The Nasal and Sinus Microbiome in Health and Disease

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Abstract There has been great interest in unraveling the complex inter-relationships between microbes and humans as they relate to human health and disease. This review will focus on recent advances in the appreciation and understanding of these relationships in terms of the upper respiratory tract, specifically the nose and paranasal sinuses.

Keywords Nasal · Microbiome · Bacterial · Chronic rhinosinusitis

Abbreviations

CNS	Coagulase-negative Staphylococcus
CRS	Chronic rhinosinusitis
CSLM	Confocal scanning laser microscopy
FISH	Fluorescence in situ hybridization
HC	Healthy control(s)
hBD	Human beta defensin
IESA	Intraepithelial *Staphyloccus aureus*
MRSA	Methicillin-resistant *Staphylococcus aureus*
NO	Nitric oxide
OTU	Operational taxonomic unit
SEM	Scanning electron microscopy
T2R38	A bitter taste receptor
TAS2R38	A bitter taste receptor polymorphism
TEM	Transmission electron microscopy
TLR	Toll-like receptor

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Introduction

The healthy human microbiome is the consequence of highly regulated host-microbial interactions. These interactions have been a mystery for years and are only recently being unraveled in mechanistic terms. The very existence of "commensal" bacteria in the gut, oropharynx, and nasal cavity implies that these bacteria subserve some useful purpose. This has now been confirmed with respect to such processes as digestion of food and development of immune tolerance toward certain autoimmune and allergic diseases. The confirmation of active regulatory processes governing the relationship between commensal bacteria and the host also implies that disturbances in this regulation may lead to disease. There is now some data to support this assertion in upper airway diseases, such as chronic rhinosinusitis (CRS).

The evolving view of bacterial involvement in CRS has expanded beyond that of infection with individual pathogens to considerations of increased bacterial burden, biofilm formation, intracellular bacteria, and alterations in the microbial community ("microbiome"). "Unbiased" analysis of the microbiome has gained much recent attention as a novel means of studying host-microbial relations in various anatomic sites. This has been made possible by the advent of broad platform molecular techniques, including methods that allow for culture-independent identification of the microbiome based on microbial RNA.

This review will summarize knowledge of host-microbial interactions elucidated in relation to normal sinus physiology and in relation to CRS and focus on studies of the bacterial microbiome, since there is limited information regarding the fungal microbiome and virtually no studies of the viral microbiome in CRS and limited data from healthy normal controls.

Defining the Microbiome

The microbiome (or "microbial community") is the universe of cultivable and uncultivable microorganisms present in a specific ecologic niche, such as the gastrointestinal tract or the nasal mucosa. Analysis of the human microbiome offers a novel means of studying host-microbial interactions in various anatomic sites. This has been made possible by the advent of broad-platform molecular techniques, including microarrays and sequencing methods based on microbial RNA that allow for identification of culturable and non-culturable organisms in the community. Techniques to study the microbiome are rapidly evolving but include methods to quantify bacterial diversity, representation of specific bacterial species, bacterial "load" or "burden," and metabolic pathways used by the microbiome.

Terminology and Methodology for Assessing the Microbiome

Within a microbial community, bacteria are typically described in terms of operational taxonomic units (OTUs) as well as in terms of specific taxa, genera, or species. Microbial abundance is simply a quantification of the amount of bacterial nucleic acid present in a community. Alpha diversity is an estimate of the within-community diversity, i.e., the number of species and the proportion of each species in the community. Beta diversity is an estimate of the diversity in samples from the same habitat among different subjects. Microbial diversity is also quantified in terms of richness and evenness. Richness represents the number of species present in a sample with greater numbers of species signifying greater richness. Evenness is a measure of the relative abundance of the different species making up the richness in a sample. Various indices are used to characterize species diversity in a community, such as the Shannon diversity index or "H" which takes into account species abundance and evenness in the community.

Methodologies used to assess the microbiome include the following: (a) phylogenetic oligonucleotide arrays (POAs), (b) 16S ribosomal RNA (rRNA) gene clone libraries, (c) analysis of functional gene arrays (e.g,. the GeoChip), (d) next-generation sequencing technologies, (e) sequencing by mass spectrometry (e.g., Ibis T5000 analysis platform), and (f) random "shotgun" metagenomics.

Phylogenetic oligonucleotide arrays such as the PhyloChip® use short oligonucleotides designed against a phylogenetic marker gene, such as the 16S rRNA gene. Multiple DNA probes on the PhyloChip are used to identify sequence variations in the 16S rRNA gene, and each probe is paired with a single-base mismatch control probe to minimize the effect of nonspecific hybridization (http://esd.lbl.gov/research/facilities/andersenlab/phylochip.html). Amplified DNA or RNA from the 16S rRNA gene of a sample hybridizes to exact DNA sequence matches from specific 25 nucleotide probes. In the PhyloChip, a taxon is broadly defined as a cluster of 16S rRNA gene sequences with <3 % divergence. The PhyloChip G3 is able to categorize all known bacteria and archaeal OTUs into over 50,000 taxa using 1,100,000 probes to identify variations in the 16S rRNA gene.

