

Decreased diversity of nasal microbiota and their secreted extracellular vesicles in patients with chronic rhinosinusitis based on a metagenomic analysis

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Abstract

Background: Chronic rhinosinusitis (CRS) is an inflammatory process in the nasal cavity and paranasal sinuses, and bacteria have been considered to be a cause. Indeed, recent evidence indicates that bacteria-derived extracellular vesicles (EV) appear to be an important causative agent of inflammatory diseases. Here, we aimed to evaluate the diversity of nasal microbiota and their secreted EV in patients with CRS.

Methods: Nasal lavage (NAL) fluid samples were obtained from five patients with CRS with polyposis, three patients with CRS without polyposis, and three non-CRS controls. After preparation of bacteria and EV from samples using differential centrifugation, genomic DNA was extracted and 16S-rDNA amplicons were subjected to high-throughput pyrosequencing on a Roche 454 GS-FLX platform.

Results: Metagenomics showed that bacteria composition was positively correlated with EV composition. Samples from patients with CRS had greater bacterial abundance and lower diversity, both from bacteria and the EV portion of samples, compared with non-CRS samples. At each phylogenetic level, Bacteroidetes decreased while Proteobacteria increased in the CRS group at the phylum level. At the genus level, *Prevotella* spp. decreased in the CRS group, while *Staphylococcus* spp. increased from both bacteria and EV. Moreover, *Staphylococcus aureus* and its secreting EV compositions were higher in samples from CRS with polyps compared with CRS without polyps.

Conclusions: These results suggest that patients with CRS have altered nasal microbiota and decreased diversity in bacterial compositions as well as increased *S. aureus* abundance in those patients with polyps.

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nasal and paranasal sinus mucosa, which persists for at least 12 weeks. Traditionally, CRS is classified into CRS with nasal polyps (CRSwNP) and without nasal polyps (CRSsNP), which differ in their immunological patterns (1). Chronic rhinosinusitis is described as a dysfunctional host–environment interaction at the site of the interface, and bacteria have a demonstrated role in the etiology of acute rhinosinusitis, but not in an initiating role in CRS. Previous studies proposed the

staphylococcal superantigen, immune barrier, and biofilm hypotheses, but the exact role(s) of bacteria in CRS remain(s) controversial (2–4).

Conventional bacteria culture techniques have drawbacks, showing only a small proportion of true bacterial diversity, but metagenomics permits large-scale pyrosequencing of 16S rRNA transcripts as markers of the structure of the bacterial community to represent the microbiota's functional landscape (5). Recent studies performed metagenomic analyses in

Caucasian CRS populations, but produced inconsistent results (6, 7). Thus, evidence for a definitive role of the nasal microbiota in CRS pathogenesis is still lacking.

Extracellular vesicles (EV), which have recently emerged as another mechanism of cell communication, are released by host cells and bacteria (8). Because EV contains much biological information, such as receptors, bioactive lipids, proteins, and nucleic acids, they can deliver signals to recipient cells. In our previous studies, we revealed important roles for EV from *Staphylococcus aureus* in atopic dermatitis and neutrophilic pulmonary inflammation (9, 10). Although a previous study demonstrated a virulence mechanism involving EV in human acute rhinosinusitis, to our knowledge, no study has reported on any association between bacterial EV and CRS.

The 'biodiversity hypothesis' explains recent changes in disease patterns, that is, biodiversity loss, secondary to human activities, is associated with various adverse human health effects (11). Metagenomics of healthy and diseased individuals has revealed that reduced biodiversity and alterations in the composition of gut and skin microbiota are associated with various inflammatory conditions, such as inflammatory bowel disease and atopic dermatitis (12, 13). In this study, we started with the notion that the ecosystem in the nasal cavity and paranasal sinuses helped to maintain immune homeostasis and prevented the development of CRS and that disrupted or reduced microbial diversity may cause or aggravate CRS and nasal polyposis (14). To test this idea, we investigated bacterial abundance and diversity in patients with CRS and the relationship between composition in nasal microbiota and their secreting EV and clinical manifestation of CRS. To do this, we performed a metagenomic analysis of nasal lavage (NAL) fluids from the controls and patients with CRSwNP or CRSsNP.

Materials and methods

Study population

From May 2012 to September 2013, consecutive patients who visited the Department of Otorhinolaryngology, Head and Neck Surgery, at Hanyang University Hospital were enrolled. The diagnosis of CRS was made according to the criteria (15). Patients with septal deviation were eligible as controls if they showed no sign of mucosal inflammation on nasal endoscopy and computed tomography (CT). Symptoms were scored using the Sinonasal Outcome Test-20 (SNOT-20). Severity of rhinosinusitis was evaluated with CT score (16). Clinical data including demographic information, relevant medical and surgical history, atopy, asthma, and aspirin intolerance were recorded. Atopy was assessed by a skin prick test to common inhalant allergens. No patient was treated with antibiotics, systemic or topical corticosteroids, or other immunomodulating agents for at least 4 weeks before surgery. Exclusion criteria included children, pregnancy, immunocompromised patients, and other sinonasal diseases, such as acute rhinosinusitis, fungal sinusitis, and tumors. This study was approved by the Hanyang University Hospital Institutional Review Board (No. HYI-11-044).

