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Medical therapy reduces microbiota diversity and evenness in surgically recalcitrant chronic rhinosinusitis

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Abstract

Background—Chronic rhinosinusitis is a highly prevalent and heterogeneous condition frequently treated with antibiotics and corticosteroid therapy. However, the effect of medical therapy on sinus microbiota remains unknown.

Methods—We enrolled CRS participants (n = 6) with patent maxillary antrostomies and active mucosal inflammation, who had not received antibiotics or corticosteroids in the previous eight weeks. A pre- and post-treatment maxillary sinus swab was collected, from which DNA was extracted, pyrosequenced, and analyzed using Naïve Bayesian Classifier and ecological analyses.

Results—Four participants showed significant improvement in endoscopic appearance. The shifts in microbiota in response to therapy were highly individualized. There was no single common microbiota profile among participants with similar clinical outcomes, but overall there was significant decrease in microbiota diversity ($t(5) = 2.05$, $p = 0.10$) and evenness ($t(5) = 2.28$, $p = 0.07$) after treatment.

Conclusions—Our findings strongly correlate with earlier studies that examined the impact of antibiotics on human microbiota. We observed that post-treatment, participants frequently became colonized by taxa that are less susceptible to the prescribed antibiotics. Our findings highlight the challenge in seeking generalizable diagnostic and therapeutic options in CRS, particularly regarding microbiological response and outcomes.

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Keywords

Bacteriology; Chronic Rhinosinusitis; Medical Therapy of Chronic Rhinosinusitis; Steroid therapy; Eosinophilic rhinitis and nasal polyposis; Sinusitis; Rhinosinusitis; Sinus microbiota; Sinus microbiome; Sinus microbiology; 16S rRNA gene; 16S rRNA gene-based pyrosequencing; Antibiotics; Antimicrobial; Corticosteroids; Chronic Rhinosinusitis; Maximum medical therapy

Introduction

Chronic rhinosinusitis (CRS) affects 31 million Americans every year, leading to 12.5 million physician office visits¹ and an annual healthcare expenditure at \$4.3 billion². A heterogeneous condition, CRS is diagnosed clinically based on a constellation of symptoms lasting more than 12 consecutive weeks, confirmed by evidence of sinus mucosal inflammation³. Phenotypically and immunologically, CRS can be further grouped into CRS with nasal polyposis (CRSwNP) and without nasal polyposis (CRSsNP)⁴⁻⁶. Nevertheless, the etiology of both groups is considered to be multifactorial and comprised of both endogenous and exogenous factors.

Although the precise role of microbes in CRS pathogenesis remains unclear, systemic antibiotics and anti-inflammatory therapy remain an integral strategy for managing the disease. But increasing evidence suggests that antibiotics have limited efficacy^{3,7,8}. This may in part be due to the development of bacterial biofilms in the sinus⁹⁻¹¹. Thus far, the use of systemic antibiotics to eliminate cultured pathogens, such as *S. aureus*, had shown mixed results and unintended side effects. Intra-nasal mupirocin in surgically recalcitrant CRS patients^{12,13} and mupirocin irrigation during endoscopic sinus surgery¹⁴ were efficacious in short-term *S. aureus* clearance. However, a four-week course of mupirocin washes to decolonize antibiotic-resistant *S. aureus* resulted in a high rate of microbiological failure (73.7%) and the selection of non-*S. aureus* pathogens¹³. Likewise, the increases in bacterial resistance against erythromycin¹⁵, quinolones, and aminoglycosides in CRS¹⁶ have also been attributed to the frequent use of antibiotics. This highlights the importance of fully evaluating the microbiological outcomes of maximum medical therapy..

A seemingly paradoxical observation in CRS microbiology had been the detection of many putative sinus pathogens in both healthy and diseased individuals, including *S. aureus*, *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and anaerobic bacteria such as *Prevotella spp.*¹⁷⁻²³. These findings, together with the earlier identification of complex sinus bacterial communities (i.e., microbiota)²⁴⁻²⁶ indicate that an ecological framework is needed to fully assess the impact of medical therapy on sinus microbiology.

To our knowledge, there have been no reports that assessed the impact of maximum medical therapy on the sinus microbiota, even though antibiotics had been shown to have pervasive effects on many aspects of the human microbiome²⁷. In the current study, we report our comparison of sinus microbiota in six CRS patients before and after maximum medical therapy using 16S rRNA gene-based pyrosequencing analysis.

