The Laryngoscope

Different Biofilms, Different Disease? A Clinical Outcomes Study

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Objectives/Hypothesis: A potential role for biofilms in Chronic Rhinosinusitis (CRS) has been proposed, and the adverse impact they have on disease severity and postoperative outcomes has also been well described. Recent advances have allowed the species within the biofilms of CRS patients to be clearly characterized. This study investigates whether different biofilm species have different disease outcomes.

Study Design: Retrospective review.

Methods: Twenty-four patients with medically recalcitrant CRS undergoing Endoscopic Sinus Surgery (ESS), in whom we had previously characterized their biofilms using fluorescence in situ hybridization (FISH), were reviewed a median of 11 months after their surgery. They were evaluated for preoperative disease markers and evidence of on-going disease in the postoperative period.

Results: Thirty-seven biofilms were identified in the 24 patients. Almost half had polymicrobial biofilms. The presence of polymicrobial, rather than single-species biofilms adversely affected preoperative disease severity but did not alter postsurgical outcome. Patients with single organism *Haemophilus influenzae* biofilms presented with mild disease symptomatically and radiologically and achieved normal mucosa a short time after their surgery. Conversely, patients with *Staphlococcus aureus* in their biofilm makeup had more severe disease and a more complicated postoperative course. The effect of *Pseudomonas aeruginosa* and fungal biofilms is less clear.

Conclusions: Different biofilm species are associated with different disease phenotypes. *H. influenzae* biofilms are typically found in patients with mild disease, whereas *S. aureus* is associated with a more severe, surgically recalcitrant pattern.

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INTRODUCTION

Chronic Rhinosinusitis (CRS) is a heterogeneous inflammatory disorder with a poorly understood etiopathogenesis. Many patients will respond to medical therapy; however, a subset will fail this treatment and require surgical intervention. Endoscopic sinus surgery (ESS) is one of the most commonly performed surgical procedures in our discipline,[1] and although the success rate of ESS is high,[2] a number of patients will have on-going disease despite well-performed surgery. Predicting which patients will have surgically recalcitrant disease has yet to be completely defined in the literature. Factors such as depression,[3] smoking,[4] nasal polyposis,[5] aspirin sensitivity,[3] and gastroesophageal reflux[6] have all been demonstrated to adversely affect post-ESS outcomes. Early identification of patients at risk of on-going disease after surgery may facilitate a more aggressive and targeted approach to perioperative medical treatment of this group and possibly allow us to improve their postsurgical outcomes.

Since 2004, biofilms have been consistently demonstrated on the mucosal surface of CRS patients by a number of authors.[7–10] Furthermore, their presence has been associated with more severe disease preoperatively and poorer evolution following ESS in both retrospective[11] and prospective[12] trials. These two studies provide convincing evidence of a difference in disease characteristics and surgical response between patients with and without biofilms. However, no species information is provided by the biofilm identification technique used in these trials, so the question remains as to whether or not the particular species forming the biofilm has any bearing on postoperative evolution.

Recently, investigators have been able to identify the bacterial species that form biofilms in CRS patients using fluorescence in situ hybridization (FISH) with species specific probes. *Staphlococcus aureus*[13] and *Haemophilus influenza*[14,15] have been characterized as the most common biofilm-forming organisms in two separate CRS populations. Fungal biofilms have also been identified in these patients.[13] Thus, FISH can assist us to

investigate the impact different species have in CRS. Are all biofilms the same or do different biofilms result in different disease characteristics? We set out to answer this question in the current study.

MATERIALS AND METHODS

Study Design

A retrospective chart review of CRS patients undergoing ESS in the tertiary Rhinology practice of the senior author (P.J.W.) was performed. Our institution's Human Ethics Committee approved the study. All patients fulfilled the criteria for CRS diagnosis as outlined by the Rhinosinusitis Task Force[16] and had failed maximal medical therapy prior to being considered for surgical intervention. To be included, patients must have had previous biofilm determination and characterization performed (see below). Given our express interest in assessing the influence of different biofilm species on postoperative course, only biofilm positive patients were included. The differences between those patients with and without biofilms have already been well described.[11,12] Second, the operating surgeon must have seen the patient at least twice in the 3 months following their surgery. This practice receives a large number of tertiary referrals from interstate Otolaryngologists, most of who are followed up by the referring physician rather than the operative surgeon. Thus, these patients had to be excluded from this study. Patients were also excluded if they were under 18 years of age, pregnant, immunocompromised, suffered ciliary dysmotility, or if they had taken antibiotics or oral steroids in the three weeks prior to surgery.

