ORIGINAL CONTRIBUTION

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Biofilm in nasal polyps*

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SUMMARY

Introduction: *Bacterial biofilms are involved in many human bacterial infectious processes and in chronic rhinosinusitis as well. The aim of this study was to determine whether biofilm exists in nasal polyps, both in diffuse nasal polyposis (DNP) and antrochoanal polyps (ACP).*

Method: *Tissue samples were taken from seven patients suffering from DNP and three patients suffering from ACP, based on the defined patient inclusion and exclusion criteria. After the preparation, the tissue samples were analyzed by means of scanning electron microscopy (SEM) for signs of biofilm formation.*

Results: *Signs of biofilm presence were found in all DNP patients. In ACP cases, biofilm was found in the stalk and nasal, polypoid part of the ACP, whereas there were no signs of biofilm on diseased mucosa of the posterior wall of the maxillary sinus.*

Conclusions: *Our preliminary study showed a possible role of bacterial biofilm in pathogenesis and maintenance of both DNP and ACP. There are no obvious differences in the SEM appearance of biofilms in DNP and ACP. Future research is needed to explain why biofilm is present in cases of diffuse nasal polyposis and at the nasal part of ACP, but not on the maxillary sinus part of ACP.*

Key words: biofilm, nasal polyps, antrochoanal polyps

INTRODUCTION

Bacterial biofilms have increasingly been recognized as important factors in the pathogenesis of numerous chronic infections. According to the Centers for Disease Control and Prevention, biofilms are involved in at least 65% of all human bacterial infectious processes [(1)].

A biofilm is an aggregation of communicating microorganisms embedded in a protective self-produced polysaccharide matrix that can adhere to both living and inert surfaces. Biofilm bacteria interact within the matrix, which is nothing else but well hydrated extracellular polymeric substance (EPS). The microbes entrapped in biofilm EPS are considered to be dormant, meaning that their requirements for nutrients and oxygen are reduced. The other form (phenotype) of bacteria are planctonic (free-floating) ones which can normally be found in mucus in acute infections. Studies on micro-catheters showed that metabolic changes (i.e. oxygen and glucose gradients) are responsible for phenotypic change of bacteria [(2,3)] that enables biological differences between planctonic and sessile (entrapped) form of bacteria. It makes the development of bacterial most "defensive" life-strategy possible [(4)].

According to Becker et al. [(5)] and the most recent study by Babic et al. [(6)] the genetic exchange between planctonic bacteria and those entrapped within the biofilm might give rise to the development of antibiotic resistance. This genetic diversity is possible because of mutual exchange of plasmids between two bacteria through very special extensions of their "body"- so called pila. Most recent investigations have clearly shown a real "sexual relationship" between particular bacteria. It was shown that conjugation allows bacteria to acquire genes for antibiotic resistance, novel virulence attributes, and alternative metabolic pathways [(6)]. Using a fluorescent protein fusion, SeqA-YFP, the authors have visualized this process in real time and in single cells of Escherichia coli. They found that the F pilus mediates DNA transfer at considerable cell-to-cell distances. Integration of transferred DNA by recombination occurred in up to 96% of recipients.

The bacteria in biofilms can be up to 500-1000 times more resistant to antibiotics than free-floating bacteria. Whereas an appropriate antibiotic therapy (based on the antibiogram analysis) and activated host defense mechanisms can eliminate the planctonic cells derived from biofilms, they cannot kill the bacteria in biofilms due to the protective nature and the physical barrier of the matrix, reduced bacterial metabolism and the above mentioned genetic diversity. In this way, the bacteria within the biofilm remain intact, refractory to antibiotics, and become the foci of chronic infection.

