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Biofilms in chronic rhinosinusitis with polyps: is eradication possible?

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Abstract The objective of the study was to reveal if mometasone furoate nasal spray as monotherapy or combined with long-term oral clarithromycin have influence on biofilms in chronic rhinosinusitis with polyps. The study is a randomized controlled trial in a tertiary referral hospital. Thirty-four patients with chronic rhinosinusitis completed the study. In the first group, 19 patients received mometasone furoate nasal spray 200 μg once daily for 8 weeks. In the second group, 15 patients received oral clarithromycin 500 mg twice daily for 2 weeks and continued once daily 250 mg tablet for subsequent 6 weeks, plus mometasone furoate. Scanning electron microscopy was the primary outcome measure. Secondary outcome measures included computerized tomography and sinonasal outcome test-20 items. Mucosal biofilms were detected in 23 of 34 (68 %) patients on pretreatment polyp samples. After the treatment, biofilms disappeared in 1 of 11 patients in the first group, whereas the eradication of biofilms was evident in 6 of 12 (50 %) patients in the second group. Tomography scores improved in eight patients of each group (42.1 and 53.4 %, respectively). The comparison of improvements did not reveal significant difference between the groups. The overall symptom scores improved compared to the baseline levels. The mean changes of −8.8421 and −11.4000 in the first and second group, respectively, were not statistically different. Adding long-term low-dose oral macrolides to nasal steroids was effective in the eradication of biofilm. However, we were not able to demonstrate that combined therapy was superior in terms of the improvement in tomography and symptom scores.

Keywords Nasal polyps · Biofilm · Scanning electron microscopy · Clarithromycin · Nasal steroid

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Introduction

Chronic rhinosinusitis (CRS) has been considered as a spectrum of disease entities which brings significant health and socioeconomic burden to large populations of people over the world. The disease represents a persistent inflammation of sinonasal mucosa that is believed to be the endpoint of different pathophysiologic pathways. Since infectious elements have been frequently observed in CRS, several trials attempted to find out the role of infection as either a trigger of inflammation or assistance in its persistence [1].

Since the relationship between CRS and biofilms was first described in 2004, several animal and human based studies addressing the issue of CRS have been published in which the biofilm was considered as playing a role in the recalcitrant nature of disease [2–4].

Anti-inflammatory therapies, favorably topical nasal corticosteroids have been recommended as a first-line of treatment for CRS patients according to recent consensus

and task force groups [5]. Long-term low-dose macrolide antibiotics have received an increasing interest in terms of their anti-inflammatory properties in recent years. In many studies questioning the efficacy of long-term macrolides in CRS patients, authors concluded that macrolides had some influence on polyp size and patient symptoms [6, 7]. Additionally, previous data proposed that macrolides below minimum inhibitory concentration with continued dosing affect the biofilm formation in many steps [8].

The objective of this study was to compare the efficacy of topical nasal steroids either as monotherapy or combined with long-term oral clarithromycin in eradicating the biofilm of nasal polyp samples. Therefore, scanning electron microscopic (SEM) examination was the primary outcome measure in this study. Secondary outcomes included paranasal computerized tomography (CT) and sinonasal outcome test 20 items (SNOT-20) scores.

Methods

Forty-four CRS patients with nasal polyps (CRSwNP) were enrolled in a single center-parallel group randomized study. The study was approved by the local ethical committee of Dışkapı Yıldırım Beyazıt Training and Research Hospital and written informed consent was obtained for all participants before enrollment in the study.

Diagnosis made on the basis of information obtained by patient history, nasal endoscopy and paranasal sinus CT according to the criteria of European Position Paper on Rhinosinusitis and Nasal Polyposis 2007 [5].

All subjects underwent nasal endoscopic examination and those with nasal polyps extending beyond the middle meatus were included in the study. Patients with marked septal deviation that prevent appropriate application and distribution of topical nasal corticosteroid sprays were excluded.

Subjects either on or have received antibiotic and/or corticosteroid treatment within the past 1 month were excluded.