Biofilm Determination and Characterization

Our FISH protocol has been described previously,[13] and uses species-specific probes for *S. aureus*, *H. influenzae*, and *Pseudomonas aeruginosa*, as well as a universal fungal probe. All patients had sinonasal mucosa harvested from the ethmoid cavity at the time of their surgery and transported on ice to our laboratory in Dulbecco's Modified Eagle medium (Gibco, Invitrogen Corp., Grand Island, NY), without antibiotics or Amphotericin B, where it was cryopreserved for delayed processing. The hybridized slides were analyzed at Adelaide Microscopy using the Leica TCS SP5 Confocal Scanning (Leica Microsystems, Wetzlar, Germany). The definitions used for bacterial and fungal biofilms are contained elsewhere.[13]

Data Collection

Preoperative and intraoperative data was collected at the time of surgery and enabled us to stratify disease severity. This data included demographics, symptom scores, Lund-Mackay Computed Tomography (CT) scores, intraoperative endoscopy findings, and microbiological results. Symptoms of nasal obstruction, rhinorrhea, postnasal drip, facial pain/headache, and change in smell were patient derived and surgeon recorded on a 1–5 scale to give a total out of 25. Postoperative details such as length of follow-up, number of follow-up visits, time to achieve objectively normal mucosa (measured in months), and microbiological culture results were all accessed during the chart review. Given the retrospective nature of this study, symptom scores in the postoperative period were not available, so the outcome measures remained purely objective.

Data Analysis

Data analysis was undertaken in three different ways. First, patients were grouped based on whether or not they had persisting disease at the end of the follow-up period to try to identify risk factors for poor postsurgical evolution. Patients were defined as having persisting disease if they had objective evidence of abnormal mucosa including mucosal oedema, polyposis, crusting, granulation tissue, thick mucus, or frank pus. Further to this, patients were defined as having a postoperative sinonasal mucosal infection if they had any of these features of abnormal healing as well as a positive microbiology result. Second, patients were grouped based on the number of species contained within their biofilm makeup, to determine whether number of species within a biofilm is related to disease burden and postoperative course. Finally, patients were analyzed based on specific species contained within their biofilms. Each species was divided in three different ways and compared to all remaining patients: 1) all occurrences of that species, regardless of whether this was a unimicrobial biofilm or was contained within a polymicrobial biofilm; 2) that species present only as a single organism biofilm; 3) that species when it existed in a polymicrobial biofilm.

Statistical Analysis

Our results were analyzed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Significance values of $\alpha = 5\%$, $\beta = 20\%$ and $P \leq .05$ were used. We considered our data to be nonparametric, so median and interquartile range (IQR) were reported. Fisher's exact test was used for dichotomous data and the Mann-Whitney U test was used for analysis of ordinal data. The log rank (Mantel-Cox) test was used to calculate survival curves for persisting disease in the postoperative period.

RESULTS

Demographic Analysis

Twenty-four patients fulfilled the inclusion and exclusion criteria for analysis. The study group consisted of 13 males and 11 females with a median age of 48 (IQR 39.5–56). Phenotypically, our patients could be subdivided into 9 patients with nasal polyposis and 15 patients without polyposis. On history, there were four smokers and four patients with aspirin sensitivity but no patients with either depression or gastroesophageal reflux disease. Each of these previously described outcome predictors were analyzed. There were no significant associations between any of these factors and the presence of particular biofilm species. Thus, we believe the impact of specific biofilm species on postoperative outcome is an independent observation.

Disease Characteristics

In terms of preoperative disease severity, the median symptom score was 17.5/25 (IQR 16–20.75) and the median radiology score was 14.5/24 (IQR 10.25–19.75). These patients were followed up for a median of 11 months (IQR 5–13). It is important to note that when a patient is asymptomatic and achieves normal mucosa they may be discharged from the clinic. This explains some of the patients with short follow-up times.