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Biofilms have been implicated in the chronic nature of several infections of the head and neck region such as dental and periodontal disease, otitis media with cholesteatoma, tympanostomy tube otorrhea, chronic tonsillitis and cystic fibrosis pneumonia [7-9]. The recurrent, recalcitrant course of disease and resistance to antibiotic treatment suggested the role of biofilm in chronic rhinosinusitis, which has been supported by various recent publications. For instance, Cryer in 2004, who used scanning electron microscopy (SEM), was the first to report on biofilm formation in patients who had FESS and continued to have symptoms despite medical treatment. Their patients had mainly Pseudomonas aeruginosa infection [10]. The SEM study performed by Sanclement [11] on biopsies of the ethmoid sinus mucosa taken during FESS showed the presence of biofilms in 24 out of 30 patients and in none of the controls. The presence of biofilms in chronic sinusitis has been demonstrated by other methods, such as transmission electron microscopy (TEM) [12] and confocal scanning laser microscopy (CSLM) [13].
The signs of biofilm were also found in animals in which an experimental sinusitis was produced by inoculating Pseudomonas aeruginosa species [14]. Bendouah and his collaborators have shown that biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosa is associated with an unfavorable clinical evolution in patients after endoscopic surgery for chronic sinusitis either with or without nasal polyposis [15].

The question arises here whether biofilms also exist in nasal polyps. If so, are they present in all of them or in some of them, depending on their morphological and clinical appearance?
According to Stammberger, there are five types of nasal polyps: type 1 - antrochoanal polyp, type 2 - choanal polyp, type 3 - nasal polyposis in chronic sinusitis with no eosinophilia, type 4 - nasal polyposis in chronic sinusitis with dominant eosinophilia (NARES, PARES, ASA intolerance, fungal sinusitis) and type 5 - systemic disease polyposis (cystic fibrosis, Kartagener's syndrome, primary ciliary dyskinesia) [16].

From the morphologic point of view, there is a great difference between standard, diffuse nasal polyposis (DNP), mostly originating in the ethmoidal sinuses, and antrochoanal polyps (ACP). ACP has three morphologically different parts in the vast majority of patients: 1) an emphasized cystic formation arising at the bottom of the posterior wall of the maxillary sinus and confluencing in a 2) gracile stalk, which passes through the defect in the posterior nasal fontanellae, transforming itself in 3) edematous mass, which clinically resembles very much an usual nasal polyp.
The aim of this study was to find out whether we can expect biofilms both in diffuse polyposis, which we know is connected with chronic sinusitis, and in antrochoanal polyps, which are said not to be connected with chronic sinusitis. In addition, we wanted to know whether these two entities have anything in common from the scanning electron microscopical point of view.

MATERIALS AND METHODS

Patients

Tissue samples taken from seven patients suffering from DNP and three patients suffering from ACP were analyzed.
Inclusive criteria for this study were as follows: adult patients (> 18 yrs of age) suffering from diffuse bilateral nasal polyposis for DNP group, and patients of whichever age suffering from ACP. Exclusive criteria were as follows: allergy to either inhalatory or alimentary allergens (RIST and RAST positive), previous operations for nasal polyposis, positive prick-tests on the most frequent allergens, presence of Sammter's triad or asthma and a course of antibiotic therapy during the last seven weeks.
The group of DNP patients consisted of 5 males and 2 females, aged 21-57 yrs (mean value 41.4). All of the subjects have been receiving steroid drugs perorally for three weeks before the surgery (prednisolone): 10 milligrams every morning for the first seven days and 5 milligrams for the following two weeks. They also used steroid nasal spray (mometasone) once a day, one puff in each nostril.