Gene clone libraries are typically generated from samples by PCR amplification and purification of 16S rRNA genes using a bacteria-specific 16S primer set. Amplified products are then pooled, purified, and then ligated and cloned using standard cloning techniques. Individual cloned 16S rRNA gene sequences are then amplified using specific primers and sequenced. Sequences with a minimal length of 500 base pairs are then grouped into taxa based on >97 % sequence identity (corresponding to the definition of a taxon in the PhyloChip) and then aligned using software programs for comparison to known sequence databases to identify the best-named matches [1]. Clone libraries are not ideally suited for in-depth sampling of microbial communities. By comparison, the PhyloChip is orders of magnitude more sensitive in its ability to identify diversity and for detecting low-abundance taxa [1].

Functional gene arrays (FGAs detect selected genes or gene families that encode key enzymes diagnostic for a certain metabolic pathway [2•]. For example, the GeoChip is based on 8 degenerate probes for the 16S rRNA gene used for positive controls, and hundreds of unique probes designed from hypothetical genes of 7 sequenced genomes of hyperthermophiles used as negative controls [3•]. In GeoChip 3.0, 292 key enzymes/genes (with 27,812 probes) are used to target a variety of microbial-mediated processes, including enzymes/genes involved in the carbon cycle, nitrogen cycling, sulfur cycle, phosphorous cycling, energy metabolism, and various resistance mechanisms. GeoChip 4.0 contains genes that target 539 functional gene families with 120,054 probes. These arrays are best suited to diversity analysis of selected microbial guilds, whereas PhyloChip and next-generation sequencing methods are best suited for detecting changes in the taxonomic composition of microbial populations.

Next-generation sequencing (NGS) technologies represent a major advance over earlier conventional Sanger sequencing. The main principle involves DNA molecules that are being sequenced in a massively parallel fashion in a flow cell. NGS methods allow each clonal template or single molecule in a sample to be independently sequenced and counted among the total sequences generated [2•]. It has been shown that sequences of 500–700 bp are required for phylogenetic

discrimination at the species levels, although not all commercial NGS methods sequence to this depth. This was a limitation of some NGS methods, but newer methods have overcome this problem. Various regions of the 16S gene (e.g., V1, V2, and V3, V4–V6, V7–V8) can be sequenced with some advantages or disadvantages for each region depending on the application and the heterogeneity of the community being studied. Numerous NGS technologies have been developed [2•].

The Ibis T5000 analysis platform uses a panel of broad-range primers to amplify PCR products from broadly conserved regions of the bacterial genome, following which mass spectrometry is used to determine the base compositions of the amplified PCR products [4]. A software program matches base composition with specific bacterial species. Although this technique was developed primarily to identify bacterial pathogens, it has been used to study the CRS microbiome (discussed below).

Random shotgun metagenomics is a method by which total DNA is isolated from a sample and then sequenced to profile of all genes within the community [5]. This approach has been applied to many environmental samples, such as marine ecology but has also been applied to the Human Genome Project (see below). One potential advantage of this approach is the ability to identify specific bacteria based on unique genes other than 16S rRNA gene, but the tradeoff is shorter "read" lengths for each distinct 16S rRNA gene present in the sample.

The Normal Anterior Nares Microbiome The Human Microbiome Project (HMP) laid the foundation for systematic evaluation of microbial diversity in distinct human anatomic locations [6, 7••]. For analysis of the microbiome of the anterior nares, one specimen from the nares was collected by swabbing each anterior naris and pooling for extraction. Samples were subjected to 16S ribosomal RNA gene NGS (pyrosequencing), and a subset were shotgun-sequenced for metagenomics [7••]. 16S data processing and diversity estimates were performed using established software platforms, and metagenomic data were similarly taxonomically profiled. Data, protocol details and full methodology is available at http://hmpdacc.org and in published studies [7••].

Examination of the anterior nares microbiome of normal subjects (using pooled swabs of each naris) confirmed the absence of National Institute of Allergy and Infectious Diseases (NIAID)-defined class A–C pathogens above 0.1 % abundance except for *Staphylococcus aureus* and *Escherichia coli*, but near ubiquity and broad distribution of opportunistic "pathogens" as defined by PATRIC (http://patricbrc.org/portal/portal/patric/Home) [8], including organisms such as *Staphylococcus epidermidis*, *S. aureus*,

Propionibacterium acnes, *Klebsiella pneumoniae*, *E. coli*, *Streptococcus pneumoniae*, and many others.

P. acnes and *S. epidermidis* were the most abundant species in the nasal microbiome (42.5 and 12.7 %, respectively). The abundance of *S. aureus* was 5.0 %. In total, 56 of 327 PATRIC pathogens were detected in the healthy nasal microbiome (at >1 % prevalence of >0.1 % abundance). Methicillin-resistant *S. aureus* (MRSA) presence was detected in 4 % and 29 % of skin and nasal samples, respectively, whereas the close phylogenetic relative *S. epidermidis* (considered commensal) was detected in nearly all skin samples and 93 % of nares samples. In contrast, *Pseudomonas aeruginosa* was completely absent from both the skin and nasal habitats (0 % at this level of detection).

The HMP also studied the normal nasal microbiome by analyzing the relative abundances of pathways in community metagenomes. These were found to be much more constant and evenly diverse than microbial abundances confirming that the metagenome is an ecological property of each human microbiome studied.