Materials and Methods

Study design and subjects

Adult participants of both sexes were screened, consented, and enrolled. Patients who reported using oral antibiotics or corticosteroids in the previous eight weeks or had a known history of immune or autoimmune diseases were excluded. The American Academy of Otolaryngology-Head and Neck Surgery diagnostic guidelines for CRS were used in screening and enrollment³. All patients had a history of endoscopic sinus surgery greater than 1 year prior to enrollment, with patent maxillary anastomoses. Active sinus disease was diagnosed by the presence of mucosal inflammation on endoscopic exam findings at the time of pre-treatment sample collection, and the presence of inspissated or mucopurulent secretions was noted. The post-treatment samples were collected after the patient had completed the appropriate course of maximum medical therapy. At each collection, the maxillary sinus mucosa was sampled using a calcium alginate swab (MPC, Amarillo, CA) under endoscopic guidance and were immediately placed into an empty sterile 15ml conical and flash frozen on dry ice, then stored at -80°C until analysis. Medication and culture result data were also collected, when applicable. The study was approved by three institutional review boards: the Northern Arizona University Institutional Review Board (Flagstaff, AZ), the Johns Hopkins Medicine Institutional Review Board (Baltimore, MD), and the Western Institutional Review Board (Olympia, WA), the IRB of record for the Translational Genomics Research Institute.

Sample processing and pyrosequencing analysis

All sinus swabs (n = 12) were thawed and individually eluted in 1ml of molecular-grade phosphate buffered saline. Cell lysis was performed using 100ul of the swab eluent and a combination of enzyme-free chemical and mechanical lysis and the nucleic acid purified using the Qiagen AllPrep Mini Kit (Qiagen, Valencia, CA, USA). Barcoded amplicons of the V3V6 hypervariable region were generated using broad-coverage fusion PCR primers (Forward primer: 5'- CCATCTCATCCCTGCGTGTCTCCGACTCAG~~nnnnnnnn~~- **CCTACGGG****DGGC**WGCA-3' and reverse primer: 5'- CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCT**GACGACRRCC**RTGCA-3'), with the underlined portion denoting FLX Lib-L adapter sequence, italicized portion denoting the sample-specific 8-nt barcode sequence, and bolded portion denoting 16S rRNA gene primer sequence. The fusion PCR amplicons were quantified and pooled for sequencing on the Genome Sequencer FLX (Roche Applied Sciences, Branford, USA). The resultant pyrosequence were chimera-checked²⁸, demultiplexed, and quality-checked²⁹. Taxonomic classification was performed using Ribosomal Database Project Naïve Bayesian Classifier³⁰. Pyrosequencing analysis yielded a total of 70,526 16S rRNA gene sequences (*mean* per sample = 2939, *SD* = 3985). Sequences that were assigned to taxa at below the 80% bootstrap confidence level threshold were noted as "C<0.8".

Comparative analysis of the sinus microbiota

All sinus microbiota comparisons were performed in R version 2.13.1³¹. The package *vegan* was used to generate the pie chart, box plots, and heatmap visualization³². To focus our analysis, we only included the 60 sinus bacterial taxa with the highest number of total

sequences. The pre- and post-treatment sinus microbiota were compared based proportional abundances of sinus bacteria and biodiversity metrics. Proportional abundances were calculated using: $(\text{Total number of 16S rRNA gene sequences identified as genus A from the sample})/(\text{Total number of 16S rRNA gene sequences identified as bacteria from the sample})$ [Eq. 1]. Heatmap visualization plots were generated based on proportional abundances and the changes in proportional abundance with treatment. Microbiota biodiversity metrics were calculated as follows: diversity (D), calculated as $D = \text{Shannon's diversity index}$, and evenness (E), calculated as $E = D/S$, where $S = \text{richness}$ ³³. Evenness reflects the dominance by many (i.e., high evenness) versus few (i.e., low evenness) different types of sinus bacteria, whereas richness is the total number sinus bacterial genera present. The changes in Shannon's diversity indices and evenness were compared using paired two-tailed t-test. A significance level of $\alpha = 0.10$ was used. The biodiversity plots and collector's curves were plotted in R.

Results

Participant demographics and clinical response

Six CRS patients with patent maxillary antrastomies, who had not received systemic antibiotics or corticosteroids in the previous eight weeks were enrolled. The group was comprised of four female and two male participants, with a mean age of 54.7 years (SD = 7.2, range = 49-68) (Table 1). Seasonal allergy ($n = 1$) and asthma ($n = 1$) were infrequent among the participants.

Prior to receiving their respective clinically indicated maximum medical therapy, all participants had evidence of mucosal inflammation on endoscopy. Otherwise, the constellation of symptoms varied among the participants. Four participants had polyps. Inspissated secretions ($n = 3$) or mucopurulence ($n = 3$) was observed in five of the six participants. After the four-week treatment regimen, two patients' symptoms completely resolved (Participants 1 and 2) and significant improvements were seen in Participants 4 and 6. Participants 3 and 5 both showed residual polyps, and mucopurulence developed in Participant 3 (Table 2).