Image analysis

CT-images were performed at the 19^{th}-21^{st} day after starting steroid treatment, i.e. just a couple of days before the surgery itself. The images were evaluated after Lund-Mackay's scoring system17. They showed in four out of seven patients bilateral total opacifications of both ethmoidal sinuses, ostiomeatal complex, sphenoidal and maxillary sinuses (score 2), whereas frontal sinuses were still patent despite minor changes in the naso-frontal recess (score 1). Three patients showed the opacifications also in the frontal sinuses (score 2).
Rhinoscopic and endoscopic appearance of the polyps was evaluated after the 3-weeks steroid treatment. It was done according to Malms staging system (18). Five of the patients showed stage III nasal polyps according to Malm, while 2 showed stage II.
Among ACP patients, there were two females, one aged 14, other one 27 and one male aged 33. They did not receive any therapy since diagnosis of ACP by means of nasal fiberendoscopy has almost 100% accuracy.
CT-scanning showed no additional pathological changes of the other sinuses besides typical appearance of the solitary polypous mass in the nasal cavity, typical stalk in the region of the posterior fontanel and the edematous maxillary sinus mucosa. All of them were scored 2 for the particular maxillary sinus according to Lund-Mackay's scoring system.
Regarding endoscopic appearance, all of them were very similar to the Malm's stage III in DNP patients, in these cases occluding ipsilateral choanae.

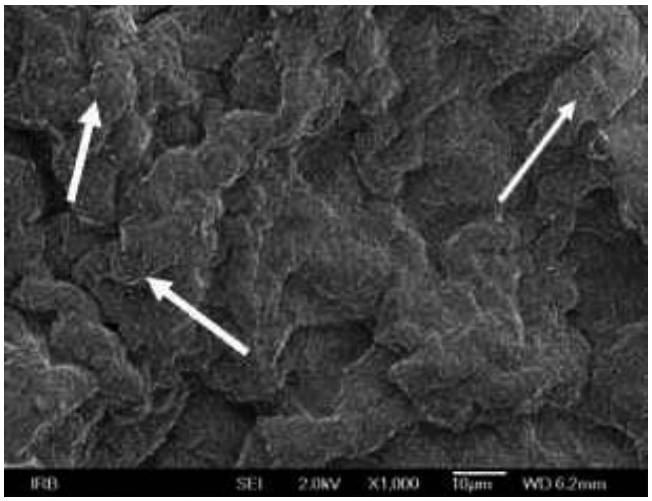

Figure 1. A SEM micrograph of the nasal polyp surface. Magnification 1,000X. Typical clouds-like formations of EPS cover the underlying cilia. Sporadical "towers" of EPS are marked by white arrows.

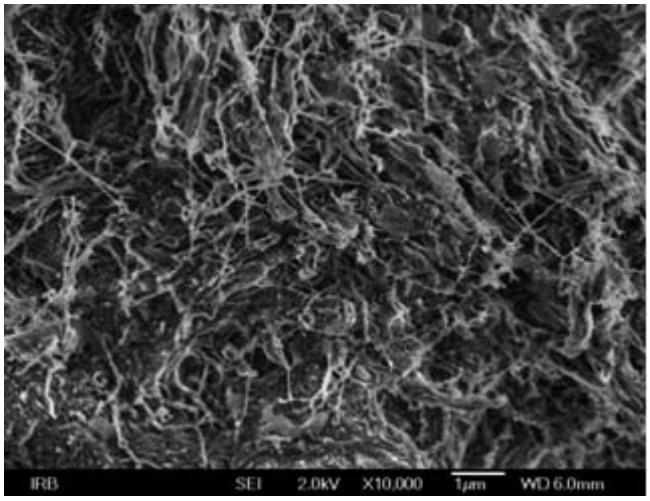

Figure 2. A SEM micrograph of the nasal polyp surface. Magnification 10,000X. The ultrastructure of the cloud-like formations very much resembles a cob-web.

