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A comprehensive review of the nasal microbiome in chronic rhinosinusitis (CRS)

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Abstract

Chronic rhinosinusitis (CRS) has been known as a disease with strong infectious and inflammatory components for decades. The recent advancement in methods identifying microbes has helped implicate the airway microbiome in inflammatory respiratory diseases such as asthma and COPD. Such studies support a role of resident microbes in both health and disease of host tissue, especially in the case of inflammatory mucosal diseases. Identifying interactive events between microbes and elements of the immune system can help us to uncover the pathogenic mechanisms underlying chronic rhinosinusitis. Here we provide a review of the findings on the complex upper respiratory microbiome in CRS in comparison to healthy controls. Furthermore, we have reviewed the defects and alterations of the host immune system that interact with microbes and could be associated with dysbiosis in CRS.

Introduction

Chronic rhinosinusitis (CRS) is a common inflammatory disease of the upper airways that afflicts about one tenth of US adults[1, 2]. The chronic inflammation in the sinus and nasal mucosa in CRS results in a constellation of symptoms that can have a significant negative effect on an individual's quality of life [3].

It has been known for a long time that the membranes of the nasal mucosa are home to a large number of microorganisms such as fungi, bacteria and viruses that are found in subjects both with and without sinus disease. CRS is regarded as a disease of inflammation rather than infection. However, numerous studies have demonstrated roles for commensal resident microbes and pathogens, or their associated products, in the initiation and/or progression of mucosal inflammation[4, 5].

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Microbes in the human body, like microbes in any other living organism, tend to adapt to their environment and to changes in that environment. Commensal microorganisms and their metabolites maintain the stability of their habitat and the health of their host in what is often viewed as a symbiotic system [6],[7]. For example symbiotic microbiota interact with gut immune cells to regulate intestinal homeostasis [8]. It is noteworthy that the effects of the microbiota are not limited to its immediate location but rather can impact the entire system of the host. For example, a healthy microbiome in the gastrointestinal system is necessary for development and training of a normal immune system[9, 10]. A disrupted microbiome in the gut, especially early in life, has been linked to the development of obesity and multiple inflammatory conditions in the rest of the body [11]. Of note in the context of this review, the gut microbiome has also been linked to multiple allergic diseases including asthma and eczema [12–14].

Multiple environmental factors – including birth mode, feeding patterns, diet, hygiene, and other life style measures – can alter the microbiome and affect its influence on the development of inflammatory conditions[15]. It is noteworthy that development of a healthy microbiome in the body is dependent on the individual's exposure to a surrounding environment rich in microbes. Moreover, diversity of microbial exposure is inversely correlated with development of allergic and inflammatory conditions such as asthma and allergic rhinitis[16–18]. Various studies have reported findings that microbes are protective against allergic diseases, which was in part the basis for the "hygiene hypothesis"[19]. The protective effect of living on a farm against the development of allergy and asthma has been known for decades [20]. This protective role has been suggested to occur at least partially via exposure to a more diverse group of microorganisms in the farm environment[16, 21, 22]. In mouse models, administration of certain bacteria, including ones that were isolated from farm environments, produced a significant reduction in the allergic inflammatory reaction in lungs, with a decrease in the number of eosinophils and mucus-producing goblet cells in airway, and reduction in allergy-induced airway hyperreactivity[23–25]. Also, exposure to dust from animal-enriched environments has been shown to change the gut microbiome in mice and to consequently protect them from allergen-mediated airway disease. Fujimura et al confirmed that the bacteria from dust from houses associated with a dog are responsible for this effect[26].

A disturbance in the composition of the local microbiome has been shown in asthma and COPD[27, 28]. Multiple studies have shown major differences in the microbiome of the lung captured by bronchoscopy or induced sputum in asthmatic versus non-asthmatic individuals[28–30]. Furthermore, the airway microbiome was shown to affect the response to corticosteroid therapy in asthma[31].

It is possible that the local microbiome could be an epiphenomenon.

In this scenario, the inflammation, or components of host defense, elicited as a result of the disease condition create an environment in which certain microbes can or cannot grow and reproduce. Consequently, the microbiome can sustain and promote a pre-existing inflammation that has been induced primarily by the underlying disease [32]. Whether microbiome changes are a cause or an effect of inflammatory disease remains to be

answered for multiple chronic inflammatory conditions associated with disrupted epithelia. For example, superinfection with *S. aureus* is one of the most common complications seen in atopic dermatitis. However, it is not clear whether skin inflammation triggers the changes in composition of colonizing microorganisms, allowing overgrowth of *Staphylococcus*, whether *Staphylococcus* overgrowth is an initiating event that affects the abundance of other species and triggers the inflammation, or both.

In the case of CRS, pathogen colonization and microbiota imbalance might be initial causes of the chronic immune response and inflammation. On the other hand, a dysfunctional immune barrier, inflamed mucosal epithelium and obstructed sinuses resulting from allergic or non-allergic inflammation may promote a condition suitable for secondary bacterial overgrowth and dysbiosis, setting the stage for chronic rhinosinusitis.

Studying the microbiome might be a useful approach for finding answers to some of the unsolved questions about CRS pathogenesis and for improving our knowledge about the inflammatory processes in this disease. Multiple studies have investigated the association of CRS with local microbial structures isolated from the sinuses or nasal cavities. Also, recent studies have used genomic analyses to evaluate nasal and sinus bacterial and fungal microbiomes in CRS, and have found associations between certain microorganisms and CRS. However these associations do not establish causality between the presence of these microbes in the airways and the development of disease. The present review aims to provide a summary of these findings by discussing the results of studies on the microbiome in CRS. We also briefly review the literature on the microbiome in asthma, another respiratory disease that is often comorbid with CRS.

The upper respiratory tract microbiome in chronic sinusitis

The association between CRS and commensal or pathogenic microbes cultured from the nasal cavity and paranasal sinuses has been under investigation for many decades. The sinuses were found to be colonized by microbial flora in both normal and disease conditions in the 1990s[33]. Multiple microorganisms isolated from sinus and nose in CRS patients, such as coagulase-negative staphylococci [34] have been identified and have been proposed as pathogens that mediate infectious processes in CRS and that result in chronic inflammation in nasal and sinus mucosa. This view has led to sinusitis (including both acute and chronic) to be the number one indication for the use of antibiotics. Furthermore, some studies have evaluated possible mechanisms by which selected bacteria could influence or initiate an immunologic cascade resulting in the chronic mucosal inflammation seen in CRS. The association between *Staphylococcus aureus (S. aureus)* superantigen specific IgE and skewing towards a Th2 milieu and eosinophilia in CRS is a notable example[35].

During the past two decades, it has been shown that conventional culture-based methods are not sufficient to elucidate the complex ecology of the bacterial populations of the human body. This was based, to a large extent, on studies in the mid-1980s when a group of investigators amplified the highly conserved 16S ribosomal ribonucleic acid (rRNA) bacterial gene from samples of organisms isolated from deep sea regions. They found that less than 1% of the organisms from those isolations could be cultured[36]. This method was

later applied to microbiota studies in humans and other mammals, uncovering a vast and impressive ecosystem of microorganisms in virtually all epithelia.

Here we will focus on the recent studies in which genetic techniques have been used to characterize the local microbiome of the nasopharynx or sinuses with the goal of discovering an association with CRS. It is notable that such studies are fraught with challenges like recent use of antibiotics and steroids, exacerbations and sampling techniques. Another significant challenge results from a recent appreciation of the variability in constitutive epithelial host defense molecule secretion based on the region of the nose and sinuses[37]. Consistently, Yan et al have shown a significant variation in the microbial composition of anterior nares compared to middle meatus and sphenoethmoidal recess in healthy individuals[38]. Interestingly, they found no difference in the bacterial composition of the two latter sites. Relatively little is stated in published studies about the region from which microbiome samples were collected, so it is difficult to evaluate to what extent this concern applies to the available literature. Such challenges to the interpretation of findings must be kept in mind while considering the complex relationship between disease and the microbiome in CRS.