Comparative Studies of the Nose Versus Throat

Lemon et al. performed a comparative study of the normal nasal cavity and oropharynx microbiome of 7 healthy adults, ages 26–45 who had not taken antibiotics in the previous 2 months using 16S pyrosequencing (PhyloChip®) [1]. Material for analysis was gathered by swabbing one nostril and the posterior wall of the oropharynx of each participant. At both sites, they found an inverse correlation between the prevalence of Firmicutes, which includes important pathogens such as the Staphylococcaceae family, and another phylum. In the anterior nares, there was an inverse correlation between Firmicutes and the Corynebacteriaceae and Propionibacteriaceae families (both Actinobacteria), whose members are more commonly benign commensals, suggesting potential antagonism between these groups [1].

The Nose in Disease

The microbiome of the "diseased" anterior nares has been studied primarily with regard to Staphylococcal carriage [9•, 10, 11•, 12•].

The "Healthy" Middle Meatus and Sinus Cavity Microbiome

A cross-sectional analysis of 28 consecutive healthy adults sought to characterize the microbiome in the middle meatus in the absence of chronic inflammation or antibiotic exposure [13•]. Patients undergoing endoscopic sinus surgery from

septoplasty, skull base, or orbital procedures were analyzed. Samples were collected at the onset of surgery from deep within the middle meatus, and swabs were used to obtain material. Use of intranasal medication, antibiotics, immune, inflammatory, or allergic conditions and the presence of an abnormal sinus CT scan were all exclusion criteria.

Quantitative PCR using pan-bacterial primers confirmed the presence of bacteria in all samples. Population analysis was performed with broad range PCR and pyrosequencing of bacterial 16S rRNA gene sequencing. Firmicutes, Proteobacteria, and Actinobacteria were detected in all subjects. *Bacteroides* spp. were detected in 83 % of subjects but were more scarce in abundance (~2.5 % of rRNA sequences). *S. aureus* was present in 67.9 % of subjects at a relative abundance of 8.3 %. Several PATRIC pathogens were present as well.

Demographic information was applied to inter-individual variability in microbiome composition, and subject age and geographic residence (zip code) were correlated with different constituencies at the species level. Smoking also demonstrated a qualitative alteration in the microbiome composition based on species distribution with the abundance of the phylum Firmicutes being enriched in smokers and Actinobacteria being decreased.

Data regarding the "healthy" sinus microbiome comes from the study of Abreu et al. [14••]. In this study, the authors obtained sinus brushings from 10 healthy controls (patients undergoing skull-based surgery for a non-sinus indication) for comparison to brushings obtained at the time of functional endoscopic sinus surgery (FESS) in patients with CRS. The healthy sinus microbiome is discussed below in comparison to the microbiome of CRS. A key finding from this study was that the taxon Lactobacillales (that includes *Lactobacillus sakei*) appeared to play a protective role in the normal sinus microbiome.

The Sinus Cavity in CRS

Chronic rhinosinusitis (CRS) is recognized as a persistent inflammatory disorder of the paranasal sinuses in which the presence of bacteria can be demonstrated by microbial culture of sinus secretions or molecular probes of secretions or tissue samples. One paradigm of CRS is that persistence of bacteria on sinus mucosal surfaces is an important predictive factor of disease that responds poorly to surgical and/or medical intervention (reviewed in [15•]).

Bacterial Involvement in CRS Using Conventional Culture and Biofilm Detection Techniques

Recent studies in adult CRS patients employing conventional intraoperative sinus cultures with simultaneous analysis of biofilm reported positive cultures in 72.6–80 % of cases with

a predominance of *S. aureus* and *P. aeruginosa* in the isolates [16–18]. The frequency with which anaerobic organisms have been recovered from adult CRS patients has varied widely. In studies employing special techniques to optimize their recovery, several anaerobic species were isolated, including pigmented Prevotella, Fusobacterium, Bacteroides species, and Peptostreptococcus species [19, 20]. The highly variable yield of anaerobic cultures in CRS patients highlights a limitation of conventional cultures at understanding the complete picture of the CRS microbiome. However, even more generally, it is estimated that 20–60 % of the human microbiome, depending on the body site, is uncultivable [6]. This fact plus the insight that can be gained by studies of the metabolic properties of the microbiome provides strong justification for moving the science of discovery of CRS pathomechanisms beyond that gleaned from conventional cultures.

Studies of bacterial biofilm have contributed to a greater understanding of CRS pathogenesis. Biofilm formation is an important survival mechanism for microorganisms through attachment to surfaces, including viable tissue [21]. Biofilm formation on sinonasal mucosal surfaces was first described in 2004 [22] and later confirmed in several studies (reviewed in [15•]). Studies employing either scanning electron microscopy, transmission electron microscopy, or confocal scanning laser microscopy (excluding one study confined to patients with NP), have collectively reported the prevalence of biofilm in the CRS as 56.3 %. The extent to which bacteria in biofilms is cultivable versus uncultivable is, in general, still not well studied.