Eleven (46%) patients had complete resolution of their disease by the end of the follow-up period. This left 13 (54%) patients with persisting disease and 10 (77%) of this group had *S. aureus* in their biofilm makeup. Not surprisingly, the group with persisting disease required more postoperative visits (median = 6 and IQR = 3.5–8.5 compared with median = 3 and IQR = 3–5, $P = .019$)

TABLE I.
Biofilm Characterization. *S. aureus* Is the Most Common Biofilm-Forming Organism and 11 of 24 (46%) of Patients Had Polymicrobial Biofilms.

SPECIES	One (n = 13)	Two (n = 9)	Three (n = 2)	TOTAL (n = 24)
S. aureus	6	8	1	15
H. influenzae	5	4	1	10
P. aeruginosa	0	4	2	6
Fungal	2	2	2	6
TOTAL biofilm organisms	13	18	6	37

and had more sinonasal infections in the postoperative period (median = 2 and IQR = 1–4.5 compared with median = 0 and IQR = 0, $P = .0015$). However, it was not possible to predict this group based on their preoperative symptoms or Lund-Mackay CT scores.

Postoperative Infection

Thirteen of 24 patients had their postoperative course complicated by sinonasal infection, as defined by having evidence of infection clinically and a positive microbiology result. In line with our previous work,[17] postoperative infections are significantly associated with positive intraoperative cultures (Fisher's exact test, $P = .03$). Analyzing all other factors, the only other influence on postoperative infection is the presence of single-species *H. influenzae* biofilms, which reduced its incidence. There were no significant differences noticed within the other species groups, and the number of species within a biofilm did not influence the frequency of postoperative infections.

Biofilm Results

The biofilm characterization results are demonstrated in Table I. Thirty-seven biofilm species were identified in the 24 patients. Eleven of 24 (46%) patients had polymicrobial biofilms. *H. influenzae* appeared more commonly as a single organism biofilm compared with the other species, which were more commonly seen in polymicrobial biofilms. The number of species contained within a biofilm affected disease severity but not postoperative outcomes; the polymicrobial biofilm group had higher symptom scores (median = 19.5 and IQR = 18.25–21 compared with median = 16 and IQR = 15–17, $P = .0065$) and higher Lund Mackay CT scores (median = 19.5 and IQR = 12.75–22 compared with median = 12 and IQR = 6.5–16.5, $P = .0082$) than did those patients with single-species biofilms. However, this did not affect the time to normal mucosa or likelihood of the patient having a postoperative infection.

H. influenzae *Biofilms*

Five patients had *H. influenzae* as a single organism biofilm (Table I). When these patients were compared to the remaining 19, a number of significant results were observed (Table II). The *H. influenzae* group had significantly less severe disease symptomatically (median = 15 and IQR = 13–17 compared with median = 19 and IQR = 16–21, $p = .015$) and radiologically (median = 6 and IQR = 4–14 compared with median = 17 and IQR = 12–22, $P = .0015$). They then had rapid resolution of disease following their surgery, achieving normal mucosa significantly earlier than the remaining patients (median = 0 and IQR = 0–6 compared with median = 13 and IQR = 8.5–16, $P < .0001$), with less postoperative infections (median = 0 and IQR = 0–0.5 compared with median = 3 and IQR = 1–6, $P = .03$). Their rapid disease resolution is also confirmed with the Mantel-Cox test ($P = .0127$, see Fig. 1a).

When the entire *H. influenzae* group was analyzed (n = 10) and when just the polymicrobial *H. influenzae* biofilms were analyzed in isolation (n = 5), these significant results were lost (Table II). This may be due to the influence of the other species within the patient's biofilm makeup.

S. aureus *Biofilms*

Six of 15 (40%) patients had single-species *S. aureus* biofilms (Table I). Analysis of this group identified no significant results either preoperatively or postoperatively (Table II). However, when *S. aureus* was involved in a polymicrobial biofilm (9 of 15 patients), affected patients were identified as having more severe disease prior to

TABLE II.
Summary of Results for Species–Specific Disease Severity and Postoperative Evolution Outcome Measures.