Summary of data from culture studies

A diverse group of bacteria have been identified in chronic rhinosinusitis based on culture methods. We have briefly reviewed the results of some of these studies below.

In 1998, Beil et al. published the results of bacterial cultures from endoscopically collected samples at the time of surgery in 174 CRS patients. The most commonly cultured bacteria were Coagulase-negative *staphylococci* (36%), followed by *S. aureus* (25%), *Streptococcus viridans* (8.3%), *Corynebacterium* (4.6%), and anaerobes (6.4%)[34]. Jiang et al. obtained culture samples from the ethmoid sinus by inserting a cotton-tip stick through a cannula placed in the ethmoid cavity after removing the ethmoid bulla. In this study, 39 CRS patients from Taiwan were included. They reported a positive culture rate of 60.9%. The most frequently isolated bacteria in this study were *Streptococcus viridans, Klebsiella pneumoniae, Proteus mirabilis, and Haemophilus parainfluenzae* [39].

In a retrospective study of nasal and sinus culture data obtained from 83 patients with CRS, positive cultures were reported in 71% of cases. The most common bacteria were reported as coagulase-negative *staphylococci* (31% of isolates), *Haemophilus influenzae (H. Influenza)* (25%), *Streptococcus pneumoniae (S. pneumoniae)* (12%), *Moraxella catarrhalis (M. catarrahalis)* (10%), *Pseudomonas aeruginosa (P. aeruginosa)* (7%), alpha-hemolytic streptococci (5%), and *S. aureus* (3%)[40].

Brook et al reported the results of cultures from sinus aspirates obtained during surgery in 6 patients with CRS. Polymicrobial flora was found in all specimens. The predominant organisms in this study were *Prevotella* spp.(13 isolates), followed by *Fusobacterium nucleatum (11), Peptostreptococcus* spp. (6), *Staphylococcus* spp (5), *H. Influenza* (2) and *S. pneumoniae* (2)[41].

A more recent study on a cohort of CRSwNP patients from Korea showed 58.0% positive aerobic and 8.6% positive anaerobic cultures in samples from the middle meatus, and 48% and 18.5% positive aerobic and anaerobic cultures in samples of maxillary sinus. The most commonly isolated species were *S. aureus, H. influenzae*, and *S. pneumoniae* and the predominant anaerobic organisms were *Prevotella* and *Peptostreptococcus*[42].

Despite the discrepancies among these results, which could be due to differential culture(including use of anaerobic cultures) and sampling methods, most of these culture-based analyses have reported evidence for *staphylococci* and *streptococci spp.* along with *H. Influenza* as predominantly detected bacteria in CRS. In the studies that have also evaluated anaerobes in sinus, *Prevotella* and *Peptostreptococcus* were commonly found.

It is noteworthy that multiple studies have shown a lack of correlation between nasal cultures with sinus cultures, which further implicates differences in sampling region as a major cause of discrepancy seen in microbial studies in CRS, regardless of analytical method[43]. Furthermore, as mentioned previously, a significant proportion of organisms from different isolations can not be cultured[36]. This makes culture-based studies less informative compared to PCR-based analyses.

Bacterial microbiome

The initial studies that used PCR-based methods to analyze bacteria-specific 16s ribosomal deoxyribonucleic acid (DNA) fragments and coding rRNA in CRS were limited by their use of a small number of primer sets focused on the capture of known bacterial flora of the upper respiratory tract. Moreover, these methods were time consuming and labor intensive [44],[45]. However, these studies expanded our knowledge beyond studies that were based on culture. Paju et al used 3 primer sets and found positive bacterial DNA results in 5 out of 11 CRS patients [45]. Keech et al, analyzed 64 samples from 32 CRS cases and focused on primer sets capturing DNA from *Staphylococcus aureus (S. aureus), Staphylococcus epidermidis (S. epidermidis), Streptococcus pneumoniae (S. pneumoniae), Pseudomonas aeruginosa (P. aeruginosa), Moraxella catarrhalis, Haemophilus influenzae (H. influenzae), and Streptococcus pyogenes*. They found that 62% of CRS patients were positive for at least one of these bacteria, while parallel culture method had found positive growth in 50% of cases[44]. In addition, except for *Pseudomonas aeruginosa*, all the tested bacteria were found in at least 3 cases[44]. Power et al used PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and species-specific primers analyzing middle meatus aspirates from 6 patients with chronic rhinosinusitis. This study detected *S.aureus* and *S. pneumoniae* in 4 out 6 patients, the parallel cultures showed positive culture for at least one bacterium in all samples and was consistent with the PCR-DGGE results. The comparison between PCR-DGGE and cultures showed that PCR based method was able to detect a greater diversity of bacteria [46].

Since 2010, more advanced microbiome analytical methods, ones that are able to detect multiple bacteria simultaneously, have been used to study CRS [47][48]. Results of these studies are summarized below and in Table I.

In a study by Stephenson et al, mucosal biopsies were assessed using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) of species-specific 16S rRNA. This method was able to identify up to 20 microorganisms in some individuals and all 18 patients included in the study showed evidence of multiple microbial colonization. Anaerobic bacteria, including *Diaphorobacter* and *Peptoniphilus*, were identified as predominant species, found in 78% and 72% of CRS cases, respectively, but not identified in control cases. Interestingly, *S. aureus* was found in half of the CRS cases, while it was detected in all controls[48].

Stressmann et al used 16S rRNA sequencing and terminal restriction fragment length polymorphism (T-RFLP) to analyze bacterial profiles in 70 tissue and mucus samples obtained from 43 CRS cases. This study had no control subjects. Species belonging to 34 different bacterial genera were identified in the CRS series. The most commonly found bacteria belonged to the following genera: *Pseudomonas*, *Citrobacter*, *Haemophilus*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus*, with *Pseudomonas aeruginosa* being the most frequent species[49].

Boase et al used sinus mucosal tissue harvested from the ethmoid sinuses to investigate the microbiome at multiple levels by bacterial and fungal cultures, Ibis T5000 analysis (PCR coupled with electrospray ionization mass spectrometry) as well as biofilm analysis[50]. Thirty-eight CRS and 6 control subjects were studied. Using the Ibis method they were able to identify 33 different bacterial species in CRS patients, with a mean of 3.0 (2.0–4.0) species per patient, and 5 different organisms in controls, with a mean of 2.0 (1.0–3.0) per subject. CRS patients had significantly increased bacterial abundance compared to control patients. *S. aureus* was the most prevalent organism in CRS patients, followed by *S. epidermidis* and *Propionibacterium acnes (P. acnes)*. *S. aureus* had a ten-fold increased abundance in CRS patients; *S. epidermidis* and *P. acnes* had similar abundances in the microbiome of CRS and control subjects. In control patients, *P. acnes* was the most commonly detected organism (5/6, 85%), followed by *S. epidermidis*, which was present in 4/6 (67%). Anaerobic species were detected in 47% of CRS cases, and 83% of controls. Furthermore, this study showed poor agreement between the Ibis biosensor and culture data for anaerobes. They concluded that conventional culture methods are selective for more abundant, rapidly growing aerobic organisms like *S. aureus* and *S. epidermidis*[50].