Subjects were also excluded if they had pregnancy, allergy to the study medications, history of cystic fibrosis, congenital or acquired immune deficiency, primary ciliary dyskinesia, non-invasive fungus ball or invasive fungal rhinosinusitis, neoplasia, systemic vasculitis, granulomatous disease and impairment of liver or renal function.

The patients were randomized into two groups who received mometasone furoat nasal spray (MFNS) in the 1st group and clarithromycin tablet plus MFNS in the 2nd group. Following the randomization, nasal polyp samples were obtained for SEM investigation of biofilm structure from each participant under topical and infiltration anesthesia.

Samples, approximately 4 × 4 mm in size each were taken by otologic punch forceps without any surface injury to avoid biofilm disruption. In the first group, MFNS was administered for 8 weeks in a single dose 200 μg/day schedule. In the second group, in addition to the identical MFNS administration, patients also received clarithromycin tablet 500 mg/bid for 2 weeks and then 250 mg/day for the following 6 weeks.

At the end of the medical treatment period, patients underwent endoscopic sinus surgery (ESS). At the initial step of surgery, polyp samples obtained from each patient once again to assess the end-point biofilm presence on SEM. ESS was then performed as in the usual manner.

SEM analysis

The fresh specimens were immediately fixed in 2.5 % glutaraldehyde for 24 h, washed in phosphate buffer (pH 7.4), postfixed in 1 % osmium tetroxide in phosphate buffer (pH 7.4), and dehydrated in increasing concentrations of alcohol. After dehydration, the specimens underwent drying to a critical point and were mounted on metal stubs with double-sided adhesive tape. Then, the samples were sputtered with 150-Å thick layer of gold in a BIO-RAD (Hercules, CA) sputter apparatus. The images were taken by JEOL SEM ASID-10 (Tokyo, Japan) and LEO 4.3 HVP SEM (Oberkochen, Germany) electron microscope.

We obtained SEM images within a voltage range of 5–80 kV and within a magnification range of 50×–5,000×. We identified the biofilms existence using SEM morphological findings as described in the literature, such as 3-dimensional structure, variable size of microorganisms embedded in polysaccharide matrix, and multilayered remnants of tissue and microorganism (Fig. 1).

Main outcome measures

As primary outcome, pretreatment and end-point polyp tissue samples were investigated by using SEM for biofilm presence. Specimens were mainly graded according to biofilm presence as positive or negative samples without taking cilial destruction into account (Figs. 1 and 2).

Treatment outcomes were also evaluated by using paranasal CT examination that was performed at the start and end-point of the intervention. We used a scoring system which was published by Dursun et al. previously [9].

The scoring system consisted of four main stages depending on the degree of sinus opacifications observed in coronal scans; stage 0, no opacification in any of the sinuses; stage 1, bilateral opacification in only the ostiomeatal region or opacification in the ostiomeatal region and an adjacent sinus or opacification in only one sinus without any opacification in the ostiomeatal region; stage 2, unilateral or bilateral opacification in the ostiomeatal region and in more than one adjacent sinus or opacification

in more than one adjacent sinus without any opacification in the ostiomeatal region; stage 3, unilateral or bilateral opacification in all sinuses.

As the third outcome all of the patients were asked to score their sinonasal symptoms and related emotional and social consequences with SNOT-20 questionnaire. Each question was graded on a 5-point scale of increasing severity from "no problem" to "problem as bad as it can be". The questionnaire was applied before and at the end of the treatment period.

Since we did not administer a placebo identical to clarithromycin tablet, patients were aware of the treatment arms. Instead, we kept investigators and assessors blind to treatment allocation. Patients were enrolled in the study and were randomized to the treatment arms by B.O. who was blinded to the outcome assessments. Premedical treatment and operative samples were examined for biofilms, blindly to both treatment arms and treatment status of the samples by two authors (I.T. and H.H.C.). Initial polyp biopsies and final endoscopic sinus surgeries were performed and SNOT-20 questionnaires were conducted by E.C.T. and CT scoring of patients were generated by G.S., who were all blinded to both treatment arms.