Highly individual-specific microbiota changes after maximal medical therapy

There was a wide range of sinus microbiota profiles prior to treatment. Participants 2, 3, and 4 each had a unique dominant sinus bacterial taxon (i.e., defined as a taxon that comprises greater than 50% of the sinus microbiota), which were *Prevotella*, *Pseudomonas*, and *Haemophilus*, respectively (Table 2, Figure S1).

The changes in microbiota after treatment were individual-specific. Substantial proportional abundance decreases and increases were both observed in *Prevotella*, *Pseudomonas*, *Haemophilus* (Figure 1). Smaller shifts were seen in many other taxa, including *Staphylococcus*, *Corynebacterium*, *Serratia*, *Propionibacterium*, *Delftia*, and *Rothia*. The sinus microbiota did not converge in response to antibiotics and anti-inflammatory therapy, and the post-treatment microbiota remained highly heterogeneous (Table 2, Figure S1). In addition, no common microbiota changes in participants with abated or persistent

symptomatic findings were observed (Figure 1). Between the two CRS participants with persistent symptoms, one shifted from a *Pseudomonas*-dominated sinus microbiota to a *Haemophilus*-dominant microbiota (Participant 3), while Participant 5 shifted from a multi-dominated sinus microbiota to one dominated by *Delftia* (Table 2). Comparison with culture results showed that culturing was limited against *Haemophilus* and a wide range of anaerobic bacteria, including *Prevotella*, *Anaerococcus*, and *Finegoldia*, (Table 2, Figure S1).

Maximum medical therapy significantly reduces sinus microbiota diversity and evenness

A consistent effect of maximum medical therapy was the diminution of microbiota biodiversity (Figure 2A-B). But these biodiversity changes were not conserved among participants with similar post-treatment endoscopic findings. For example, in both Participants 1 and 2, mucopurulence resolved completely, but the decrease in microbiota diversity and evenness was more precipitous in Participant 1 (*Diversity* = -1.29; *Evenness* = -0.39) than in Participant 2 (*Diversity* = -0.09; *Evenness* = -0.03). The changes in biodiversity among individuals with residual symptomatology were scattered between what was observed in Participants 1 and 2.

Treatment decreased the total number of unique bacterial taxa among the participants (Figure 2A). And all, except for Participant 4, showed decreased evenness and Shannon's diversity index after treatment (Figure 2B). The decline in evenness was statistically significant ($t(5) = 2.28, p = 0.07$), while the decrease in Shannon's diversity index approached statistical significance ($t(5) = 2.05, p = 0.10$).

Discussion

Our study revealed individual-specific alterations in sinus microbiota after maximum medical therapy, which highlights the challenge in identifying a unifying theme in CRS pathogenesis, management, and clinical and microbiological outcomes. It further illustrates the nature of antibiotics as a blunt tool, which is effective for treating acute, single-organism infections, but should be utilized with caution in chronic conditions. The wide range of microbiota profiles prior to treatment may contribute in part to the highly individualized outcomes observed in this study.

The absence of a common post-treatment microbiota profile or of correlations between microbiota shifts and clinical outcomes suggest the importance of bacterial strains in CRS. For example, the *Haemophilus* and *Prevotella* strains that emerged after treatment may have different virulence profiles from the strains that dominated in untreated CRS participants. Thus, studies that merge the community-level and strain-level perspectives will offer important insights.

Differentiating the effect of antibiotics from the corticosteroid therapy is challenging. In our study, the two participants without polyposis improved symptomatically, but their microbiological outcomes demonstrated shifts toward taxa that are less susceptible to the antibiotic prescribed. In Participant 1, although the patient was treated with Moxifloxacin, a microbiota profile dominated by the frequently biofilm-forming *Pseudomonas* emerged

post-treatment despite antipseudomonal treatment, a paradoxical phenomenon previously reported in the respiratory tract of intubated patients³⁴. The use of TMP-SMZ in Participant 2 reduced *Prevotella* but selected for Coagulase-negative *Staphylococcus*³⁵. There was no evidence of normalization to the most common non-diseased microbiota profile (i.e., co-dominated by *Corynebacterium* and *Propionibacterium*). This suggests that while their symptoms resolved, they were likely not cured microbiologically.

In nasal polyposis patients who responded to medical therapy, Participants 4 and 6 resulted in a *Corynebacterium*- and a *Propionibacterium*-dominated microbiota, respectively. Interestingly, this microbiota restoration occurred in Participant 6 with solely corticosteroid therapy and without the use of antibiotics. In the nasal polyposis patients with persistent symptoms, Participant 3 had a *Pseudomonas*-dominated microbiota that shifted to one that is *Haemophilus*-dominated. As Cefuroxime should have been effective against *Haemophilus*, this finding suggests that the persistently inflamed mucosa may have been ineffective immunologically and permissive of pathogen succession. In participant 5, the shift from a multi-dominated to a *Delftia*-dominated again mirrors the selection of less susceptible taxa, as *Delftia* had shown to be frequently resistant to Cefuroxime³⁶.

