

# Remodeling of Paranasal Sinuses Mucosa Functions in Response to Biofilm-Induced Inflammation

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**Abstract:** Rhinosinusitis (RS) is an acute (ARS) or chronic (CRS) inflammatory disease of the nasal and paranasal sinus mucosa. CRS is a heterogeneous condition characterized by distinct inflammatory patterns (endotypes) and phenotypes associated with the presence (CRSwNP) or absence (CRSSNP) of nasal polyps. Mucosal barrier and mucociliary clearance dysfunction, inflammatory cell infiltration, mucus hypersecretion, and tissue remodeling are the hallmarks of CRS. However, the underlying factors, their priority, and the mechanisms of inflammatory responses remain unclear. Several hypotheses have been proposed that link CRS etiology and pathogenesis with host (eg, “immune barrier”) and exogenous factors (eg, bacterial/fungal pathogens, dysbiotic microbiota/biofilms, or staphylococcal superantigens). The abnormal interplay between these factors is likely central to the pathophysiology of CRS by triggering compensatory immune responses. Here, we discuss the role of the sinonasal microbiota in CRS and its biofilms in the context of mucosal zinc (Zn) deficiency, serving as a possible unifying link between five host and “bacterial” hypotheses of CRS that lead to sinus mucosa remodeling. To date, no clear correlation between sinonasal microbiota and CRS has been established. However, the predominance of Corynebacteria and Staphylococci and their interspecies relationships likely play a vital role in the formation of the CRS-associated microbiota. Zn-mediated “nutritional immunity”, exerted via calprotectin, alongside the dysregulation of Zn-dependent cellular processes, could be a crucial microbiota-shaping factor in CRS. Similar to cystic fibrosis (CF), the role of SPLUNC1-mediated regulation of mucus volume and pH in CRS has been considered. We complement the biofilms’ “mechanistic” and “mucin” hypotheses behind CRS pathogenesis with the “structural” one – associated with bacterial “corncob” structures. Finally, microbiota restoration approaches for CRS prevention and treatment are reviewed, including pre- and probiotics, as well as Nasal Microbiota Transplantation (NMT).

**Keywords:** rhinosinusitis, chronic rhinosinusitis, nasal polyps, rhinosinusitis pathophysiology, rhinosinusitis pathophysiology, microbiota, nutritional immunity

## Introduction

Rhinosinusitis (RS) is an inflammatory disease of the nasal and paranasal sinuses. According to the European Position Paper on Rhinosinusitis and Nasal Polyps 2020 (EPOS 2020), RS is classified into acute (ARS) or chronic (CRS).<sup>1</sup> The categorizing factors involve (i) the presence and type of symptoms, such as nasal blockage, facial pain, cough, reduction or loss of smell, and (ii) their duration, ie,  $\leq 12$  weeks for ARS and  $\geq 12$  weeks for CRS. Both types of RS are among the most common health issues worldwide. It is estimated that 6–15% of the general population suffers from ARS, one of the most common diagnoses for antibiotic prescriptions. Similarly, CRS affects 5–12% of the global population, and differences in risk factors such as smoking or environmental irritants may account for some geographical variation.<sup>1–4</sup> The overall prevalence of CRS in the general population in Europe and the United States was estimated to be 10.9% and 11.9%, respectively.<sup>1</sup> Comparable percentages were found in the adult general population of other countries, such as South Korea (8.4%), China (8.0%), and Brazil (5.5%).<sup>4</sup> CRS significantly reduces the health-related quality of life (HRQoL), mainly due to nasal obstruction, rhinorrhea, and olfactory impairments, as well as substantially burdens the health care system, even more costly than asthma or peptic ulcer disease.<sup>1,4–7</sup> The implementation of HRQoL

questionnaires demonstrates clinical and predictive treatment efficacy and enhances patient care. The Sino-Nasal Outcome Test-22 (SNOT-22) is the most widely utilized and most suitable questionnaire among those frequently employed in rhinology due to its validity, reliability, and usability.<sup>7</sup>

Chronic rhinosinusitis (CRS) is a complex condition characterized by numerous variants (phenotypes) with distinct pathophysiologies (endotypes).<sup>8</sup> Clinically, the presence or absence of nasal polyps (NPs) has been used to differentiate between the two primary CRS phenotypes – (i) with NPs (CRSwNP) and (ii) without NPs (CRSsNP).<sup>9,10</sup> Other CRS phenotypes entail (i) infectious, (ii) fungal, (iii) pediatric, and associated with (iv) cystic fibrosis, (v) aspirin-exacerbated respiratory disease, and (vi) systemic diseases.<sup>9</sup>

A critical aspect of CRSwNP is the restoration of recovery of olfactory function. The standard of care for CRSwNP, consisting of intranasal corticosteroids (INCS) and short repeated courses of oral corticosteroids (OCS), produced mixed results of olfactory function recovery. In addition, in subjects with CRSwNP, OCS has non-durable effects on olfactory function, often leading to the consumption of high cumulative doses. Therefore, biological medications targeting the IL-4/-13 receptor, such as dupilumab, have been proposed as promising treatments for improving olfactory outcomes in CRSwNP patients with type 2 inflammation.<sup>11</sup>

In both CRSsNP and CRSwNP, inflammation is highly heterogeneous and manifests as one of three major inflammatory endotypes characterized by various T cell cytokine patterns, including type 1 (T1) with Th1 cytokine IFN- $\gamma$ , type 2 (T2) with Th2 cytokines IL-4, IL-5, IL-9 and IL-13, and type 3 (T3) with Th17 cytokines IL-17 and IL-22.<sup>12</sup> Since type 2 inflammation is distinctive of CRSwNP, CRS is also often divided into type 2 and non-type 2 categories.<sup>1,13</sup> Depending on the degree of eosinophilia in sinuses, idiopathic eosinophilic CRS (E-CRS) and non-eosinophilic CRS (NE-CRS) subsets of CRS are also distinguished. Severe tissue eosinophilia is usually associated with more severe symptoms and poorer treatment outcomes.<sup>14</sup> Furthermore, CRS is subdivided into primary and secondary, as well as localized and diffused, based on anatomical distribution. Up to 30% of patients with CRS develop NPs, typically as a Th2-dominant eosinophil-driven disease. However, type 1 mediators may also contribute to NPs.<sup>15</sup> Consequently, remodeling of the sinus mucosa matter is a common, but not unique, feature of diffused primary CRSwNP. Remodeling of the sinus mucosa may activate several cellular processes such as epithelial-to-mesenchymal transition (EMT) and autophagy.<sup>14</sup>

Chronic inflammation in CRS generally results in mucosal barrier dysfunction, inflammatory cell infiltration, impaired mucociliary clearance, mucus hypersecretion, and tissue remodeling. However, the stimuli and mechanisms underlying these inflammatory processes remain unclear. CRS etiology has been linked with multiple host and exogenous factors, such as dysfunction of the epithelial barrier, mucociliary clearance and immune responses, environmental exposures, specific bacterial or fungal organisms and their toxins, microbial biofilms, and sinonasal microbiota imbalance (dysbiosis).<sup>12,16</sup>

Accordingly, several hypotheses underlying CRS development have been proposed, including (i) “immune barrier” hypothesis, (ii) “eicosanoid” hypothesis, (iii) “fungal” hypothesis, (iv) ‘staphylococcal superantigens’ hypothesis, (v) “biofilm” hypothesis, and (vi) “microbiota/microbiome” hypothesis.<sup>4,12,17,18</sup> However, the current view of CRS assumes an aberrant interplay between the host and exogenous factors rather than a single cause.<sup>17</sup> The hypotheses have been discussed throughout the manuscript. It should be emphasized that single-nucleotide polymorphisms (SNPs) may increase the likelihood of developing CRS.<sup>19</sup> Barrier deficiencies, alterations in ion channels, or mutations in genes associated with the TH2 inflammatory response are the major mechanisms that contribute to the development and maintenance of chronic inflammation in CRS. Therefore, understanding the pathophysiology of CRS is improved through genetic analysis of SNPs in patients with CRS.<sup>19</sup>

The “immune barrier” hypothesis is the most comprehensive and coherent with the other theories’ view on the etiology and pathogenesis of CRS. It is assumed that mechanical or immune-mediated disruption of the mucosal barrier and triggered inflammation enhances exposure to external stimuli and induces dysbiotic changes in biofilms, resulting in excessive compensatory immune responses. Genetic and epigenetic factors are also involved, such as bitter taste receptors (T2Rs) polymorphism, that is receptors that play an essential role in microbiota surveillance by sensing mediators of bacterial quorum sensing (QS) communication.

Recently, a link between CRS and low zinc (Zn) levels in the nasal mucosa of CRS patients, in particular with nasal polyps (CRSwNPs), has been suggested.<sup>20</sup> Mucosal zinc (Zn) deficiency may induce several typical CRS pathomechanisms, such as

mucosal barrier dysfunction, inflammatory cell infiltration, impaired mucociliary clearance, mucus hypersecretion, and tissue remodeling.<sup>20</sup>

Like in other chronic diseases, the interplay between sinonasal microbiota and the immune system appears to be an essential but unrevealed trait of CRS-“microbiota” hypothesis.<sup>8,21–25</sup> These growths in biofilms microbial communities coexist in a commensal relationship with the human host (eubiosis), and contribute to a nonspecific defense due to their colonization resistance function, ie, the ability of microbiota to limit the introduction of exogenous microorganisms and pathobiont expansion.<sup>26,27</sup> If this homeostatic balance is not maintained, certain bacterial species or, more likely, clusters of bacteria (“co-occurrence” or “community” hypothesis) may overgrow and undermine others (dysbiosis) and, in turn, shift from a commensal to parasitic lifestyle. Multiple synergistic and antagonistic interspecies interactions, such as cross-feeding and competitive exclusion, regulate microbiota homeostasis and may contribute to dysbiotic transitions. This interspecies crosstalk is coordinated by quorum sensing (QS) systems. QS is a bacterial cell–cell and population density-dependent communication via signal molecules (QSM), such as *N*-acyl-homoserine lactone (AHL) and *N*-3-(oxododecanoyl)-homoserine lactone (C12-HSL), which synchronize metabolic processes and gene expression in a group of microorganisms. In addition, QS systems control several crucial bacterial–host interaction processes such as biofilm formation, expression of virulence factors, stress responses, and metal sequestration. For example, *Staphylococcus aureus* growth, virulence, and colonization abilities can be impaired by other *Staphylococcus* species through the accessory gene regulatory (*agr*) QS system.<sup>28</sup> Furthermore, QMS, such as C12-HSL, act as virulence factors and directly or indirectly may affect the epithelial barrier function, inflammation, and even intestinal carcinogenesis (reviewed by Conquant et al, 2021).<sup>29</sup> Therefore, QSS maintain a balanced microbiota and, in concert with bitter taste receptors (T2Rs), microbiota–host interactions in homeostasis.<sup>30</sup> It has been hypothesized that inflammation and sinus blockage can originate either (i) from immune dysfunction followed by microbiota dysbiosis or, conversely, (ii) that dysbiosis is responsible for immune response dysregulation.<sup>31,32</sup> Yet, no clear distinctions between the sinonasal microbiota composition (microbiotypes) of healthy and CRS subjects have been established; hence, the microbiota cannot serve as an independent CRS biomarker.<sup>8,22,23</sup>

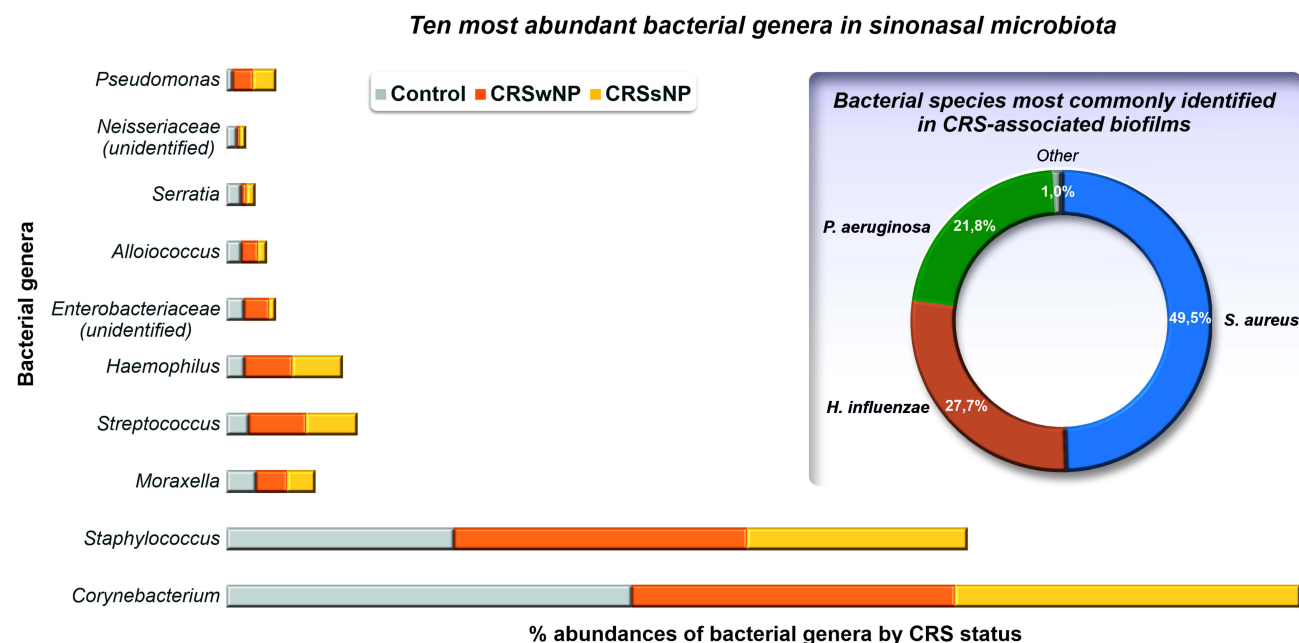
The ineffectiveness of antibiotics in treating CRS supports the “microbiota” hypothesis.<sup>33</sup> Nonetheless, like in cystitis fibrosis, antimicrobials with anti-inflammatory properties, such as doxycycline or macrolides, may be advantageous.<sup>1,34</sup> For instance, a short course of doxycycline appears beneficial in patients with polyps.<sup>1</sup>

Microbial biofilms are a ubiquitous form of microbial growth in up to 80% of microorganisms on the Earth, including microbiota in the human body. In medicine, biofilms account for 80% of chronic infections.<sup>35,36</sup> These complex and common multispecies communities of microorganisms encased in a self-produced slimy matrix of extracellular polymers (EPS) have an extraordinary capacity for growth and persistence on inanimate and living surfaces, such as mucosal epithelium.