Sample collection

To obtain NAL fluids, each nostril was flushed with 5 ml of isotonic saline and the fluid was collected from the nasal cavity with a suction collector connected to a 15-ml conical tube. To remove crust and debris, NAL fluids were passed through a cell strainer (100 μ m, SPL) and immediately centrifuged (4500 *g*, 5 min). Cell pellets were suspended in 1 ml of phosphate-buffered saline (PBS) for the bacterial and EV metagenomic study.

Bacteria and EV preparation from NAL fluids

To obtain bacterial and EV portions from NAL fluids, we performed differential centrifugation. NAL fluids were centrifuged at 3000 *g* for 20 min at 4°C, and the pellet was stored as the bacteria portion. The supernatant was subjected to ultracentrifugation (Beckman 90Ti rotor; 150 000 *g*, 3 h, 4°C) after 0.2 μ m filtration, and the pellet was stored as the EV portion.

DNA extraction and metagenomic analysis

After preparation of the bacterial and EV portions from NAL fluids, bacterial and EV DNA were extracted using a DNA extraction kit (DNeasy Blood & Tissue Kit; Qiagen, Hilden, Germany). The standard protocol for this kit was followed. With PCR, we successfully detected bacterial 16S rDNA in all samples. 16S rDNA amplicons were subjected to high-throughput pyrosequencing using the Roche 454 GS-FLX platform (DNA Link Corporation, Seoul, Korea). Each DNA sequence was identified using the ExTaxon database (17), and then, a metagenomic analysis was processed by ChunLab Corporation.

Visualization of species richness

We used the CLcommunity software provided by ChunLab (<http://www.chunlab.com>) to assess species richness and diversity. With this software, a rarefaction technique (18) is used to visualize the rate of increase in the number of operational taxonomic units (OTUs). OTUs are defined by *de novo* clustering using CD-HIT program and defined mathematically with 97% sequence similarity as a boundary. Alpha-diversity indices were calculated using the mothur package (17) to assess Shannon's diversity index (Table 1).

Generation of phylogenetic tree

Numbers of sequence reads that corresponded to each level of phylogeny were assigned to each category. Only the five major phyla are listed, and unclassified species are excluded.

Generation of heat map

First, the percentage of species for samples were normalized to have a mean of 0 and standard deviation of 1 by linear normalization, as incorporated in MatLab 2010a. Then, a heat map of species for samples was drawn using the 'imagesc' function

Table 1 Definition of Shannon diversity, abundance, richness, alpha-diversity, and operational taxonomic units (OTUs)

	Definition
Shannon diversity	One of diversity indices to quantify species diversity.
Abundance	An ecological concept referring to how common a species is relative to other in a particular ecosystem.
Richness	'Species richness' refers to the number of different species represented in a particular ecosystem.
Alpha-diversity	An estimator of within-community (alpha) diversity.
OTUs	Operational taxonomic units commonly used microbial diversity unit. Usually defined as a cluster of reads with 97% similarity.

of MatLab 2010a. Species that showed significant differences ($P < 0.05$) were chosen. The red color indicates a higher percentage, and the green color indicates a lower percentage.

Statistical analysis

To compare differences in bacterial abundance between non-CRS controls and patients with CRS for both the bacterial and EV portion, we used an integrative statistical hypothesis testing method (19) that combined adjusted P -values from paired two-tailed t -tests and the median ratio test using MatLab2010a. Pearson's correlation coefficient was also calculated using MatLab2010a.

Results

Microbial diversity and relative abundances of bacteria and EV in NAL fluids

We compared NAL fluid samples from three non-CRS subjects and eight patients with CRS aged 19–67 years old during endoscopic surgery. Demographics and characteristics of

the study subjects are shown in Table 2. Using the Roche 454 GL-FLX platform, we generated a total of 128 769 pyrosequencing reads from 22 samples: 11 samples each for the bacterial and EV portions. One indicator of biological diversity is the alpha-diversity (20). To measure it, we generated a rarefaction curve to visualize the rate of increase in the number of OTUs. The number of OTUs varied from about 100 to 500 (Fig. 1A,B), which was lower in the CRS group than in the controls for both the bacterial and EV portions, although the difference was not significant (Fig. 1C). This reduced number of OTUs corresponded to decreased microbial diversity in CRS (Fig. 1D), but this was not the result of diminished read numbers; indeed, read numbers were higher in the CRS group (Fig. 1E). As represented by Shannon's diversity index, species diversity decreased significantly in patients with CRS from both the bacterial ($P < 0.05$) and EV ($P < 0.01$) portions of the samples.

Phylogenetic analyses of metagenomes from bacteria and EV in NAL fluids

In metagenomic data, the number of sequences counted within one species represents the relative abundance of that species in the original sample (21). In Fig. 2, the total number of sequences identified is shown in the phylogenetic tree of the metagenomes of bacteria and EV from CRS and non-CRS subjects. Mean number of positive sequences was 3.63 times higher in bacteria (Fig. 2A) and 3.4 times higher in EV (Fig. 2B) in CRS compared to non-CRS control. Taken together, this result indicates that patients with CRS carry a relatively high abundance of bacteria and their secreted EV show less diversity compared to the controls.

