičnoj sluznici. Rezultati ovog istraživanja podupiru pretpostavku da stvaranje biofilma ne predstavlja početnu fazu upalnog procesa.

Ključne riječi: *Antrokoanalni polip; Biofilm; Maksilarni sinus*