Statistical analysis

Descriptive statistical analysis was conducted by using the mean ± standard deviation, median, 95 % confidence interval and percentage. McNemar–Bowker test, marginal homogeneity test and Wilcoxon signed ranks test were applied for the assessment of pre and post-treatment differences and Chi-square test (likelihood ratio, Fisher's exact test), Mann–Whitney *U* test, independent samples *t* test were performed to identify the differences between the study groups. Correlations between the parameters of pre and post-treatment differences were analyzed by Spearman's rho coefficient. Statistical significance was defined as $p < 0.05$. Statistical analysis was performed by using SPSS 10.0 (SPSS Inc, IBM company, Chicago, IL).

Results

The enrollment and study intervention took place over 2 years between April 2007 and May 2009. We asked about 67 CRS patients who met the eligibility criteria to participate in the study. Of these, 44 agreed to participate. We used a block randomization for allocation of patients to each treatment arm. Four was the selected block size that provided six different ways to allocate participants equally.

Initially, 44 patients were equally randomized into the treatment arms. Of these, two from MFNS group (group I), three from MFNS plus clarithromycin group (group II) were lost to follow-up. One patient from the first group withdrew because of not being able to follow the dose schedule. Four patients from the second group withdrew because of two declined subsequent final biopsy and surgery due to symptomatic improvement after the treatment, one had new developed pregnancy and one had mild diarrhea. Thus, 34 patients (19 in group I, 15 in group II) completed the study and their data underwent statistical assessments. Figure 3 shows the patient flow diagram.

No statistically significant difference was found between the groups in terms of age, gender, prior sinus surgery and in the baseline data of biofilm presence, CT and SNOT-20 scores (Table 1).

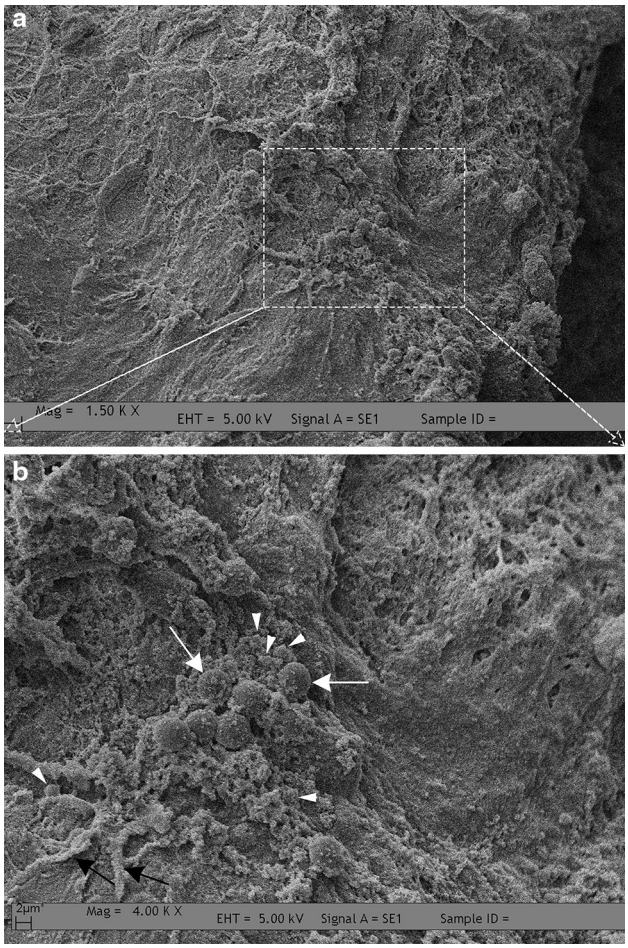

Fig. 1 SEM micrographs show **a** biofilm matrix which covers slightly more than the right half of the image and almost completely destroyed cilia. **b** At ×4,000 magnification, the *white arrows* indicates white and red blood cells (8–10 µm) and *white arrowheads points* to cocci bacteria (0.5–1 µm) embedded in biofilm matrix either next to white blood cells or remnants of destructed epithelium. *Black arrows* indicate pseudohyphae like appearance of biofilm matrix which disperses in irregular radial direction