Phylum- and genus-level comparisons of bacteria and EV compositions

To compare the bacterial composition between non-CRS and CRS subjects, we generated taxonomic composition charts for each sample. At the phylum level, Bacteroidetes decreased, from 25.42% to 7.37%, whereas Proteobacteria

Table 2 Demographic and clinical characteristics of patients with CRS

Subjects	Sex	Age	Atopy	Asthma	SNOT-20	CT score	Blood Eo (%)	Tissue Eo (%)	Pus	NAL recovery (ml)
Control 1	M	19	No	No	19	3	1.7		No	7
Control 2	M	19	No	No	11	0	2.4		No	9
Control 3	M	55	No	No	5	2	1.5		No	10
CRSsNP 1	M	50	Yes	No	2	4	1		Yes	7
CRSsNP 2	F	50	Yes	No	6	4	3.2		Yes	7
CRSsNP 3	F	53	No	No	10	6	0.1		Yes	10
CRSwNP 4	M	58	No	No	22	15	9.7	52.7	Yes	6
CRSwNP 5	M	67	No	No	5	6	1.4	0.9	Yes	8
CRSwNP 6	M	40	No	No	13	12	5.6	36.8	No	8
CRSwNP 7	F	40	Yes	No	28	20	3.6	24.6	No	8
CRSwNP 8	M	23	No	No	17	20	1.8	6.9	Yes	8

CT, computed tomography; CRS, chronic rhinosinusitis; CRSsNP, chronic rhinosinusitis without polyp; CRSwNP, chronic rhinosinusitis with nasal polyp; SNOT, Sino-Nasal Outcome Test; Eo, blood eosinophils; NAL, nasal lavage.

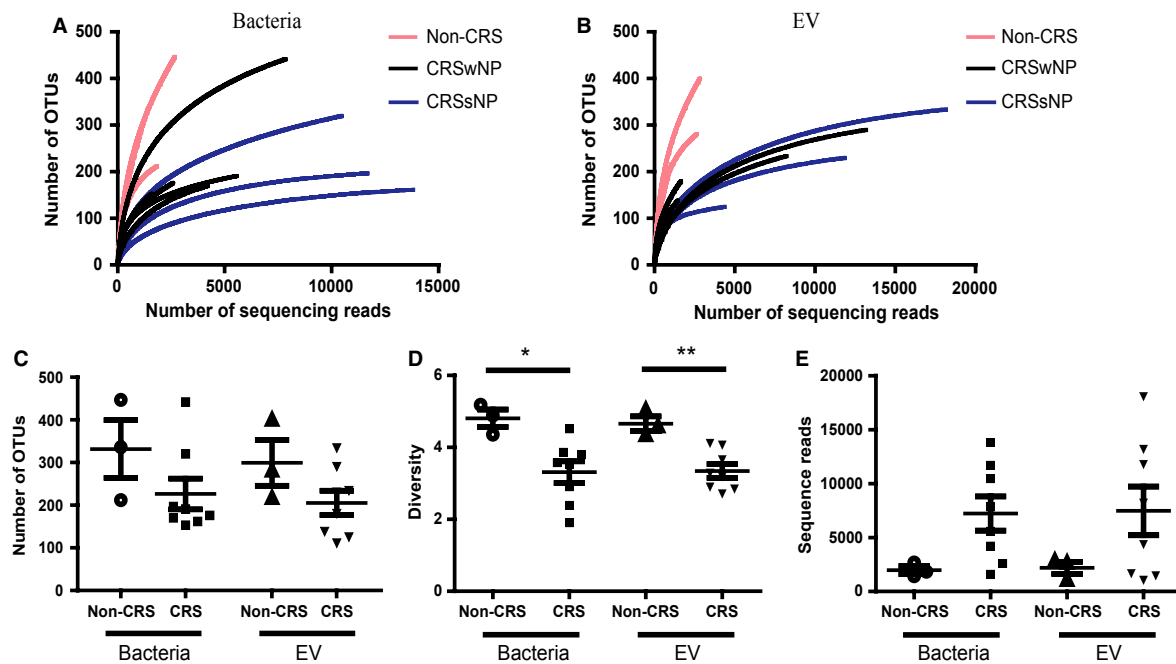


Figure 1 Diversity of microbial communities. (A–C) Number of OTUs (97% similarity) detected in (A) the bacterial portion and (B) extracellular vesicle (EV) portion of samples. (C) Scatterplot showing

the number of OTUs. (D) Shannon's diversity measure. (E) Number of reads per sample in each portion. * $P < 0.05$; ** $P < 0.01$.