In 2012, Feazel et al studied 21 middle meatus swabs from a series of 15 CRS patients and 5 controls. A total of 57,407 pyrosequencing reads were generated in all samples (interquartile range, 682–3296 per specimen). The most commonly detected sequences in all subjects were from coagulase-negative *staphylococci* (21/21 specimens) followed by *Corynebacterium* species (*Corynebacterium* spp.) (18/21), *Propionibacterium acnes* (16/21), and *S. aureus* (14/21). In the vast majority of specimens (95.2%) more than half of the sequences belonged to known upper respiratory bacterial genera[51]. All of these subjects had positive bacterial cultures and the more prevalent sequence types (>15%) corresponded to the cultured organisms in 65% of cases. In comparison to controls, CRS patients had significantly fewer bacterial types and less even distribution of genus-level sequence types. Evenness is calculated based on relative distribution of bacterial types in the samples. *S aureus* was selectively enriched in CRS in this study. Furthermore, in this study

chronic rhinosinusitis with asthma was associated with lower microbial diversity and higher abundance of *S. aureus* [51].

In another study, lavage samples from the middle meatus of 30 patients with CRS and 12 control individuals were tested for cytokine levels, immune cells, and bacterial and fungal microbiomes. In contrast to the previous study [51], the diversity of bacterial types was higher in CRS patients compared to controls. The most abundant species (in both CRS and in healthy controls) was an uncultured *Cyanobacterium*, followed by a *Curtobacterium* species, which was present in 29 patients, and present at lower abundance in the controls. *Corynebacterium accolens* was the most abundant species of bacteria (it was significantly increased in CRS relative to controls). In contrast, *Alicycliphilus* and *Cloacibacterium* were decreased in CRS patients compared to controls[52].

Abreu et al used a 16S rRNA PhyloChip to test for 8500 bacterial taxa in endoscopically guided brush samples of mucosal surfaces of the maxillary sinus. The bacterial profile was amplified sufficiently in 7 cases in each group (CRS and control) that it could be analyzed by microarray. CRS patients had significantly lower bacterial evenness, richness (number of bacterial types detected), and Shannon's diversity (a metric calculated using richness and evenness indices). They detected pathogenic members of *Pseudomonadaceae, Lachnospiraceae, Ralstoniaceae, Mycobacteriaceae*, and *Helico-bacteriaceae* in CRS as well as healthy subjects. *Lactobacillales* such as *Lactobacillus sakei (L. Sakei)*, as well as *Carnobacterium alterfunditum, Enterococcus mundtii*, and *Pediococcus pentosaceus* were reduced in abundance in CRS and there was only a single taxon, *Corynebacterium tuberculostearicum (C. tuberculostearicum)*, that was increased in abundance in CRS. Interestingly, *Lactobacillaceae, Enterococcaceae, Aerococcaceae, and Streptococcaceae*, were associated with lower symptom score (Sino-nasal outcome test, SNOT-22), while *Corynebacteria tuberculostearicum* was associated with higher SNOT-22 score [53].

In a more recent study from Korea, Choi et al used nasal lavage fluid samples from 8 CRS patients and 3 controls. Bacterial 16S-rDNA amplicons were analyzed by high-throughput pyrosequencing on a Roche 454 GS-FLX platform. They also isolated and studied the contents of bacteria-derived extracellular vesicles (EV) [54]. These vesicles contain bacterial biological components like bioactive lipids, proteins, and nucleic acids, which could be delivered to other cells[55]. EV from *Staphylococcus aureus* have been shown to have a role in pathogenesis of atopic dermatitis[56]. Choi et al showed that CRS patients had significantly greater bacterial abundance and lower diversity, which held true when analyzing the bacterial or EV portion of samples. Bacterial and EV compositions strongly correlated. At the Phylum level, CRS patients had higher *Proteobacteria* and decreased *Bacteroidetes*. An analysis of the species showed increased *S. epidermidis* and decreased *Prevotella melaninogenica* in patients with CRS compared to control cases. Regarding the EV composition, EV from *Pseudomonas monteilii, Corynebacterium spp.*, and *Propionibacterium spp*. were increased, while EV from *Prevotella spp., Streptococcus spp.*, and *Veillonella spp.*, were decreased in patients with CRS [54]. Comparison of CRSwNP and CRSsNP cases showed that CRSwNP cases had increased *Pseudomonas spp*. at the genus level and *S. aureus* at the species level compared with CRSsNP, both in bacteria and EVs [54].

Bucholtz et al studied 31 polyps, 6 turbinates, and 4 sinus biopsy samples using 16S rDNA. The bacterial DNA was rarely found inside any of these tissues. *Streptococcus spp*. DNA was seen inside the tissue in one polyp sample and *Pseudomonas aeruginosa* DNA was isolated from one maxillary sinus biopsy[57]. These negative results could be due to the sampling and analysis methods used in this study. A more recent study has found evidence of intracellular invasion by *S. aureus* in 39% of cases [58].

Recently a larger study on Microbiome of CRS was published; this study took a step forward and evaluated the microbiome not only in association with CRS, but also the phenotypic features and surgical outcome of patients[59]. Nasal swabs from the ethmoid region were examined in 56 CRS patients and 26 controls. The bacterial microbiome of CRS and control patients was similar in terms of biodiversity and at the phylum level, however there were a couple of differences at the genus level; *Propionibacterium* and *Porphyromonas* were both decreased in CRS compared with control cases. They found no difference between CRSsNP and CRSwNP cases in terms of bacterial community alterations.

The subgroup analyses showed that the sinus microbiome of CRS patients with asthma had significant differences compared with non-asthmatic CRS cases at both the Phylum and Genus levels. The first figure in that paper shows that the distribution of bacteria at the Phylum level in asthmatic CRS cases was more similar to controls than non-asthmatic patients with CRS. Furthermore at the genus level, Prevotella, which had a relative abundance of only 1.5% in controls and 0.6% in asthmatic CRS cases, had a significantly higher relative abundance of 7.7% in non-asthmatic CRS. A similar pattern was observed for *Staphylococcus*, which had a trend towards a lower abundance in CRS compared to controls, but a trend toward higher abundance in non-asthmatics within the CRS group. We find these findings rather thought-provoking as to why asthma should push back the microbiome of CRS patients closer to the healthy comparison group. However there were multiple genera that were different between asthmatic CRS and non-asthmatic CRS which were not reported to be distinguishing factors between CRS and controls, making direct comparisons difficult. Interestingly higher diversity and higher abundance of Actinobacteria at the phylum level and *Corynebacterium* at the genus level were associated with better outcomes after surgery[59].

All of the above studies showed some differences in the microbial community composition of the upper airways between CRS patients and non-CRS controls. However, there are major variabilities and discordances among the studies' results (Table 1). Overall, most studies have shown increased bacterial abundance in CRS [50, 52, 54]. The bacterial microbiome in the respiratory tract tends to have a high diversity, while the absolute quantity of each species may be low. Therefore, defining the species with higher relative abundance in the bacterial community could be as important as the overall bacterial abundance.

The diversity of the microbiome has been proposed to be essential for sustaining a healthy microenvironment. Chronic inflammation in other diseases like inflammatory bowel disease has been associated with reduced diversity in bacterial composition[60, 61]. However, the results on bacterial diversity in CRS are varied and likely to be complicated by repeated

antibiotic use in CRS patients. Two of the studies showed decreased[53, 54] diversity, while others showed either a significant increase in diversity [52], a trend toward increased diversity[50], or no difference at all[51],[59]. In the most recent study by Ramakrishnan et al., diversity was not different between CRS and controls, although in the CRS group it was predictive of better outcome[59]. The discrepancy among studies in terms of diversity might have been related to the use of antibiotics prior to and around the time of sampling for microbial analyses or based on regional microbial differences in the nasal cavity referred to above. Middle meatus and sphenoethmoidal recess regions have been shown to have bacterial composition and diversity similar to eachother but significantly higher compared to anterior nares[38]. In this regard, using lavage for microbiome analysis instead of direct sampling from a single region of the nasal cavity would complicate the analyses.