	H. influenzae			*S. aureus*			*P. aeruginosa*[1]	Fungal[2]	
	Single	Poly	All	Single	Poly	All	All	Poly	All
Symptoms	p = 0.015	NS	NS	NS	p = 0.0069	p = 0.037	p = 0.01	NS	NS
Lund-Mackay Score	p = 0.0015	NS	NS	NS	p = 0.025	p = 0.009	p = 0.04	NS	NS
Time to normal mucosa	p < 0.0001	NS	NS	NS	NS	p = 0.016	NS	NS	NS
Post-operative infection	p = 0.03	NS	NS	NS	NS	NS	NS	NS	NS

Analysis of each species was undertaken in three ways based on whether the species was present on its own or in a polymicrobial mix. The significant findings related to single species *H. influenzae* biofilms and all *S. aureus* biofilms can be appreciated.

*There were no occurrences of single species *P. aeruginosa* biofilms thus all *P. aeruginosa* were contained in a polymicrobial species mix.

†There were only two patients with fungal biofilms exclusive of bacterial species. This group was too small to analyze individually, so was excluded from analysis.

NS = not significant.

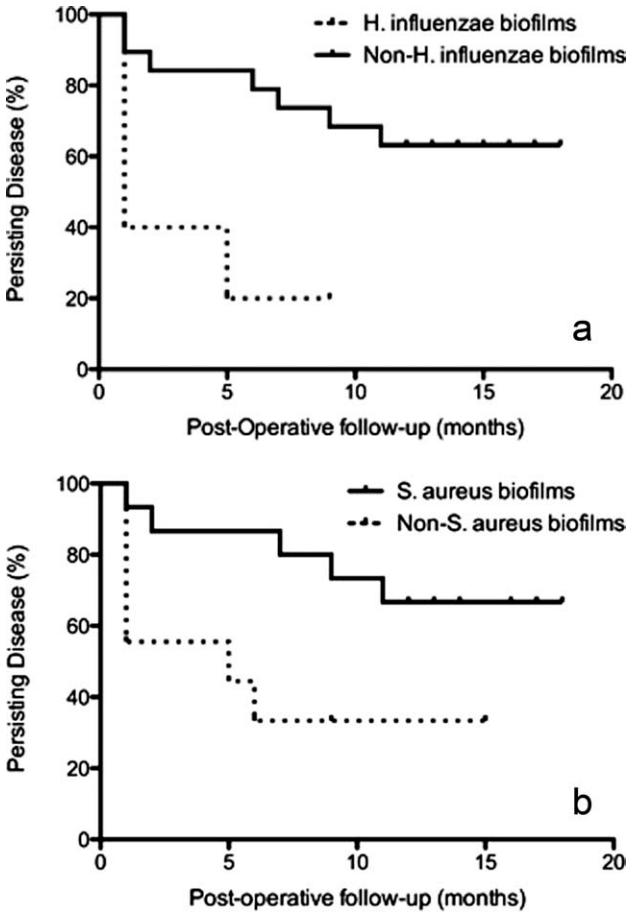

Fig. 1. Mantel-Cox survival curves. (**a**) *H. influenzae* as a single organism biofilm results in rapid disease resolution following surgery in most cases ($P = .0127$). (**b**) Significantly greater percentage of patients with persisting disease, despite surgery, in those with *S. aureus* in their biofilm makeup ($P = .0274$).

surgery. They had higher symptom scores (median = 20 and IQR = 18.5–21 compared with median = 16 and IQR = 15–19, $P = .0069$) and higher CT scores (median = 22 and IQR 12.5–22 compared with median = 12 and IQR = 7–18, $P = .025$). When these two groups were combined (i.e., all patients with *S. aureus* in their biofilm makeup), the deleterious effect of *S. aureus* on both preoperative disease and postoperative outcome can be fully appreciated (Table II). This group was symptomatically worse (median = 19.5 and IQR = 16.25–21 compared with median = 16.5 and IQR = 15–18.5, $P = .037$) and radiologically worse (median = 17 and IQR = 12.25–22 compared with median = 8.5 and IQR = 4.5–17.25, $P = .009$). They took significantly longer to achieve normal mucosa (median = 13 and IQR = 9–16 compared with median = 5 and IQR = 0–12, $P = .016$). This point is also demonstrated in Figure 1b using the Mantel-Cox log rank test ($P = .0274$). Furthermore, the *S. aureus* biofilm group had a trend toward requiring more postoperative visits (median = 5 and IQR = 3–8 compared with median = 4 and IQR = 2.5–5.5, $P = .097$) and having their postoperative course complicated by infections (median = 1 and IQR = 0–3 compared with median = 0 and IQR = 0–1.5, $P = .078$).