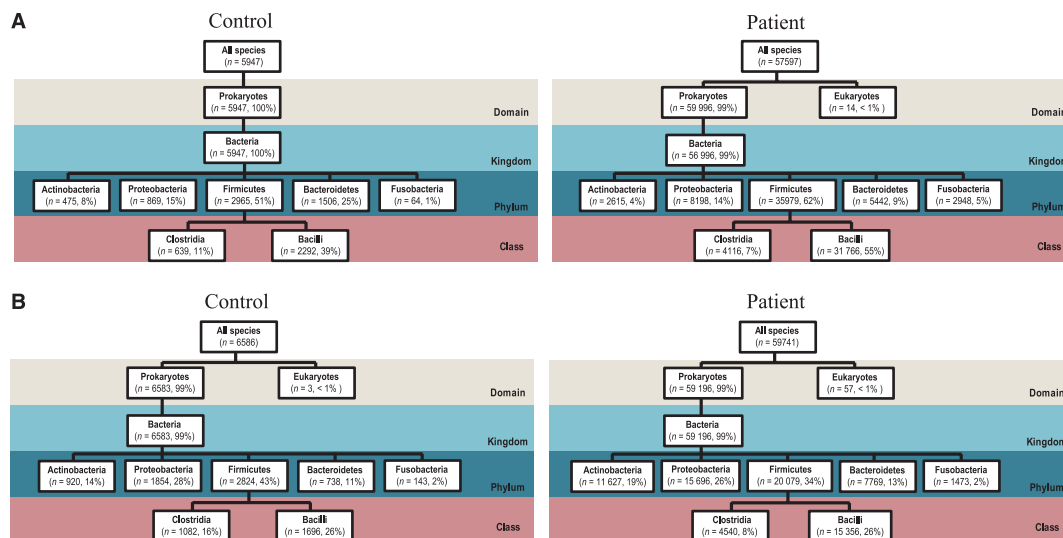


Figure 2 Phylogenetic metagenomic classification of bacteria and extracellular vesicles (EV) in nasal lavage fluids. Phylogenetic tree of metagenomes of bacteria (A) from nonchronic rhinosinusitis (CRS) control (left panel) and patients with CRS (right panel), and of

EV (B) from non-CRS controls (left panel) and patients with CRS (right panel). Numbers of sequences in metagenomics that corresponded to each phylogenetic category are listed.

increased, from 14.33% to 26.51%, in patients with CRS compared with non-CRS controls (Fig. 3A). In the EV comparison, Bacteroidetes decreased, from 13.43% to 7.15%, in the former group compared with the latter (Fig. 3B). At the

genus level, the bacterial and EV compositions of *Prevotella* spp., belonging to the phylum Bacteroidetes, decreased, from 19.84% to 3.54% and from 11.05% to 3.29%, respectively, in patients with CRS compared with non-CRS controls

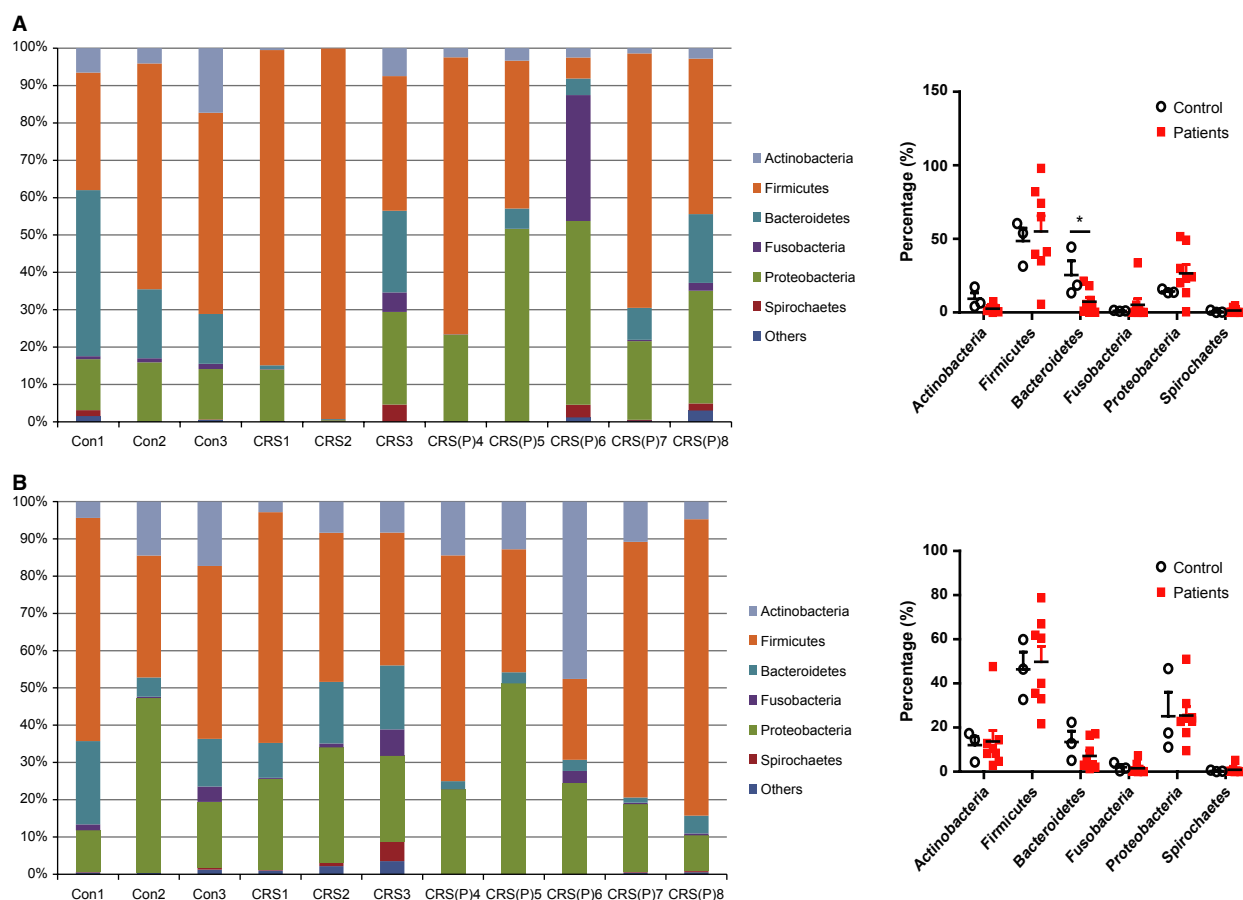


Figure 3 Phylum-level differences in compositions of bacteria and extracellular vesicles (EV) in nasal lavage fluids. (A) Bar and dot graphs of relative abundance of major phylum classification from

bacterial portion of samples. (B) Bar and dot graphs of the relative abundance of major phylum classification from the EV portion of samples. * $P < 0.05$.

