Seasonal allergic rhinitis affects sinonasal microbiota

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ABSTRACT

Background: *Microbes and allergens can stimulate the nasal mucosa, potentially leading to the development of acute bacterial rhinosinusitis (ABRS). This study was designed to determine if allergen exposure alters the sinonasal microbiome.*

Methods: *We performed a parallel observational study of healthy adults with seasonal allergic rhinitis (SAR; grass or tree, n = 20) or nonallergic subjects (n = 19). Microbiota specimens were obtained by endoscopy from the middle meatus and vestibule before and during the relevant season and were analyzed by terminal restriction fragment length polymorphism analysis. Differences in bacterial microbiota were assessed by standard ecological measures of bacterial diversity. Quality of life and symptom scores were recorded, and nasal lavages for eosinophils were performed.*

Results: *SAR subjects had increased nasal symptoms in season, impaired disease-specific quality of life, and increased nasal eosinophils, compared with no changes in nonallergic subjects. During the season, SAR subjects had a significantly greater variety of organisms in the middle meatus compared with nonallergic subjects ($p < 0.036$) and increased bacterial diversity (Shannon index, $p < 0.013$). We found a significant positive correlation between bacterial diversity in the middle meatus during the season and the nasal lavage eosinophil count of SAR subjects. There were no significant changes in the nasal vestibule ($p > 0.05$, all comparisons).*

Conclusion: *The interaction of allergy and microbiota may affect the sinonasal physiology, with broad implications for several airway diseases. Characterization of the specific organisms involved using next-generation sequencing may clarify the relationship between allergic inflammation and ABRS. This finding may help explain why allergic inflammation predisposes to ABRS.*

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Acute rhinosinusitis (ARS) is one of the most common diseases in the United States, affecting ~32 million adults annually, with a direct cost of over $2.4 billion.[1] Indirect societal costs such as absenteeism and presenteeism, with related reduced productivity, are likely to be even higher, on the order of $25 billion annually.[2,3] Understanding factors that predispose to or exacerbate this condition, therefore, would provide an enormous public health benefit.

Allergic rhinitis is considered a major risk factor for acute bacterial rhinosinusitis (ABRS).[4,5] Although there are several theories to explain the mechanism of this relationship, one unifying idea is that allergic inflammation creates an environment suitable for bacterial infection, *e.g.*, through skewing of mucosal inflammation toward a Th2-type response, disruption of immune defense, alteration in epithelial barriers, or mechanical ostial obstruction. Interestingly, adults with upper respiratory tract infections and allergic rhinitis have increased sinus inflammation by computed tomography than do patients with upper respiratory tract infections who are not allergic.[6] Similar studies in children have found a higher prevalence of ARS in children with allergic rhinitis compared with those who are not allergic.[7] However, the specific underlying cause of increased risk of either ARS or ABRS in patients with allergic rhinitis remains unclear.

Another major paradox in the field is that ~60% of cases of ABRS resolve with no treatment.[8] In many cases, no pathogen is identified in culture, leading to empiric therapy, inappropriate antibiotic use for viral causes, and resultant antibiotic resistance. ARS, therefore, is a major driver of important public health burdens, in terms of human suffering, societal cost, and infection risk *via* the development of resistant organisms.[9] Thus, documenting the association between bacterial flora and allergic inflammation would potentially lead to progress toward understanding this mechanism.

Previous studies have implicated *Streptococcus pneumoniae* and *Haemophilus influenzae* as the main pathogens associated with ABRS,[10] with confirmatory studies in animal models.[11,12] Many cases of ARS do not grow any bacteria when measured with culture-based assays, suggesting the possibility that bacteria that we are unable to cultivate by using conventional microbiological methods may be involved in this disease (although a viral etiology is not precluded in some cases). Disruption of the normal sinus microbial ecosystem by environmental perturbation may therefore result in the emergence of increased numbers of pathogenic flora, leading to disease. Specifically, allergic rhinitis could predispose to ABRS by altering the balance of microbial flora in the sinonasal tract.

