The Association Between Disease Severity and Microbiome in Chronic Rhinosinusitis

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INTRODUCTION

Chronic rhinosinusitis (CRS) is a heterogeneous disorder of the sinonasal mucosa persisting longer than 12 weeks.1 Traditionally, CRS has been classified based on phenotype, with the two major categories being the presence or absence of nasal polyps (CRSwNP and CRSSsNP, respectively). More recently, an alternative classification has been postulated based on inflammatory cell predominance, or endotype, with varying degrees of eosinophilic inflammation.2,3 Eosinophilic CRS is associated with a T-helper cell (Th2) inflammatory response and has been demonstrated to correlate with greater clinical severity, higher recurrence rates of disease following surgery, and resistance to steroid and macrolide therapy.4

The role of bacteria in the pathogenesis of CRS has long been debated; however, few common causative pathogens have been identified. Additionally, antibiotics are not an effective long-term treatment in CRS.5 Conventional culture-sensitive techniques have implicated various species of bacteria, including Staphylococcus aureus, Pseudomonas aeruginosa, and Haemophilus influenzae; however, several dilemmas compound the simplified causative model.6,7 The presumed putative pathogens have also been identified in non-diseased, cultivated samples, suggesting their contribution to the healthy sinonasal microbiome as well.8

With the advent of newer culture-independent techniques such as bacterial 16S ribosomal RNA (rRNA) sequencing, greater detection in bacterial presence, abundance, richness, and diversity indicates that traditional culture-based methods significantly underrepresent the

OBJECTIVE: The role of the microbiome in the etiology of chronic rhinosinusitis (CRS) is still in debate. Reductions in richness and diversity have been implicated in CRS; however, limited knowledge exists regarding the impact of the severity of disease on the microbiome. The associations between constituents of the microbiome and the degree of mucosal inflammation and tissue eosinophilia are described.

METHODS: A cross-sectional study of CRS and non-CRS patients who underwent endoscopic sinus surgery was performed. Sinus mucosal biopsies were assessed for the degree of inflammation and tissue eosinophilia. Middle-meatal swabs were subjected to 16S rRNA gene sequencing, which quantified the prevalence, mean relative abundance, richness, and diversity. Comparisons between the microbiome at the genus level and degree of inflammation (absent, mild, moderate, severe) and tissue eosinophilia (absent, < 10, 10–100, > 100 per high-powered field) were performed.

RESULTS: Eight-nine patients (52.8 ± 14.21 years, 64.0% male) were assessed. Of those, 52 had CRS and 37 were controls. Corynebacterium and Staphylococcus were the most abundant genera in both the CRS (29% and 16%) and non-CRS groups (40% and 20%). Richness decreased in more severely inflamed patients (23.2 ± 13.9 vs. 18.1 ± 16.1 vs. 16.8 ± 12.3 vs. 14.7 ± 10.9; P < 0.01), as did diversity (1.4 ± 0.7 vs. 1.2 ± 1.0 vs. 1.2 ± 0.8 vs. 0.9 ± 0.7; P = 0.05). Richness was associated with higher tissue eosinophilia (23.2 ± 13.9 vs. 19.3 ± 17.2 vs. 15.9 ± 11.6 vs. 13.4 ± 6.6; P < 0.01).

CONCLUSION: The loss of richness and diversity seen in the CRS microbiome appears to be a product of severity of inflammation and tissue eosinophilia. Whether this dysbiosis is causative or a result of the disease with impaired epithelial integrity requires ongoing research.

KEY WORDS: Sinus, microbiome, richness, tissue eosinophilia.

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microbial community. Emerging from recent studies is a shift toward the dysbiosis hypothesis whereby an imbalance in the sinus microbiome leads to an overabundance of opportunistic pathogens and loss of key commensal organisms. This sets the stage for a chronic inflammatory state.

To date, there is incomplete insight into the CRS microbiome, and its complexity is emphasized by variation in taxa not only among patients but between sinus cavities of the same individual. Despite this, there are certain ecological features that differentiate the CRS and non-CRS microbiota. When compared to healthy individuals, CRS patients have shown a reduction in bacterial richness (total number of bacterial species) and evenness (relative proportion of each species), together defined as the diversity. Although notable depletions in common commensal organisms such as Lactobacillus spp., Corynebacterium, and Acinetobacter johnsonii are also apparent, enrichment of other species such as Staphylococcus aureus, Propionibacterium acnes, and Pseudomonas aeruginosa have been reported. Together, this indicates that fewer species of bacteria are present within the CRS microbiome, which is further characterized by a depletion of protective species and an overabundance of disease-causing organisms.