In biofilms growing on the sinus mucosa of patients with CRS, well-recognized pathogens include *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. In particular, *S. aureus*, *H. influenzae*, and *P. aeruginosa* are typically identified in CRS-associated biofilms (Figure 1).<sup>37–39</sup> In addition, various opportunistic pathogens such as coagulase-negative staphylococci, Corynebacteria, *Streptococcus viridans*, *Enterococcus faecalis*, anaerobes, *Cutibacterium (Propionibacterium)*, *Porphyromonas*, *Prevotella*, *Bacteroides*, and *Peptoniphilus (Peptostreptococcus)*. However, comparable biofilm detection rates in patients with CRS and healthy controls raise questions about their role in this disease.<sup>40</sup>

However, comparable biofilm detection rates in CRS patients and healthy controls raise questions about the role of these structures in this disease.<sup>40</sup> Nonetheless, biofilms in CRS patients are associated with more severe symptoms, poorer outcome, complicated postoperative course, and increased likelihood of revision surgery.<sup>41–46</sup> Alterations in the structure and function of the paranasal sinuses associated with epithelial barrier disruption, impaired mucociliary clearance, elevated inflammatory response, fibrosis, hyperplasia, and abnormal collagen deposition, resulting in tissue remodeling, and ultimately, formation of nasal polyps, are also typical manifestations of biofilm-induced sinusitis.<sup>47</sup> A “mechanistic” and “mucin” hypotheses have been proposed to explain the potential role of such “pathologic” or “dysbiotic” biofilms in CRS pathogenesis.

In this review, we discuss the potential role of sinonasal microbiota in CRS based on recent metagenomic findings in the context of mucosal zinc deficiency as a possible link between the “host” and “bacterial” hypotheses of CRS. For instance, the potential function of Zn-mediated “nutritional immunity” and calprotectin, a metal-binding antimicrobial peptide (AMP), as microbiota-shaping factors in CRS has been addressed. In addition, another AMP-SPLUNC1, acting as an epithelial Na<sup>+</sup> channel



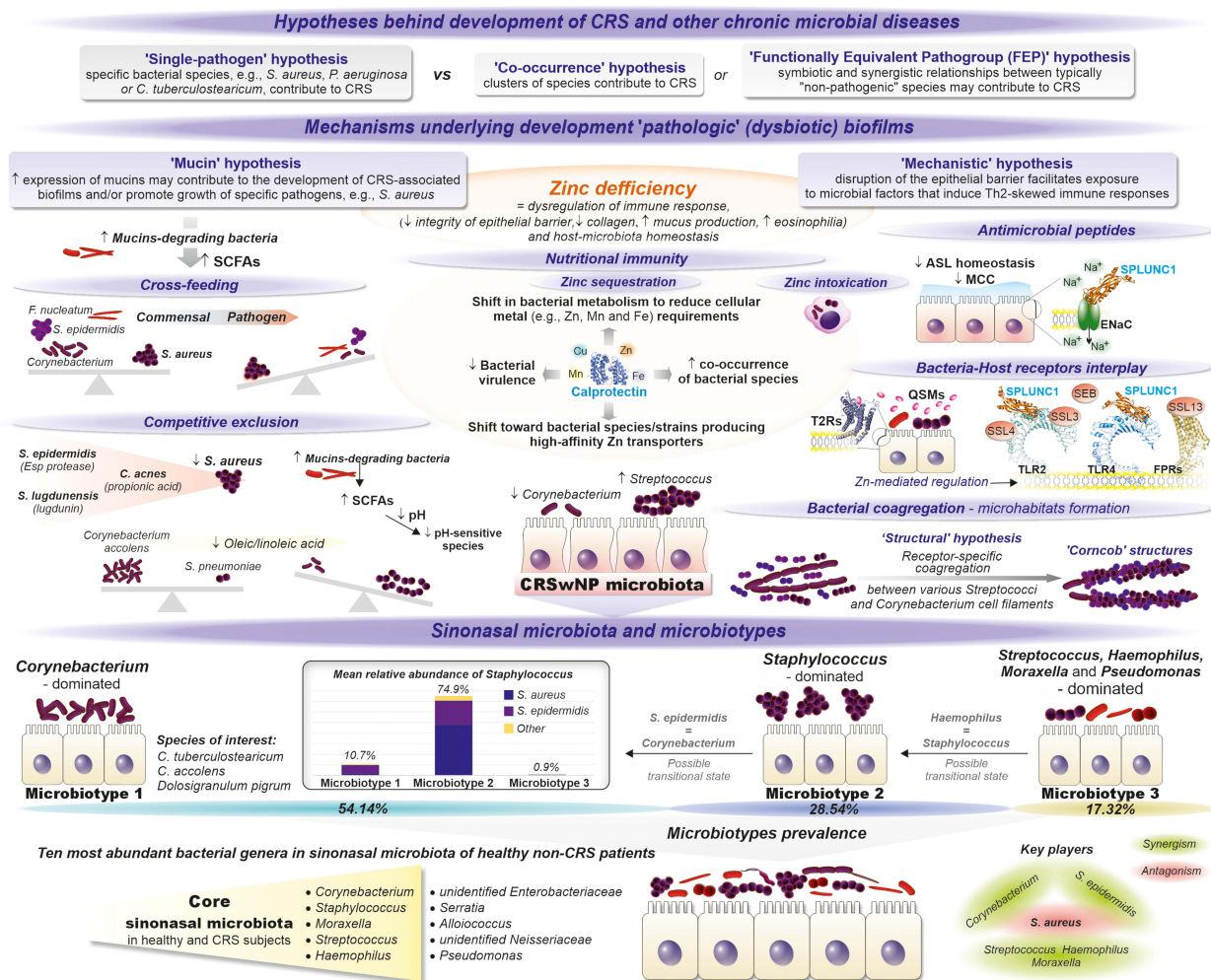
**Figure 1** Ten most abundant bacterial genera in sinonasal microbiota (Data from Paramasivan S, Bassiouni A, Shiffer A, et al. The international sinonasal microbiome study: a multicentre, multinational characterization of sinonasal bacterial ecology. *Allergy*. 2020;75(8):2037–2049.<sup>23</sup>) and bacterial species most commonly identified in CRS-associated biofilms. Data from.<sup>37–39</sup>

(ENaC) pH-dependent inhibitor, may link microbiota via pH with airway surface liquid (ASL) homeostasis and impaired mucociliary clearance (MCC), as shown in cystic fibrosis. In addition, we consider mechanisms behind biofilms’ “mechanistic” and “mucin” hypotheses in the pathogenesis of CRS. Furthermore, alterations in bacterial genetic background as well as inter- and interspecies interactions and processes underlying the transition from “healthy” or eubiotic biofilms toward “pathologic” or “dysbiotic” biofilms are confronted with the host immune responses. We believe that our findings will aid in a better understanding of the biofilm-mediated pathomechanism in CRS and in developing new diagnostic and treatment methods based on microbiota restoration, including nasal microbiota transplantation (NMT).

## Role of Sinonasal Microbiota in CRS

Although a metagenomic approach to explore microbial diversity has significantly improved our understanding of the human microbiota, several variables, such as (i) sample type and collection method, (ii) bacterial nucleic acid isolation methodology, and (iii) sequencing technology limitations, may still influence the results and their interpretation. Additional confounders included significant interpersonal, geographical, and racial heterogeneity in the sinonasal microbiota composition. Subsequently, data collected in metagenomic studies on sinonasal microbiota should be carefully interpreted.<sup>23–25,40,48</sup> For instance, atypical or unusual microorganisms identified as causative agents of CRS that are not part of the sinonasal microbiome should be thoroughly evaluated.<sup>23</sup>

Nevertheless, specific sinonasal microbiota patterns and/or interbacterial relationships have been linked to the onset and progression of CRS. Specifically, the predominance of *Corynebacterium* and *Staphylococcus*, which likely live in reciprocal or antagonistic relationships, was observed.<sup>24,31</sup> Briefly, five genera, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella*, represent the core sinonasal microbiome in both healthy and CRS subjects according to a recent international sinonasal microbiome study involving 461 subjects.<sup>23</sup> However, *Corynebacterium* and *Staphylococcus* were the far most abundant genera, occurring in samples with a mean relative abundance of 44.02% and 27.34%, respectively (Figure 2). Furthermore, in CRSwNP patients, *Corynebacterium* abundance was significantly decreased (40.29% vs 50.43%;  $p = 0.02$ ) and correlated with increased abundance of *Streptococcus* genus, compared to healthy controls. In contrast, no significant differences were observed between CRSsNP patients and control subjects in the predominance of any of the top ten most



**Figure 2** The sinonasal microbiota and microbiotypes in patients with CRS and healthy individuals (adapted from Paramasivan et al and Bassiouni et al) and potential hypotheses and mechanisms underlying the development of "pathologic" (dysbiotic) biofilms. Data from <sup>22,23</sup>.

abundant bacterial genera (Figure 2). Interestingly, the distributions of the two most frequently isolated pathogens from CRS-biofilms biofilms, *Staphylococcus* and *Pseudomonas*, were similar among all the tested groups.<sup>23</sup>

The sinonasal microbiome appears to be most similar to the microbial community of anterior nares, which could explain the abundance of typical respiratory tract pathogens, *Streptococcus*, *Haemophilus*, and *Moraxella*, and implicates sinuses as a potential reservoir for other respiratory tract diseases, such as acute otitis media.<sup>49</sup> On the other hand, *Streptococcus*, *Haemophilus*, and *Moraxella*, as a part of the core sinonasal microbiota, likely emphasizing their importance in maintaining sinonasal physiology as commensals.

Furthermore, Bassiouni et al distinguished in this core sinonasal microbiota three major sinonasal "microbiotypes", ie, *Corynebacterium*-dominated (microbiotype 1), (ii) *Staphylococcus*-dominated (microbiotype 2), and (iii) *Streptococcus*, *Haemophilus*, *Moraxella*, and *Pseudomonas*-dominated (microbiotype 3).<sup>22</sup> The prevalence of these microbiotypes was not correlated with healthy and diseased sinuses; however, geographical differences in their distribution were reported.<sup>22</sup> The microbiotype 1 was overrepresented in Asia and Australasia, while the prevalence of microbiotype 2 was higher in Europe. However, Europe was represented only by one location (Amsterdam); therefore, it is uncertain whether these results apply to other European areas.

Hoggard et al found a similar sinonasal microbiota profile in controls and idiopathic CRS subjects, with a predominance of *Corynebacterium* and *Staphylococcus* genera and lower abundances of *Streptococcus*, *Moraxella*, and *Haemophilus*.<sup>40</sup> In

contrast, members of the genera *Staphylococcus*, *Streptococcus*, *Haemophilus*, *Pseudomonas*, *Moraxella*, and *Fusobacterium* dominated at various levels in dysbiotic communities, along with a reduced abundance of the dominant *Corynebacteria*.<sup>40</sup>

Due to its probiotic potential, *Corynebacterium* is referred to as a “gatekeeper”, “keystone”, or “cornerstone” member of the upper respiratory tract microbiota that is essential for respiratory health and competitively excludes potential pathogens, such as *S. pneumoniae*.<sup>22,50</sup> Interestingly, *Corynebacterium* is also the most prevalent and abundant bacterial genus in another chronic condition – diabetic foot ulcers (DFUs).<sup>51</sup> In contrast, a high abundance of staphylococci (coagulase-negative commensal species and *S. aureus*) in the sinonasal microbiota of healthy subjects likely indicates their dual, associated with the ability to shift lifestyle, commensal-pathogen role. The exact factors and mechanisms underlying *S. aureus* transition from a commensal to pathogenic lifestyle are unknown, but reciprocal or antagonistic interactions via QS systems with *Corynebacteria* and other *Staphylococci* are likely involved.<sup>22</sup> Indeed, a positive and negative correlation was reported between the co-occurrence of *Corynebacterium* and *S. epidermidis* and between this bacterial pair and *S. aureus*, respectively.<sup>22</sup> For example, a serine protease Esp of *S. epidermidis* disassembles *S. aureus* biofilms and inhibits its colonization, degrading ~75 *S. aureus* proteins, including 11 biofilm formation- and colonization-associated proteins as well as several human receptors, such as fibronectin, fibrinogen, and vitronectin, utilized by *S. aureus* during host colonization or infection.<sup>52</sup> Likewise, *S. lugdunensis* produces lugdunin – a bactericidal peptide that prevents *S. aureus* nasal colonization.<sup>53</sup> Moreover, *C. accolens* and *C. pseudodiphtheriticum* are the two most significant determinants of *S. aureus* nasal carriage, likely due to mutually cooperative - *C. accolens* and mutually competitive - *C. pseudodiphtheriticum* interactions. The competitive interactions appear to be microenvironment-dependent, ie, restricted to mucosal sites lined by ciliated pseudostratified columnar epithelium (but not with lined by skin-like squamous epithelium of the anterior nares), possibly as nutrient or other niche-related limitations supporting competitive behavior.<sup>54</sup> However, an opposite relationship between *C. accolens* and *S. aureus* was reported in other studies, where *C. accolens* cell-free culture supernatants appear to inhibit the growth of both planktonic and biofilm forms of *S. aureus* oleic acid.<sup>55,56</sup> Subsequently, Tween-80, metabolized by *C. accolens* to oleic acid, has been proposed as a prebiotic to promote microbiota homeostasis in patients with CRS.<sup>57</sup> Also, *Dolosigranulum pigrum*, as a nasal health-promoting species with immunomodulatory and nasal barrier-enhancing potential as well as antimicrobial activity against *S. aureus*, was indicated candidate for development into a topical next-generation probiotics or live-biotherapeutic products.<sup>58</sup> In addition, Huang et al demonstrated that *C. accolens* has the potential to mitigate *S. aureus*-induced mucosal barrier damage.<sup>56</sup> Notably, *C. accolens* antagonizes with *S. pneumoniae* via oleic and linoleic acid, which likely also explains *Streptococcus* depletion in *Corynebacterium*-dominated microbiota in approximately one-fifth of CRS patients.<sup>8</sup>

Also, antagonism between *S. aureus* and *S. pneumoniae*, *H. influenzae*, or *M. catarrhalis* is well documented and probably reflects its drastically low abundance (0.53%) in the microbiotype 3 (Figure 2).<sup>59–61</sup> Accordingly, Pettigrew et al reported that the elimination from the nasopharyngeal carriage of *S. pneumoniae* and *H. influenzae* increases the risk of *S. aureus* colonization.<sup>60</sup> Killing *S. aureus* by pneumococcal hydrogen peroxide through activation of lytic bacteriophages can partially explain their negative co-occurrence.<sup>50</sup> In contrast, positive associations have been identified among *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.<sup>62</sup>

The abundance of *Corynebacteria* or specific *Corynebacterium* species, such as *C. tuberculostearicum*, was also positively correlated with CRS in certain other studies.<sup>8,24,31,63</sup> Accordingly, Cope et al distinguished four central taxonomic states in CRS microbiota, characterized by specifically enriched gene pathways and immune response patterns: (i) Streptococcaceae (DSI) – ansamycin biosynthesis, (ii) Pseudomonadaceae (DSII) – tryptophan metabolism, (iii) Corynebacteriaceae [DSIII(a)] – with enriched the PPAR- $\gamma$  signaling pathway, and (iv) Staphylococcaceae [DSIII(b)] – two-component response.<sup>8</sup> Corynebacteriaceae [DSIII(a)] state was detected in the microbiota of approximately one-fifth of CRS patients and positively correlated with increased expression of IL-5 and IFN- $\gamma$  and a higher risk for developing polyposis.<sup>8</sup> In contrast, Pseudomonadaceae (DSII) and Staphylococcaceae [DSIII(b)] states characterized an increase in a variety of pro-inflammatory Th1 responses.