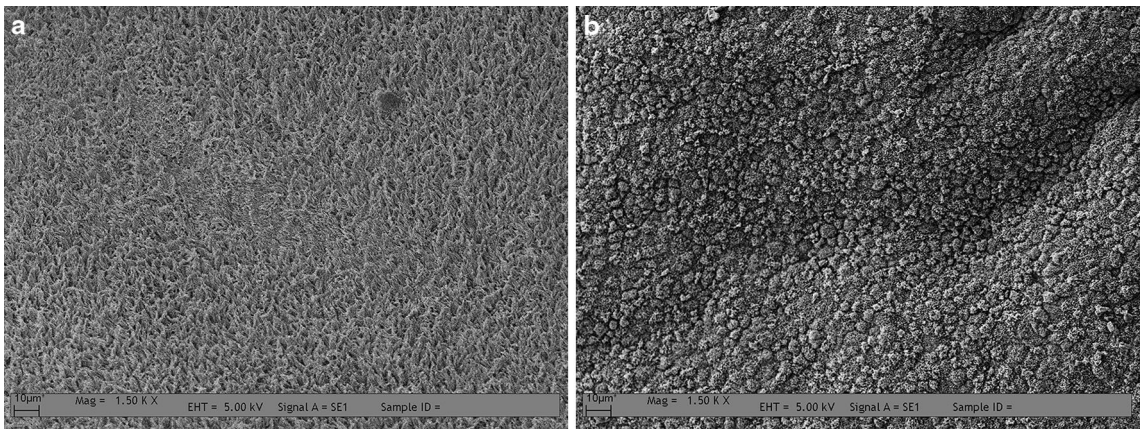

Fig. 2 SEM micrographs of nasal polyp surface without biofilms (**a**). A healthy ciliated respiratory epithelium (**b**). Decreased cilia structures and partially denuded epithelium