Noncultivation-based methods of assessing bacteria are now available to address this question.[13] Importantly, environmental effects on human microbiota (the collection of microbes that live on and inside humans, including the nose and upper airway) remain an underexplored arena with important implications for human health and disease. The fact that microbial cells outnumber human cells by 10 to 1[14] and that, in the gut at least, they provide symbiotic metabolic functions that have been shown to affect physiology[15] and disease,[16] provides proof of principle of this concept. Nevertheless, environmental effects on the microbiome outside of diet have been poorly characterized.[17] In the airway, *e.g.*, little is known about the effects of important environmental influences such as pollution, smoking, temperature, medication use, or allergen exposure.

In this work, we hypothesize that allergens can stimulate the nasal mucosa of allergic subjects to affect the composition of the microbes at the mucosal surface, potentially affecting the development of ABRS.

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Table 1 **Subjects**

	Nonallergic Subjects	SAR Subjects
Sex		
Males/females	12/8	11/8
Race		
Caucasian	14	8
African American	1	6
Asian	5	4
Hispanic	0	1
Age (yr)		
Mean	26	27
Range	18–48	18–39
Allergen sensitivity		
Tree	0	6
Grass	0	13

METHODS

Study Design

We performed a 2-week, single-center, parallel observational study during tree or grass allergen seasons in Chicago in 2011.

Subjects

All subjects were recruited, screened, and enrolled at the Nasal Physiology Laboratory at The University of Chicago, Chicago, IL, before the start of the relevant allergy season. Healthy subjects with a minimum 2-year history of seasonal allergic rhinitis (SAR) and confirmatory positive skin tests to trees or grasses ($n = 19$) were enrolled along with healthy nonallergic subjects who had negative skin tests ($n = 20$). All subjects were not taking any medications other than oral contraceptives for female subjects. C.H. Choi, V. Poroyko, and S. Watanabe contributed equally to this work. The study protocol was approved by the Institutional Review Board of The University of Chicago, and written informed consent was obtained from all subjects.

At the subjects' first visit before the allergy season, we used flocked swabs (Puritan 25-3316 -1PN; Puritan Medical, Guilford, ME) to sample the osteomeatal unit and the nasal vestibule on both sides by using a rigid, 30° nasal endoscope (Karl Storz) for microbiome analysis (see later in text). Subjects then underwent nasal lavage for quantification of eosinophils.[18] A baseline disease-specific Rhinitis Quality-of-Life Questionnaire (RQLQ) was completed before swab or lavage collection each time.[19] Subjects went home with diary cards on which to record nasal symptoms twice daily when the allergy season began, as in prior work.[18] We used the diaries to rank four symptoms (sneezing, runny nose, nasal congestion, and other symptoms) on a scale of 0 to 3 (0 = no symptoms and 3 = severe symptoms). Once respective pollen counts were determined to be elevated for at least 3 consecutive days by the study staff, subjects were contacted to begin their symptom diaries as well as to schedule their second visit in 2 weeks. Median daily total nasal symptom scores were calculated across the 2-week period and were analyzed. When the subjects returned to the laboratory, swabs for microbial evaluation were obtained again, followed by a second nasal lavage. Subjects completed an in-season RQLQ at this second visit. Subject demographics are presented in Table 1.

Subjects were permitted to use over-the-counter rescue oral medicines during the allergy season, which consisted of nonsedating antihistamines, decongestants, and acetaminophen. The use of these medicines was recorded in the symptom diary. If symptoms persisted without sufficient relief, subjects reported to the laboratory for a final visit and were offered treatment with an intranasal steroid.

Microbial Analysis

Genomic DNA was extracted from microbiome samples by use of a commercial kit (Biostic Bacteremia kit; Mo Bio Laboratories, Carlsbad, CA). The samples were then amplified by polymerase chain reaction for the near full length of the V4 region of the 16S rRNA gene with 6-FAM tagged primers: 27F 5′ (6-FAM) AGAGTTTGATCCTGGCTCAG-3′, 1492R 5′-GGTTACCTTGTTACGACTT-3′. After Msp1 digestion, the 16S rRNA gene was then analyzed with terminal restriction fragment length polymorphism (T-RFLP) analysis,[20] with use of a capillary-based automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA), which separated fragments based on size. T-RFLP allows the assessment of bacterial diversity by comparing DNA fragment patterns after restriction digest that vary based on genetic variation of the 16S rRNA gene because of evolutionary differences in genetic sequence among organisms present in the sample.