(Fig. 4A–C). In contrast, the bacterial and EV compositions of *Staphylococcus* spp., belonging to the phylum Firmicutes, increased, from 1.03% to 46.11% and from 15.03% to 39.08%, respectively, in the former group vs the latter (Fig. 4A,B,D).

Correlation between bacterial and EV compositions at the species level

A strong correlation was observed between bacterial and EV compositions ($r = 0.550$, $P = 0.001$; Fig. 5A). Species showing strong correlations between bacterial and EV compositions included *Rothia mucilaginosa*, *Streptococcus australis*, *Streptococcus mitis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Staphylococcus warneri*, *Corynebacterium macginleyi*, and *Corynebacterium accolens* (Fig. 5B).

Species-level comparison of bacteria and EV compositions

To visualize which species increased or decreased in patients with CRS at the species level, we generated heat map charts indicating the relative abundance of bacterial species. The

bacterial composition of *Staphylococcus epidermidis* increased, whereas other species, including *Prevotella melaninogenica*, decreased in patients with CRS vs non-CRS controls (Fig. 6A). For EV compositions, EV from *Pseudomonas monteilii*, *Corynebacterium* spp., and *Propionibacterium* spp. increased, whereas other EV, including *Prevotella* spp., *Streptococcus* spp., and *Veillonella* spp., decreased in patients with CRS compared with non-CRS controls (Fig. 6B).

Comparison of bacteria and EV compositions between patients having CRS with and without polyps

Finally, bacterial and EV were compared in patients who had CRS with and without nasal polyps. At the genus level, the bacterial composition of *Pseudomonas* spp. increased in CRSwNP compared with CRSsNP (Fig. 7A). For the EV composition, EV from *Pseudomonas* spp. and *Propionibacterium* spp. increased in the former group vs the latter (Fig. 7B). At the species level, the *S. aureus* increased significantly in CRSwNP compared with CRSsNP (8.83% vs 0.05%; Fig. 7C). Moreover, EV from *S. aureus* also increased significantly in the former vs the latter group (3.96% vs 0.53%; Fig. 7C).