Fig. 3 Patient flow diagram

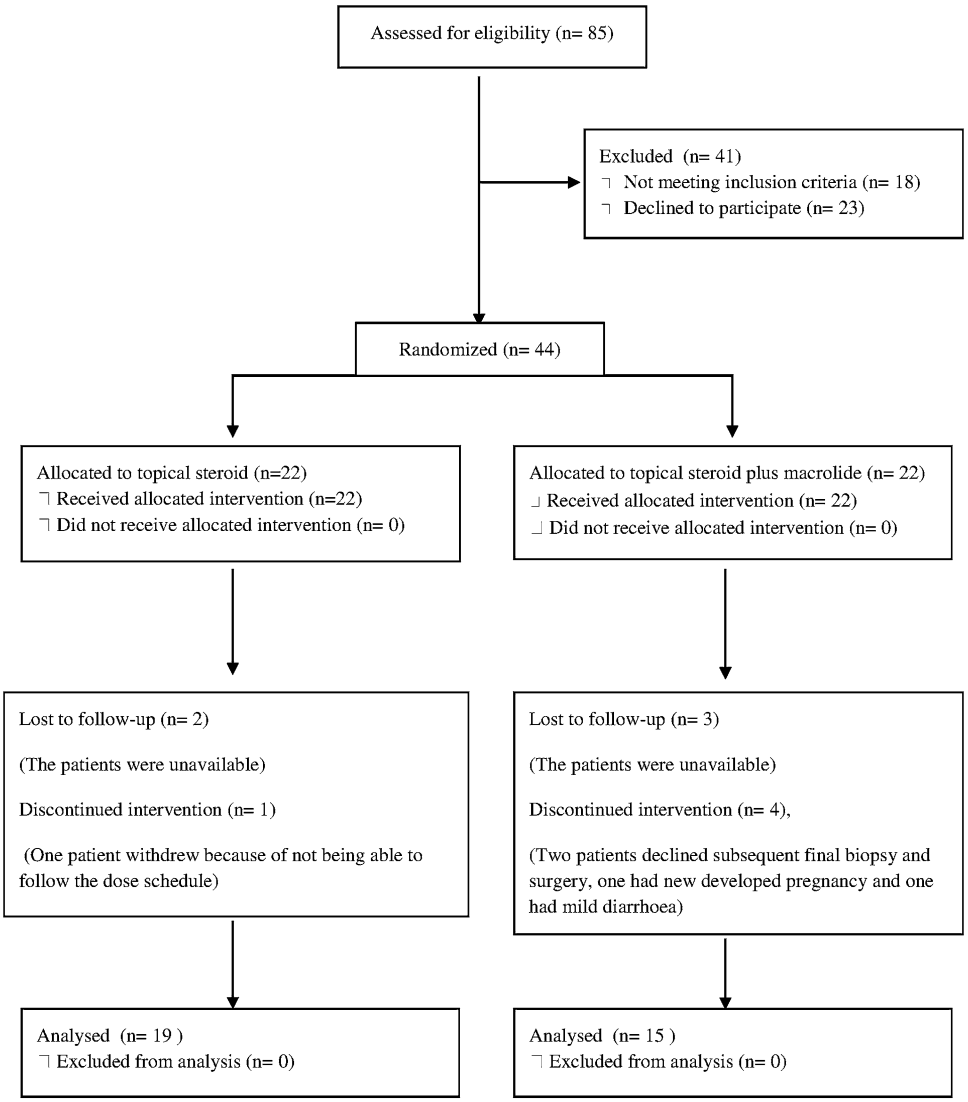

Table 1 Patient demographic characteristics and baseline data

Variable	Group I: topical steroid	Group II: topical steroid + macrolide	*p* value
Age, mean [SD] years	44.0 [±15.8]	42.8 [±9.5]	0.795
Male, no. (%)	16 (84.2)	10 (66.6)	0.417
Revision surgery, no. (%)	7 (36.8)	7 (46.7)	0.820
Patients have biofilm, no. (%)	11 (57.9)	12 (80.0)	0.271
CT grade, median (min–max)	3 (1–3)	3 (2–3)	0.973
SNOT-20 scores, mean [SD]	26.6 [±11.5]	32.6 [±12.3]	0.145
CT grade of patients with biofilm, no. for each grade	1/2/8	0/4/8	0.364

CT computerized tomography, *SNOT-20* sinonasal outcome test 20 item

Biofilm prevalence and regression after the therapy

Overall biofilm prevalence was found to be 68 % (23/34) in the initial baseline SEM examination. Pretreatment biofilm negative samples were not significantly different between the groups: 8 of 19 samples in group 1, 3 of 15 samples in group 2 (Fisher's exact test, $p = 0.271$). In group I, subsequent to 8 weeks MFNS administration, biofilm disappeared in only one patient. In 10 patients, biofilm structures did not regress when compared to pretreatment status. This improvement (in 1 of 11 samples) did not reach statistical significance (McNemar test, $p = 1.00$). In group II, after MFNS plus clarithromycin tablet administration, 6 of 12 biofilm-positive samples turned into negative. The other six positive samples remained the same. The improvement (in 6 of 12 samples) reached statistical significance (McNemar test, $p < 0.05$). The biofilm-improved six samples were obtained from three patients who had no previous sinus surgery and from three patients who underwent revision surgeries (Table 2).

As seen above results, a significant improvement was achieved in terms of biofilm regression by using combined therapy compared with MFNS alone.

CT score changes after the treatment

All subjects had abnormal CT scan (CT stage ≥1) ranged from 1 to 3 at baseline.

In the 8-week end-point setting, the improvement in CT scores was significant for both groups.

In group I, posttreatment CT scores improved in 8 of 19 (42.1 %) patients. In this group, CT scores of 10 patients remained the same, whereas we observed a worsening in one of them after the therapy. The improvement in 42.1 % of patients reached statistical significance (marginal homogeneity test, $p < 0.05$) (95 % CI, −0.65 to −0.08) (Table 3).

In group II, posttreatment CT scores improved in 8 of 15 (53.4 %) patients. None of the patients had worsening and posttreatment scores of seven did not change compared with the baseline levels. The improvement in 53.4 % of patients was statistically significant (marginal homogeneity test, $p < 0.05$) (95 % CI −0.95 to −0.025).