Outcome Measures

Clinical Measures. To confirm exposure to allergen and development of SAR, RQLQ measures and daily symptom scores were recorded before and in season. Nasal lavage was performed for analysis of nasal eosinophilia.

Microbiome

Sinonasal samples were processed for T-RFLP analysis for determination of differences in bacterial microbiota across groups by three complimentary measures: number of distinct organisms (proxy by number of separate peaks by T-RFLP) and two standard ecological measures of bacterial diversity (Shannon and Simpson's indices).[13] The Shannon index is an ecological measure based on the weighted geometric mean of the proportional abundances of the types present in a sample; the Simpson's index represents the probability that two entities taken at random from a sample represent the same type. Both measures are standard measures in microbiome studies (see the study by Hamady and Knight[13]) These three measures were chosen because of the complexity of the data analysis. Both the fragment length and the abundance of the terminal fragment were measured using GeneMapper software (Applied Biosystems, Inc.) and data were processed as described.[21] For standardization, each peak was expressed as the fraction of the sum of all peaks for each sample. Peak sizes were then calibrated to be comparable across samples. The number and height of the peaks correspond to the number and abundance of bacterial phylotypes represented in the samples. Transformation was performed by expressing the value of each metric as the square root of itself, standard for such analyses. Initial analyses showed no significant differences between the left and right sides; therefore, these data were combined, and the averages were analyzed.

Statistical Analysis

The electropherograms obtained were then analyzed as follows: the two different sites (vestibule and middle meatus) on each individual, the two groups (allergic versus nonallergic), and exposure (before and during allergen season) were compared. Numbers of organisms and diversity measures were compared by nonparametric testing. In this analysis, Simpson's index was inverted such that increased Simpson's index corresponded to a decreased diversity level. Finally, we performed linear discriminant analysis (LDA) to determine which specific operational taxonomic unit (OTU) patterns (here, referring to T-RFLP fragment pattern) were characteristic of each group. LDA is a statistical tool for finding linear combinations of variables that best separate classes of observations. We used LDA to identify OTU patterns that differentiate between the four allergy groups (nonallergic/allergic subjects before/during allergy season) for each of the two sites (vestibule and middle meatus). To adjust for demographic variables, we first fitted linear models for proportional intensities of the

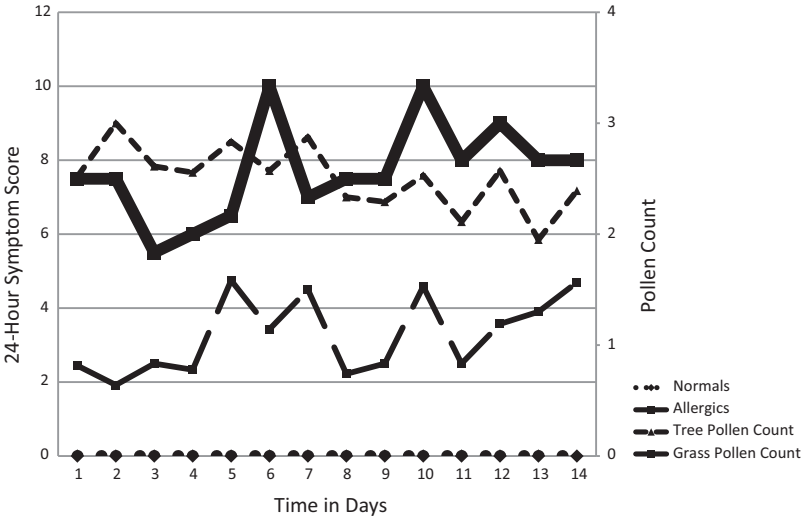

Figure 1. Symptoms and pollen count during the study. The x-axis shows time in days during the allergen season. The left y-axis shows 24-hour total nasal symptom score. The right y-axis shows pollen count (recorded at Chicago National Allergy Bureau station).