The presence of intracellular (intraepithelial) *S. aureus* (IESA) in the sinus epithelium was also reported by Corriveau et al. 2009 using a peptide nucleic acid-fluorescence in situ hybridization (FISH) assay [23]. In this study, IESA were found in the epithelium of CRS patients but also seen in some healthy controls raising question of the pathologic significance. In a recent study using a similar *S. aureus* fluorescence in situ hybridization probe with propodium iodide counterstain and confocal scanning laser microscopy (CSLM), IESA were detected in 56 % of CRS patients undergoing sinus surgery but in none of 8 healthy controls [24•]. Although it is unknown whether the presence of IESA has any prognostic or pathologic significance beyond that afforded by the presence of biofilm in CRS patients, intracellular bacteria such as IESA represent another microbial compartment or "niche" inaccessible to conventional culture that may play a significant role in CRS pathogenesis. This niche is amenable to study by molecular techniques used for microbiome analysis but requires processing of tissue.

The Microbiome in the Paranasal Sinuses in CRS

In an attempt to more comprehensively characterize the microbial constituents in CRS and compare them to species

identifiable by conventional culture techniques, authors have begun using modern techniques for microbiome analysis in the setting of CRS.

Stephenson et al. [18] employed pyrosequencing using modified 16S eubacterial primers to amplifying the 600 bp region of 16S rRNA genes [18]. Bacterial genetic material was obtained through mucosal biopsy of the anterior ethmoid sinus. Culture material was gathered through a swab directed to the area of maximal purulence or the surface of the maxillary sinus in the absence of purulence. Eighteen CRS patients (83 % with nasal polyps) were compared to 9 control subjects. Conventional cultures identified bacterial growth in 82 % of CRS patients (the % positive in control subjects was not mentioned) with an average of 1.4 isolates per sample. In contrast, pyrosequencing was positive in 100 % of CRS patients, with a mean of 10 isolates/sample and range of 1–20. The most prevalent organisms in CRS patients were anaerobic bacteria, Diaphorobacter, and Peptoniphilus, whereas coagulase-negative Staphylococcus was identified in 50 % of samples. S. aureus, Corynebacterium, and Propionibacterium were also identified in control subjects. In general, this study highlighted the presence of anaerobes in samples from patients with CRS undergoing surgery and confirmed the presence of PATRIC pathogens in the microbiome of the normal controls.

Feazel et al. obtained middle meatus swabs and compared bacterial culture and pyrosequencing results in 15 patients with CRS (including 2 with NP) versus 5 healthy controls [25••]. Standard bacteriologic cultures were positive for all 15 CRS subjects and 5 controls, with an average of 2.8 isolates reported per subject (range, 1–5). No statistically significant differences were found between the number of isolates obtained from CRS patients and controls. The most commonly cultured organisms were coagulase-negative staphylococci (75 %), S. aureus (50 %), and P. acnes (30 %). Using pyrosequencing, the most prevalent species detected were coagulase-negative staphylococci (100 %), Corynebacterium spp. (85.7 %), P. acnes (76.2 %), and S. aureus (66.7 %). In general, cultured organisms comprised <15 % of the organisms identified by sequencing, but bacteria identified by culture were identified by pyrosequencing in most cases. Some of the nonspecific CRS culture results, such as "mixed Gram-negative rods," were associated with pyrosequencing detection of moderate mixed anaerobes representing 75 % of the total sequences identified. This is in agreement with Stephenson et al. [18] regarding the high prevalence of anaerobes in the microbiome of CRS patients. These studies also call into question the validity of earlier culture-based investigations that reported a low prevalence of anaerobes in CRS samples owing to methodologic flaws related to sample collection and/or anaerobic culture conditions.

In the study by Feazel et al., S. aureus was selectively enriched in CRS patients by culture (10 of 16 CRS versus 0 of 5 controls). DNA sequences representative of S. aureus were detected in a similar percent of CRS patients (11 of 16, 69 %) and controls (3 of 5, 60 %), but a higher abundance ("bacterial burden") of S. aureus sequences was found in CRS patients. Both antibiotic exposure and asthma were associated with reduced microbial diversity and increased S. aureus abundance.

A study by Boase et al. sought to determine the relationship between disease manifestations, microbial biodiversity, and microbial abundance of mucosal microorganisms in patients with CRS (N=35) and healthy control subjects (N=6) using the Ibis T5000 analysis platform and conventional bacterial and fungal cultures [26•]. The key finding in the study is that there is an increased microbial burden of S. aureus in CRS, confirming results from the study by Feasel et al. [25••]. The number of S. aureus genomes per sample (Ibis) was significantly higher in those specimens testing positive for S. aureus by biofilm analysis (FISH) or conventional culture demonstrating reasonably good agreement between methods.

In a study by Abreu et al., sinus tissues from 7 CRS patients were obtained by sinus brushing at the time of functional endoscopic sinus surgery (FESS) and compared to 10 healthy controls (patients undergoing skull-based surgery for a non-sinus indication) [14••]. Routine bacteriologic cultures from the tissues were not performed. Samples were analyzed using the standardized phylogenetic microarray, the 16S rRNA PhyloChip (Affymetrix G2 PhyloChip) using 250 ng of purified pooled 16S rRNA amplicon per sample generated using 27F and 1492R universal primers to compare the presence and relative abundance of approximately 8900 bacterial taxa (OTU).