P. aeruginosa *Biofilms*

There were no single-species *P. aeruginosa* biofilms observed in our small sample group (Table I). Of the six *P. aeruginosa* biofilms in this study, four were associated with *S. aureus*. This makes interpretation difficult because it is unclear whether these results are truly reflective of *P. aeruginosa* biofilms or influenced by the presence of *S. aureus*. Nevertheless, analyzing this group as a whole (all *P. aeruginosa* cases were contained within polymicrobial biofilms), we found this group to have higher symptom scores (median = 21 and IQR = 18.74–21 compared with median = 17 and IQR = 15.75–20, $P = .01$) and higher radiology scores (median = 19.5 and IQR = 16–22 compared with median = 12 and IQR = 9.25–18.25, $P = .04$). No significant differences were found for this group in the postoperative period (Table II).

Fungal Biofilms

There were only two patients in whom the fungal biofilms were not associated with bacterial species (Table I). This number was too small for statistical analysis. Analysis of the remainder identified no significant results to suggest fungal biofilms affect CRS disease severity or postoperative outcomes in this cohort (Table II).

DISCUSSION

Since ESS was introduced over 20 years ago, significant technical refinements have occurred, taking it from a subspecialty procedure performed by relatively few surgeons to one comfortably undertaken by most Otolaryngologists. Despite improvements in the understanding of sinus anatomy, surgical technique, and instrumentation, a group of patients will still have symptoms after well-performed ESS. All sinus surgeons are faced with a spectrum of disease severity and recognize that this significantly impacts both their intraoperative findings and postoperative course. However, stratifying this heterogeneous group of patients remains a significant challenge. This is partly because the underlying pathophysiologic mechanisms of CRS are poorly understood. Many factors reported to affect post-ESS outcomes cannot be ameliorated prior to surgery, such as unfavorable sinus anatomy and aspirin sensitivity. However, a number of other factors can, and if these reversible causes can be identified this can provide the key to improving postoperative outcomes. Although the research is in its formative years, biofilms (specifically *S. aureus* biofilms) may well prove to be one such factor that can be successfully eradicated, thereby improving postoperative evolution.

This study builds on current knowledge of the clinical relevance of biofilms in CRS. It is well established that the presence of biofilms is a predictor of more severe disease and poorer outcomes following endoscopic sinus surgery. But until now, it had been unclear whether the impact of biofilms on disease outcomes could be further stratified based on the different species contained within them. The results of this study suggest that different biofilm species are indeed associated with

different disease characteristics. *H. influenzae*, when contained within a single organism biofilm, produces mild disease with a predictably favorable outcome after surgery. Conversely, the presence of *S. aureus* seems to predict more severe disease and a prolonged, complicated postoperative course.

S. aureus is recognized as a significant pathogen in CRS, and its multitude of roles are being constantly refined.[18] It is the most frequently cultured organism at the time of surgery[19,20] and the most common biofilm-forming organism found on intraoperative mucosal specimens.[13] In the postoperative period, *S. aureus* is again the most commonly cultured pathogen[17] and its in vitro biofilm-forming capacity has been correlated with unfavorable evolution following ESS.[21] We have confirmed this association through the use of direct biofilm detection techniques. Comparison of all patients with *S. aureus* biofilms with remaining patients (Table II) yielded a number of significant results that suggest *S. aureus* adversely affects both preoperative disease severity and progression after surgery. However, these effects are lost when this broad group is subdivided to analyze for our secondary analysis of the bacteria within only unimicrobial or polymicrobial biofilms. In these analyzes the *S. aureus* biofilms are spread across the comparison groups, resulting in a convergence of results and a loss of the significant differences. This would suggest that *S. aureus* plays a dominant role in determining disease severity and guiding postoperative course.

The role of *H. influenzae* in CRS is less well defined. Two previous articles that used FISH identified it as the most common biofilm forming organism.[14,15] Indeed, the results of our previous work also found *H. influenzae* was commonly found in the biofilm form.[13] But, despite its frequency, it appears that the presence of *H. influenzae* in a unimicrobial biofilm is actually associated with mild disease and rapid resolution of signs and symptoms following surgery. Of note is the fact that when *H. influenzae* is combined with other biofilm species in polymicrobial biofilms (Table II), these significant effects are lost, and this might suggest that the effect of *H. influenzae* is weak and that these patients have a disease course that more closely reflects the biofilm negative patients we have seen in other studies,[11,12] rather than refractory disease characteristics seen with other bacteria such as *S. aureus*.