Similarly, a meta-analysis of sinonasal microbiome studies revealed *Staphylococcus*, *Propionibacterium*, *Corynebacterium*, *Streptococcus*, and unclassified *Actinobacteria* were the most abundant bacteria across all subjects. However, only a higher abundance of members of the genus *Corynebacterium* was consistently linked to CRS; hence, it was proposed as a potential biomarker of CRS-associated sinonasal microbiota.<sup>31</sup> Additionally, bacterial communities in this group were characterized by significantly lower diversity and increased dispersion. Among the remaining genera, positive

correlations with CRS were observed for *Parvimonas*, *Porphyromonas*, *Citrobacter*, *Prevotella*, *Curtobacterium*, and *Fusobacterium*. Notably, a negative correlation was observed between the abundance of *Corynebacterium* and *Fusobacterium*, indicating that these bacteria may compete for the same niche. In addition, the importance of *Burkholderia* and *Propionibacterium* (*Cutibacterium*) in regulating the microbiota communities in healthy sinuses has been suggested. Subsequently, depletion of these “protective” or “gatekeepers” genera significantly increased the susceptibility of the sinonasal microbiota to disturbance.<sup>31</sup> This “protection” could be mediated by *Cutibacterium acnes* (*Propionibacterium acnes*) via propionic acid-mediated attenuation of macrophage-induced inflammation and inhibition of *S. aureus* growth.<sup>64</sup> Strikingly, *Burkholderia* and *Corynebacterium* were found to be negatively correlated. In addition, Abreu et al reported an increase in the relative abundance of a single species, *C. tuberculostearicum*, concomitant with the depletion of distinct lactic acid bacteria in CRS patients.<sup>24</sup> Likewise, Aurora et al reported a statistically significant increase in *C. accolens* in patients with CRS compared to controls.<sup>63</sup>

Notably, in dental plaque biofilms, so-called “corncob” or “hedgehog” structures are formed by filaments of *Corynebacteria* cells (~10–20 µm long) coaggregating with Streptococci, and to a lesser extent with members of *Porphyromonas* genus and Pasteurellaceae family (*Haemophilus* and *Aggregatibacter*) in a specific order.<sup>65</sup> Corncobs are well-defined microhabitats within biofilms, where the spatial proximity of their inhabitants facilitates the cross-feeding phenomenon, ie, nutrient exchange between bacteria, where the metabolic products of one species are utilized in the nutrition of another species.<sup>65</sup> In this view, *Corynebacteria* may be essential to the architecture of sinonasal biofilms, and their transition to a dysbiotic state may be associated with structural disturbances affecting corncobs.<sup>65</sup> Hence, expanding the mechanistic and mechanistic mucin and mucin hypotheses of biofilms’ role in CRS by the structural hypothesis.

The formation of “corncobs” is a well-described trait of *Fusobacterium nucleatum* in the context of dental plaque-associated diseases, such as dental caries and periodontitis. In dental plaque, *F. nucleatum* co-aggregates through receptor-ligand interactions (via RadA and Fap2 fusobacterial receptors) with multiple early (aerobic) and late (anaerobic) colonizers. The receptor–ligand mechanism is likely utilized by *Corynebacteria*, since co-aggregation with several streptococcal species (*S. cristatus*, *S. mitis*, *S. oralis*, or *S. infantis*), except *S. gordonii*, was observed.<sup>66</sup> Importantly, a negative correlation between *Corynebacterium* and *Fusobacterium* in sinonasal microbiota reported by Mackenzie et al may suggest their distinct roles in sinonasal biofilm formation or participation at various stages of its development.<sup>31</sup> For instance, *F. nucleatum* as an aerotolerant organism may promote the growth of strict anaerobic species. In contrast, a limited number of potential co-aggregations with *Corynebacterium* species may contribute to the long-term stability of biofilms.<sup>65</sup>

## Role of Zinc in CRS

Zinc is localized in the basal bodies of cilia in airway epithelial cells, and its chelation in human nasal and bronchial airway cells slows ciliary beating by 25%, suggesting that mucociliary function depends on Zn.<sup>20</sup> Furthermore, Th2-polarization with elevated production of proinflammatory cytokines (IL-6 and IL-8), tissue eosinophilia, and reduced collagen synthesis were observed under zinc depletion-linking Zn with “immune barrier” hypothesis.<sup>67</sup> Suzuki et al hypothesized that efficient Zn sequestration by mucins in the submucosal glands, followed by the release of mucin-Zn complexes in nasal discharge, is responsible for its deficiency. In addition, the authors suggested that this process is continually driven by inflammation in a “vicious cycle” mechanism, where inflammation-induced upregulation of mucins results in hypersecretion of mucus and Zn loss from the mucosa, which, in turn, promotes further Zn depletion and maintains inflammatory process.<sup>20,67</sup>

Zn is an essential micronutrient required for the activity of >300 enzymes and 1000 transcription factors. For example, intestinal alkaline phosphatase, an enzyme involved in maintaining intestinal barrier integrity and associated with tissue remodeling matrix metalloproteinases (MMPs), are zinc-dependant enzymes.<sup>68</sup> Likewise, Zn-dependent proteins account for ~4–8% of the bacterial proteome, ie, ~100–200 *S. aureus* proteins, including most superantigens. Furthermore, Zn regulates superantigens’ cellular receptor activity, T cell receptors (TCR) signaling activation, and the surface expression of major histocompatibility complex (MHC) class II – linking Zn with “staphylococcal superantigens” hypothesis.<sup>69</sup> More importantly, Zn controls the activity of microbial pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), receptor for advanced glycation endproducts (RAGEs), and G-protein-coupled receptors (GPRPs), the first line of defense against invading

microbial pathogens, recognizing conserved microbial molecules, ie, microbial- or pathogen-associated molecular patterns (MAMPs/PAMPs). For example, Zn modulates LPS-induced and TLR-4-dependent inflammation.<sup>68,70</sup>

Consequently, a zinc deficiency may serve as an intermediary between exogenous and endogenous factors that contribute to the development and progression of CRS. Indeed, Zn deficiency was connected with hyperpermeability of the airway epithelium in other inflammatory diseases, such as atopic dermatitis and bronchial asthma.<sup>20,67</sup> Similarly, low zinc levels impair the stability of intestinal mucosa and can induce gut dysbiosis, whereas adequate Zn levels promote the formation of “healthy” gut microbiota.<sup>71</sup> Recently, Yunker et al stated that Zn homeostasis is necessary to regulate host-microbiome interaction via modulation of intestinal barrier permeability and maintenance of Paneth cells.<sup>72</sup> Subsequently, Zn homeostatic imbalance was reported in various chronic intestinal diseases, including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and colorectal cancer (CRC). Moreover, zinc deficiency in elderly individuals is positively correlated with an increased risk of pneumonia caused by *S. aureus*, *S. pneumoniae*, *H. influenzae*, *K. pneumoniae*, *L. pneumophila*.<sup>73</sup>

It may be assumed that CRS-associated microbiota are under permanent Zn-mediated “nutritional immunity”, that is, the host’s mechanism for inhibiting bacterial growth by restricting the availability of essential metal ions. This process may shape the microbiota composition based on the Zn requirements and tolerances of individual microbiota members and/or promote specific bacterial species or strains adapted to Zn-deprived conditions, for example, equipped with high-affinity Zn transporters such as ZnuABC or less Zn-dependent.<sup>73–75</sup>

For instance, a high-affinity ZnuABC transporter is crucial for *Campylobacter jejuni* to survive in chicken intestines colonized with normal microbiota but not with a limited one.<sup>74</sup> Likewise, *Salmonella enterica* can outcompete and survive in the inflamed intestine by overcoming calprotectin-mediated zinc sequestration due to ZnuABC transporters.<sup>76</sup> Inactivation of these transporters significantly reduces bacterial virulence. Hence, competition for Zn is part of the host–microbe interplay regulatory network.<sup>70,77</sup>

## Tissue Remodeling

Tissue remodeling is the improper regeneration or restoration of damaged tissues owing to inflammation or mechanical injury. This dynamic process is characterized by both temporary and permanent changes observed in CRS and other inflammatory diseases such as asthma, chronic obstructive pulmonary disease, and bronchiectasis. Tissue remodeling has traditionally been regarded as secondary to persistent inflammation. However, this process has recently been shown to occur concurrently with inflammation after an injury rather than its consequence.<sup>78</sup> Therefore, tissue remodeling might be an early stage of CRS, and recognition of its features can improve the identification of patients with a higher risk of recurrent CRS.

However, there are no strict criteria for defining tissue remodeling. In addition, in patients with CRSwNP or CRSsNP the remodeling pattern does not always show a consistent profile. NPs are grape-like, translucent, edematous structures, whereas the ethmoidal mucosa of CRSsNP patients is firm. These differences suggest different types of mucosal remodeling, which are regulated by factors such as the TGF-family and their receptors, matrix metalloproteinases (MMPs; enzymes responsible for extracellular matrix degradation contributing to edema formation), and tissue inhibitors of metalloproteinase (TIMP).<sup>10</sup>

Epithelial hyperplasia, metaplasia, subepithelial edema, basement membrane thickening, extracellular matrix protein deposition, extracellular matrix degradation, fibrosis, subepithelial collagen deposition, osteitis, and angiogenesis are all commonly involved in this process.<sup>79–85</sup> In CRSsNP, tissue remodeling is characterized by transforming growth factor  $\beta$  (TGF- $\beta$ )-driven fibrotic remodeling of the lamina propria, hyperplasia, excessive collagen production, and thickening of the extracellular matrix.<sup>47</sup> Since TGF- $\beta$  levels are significantly lower in the mucosa of CRSwNP patients, tissue remodeling in this condition is defined by less dense collagen deposition and barrier disruption, as well as by less membrane thickening and interstitial edema, less fibrotic alternations, and glandular hyperplasia.<sup>16,47,86</sup>

TGF- $\beta$  is a pleiotropic and multifunctional growth factor that promotes fibroblast proliferation, differentiation, and fibrosis and prevents extracellular matrix (ECM) degradation by inducing tissue inhibitors of metalloproteinase 1 (TIMP-1). Therefore, tissue repair is compromised, which is reflected by the presence of loose connective tissue and edema formation in the severely inflamed tissues in CRSwNP.<sup>14</sup> In addition, several other tissue remodeling factors, such as platelet-derived growth factor (PDGF), periostin, and vascular-endothelial growth factor (VEGF), are dysregulated in the CRS mucosa. VEGF



can promote nasal epithelial cell growth and resistance to apoptosis, which in turn may play a significant role in nasal polyp formation.<sup>47</sup>

Tissue remodeling features are also endotype-dependent in CRS. CRSwNP is characterized by a Th1/Th2 cytokine pattern and eosinophil-dominant inflammation, which is typically mediated by *S. aureus* superantigens, fungi, and allergens. In contrast, neutrophilic infiltration and various Th1, Th2, and Th17 cytokine patterns are the hallmarks of CRSsNP. Clinically, these differences are reflected by a relatively good response to corticosteroid therapy, which effectively reduces Th2-driven eosinophilic inflammatory processes and nasal polyp formation. Conversely, corticosteroids, in general, do not suppress Th17-dependent inflammation.<sup>47</sup>

Furthermore, the epithelial-to-mesenchymal transition (EMT) and autophagy processes may be activated during sinus mucosa remodeling in CRSwNP and nasal polyp formation.<sup>14</sup> EMT is the transformation of adherent epithelial cells into migratory cells that invade the extracellular matrix, resulting in the loss of E-cadherin, a cell-cell adhesion molecule with pivotal roles in epithelial cell behavior. EMT causes neutrophilic inflammation, fibrosis, and tissue remodeling owing to the loss of epithelial mucosal properties.<sup>87–91</sup> Factors triggering EMT include microbial infections, hypoxia, allergens, and air pollutions.<sup>87–91</sup> EMT is triggered by the disassembly of tight junctions associated with changes in the distribution of occludin, claudin, and zonula occludens 1 (ZO-1), as well as disruption of cell polarity and reorganization of the cytoskeleton. Elevated expression and activity of extracellular proteases, such as matrix metalloproteinase 9 (MMP-9), result in the degradation of ECM proteins.<sup>92</sup> Subsequently, epithelial cells acquire mesenchymal characteristics, which are linked with increased levels of N-cadherin, vimentin,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), collagen, fibronectin, and other extracellular matrix proteins.<sup>92</sup> EMT is mediated and regulated by several signaling pathways, including TGF- $\beta$ /Smad, Wnt/ $\beta$ -actin, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ).<sup>92</sup> Autophagy is a process essential for cell survival, differentiation, development, and homeostasis and plays a vital role in some chronic obstructive pulmonary disease (COPD).<sup>93</sup>

## Biofilm Impact on Sinuses Mucosa

The “mechanistic” and “mucin” hypotheses of biofilms’ contribution to CRS have been suggested.<sup>94</sup> The former assumes that disruption of the epithelial barrier results in enhanced exposure to microbial factors and overstimulation of the inflammatory response. The “mucin” hypothesis is based on the dysregulated expression of multiple mucins in CRS, which act as attachment ligands for bacterial adhesins initiating bacterial colonization - the first step in biofilm formation.<sup>95</sup> Both hypotheses intersect with Zn deficiency due to its pro-inflammatory and barrier-impairing action, mucins’ Zn-binding properties, and their upregulation at low Zn levels.<sup>96</sup> In addition, mucins are strictly connected with the “microbiota” hypothesis, being implicated in bacterial cross-feeding and as AMPs binding factors.