The authors claim their results were not affected by prior antibiotic use, but in fact, 6 of 10 CRS patients had received antibiotics prior to sinus surgery, whereas only 1 of 10 controls had received an antibiotic. This conclusion was based on a comparison of the 6 patients receiving prior antibiotics compared to the 4 CRS patients who had not. Specifically, 2 CRS patients who had not received long-term antibiotics exhibited significantly lower sinus microbial diversity compared to the other CRS patients who had received long-term prophylactic antibiotic administration.

The "bacterial burden" was found to be similar in CRS and healthy control subjects. However, 1482 bacterial taxa were found to be in lower abundance in CRS. These data were also expressed in terms of classic microbiome parameters, including the following: microbial richness, evenness, and microbial diversity, all three of which were significantly reduced in CRS. The most significant reduction in abundance was found in Lactobacillales (the taxon that includes L. sakei). In contrast, only a single taxon was overrepresented in CRS with the representative organism Corynebacteium tuberculostericum (Ct) being found with increased abundance. The relative abundance of Corynebacteriaceae correlated with CRS

symptom severity as assessed by the Sino-Nasal Outcome Test 20 (SNOT-20).

Certain taxa were not discriminatory of CRS versus healthy controls. These included several known pathogens, including known pathogenic members of Pseudomonadaceae, Lachnospiraceae, Ralstoniaceae, Mycobacteriaceae, and Helicobacteriaceae. However, 1482 taxa were detected with relatively low abundance in CRS, including 228 taxa that were significantly ($P<0.05$, Welch's t test) correlated with lower SNOT-20 scores (indicative of healthy sinuses). Among these "protective" taxa were members of the Lactobacillaceae, Enterococcaceae, Aerococcaceae, and Streptococcaceae.

In companion experiments, the authors created a mouse model of acute bacterial sinus infection in C57Bl6/J mice and confirmed that depletion of the sinus microbiome with amoxicillin/clavulanic acid for 5 days followed by instillation of *Corynebacterium tuberculostearicum* contributed to the development of marked goblet cell hyperplasia and mucus hypersecretion, features reminiscent of human CRS. By comparison, instillation of large numbers of *C. tuberculostearicum* in the presence of a replete sinus microbiome elicited only a modest increase in mucin-secreting goblet cells. The authors further showed using this model that instillation of *L. sakei* alone failed to induce goblet cell hyperplasia, and co-instillation of *L. sakei* in conjunction with *C. tuberculostearicum* was sufficient to protect against the goblet cell hyperplasia induced by *C. tuberculostearicum* alone. These studies support the interesting paradigm of CRS as a disease characterized by reduced microbial diversity, depletion of certain "protective" bacterial taxa, and emergence of other "disease-producing" taxa.

Interestingly, in the Ramakrishnan study characterizing microbiome constituents at the site of the middle meatus in healthy adults, Lactobacilli were a very minor component with relative abundances ranging from 0.0 to 0.3 % of rRNA sequences. Furthermore, in that study, Corynebacterial species had a prevalence of 92.9 % among the healthy subjects and *C. tuberculostearicum* was the numerically dominant corynebacterial species (2.0 % relative abundance) [13•].

Further evidence for a correlation between increased abundance and decreased bacterial diversity in CRS comes from Choi et al. [27•]. They sought to analyze not only bacterial composition but also bacterial extracellular vesicles (EVs) in the CRS microbiome. Outer membrane EVs from certain bacterial pathogens contribute to the virulence by delivering virulence factors, such as beta-lactamase, over long distances. EVs also carry RNA and DNA and have been shown to facilitate horizontal gene transfer among bacteria [28, 29]. A previous study by Choi et al. demonstrated that EVs from *S. aureus* induce inflammatory responses in airway epithelial cells and alveolar macrophages (production of IL-6) and

induce neutrophilic pulmonary inflammation after ovalbumin challenge, mainly in a Toll-like receptor 2 (TLR2)-dependent manner [30•].

In this study, Choi et al. performed a metagenomic analysis of nasal lavage (NAL) fluids from 3 'healthy controls', 3 patients with CRS with nasal polyposis (CRSwNP), and 3 patients with CRS without nasal polyposis (CRSsNP). Control patients ($N=3$) had septal deviation but no sign of mucosal inflammation on nasal endoscopy and computed tomography (CT). All patients were adults. NAL fluid samples were obtained during endoscopic sinus surgery by flushing each nostril with 5 ml of isotonic saline and collecting the fluid with a suction collector. Differential centrifugation and ultracentrifugation were used to obtain bacterial and EV portions from the fluids.

Metagenomic analysis (conventional bacterial 16S ribosomal RNA pyrosequencing) confirmed the presence of an increase in bacterial burden quantitatively but a decrease in diversity among CRS patients compared to healthy control nasal lavage samples both in the bacterial and EV fractions. Similar to previous studies, they found enrichment of specific species as well and demonstrated an increase in *S. aureus* abundance or "burden" from both the bacterial and EV fractions among samples from CRS with polyps compared to CRS without polyps.

Specifically, the authors found a higher bacterial abundance or "burden" in CRS samples. Despite this, they found a decrease in alpha diversity (number of OTUs) in both CRSwNP and CRSsNP, although the greatest reduction was seen in CRSsNP. However, compared to controls, this difference was not statistically significant. Analyzed by the Shannon diversity index, there was a statistically significant decrease in diversity in CRSsNP in both the bacterial and EV portions of the samples.