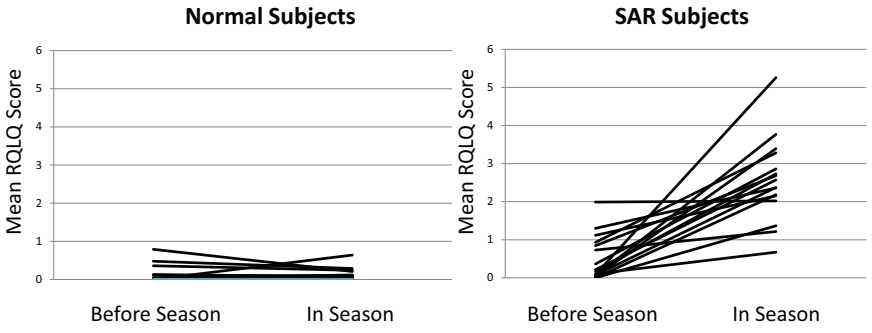

Figure 2. Disease-specific quality of life (Rhinitis Quality-of-Life Questionnaire [RQLQ]) before and during allergy season. SAR, seasonal allergic rhinitis.

OTUs against age, sex, and race. We then performed LDA on the residuals of the linear regressions, instead of the original OTU intensities.

Symptoms and nasal lavage cell counts were analyzed by use of nonparametric tests (Mann-Whitney), and RQLQ was analyzed by use of parametric testing. Correlations were obtained by use of Pearson's test. A value of $p < 0.05$ was statistically significant.

RESULTS

Clinical Measures

The pollen counts were typical for the Chicago tree/grass seasons (Fig. 1). The mean RQLQ scores for the nonallergic subjects stayed below 1 for both visits ($p = 0.473$), whereas all of the mean RQLQ scores for the seasonal allergic subjects rose significantly from baseline, reflecting a worse quality of life during the allergy season ($p < 0.05$; Fig. 2). The mean value during the season was fairly typical for subjects entering an SAR trial.[22] Only one patient used rescue medication (an oral antihistamine) on days 3–5 during the season.

Medians from nasal symptom scores from each group are shown in Fig. 1. Nasal symptom scores were minimal in nonallergic subjects and stayed low during the allergy season. In contrast, subjects with SAR had a notable increase in the total nasal symptom score during the allergy season (Fig. 1), paralleling the recorded pollen levels.

The percentage of eosinophils in nasal lavage from nonallergic subjects was low outside of season and did not change during the allergy season (data not shown; $p > 0.05$). Subjects with SAR showed a clear elevation of median eosinophils in nasal lavage in season compared with before season (data not shown; $p < 0.05$). Thus, the clinical measures show that our allergic subjects showed typical responses for an SAR trial.

Microbial Measures

Numbers of Organisms. We used number of distinct T-RFLP peaks as a proxy for number of different types of organisms as genetic differences in different types of organisms would result in different peaks. There were significantly more different types of organisms present in the middle meatus in subjects with SAR compared with nonallergic subjects during the allergy season ($p < 0.036$) but there were no differences outside of the season ($p < 0.14$). Within-subject comparisons showed no differences in either anatomic site either before or during the allergy season ($p > 0.05$ for all). In contrast, comparisons of the number of different types of organisms present in the nasal vestibule in allergic subjects versus nonallergic subjects showed no differences (Fig. 3 *A*), both before and during the allergy season.

Bacterial Diversity. Bacterial diversity, measured by both the Shannon and the Simpson's indices, reflected global composition of the community of organisms (see Methods section for details) and was elevated in the middle meatus of subjects with SAR during the allergy season (Fig. 3, *B* and *C*; $p < 0.013$ and $p < 0.023$, respectively) compared with nonallergic subjects. In contrast, there were no differences between these groups before the allergy season and there were no significant changes in the nasal vestibule for all comparisons ($p > 0.05$ for all). Within-subject changes comparing before and during the allergy season were not different at either anatomic site in both groups ($p > 0.05$ for all).