Furthermore, the “mechanistic” and “mucin” hypothesis concepts assume the formation of “pathologic” or dysbiotic biofilms, characterized by specific qualitative and/or quantitative changes in the proportion of bacterial species resulting from dysregulated reciprocal interactions. The latter are strictly associated with niche nutritional resources and are typically coordinated by QS communication systems, allowing unicellular organisms to function as multicellular entities, improving their chances of survival, competition, and cooperation in complex microbial environments.<sup>30,36,97</sup> However, QS signal molecules (QSMs), such as acyl-homoserine lactones (AHLs), can be disrupted by several bacterial enzymes, such as lactonases, acylases, or oxidoreductases.<sup>98</sup> Additionally, antagonism between QS signal-sensing molecules, such as auto-inducers 2 (AI-2) and auto-inducing peptides (AIPs), may affect interspecies QS communication. For instance, four groups of *S. aureus* species are distinguished based on differences in AIPs, where APIs of one group inhibit QS signaling in the other *S. aureus* groups.<sup>98</sup> In this context, producing antimicrobial substances such as bacteriocins or competition for space, which can be viewed as mechanisms that maximize nutrient absorption in one species while restricting it in another. Therefore, QSMs maintain the balance of the microbial community (eubiosis), which ensures colonization resistance and microbiota–host interactions in homeostasis. Subsequently, QSM-based communication is vital to maintain a balanced microbiota (eubiosis).<sup>30</sup> In addition, QSM may act as virulence factors and directly or indirectly influence intestinal epithelial barrier function, inflammation, and intestinal carcinogenesis (reviewed by Conquant et al, 2021).<sup>29</sup>

Additionally, competitive mechanisms in biofilms can be mediated through so-called public goods, ie, products of one species (siderophores, proteases) beneficial for other community members, eg, siderophore production as competition for iron between *S. aureus* and *P. aeruginosa*, supporting kin selection and social cheaters’ interactions.<sup>99</sup> Furthermore, lifestyle

transition, eg, from commensalism to parasitism or dormancy (persister or VBNC state), is a common bacterial strategy of response to changes in environmental conditions.<sup>100</sup> For example, a shift of *S. aureus* from a virulent to a commensal state can be induced by other Staphylococci and bacteria via QS accessory gene regulator (Agr) – a major regulator of virulence controlling over 70 genes, including 23 virulence factors.<sup>28,101,102</sup> Accordingly, *S. aureus* co-culture with *C. striatum* causes alterations in *S. aureus* transcriptome involving downregulation of virulence genes, eg, hemolysins, and upregulation of nasal colonization factors, eg, SpA, enhancing *S. aureus* adhesion to epithelial cells.<sup>103</sup> Likewise, *S. epidermidis* ability to inhibit *agr* activity of *S. aureus* via AIP peptide likely explains its domination over *S. aureus* on the human skin.<sup>28</sup> Subsequently, staquorsin (an analog of savarin-triazoloquinazoline derivative), a potent Agr inhibitor, has been proposed as a promising drug candidate for reducing the symptoms of *S. aureus* infections, including weight loss and the severity of inflammation in the tissues.<sup>104</sup> Similarly, in *P. aeruginosa*, inactivation or loss of QS receptor LasR is a possible indicator of the initial phase of cystic fibrosis (CF) via promoting production of several toxic for other microbes and the host molecules, such as pyocyanin proteases and hydrogen cyanide, resulting in the exaggerated inflammatory response.<sup>105–107</sup>

From this perspective, positive co-occurrence of *Corynebacterium* and *S. epidermidis* in “microbiotype 1” may indicate that both may act as probiotics impeding *S. aureus* growth (Figure 2). Significantly, bacterial mucin-derived metabolites, eg, short-chain fatty acids (SCFAs), can induce similar changes in bacterial transcriptomes and simultaneously drive cross-feeding interactions, regulate mucosal pH, and upregulate mucin expression (Figure 3).<sup>108,109</sup> Therefore, bacterial biofilms should be perceived as a network of reciprocal interactions between coordinated bacterial consortia, actively sensing and responding to changes in the inhabited niche as well as maximizing nutrient acquisition via numerous cooperative and competitive, such as (i) competitive exclusion, (ii) contest and scramble competition, (iii) negative-frequency dependent and Kin selection as well as (i) cross-feeding, commonly associated with lifestyle transitions, rather than passive nutrient exploiters.<sup>36,98,110</sup> Also, the spatial structure-dependent coexistence in biofilms, such as bacterial co-aggregation into “corncocks” structures driving interspecies cross-feeding or excessive EPS production elevating bacteria toward oxygen-rich regions at the air-liquid interface, is important mechanisms shaping the composition and structure of bacterial biofilms.<sup>65,111</sup>

This viewpoint on bacterial biofilms is adapted to the functionally equivalent pathway (FEP) hypothesis to describe the mechanisms of microbiota-associated chronic infections.<sup>51</sup> The FEP postulating that symbiotic and synergistic relationships between typically “non-pathogenic” species (pathobionts) may form communities or consortia (“pathogenic” biofilms), ie, FEPs with pathogenic potential comparable – “functional equivalence” to well-established pathogens, such as *S. aureus* or *P. aeruginosa*.<sup>51</sup>

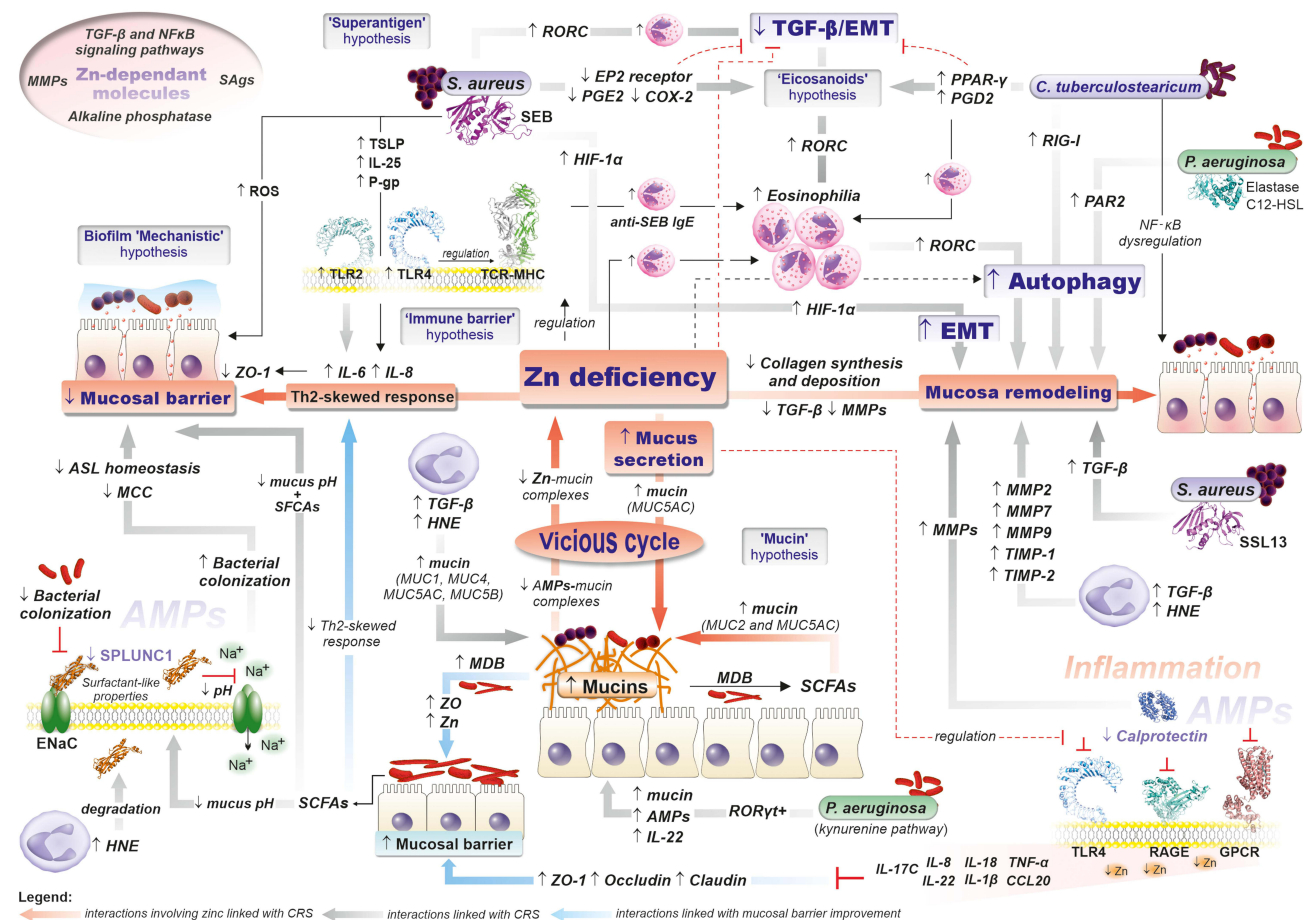
## Role of Epithelial Cell Barrier in CRS

Sinonasal epithelial cells (ciliated, non-ciliated, goblet, and basal cells) are the first line of defense against potentially harmful environmental factors and play a pivotal role in the onset and progression of CRS. Impairment of mucociliary clearance (MCC) and cell–cell contact in the sinonasal epithelium promote bacterial colonization, biofilm formation, dysbiosis, and mucositis. In addition, the sinonasal epithelium, via microbial pattern-recognition receptors (PRRs) and release of cytokines, complements system proteins and exosomes, coordinates the innate and adaptive immune responses and actively participates in the inflammatory process.

In general, the sinonasal mucosa of CRS is characterized by increased ion permeability and decreased tight junctions (TJ) due to decreased expression of TJ proteins occludin-1, claudin-1, and zonula occludens 1 (ZO-1), as well as iE-cadherin and desmosome shortening.<sup>12</sup> The epithelial barrier can be compromised by several immune system molecules, eg, IL-4 and IL-13, and IFN- $\gamma$  released under T2 and T1 conditions, respectively. Granule proteins and eosinophil extracellular traps (EETs) produced by eosinophils and other inflammatory mediators, such as oncostatin M (OSM), are significant epithelial barrier-affecting factors in CRSwNP.<sup>12,112</sup>

The “vicious cycle” associated with Zn deficiency may be responsible for the enhanced and sustained bacteria-mediated impairment of the epithelial barrier. Low Zn concentrations downregulate ZO-1 expression, likely via significant upregulation of the proinflammatory cytokines IL-6 and IL-8 (Figure 3).<sup>20,67</sup>

CRS-associated pathogens such as *S. aureus*, *S. pneumoniae*, *P. aeruginosa*, and *H. influenzae*, produce multiple virulence factors that affect transmembrane proteins (reviewed by Gao et al, 2022).<sup>37–39,113</sup> For example, *P. aeruginosa*



**Figure 3** Mechanisms of CRS pathogenesis in the light of various hypotheses underlying CRS development, (i) "immune barrier" hypothesis, (ii) "eicosanoid" hypothesis, (iii) "staphylococcal superantigens" hypothesis, (iv) "biofilm" hypothesis, (v) "microbiota" hypothesis, and "Zinc deficiency hypothesis (adapted from Suzuki et al) as well as "mechanistic" and "mucin" hypotheses of biofilms" role in CRS pathogenesis (details are included in the text).<sup>20,67</sup>

**Abbreviations:** MDB, Mucin-Degrading Bacterial; SCFAs, Short-chain fatty acids; TLRs, Toll-like receptors; PPRs, N-formyl peptide receptors; T2Rs, Taste Receptor Family 2 receptors; RAGE, Receptor for Advanced Glycation Endproducts; GOCRs, G-protein-coupled receptors; HNE, Human neutrophil elastase; RORC, Retinoic Receptor-Related Orphan Receptor C; HIF-1 $\alpha$ , Hypoxia-Inducible Factor 1 $\alpha$  Receptor; PAR2, Protease-Activated Receptor 2; PPAR- $\gamma$ , Peroxisome Proliferator-Activated Receptor Gamma; RIG-I, Retinoic Acid-Inducible Gene I; ENaC, Epithelial Na<sup>+</sup> Channel; TGF- $\beta$ , transforming growth factor  $\beta$ ; TIMP-1, tissue inhibitors of metalloproteinase 1; MMPs, matrix metalloproteinases; HNE, Human neutrophil elastase; ASL, airway surface liquid; QSM, quorum sensing molecules; ASL, epithelial-to-mesenchymal transition; SEB, *S aureus* superantigenic enterotoxin B; SSL, *S aureus* superantigen-like (SSL) toxins; C12-HSL, N-3-(oxododecanoyl)-homoserine lactone; SPLUNC1, Short-palate lung and nasal epithelial clone 1; ZO-1, zonula occludens; MCC, Mucociliary clearance.

elastase downregulates the expression of claudin-1 and -4, occludin, and tricellulin, as well as protease-activated receptor 2 (PAR2), which regulates the expression of TJ proteins and epithelial permeability via autophagy.<sup>114,115</sup> *P. aeruginosa* exoenzymes (Exo) and rhamnolipids also disrupt the actin cytoskeleton and TJ structure.<sup>113</sup> Similarly, *S. aureus* superantigenic enterotoxin B (SEB) and V8 protease downregulate expression of ZO-1, occluding, phospho-occludin, claudin-1,<sup>113</sup> whereas *S. aureus*  $\alpha$ -toxin activates the metalloproteinase domain-containing protein 10 (ADAM10), resulting in the cleavage of E-cadherin as well as compromises the actin cytoskeleton.<sup>113</sup> Furthermore, several QSMs, eg, *P. aeruginosa* 3-oxo-C12-HSL, directly or indirectly may disrupt TJ.<sup>29</sup> On the other hand, numerous commensal mucin-degrading bacteria, such as *Fusobacterium*, *Prevotella*, and *Streptococcus*, can stabilize the TJ barrier, eg, regulating the expression of ZO-1, occludin, claudin-1, and E-cadherin.<sup>116–118</sup>