There was an increased abundance of bacteria (sequences) in CRS (mean 3.6-fold higher in the bacterial and 3.4-fold higher in EV) compared to control including all five phyla (Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes, and Fusobacteria). However, the taxonomic composition chart revealed that Bacteroidetes decreased, from 25.42 to 7.37 %, whereas Proteobacteria increased from 14.3 to 26.5 %, in CRS patients compared with controls. As a caveat, the data in Figs. 2 and 3 in [27•] do not appear to be consistent with regard to the percent distribution of the phyla across bacterial and EV samples. Also, phylogenetic differences were not as dramatic in the EV samples and were not statistically significant.

At the genus level, the bacterial and EV compositions of *Prevotella spp.* (in the phylum Bacteroidetes) was more consistently decreased from 19.8 to 3.5 and 11.0 to 3.3 %, respectively, in patients with CRS compared with controls. In contrast, the bacterial and EV compositions of *Staphylococcus* spp., (in the phylum Firmicutes) increased, from 1.0 to 46.1 %

and from 15.0 to 39.1 %, respectively, in the CRS patients versus controls.

Additionally, bacterial and EV were compared in patients who had CRSwNP versus CRSsNP. At the genus level, *Pseudomonas* spp. was increased in CRSwNP compared with CRSsNP. In the EV composition, *Pseudomonas* spp. and *Propionibacterium* spp. were increased in CRSwNP versus CRSsNP. Finally, at the species level, *S. aureus* was increased significantly in CRSwNP compared with CRSsNP in the bacterial and EV samples (8.8 versus 0.05 and 4.0 versus 0.5 %, respectively). The results of this study are consistent with previous reports indicating an increased bacterial abundance in CRS and a decrease in bacterial diversity [14••, 26•]. An increase in abundance of *S. aureus* is also consistent with previous reports [18, 25••, 26•].

The most novel aspect of this study was the analysis of the microbial composition of EVs in nasal fluids. The presence of bacterial EVs in nasal fluid should not come as a surprise, since EVs have been found in respiratory tract fluids. The interesting possibility that EVs may serve a functional role in CRS remains to be explored and, ultimately, will require analysis of EVs in sinus (as opposed to nasal) secretions and analysis of the relationship between EVs and the inflammatory response as well as the presence or absence of bacterial biofilms.

Finally, data from Aurora et al. offer a contrasting picture [31•]. Using next-generation sequencing, they compared the microbiomes of 30 CRS patients and 12 controls undergoing surgery. While they did confirm the previous findings of increase microbial burden quantitatively among the CRS patients, they found no significant differences in composition of the microbiome from a qualitative standpoint between patients and controls. They performed a phenotypic analysis by characterizing immune cells isolated from the nasal lavage as well as functional studies stimulating peripheral blood lymphocytes with lavage material to propose that, in fact, the defect in CRS patients was a hyper-responsiveness to normal microbial constituents.

In comparing the published studies of the CRS microbiome, there are significant differences in over- or under-representation of taxa or species not easily reconciled.

What Lessons Have We Learned from the Anterior Nares and Sinus Microbiomes?

The key findings from studies of CRS are summarized in Table 1. In general, the microbiome of CRS is characterized by less richness, evenness, and diversity compared to healthy controls. While the total bacterial burden in CRS is similar to controls, an increase in burden for certain organisms, most notably, *S. aureus* has been observed in several studies. Certain bacterial taxa or species may be protective against

Table 1 Key findings from studies of the microbiome in CRS patients

Less richness, evenness, and diversity in CRS than controls

Total bacterial burden in CRS is similar to controls

Increased burden for certain organisms (e.g. *Staphylococcus aureus*)

Certain bacterial taxa or species may be protective (e.g., the order Lactobacillales or the species *Lactobacillus sakei*), although this has not been supported in all studies

Some taxa or species may be uniquely harmful (e.g. the family Corynebacteriaceae and the species *Corynebacterium tuberculostearicum*), although this has not been supported in all studies

the development of CRS, most notably those of the order Lactobacillales and the species *L. sakei*, although this has not been supported in all studies [13•]. Conversely, certain taxa or species may be uniquely harmful, most notably members of the family Corynebacteriaceae and the species *C. tuberculostearicum*, although these observations were derived from one study and were not observed in other studies. Clearly, more studies are needed, particularly in larger, well-characterized CRS patient populations. Furthermore, more work is needed to assess the microbiome in relation to the presence of biofilm or intraepithelial bacteria.

It could be hypothesized that the healthy middle meatus and sinus cavity microbiome may be heavily dependent on high constitutive levels of NO in the sinus gaseous compartment or other antimicrobial proteins/peptides in sinus fluid. This, however, has not been studied. A study correlating NO levels and microbiome characteristics in healthy subjects would serve as an important reference point for interpreting whether abnormalities observed in CRS represent primary defects or defects secondary to chronic inflammation, infection, and/or antibiotic use. Such a study could also serve as a "proof of concept" for better understanding the importance of innate immune pathways, such as bitter taste receptors, in maintaining constitutive levels of sinus NO and maintaining the middle meatus and sinus cavity free of putative CRS pathogens (e.g., *C. tuberculostearicum*, *S. aureus*, or *P. aeruginosa*), since it has been reported that CRS patients with nonfunctioning bitter taste receptors (TAS2R phenotype) have a higher burden of infection with *P. aeruginosa* and more severe sinus mucosal inflammation [32•].