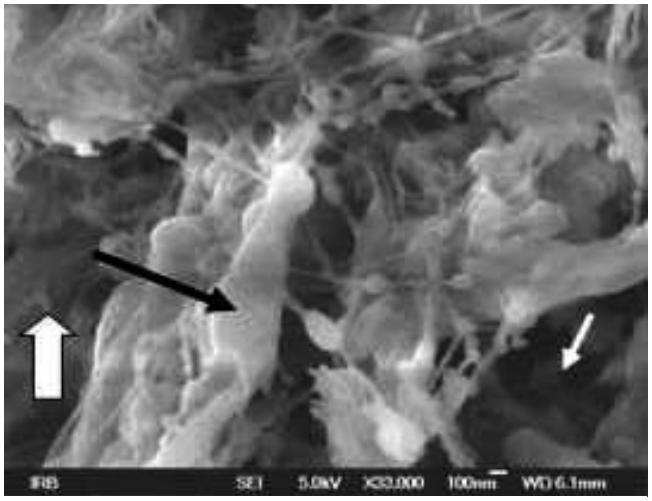

Figure 3. A SEM micrograph of the nasal polyp surface. Magnification 33,000X. It seems that some scarce bunches of residual cilia can be seen under the cob-web formation (wide white arrow) and some areas with no cilia at all but only a few, compund microvilae (thin white arrow). Black arrow indicates one of the EPS "towers".

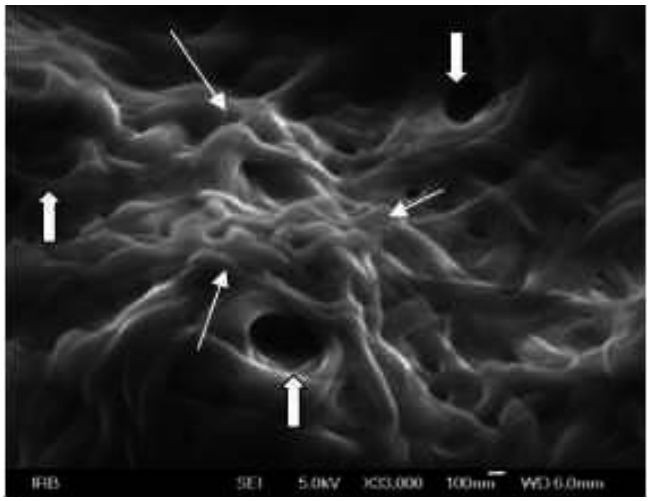

Figure 4. A SEM micrograph of the nasal polyp surface. Magnification 55,000X. Typical finding of inter-bacterial pila connecting one bacteria to another. An ampular dilatation of the particular pilus can be seen at the donor-bacteria's side (white thin arrows). Wide water channels are also very frequently found (wide white arrows). All the bacteria that can bee seen at this micrograph are typically rod-shaped

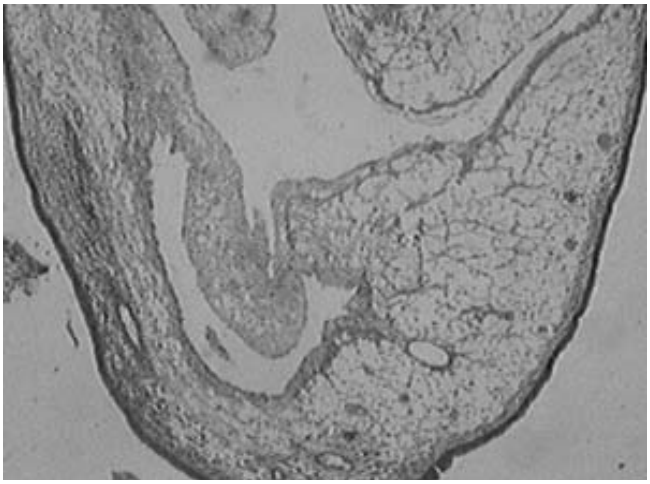

Figure 5. Light-microscopy image of the mucosa from the posterior wall of the maxillary sinus. Light-microscopy by LEITZ DIAPLAN microscope, magnification 0.8 x 2.