However, these changes in the microbiome do not imply causation. As the mucosal barrier becomes impaired, so do normal, innate immune functions that regulate the healthy microbiome state. Such an example can be seen in atopic dermatitis or burns patients; primary inflammation and disruption of the normal epithelial barrier result in significant secondary distortion of the microbiome. In CRS patients, the critical question still remains as to whether the microbiome is secondary to the inflammation and breakdown of the epithelial barrier or is causing it.

To date, the association between bacterial pathogens and histopathology is limited to simple culture-based studies, with conflicting data thus far. Ba et al. reported that Th2-mediated inflammation was associated with greater gram-positive colonization; however, this was not reproduced in a subsequent study. Investigation into the association between the microbiome and histopathology in CRS is lacking. This study aimed to assess whether the severity of mucosal inflammation and the extent of tissue eosinophilia in CRS was associated with changes in the microbiome.

Study Participants

Patients greater than 18 years of age with CRS undergoing endoscopic sinus surgery (ESS) in a tertiary referral hospital were included in the study. Patients with CRS were defined based on the European Position Paper on Rhinosinusitis and Nasal Polyposis. All patients had failed maximal medical therapy prior to ESS. Patients who had received antibiotics or oral steroids less than 4 weeks prior to surgery were excluded. The control group included patients undergoing ESS for pituitary tumor excision or repair of a CSF leak and showed no radiological or endoscopic evidence of CRS. Any patient with established immunodeficiency, pregnancy, coagulation disorder, Churg-Strauss syndrome, or cystic fibrosis were excluded from the study.

Histopathological Assessment for Inflammatory Severity and Tissue Eosinophilia

Sinus mucosal tissue was biopsied from the maxillary or ethmoid sinuses in both CRS and control patients. Specimens were fixed in 10% buffered formalin, processed, and embedded in paraffin wax. The formalin-fixed and paraffin-embedded tissue was cut at 3 μm thickness, and sections were mounted on standard glass slides for hematoxylin and eosin (H&E) staining. Three levels of H&E-stained sections were prepared on each specimen and examined. All specimens were scored at the same pathology institution (SydPath, St Vincent’s Hospital) by the same assessor using a standardized CRS structured reporting pro forma. The assessor was blinded to the disease state of the patient. The scoring criteria included the overall degree of tissue inflammation (absent, mild, moderate, severe) and tissue eosinophilia (< 10 eosinophils per high-powered field [HPF], 10–100 eosinophils per HPF, > 100 eosinophils per HPF), as expressed in Figure 1.

Microbiome Analysis

Sample Collection. At the time of surgery, endoscopically directed guarded swabs (Transwab ENT for Aerobes and Anaerobes, ENT Amies Plain, Sigma-Aldrich, St. Louis, MO) were collected from the middle meati of all control and CRS patients. The middle meatus was chosen as a fair representation of the underlying sinuses. Care was taken to avoid contact with non-target surfaces, and any contaminated samples were discarded. Once retrieved, samples were placed in a sterile container and transported immediately on ice to the laboratory for storage at ~80°C.

DNA Extraction. DNA extraction was carried out using PowerLyzer Power-Soil DNA Isolation Kit (MoBio Laboratories, Salona Beach, CA). Swab heads and tissue were thawed on ice and placed directly onto the beads for homogenization. Total DNA was extracted from all clinical samples and two DNA extraction negative controls containing extraction reagents only. The remainder of the extraction protocol was performed as per the manufacturer’s protocol. Extracted DNA was stored at ~80°C until sequencing.

Polymerase Chain Reaction Amplification of the 16S rRNA Gene and Sequencing. Polymerase chain reaction (PCR) amplification and sequencing was performed by the Australian Genome Research Facility. Libraries were generated by amplifying the V3 to V4 (341F–868R) hypervariable region of the 16S rRNA gene. PCR amplicons were generated using the primers CCAATAYGGGRBCAGCACG in the forward sequence and GGACTACNNGGATATGCTATATTAT in the reverse sequence, using AmpliTaq Gold 360 Master Mix (Life Technologies, Mulgrave, Victoria, Australia) and a CFX96 (Bio-Rad, Hercules, CA) thermal cycler. A negative template control was included in each reaction and all sequences were aligned using MOTHUR (v. 1.31.2) and analyzed with QIIME (v. 1.9.1).
Australia) and following local protocol. The resulting amplicons were measured by fluorometry (Invitrogen PicoGreen; Thermo Fisher Scientific, Waltham, MA) and normalized. The equimolar pool was then quantified by quantitative PCR (KAPA Biosystems, Capetown, South Africa) and set up for sequencing on the Illumina MiSeq (Illumina Inc., San Diego, CA) with 300 base-paired end chemistry. Reads from Illumina sequencing were used as raw data for bioinformatics analyses.