Predicting which patients will have their postoperative course complicated by sinonasal mucosa infection and what the significance of this is remains debatable. We did not demonstrate a significant association between the patient's intraoperative biofilm status and their likelihood of have an infection in the postoperative period. This finding would be consistent with the biofilm paradigm, which dictates that these genotypically altered bacteria are difficult to culture using standard microbiologic techniques.[22] Conversely, positive culture of planktonic bacteria at the time of surgery did predict which patients suffered sinonasal infections following ESS. In line with our previous work we have reinforced that those who culture *S. aureus* intraoperatively are a "high-risk" group for postoperative mucosal infections.[17] It is most likely that the bacterial populations we see in CRS are polyclonal in nature, some favoring planktonic growth, whereas others will be stressed into the biofilm mode. We would hypothesize, from the results of this study, that the biofilm-forming subgroup may mediate the persisting inflammation seen postoperatively, whereas the planktonic clones will be responsible for inducing signs and symptoms of mucosal infection. This hypothesis requires on-going investigation.

As with any study, this is not without its methodologic limitations. First, this was a retrospective review of a relatively small number of patients. To confirm these preliminary findings, a large prospective study would need to be undertaken. Any prospective trial undertaken in this area should employ validated, ordinal scales to avoid biasing toward an unfavourable outcome as may have occurred with the nominal measures used in our study design. It should also include validated symptom scores to gain a true appreciation of the impact the abnormal mucosal outcomes we observed have on our patients. It is interesting to note that Wang et al.[23] found a significant correlation between subjective scores and objective evaluation, suggesting that our objective results may be able to be extrapolated to provide an indication of the impact these findings have on the patient. Second, the use of FISH for biofilm detection has technical limitations, specifically the inability to be sure of sampling all relevant species. *S. pneumoniae* and *M. catarrhalis* are the most likely biofilm-forming organisms to have been excluded. The prevalence and effect on disease characteristics that these biofilm-forming organisms have in CRS remains to be fully understood. Finally, the small numbers in the *P. aeruginosa* biofilm and fungal biofilm groups make it impossible to draw definitive conclusions about the effect of these bacteria on CRS as a disease.

Nevertheless, we believe that this study highlights some clinically relevant, novel concepts in the area of biofilm research. Our results suggest that different biofilms are associated with different disease patterns—both disease severity and surgical responsiveness. This has implications both for further research into understanding the role of biofilms in the aetiopathogenesis of CRS as well as for developing biofilm eradication agents. If all biofilms were different it would be reasonable to suggest that species-specific biofilm investigations are more relevant than a general approach to all biofilms. Thus, the focus should be on the clinically relevant biofilm species such as *S. aureus*. FISH is a good technique for this.[24] Similarly, the evidence for biofilms, and in particular those involving *S. aureus*, as one predictor of severe disease is mounting. Thus, we may be able to identify an unfavorable group who actually have a potentially reversible aetiology. Biofilm research in all disciplines is progressing rapidly, and research energy is increasingly being channeled toward biofilm eradication strategies. Much of this work is now directed at finding components of biofilms formed by specific organisms (including *S. aureus*), which can then be targeted with novel treatments,[25] further highlighting the potential

relevance of our findings. This advancing knowledge will enable us to aggressively treat these patients who will progress poorly after surgery with directed antibiofilm agents in the perioperative period in the hope of improving their outcome following ESS.

CONCLUSION

Within the limitations of a retrospective review, this study demonstrates that different biofilms probably are associated with different disease characteristics and surgical responsiveness. Specifically, *H. influenzae* biofilms convey mild disease that is highly surgically responsive whereas *S. aureus* biofilms are associated with more severe, surgically recalcitrant disease, and appear to play a dominant role over other species in polymicrobial biofilms. These new findings have relevance both in guiding future laboratory research in this area as well as identifying targets for directed biofilm eradication strategies that may improve patients outcomes following sinus surgery.

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