Role of S. Aureus in CRS

A meta-analysis of literature from 1990 to 2006 on culture-based bacterial analyses in rhinosinusitis revealed *S. Aureus* as one of the three most common pathogens cultured in acute rhinosinusitis[62]. Similarly *S. aureus* is a commonly found organism in chronic rhinosinusitis, observed consistently in multiple culture-based and PCR-based microbial studies[50, 51, 63, 64]. *S. aureus* frequently colonizes the nose of healthy subjects as well[38, 48, 65]. However, the abundance of this bacterium is increased in CRS[50]. Therefore, it seems the contribution of *S. aureus* to pathogenesis of CRS goes beyond just colonization of the nasal mucosal membranes. In one study, *S. Aureus* was found more frequently in nasal mucosa of CRSwNP compared to CRSsNP [54], the increased *S. aureus* colonization was observed in the nasal polyps in previous culture based reports[35]. In contrast, Ramakrishnan et al. showed a higher abundance but similar frequency of *S. Aureus* in CRSwNP compared to CRSsNP[66]. Sachse et al. used peptide nucleic acid-fluorescence *in situ* hybridization for detection of *S. aureus* and showed evidence for invasion of *S. aureus* into the epithelial layer in CRSwNP but not CRSsNP patients[67]. In context of loss of epithelial integrity seen in chronic rhinosinusitis[68, 69], the authors hypothesized that *S. aureus* can invade the epithelial layer and move into the submucosal space and potentially play a pathogenic role in formation of nasal polyps in CRSwNP patients. The authors also showed that *S. aureus* Newman could survive intra-cellularly for 48 hours in nasal polyp epithelial cells and induce the production of IL-6 from these cells in an in vitro cell culture model[67]. Tan NC. et al. showed evidence for intracellular *S. aureus* in 20 of 51 (39%) of patients. Intracellular *S. aureus* was associated with higher risk of late clinical and microbiological relapse[58]. How *S. aureus* can survive in nasal polyp cells in vivo and contribute to the pathogenesis of CRSwNP requires further investigation.

It has been proposed that *S. aureus*-derived enterotoxins can possibly act as super-antigens and induce a polyclonal stimulation resulting in eosinophilic inflammation and IgE formation. In a study by Bachert et al, almost half of the nasal tissue homogenates derived from NPs had detectable concentrations of specific IgE to *S. aureus*-derived enterotoxins, including A and/or B(SEA and SEB). The presence of sIgE to SEA and SEB were reportedly associated with total IgE concentration in tissue and local polyclonal IgE against inhalant allergens. The *S. aureus*-derived enterotoxins-specific IgE antibodies were also linked to higher eosinophilic inflammation, asthma[35],[70] and aspirin sensitivity [70].

Clark et al found an association between positive *S. aureus* cultures and higher total serum IgE and severity of disease in CRSwNP based on Lund-Mackay tomography score[64]. Another possible contributing factor to invasion of *S. aureus* could be pre-infection with herpes simplex virus type 1 (HSV1). Wang et al showed evidence suggestive of a significant damage to the nasal epithelium by HSV1 infection that could consequently facilitate invasion of S. aureus into the nasal mucosa [71].

All of this evidence supports a contribution of *S. aureus* in pathogenesis or worsening of CRS, especially in CRSwNP, which suggests that this bacterium could be a potential therapeutic target in CRSwNP. However it seems that a defect in mucosal barrier and the innate immune system in CRS may predispose individuals to *S. aureus* invasion and potentially increased enterotoxin production, further promoting inflammation, thus closing a positive feedback loop. The fact that treatment of CRS with prolonged antibiotics that cover *S. aureus* is only a partial remedy for CRS, and does not improve multiple symptoms like congestion or loss of smell[72], shows that merely eliminating *S. aureus* temporarily is not sufficient to break the cycle.

Biofilm formation in rhinosinusitis

A biofilm is a complex polymicrobial community of fungi or bacteria surrounded by an exopolysaccharide matrix produced by these microorganisms. Formation of biofilms is a defense mechanism for microbes that can protect themselves from antibiotics and from the host immune system[73]. Biofilm formation in sinonasal mucosa has been studied in CRS in multiple studies[74, 75]. It is associated with recurrence of disease, poor response to treatment and unfavorable outcome after surgery[75],[76, 77]. Biofilm formation has also been linked to environmental factors such as smoking[78]. Furthermore, diverse bacterial biofilms have been attributed to the pathogenesis and phenotypes of CRS [79]. Study of biofilms requires specialized techniques and most of the bacterial species that form them do not grow well in culture[80].

Boase et al compared a molecular PCR-based method, culture and FISH (fluorescent *in situ* hybridization) for detection of bacteria in sinonasal mucosal tissue. They used *S. aureus* as a comparitor and showed that use of a PCR-based method (as opposed to conventional cultures) could sensitively detect *S. aureus*, which was also present in biofilms in these cases[50]. It is not clear how the presence of biofilms will affect the microbiota and microbiome analyses of the nasal cavity and sinuses. More studies are needed to determine whether different sampling and digestion methods and advanced microbiome analysis are able to capture the microbial DNA from biofilms for detection and inclusion of these microorganisms in the analysis.

In 2008, use of baby shampoo nasal irrigation was proposed as a potential treatment for CRS. The proposed mechanism was based on its ability as a surfactant to inhibit biofilm formation. One percent Johnson’s Baby Shampoo was found to inhibit *in vitro* formation of *P. aeruginosa* biofilms; however, it had no ability to eradicate preformed Pseudomonas biofilms[81]. Furthermore, a study in healthy individuals showed that this treatment could increase the mucociliary clearance time (MCT)[82]. The first study in 2008 showed a

significant improvement in symptoms of thickened nasal discharge and postnasal drainage in patients who remained symptomatic despite medical and surgical therapy for CRS and drew attention to this therapy as a mucolytic agent [81]. However another study in 2013 found no significant differences in overall subjective symptoms between patients who used diluted shampoo as surfactant versus the group who had used hypertonic saline irrigation solution in the early postoperative period[83]. Furthermore a higher number of patients reported significant side effects with surfactant[83].

The fungal microbiome (mycobiome) in sinusitis

Study of nonbacterial microbiota in the nose has been an area of interest in recent years. The association of fungi with CRS has been investigated in multiple studies, with controversial results. In the late 1990s, fungal cultures identified colonization in more than 90% of CRS patients and drew significant attention to this field[84]. In this way, several fungal species were identified in CRS[84]. But further studies showed evidence of fungal colonization in the nose in more than 90% of healthy controls from the same population [85]. Interestingly, studies in other populations showed a significantly lower level of fungal colonization in CRS. Similar fungal culture techniques yielded positive results in 13% of a Turkish[86] and 36% of a Malaysian [87] CRS series. Fungal organisms have also been found in biofilm formations in CRS [75]. Studies using antifungal treatments in CRS have primarily failed to support a clinical use of this approach[88, 89].