Importantly, the sinonasal epithelium acts not only as a passive barrier but also actively coordinates both innate and adaptive responses through pathogen-recognition receptors (PRRs), such as Toll-like receptors (TLRs), N-formyl peptide receptors (FPRs), and Taste Receptor Family 2 receptors (T2Rs).<sup>12</sup> It should be emphasized that the function of PPRs is far beyond pathogen recognition. As part of the cell signaling pathways, PPRs promote the secretion of proinflammatory cytokines, TSLP, IL-25, and IL-33, which recruit multiple immune cells and promote the production of different Th2 cytokines, such as IL-4, IL-5, and IL-13, by activating Th2 cells and ultimately inducing type 2 inflammatory responses.<sup>12,119</sup>

Moreover, due to recognition by T2Rs bacterial quorum sensing molecules (QSMs), eg, 3-oxo-C12-HSL (C12-HSL), T2Rs provide information about the population density of microorganisms based on the chemical composition of the extracellular environment.<sup>120</sup> In addition, T2Rs may mediate tumor-microbiome crosstalk via T2R-driven apoptotic pathways.<sup>120</sup> Overall, T2Rs activation led to enhanced bacteria phagocytosis by airway ciliated cells through T2R-eNOS pathway and control AMP secretion. For instance, activation of T2R38 by *P. aeruginosa* C12-HSL results in a signaling cascade with resultant Ca<sup>2+</sup>-dependent release of bactericidal nitric oxide and increased ciliary beat frequency.<sup>121</sup> Moreover, C12-HSL induces also cell-dependent dysregulation of mucin expression, upregulating of MUC2 and down-regulating of MUC3 in the LS174T Caco-2 cell lines, respectively.<sup>29</sup> C12-HSL also stimulates MMP-2 and MMP-3 activation via the protease-activated receptor (PAR2) and shows several immunomodulatory properties, eg, inhibition of TNF- $\alpha$  and IL-12 production, promotion of T lymphocytes differentiation toward Th1-like phenotype, and inhibition of the differentiation of Th1 and Th2 T lymphocytes.<sup>122</sup> Hence, T2Rs may play a pivotal role in microbial surveillance and host-bacteria interactions that may be augmented by T2Rs gene polymorphisms, eg, altering T2Rs response to sugars and, in turn, T2R-mediated AMP secretion.<sup>30,120,123</sup> For example, in CRS patients, glucose concentration in nasal secretions is 3- to 4-fold higher than in healthy individuals, resulting in lower AMP levels.<sup>121</sup>

In contrast, activation of FPRs (FPR-1, FPR-2, and FPR-3) by N-formylated peptides present in bacterial cells or *S. aureus* superantigen-like (SSL) toxin 13 (SSL13) is associated with increased release of molecules responsible for tissue remodeling by epithelial cells, such as VEGF-A, TGF- $\beta$ , and MMPs (MMP-2, MMP-7, and MMP-9), parallel to the reduced expression of tissue inhibitors of MMPs (TIMP-1 and TIMP-2), indicating their role in nasal polyp formation (Figure 3).<sup>47,124,125</sup>

## Role of *S. Aureus* Superantigens in CRS

*S. aureus* SEB is the single bacterial toxin most frequently linked to CRS, that is, the “superantigen” hypothesis. SEB induces the local production of Th2 cytokines, regulatory T-cell inhibition, and increased eosinophil and mast cell activity, which leads to tissue injury, remodeling, and polyp formation (Figure 3).<sup>126</sup>

Superantigens (SAGs) are a class of immunostimulatory exotoxins that directly bind to the  $\alpha\beta$  T-cell receptor (TCR) and major histocompatibility complex (MHC) molecules that bypass the antigen recognition process, resulting in polyclonal T-lymphocyte proliferation and massive cytokine release. At least 80% of *S. aureus* clinical strains carry at least one SAG gene, and more than 20 different SAGs have been identified in *S. aureus*.<sup>127</sup> Notably, Rha et al reported that among eight studied *S. aureus* enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, and SEI), the *sei* gene was present in all tested patients with nasal polyps (NPs). Subsequently, the fraction of SEI (and SED) SED-responsive TCRV $\beta$ + (TCRV $\beta$ 1+ and V $\beta$ 5.1+) CD4+ T cells was significantly increased in the ethmoidal mucosa of CRSwNP subjects.<sup>126</sup> Therefore, SEI was suggested as a leading *S. aureus* SAGs driving TCRV $\beta$ 1+ or V $\beta$ 5.1+ CD4+ T cell expansion.<sup>126</sup> Interestingly, despite the detection of the *sei* genes in 95% of CRSwNPs and 90% of controls, the skewed distribution of TCRV $\beta$  was not observed in these groups, implying the involvement of other factors or mechanisms underlying this process.<sup>126</sup> This may be associated with promoting MHC-II surface expression on dendritic cells by a decrease in intracellular zinc, which can be inhibited by artificially increasing intracellular zinc levels.<sup>128</sup>

SAGs are classified into three groups based on their binding mechanisms with MHC: (i) binding only the MHC  $\alpha$ -chain through the low-affinity site (SEB, SEC1-3, SEG, TSST-1, SPEA, SSA); (ii) binding only the MHC  $\beta$ -chain through the zinc-dependent and high-affinity sites (SEH, SEI, SEJ, SEK, SEL, SEM, SPEC, SPEG, SPEH, SPEJ, SMEZ); and (iii) crosslinking two MHC molecules by simultaneously binding the  $\alpha$ -chain of one MHC and the  $\beta$ -chain of another MHC via the low- and high-affinity sites, respectively (SEA, SED, SEE).<sup>129</sup> Moreover, SAGs show  $\beta$ -chain (V $\beta$ ) specificities, for example, SEA and SEB, and preferentially promote the expansion of V $\beta$ 11 and V $\beta$ 8.1,8.2 T cell subsets, respectively. Importantly, zinc-binding superantigens remain on the cell surface for at least 40 h, whereas single-binding-site superantigens diminish within four hours, which explains the enhanced T cell activation by SEA and other zinc-dependent and cross-linking SAGs.<sup>130</sup>

The importance of Zn in the action of SAGs was emphasized by Fraser et al.<sup>131</sup> The authors demonstrated that SEA and SEE binding to HLA-DR, but not TSST-1, were entirely abolished by low levels of EDTA and that the addition of Zn<sup>2+</sup>, over EDTA as low as 2  $\mu$ M, completely reversed this inhibition.<sup>131</sup>

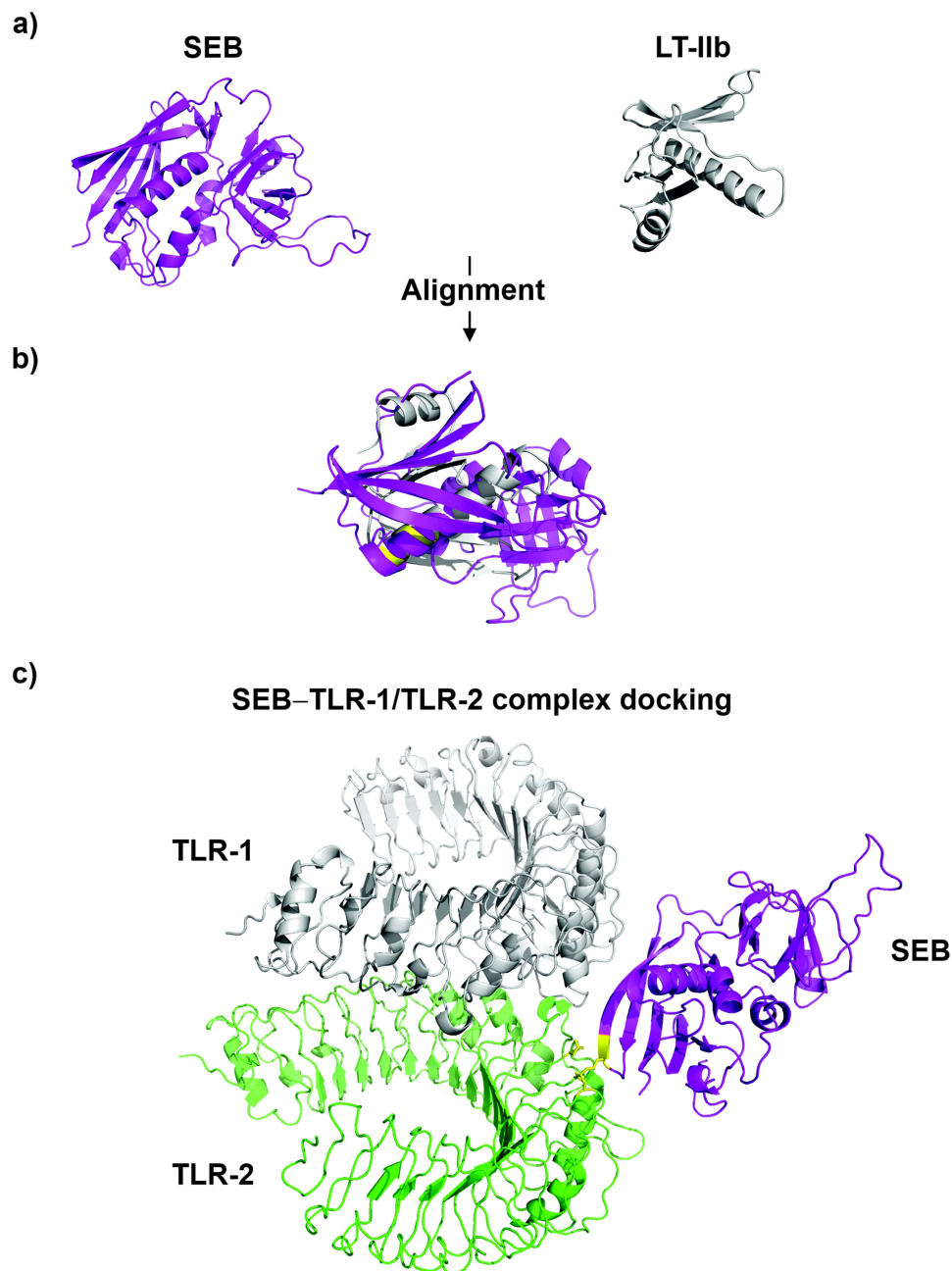
Furthermore, TCR function is regulated by intracellular zinc levels.<sup>132</sup> Briefly, antigen recognition by TLR increases cytoplasmic uptake of extracellular zinc by Zn transporter – ZIP6, which is crucial for TCRs signaling activation.<sup>20</sup> As a result, elevated extracellular Zn bioavailability promotes the induction of T cell proliferative responses to suboptimal antigen concentration through amplifying TCR signaling.<sup>69</sup> For example, the frequencies of T cells that responded to the stimulation with mature dendritic cells (DC) and 0.04 ng/mL of TSST-1 increased ~5-fold in medium containing Zn at concentrations of 3  $\mu$ M, 15  $\mu$ M, 30  $\mu$ M, 45  $\mu$ M, and 75  $\mu$ M.<sup>69</sup> Furthermore, Zn enhances TCR signal antigen reactivity (avidity), ie, lowers TCR activation, eg, TSST-1, which typically activates only  $V\beta^{2+}$  T cells, under Zn-optimized conditions, can also activate a small number of  $V\beta^{2-}$  T cells, likely due a low affinity of TSST-1 to  $V\beta^{2-}$ .<sup>69</sup> On the contrary, a low level of intracellular Zn stimulates the surface expression of MHC-II in TLR4-mediated mechanism involving activation of TRIF and Zn transporters (ZIP and ZNT).<sup>128</sup> Notably, SEB upregulates the expression of TLR4 and TLR2, and likely directly interacts with the latter receptor.<sup>133</sup> Whereas high Zn levels suppress TLR2 and TLR4.<sup>134</sup>

Overall, SEB contributes to CRS pathogenesis through several mechanisms, including TLR2-mediated disruption of epithelial barrier integrity and a local Th2/Th17 response, which causes eosinophil degranulation, the release of antimicrobial proteins (AMPs), and localized eosinophilic inflammation. The induction of Th2 cytokines, such as IL-2, IL-4, IL-5, IL-6, and IL-8, is preferred over IL-10 and TGF- $\beta$ 1, and SEB does not appear to cause allergic reactions in the nasal tissue. Additionally, TSLP and IL-25 levels were upregulated by SEB. The former is essential for promoting Th2 cytokines and inhibiting IFN- $\gamma$  production, whereas IL-25 is associated with the production of multiple inflammatory markers, including Th1/Th2/Th17 inflammation. TSLP upregulation is likely associated with P-glycoprotein (P-gp), an efflux pump that participates in cytokine expression and transport in the sinonasal mucosa.<sup>135</sup>

Furthermore, SEB-mediated disruption of TJs depends on the activation of TLR2 and triggering the production of pro-inflammatory cytokines IL-6 and IL8 as well as the induction of reactive oxygen species (ROS) and endoplasmic reticulum stress-induced inflammation in CRSwNP.<sup>133</sup> Recently, Martens et al revealed in SEB-stimulated polyp epithelial cells from CRSwNP patients a dose-dependent decrease of trans-epithelial electrical resistance (TEER) and an increase of FD4 (Fluorescein isothiocyanate dextran 4 kDa) permeability. Interestingly, SEB did not affect the TEER of primary nasal epithelial cells in the control subjects. Therefore, SEB appears to be a driving factor, rather than a primary cause, of epithelial barrier impairment. The fact that SEB binds to TLR2 and elevates TLR2 expression could explain this observation, because anti-TLR2 antibodies prevent SEB-induced mucosal barrier disruption.<sup>135</sup> Subsequently, TLR2-directed treatment may offer a novel therapeutic strategy for preventing the pathophysiological effects of *S. aureus* in CRSwNP.<sup>133</sup> Furthermore, Wang et al reported significantly higher expression of TLR2, TLR4, and TGF- $\beta$ 1 in CRSsNP patients than in CRSwNP patients.<sup>136</sup> In addition, in CRSsNP, the expression of TLR2 in patients with severe epithelial damage (score 3) was significantly higher than that in patients with mild epithelial damage (score  $\leq$ 2). Moreover, the expression of TLR2 correlated negatively with squamous hyperplasia in CRSsNP, and positively correlated with gland hyperplasia in CRSwNP.<sup>136</sup> Interestingly, TLR2-induced inflammation is also a property of *C. tuberculoesteiricum*, and Abreu et al demonstrated in a mouse model that the pathogen *C. tuberculoesteiricum* induces goblet cell hyperplasia and mucin hypersecretion.<sup>24,125</sup>