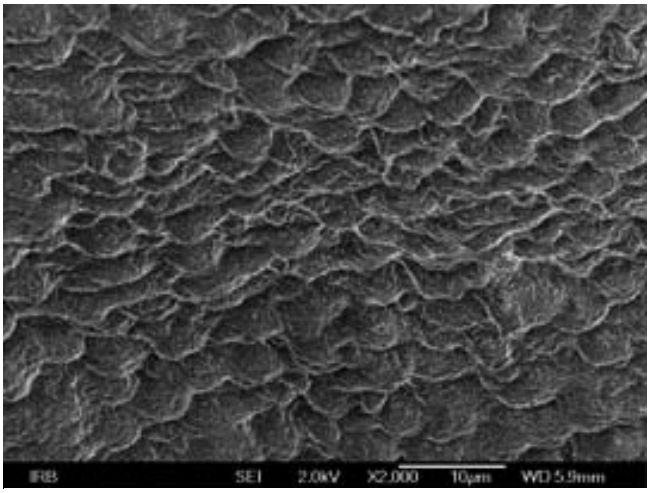

Figure 6. A SEM micrograph of the posterior wall of the maxillary sinus mucosa surface in ACP. Magnification 2,000X. Typical appearance of the rippled water surface.

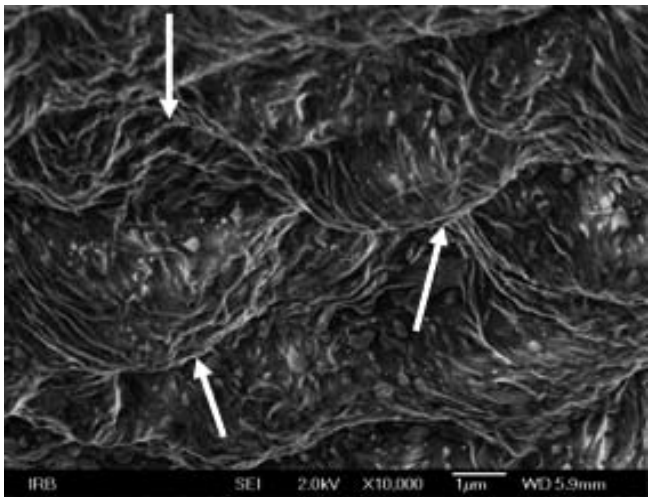

Figure 7. A SEM micrograph of the posterior wall of the maxillary sinus mucosa surface in ACP. Magnification 10,000X. Typical appearance of the rippled water surface now transferred produced by compound cilia, typical of chronic inflammation (white arrows).

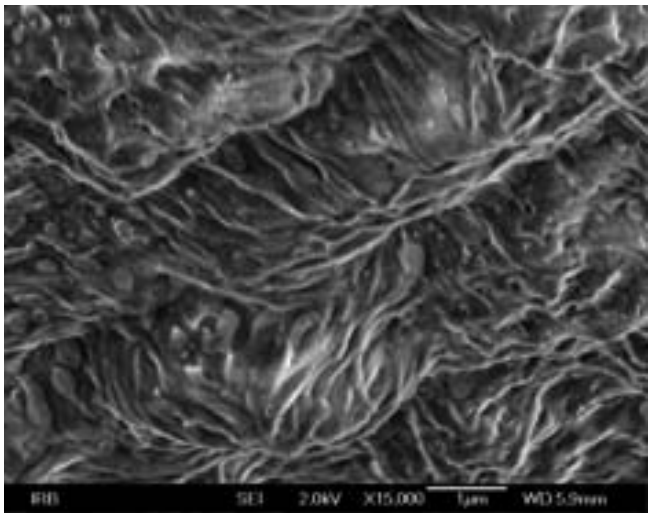

Figure 8. A SEM micrograph of the posterior wall of the maxillary sinus mucosa surface in ACP. Magnification 15,000X. A better view of the compound cilia.