More recent studies that used organism-specific primers for PCR analyses of fungi were able to give a more complete picture of the fungal microbiome (mycobiome) in the nose and paranasal sinuses, but the results of these studies have engendered controversies as well. Cleland et al. analyzed nasal swabs of 23 CRS patients and 11 controls. They used 18S ribosomal DNA (rDNA) fungal tag-encoded FLX amplicon pyrosequencing [90]. They showed that CRS cases had greater fungal richness than controls (mean sample richness of 12.14 vs. 8.18). This richness decreased significantly following sinus surgery. In that study, *Malassezia* was the most common and abundant species and was found in all CRS cases at surgery [90]. Aurora et al. [52] analyzed the mycobiome by deep sequencing of 18S rDNA. They found qualitatively similar mycobiomes in CRS and controls, but greater diversity and abundance of fungi in the CRS group. *Cryptococcus neoformans* was the most abundant fungus in both CRS and control cases, although it was more prevalent in the CRS group (90% vs 61%). In their study, *Malassezia* was the 3rd most common species and was seen more commonly in controls (4.7% vs. 1.4%). Boase et al. [50] focused on pathogenic taxa using 16 primer pairs for PCR, which could survey nearly all pathogenic fungal species, but could not find evidence of pathogenic fungal DNA in most of the cases studied. Three CRSwNP cases were positive (two for *Aspergillus fumigatus* and one for *Bipolaris papendorfii*), while all CRSsNP cases and healthy controls were negative.

Viral microbiome and CRS

A majority of CRS exacerbations occur in seasons with a high prevalence of viral infections [91]. Using multiplex PCR for respiratory viruses, Cho et al [92] found viral nucleic acid sequences in 64% of sinus scrapings and 50% of nasal lavage samples in the CRS group,

which was significantly higher than controls (30% and 14% in scraping and lavage respectively). In their study, rhinovirus (RV) was the most frequently detected virus. In a study from China, test of the scraped epithelial cells from the middle meatus revealed an evidence for respiratory viruses in 68.66%, 73.77% and 75.47% of CRSwNP, CRSsNP and control cases, which was not statistically different[93]. In 2006 Jang et al [94] showed that 21% of turbinate epithelial cell samples from CRS patients were positive for rhinovirus detected by reverse-transcription PCR. In that study, RV was not detected in the lavage of any of these patients. In contrast, Wood et al [95]did not find any evidence for respiratory viruses in 13 CRS patients tested by PCR for common respiratory viruses including, but not limited to, rhinovirus, parainfluenza viruses 1, 2, and 3, and respiratory syncytial virus (RSV).

It is noteworthy that studies of experimentally-induced viral infections in CRS are limited. In an *in vitro* study, NPs and turbinate epithelial cells from CRSwNP patients and healthy controls did not show any differential response in induction of IL-6 and IL-8 upon infection with RV [96]. However, another study showed that stimulation of cultured epithelial cells from CRSsNP cases with toll-like receptor (TLR) 3 agonist in the presence of cigarette smoke extract causes an exaggerated production of chemokine (C-C motif) ligand 5 (CCL5) and human beta-defensin-2 (hBD-2). These researchers proposed that conjoint cigarette smoking and viral infection could contribute to CRS exacerbation and eosinophilic inflammation[97].

The role of rhinovirus infection in asthma exacerbation has been well established and the similarities between the two diseases suggest a role for viral infection in triggering exacerbations of CRS, a hypothesis that calls for further investigation. However, current literature in this field is not conclusive.

Analyzing the sino-nasal microbiome: the disparity and the controversies

The disparities in organism profiling results found in the above studies might be reflective of differences in methods of collection or analysis, which can influence the results of a microbiome study. Controversies were also seen in previous decades in the literature of studies using culture-based methods, mainly due to insensitivity and variability of these methods in capturing microorganisms. This insensitivity is attributed to non-optimal culture conditions, oxygen exposure, or disruption of symbiotic interactions while culturing organisms.

As briefly discussed above, multiple factors could influence the microbiome studies that include, but are not limited to, regional variations of the microbiome (nasal cavity vs. sinuses) [98],[37], antimicrobial use around the time of sampling [53, 99], sampling methods (lavage, tissue biopsy, swab etc.), disease subgroups [54], age [65], severity and phenotypes of included cases or genetic background of patients. The area in the nasal cavity where the swab or biopsy is taken is of utmost importance. As the goal of most research studies of the microbiome is to elucidate the pathogenesis of disease, it is important to sample the microbes from a region that is most involved or representative of the disease. For example lavage would provide a diluted sample containing the microbes from almost all

areas in the sino-nasal cavity, and potentially pharyngeal area, and could even be contaminated by skin microbes. Similarly, samples from the anterior nares and inferior turbinate are likely not representative of the sinus microbiome. The differences in sampling techniques could significantly influence the findings including the detection of anaerobes, which could have different abundance in various locations of sinonasal cavity.

Although surgically collected sinus tissue is the best representation, collecting a large series of cases along with healthy controls is more difficult and would limit that data primarily to surgical patients with more severe disease. Potentially, careful swab sampling of the middle meatus with the guidance of endoscopy could be an acceptable alternative.

Furthermore, there are critical factors – nucleic acid extraction, amplicon sequencing, and data analysis – that can influence the accuracy of metagenomic studies based on 16S ribosomal RNA gene (rDNA) sequencing [100]. In the 1970s it was discovered that the 16S ribosomal RNA (16S rRNA), which is a component of the 30S small subunit of prokaryotic ribosomes, could be used for phylogenetic classification. The 16S rRNA is present in all bacteria and contains highly conserved regions flanked by variable regions, which permit phylogenetic classification of the different microbial species[101]. With methods focused on analyzing 16S rDNA (the rRNA gene) for bacteria and 18S rDNA for fungi, the bacterial and fungal DNA base sequences can be analyzed without including human genomic DNA sequences. Although the results of older studies might vary depending on the primer sets, most above mentioned studies in the past 3–4 years have implemented more advanced methods that have the capacity to capture most bacterial or fungal DNA in the sample. However, major differences, including sampling techniques and data analyses mentioned above, exist that could contribute to the variability observed in these studies. The most advanced methods for studying the microbiome based on next generations sequencing are currently available; these methods do not require a cloning step before sequencing analysis and are the basis for high throughput parallel sequencing. However no CRS microbiome studies have yet used these advanced methods. The statistical method used for analysis of data is another important variable in accuracy of data. The data generated from microbiome experiments are multi-dimensional resulting from the large volume of variables. The statistical models are used to explain a clinical or biological endpoint in association with this high-dimensional data. Therefore, the data analysis method could be one of the most important factors playing a role in the discrepancies mentioned above.

Another reason for differences in published findings could be regional variation of the microbiota in the sinonasal cavity. The airway epithelium secretes multiple antimicrobial peptides (AMP); these AMPs are secreted both constitutively and in response to bacteria-derived and other pathogen-derived components; in return, host AMP can affect the microbiota on the adjacent mucosal surface. Different regions of the nasal cavity – inferior turbinate (IT) and uncinate tissue (UT) – have significantly different profiles of innate host defense molecules[37]. IT is the proximal point of contact of inhaled air containing microbial and pathogen components in close proximity to the nares; in contrast, the uncinate process is exposed to drainage of sinuses and is representative of sinus epithelium. The increased expression of gland-derived antimicrobial proteins – e.g. short palate lung and nasal epithelium clone -1 (SPLUNC1) and lactoferrin in uncinate tissue – could be due to

differences in the microbiota in the proximal (IT) and distal (UT) areas of the sinonasal mucosa, or alternatively may present a highly regionalized mucosal surface that is inhospitable to many microorganisms.

The respiratory microbiome in asthma

Chronic rhinosinusitis and asthma are both chronic diseases characterized by respiratory mucosal inflammation, often rich in eosinophils. Approximately half of CRS patients also suffer from asthma, asthmatics are much more likely to have CRS than the general population, and the two diseases share an association with allergic diseases. There are similarities in the deviations of the microbiome from control subjects in these diseases as well.