The difference of CT improvement scores between the treatment groups was statistically insignificant ($p > 0.05$) (Table 3).

When we focused on the posttreatment biofilm status of the patients in whom CT scans improved to the lower stages after the treatment, in group II we noticed that biofilm structures cleared away in six patients' samples (in 6 of 8, 75 %). However, in group I, biofilm status did not change among the patients in whom posttreatment CT scores improved (0/8, 0 %). The difference between the groups regarding the positive correlation of the presence of biofilm and CT scores was statistically significant (Chi-square test, $p < 0.05$).

Changes in SNOT-20 scores

Overall mean SNOT-20 scores improved in all patients in both groups after the treatment (Wilcoxon signed ranks test, $p < 0.05$). The mean change of total SNOT-20 scores from

Table 2 Changes in biofilm prevalence with the therapy

Biofilm-positive cases	Group I		Group II	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
No. (%)	11 (57.9)	10 (52.6)	12 (80.0)	6 (40.0)
95 % CI	36.3–78.9	31.7–72.7	54.8–92.9	19.8–64.3
p value	>0.05 (within group I)		0.031 (within group II)	

CI confidence interval

Table 3 Changes in CT scores with the therapy

CT score	Group I			Group II		
	Pretreatment	Posttreatment	Difference	Pretreatment	Posttreatment	Difference
Mean [SD]	2.58 [±0.61]	2.21 [±0.71]	−0.36 [± 0.60]	2.60 [±0.51]	2.00 [±0.76]	−0.6 [0.63]
95 % CI	2.29–2.87	1.87–2.55	−0.65 to −0.08	2.32–2.88	1.58–2.42	−0.95 to −0.025
Median (min–max)	3 [1–3]	2 [1–3]	0 [−1 to 1]	3 [2, 3]	2 [1–3]	−1 [−2 to 0]
p (within)	0.02 (within group I)			0.007 (within group II)		
p (between)	0.760 (between two groups)					

CT computerized tomography, *CI* confidence interval

Table 4 Changes in SNOT-20 scores with the therapy

SNOT-20 score	Group I			Group II		
	Pretreatment	Posttreatment	Difference	Pretreatment	Posttreatment	Difference
Mean [SD]	26.5 [±11.5]	17.6 [±10.0]	8.8 [±6.6]	32.6 [±12.3]	21.2 [±9.7]	11.4 [±4.4]
Median (min–max)	23 (8–48)	13 (4–34)	8 (1–29)	30 (17–55)	18 (11–43)	12 (5–21)
95 % CI	20.9–32.0	12.8–22.5	5.7–12.0	25.8–39.4	15.9–26.6	9.0–13.8
p (within)	<0.001 (within group I)			0.001 (within group II)		
p (between)	0.056 (between two groups)					

SNOT-20 sinonasal outcome test 20 item, *CI* confidence interval

baseline in the patients treated with MFNS alone (group I) was −8.8421 (SD ± 6.602) (95 % CI −5.7 to −12.0). In group II, the mean change was determined as −11.4000 (SD ± 4.421) (95 % CI −9.0 to −13.8) at the end-point assessment. Although, the difference between the groups regarding the improvement in SNOT-20 scores did not reach statistical significance (Mann–Whitney *U* test, $p = 0.056$), there seemed to be a trend toward significance (Table 4).

Discussion

The prevalence of biofilm has been reported in a wide range of spectrum which basically depended on the methods used for biofilm detection, number of samples, and varying definitions of CRS [2–4, 10, 11].

We investigated the prevalence of biofilm by using SEM in patients with the diagnosis of CRSwNP. The presence of mucosal biofilm was demonstrated in 23 of 34 (65 %) patients, which was consistent with a large number of previous reports [12, 13]. However, this finding is not able to answer whether the biofilm initiates sinonasal inflammation or contributes to its persistence.