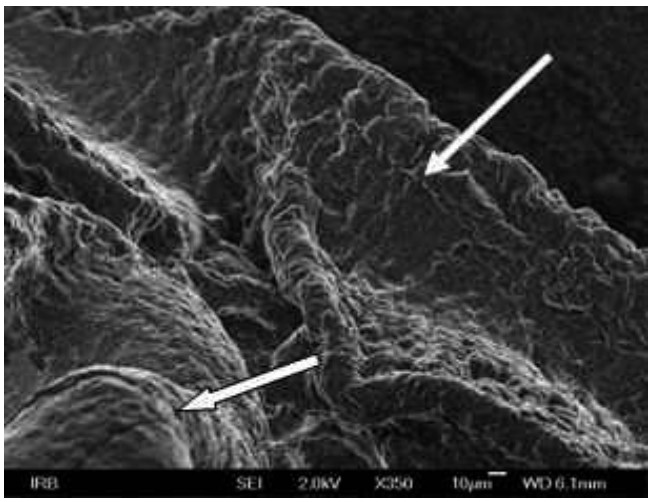

Figure 9. A SEM micrograph of the ACP stalk mucosal surface. Magnification 350X. Typical appearance of the biofilm on the left 2/3 of the image. White arrows indicate the EPS "towers".

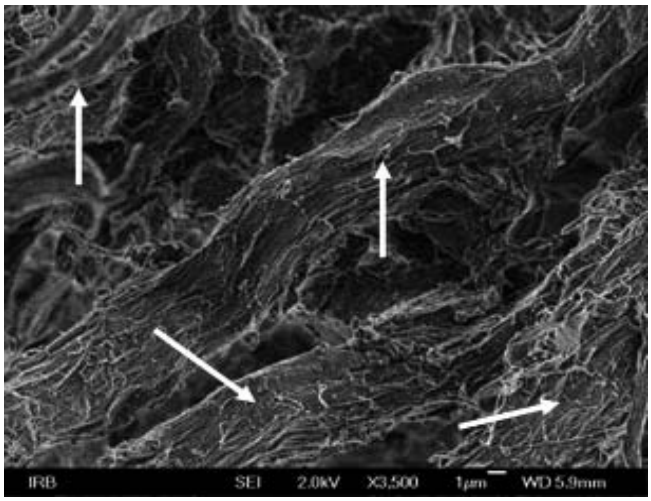

Figure 10. A higher magnification SEM micrograph of the ACP stalk mucosal surface from the part suspected of biofilm presence. Magnification 3,500X. There are relatively scarce signs of the biofilm presence (white arrows).

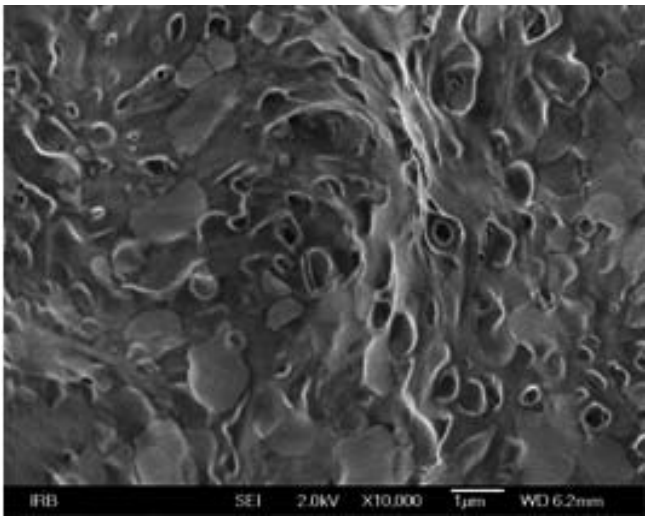

Figure 11. A SEM micrograph of the ACP stalk mucosal surface from the "microvilli" part. Magnification 10,000X. There are no typical signs of biofilm presence. The appearance of the mucosa resembles satellite photographs of the surface of the Moon or Mars, with many openings that could be either water channels (belonging to the biofilm community) or vascular elements (lymph and blood capillaries).

Surgery

All the patients were operated on by the same surgeon by means of FES surgery. During the surgery, tissue samples were taken for SEM analysis.