**Bioinformatics Pipeline.** The paired-end read merger (PEAR)\(^2\) version 0.9.5 was used to pair the forward and reverse reads in each sample. Only reads that joined successfully were followed through to downstream analysis. These reads were quality-filtered using Quantitative Insights Into Microbial Ecology (QIIME) (University of Colorado, Boulder, CO) split_libraries_fasta.py command default settings (QIIME version 1.9.1). Reads were then clustered into operational taxonomic units (OTUs) at 100% similarity (i.e., full-length deduplication) using vsearch version 1.9.9. Taxonomy assignment for the deduplicated OTU reads was performed using QIIME taxonomy open-reference assignment command, UCLUST, and searching against the Greengenes version 13.8 (August 2013) 97% representative database.

**Statistical Analysis**

All tests were performed at the taxonomic rank of genus. Pursuant to rarefaction, all samples were subsampled to 800 reads. Statistical analysis was performed using SPSS version 23 (IBM Corp., Armonk, NY) and R statistical software (R 3.5.0 Foundation for Statistical Computing, Vienna, Austria). Hypothesis testing used two-sided significance tests, and the

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**Fig. 1.** Structured histopathological scoring pro forma for patients with chronic rhinosinusitis. CRS = chronic rhinosinusitis; HPF = high-powered field. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]
alpha level was taken at 0.05. Continuous data were summarized as mean with standard deviation and categorical data by proportions. A two-sample t-test was used to compare continuous scales, and Man-Whitney U test was used when the normality assumption was not met. Pearson correlation coefficients were performed to examine the association between two continuous variables, and Spearman rank order test was used when the normality assumption did not meet. Chi-square test or Fisher exact test were used to examine the association between two categorical variables. Kendall tau-b test was employed to examine the correlation between two ordinal measures. Comparisons of following components of the microbiome were firstly assessed between the CRS and control groups:

1. Prevalence (presence or absence of particular genus): compared using chi-square or Fisher exact test
2. Mean relative abundance (MRA) (proportional presence of particular genus compared to others in the same sample): compared using a one-way analysis of variance. P-values were adjusted using the false discovery rate (FDR) approach based on the Benjamini and Hochberg step-up FDR-controlling procedure, and conducted in R using mt.rawp2adjp function of Bioconductor package.
3. Richness (number of unique genera per sample expressed as an index): CRS and control groups were compared using a Mann-Whitney U test.

Correlation between microbiome outcomes (richness, diversity) and histopathological outcomes (degree of inflammation, tissue eosinophilia) was then performed using the Spearman rank test of correlation. Multivariate linear modelling was performed to explore the effect of confounding covariates, using the lmp function from the R package lmPerm, which employs linear modelling functions using permutation tests as a basis.

**RESULTS**

There were 89 patients (mean age 52.8 ± 14.2 years, 64.0% male) included in this study, 52% (n = 46) from St Vincent’s Hospital and the remaining patients from the Queen Elizabeth Hospital. Of these, 58% (n = 52) of all patients had CRS, with the remaining patients as non-CRS controls. Nasal polyps were present in 88% (n = 46) of patients, and 12% (n = 6) were CRSsNP. No patient was known to have had an exacerbation or acute flare of their condition at the time of tissue sampling. Clinical
and histopathological outcomes are summarized in Table I. Among the 17 patients with severe inflammation, 82% (n = 14) were found in the Sydney center (P = 0.04). Other demographic data expressed according to diagnosis is represented in Table II.

**Microbiome Outcomes**

**Prevalence and Mean Relative Abundance.** A total of 299 different genera were identified among all patients in the study. The 13 most common genera are expressed in Table III in terms of prevalence and MRA. Overall, *Corynebacterium* was the most prevalent genus (94%), followed by *Staphylococcus* (91%), which was consistent between institutions (98% and 89% for Sydney samples, and 91% and 93% for Adelaide samples, respectively). *Corynebacterium* and *Staphylococcus* were the most abundant genera among all CRS patients (29% and 16%, respectively) as well as between institutions (26% and 15% for Sydney, and 33% and 18% for Adelaide, respectively). The composition of the microbiome among different degrees of inflammation and tissue eosinophilia was also analyzed in terms of MRA. These findings are summarized in Figure 2.