Bacterial diversity was then correlated with allergic inflammation. A significant correlation was found between bacterial diversity and nasal eosinophil count during the season in the middle meatus of SAR subjects (number of types of bacteria, $\rho = 0.35$ and $p < 0.033$; Shannon's index, $\rho = 0.46$ and $p < 0.005$; Simpson's index, $\rho = 0.45$ and $p < 0.005$). In contrast, there was no correlation between eosinophilia and

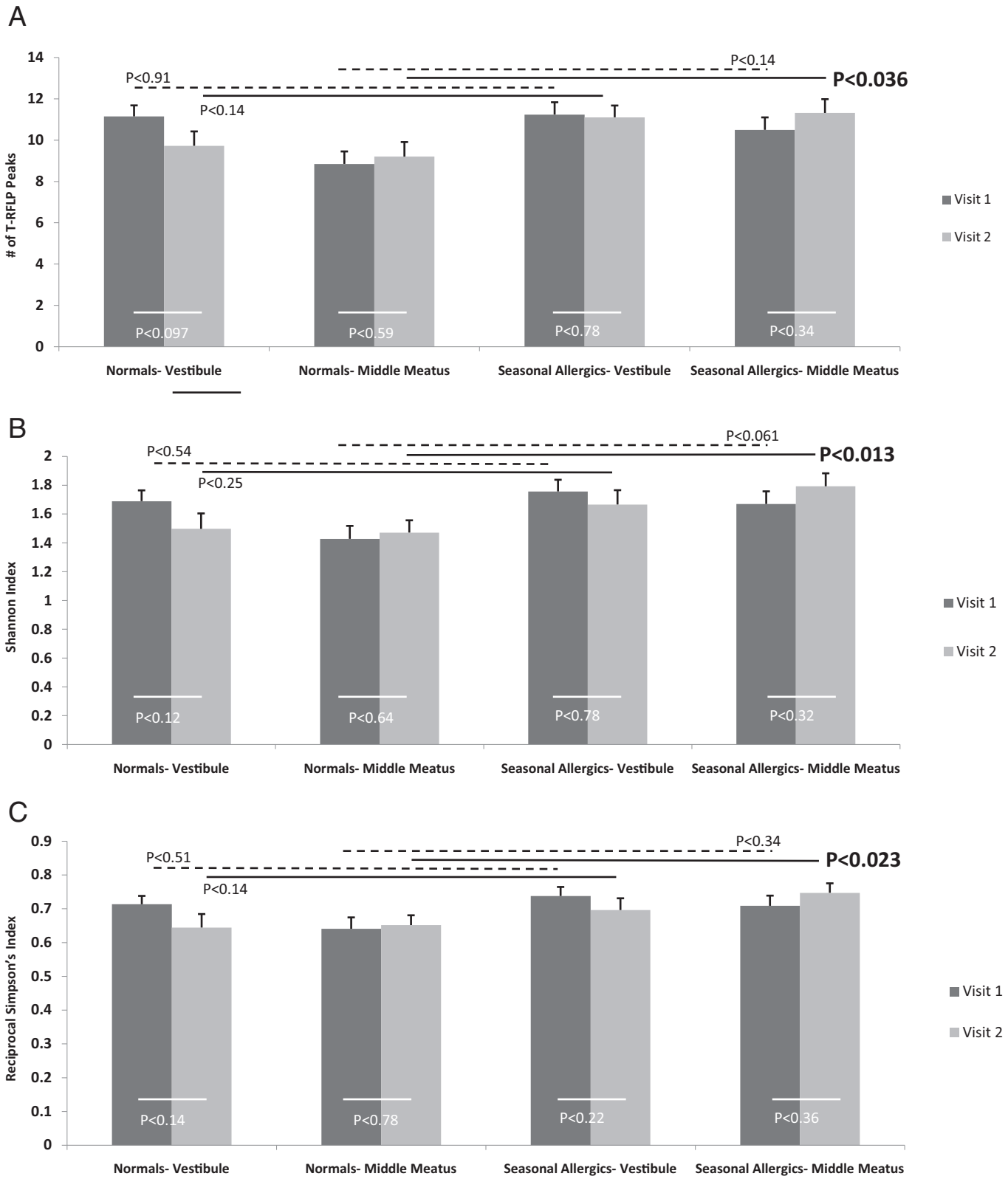

Figure 3. *(A) Increased number of T-RFLP peaks in SAR compared with nonallergic subjects in the middle meatus in season. The y-axis shows the number of T-RFLP peaks. Visit 1 = before season and visit 2 = during allergy season. (B) Increased bacterial diversity in SAR compared with nonallergic subjects in the middle meatus in season. The y-axis shows Shannon diversity index. Visit 1 = before season and visit 2 = during allergy season. (C) Increased bacterial diversity in SAR compared with nonallergic subjects in the middle meatus in season. The y-axis shows reciprocal Simpson's diversity index. Visit 1 = before season and visit 2 = during allergy season. T-RFLP, terminal restriction fragment length polymorphism; SAR, seasonal allergic rhinitis.*

number of types or bacterial diversity in the vestibule ($p > 0.05$ for all).

Linear Discriminant Analysis. Finally, we performed LDA to identify microbiome patterns that differentiate between allergic and nonallergic groups (online supplement Fig. E1). After adjusting for clinical variables for both the vestibule and the middle meatus, we obtained three uncorrelated linear combinations of the relative intensities of the OTUs that explain a large part of the between-group variability (see online supplemental data). Thus, we were able to identify three discriminants or T-RFLP patterns that differentiate our groups (online supplement Table E1), evidence that specific differences in bacterial patterns exist between allergic and nonallergic groups (see online supplemental data).

DISCUSSION

Our data suggest that alterations in the sinonasal microbiome might be one mechanism that predisposes allergic subjects to ABRS. These novel findings suggest a new paradigm in airway biology, *viz.*, that environmental determinants can affect the nasal microbiome, with potential effects on human health and disease.