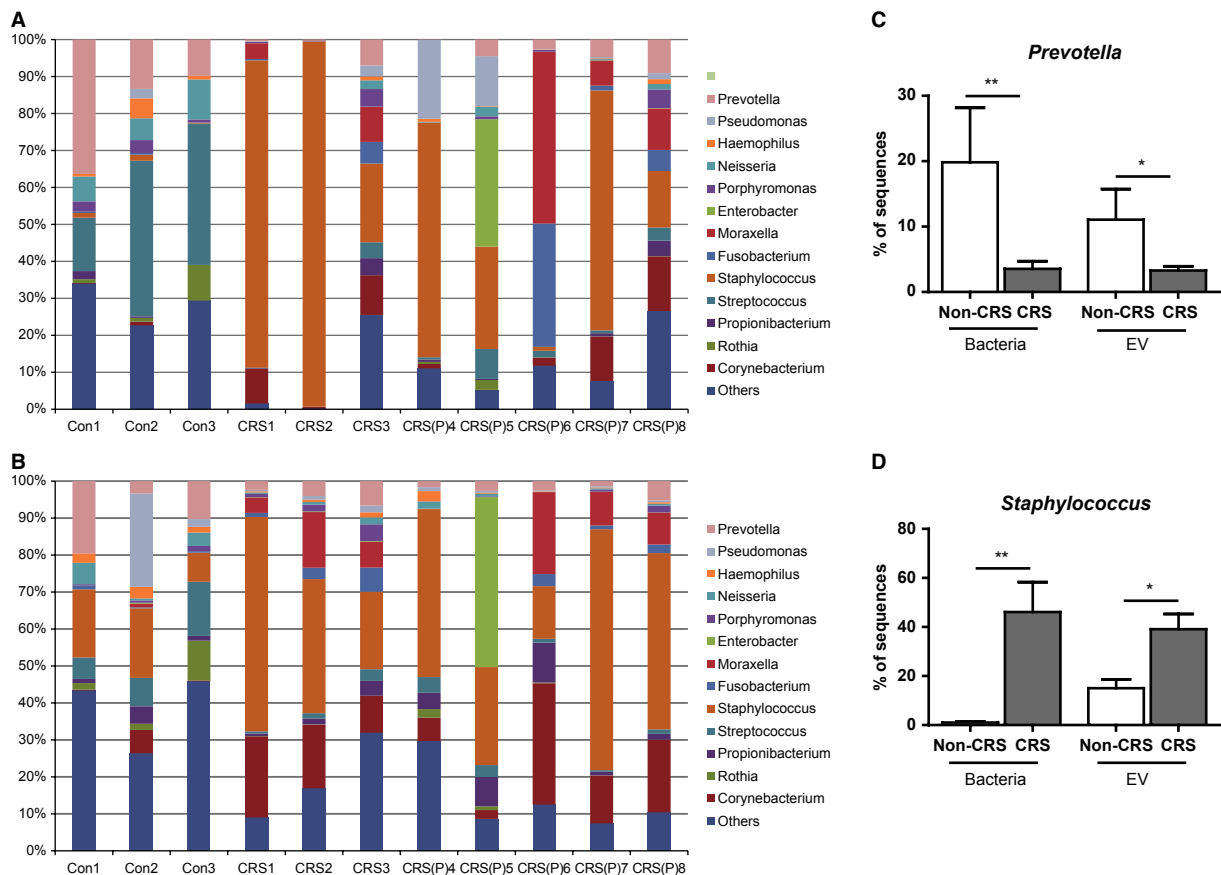


Figure 4 Genus-level differences in the compositions of bacteria and extracellular vesicles (EV) in nasal lavage fluids. Bar graph of the relative abundance of major genus classification from bacteria (A) and EV (B) portions of samples. (C) Composition of *Prevotella*

spp. and their secreted EV in patients with chronic rhinosinusitis (CRS) and non-CRS controls. (D) Composition of *Staphylococcus* spp. and their secreted EV in patients with CRS and non-CRS controls. * $P < 0.05$; ** $P < 0.01$.

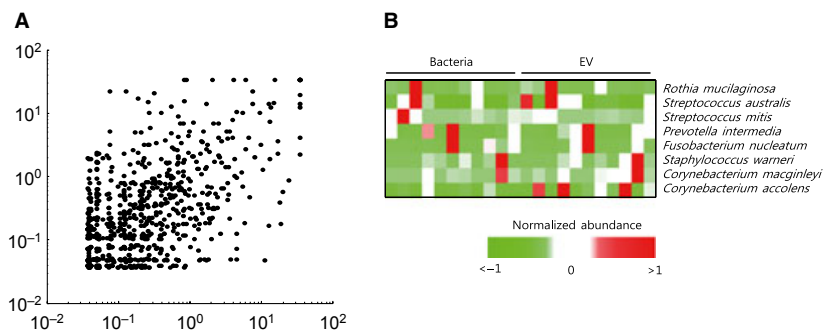


Figure 5 Correlation between bacteria and extracellular vesicles (EV) compositions in nasal lavage fluids. (A) Graph showing overall correlation between bacteria and EV compositions (correlation coefficient = 0.299; Pearson's correlation coefficient = 0.550). (B) Heat

map indicating abundant species that had positive correlations between bacterial and EV compositions (correlation coefficient > 0.8 and $P < 0.01$ by Pearson's correlation coefficient).

Discussion

Recent advances in culture-independent technologies have provided knowledge about microbial diversity that colonize specific host sites as well as the relationships between

alterations in microbiota and disease states (12, 22). However, only a few studies have shown a relationship between nasal microbiota composition and the pathogenesis of CRS, mostly in Caucasian populations (6, 7). A previous study observed significantly reduced bacterial diversity in patients with CRS (23),

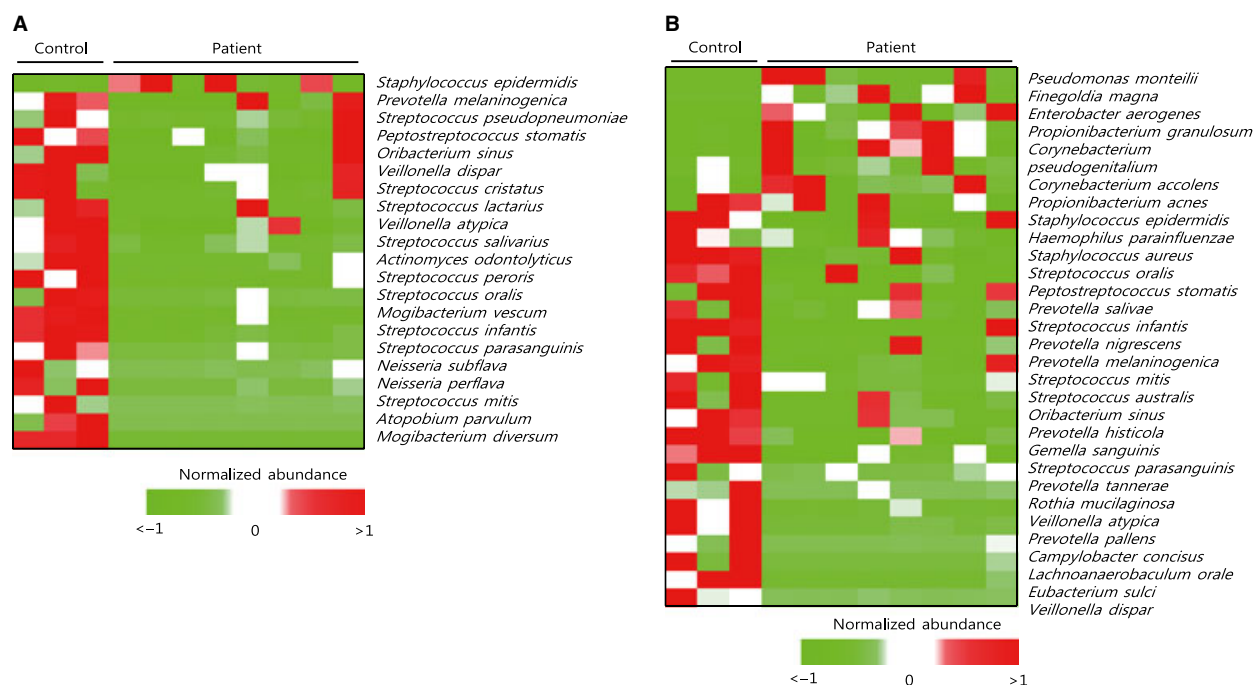


Figure 6 Species-level differences in the compositions of bacteria and extracellular vesicles (EV) in nasal lavage (NAL) fluids. (A) Heat map comparing the relative abundances of bacterial species in NAL fluids from nonchronic rhinosinusitis (CRS) controls and patients