However, the significance of these SEB-mediated mechanisms in overall CRS pathogenesis.<sup>137–144</sup> Moreover, the mode of SEB interaction with TLR2 remains to be elucidated. To predict potential SEB-TLR2 binding sites, we searched the protein 3D model databases for other TLR2 binding proteins sharing structural similarity with SEB (Figure 4).<sup>145</sup> For instance, the B subunit of a type II heat-labile enterotoxin of *E. coli* (LT-IIb-B5) was matched. LT-IIb is a potent immunomodulatory molecule with adjuvant properties and, similar to SEB, induces local production of pro-inflammatory cytokines.<sup>144,146–148</sup>

It is also reasonable to investigate the role of *S. aureus* superantigen-like (SSL) toxins such as SSL3 and SSL4-TLR2 antagonists, in CRS pathogenesis. SSLs are an under-examined family of 14 exotoxins that share structural similarity with superantigens, but lack superantigenic activity. Nonetheless, SSL proteins can modulate immune responses by binding to various host receptors.<sup>149</sup> For instance, SSL13 is a potent neutrophil activator that interacts with formyl peptide receptor 2 (FPR2).<sup>150,151</sup> Accordingly, whole-genome sequencing (WGS) analysis of *S. aureus* isolates collected from 28 CRSsNP and 30 CRSwNP patients revealed SSL proteins as the only virulence factors correlated with the CRS type. SSL5 was more prevalent in the CRSsNP cohort, whereas SSL14 was more prevalent in the CRSwNP cohort. Notably, SSL5 inhibited neutrophil extravasation by blocking the interaction between PSGL-1 and P-selectin and the enzymatic activities of MMP8 and MMP9. Moreover, SSL5 modulates the host immune defense by interfering with C1Inh in the complement system.<sup>152</sup>



**Figure 4** 3D models *S. aureus* enterotoxin type B - SEB (3seb.pdb) and B subunit of the type II heat-labile enterotoxin of *E. coli* monomer (LT-IIb; qb5.pdb) (panel (A)); SEB and LT-IIb alignment (TM-score = 0.43; RMSD = 4.44) (panel (B)), LT-IIb residues (Met69, Ala70, Leu73 and Ser74) which responsible for binding with TLR-2 (residues Asp235 and Asn290)<sup>153</sup> are highlighted on yellow color; SEB and TLR-1 – TLR-2 complex (2z7x.pdb) docking model (panel (C)), the predicted SEB residues (Lys141 and Asn142) responsible for interaction with TLR-2 (residues Asp235 and Asn290) are highlighted on yellow color. Protein alignment was performed with TM-align tool (<https://zhanggroup.org/TM-align/>), Protein-docking was predicted using ZDOCK tool (<https://zdock.umassmed.edu/>).

The connection between superantigens and the “eicosanoids” hypothesis is also notable. Eicosanoids, such as prostaglandins (PGs) and leukotrienes (LTs), are lipid-based signaling molecules that play a unique role in innate immune responses through cyclooxygenase (CO)-mediated metabolism of arachidonic acid. Patients with CRSwNP commonly demonstrate increased levels of proinflammatory leukotrienes alongside the downregulation of cyclooxygenase-2 (COX-2) and reduced levels of prostaglandin E2 (PGE2).<sup>154</sup> SEB can affect eicosanoid metabolism via downregulation of PGE2, COX-2, and E-prostanoid-2 (EP2) receptor expression resulting in enhanced eosinophilic inflammation.<sup>154</sup> This process can be reversed by PGE2 that acts as a dose-dependent inhibitor of SEB-induced eosinophilic inflammation primarily through the EP2-mediated pathway.<sup>154</sup> Conversely, this process may downregulate EMT since PGE2 is a potent inhibitor of TGF- $\beta$ 1-induced EMT.<sup>155</sup>

Hence, PGE derivatives, such as misoprostol, have been indicated as efficient treatment method for CRS and other eosinophilic airway diseases (eg, bronchial asthma or allergic rhinitis).<sup>154</sup> On the other hand, this therapy might not be applicable for *Corynebacterium tuberculo*stearicum-induced eosinophilia, which possibly occurs via activation of PPAR- $\gamma$  receptor and upregulation of PGD2, a potent chemoattractant for eosinophils, and in turn, downregulation of TGF- $\beta$  production and Th2 cytokines.<sup>8,156</sup> Since PGD2, like PGE2, is a potent inhibitor of TGF- $\beta$ 1-induced EMT, an activation of PPAR- $\gamma$  may affect EMT and tissue remodeling, as reported in a mouse asthma model.<sup>155,156</sup> For instance, Asaka et al reported expression PPAR- $\gamma$  and PGD2 synthase in eosinophils and T cells infiltrating the nasal polyps and administration of PPAR- $\gamma$  agonist diminishes nasal allergy symptoms and eosinophilic infiltration of the nasal mucosa.<sup>157</sup>

Furthermore, the biological activity of SEB may be enhanced by other *S. aureus* virulence factors and may trigger intensive inflammation, resulting in the disruption of epithelial cells and ultimately nasal polyp formation.<sup>135</sup> For instance, *S. aureus* surface protein A (SpA) induces an allergenic response, increasing the release of histamine, LTC<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub>, and PGD2 in nasal tissue, contributing to inflammation in CRS. Additionally, certain *S. aureus* proteases, such as serine protease-like protein (Spl) D, act as allergens and induce the release of IL-33, production of specific IgE and eosinophils, bronchial hyperreactivity, and airway goblet cell hyperplasia response.<sup>158</sup> In addition, delta-toxin and lipoteichoic acid (LTA) have been linked to mast cell degranulation and epithelial colonization, respectively. Finally, *S. aureus*-induced Th1 and Th17 inflammation via the release of extracellular membrane vesicles (EV) may contribute to the development of CRSnPs.

## Role of Mucins in CRS

Mucus hypersecretion is a hallmark of CRS owing to the dysregulation of mucin expression.<sup>95</sup> Neutrophils regulate this process through signaling pathways involving TNF- $\alpha$ ,  $\alpha$ -convertase, TGF- $\beta$ , epidermal growth factor receptor (EGFR), and human neutrophil elastase (HNE). For example, HNE and TGF- $\beta$ 1 regulate the expression of several mucins, mainly MUC1, MUC4, MUC5AC, and MUC5B (Figure 3).<sup>95</sup> Also, HNE triggers autophagy in nasal epithelial cells - a crucial process for the subsequent HNE-induced mucus hypersecretion through upregulation of MUC5AC via JNK-AP-1 pathway.<sup>93</sup>

Mucus hypersecretion from the nasal epithelium may contribute to zinc (Zn) depletion due to the efficient Zn-binding properties of mucins and the “vicious cycle” mechanism.<sup>67</sup> Accordingly, an inflammation-induced upregulation of mucins results in hypersecretion of mucus and a net loss of Zn (via Zn-mucin complexes) from the mucosa, which promotes further Zn loss, contributing to CRS progression (Figure 3).<sup>20,67</sup> In human nasal epithelial cells (HNECs), Zn depletion significantly upregulated the expression of Muc5AC and proinflammatory cytokines IL-6 and IL-8. Notably, both cytokines may be directly responsible for the elevated *Muc5AC* expression. Neveu et al demonstrated that IL-6 can directly induce the mucin genes *Muc5AC* and *Muc5B* in human lung epithelial cell lines.<sup>159</sup> Similarly, Bautista et al proposed a novel function for IL-8, that is, maintaining *MUC5AC* (and *MUC5B*) gene expression in inflamed airways through a post-transcriptional mechanism based on increasing the abundance of *MUC5AC* mRNA.<sup>160</sup> Furthermore, Zn deficiency can impair mucus stability by interfering with mucin O-glycosylation and their secretion by goblet cells.<sup>161</sup>

Mucins serve as receptor-binding sites for bacterial adhesion and nutrient sources and are implicated in bacterial cross-feeding interactions. Importantly, the latter influence several virulence-associated processes, such as biofilm formation and motility, as well as may alter the lifestyle of bacteria, hence contributing to maintaining commensal relationships with microbiota.<sup>162</sup> Moreover, mucin degradation products, such as SCFAs, are vital for mucus pH homeostasis and “colonization resistance” processes.<sup>116,163</sup>

Specifically, mucins may contribute to developing CRS-associated biofilms and/or supporting the growth of particular species such as *S. aureus*.<sup>164</sup> Psaltis et al reported a significant enrichment of the CRS-associated microbiome with mucin-degrading genes produced by various mucin-degrading bacteria (MDB), including *Prevotella*, *Fusobacterium*, and *Streptococcus*.<sup>108,165</sup> Degradation of mucin generates carbon-source nutrients for pathogens such as SCFAs (propionate, butyrate, and acetate) and other mucin-derived metabolites utilized, for example, by *P. aeruginosa*, or can shift bacterial lifestyle, for example, from commensalism to parasitism, by interfering with the expression of key metabolism- and virulence-associated pathways.<sup>165</sup> Specifically, SCFAs (propionate and butyrate) directly impede *S. aureus* growth and lead to reduced expression of the accessory gene regulator (*agr*) quorum sensing system and induction of fatty acid degradation operon (*fadXEDBA*), likely as an SCFA-mediated stress response.<sup>166</sup> In addition, SCFAs are toxic to many bacteria, including antibiotic-resistant Enterobacteriaceae, and can lower the pH. The latter shape microbiota composition and prevent overgrowth by pH-

sensitive pathogenic bacteria.<sup>116,163</sup> SCFAs also contribute to epithelial barrier stability, promoting ZO-1 and mucin (*MUC2* and *MUC5AC*) expression.<sup>116,117</sup> However, a combination of low pH and SCFAs may disrupt TJ and impair barrier function.<sup>116,167</sup> Recently, Yuan et al reported that intestinal microbiota-derived SCFAs might attenuate allergic responses by maintaining stable IGF-1 expression. Furthermore, SCFAs-RAR $\alpha$  (retinoic acid receptor  $\alpha$ ) interaction in dendritic cells has been suggested as a crucial immunomodulator of Th2 cell differentiation in a dysbiotic microbiota state.<sup>168</sup> Therefore, SCFA deficiency in allergic diseases enhances a Th2-skewed immune response and contributes to allergy development.<sup>169</sup>

In addition, tryptophan-mediated cross-feeding relationships may link the co-colonization of *Pseudomonas* and tryptophan-producing bacteria, such as, *Achromobacter* in Pseudomonadaceae-dominated microbiota, recognized by Cope et al in patients with CRS.<sup>8</sup> Products of tryptophan catabolism (anthranilate, kynurenine, and kynurenic acid) possess immunomodulatory properties. For instance, *Pseudomonas* species catabolize tryptophan to pro-inflammatory kynurenine via the kynurenine pathway, which also provides anthranilate, a precursor of several *P. aeruginosa* virulence factors.<sup>8,170</sup> As a result, the increased capacity for tryptophan production and metabolism by Pseudomonadaceae-dominated microbiota may contribute to enhanced antimicrobial resistance and pathogenicity by upregulating biofilm formation and virulence gene expression.<sup>8,170</sup> In the intestinal tract, microbiota via tryptophan-indole derivatives can regulate Th2-driven immunity, inducing Th17 cells and a subset of Treg cells expressing the retinoid-related orphan receptor (ROR $\gamma$ <sup>+</sup>), and stimulate the production of IL-22. IL-22 aids intestinal homeostasis and promotes epithelial cell regeneration, AMPs, and mucus secretion.<sup>47,171</sup>

## Role of AMPs in CRS

Sinonasal mucus improves the retention of AMPs owing to its direct binding to mucins; for example, MUC5B binds histatin-1, -3, and -5 and statherin.<sup>172</sup> Therefore, it might be assumed that mucus hypersecretion in CRS results in a more significant loss of AMPs. AMPs play a pivotal role in several aspects of CRS pathogenesis, including biofilm formation, tissue remodeling, and EMT.

