Impact of mucosal biofilm and bony osteitis on chronic rhinosinusitis with nasal polyps

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Abstract

Background: Chronic rhinosinusitis (CRS) is a common inflammatory disorder whose underlying etiopathogenesis has not yet been completely understood and appears to be multifactorial. Microbial biofilms and bony osteitis are gaining an increased concern as they are considered to be among the possible factors that contribute to the overall local inflammatory load in chronic rhinosinusitis (CRS). This study investigated the impact of mucosal biofilm and bony osteitis on the pathophysiology and severity of chronic rhinosinusitis with nasal polyps (CRSwNP).

Results: Forty-five CRSwNP patients performing functional endoscopic sinus surgery (FESS) and 10 control patients were involved in this cross-sectional study. Mucosal and bony specimens from ethmoid sinus were obtained for both light and scanning electron (SEM) microscopic examination. The histopathologic bony grade was positive in 40/45 of CRSwNP patients versus 6/10 of the control patients ($P = 0.300$); histopathologic mucosal grade was 44/45 versus 4/10 ($P < 0.001$), and tissue eosinophilia was 45/45 versus 6/10 ($P < 0.001$); biofilm was positive in 37/45 versus 4/10 ($P = 0.012$). The mean of the sinonasal outcome treatment score (SNOT)-22 is 39.8 versus 50.5 ($P = 0.067$); Lund-Mackay score (LMS) is 19.6 versus 3.1 ($P < 0.0001$).

Conclusion: (1) Mucosal biofilms and osteitis were detected in patients undergoing FESS for CRSwNP and also in controls without CRS. This suggests that mucosal biofilms and osteitis may not alone be the etiology of CRS without other cofactors. The pathogenesis of biofilms could be related to host factors. (2) The high odds ratio and wide confidence interval in our study suggest that there is a statistically significant association between biofilm formation and CRSwNP. (3) The high grade of mucosal inflammation and tissue eosinophilia suggests the inflammatory load added by osteitis and bacterial biofilm (BBF).

Keywords: Chronic rhinosinusitis, Chronic rhinosinusitis with nasal polyps, Biofilm, Osteitis

Background

Mucosal biofilms and bony osteitis are gaining an increased concern as they are considered to be among the possible factors that contribute to the total local inflammatory load [1] in CRS. Bacterial biofilms (BBF) are highly organized 3-dimensional bodies constituted of groups of bacteria encased inside a protective extracellular polysaccharide matrix, which are recalcitrant to both antibiotic therapy and host immune mechanisms [2]. With the use of a variety of techniques, BBF have been consistently showed on the mucosa of patients with CRS among an ever-expanding research body, with a prevalence of 30 to 80% [3–5], and their existence has been correlated with worse parameters of the disease, post-surgical results, a greater prevalence of infection recurrence, and an elevated sinonasal outcome test (SNOT)-22 score [4–8]. Their existence has also been associated with increased disease severity that is in mostly refractory to present treatment paradigms [4].
Osteitis is the bone inflammation that is devoid of marrow space [9]. In CRS, it is still uncertain whether osteitis is triggered via direct bacterial invasion, which is still not elucidated in researches or possibly is secondary to mediators of inflammation [9, 10]. The prevalence of osteitis is between 36 and 79% in patients with CRS depending on radiographic criteria, findings of single-photon emission computerized tomography, or pathologic results [11–13]. The degree of bony osteitis was correlated with disease severity and results after medical and surgical management [13, 14]. Moreover, the existence of bony osteitis is correlated with a decreased potential for a better change in certain quality-of-life outcome values [9]. The aim of this study was to assess the impact of mucosal biofilm and bony osteitis on the pathophysiology and severity of CRSwNP.

Methods

Study design and population
This is a cross-sectional study that was done in the Otorhinolaryngology Department between 1/2018 and 5/2019 which was formally approved by the Ethics Committee (registration-number: FMASU MD 466/2017). Informed consent was obtained from all the participants.