Conclusion

At present, the field is in the beginning stages in the generation of a cast of characters that may play roles in maintaining mucosal homeostasis. Homeostatic pressures may involve not only pathogen exclusion and niche security but also cross talk with the host immune system to influence generation of

tolerance and regulatory mechanisms. Roles for the key players in the cast will likely prove difficult to assign definitively as the cast is likely to change based on several host factors including but not limited to genetic makeup, birth route, age, geographic location, and antibiotic/probiotic exposures. However, a more sophisticated understanding of microbiome constituents in health and disease holds significant promise in terms of promoting the cultivation of optimal bacterial communities for the restoration of a normal host-microbiome relationship.

Compliance with Ethics Guidelines

Conflict of Interest   Michael T. Wilson and Daniel L. Hamilos report no conflict of interest.

Human and Animal Rights and Informed Consent   This article does not contain any studies with human or animal subjects performed by the authors.

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• Of importance
•• Of major importance

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2.• Nikolaki S, Tsiamis G. Microbial diversity in the era of omic technologies. BioMed Res Int. 2013;2013:958719. *This is a excellent review of molecular tools used to study microbial communities.*

3.• He Z, Deng Y, Zhou J. Development of functional gene microarrays for microbial community analysis. Curr Opin Biotechnol. 2012;23(1):49–55. *This is a review of functional gene arrays (FGAs), a special type of microarrays containing probes for key genes involved in microbial functional processes, and the application of FGAs to examine the human microbiome.*

4. Ecker DJ, Sampath R, Massire C, Blyn LB, Hall TA, Eshoo MW, et al. Ibis T5000: a universal biosensor approach for microbiology. Nat Rev Microbiol. 2008;6(7):553–8. *This study described the Ibis T5000, a platform that couples nucleic acid amplification to high-performance electrospray ionization mass spectrometry and base-composition analysis to identify pathogens in clinical and environmental samples.*

5. Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem Biol. 1998;5(10):R245-9. *This paper described the use of random shotgun metagenomics, a method by which total DNA is isolated from a sample and then sequenced to profile of all genes within the community, as a means of accessing the collective genomes and the biosynthetic machinery of soil microflora.*

6. Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, et al. The NIH Human Microbiome Project. Genome Res. 2009;19(12):2317–23. *This study described the Human Microbiome Project (HMP), funded as an initiative of the NIH Roadmap for Biomedical Research, and describes the various types of data and database resources it provides.*

7.•• The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207–14. *This paper summarized the Human Microbiome Project's analysis of the largest cohort and set of distinct, clinically relevant body habitats so far and delineating the range of structural and functional configurations in the microbial communities of a healthy population.*

8. Gillespie JJ, Wattam AR, Cammer SA, Gabbard JL, Shukla MP, Dalay O, et al. PATRIC: the comprehensive bacterial bioinformatics resource with a focus on human pathogenic species. Infect Immun. 2011;79(11):4286–98. *This paper described the Pathosystems Resource Integration Center (PATRIC), a genomics-centric relational database and bioinformatics resource that provides scientists with (i) a comprehensive bacterial genomics database, (ii) a plethora of associated data relevant to genomic analysis, and (iii) an extensive suite of computational tools and platforms for bioinformatics analysis.*

9.• den Heijer CD, van Bijnen EM, Paget WJ, Pringle M, Goossens H, Bruggeman CA, et al. Prevalence and resistance of commensal *Staphylococcus aureus*, including meticillin-resistant *S aureus*, in nine European countries: a cross-sectional study. Lancet Infect Dis. 2013;13(5):409–15. *This study examined the prevalence of nasal S aureus carriage and antibiotic resistance, including meticillin-resistant S aureus (MRSA), in healthy patients across nine European countries using nasal swabs.*

10. Frank DN, Feazel LM, Bessesen MT, Price CS, Janoff EN, Pace NR. The human nasal microbiota and *Staphylococcus aureus* carriage. PLoS One. 2010;5(5):e10598. *This study examined nasal specimens from 5 healthy adults and hospitalized patients (including 26 S aureus carriers and 16 non-carriers) using 16S rRNA sequences and demonstrated a negative association between S aureus, S epidermidis, and other groups suggests microbial competition during colonization of the nares.*

11.• Gosbell IB, van Hal SJ. *Staphylococcus aureus* colonisation: some questions answered. Lancet Infect Dis. 2013;13(5):380–1. *This is an editorial regarding the den Heijer paper (reference 9) and the prevalence of S aureus and MRSA carriage across the world and their implications for public health measures.*

12.• Yan M, Pamp SJ, Fukuyama J, Hwang PH, Cho DY, Holmes S, et al. Nasal microenvironments and interspecific interactions influence nasal microbiota complexity and *S aureus* carriage. Cell Host Microbe. 2013;14(6):631–40. *This study examined healthy subjects, both S aureus carriers and noncarriers, at the anterior naris, middle meatus, and sphenoethmoidal recess using phylogenetic compositional and sparse linear discriminant analyses and identified microbial community determinants of S aureus carriage at sites with ciliated pseudostratified columnar epithelium.*