Allergic subjects showed a decreased quality of life, increased nasal symptoms, and increased nasal eosinophils, confirming allergic disease during the allergy season in these subjects, whereas there were no differences in nonallergic controls. Thus, our human model, in which we used a parallel observational study design, functioned as we intended.

Screening analysis by T-RFLP showed a significant difference in three different measures of bacterial diversity between the microbial communities of subjects with SAR compared with nonallergic subjects. We chose to compare allergic and nonallergic subjects. Thus, our design allowed us to assess whether the allergic state was associated with microbial changes as well as allergen exposure affecting them. Most importantly, subjects with SAR show increased bacterial diversity (in bacterial number and ecological diversity indices) during the allergy season at the anatomic site (the middle meatus) relevant for ARS, but not consistently at a site not thought to be involved in this disease (the vestibule). This is consistent with recent findings showing increased diversity in sputum from asthmatic subjects compared with nonallergic subjects[23] but in contrast to other diseases, mainly those of chronic rather than acute inflammation (see later in text). Additionally, eosinophil counts were correlated with altered sinonasal microbiota in subjects with SAR, but not in nonallergic subjects, suggesting that allergic inflammation, an extrinsic factor caused by seasonal exposure, increases microbial diversity at the opening to the sinuses in sensitized subjects but not in subjects without allergic inflammation. This could potentially explain the increased number of sinus infections in allergic subjects.

Alternative explanations of our findings include innate differences in nasal physiology that are different in allergic compared with nonallergic subjects. One example in support of intrinsic differences in nasal physiology between allergic and nonallergic subjects is water transport, which is markedly different between these two groups.[24] Such mechanisms underlying these phenomena may cause physiological differences in subjects with SAR, leading to alterations in the epithelial milieu that favor particular organisms or sets of organisms. However, this might be expected to occur outside of the allergy season unless synergy with allergen stimulation was required to unmask the effects.