The study involved 2 groups; the first group was the CRSwNP group which included 45 patients who were diagnosed according to the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines [15], had failed optimal medical treatment, and underwent functional endoscopic sinus surgery (FESS). The second group included 10 control subjects with negligible or no subjective or objective evidence of CRS nor nasal polyps scheduled for nasal surgery (septoplasty and/or inferior turinate resection for nasal obstruction) were recruited as well. Exclusion criteria were patients with acute rhinosinusitis, fungal sinusitis, chronic rhinosinusitis without nasal polyps (CRSsNP), Wegener’s granulomatosis, and other granulomatous diseases, cystic fibrosis, Kartagener syndrome, and other genetic and immune disorders and who underwent previous endoscopic sinus surgery.

Collection of clinical data
Preoperative collection of data included demographic features, Lund-Mackay computed tomography (CT) score, Rasp endoscopic grade, SNOT-22 score, aspirin sensitivity, systemic (granulomatous) diseases, previous FESS, smoking habit, allergic rhinitis, and bronchial asthma.

Tissue preparation for osteitis and mucosal assay
A tissue biopsy was taken from the ethmoidal bone with the overlying mucosa and polypoidal tissue during FESS, which were fixed into formalin (10%) for 2 h followed by paraffin inclusion. Paraffin sections were cut at 4 microns thick and subjected to routine hematoxylin and eosin staining. Microscopic review was performed using a binocular light microscope. The microscopic slides were examined to assess the degree of mucosal and bony affection according to the criteria published by Biedlingmaier et al. [16] and to detect the number of present mucosal eosinophils. Eosinophils were calculated in the areas of richest cellular infiltrate to be sure that patients were categorized consistently based on the foci of highest inflammation. The eosinophil count was reported in each reticle field at 400× power and recorded as an absolute number per high power field. The degree of eosinophilia was classified according to Snidvongs et al. [17] as low eosinophilia (< 5 per high power field [HPF]), moderate eosinophilia (5 to 10 per HPF), and high eosinophilia (> 10 per HPF).

Biofilm assay
Biofilm was detected by means of SEM. Blocks of nasal samples were prepared for examination. Fixation and dehydration techniques were done using the tissue processor model (A-1170), where 6 to 8 mm² of nasal specimens was cut. The squares were then fixed by submerging in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) at 4 °C overnight. Fixed substance was allowed to acquire the temperature of the surrounding room and then washed in PBS (3 times, 10 min each) to take out the excess amounts of the fixative material. Substances dehydrated and were submerged via a graduated, 10% rising steps of ethanol, starting from 10 to 90%, and lastly absolute ethanol. Desiccated samples were dried at the critical point. Then, the dried samples were fixed to 0.9 mm diameter stubs of copper using an electron microscopy-specific, conductive, double-adhesive carbon tape. Samples were subsequently coated with gold (about 50 nm thickness) via a Sputter Coater and then viewed by the high-vacuum mode of a JEOL JSM-5500LV Scanning.

Structures characterized by 3-dimensional (3D) configuration and amorphous matrix that takes the shape of spherical or elliptical bodies were described as biofilms’ evidence.

Statistical analysis
Data were analyzed using IBM® SPSS® Statistics version 23 (IBM® Corp., Armonk, NY). Numerical variables were described as mean and standard deviation (SD), and inter-group differences were compared using the unpaired t test. Categorical variables were shown as number and percentage, and differences between groups were compared using Pearson chi-squared test or Fisher’s exact test. Ordinal data were compared using the chi-squared test for trend. Associations between nominal dichotomous were tested using the phi coefficient (φ). Point biserial correlation was used to evaluate the
correlation between continuous variables and dichotomous variables. Rank biserial correlation was used to evaluate the correlation between ordinal variables and dichotomous variables. The Spearman rank correlation was used to test the correlation between ordinal variables. The point biserial (r\textsubscript{pb}), rank biserial (r\textsubscript{pb}), or Spearman's (\rho, \rho) correlation coefficients are interpreted.

**Results**

As regards basic characteristics in CRSwNP patients and control group, there was only difference in the age distribution while there were no differences in the distribution of gender, allergy, asthma, aspirin sensitivity and smoking status, and SNOT-22 score between the two groups, but CRSwNP patients had statistically significant higher LMS (Tables 1, 2, and 3).