For instance, lower expression of PLUNC family proteins, SPLUNC1 (BPIFA1) and LPLUNC2, has been reported in CRS patients.<sup>173</sup> SPLUNC1, in addition to its antibacterial activity, acts as an LPS scavenger to suppress the LPS-induced inflammatory response.<sup>174,175</sup> In addition, SPLUNC1 modulates airway surface liquid (ASL) volume, acting as an endogenous inhibitor of the epithelial Na<sup>+</sup> channel (ENaC) in a pH-dependent.<sup>174</sup> As a result, hyperactivity of ENaC, hence Na<sup>+</sup> hyperabsorption and ASL dehydration in cystic fibrosis (CF) airways, is related to low ASL pH, which prevents binding of SPLUNC1 to ENaC. Hence, SPLUNC1 represents a soluble mucus volume sensor, and its low levels may affect MCC.<sup>176</sup> On the other hand, SCFAs (butyrate), by increasing ENaC transcription and activity, regulate colonic Na absorption.<sup>177</sup> Furthermore, the SPLUNC1-ENaC complex protects normal airway epithelia from *Burkholderia cenocepacia* invasion, and degradation of SPLUNC1 by HNE and MMPs impairs airway epithelial defense against bacteria (Figure 3).<sup>178,179</sup> Subsequently, SPLUNC1 was proposed as a novel marker of disease severity and airway infection in bronchiectasis and CF exacerbations, and ENaC antagonists, for example, SPLUNC1-derived S18 peptide, have been suggested as a therapeutic option for the treatment of CF lung disease.<sup>180–182</sup>

In addition, significantly lower expression of SLPI and clusterin (CLU) antimicrobial peptides in the nasal tissues of CRSwNP patients was correlated with a low number controlled by Th2 cytokine submucosal glands.<sup>183</sup> Th2 inflammatory conditions in CRS reduced SLPI expression, but not CLU, and S100A8/A9 (BPIFA1) were specifically attenuated by IL-13 in eosinophilic CRSwNP.<sup>183,184</sup> Moreover, the expression of SLPI and CLU was affected by TGF- $\beta$ 1, an essential factor in the development of EMT and tissue remodeling, and the regulation of various mucins (Figure 3).<sup>95,183</sup> Notably, in human airway epithelial cells, CLU also induces MUC5AC expression via the activation of the NF- $\kappa$ B signaling pathway.<sup>185</sup>

The S100 family proteins, psoriasin (S100A7), calgranulins A (S100A8), B (S100A9), C (S100A12), and calprotectin (S100A8/A9), are examples of other AMPs that are abnormally expressed in CRS. S100 proteins show different expression patterns concerning the location of tissue sampling and tissue subsite, which may explain the disagreement in the literature about whether S100 proteins, such as calprotectin, play a protective or pathological role in patients with CRS.<sup>186,187</sup> In contrast, this observation implies that CRS inflammation may manifest differently depending on the sinus location.<sup>187</sup> For example, the epithelium of CRS patients expresses significantly less psoriasin and calprotectin, which may contribute to a diminished innate immune response and barrier function. Similarly, Viksne et al reported decreased HBD-2, HBD-3, and LL-37 concentrations in the epithelium of patients with nasal polyps and elevated levels of HBD-3



in subepithelial connective tissue, suggesting its role in the pathogenesis of primary nasal polyps.<sup>188</sup> Conversely, elevated calprotectin levels in nasal polyp tissues may reflect neutrophil recruitment as a compensatory mechanism.<sup>189</sup>

Calprotectin (CP) exerts proinflammatory responses via TLR4, IL-8, and TNF- $\alpha$ , as well as anti-inflammatory and tissue-protective functions, such as inhibition of MMPs by sequestration of zinc and PAR-2-mediated antioxidative effects.<sup>190,191</sup> CP is a Mn-, Fe-, Ni-, and Zn-binding antimicrobial protein produced by neutrophils that inhibits the growth of pathogenic microorganisms by sequestering essential metal nutrients and reprogramming bacterial metabolism, for example, toward utilization of Fe- and Zn-dependent cofactors, to overcome their deficiency. For example, in a systemic infection model, inhibition of *S. aureus* Mn-dependent superoxide dismutase (SOD) by CP increases *S. aureus* sensitivity to neutrophil-mediated killing and attenuates virulence.<sup>192</sup> On the other hand, *S. aureus* and *P. aeruginosa* enhance heme utilization via upregulation of heme uptake systems and siderophores to overcome Fe depletion by CP.<sup>193</sup> Additionally, CP-mediated repression of *P. aeruginosa* Zn- and Mn-dependent anti-staphylococcal factors promotes its co-colonization in biofilms with *S. aureus*, which may account for their frequent co-infections.<sup>194</sup> Similarly, *Acinetobacter baumannii*, in response to CP-mediated Fe and Zn starvation, upregulates flavin biosynthesis pathways, which increases the use of flavodoxins as substitutes for ferredoxins and decreases the cellular metabolic Fe requirement.<sup>195</sup> Furthermore, *Helicobacter pylori* circumvents metal sequestration by CP by modifying the outer membrane and biofilm formation.<sup>196</sup> Interestingly, by reducing sensitivity to zinc poisoning, CP may inadvertently benefit bacterial pathogens instead of the host, eg, via sequestration of Zn from high-affinity manganese transporter PsaA of highly sensitive to zinc poisoning pathogen *S. pneumoniae*.<sup>197</sup> Finally, production of high-affinity Zn transporter ZnuABC transporter allows *Salmonella enterica* to overcome CP-mediated Zn sequestration, and, in turn, to outcompete intestinal commensals and survive in the inflamed intestine.<sup>76</sup>

## Nasal Polyp Development

Nasal polyps (NP) are benign, hyperplastic, inflammatory edema lesions resulting from mucosal linings of the sinuses.<sup>84,198–200</sup> Although the formation of NP is still mostly enigmatic, their classification is based on etiology (inflammatory, neoplastic, atopy, *Staphylococcus* super-antigens) as well as type and area covered (localized, diffused, systemic).<sup>199</sup> In general, dysregulated and intense local immune response, triggered by extrinsic factors (fungi, staphylococcal superantigens, biofilms, and imbalanced microbiota) and intrinsic factors like (i) induced by hypoxia EMT, (ii) depletion of Treg lymphocytes, (iii) low local vitamin-D levels, (iv) high levels of leukotrienes, and (v) altered levels of NO have been associated with pathogenesis of nasal polyposis.<sup>201</sup> Nevertheless, it appears that the damage of the epithelial barrier, regardless form intrinsic or extrinsic etiology, is a precondition increasing the susceptibility of subepithelial layers to invasion by pathogens that trigger a Th-2-skewed response. Subsequently, Th2 cytokines induce the accumulation of eosinophils and IgE, as well as remodeling of the sub-epithelial stroma, resulting in the formation of nasal polyps.<sup>201</sup>

Pseudocyst formation, involving edema formation with albumin accumulation and collagen depletion within the extracellular matrix, is a typical remodeling feature of the mucosa in CRSwNP.<sup>136</sup> Damage to the epithelial barrier results in an influx of inflammatory cells, tissue swelling, and fibrin deposition. The nasal polyp tissues accumulate dendritic cells (DCs) expressing IL-17Rb, ST2, and TSLPR.<sup>14</sup> Exosomes have also been found to be associated with nasal polyp formation via upregulation of coagulation and downregulation of fibrinolysis cascades, causing retention of plasma proteins, intense edema, excessive fibrin deposition in nasal polyps, and subsequent tissue remodeling.<sup>14</sup> A nasal polyp forms without submucosal glands through tissue remodeling and intensified inflammation. Therefore, lower expression of specific AMPs, such as SLPI, SPLUNC1, and LPLUNC2, associated with decreased numbers of submucosal glands is likely most frequently linked to nasal polyps in CRS.<sup>183</sup> Proinflammatory IL-6 and Oncostatin M (OSM) are highly expressed in CRSwNP tissue and may cause an overreaction to external stimuli, hence contributing to the formation of nasal polyps.<sup>14</sup> In addition, OSM can disrupt the TJs of epithelial cells and increase their permeability, leading to epithelial barrier dysfunction.<sup>92</sup> Since OSM expression is induced by lipopolysaccharide (LPS), it has been suggested as a therapy target to prevent the progression and exacerbation of severe asthma caused by bacteria.<sup>202</sup>

Likewise, Zn affects the expression and enzymatic activities of zinc-dependent MMPs that are responsible for collagen removal and are dysregulated in CRS, for example, elevated expression of MMP9 in nasal polyps is well documented. However, the relation between zinc and MMPs is equivocal; according to the literature review by Nosrati et al inhibitory effect of Zn on MMPs production and activity was reported by ~62% (29/47), whereas the remaining

papers showed null or opposite action<sup>203</sup> The epithelial barrier can also be impaired by decreased expression of tight junction protein - ZO-1 under low Zn concentrations.<sup>20</sup>

A so-called “staphylococcus superantigen” hypothesis has been suggested, where by *S. aureus* superantigenic enterotoxins induce local production of Th2 cytokines, regulatory T-cell inhibition, and increased eosinophil and mast cell activity, which led to tissue injury and remodeling and the formation of polyps.<sup>126</sup> Indeed, the severity of CRSwNP correlates with the expansion of CD4<sup>+</sup> T cells differentiated into the Th2 subset with proliferative features in response to a superantigen that appears to play a vital role in Th2-skewed inflammation.<sup>126</sup> In addition, specific anti-superantigen IgE is present in almost half of the nasal tissue homogenates from nasal polyps<sup>135,204–206</sup> SEB may play a role in the upregulation of retinoic acid receptor-related orphan receptor C (RORC) and hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) receptor expression in Tregs and by maintaining inflammation in the sinonasal mucosa, contributing to the pathogenesis of nasal polyposis.<sup>133,135</sup> RORC is a multifunctional nuclear receptor on mucosal surfaces that maintains homeostasis with the symbiotic microbiota, synchronizes defenses against extracellular microbes, regulates allergic responses, and induces autophagy. RORC+ Tregs may be associated with eosinophilic nasal polyposis. In addition, it negatively regulates oncogenic TGF- $\beta$ /EMT and induces autophagy.<sup>207–209</sup> HIF-1 $\alpha$  induces an imbalance between Treg and Th17 activity and can lead to autoimmune disorders and chronic inflammation.

In CRSwNP, TGF- $\beta$ 1 can induce EMT by modulating various microRNAs, resulting in downregulation of E-cadherin, an epithelial biomarker, and upregulation of mesenchymal biomarkers, such as vimentin and N-cadherin.<sup>14</sup> For example, macrophage-derived mucin domain 4 (TIM-4) promotes transforming growth factor- $\beta$  (TGF- $\beta$ 1)-mediated EMT in nasal epithelial cells, facilitating nasal polyp formation.

Likewise, the abundance of Corynebacteriaceae in CRS-associated microbiota is linked with a higher risk of developing polyposis, possibly via the activation of PPAR- $\gamma$  and RIG-I receptors.<sup>8</sup> PPAR- $\gamma$  is a lipid-sensing receptor that has recently been shown to regulate eosinophil activation in polyp tissues of CRS patients<sup>157</sup> and airway remodeling following allergic inflammation in mice. In contrast, RIG-I is an intracellular sensor of viral DNA that is elevated in nasal polyp tissue [43] and is induced by IFN- $\gamma$  [53]. In addition, *C. tuberculo* may be responsible for the NP formation. This lipophilic skin inhibitor shows potent immunomodulatory properties via NF- $\kappa$ B dysregulation.<sup>125,210</sup> As NF- $\kappa$ B dysregulation is associated with chronic inflammatory skin diseases, Altonsy et al suggested that this pathogen can initiate and/or sustain a chronic inflammatory process or skin cancers, and its higher occurrence in keratinocyte carcinomas has been reported.<sup>125</sup>

In other studies, the development of CRSwNP has been described as a complex process, represented mainly by increased epithelial damage and expression of fibronectin.<sup>83</sup>

## Future Perspectives

To date, no clear link has been found between CRS and sinonasal microbiota composition or specific species or toxins, that is, single-species or superantigen hypotheses. Moreover, the lack of validated animal models and the disease’s complexity have hampered research into CRS disease mechanisms.<sup>211</sup> Nonetheless, investigating the hypotheses leads to greater understanding of CRS mechanisms.<sup>211</sup> Likewise, identifying and characterizing CRS endotypes could ultimately enhance patient care.<sup>7,11,211</sup> For instance, the identification of immune factors that regulate the process by which neutrophils influence polyp formation, tissue remodeling, and mucosal barriers has the potential to facilitate diagnostic and therapeutic applications. Recent discoveries suggest that IL-6, IL-8, IL-1 $\beta$ , IL-36 $\gamma$ , TGF- $\beta$ 1, and oncostatin M may be implicated in the pathophysiology of neutrophilic inflammation in CRS. A better understanding of these pathways may guide future research into the use of monoclonal antibodies targeting neutrophilic pathways in CRS.<sup>7,11,211</sup>

Furthermore, confounding results regarding the importance of certain microorganisms in CRS have been reported in literature. These discrepancies are most likely due to several methodological issues with 16S rRNA sequencing and the significant inter-individual, geographical, and racial heterogeneity of sinonasal microbiota. In addition, it is unclear whether the reported differences in the composition of the sinonasal microbiota, eg, “microbiotypes”, represent specific microbiota community “types” or rather temporary “states”. In addition, transitional or intermediate states between specific microbiotypes are unknown because of the lack of longitudinal data showing their stability over time.<sup>22</sup> Also, it cannot be excluded that chronicity of inflammation alone does not predict a dysbiotic microbiome, rather, the presence of

a clinically apparent “sinus infection” at the time of sample collection.<sup>22</sup> Nevertheless, exploring the hypotheses provides insights into disease mechanisms.

Furthermore, the discriminatory power of the current sequencing technology, based on short 16S rRNA amplicons, ie, generally genus-specific, is too low to resolve mainly species-specific relationships between the microbiota members.<sup>22</sup> For instance, *S. epidermidis* appears to be a mutually cooperative partner for Corynebacteria, whereas mutually competitive for *S. aureus*.<sup>22</sup> Overall, interspecies interactions rely on a delicate balance of competition and cooperation between species and may be substantially affected by environmental physicochemical factors, such as pH or essential micronutrient fluctuations and the genetic background of the interacting species. At the molecular level, these interactions may involve substantial alterations at the transcriptome level, resulting in different metabolic or virulence features, including the transition from a commensal to a pathogenic or dormant lifestyle. For instance, both synergy and antagonism are possible between *S. pneumoniae* and *H. influenzae*, depending on the pH and phase of growth. Low pH and stationary growth phases trigger *H. influenzae* to a viable but nonculturable (VBNC) state.<sup>110</sup>

In this view, the role of *S. aureus* (or its superantigens) in CRS should be perceived as a derivative of its genetic and the biofilm “background” as well as physicochemical parameters of the sinonasal mucosa promoting *S. aureus* lifestyle toward commensalism or parasitism and vice versa - “co-occurrence” hypothesis. Analogically, according to the functionally equivalent pathogroups (FEP) hypothesis, CRS can be triggered by symbiotic/commensal species without the contribution of well-known pathogens such as *S. aureus* and *P. aeruginosa*.

This view intersects with “mucin” and “mechanistic” hypotheses of CRS-associated biofilms through mucin-degrading bacteria and mucin-derived metabolites, eg, SCFAs. SCFAs have been implicated in cross-feeding multifunctional molecules that act on both the bacteria and the host. In the intestine, SCFA-mediated lower pH shapes the composition of gut microbiota and prevents the overgrowth of pH-sensitive species. Low mucus pH may interfere with the pH-dependent regulation of ASL homeostasis via inhibition of the Na(+) channel (ENaC) by SPLUNC1, an antimicrobial peptide that is dysregulated in CRS. Hence, as in CF lung disease, therapies based on ENaC antagonists may be beneficial in patients with CRS.