Sample preparation for microscopy

Samples for electron microscopic analysis were obtained and conserved in formaldehyde solution. In the first step, the samples were separated from the formaldehyde solution. 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; the following volume ratio twice distilled water to ethanol was used: 75/25, 50/50, 25/75. Then the samples were washed two times with absolute ethanol, supplied by Kemika. During the washing procedure with the mixtures of water and ethanol, the samples were slightly shaken and left for 30 min at each volume ratio. The samples could stay in absolute ethanol for up to 24 h after the last washing with no effect on their integrity. Finally, the samples were separated from the ethanol, put in a Petri dish, covered with porous cellulose paper and dried for 24 h. The dried samples were sharp cut, and the specimen was placed on carbon substrate for inspection with a thermal field emission electron microscope.

Electron microscopy

A thermal field emission electron microscope (FE SEM, model JSM-7000F, manufactured by JEOL Ltd.) was used. The specimens were not coated with an electrically conductive metal coating. The electron accelerating voltage was kept as low as possible so as to prevent eventual charging/discharging effects during FE-SEM inspection of the specimens.

The two experts who performed SEM analysis of our samples were absolutely blinded since the samples were sent under a code. Both of them have remarkable international reputation in SEM analysis of various materials.

RESULTS

SEM analysis showed the presence of biofilm in all samples taken from the DNP patients (Figures 1-4).

The SEM profile in the ACP patients varied according to the site from which we took the sample: mucosa of the posterior wall of the maxillary sinus, the stalk (pedunculum) or the polyp itself. The SEM micrographs of the mucosa of the posterior wall of the maxillary sinus showed typical signs of chronic inflammation of the respiratory mucosa with numerous nests of compound cilia (Figures 5-7); however, no signs of biofilm formations were observed in any of the three ACP patients. On the other hand, the SEM micrographs of the other two parts of the antrochoanal polyp, i.e. the stalk (pendunculum) and the polyp itself, showed a completely different appearance, a graphically autonomous image (Figures 8-11), in all our ACP patients. The SEM appearance of both these parts was the same as in the polyps in the DNP patients: a hard blanket of the biofilm covering the infrastructure (cilia and microvillae).

DISCUSSION

Since 2004, it has become obvious that we have to take into consideration the presence of biofilms in patients suffering from chronic sinusitis [10-15]. It is also well known that the vast majority of DNP patients suffer from chronic rhinosinusitis as a background to the polyps [19]. Therefore, we can expect biofilms in nasal polyps. Indeed, our study showed the presence of biofilms on SEM micrographs in each of the seven DNP patients, which would, according to the literature, indicate the presence of chronic inflammation in this disease.

There is also good reason to expect biofilm in ACP patients, since the “internal part” of the antrochoanal polyp, i.e. the swollen mucosa of the posterior wall of the maxillary sinus, always shows undoubted signs of chronic inflammation at light microscopy. This suggests that ACP generally begins as an inflammatory process located at the posterior wall of the maxillary sinus, which grows until it reaches the posterior fontanel of the lateral nasal wall, when the process starts to break through the fontanel and finally comes out into nasal cavity, presenting as a solitary polyp. However, we did not find any evidence of biofilm in the mucosa of the posterior wall of the maxillary sinus. The reason for this remains unclarified.

In addition, it is well known that evidence of biofilm is very frequently found on materials which cannot be inflamed simply because they are not organic tissues (metallic pipes, filters in swimming pools, indwelling catheters and other plastic, inorganic medical devices applied or inserted in the human body, etc.) [20-24].

It seems that we are talking about two different kinds of biofilm, one that develops on organic materials, i.e. biological tissues, and another that develops on the surfaces of inorganic materials. It seems quite unclear whether or not the biofilm has anything to do with the onset of chronic inflammation, or it just serves as a maintainer of an already existing infection and is thus rather the consequence than the cause of the disease in cases of DNP biofilm.

In addition, the absence of biofilm on the surface of swollen mucosa of the maxillary sinus in ACP patients also strongly suggests the same idea.

At the moment, it is evident that biofilm can be found in cases of diffuse nasal polyposis and on the stalk and polyp itself in cases of ACP, but not on the diseased mucosa of the posterior wall of the maxillary sinus. There are no obvious differences in the SEM appearance of biofilms in DNP and ACP.

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