Although acute viral and bacterial infections of the respiratory tract are long-known triggers of asthma exacerbations, the existence of a lower airway commensal microbiome was not discovered until recently [28]. Since then, multiple studies have evaluated the lung microbial residents in asthmatics in search of a causal relationship between these microorganisms and asthma[102]. However, the results of culture-based analyses were inconclusive and controversial, most probably due to the reasons described above in CRS studies, such as sampling technique and insensitivity of the methods. More recently, some studies have applied PCR-based methods aimed at detecting species-specific nucleic acids, and have supported an association between asthma and the increased presence of certain bacteria. For example, the levels of atypical bacteria like *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* in induced sputum or bronchoalveolar lavage are significantly higher in asthmatic patients compared to controls[103, 104]. Moreover the lung microbiota composition and diversity, specifically the abundance of certain phylotypes such as *Comamonadaceae*, *Sphingomonadaceae* and *Oxalobacteraceae*, have been correlated with the degree of bronchial hyperresponsiveness. [29].

Colonization with *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* or a combination of these organisms in neonatal samples from lung is associated with increased risk of asthma later in life[105]. This provides evidence of a persistent effect of microbial colonization of airways on initiation or progression of inflammatory respiratory disease. Along the same lines, multiple longitudinal studies have shown an association between viral respiratory illness with wheezing due to RSV and RV and subsequent development of asthma later in life[106–109].

Comparing CRS and asthma microbiomes

Similar to the finding of Choi et al. for upper airways of CRS cases[54], an increased abundance of bacteria of the phylum Proteobacteria [28, 30] and a decreased abundance of bacteria of the phylum Bacteroidetes has been reported for asthmatic lungs [28]. The number of bacteria of the phylum Bacteroidetes is also decreased in the upper airways of asthmatics compared to healthy controls[110]. A genus of bacteria found more abundantly in asthma was *Haemophilus*, which belongs to the phylum *Proteobacteria. Haemophilus* was more frequently detected in the lower airways of adult asthmatics [28] and in the oropharynx of toddlers with wheezing[111]. In CRS studies, *Haemophilu*s was found in 13% [50] and

17% [48]of CRS cases but none of control cases[50]. H. Influenza was also one of the most frequently cultured bacteria in earlier culture-based studies of CRS[40],[41].

There are similar changes in the fungal microbiome in the two diseases. Mycobiome analysis of induced sputum in asthmatics has shown an association with the abundance of Malassezia pachydermatis[112]. As mentioned previously, *Malassezia* genus was found in the upper airways of CRS patients. However, the results are controversial: in one study *Malassezia* was detected in all CRS cases at time of surgery [90], while in another report it was found at a higher frequency in healthy upper airways [52].

In asthma, different bacteria have been associated with specific phenotypes of disease; pathogens like *Moraxella catarrhalis* and members of *Haemophilus* or *Streptococcus* genera have been associated with steroid resistant neutrophilic asthma[113]. The only species that was found to be associated with nasal polyposis, an important hallmark of disease phenotype in CRS, is *S. aureus*, which has been found more frequently in CRSwNP compared to CRSsNP [54]. *S. aureus* has also been associated with asthma in a series of CRS patients[51]. These findings from microbiome analyses are in line with previously found associations of *S. aureus* and specific IgE to its enterotoxins in nasal polyposis and asthma[35]. Ramakrishnan et al. showed that the relative abundance of genera *Provotella*, *Fusobacterium* and *Campylobacter* in upper airways was decreased in CRS with asthma compared with CRS without asthma, while *Acinetobacter* and *Ralstonia* were relatively increased in nasal microbiome of asthmatics CRS cases[59].

Genetic factors influencing microbiomes

The effect of host genetic factors on Microbiota is another area of unmet research need. Diversity in host genetic factors can influence recognition and binding of microbes to the body surfaces, which can provide differential growth conditions[114]. Furthermore the intensity of immune reactions to microorganisms could be different based on genetic factors[115]. There might be individualized predispositions to development of inflammation in response to microbes or to microbial components based on an individual's immune response, which may result in the initiation or progression of inflammation and development of diseases like CRS or asthma. Hsu et al comprehensively reviewed the data on genetic polymorphisms and variants associated with CRS [116]. There are a few genetic variants in elements of the innate immune system associated with CRS – including the Nitric Oxide Synthase (NOS) pathway [117] and TLR-2 [110] – that could directly or indirectly influence the microbiome.

Host factors and the microbiome in CRS

The interplay between the innate and adaptive immune systems, the microbial communities and inflammation in sinonasal mucosa is rather complex and not completely understood yet. Th2 inflammation in eosinophilic CRS has been associated with down regulation of elements of innate immunity. Reductions in antimicrobial peptides like human beta-defensin 2(hBD-2) and surfactant protein-A (SP-A) and decreased expression of TLR9 have been described in CRS. These alterations can influence the defense against microbial pathogens and affect microbial colonization[118]. On the other hand, colonization with fungi and

specific bacteria like *S. aureus* has been linked to maladaptive Th2 responses[35]. Microbes and their products are ligands for the innate immune receptors that are also present on inflammatory granulocytes like basophils and mast cells that are found in CRSwNP[119] [120]. Activation of these cells through these receptors could result in recruitment of other inflammatory cells involved in Th2 response and progression of the inflammation in CRS.

In the following paragraphs, we have briefly summarized the findings regarding changes in the mucosal immune system in CRS that can influence the microbial communities in the sinonasal cavity.

Multiple elements of the innate immune system, including antimicrobial peptides and proteins, and pattern recognition receptors, have been studied in CRS[121],[122]. Sinus mucosal cells including submucosal mixed seromucinous glands, and epithelial cells produce a host of proteins and peptides with antimicrobial functions[123]. These molecules include enzymes (e.g. lysozyme and complement components), permeabilizing peptides (e.g. defensins, cathelicidins, bacterial permeability-increasing protein [BPI], and palate, lung, and nasal epithelium clone [PLUNC]), collectins (e.g. mannose-binding lectin[MBL], surfactant protein-A[SP-A] and surfactant protein-D[SP-D]), and binding and neutralizing proteins (e.g. lactoferrin and serum amyloid A [SAA])[124].

In normal conditions, one would anticipate an increase in the inducible peptides in response to increased microbial colonization or infection. Any reduction in the level of these molecules can potentially influence microbial colonization. Human sinonasal epithelial cells isolated from CRS patients were shown to have decreased expression of human beta-defensin 2(hBD-2), which belongs to the defensin family[118]. Defensins are one of the main antimicrobial peptide families involved in the microbiome of the respiratory epithelium[125]. And the reduced expression of hBD-2 could potentially affect the abnormal microbial colonization and immune responses in CRSwNP.

Furthermore, multiple members of the PLUNC family, including SPLUNC1 and long palate lung and nasal epithelium clone-2 (LPLUNC2), were reduced, along with reduced lysozyme and lactoferrin, in nasal polyps relative to uncinate tissue of healthy controls[126], which is reflective of the decreased number of submucosal glands in NP [126]. These findings suggest that decreased numbers of glands in nasal polyp leads to a localized defect in the production and release of glandular innate defense molecules, which can subsequently affect the microbial colonization of nose and sinuses in these individuals.

S100 proteins are involved in epithelial defense and repair, and have antimicrobial functions[127]. A few studies have shown a significant reduction in S100 proteins including S100A7 and S100A8/A9 in CRSsNP and CRSwNP [69, 128], which can lead to a diminished innate immune response.