Likewise, because SCFAs commonly modify bacterial transcriptomes, QS-directed treatments interfere with these bacterial communication systems, such as the accessory gene regulator (*agr*) locus, which is the central *S. aureus* virulence regulator. The activation of *agr* results in biofilm dispersion, infection dissemination, and the upregulation of several toxins, including superantigens. Therefore, loss of the *agr* or its dysfunction enhances *S. aureus* adhesion and biofilm formation, which likely explains the common association (13–82%) of *S. aureus agr*-defective strains with hospital settings or hospitalization, where most infections are biofilm-associated, eg, catheter-associated.<sup>212–214</sup> Also, the *agr* is repressed by mildly acidic conditions (pH ~ 5.5) that may facilitate colonization of low pH niches, such as the skin or the vagina.<sup>212,215</sup> Thus, the Agr system and its regulation represent promising therapeutic targets, and screening the sinonasal *S. aureus* population for *agr* dysfunction or dysregulated expression appears relevant.<sup>216</sup>

Due to immunomodulatory traits, tryptophan metabolites, such as kynurenine, may contribute to CRS pathogenesis in Pseudomonadaceae-dominated microbiota.<sup>8</sup> Notably, tryptophan is the leading mediator in the gut-brain axis (GBA), that is, a bilateral communication network between the gastrointestinal (GI) tract microbiota and the central nervous system.<sup>217</sup>

In CRS, Zn-mediated nutritional immunity may select specific species and force or drive bacterial adaptation to overcome the Zn limitations. Hence, differences in the genetic background of “healthy” and CRS-microbiota may concern the prevalence and the type of high-affinity Zn transporters, lower number of Zn-dependent proteins, metabolic pathways as adaptations to Zn-depleted mucosa as well as QS and two-component signal transduction system (TCS) patterns along with T2Rs polymorphism. Accordingly, TCS-enriched gene content characterizes the Staphylococcaceae-dominated sinonasal microbiota in CRS patients.<sup>8</sup> This assumption is also supported by the observation that the nasal carrier strain of *S. aureus* shows significantly greater attachment and growth on nasal epithelial cells (NEC) than the non-carrier (laboratory) *S. aureus* strain.<sup>218</sup> Additionally, the susceptibility of the non-carrier *S. aureus* strain to nasal fluid collected from *S. aureus* carriers is likely explained by its inability to induce HBD-2 and HBD-3 and its delayed upregulation of TLR2 in NEC.<sup>218</sup>

Similarly, low Zn levels should be considered in studies on the role of SEB and other superantigens in CRS pathogenesis. Interestingly, upregulation of IL-6 and IL-8, along with downregulation of ZO-1 expression, is a hallmark of the Th2-skewed pattern induced by both Zn depletion and SEB. Furthermore, investigating the role of *S. aureus* SSL3 or SSL4-TLR2 antagonists or low intracellular Zn concentrations for enhanced MHC-II surface

expression may help decipher differences between SEB action in CRS patients and healthy controls as well as create a basis for future TLR2-directed therapies.<sup>128,133</sup>

Competitive exclusion mediated by anti-streptococcal action of *Corynebacterium*-produced oleic and linoleic acids was suggested to explain a negative correlation between *Corynebacterium* and *Streptococcus* abundance in CRS-associated microbiota.<sup>23</sup> However, structural characteristics of CRS-associated biofilms, eg, formation of “corncob” structures via co-aggregation of these bacteria, may also underlay this observation. Therefore, *Corynebacteria* may be vital compositional and structural members of sinonasal biofilms responsible for their long-term stability, and the “mechanistic” and “mucin” hypotheses of biofilms’ role in CRS pathogenesis may be complemented by the “structural” one.

Importantly, *F. nucleatum*-mediated co-aggregation is a receptor-ligand-specific interaction that can be inhibited by relatively common compounds. For example, co-aggregation with early colonizers, including *Streptococcus* and *Actinomyces*, can be suppressed by L-arginine and L-lysine (via the RadD receptor), whereas D-galactose inhibits (via the Fap2 receptor) the interaction with late colonizers (*Actinobacillus* and *Porphyromonas*). In addition, L-arginine- and L-lysine-inhibitable adhesion preferentially affects gram-positive and gram-negative species, respectively. Furthermore, this process is controlled by a two-component signal transduction system CarRS, controlling lysine metabolism and several virulence genes.<sup>219</sup> Overall, this system, similarly to the Agr system of *S.aureus*, may function as a mechanism controlling planktonic – biofilm (co-aggregation) state or spreading – localized infection biofilm, where a lack of lysine promotes *F. nucleatum* planktonic (spreading) form via downregulation by CarRS of RadD, which acts as a lysine receptor.<sup>219</sup> Consequently, understanding the mechanisms underlying *Corynebacteria*-mediated “corncobs” could be useful for developing CRS therapies, as shown for staquorsin – the Agr inhibitor (Figure 5).

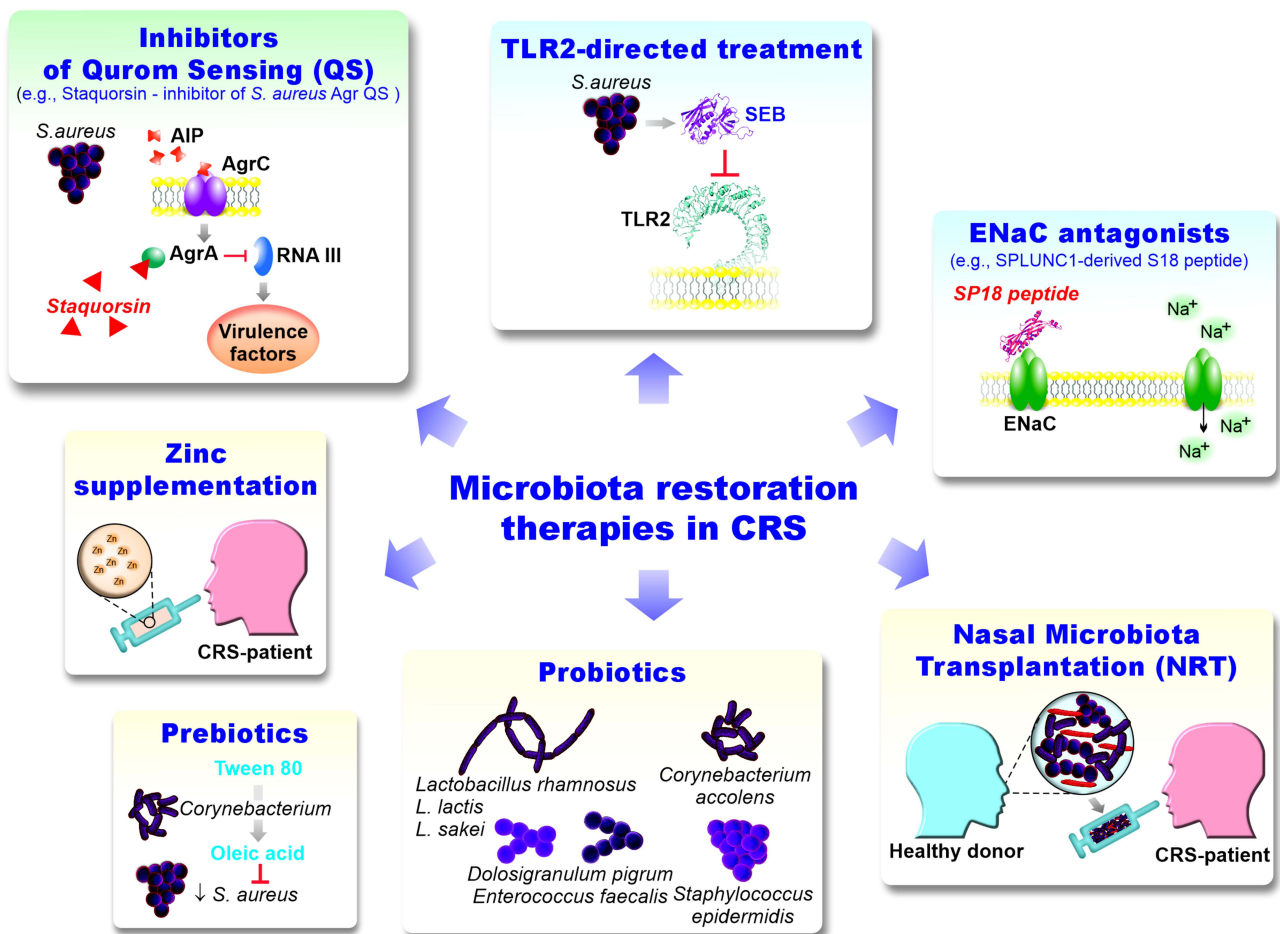


Figure 5 Microbiota restoration therapies in CRS.

## Conclusion

In conclusion, biofilm formation depends on various environmental physicochemical parameters such as pH, ion concentration, temperature, and specific nutrients. Fluctuations in the biofilm pH are commonly associated with dysbiotic changes in these bacterial communities. Glycogens drive *Lactobacillus*-mediated eubiosis associated with low vaginal pH, and an increase in vaginal pH results in dysbiosis and vaginitis. In contrast, sugar-rich saliva and an abundance of streptococci and other lactic acid bacteria result in pH acidification, leading to dental caries. Acidification of the local environment by *Dolosigranulum* has also been suggested as a factor facilitating the expansion of lipophilic Corynebacteria.<sup>220</sup> In comparison, protein-rich gingival fluid promotes the growth of acquiring energy from the metabolism of amino acids periodontal pathogens, such as *Porphyromonas gingivalis*, and hence a neutral to weakly alkaline pH environment. Identification of the physicochemical characteristics of sinonasal biofilms may be essential for deciphering their compositional and architectural features, which is indispensable for understanding biofilm-associated CRS pathogenesis and developing appropriate therapies to reestablish eubiotic biofilm microbiota restoration therapies, for example, by supporting competitive exclusion, interfering with bacterial QS communication, host receptors, and microbiota transplantation. Successful nasal microbiota transplantation (NMT) using nasal lavages from healthy donors was recently performed in patients with CRSnNP, resulting in significant and long-lasting improvements in symptoms along with a stable increase in the abundance and diversity of resident bacteria.<sup>221</sup> These results have now been verified in the Phase II Randomized Control Trial of Nasal Microbiota Transplant Therapy in Chronic Rhinosinusitis Without Nasal Polyps (CRSsNP) (ClinicalTrials.gov Identifier: NCT03122795). The concept of Nasal Microbiota Transplantation (NMT) is analogous to fecal microbiota transplantation (FMT) or Oral Microbiota Transplantation (OMT), that is, transferring bacteria from a healthy donor to a patient.<sup>222,223</sup> FMT as a therapy for *Clostridioides difficile* (CDI) diseases has been used for over two decades, and recently, the US Food and Drug Administration (FDA) approved Vowst capsules, the first oral fecal microbiota formulation (ClinicalTrials.gov Identifier: NCT03183128). OMT treats oral diseases such as dental caries and periodontitis.<sup>222,223</sup> Currently, OMT therapy has only been applied to treat periodontitis in dogs.<sup>224</sup> Therefore, NMT likely supported by local zinc administration, for example, via nasal irrigation or surgical hydrogels, could be a promising CRS treatment.<sup>20</sup>

## Abbreviations

RS, rhinosinusitis; CRS, chronic rhinosinusitis; NPs, nasal polyps; CRSwNP; chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps; E-CRS, idiopathic eosinophilic chronic rhinosinusitis; NE-CRS, and non-eosinophilic chronic rhinosinusitis; EMT, epithelial-to-mesenchymal transition; QS, quorum sensing; QSM, quorum sensing molecules; TCR, T cell receptors; PRRs, pattern-recognition receptors; MAMPs/PAMPs, pathogen/microbe-associated molecular patterns; AHL, *N*-acyl-homoserine lactone; C12-HSL, *N*-3-(oxododecanoyl)-homoserine lactone; AMP, antimicrobial peptide; ASL, airway surface liquid; ENaC, epithelial Na<sup>+</sup> channel; SPLUNC1, Short-palate lung and nasal epithelial clone 1; NMT, Nasal microbiota Transplantation; DFUs, diabetic foot ulcers; TGF-β, transforming growth factor β; TIMP-1, tissue inhibitors of metalloproteinase 1; VEGF, vascular-endothelial growth factor; PDGF, platelet-derived growth factor, TJ, tight junctions; ZO-1, zonula occludens 1; COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; AI-2, autoinducers 2; AIPs, auto-inducing peptides; VBNC, viable but non-culturable cells; EPS, extracellular polymeric substances; FEP, Functionally Equivalent Pathogroups (FEP) hypothesis; EETs, eosinophil extracellular traps; OSM, oncostatin M; SEB, *S. aureus* superantigenic enterotoxin B; MMPs, matrix metalloproteinases; SAGs, superantigens; SSL, *S. aureus* superantigen-like (SSL) toxins, ROS, reactive oxygen species; LT-IIb, type II heat-labile enterotoxin of *E. coli*; PGs, prostaglandins; LTs, leukotrienes; PGE2, prostaglandin E2; COX-2, cyclooxygenase-2; EP2, E-prostanoid-2; PGD2, prostaglandin D2; HNE; human neutrophil elastase; EGFR, epidermal growth factor receptor; LPLUNC2, long-palate lung and nasal epithelium clone 2; CLU, clusterin; SLPI, secretory ILeukocyte peptidase inhibitor; HBD, human beta-defensin; CP, calprotectin; NEC, nasal epithelial cells; TCS, two-component signal transduction system; TLRs, Toll-like receptors; FPRs, N-formyl peptide receptors; T2Rs, Taste Receptor Family 2 receptors; RAGE, Receptor for Advanced Glycation Endproducts; GOCRs, G-protein-coupled receptors; RORC, Retinoic Acid Receptor Related Orphan Receptor C; HIF-1α, Hypoxia-Inducible Factor 1α Receptor; PAR2, Protease-Activated Receptor 2; PPAR-γ, Peroxisome Proliferator-Activated Receptor Gamma; RIG-1, Retinoic Acid-Inducible Gene I; ENaC, Epithelial Na<sup>+</sup> Channel; TSPL, Thymic Stromal Lymphopoietin; MDB, Mucin-Degrading Bacterial; SCFAs, Short-chain fatty acids; MCC, Mucociliary clearance; *agr*, the accessory gene regulatory (*agr*) system; PSGL-1, P-selectin glycoprotein ligand-1.

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## Disclosure

The authors report no conflicts of interest in this work.

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