with CRS. (B) Heat map comparing the relative abundances of EV at the species level in NAL fluids from non-CRS controls and patients with CRS.

consistent with our results. Moreover, we demonstrated for the first time that lower diversity in bacterial EV occurred simultaneously in NAL fluids from CRS patients. A major strength of the present study is the metagenomic analysis using bacteria and also EV from NAL fluids with good recovery rates.

Characterization of microbiota habitats by alpha-diversity index provides information about community structure and can provide a quantitative method to follow changes in specific host sites (21). In the present study, patients with CRS exhibited less alpha-diversity and a lower Shannon's diversity index for both the bacterial and EV. On the other hand, a phylogenetic chart showed higher abundances of bacteria and EV in the NAL fluids from patients with CRS compared with non-CRS subjects. Similar to a recent report (24), the Firmicutes phylum was the most dominant of the five major phyla in the nasal microbiota in all groups. However, the Bacteroidetes phylum, a major member of the normal bacteria community, significantly decreased in patients with CRS vs the non-CRS controls. Thus, we suggest that this reduced abundance of Bacteroidetes may contribute to the pathogenesis of CRS.

Previous studies have suggested that the genus *Prevotella* was the main anaerobic isolate in pediatric and adult maxillary sinusitis, acute exacerbation of CRS, and CRSwNP (13, 25). Thus, *Prevotella* may be an important pathogen in CRS. However, a major limitation of the previous studies is the lack of comparison with a non-CRS control. *Prevotella* is a genus of Gram-negative bacteria and a member of the Bacteroidetes phylum. *Prevotella* spp. are members of the normal

flora and are recovered from anaerobic infections of the respiratory tract, including chronic otitis media and sinusitis (26). The present study showed that *Prevotella* spp. significantly decreased in patients with CRS compared with the non-CRS controls. This suggests that the reduced numbers of *Prevotella* spp. may contribute to the pathogenesis of CRS. These somewhat conflicting data point to the need for future functional studies to discriminate 'good' from 'bad' bacteria in CRS pathogenesis.

Among the phylum Firmicutes, the present data showed that *Staphylococcus* spp. and their secreted EV were elevated in patients with CRS. Previous studies reported a positive association between *S. aureus* and CRS (7, 27), which is consistent with our results. The present data also showed that patients with CRS had increased *S. epidermidis*, which is a coagulase-negative staphylococcus and usually considered as a nasal commensal and low-virulence organism (28). Although pathogenic roles of *S. epidermidis* have been reported in infections in other body sites (sepsis, infections of indwelling catheters, and in burn patients), further studies will be needed to determine whether *S. epidermidis*-derived EV are pathogenic in CRS pathogenesis.

Although many studies have reported on the microbiology of CRS, no comparison study had examined CRS with and without nasal polyps. Moreover, previous studies were performed in settings without non-CRS controls (6, 29, 30). Over the last several decades, *S. aureus* has emerged as an important pathogen and staphylococcal superantigen has