In support of this concept, previous studies have found an increased abundance of *Staphylococcus* on the skin of individuals with atopic eczema.[25] An example of such a mechanism might also be extrapolated from recent work *in vitro* showing that matrix metalloprotease-9, a mediator present in the allergic nasal mucosa,[26] can cleave surfactant protein D, an innate immune molecule involved in mucosal defense, thus predisposing to respiratory infection.[27] Another interesting recent discovery is that pollen itself harbors microbes that can cause inflammatory responses[28]; this could be another explanation of our findings, although the lack of change in nonallergic subjects indicates, at best, that allergic subjects differ in their susceptibility to these phenomena (perhaps by barrier permeability changes at the mucosal surface induced by inflammation). Absence of microbes also causes derangements of the mucosal immune response in animal models, again highlighting the interrelationship of allergic inflammation and bacteria.[29] Further study of these findings in a larger sample is warranted to confirm our findings and to test these hypotheses to determine the mechanism.

The finding of increased sinus bacterial diversity in allergic subjects contrasts other models (primarily in the gut) showing decreased diversity with active infections.[30] This may reflect differences in body site or specifics of disease; bacterial load is considerably higher in the gut, which also has a different ecosystem than the respiratory tract. However, our data do support the concept of allergy changing the sinonasal microflora perhaps *via* expansion of the sinonasal microbiome by creating conditions to allow a greater diversity of bacteria to exist. Such a disruption then might set the conditions for a "second hit" to cause acute infection, with subsequent predominance of a pathogenic organism. We speculate that this could be caused by reductions in protective flora, changes in virulence of colonized organisms, alterations in physical conditions (*e.g.*, hypoxia related to ostial obstruction), or local changes in immune defense. Our study design did not allow us to address these possibilities. We note that none of our subjects developed acute sinusitis and there was no frank pus present on follow-up endoscopy during swab collection at visit 2, consistent with the fact that, despite increased risk of this condition in allergic subjects, only a small fraction of patients develop a sinus infection, similar to other risk factors (*e.g.*, viral infections[31]). We also acknowledge that not all in the field consider allergy a risk factor for the development of a sinus infection. Additional studies of bacterial diversity before and during episodes of acute sinusitis in allergic subjects and assessment of subsequent risk of infection can address this issue.

Finally, perhaps the most interesting finding is that specific patterns of organisms are associated with allergic status and season by LDA. Although sequencing of the 16S polymerase chain reaction products will provide information on the specific underlying organisms, the findings from this study provide evidence that a particular microbial signature in the nose is associated with allergic status and allergic exposure. The driver(s) of this microbial community is presently unknown, as is whether these microbes can influence the immune function of the nose itself. One can envision a complex interplay of signals between mucosal immune responses and the commensal bacteria in the nose. This complexity extends to the physical environment and population level, because geographic environmental biodiversity has been linked to the skin microbiome and the presence of atopy.[32]

The main limitations of this study stem from a small sample size and from derivation of data at a single-center study. All of the seasonal allergy subjects were either allergic to trees or grass, both of which are found ubiquitously; we expect that studies with other allergens would show similar results. Additionally, the small sample size makes it possible that some interesting suggestions present in our data (*e.g.*, nonsignificant lower diversity in the middle meatus in nonallergic subjects compared with all other comparisons) may require larger samples to confirm. We do not feel that allergic exposures in the preseason could have affected our results: subjects reported major symptoms consistent with the relevant season (tree or grass in Chicago), and only four were sensitized to both allergens (one studied in grass season; three studied in tree season). Sensitivity to multiple allergens is a potential problem, however, in all studies of SAR. Additionally, they were asymptomatic out of season, so perennial allergens were likely irrelevant.

There are several implications of these data that could translate to clinical medicine and provide information on airway physiology.

Alterations in commensal bacteria in allergic subjects could cause effects on sinonasal epithelia, enhancing or blocking immune responses to allergen or other stimuli, with effects on lower airway disease. Controlling inflammation may prevent change of sinonasal microbiota and decrease number of acute bacterial sinus infections. For example, steroids can effectively reduce bacteria-induced mucin expression in the airways[33] and can mitigate the proinflammatory responses to pathogens.[34] Dissecting these mechanisms would have broad implications for a range of diseases related to SAR, including asthma, perennial allergic rhinitis, and nonallergic rhinitis, in addition to ARS.

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