**Mean Richness and Diversity.** The mean sample richness was 19.3 ± 13.7 overall. Control patients demonstrated a significantly richer microbiome compared to those with CRS (23.2 ± 13.7 vs. 16.4 ± 12.7; P < 0.01). There was no significant difference in richness between CRSwNP and CRSsNP (17.3 ± 13.0 v. 9.7 ± 10.1; P = 0.08). Both CRS and control patients from the Adelaide center had a richer microbiome compared to those from the Sydney center (21.6 ± 16.2 vs. 12.3 ± 7.5, P = 0.01; 30.0 ± 14.3 vs. 30.0 ± 14.3, P < 0.01). The Shannon-Weiner index for diversity was similar in patients with CRS compared with the control group (1.4 vs. 1.1; P = 0.07) as well as CRSwNP compared to

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**Fig. 2.** Stacked bar-plot of the mean relative abundance of common genera by degree of inflammation (A) and tissue eosinophilia (B). HPF = high-powered field. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

**Fig. 3.** Box-plot representation of the impact of richness on degree of inflammation (A) and tissue eosinophilia (B). HPF = high-powered field. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]
CRSsNP (1.2 ± 0.8 vs. 0.7 ± 0.7; \( P = 0.12 \)). Control patients from the Adelaide center were had a higher mean diversity compared to the Sydney center (1.7 ± 0.7 vs. 1.0 ± 0.6; \( P < 0.01 \)), whereas for CRS patients the mean diversity was not significantly different (1.3 ± 0.9 vs. 0.9 ± 0.6; \( P = 0.08 \)).

### Mean Richness versus Inflammation and Tissue Eosinophilia

Mean richness decreased in more severely inflamed patients (absent [23.2 ± 13.9] v mild [18.1 ± 16.1] v moderate [16.8 ± 12.4] v severe [14.7 ± 10.9]; \( \rho = -0.30, \ P < 0.01 \)). Richness decreased with increasing tissue eosinophilia (absent [23.2 ± 13.9] v < 10 per HPF [19.3 ± 17.2] v 10-100 per HPF [15.9 ± 11.6] v > 100 per HPF [13.4 ± 6.6]; \( \rho = -0.32, \ P < 0.01 \)). These findings are summarized in figure 3. A multivariate linear model was conducted to explore the effect of confounding of the asthma and smoking covariates, as these were differentially-distributed among the three study groups. The results of the model showed no significant confounding of these covariates on the significant association of tissue eosinophilia with richness (Table IV).

| Table IV. Multivariate Linear Model to Explore Cofounding of Asthma and Smoking on the Association Between Richness and Tissue Eosinophilia. |
|-----------------|------------------|
| **Standard Deviation** | **P Value** |
| Control | 23.9 | < 0.01 |
| Tissue eosinophilia | 20.6 | < 0.01 |
| (< 10 per HPF) | 17.7 | < 0.01 |
| Tissue eosinophilia | 15.4 | < 0.01 |
| (> 100 per HPF) | −1.2 | 0.70 |
| Smoker (current/ex) | −2.6 | 0.47 |

\( \text{HPF = high-powered field.} \)

Diversity versus Inflammation and Tissue Eosinophilia. Mean diversity decreased across different degrees of inflammation as seen in figure 4 (absent [1.4 ± 0.7] v mild [1.2 ± 1.0] v moderate [1.2 ± 0.8] v severe [0.9 ± 0.7]; \( \rho = -0.21, \ P = 0.05 \)). Diversity and tissue eosinophilia did not show a significant association (absent [1.4 ± 0.7] v < 10 per HPF [1.1 ± 1.0] v 10-100 per HPF [1.2 ± 0.8] v > 100 per HPF [1.0 ± 0.6]; \( \rho = -0.18, \ P = 0.10 \)).