Regarding disease severity and symptom burden, there was a statistically significant difference between the 2 groups as regards the Lund-Mackay CT score (P < 0.0001), Rasp endoscopy score (P < 0.001), but unexpectedly the mean SNOT-22 score is higher in the control group (50.5) than the CRSwNP group (39.8) with no significant difference statistically (P = 0.067) (Tables 2 and 3).

**BBF, osteitis, and mucosa in CRSwNP patients and controls**

Under SEM, 37/45 (82.2%) were positive for biofilms in the CRSwNP group and 4 (40%) in the control group (P = 0.012) (Fig. 1). CRSwNP group has a higher rate of bony osteitis than that in the control group, based on histopathological findings 40/45 (88.8%) versus 6/10 (60%), but the difference is not statistically significant (\chi\textsuperscript{2}(1) = 1.074, P = 0.300). The CRSwNP patients had more histopathological bony, mucosal changes assessed by Biedlingmaier's grade [16], and tissue eosinophilia evaluated by Snidvongs [17] grade (Table 4) (Figs. 2 and 3). The bony osteitis of histopathological grade 4 (frank osteitis and bone destruction) and mucosal grade 4 (severe significant amounts of chronic inflammatory cellular infiltrate in the lamina propria) was not found in this study; there was statistically significant difference between the two groups regarding both the mucosal grading (P value < 0.001) and tissue eosinophilia (P value < 0.001).

**Correlation between BBF and histopathologic mucosal and bony grades**

The numbers of CRSwNP patients with different statuses of BBF, histopathologic osteitis, mucosal grading, and eosinophil count were shown in Table 4. There was no significant association between BBF and osteitis (Spearman rho = 0.205, P value = 0.176). There was a weak positive correlation between bone grading and mucosal grading (Spearman rho = 0.345, P value = 0.020). There was positive correlation between bone grading and tissue eosinophilia grading (Spearman rho = 0.294, P value = 0.049). There was also a very strong positive correlation between mucosal grading and tissue eosinophilia grading (Spearman rho = 0.294, P value = 0.049).

**Discussion**

This study reports the relationship between BBF and bony osteitis in patients with CRSwNP. In our study, histopathological features of bony osteitis were found in 88.8% of CRSwNP patients, 77.7% of the CRSwNP patients were positive for both bony osteitis and BBF, and only 6.6% of the CRSwNP patients were negative for both. Bony osteitis was also found in 60% of the control group which may explain the high SNOT-22 score in this group. Further association analysis showed no correlation between the presence of BBF and osteitis grading which may be attributed to the relatively large number of patients with bony osteitis in the control group or to the relatively small study population.

The idea of biofilm-induced CRS has been suggested by a number of researches [18–23] providing evidence that the expression of proteins on the cell surface, and some cytokines are up- or downregulated in local mucosa with BBF; however, the consensus is not reached. Regarding the osteitic bone and the overlying mucosa which BBF adheres to, it seems that both are stimulated by and act like a “depot” of inflammatory cytokines [10] which is reflected in our study by high mucosal grading and tissue eosinophilia.

Furthermore, the causal relationship between the BBF, osteitis, and local immune response is still undiscovered. Also, the definite mechanism of how bony osteitis
develops is yet not fully reached. Grossly, in areas where mucosal disease is persistent, the underlying bone is rendered poorly viable, which later on becomes thickened and develops neo-osteogenesis. This consequently results in increased scarring of the mucosa and potential for bony adhesions [14].

Further investigation is worth to detect the mechanism by which biofilm triggers an inflammatory response and the role of bony osteitis in the pathogenesis of CRS. Early studies supposed that CRS resistant patients’ could make use of more extensive surgical procedures such as Denker’s operation and the nasalization [1]. Since BBF and bony osteitis were potential etiologic factors in refractory CRS [24], it is thus intriguing to postulate that for patients with such inflammatory burden, a comprehensive management plan should be considered which includes a more radical surgery together with antibiofilm therapy to remove all triggers of inflammation that maintain a vicious cycle of local inflammation [1].

The positive rate of BBF in our study was in line with some of the other studies using SEM [25–27]; the high odds ratio and wide confidence interval in our study suggest that there is a statistically significant association between biofilm formation and CRSwNP.