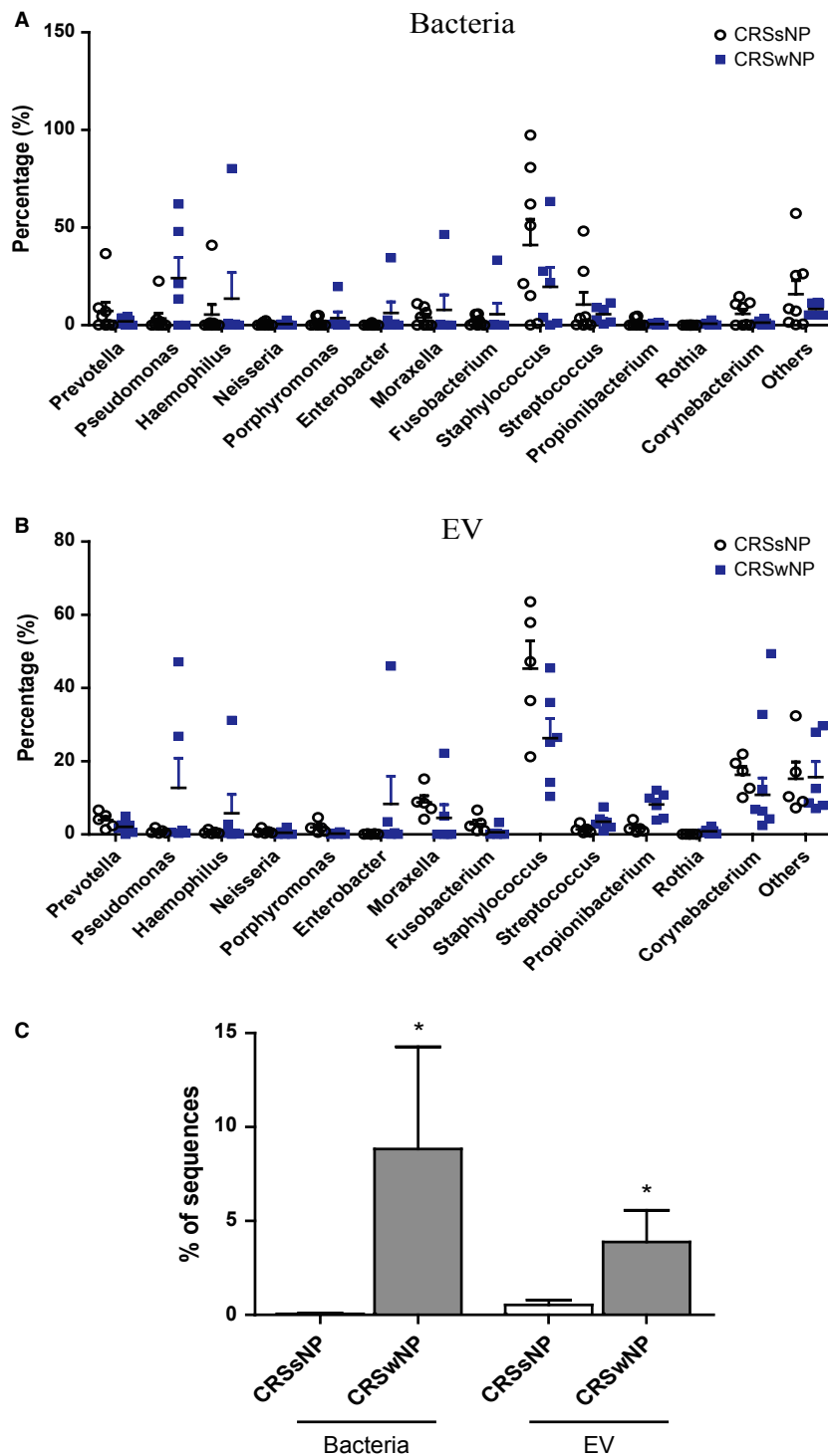


Figure 7 Compositional differences in bacteria and their secreted extracellular vesicles (EV) between chronic rhinosinusitis (CRS) with and without polyps. (A) Dot graph showing the compositions of major bacterial genera in nasal lavage (NAL) fluids from patients having CRS with nasal polyps (CRSsNP) and without polyps (CRSwNP). (B) Dot graph showing the compositions of EV derived

from major bacterial genera in NAL fluids from patients with CRS with nasal polyps (CRSsNP) and without polyps (CRSwNP). (C) Graph comparing the relative abundance of *Staphylococcus aureus* between CRS with polyps (CRSwNP) and CRS without polyps (CRSsNP). * $P < 0.05$ vs CRSsNP.

been detected in approximately 50% of Caucasian nasal polyps as well as a lower percentage of Asian polyps (1). In the present study, both *S. aureus* and its secreted EV were found more frequently in CRSwNP than in CRSsNP, supporting a pathogenic role of *S. aureus*-derived EV in the pathogenesis of CRS-associated nasal polyposis. EV are secreted by Gram-negative and some Gram-positive bacteria and provide an important machinery for cell communications playing roles as virulence factors in respiratory track diseases (31). Our previous study revealed that *S. aureus* EV induced the production of pro-inflammatory cytokines from airway epithelial cells and resultant neutrophilic airway inflammation (10). To sum up, these findings suggest that *S. aureus* EV are an important agent in development of inflammation in the upper and lower airways.

Given the strong correlation between the bacteria and EV, EV can be considered to represent the bacteria in the nasal cavity. However, inconsistencies were observed in the relative abundance of species between bacteria and EV. One possibility is that different amounts of EV are secreted by individual bacterial species, suggesting that both functional and virulent factors of specific EV may be more important than just their abundance in the pathogenesis of CRS. If we can clearly determine the pathogenic roles of EV, they may be useful as another biomarker of CRS in the future. Despite our successful demonstration of the decreased biodiversity of bacteria and EV in CRS patients compared to non-CRS controls, we acknowledge that the present study has some drawbacks, especially the small numbers of the enrolled patients. However, a previous metagenomic study also found divergent results based on small numbers of CRS (23).

In conclusion, this is the first report of a metagenomic analysis of bacteria and EV in NAL fluids from patients with CRS. Given the heterogeneous characteristics of CRS, concluding that changes in microbial composition are the direct cause of CRS and/or nasal polyps is difficult. However, our findings support the hypothesis that reduced microbial diversity is involved in the pathogenesis of CRS. Additionally, EV secreted by pathogenic bacteria in the nasal community may participate in the pathogenesis of CRS, and this issue needs further studies.

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Author contributions

The authors made the following contributions to the study: study concept and design: E.C., S.H., K.K., S.K; acquisition of data: E.C., W.H., D.K., Y.K.; analysis and interpretation of data: E.C., D.K., S.J., S.C., Y.G., Y.K.; drafting of the manuscript: E.C., S.C., Y.K.; statistical analysis: E.C., D.K. obtained funding: Y.K.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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