The clinical relevance of decreased sinus microbiota diversity and evenness after medical therapeutics is uncertain. While some body sites require diversity, less diverse states in specific body sites have been associated with better outcomes, such as in the female and male urogenital tracts³⁷⁻³⁹. Our findings are consistent with the pattern of individualized response to antibiotic therapy and the decrease in diversity and evenness seen in earlier gut microbiota studies^{34,40,41}. Here, we further demonstrated that the loss of microbiota diversity and evenness was not affected by the combination of antibiotics with corticosteroid therapy.

Conclusion

This study provides evidence that the combination of antibiotics and anti-inflammatory therapy significantly decreased sinus microbiota biodiversity in post-operative maxillary sinuses. However, the losses and gains of sinus bacteria after treatment were unique to each individual. This finding of a highly individualized sinus microbiome in CRS before and after medical therapy underscores the potential futility of the search for a universal antimicrobial strategy. Undoubtedly, the future of CRS therapy will require more effective stratifications of patient population prior to treatment. Studies involving large, prospective cohorts are needed to fully elucidate the landscape of sinus microbiota in health versus disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effects of clinically indicated maximum medical therapy on the sinus microbiota. In this heatmap visualization, each column represents the change in sinus microbiota from an individual. Along each row, the change in the proportional abundance of a bacterial taxon (e.g., *Pseudomonas*) is shown and can be interpreted using the annotated color-coding key (right, color bar), which denotes each of the color that correlates with its respective proportional abundance change.

Figure 2.

Maximum medical therapy is associated with decrease in sinus microbiota richness (Figure 2A), as well as microbiota diversity (Figure 2B, *left*) and evenness (Figure 2B, *right*). Figure 2A is the collector's curve, which represents the total number of unique bacterial taxon found in participants before (solid line) or after (dashed line) treatment as each participant is analyzed. This showed the total number of unique taxa decreased after treatment. In the two sets of plots in Figure 2B, each data point represents the evenness and Shannon Diversity value either before or after treatment in a single participant, and is labeled by the Participant study ID. At each time point, the mean value is annotated by a simple horizontal line.

Table 1

Demographics, clinical history, and the maxillary sinus sampled in the study participants.

ID	Age (years)	Sex (M/F)	Seasonal allergy	Asthma	Maxillary sinus sampled (Left/Right)
1	51	F	No	No	Right
2	68	M	No	No	Right
3	55	F	No	Yes	Left
4	49	M	No	No	Left
5	56	F	Yes	No	Left
6	49	F	No	No	Left

Table 2

Treatment regimen received by each participant, with corresponding pre- and post-treatment microbiota profile and findings on endoscopy.

ID	Medical Therapy (4 weeks)	Concurrent medications	Pre-treatment Microbiology		Pre-treatment endoscopic findings			Post-treatment Microbiology		Post-treatment endoscopic findings			
			Culture	Microbiota	Mucosal inflammation	Polyps	Inspissated secretions	Muco-purulence	Culture	Microbiota	Mucosal inflammation	Polyps	Inspissated secretions
1	Moxifloxacin + Medrol	Saline rinse, Nasonex	Very light normal skin flora	Multi-dominated	✓	-	-	✓	n/a	-	-	-	-
2	TMP-SMZ +Medrol	Pulmicort irrigations	Moderate normal skin flora (Day 2)	Prevotella - dominated	✓	-	✓	✓	n/a	-	-	-	-
3	Cefuroxime + Medrol	Saline rinse, Singulair, Guaifenesin Zyrtec D, and Rhinocort.	n/a	Pseudomonas-dominated	✓	✓	✓	-	Moderate S. aureus	Improved	✓	Improved	✓
4	Cefuroxime + Medrol	Saline rinse. Advair, Fluticasone, Pulmicort	Light Acinetobacter spp. Very light P.aeruginosa	Haemophilus dominated	✓	✓	-	✓	n/a	-	Improved	-	-
5	Cefuroxime + Medrol	Saline rinse, Claritin, Pulmicort nasal atomizer	n/a	Multi-dominated	✓	✓	✓	-	n/a	Improved	Minimal	-	-
6	No antibiotics + Medrol	Saline rinse and Rhinocort aqua	n/a	Multi-dominated	✓	✓	-	-	n/a	-	Improved	-	-

* ✓ denotes presence of specified symptom, - denotes absence of specific symptom, “Improved” denotes symptom improvement, and “Minimal” denotes minimal residual symptom.