In our study, biofilms were also found in 4 (40%) of the 10 patients in the control group; they were also detected on the nasal mucosae of normal controls in some studies [25–28], assuming that biofilms may be normal colonies of bacteria. Mladina et al. detected BBF on healthy sinus mucosa and suggested that BBF could be normal respiratory mucosal blankets that contain lots of bacteria and part of the mucociliary system [27].

It is sensible to presume that BBF may be present in nasal mucosae of all controls and CRSwNP patients as the common survival bacterial property.

Besides, the presented pathological features of bony osteitis in our study that included thickening of the periosteum, new formation of unorganized woven bone, and fibrosis were also similar to previous studies [23, 26, 29–31]. For additional correlation assessment, we used the histopathological bony grade of Biedlingmaier, which was proposed as the most beneficial system for histopathological grading by Videler et al. [24].

| Table 2 | SNOT-22 score and Lund-Mackay CT score in both study groups |
|---------|--------------------------------------------------|
| Variable | CRSwNP (n = 45) | Control (n = 10) | Difference | 95% CI | P value* |
| SNOT-22 score | Mean 39.8 | SD 17.5 | Mean 50.5 | SD 9.2 | 10.7 | −0.8 to 22.2 | 0.067 |
| Lund-Mackay CT score | Mean 19.6 | SD 5.7 | Mean 1.5 | SD 3.1 | −18.1 | −21.8 to −14.3 | < 0.0001 |

Data are mean and standard deviation (SD) 95% CI 95% confidence interval *Independent samples t test

| Table 3 | Endoscopic grading in both study groups |
|---------|------------------------------------------|
| Endoscopic grading | CRSwNP (n = 45) | Control (n = 10) | χ²(1) | P value* |
| Right side | n | % | n | % | χ²(1) | P value* |
| Grade 0 | 0 | 0.0 | 10 | 100.0 | 36.258 | < 0.001 |
| Grade I | 5 | 11.1 | 0 | 0.0 |
| Grade II | 14 | 31.1 | 0 | 0.0 |
| Grade III | 24 | 53.3 | 0 | 0.0 |
| Grade IV | 2 | 4.4 | 0 | 0.0 |
| Left side | Grade 0 | 1 | 2.2 | 10 | 100.0 | 32.083 | < 0.001 |
| Grade I | 5 | 11.1 | 0 | 0.0 |
| Grade II | 13 | 28.9 | 0 | 0.0 |
| Grade III | 22 | 48.9 | 0 | 0.0 |
| Grade IV | 4 | 8.9 | 0 | 0.0 |

Data are number (n) and percentage (%) χ² chi-squared statistic *Chi-squared test for trend
Our study displayed a positive correlation between bony osteitis and both mucosal grading (Spearman rho $= 0.345$, $P$ value $= 0.020$) and tissue eosinophilia (Spearman rho $= 0.294$, $P$ value $= 0.049$), also a positive correlation between mucosal grading and tissue eosinophilia (Spearman rho $\rho = 0.800$, $P$ value $< 0.001$), which reflects the importance of inflammatory load in the disease development and maintenance.

Moreover, the presence of a positive correlation between biofilm and both bronchial asthma (phi coefficient $\phi = 0.296$, $P$ value $= 0.047$) and allergy (phi coefficient $\phi = 0.394$, $P$ value $= 0.021$) may suggest either both are predisposing factors for biofilms development or the role of intrinsic factors in biofilm development.

Moreover, the presence of a positive correlation between biofilm and both bronchial asthma (phi coefficient $\phi = 0.296$, $P$ value $= 0.047$) and allergy (phi coefficient $\phi = 0.394$, $P$ value $= 0.021$) may suggest either both are predisposing factors for biofilms development or the role of intrinsic factors in biofilm development.

There was also a positive correlation between allergy and both the endoscopic grading (rank biserial correlation coefficient $= 0.392$, $P$ value $= 0.008$) and Lund-Mackay score (point biserial correlation coefficient $= 0.394$, $P$ value $= 0.007$) which suggests the contribution of allergy in the disease severity.

There were also technical limitations in our study. First, the immunological characteristics of the mucosa that lies between mucosal biofilm and osteitic bone were not studied. Second, Foreman et al. [32] reported other methods as the noninvasive immunofluorescent demonstration of BBF, which will allow for both, leaving intact mucosa for additional study and also identifying the microbial species in BBF.