Ficolins and collectins like MBL, SP-A and SP-D are soluble pattern recognition receptors. *In vitro* culture of human sinonasal epithelial cells with IL-4 or IL-13 reduced the expression of SP-A[118], which could compromise innate immunity in CRSwNP in which these cytokines are expressed. However, IHC staining of SP-A and SP-D in a clinical study

showed similar expression of these two molecules in CRSwNP and controls[129]. In contrast, SP-A was reported to be increased in the sinus tissue of CRSsNP patients[130].

Pattern recognition receptors, including NOD-like receptors, Toll-like receptors and bitter taste receptors are important players in host and microbial interactions in nasal and sinus mucosa. Toll-like receptors (TLR 1 through TLR10) are expressed in sinonasal epithelium [131, 132]. Signaling through TLRs results in production of cytokines and chemokines along with antimicrobial peptides. Lane et al. showed that TLR2 mRNA expression was increased in CRS in general[121]. However, expression of TLR2 and TLR9 were decreased in patients with early recurrence of polyps after surgery [121]. Furthermore, TLR9 was decreased in CRSwNP[133]. The TLR2 receptor recognizes a wide spectrum of microbial organisms including gram-negative and gram-positive bacteria, and fungi, which are found in abundance in CRS, and TLR-9 is important in recognition of DNA from bacteria and viruses. Reduced expression of these receptors may link early recurrence of disease with possible deficiency in host defense responses.

Bitter taste receptors signal by increasing intracellular calcium flux and increasing nitric oxide (NO) production and ciliary beat frequency. A common polymorphism in a bitter taste receptor (TAS2R38) is associated with gram-negative infection and biofilm formation and has been correlated with positive cultures for gram-negative bacteria like *P. aeruginosa* in CRS [134]. This polymorphism results in reduced signaling through the receptor, thus decreasing NO production and ciliary beat frequency, which are important in host immune defense against gram-negative organisms, including *P. aeruginosa* [134], which is commonly found in CRS [49, 52, 57, 76].

IL-22 plays an important role in mucosal integrity and in immunity against bacteria in the intestine and lung [135, 136]. This cytokine elicits multiple innate immune responses through epithelial cells, maintains mucosal homeostasis [137], and limits the allergic response and production of Th2 cytokines [138]. The main source of Il-22 in airways has been shown to be type 3 innate immune cells (ILC3) [138]. It was recently shown that production of IL-22 in large intestine by ILC3 is induced by commensal gut microbiota, specifically *Clostridia* [139]. A polymorphism in the IL-22 receptor (IL-22R) has been associated with severe CRS [140]. Furthermore, in patients with recalcitrant CRSwNP, the expression of IL-22R in sinus mucosal epithelial cells is significantly reduced [141]. However Delwitt et al. reported an increased level of this receptor in CRSsNP[142]. These data collectively point to mutual interactions of IL-22 and microbiota, and the reduced level of the receptor for IL-22 in CRSwNP could be an important factor in the pathogenesis of this disease.

Therapeutic use of Microbiota in CRS

Introducing microbial species in the form of probiotics, or inducing a transition in benefit of certain commensal bacteria by fecal transplant have been used in the treatment of multiple gastrointestinal diseases that are associated with altered gut Microbiota such as clostridium difficile infection and inflammatory bowel disease [143, 144]. It is not clear whether altering the microbiome of the nasal cavity towards a more sustained and symbiotic combination in

humans is feasible or has any therapeutic potential. One study tried to investigate a role for transitioning of the nasal microbiome for prevention or treatment in a mouse sinusitis model. Intranasal inoculations of *S. epidermis* plus *S. aureus* showed a significant decrease in the number of periodic acid-Schiff (PAS)-positive goblet cells in comparison with mice inoculated with *S. aureus* in this mouse model of sinusitis[145]. In a similar experimental model, Abreu et al. co-administered *L. sakei* with *C. tuberculostearicum* in mouse sinuses. They found significantly lower goblet cell hyperplasia and mucin hypersecretion in the sinus of these mice compared with those inoculated only with *C. tuberculostearicum*. This was suggestive that *L. sakei* protects the sinus epithelium, possibly through competitive inhibition of *C. tuberculostearicum* [53]. Whether these results could have any therapeutic implication in human disease remains to be investigated.

Summary and conclusions

Although the number of publications on microbiome in CRS has been increasing in the past few years, differences in experimental protocols, sampling and data analysis complicate comparisons. Except few species like S. Aureus that have been studied at length, it is rather difficult to extrapolate a consistent conclusion on the microbial changes in CRS from the existing data. However, available studies show that the microbiome of the nasal cavity differs significantly between CRS patients and controls and most studies have shown increased bacterial abundance in CRS. The results on bacterial diversity in CRS are varied, which is possibly due to antibiotic use prior to and around the time of sampling for microbial analyses or based on the regional microbial differences in the nasal cavity. Furthermore, there is a paucity of data on the viral microbiome in CRS, which can potentially affect the host inflammatory response as well as bacterial and fungal microorganisms.

Multiple inflammatory pathways and host defense elements are altered in CRS that could contribute to the observed microbiome differences. The observed decrease in number of glands and their associated antimicrobial peptides like SPLUNC1, LPLUNC2, lysozymes and lactoferrin in nasal polyps could potentiate the colonization and invasion of microbes to the adjacent mucosal membranes. Furthermore the nasal epithelium in CRS patients produces reduced amounts of other important AMPs like hBD-2, S100A7 and S100A8/9. Some of the pattern recognition receptors in charge of recognizing pathogen-associated molecules like TLR2 have also been found at reduced levels in refractory CRS. These defects, along with the loss of barrier function in the epithelium, further predispose the nasal and sinus mucosa to invasion by microbes like S. Aureus that would otherwise be non-pathogen residents.

However, it still remains to be determined if the alterations seen in nasal microbiome are cause or effect of this chronic inflammatory disease. Multiple microbial products are major triggers of inflammation; LPS and bacterial surface molecules trigger pattern-recognition receptors that are commonly found on innate immune system inflammatory cells like macrophages, epithelial cells and dendritic cells (DC). DCs have been described to be elevated in CRS tissue [146][147]. Furthermore, cells like basophils and mast cells that are found at increased numbers in nasal polyps and uncinate tissue of CRSwNP patients[119]

[120] possess pattern recognition receptors including TLR2[148][149] and formyl-methionyl-leucyl-phenylalanine(fMLP) receptors [150] that activate these cells upon microbial exposure and can potentially be a bridge between innate and adaptive immune response in CRS. These mechanisms can all link the mucosal inflammation that is the hallmark of CRS pathogenesis to the microbiome and point towards the importance of studying the nasal microbiome in association with inflammatory elements.