Prevalence and MRA Versus Inflammation and Tissue Eosinophilia. The impact of prevalence and MRA of common genera on degree of inflammation and tissue eosinophilia was investigated. *Corynebacterium* was significantly more prevalent in patients with mild inflammation (100%) compared to severe inflammation (94.1%) (\( P = 0.03 \)). There was no significant association found between the MRA of *Corynebacterium* and degree of inflammation. Similarly, there was no significant association between the prevalence or MRA of *Corynebacterium* and tissue eosinophilia. The prevalence of *Staphylococcus* was more associated with severe inflammation (78.6% vs. 82.4%; \( P = 0.02 \)) as well as higher levels of tissue eosinophilia compared to lower levels (85.7% vs. 77.8; \( P = 0.03 \)). *Anaerococcus* was more prevalent in lower levels of inflammation (64.3% vs. 29.4%; \( P = 0.05 \)), and *Acinetobacter* had higher prevalence in lower levels of tissue eosinophilia (22.2% vs. 7.1%; \( P = 0.04 \)). There were no other significant associations between the MRA of particular genera and degree of inflammation or tissue eosinophilia, as expressed in Tables V and VI.
DISCUSSION

Unravelling the microbiome in CRS using molecular techniques such as 16S rRNA sequencing is a rapidly developing field. However, observing differences between CRS and healthy mucosa patients does not imply causation. The dysbiosis in this study varied as much with the degree of tissue eosinophilia and severity of inflammation as it did between controls and CRS patients.

Among CRS patients, mean sample richness significantly decreased as severity of disease and the level of tissue eosinophilia increased. Similarly, the microbiome of CRS patients showed reduced richness in comparison to the control group. This supports the results of a meta-analysis conducted by Wagner et al., which found that bacterial richness and diversity is consistently decreased at the species level in inflammatory sinuses. Like Wagner's analysis, a significant association was also observed for mean diversity and severity of inflammation in this study but not tissue eosinophilia. This may represent a type 2 error. Richness among CRS patients also varied across institutions, suggesting the Sydney cohort have a higher severity of disease. This may be explained by differences in the tertiary referral of patients with recalcitrant disease, but the findings are in keeping with the total population. The reduced richness seen in Sydney controls was difficult to interpret and may be a type 2 error. Further analysis with a larger cohort of patients may provide additional insight.

Reduced richness and diversity support the key-stone-pathogen hypothesis whereby certain pathogenic
microbes that usually exist in low abundance have the potential to shape the microbiome in diseased states. Perturbations in the microbial community are postulated to lead to a loss of critical commensal species that under normal conditions would protect from the overgrowth of pathogenic species. However, it remains unclear whether such perturbations are a result of the presence of bacteria inducing a particular pathophysiological process or if a severely inflamed and compromised mucosal barrier merely becomes colonized with particular bacteria. This model is thought to exist in some chronic inflammatory conditions, such as inflammatory bowel disease, but has been established only in pseudo-membranous colitis.

In terms of abundance and prevalence, no single genus was significantly associated with CRS severity. Corynebacterium and Staphylococcus were the most abundant genera across both CRS and control groups, which supports Wagner’s findings in 189 patients (131 CRS and 58 healthy patients). Actinobacter was more prevalent in control patients compared with CRS and was in lower levels of tissue eosinophilia when analyzed among CRS patients. This supports the finding by Cleland et al., who found A. johnsonii to be significantly associated with healthy sinuses. Other genera implicated in the protection against CRS include Propionibacterium and Burkholderia; however, these genera were not found to be significantly abundant or prevalent in the control arm of this study.

Several limitations must be acknowledged in this study. The introduction of microorganisms into specimens at the time of endoscopic surgery is possible. Our sampling method attempted to minimize the chance of contamination by implementing carefully directed swab placement and technique to avoid contact with additional sinonasal structures. Potential for contamination at any stage during cultivation, DNA extraction or sequencing was possible. The high degree of variation in implicated organisms within CRS among the literature is partly related to small sample sizes; however, other factors need to be considered, including geographical differences and disparity in collection methods. To further assess the true microbiome in CRS, large-scale studies are required. Despite this, significant differences in mean richness were found between CRS and controls, as well as varying levels of mucosal inflammatory severity and tissue eosinophilia. To truly identify whether tissue eosinophilia is a predictor of richness, the degree of inflammation needs to be accounted for. Both inflammation and tissue eosinophilia were significantly associated with the loss of richness in the univariate analysis. Due to the high correlation between inflammation and tissue eosinophilia (73% concordance pairs, Kendall tau = 0.82), multicollinearity occurred when both the variables were fitted in the regression on richness simultaneously. The multicollinearity inflated the standard error of the predictor coefficients and as a consequence distorted the inference about the predictor; these factors could not be separated statistically on their influence on the microbiome.

CONCLUSION
The loss of richness and diversity seen in the CRS microbiome appears to be a product of severity of mucosal inflammation and tissue eosinophilia. Whether this dysbiosis is causative or a result of the disease with impaired epithelial integrity remains an area of ongoing research.

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