**Conclusion**

1. Mucosal biofilms and osteitis were detected in patients undergoing FESS for CRSwNP and also in controls without CRS. This suggests that BBF and osteitis may not alone be the etiology of CRS without other cofactors. The pathogenesis of biofilms could be related to host factors. (2) The high odds ratio and wide confidence
Table 4 Results of histopathological examination in both study groups

| Variable       | Group | CRSwNP (n = 45) | Control (n = 10) | $\chi^2$ (1) | P value* |
|----------------|-------|-----------------|------------------|--------------|----------|
|                |       | n (%)           | n (%)            |              |          |
| Bone grading   | Grade 0 | 5 (11.1)       | 4 (40.0)         | 1.074        | 0.300    |
|                | Grade I | 16 (35.6)      | 2 (20.0)         |              |          |
|                | Grade II | 19 (42.2)     | 2 (20.0)         |              |          |
|                | Grade III | 5 (11.1)    | 2 (20.0)         |              |          |
| Mucosal grading| Grade 0 | 1 (2.2)        | 6 (60.0)         | 19.636       | < 0.001  |
|                | Grade I | 8 (17.8)       | 2 (20.0)         |              |          |
|                | Grade II | 28 (62.2)    | 2 (20.0)         |              |          |
|                | Grade III | 8 (17.8)    | 0 (0.0)          |              |          |
| Tissue eosinophils | Nil | 0 (0.0)       | 4 (40.0)         | 25.834       | < 0.001  |
|                | Low | 3 (6.7)        | 4 (40.0)         |              |          |
|                | Moderate | 6 (13.3)    | 0 (0.0)          |              |          |
|                | High | 36 (80.0)      | 2 (20.0)         |              |          |
| Biofilm        | Negative | 8 (17.8)    | 6 (60.0)         | –            | 0.012#   |
|                | Positive | 37 (82.2)    | 4 (40.0)         |              |          |

Data are number (n) and percentage (%)

$\chi^2$ chi-squared statistic

*Chi-squared test for trend unless otherwise indicated

#Fisher’s exact test

Fig. 2 a Bone grade I with periosteal thickening (arrow). b Bone grade II with periosteal thickening (star) and osteoblastic activity (arrow). c Bone grade II with osteoblastic and osteoclastic activity (arrow). d Bone grade III with irregular wide bony trabeculae (arrow)
interval in our study suggest that there is a statistically significant association between biofilm formation and CRSwNP. (3) The high grade of mucosal inflammation and tissue eosinophilia suggests the inflammatory load added by osteitis and BBF.

Abbreviations
CRS: Chronic rhinosinusitis; CRSwNP: Chronic rhinosinusitis with nasal polyps; CRSsNP: Chronic rhinosinusitis without nasal polyps; BBF: Bacterial biofilm; CT: Computed Tomography; SNOT-22: Sinonasal outcome treatment score; HPF: High power field; SD: Standard deviation; SE: Standard error; SEM: Scanning electron microscope; FESS: Functional endoscopic sinus surgery; PBS: Phosphate buffered saline; 3D: 3-Dimensional; CI: Confidence interval; FESS: Functional endoscopic sinus surgery; LMS: Lund-Mackay score

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Authors’ contributions
TA: contributions to the conception and design of the work, the acquisition, collection, analyses, and interpretation of specimens and data of the work; wrote and drafted the work; and agreed both to be personally accountable for the author’s contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature. NS: contributions to the conception, examined the specimens and revised the related part in the written draft and agreed both to be personally accountable for the author’s contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The ethical committee of Faculty of Medicine, Ain Shams University, Egypt, approved this work (registration-number: FMASU MD 466/2017). Informed written consent was obtained from all the participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Fig. 3 e Mucosal grade I: mild inflammation (star) and focal degeneration of the epithelium (arrow). f Mucosal grade II: moderate inflammation (star) and hyperplastic surface epithelium (arrow). g Mucosal grade III: severe inflammatory cellular infiltrate (star). h Eosinophil rich inflammatory cellular infiltrate (arrows)
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