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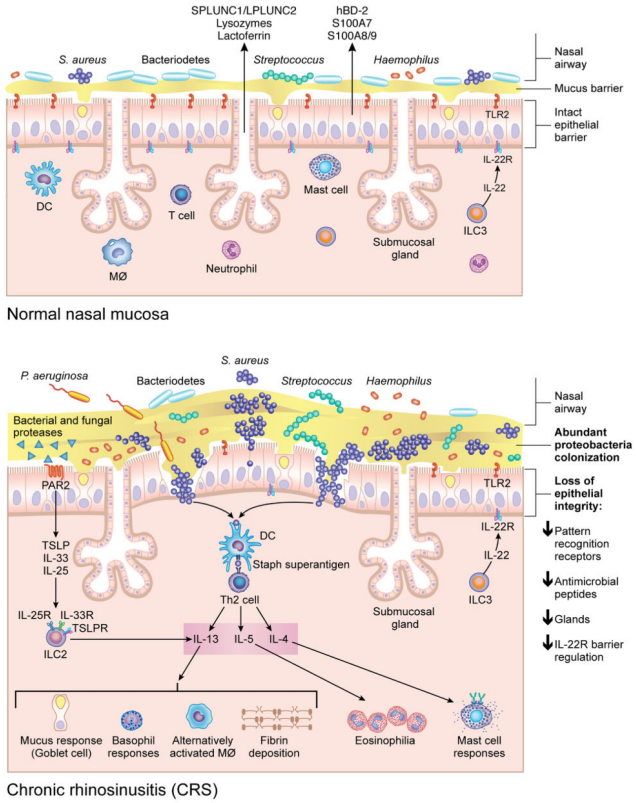

Figure 1. A Model for changes in the nasal microbiome and mucosal immune response in CRSwNP

Although multiple microorganisms colonize healthy nasal mucosa membranes, as shown in the top figure, there are changes that occur in the microbiome in patients with CRSwNP, including increased S. aureus abundance, decreased Bacteriodetes and decreased diversity, as shown in the bottom figure. These changes, along with loss of epithelial integrity, decreased pattern recognition molecules, decreased mucosal glands and decreased antimicrobial peptide production in the nasal polyp and sinus tissue, can potentially provide an environment that promotes invasion of microorganisms across the mucosal barrier. Enterotoxins produced by S. aureus can act as superantigens and promote Th-2 inflammation, resulting in production of cytokines such as IL-13, IL-4 and IL-5 that further recruit and activate inflammatory cells such as eosinophils, mast cells, basophils and alternatively activated macrophages. Bacterial and fungal proteases can induce production of thymic stromal lymphopoietin (TSLP) through Protease-Activated Receptor-2(PAR-2)[151], which will subsequently result in activation of innate lymphoid type 2 cells (ILC-2) that further produce IL-5 and IL-13.

Table 1

Results of studies on Nasal microbiome in CRS

Bacterial Microbiome Studies								
Name and year of publication	**Study participants**	**Sample type**	**Analysis method**	**Microbiome analysis Results**				
				Microbial Diversity	**Microbial Abundance**	**Differences at Phylum level**	**Differences at order, family or genus level**	**Differences at Species level**
Stephenson et al, 2010 (48)	18 CRS 9 controls	Anterior ethmoid mucosal biopsy	Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP)	-	-	-	*Propionibacterium* found in 83% of CRS vs. 67% of controls(no statistics available) *Diaphorobacter spp.* And *Peptoniphilus* only reported in CRS(78% and 72% respectively)	*Staphylococcus aureus* found in 50% of CRS vs 100% of controls (no statistics available)
Stressmann et al, 2011(49)	70 samples from 43 CRS No control	Mucosal biopsy	16S-rDNA sequencing and terminal restriction fragment length polymorphism (T-RFLP)	No control	-	-	Most abundant: *Pseudomonas, Citrobacter, Haemophilus, Propionibacterium, Staphylococcus and Streptococcus*	-
Abreu et al, 2012(53)	7 CRS 7control	Endoscopically guided maxillary sinus brushing	16S rRNA PhyloChip Phylogenetic microarray approach	Decreased in CRS: **1** Decreased richness (number of bacterial types) **2** Decreased evenness (relative distribution of bacterial types) **3** Decreased Shannon's diversity	Not different	-	Decreased:order *Lactobacillales*	Increased:*Corynebacterium tuberculostearicum* Decreased: *Lactobacillus Sakai, Carnobacterium alterfunditum, Enterococcus mundtii*, and *Pediococcus pentosaceus*
Feazel et al, 2012(51)	15 CRS (2 wNP and 13sNP) 5 controls	Middle meatus swabs	16-rDNA pyrosequencing and comparison with Silva version 104	Diversity was not different, A trend towards lower evenness in CRS	Not different	-	-	Increased: a trend towards increase for *Staphylococcus aureus*
Aurora et al, 2013(52)	30 CRS 12control	Lavage of the middle meatus	16S rRNA sequenced and submitted to NCBI; analyzed using scripts in the QIIME (Quantitative Insights into Microbial Ecology)	Increased diversity in CRS: 2333 in controls vs 3780 in CRS	Increased in CRS	No difference at phylum level	-	Increased *Corynebacterium accolens*, *Curtobacterium* species S22, *Pseudomonas* DT3-61, *Staphylococcus aureus Pseudomonas aeruginosa Decreased: Alicycliphilus* and *Cloacibacterium*
Boase et al, 2013(50)	38 CRS and 6 controls	Ethmoid sinus mucosal tissue	Ibis T5000 analysis PCR coupled with	Trends towards increased diversity; mean isolates per	Increased in CRS	-	-	Increased: *Staphylococcus aureus* in abundance and frequency

Bacterial Microbiome Studies								
Name and year of publication	**Study participants**	**Sample type**	**Analysis method**	**Microbiome analysis Results**				
				Microbial Diversity	**Microbial Abundance**	**Differences at Phylum level**	**Differences at order, family or genus level**	**Differences at Species level**
			electrospray ionization mass spectrometry	patients to controls was 2, in CRSsNP was 2.5 and in CRSwNP was 3.2				Less frequently: *Propionibacterium acnes*
Choi et al, 2014(54)	8 CRS (5wNP and 3sNP) 3 controls	Nasal lavage (NAL) fluid	16S-rDNA high-throughput pyrosequencing followed by identification using ExTaxon database.	Decreased diversity in CRS	Increased abundance in CRS	Increased: Proteobacteria Decreased: Bacteroidetes decreased, from 25.42% to 7.37%	Increased: *Staphylococcus*, *Corynebacterium* and *Propionibacterium* Decreased: *Prevotella*, *Streptococcus* and *Veillonella* **Pseudomonas* increased in CRSwNP compared with CRSsNP	Increased: Staphylococcus epidermidis, *Pseudomonas monteilii* Decreased: *Prevotella melaninogenica* *Staphylococcus aureus* increased in CRSwNP compared to CRSsNP
Ramakrishnan et al, 2015(58)	56 CRS and 26 controls	Swabs from Ethmoid region obtained during surgery	16-rDNA pyrosequencing and comparison with Silva version 111.	Not reported between CRS and Controls, Diversity was associated with optimal surgical outcome in CRS	No difference	No difference	Decreased: *Propionibacterium* and *Peptoniphilus* at genum level *Multiple Genera different when comparing CRS with and without asthma(detailed in the text)	Not reported between CRS and controls
Fungal Microbiome Studies								
	Study participants	Sample type	Analysis method	**Microbial Diversity**	**Microbial Abundance**	**Differences at Phylum level**	**Differences at order, family or genus level**	**Differences at Species level**
Aurora et al. 2013(52)	30 CRS 12 controls	Lavage of the middle meatus	18S rRNA deep sequencing	Increased diversity in CRS	-	Increased: Ascomycota Decreased: Basidiomycota	-	Increased: Cryptococcus neofromans, Rhodosporidium diabovatum, Davadiella Tassaiana Decreased: Malassezia-uncultured stramenopile
Boase et al 2013(50)	38 CRS and 6 controls	Ethmoid sinus mucosal tissue	Ibis T5000 analysis PCR coupled with electrospray ionization mass spectrometry	Fungi were only detected in 3 CRSwNP cases and in none of the control or CRSsNP	-	-	-	Only 3 positive samples; 2 CRSwNP cases had Aspergillus Fumigatus and 1 CRSwNP case had Penicilium Chrysogenum
Cleland et al. 2014(88)	23 CRS 11 controls	Middle meatus or anterior ethmoid swabs	18S rDNA tag-encoded FLX amplicon pyrosequencing	Not different	-	-	Increased: *Scutellospora* 33 genera had differential abundance in CRS vs. Controls	-