13.• Ramakrishnan VR, Feazel LM, Gitomer SA, Ir D, Robertson CE, Frank DN. The microbiome of the middle meatus in healthy adults. PLoS One. 2013;8(12):e85507. *This study examined the microbiome of the middle meatus in healthy adults using middle meatus swabs, quantitative PCR and 16S rRNA pyrosequencing thereby establishing a baseline for understanding how the sinonasal microbiome may impact diseases.*

14.•• Abreu NA, Nagalingam NA, Song Y, Roediger FC, Pletcher SD, Goldberg AN, et al. Sinus microbiome diversity depletion and

Corynebacterium tuberculostearicum enrichment mediates rhinosinusitis. Sci Transl Med. 2012;4(151):151ra24. *This study compared sinus brushings from 10 healthy non-CRS controls and 7 CRS patients using a phylogenetic microarray and identified a protective role for the taxon Lactobacillales (that includes Lactobacillus sakei) and a pathogenic role for Corynebacteriaceae (including the organism Corynebacteium tuberculostericum) in CRS.*

15.• Hamilos DL. Host-microbial interactions in patients with chronic rhinosinusitis. J Allergy Clin Immunol. 2014;133(3):640.e4–53.e4. *This is a state-of-the-art review of evidence for microbial involvement in CRS (viral, bacterial and fungal) and the role of host mucociliary clearance and mucosal innate immunity as they relate to mucosal susceptibility toward microbial infection and the pathogenesis of CRS.*

16. Psaltis AJ, Ha KR, Beule AG, Tan LW, Wormald PJ. Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. Laryngoscope. 2007;117(7):1302–6. *This study investigate biofilm presence in 38 CRS patients and 9 non-CRS controls using CSLM and identified biofilm in 44 % of CRS patients and none of the non-CRS controls.*

17. Oncel S, Pinar E, Sener G, Calli C, Karagoz U. Evaluation of bacterial biofilms in chronic rhinosinusitis. J Otolaryngol Head Neck Surg Le Journal d'oto-rhino-laryngologie et de chirurgie cervico-faciale. 2010;39(1):52–5. *This is a systematic evaluation of biofilm production by nasal swab culture of patients with CRS undergoing sinus surgery that revealed biofilm production in 9 of 13 S. aureus cultures and 6 of 10 P. auruginosa cultures.*

18. Stephenson MF, Mfuna L, Dowd SE, Wolcott RD, Barbeau J, Poisson M, et al. Molecular characterization of the polymicrobial flora in chronic rhinosinusitis. J Otolaryngol Head Neck Surgy=Le Journal d'oto-rhino-laryngologie et de chirurgie cervico-faciale. 2010;39(2):182–7. *This is a study of molecular culture of mucosal biopsies from CRS patients undergoing sinus surgery and control non-CRS surgical patients. Compared to conventional culture, molecular culture revealed a much broader array or organisms (up to 20 per sample) among the CRS patients with a predominance of anaerobic species and 50 % prevalence of S aureus.*

19. Brook I, Frazier EH. Correlation between microbiology and previous sinus surgery in patients with chronic maxillary sinusitis. Ann Otol Rhinol Laryngol. 2001;110(2):148–51. *This study perfomed culture and analysis of aspirates from 108 chronically inflamed maxillary sinuses ultimately correlating microbial results with previous sinus surgery and finding higher prevalence of P. aeruginosa and gram negative aerobic bacilli and a lower prevalence of anaerobes among patients with previous surgery.*

20. Finegold SM, Flynn MJ, Rose FV, Jousimies-Somer H, Jakielaszek C, McTeague M, et al. Bacteriologic findings associated with chronic bacterial maxillary sinusitis in adults. Clin Infect Dis Off Public Infect Dis Soc Am. 2002;35(4):428–33. *This study was an analysis of bacterial cultures from patients with bacterial maxillary sinusitis that recovered aerobes in 52.2 % and anaerobes in 47.8 % of cases and found that recurrence of sinusitis was associated with counts of anaerobes> or =10(3) cfu/mL.*

21. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15(2):167–93. *This review discussed biofilms, their structure, role in disease and microbial resistance and involvement of medical devices.*

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25.•• Feazel LM, Robertson CE, Ramakrishnan VR, Frank DN. Microbiome complexity and *Staphylococcus aureus* in chronic rhinosinusitis. Laryngoscope. 2012;122(2):467–72. *This study compared conventional culture techniques and DNA pyrosequencing of middle meatus swabs during sinus surgery on CRS patients and non-CRS controls and demonstated increased biodiversity identifiable by DNA pyrosequencing when compared to bacterial culture as well as altered microbial composition and greater abundance of S aureus in CRS patients.*

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27.• Choi EB, Hong SW, Kim DK, Jeon SG, Kim KR, Cho SH, et al. Decreased diversity of nasal microbiota and their secreted extracellular vesicles in patients with chronic rhinosinusitis based on a metagenomic analysis. Allergy. 2014;69(4):517–26. *This study evaluated the microbiota and extracellular vesicles in CRS patients with and without polyposis and non-CRS controls using pyrosequencing to quantify diversity and abundance and found decreased abundance of Bacteroidetes and increased abundance of Firmicutes in CRS patients.*

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patients and controls but an altered immune response to normal commensal bacteria among the CRS patients.

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