It is widely believed that CRS is a heterogenous condition, which may be encountered in various clinical presentations. The current guidelines have suggested classifying chronic sinus diseases simply based on polyp status. The aim of this classification is to reveal likely different underlying pathophysiologic pathways of these subtypes, which may lead to development of more precise and focused treatment for CRS [5].

To our knowledge, this study is the first to evaluate the efficacy of MFNS, either as monotherapy or in combination with long-term oral clarithromycin, regarding the eradication of biofilm and the improvement in CT and SNOT-20 scores in CRSwNP patients. We noted that adding oral macrolides to MFNS was associated with a statistically significant eradication in biofilm formation, when compared to administering MFNS alone. In our recent report, we questioned the efficacy of long-term oral clarithromycin against the biofilm structure in CRS without NP, either by using alone or combined with MFNS. Although, both modalities resulted in significant improvement in the grading scale of biofilm, adding nasal steroids to macrolides did not give any further benefit. From this point of view, when we consider the results of both studies, long-term oral clarithromycin seems to have an effect on the eradication of biofilm [13].

In the light of current literature, we are already aware of the fact that topical nasal steroids are effective in clinical settings of CRSwNP. In contrast to topical nasal steroids, for which the literature contains plentiful data stemming from numerous randomized controlled trials, the clinical efficacy of macrolides in CRS with nasal polyps has been subjected to investigation mostly in non-placebo controlled cohorts [8, 14]. These reports suggested some clinical benefits of oral macrolides by means of using various outcome measures regardless of strong comparative data.

In our study, despite the promising effect at eradication of the biofilm by way of adding long-term oral clarithromycin to MFNS, it was not able to provide similar superior effect in terms of the improvement in clinical outcome measures over MFNS alone.

The major weak point of this current study was the number of patients. We were able to obtain such a group of CRSwNP patients within 2 years of study period. Despite this limited number of sample size of 19 and 15 for group 1 and group 2, respectively, our study enabled 78 % statistical power to detect a difference of 34.7 % between the groups in terms of eradicating the biofilm.

The other limitation inherent in SEM was that some artifacts like mucus may be interpreted as biofilms as a result of protein cross-linking and dehydration. This possible confusion between the biofilm structure and mucus may raise some questions as to interpretation of treatment responses and can be overcome by using the other confirmatory modalities with florescence in situ hybridization (FISH) probes, However, it must be emphasized that each modality has its own advantages and disadvantages. SEM is a validated method for identifying the 3-dimensional structure of biofilm which has been commonly used and has yielded consistent results in previous studies [12, 13, 15].

The lack of patient blinding to treatment allocation was another limitation of study that may be associated with the performance bias. Instead, we intended to ensure blind assessment of results so as to deal with subjectivity in assessment.

Our results demonstrated that adding clarithromycin to MFNS improved biofilms when compared to MFNS alone in CRSwNP. This effect can be attributed to either antibacterial and/or anti-inflammatory effects of low-dose long-term clarithromycin, or it may be due to dual effects. However, the improvement of clinical parameters was beyond that of biofilms. With these results, we cannot speculate on the relation of biofilms and nasal polyp etiology. Furthermore, it is not possible to recommend that treating biofilms, would cure nasal polyps. It is a possibility that biofilms are secondary to nasal polyposis, rather than being the etiology. Despite this argument in the focus of our study, we believe that our results are scientifically valuable. Further studies of biofilms can be designed in CRSwNP, by using clarithromycin alone in longer periods with placebo controls. Longer follow-up studies would also reveal the recurrence of biofilms in CRSwNP patients treated with clarithromycin.

Conclusion

The current study demonstrated that biofilms existed in 68 % of CRSwNP patients. Although, MFNS alone seemed to be enable to disrupt the biofilm, adding long-term oral clarithromycin achieved further regressions in biofilm formations. However, these superior results were not reflected on the secondary outcome measures. In this sense, despite our promising results regarding the eradication of biofilm in nasal polyps, whether it is clinically relevant remains unanswered. More research is needed so as to answer this topic and confirm the effectiveness of clarithromycin on mucosal biofilms.

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Conflict of interest All authors state that they have no conflicts of interest.

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