1 ***Running title: Multi-omic analysis in chronic rhinosinusitis***

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3 **Antibiotic-dependent relationships between nasal microbiome and secreted proteome in chronic**
4 **rhinosinusitis and nasal polyps**

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47

48 **Abstract**

49 **Background:** Antibiotics are commonly prescribed to treat chronic rhinosinusitis (CRS); however,
50 the effects of antibiotics on the microbiome and secreted proteome remain unknown in regard to CRS.

51 **Objective:** We analyzed the effects of antibiotics on the nasal microbiome and secreted proteome in
52 the context of CRS using multi-omic analysis.

53 **Methods:** Nasal secretions were collected from 29 control, 30 CRS patients without nasal polyps
54 (CRSsNP), and 40 CRS patients with nasal polyps (CRSwNP). A total of 99 subjects were divided
55 into two groups that included subjects who had taken antibiotics 3 months prior to sampling (ABX)
56 and those who had not (NABX). We performed 16S rDNA sequence analyses and Orbitrap mass
57 spectrometry-based proteomic analyses in data-independent acquisition (DIA) on the nasal secretions.
58 Spearman correlation was used to assess the correlations between the nasal microbiome and secreted
59 proteome.

60 **Results:** We observed a strong association between the nasal microbiome and secreted proteome
61 according to disease status. Antibiotic use reduced differences in the microbial community and
62 secreted proteome according to disease status. Interestingly, in nasal polyp (NP) patients, antibiotics
63 exhibited strong effects not only on the nasal microbiome and the secreted proteome but also on their
64 associations. Additionally, their correlations were strengthened in subjects who had taken antibiotics.

65 **Conclusion:** Integrative analyses revealed that the correlations between the microbiome and the
66 secreted proteome could be altered and strengthened in subjects who used antibiotics. These findings
67 provide novel insight into the effects of antibiotics on the nasal environment and the host responses in
68 CRS.

69

70 **Key words**

71 Metagenomics, proteomics, anti-bacterial agents, sinusitis, microbiota, proteome, mucus

72

73 **Abbreviations**

74 CRS, Chronic rhinosinusitis;

75 CRSsNP, Chronic rhinosinusitis without nasal polyp;

76 CRSwNP, Chronic rhinosinusitis with nasal polyp;

77 ABX, the subjects who had taken antibiotics 3 months before sampling;

78 NABX, the subjects who had not taken antibiotics 3 months before sampling;

79 L-M, Lund-Mackay;

80 SPT, skin prick test;

81 PERMANOVA, Permutational multivariate analysis of variance;

82 PCoA, principal coordinates analysis;

83 LDA, Linear discriminant analysis;

84 LefSe, Linear discriminant analysis effect size;

85 OTU, Operational taxonomic unit;

86 LC-MS, Liquid chromatography-mass spectrometry;

87 DIA, Data-independent acquisition;

88 IPA, Ingenuity pathway analysis;

89 NP, Nasal polyp;

90 aSPC, adaptive sum of powered correlation

91

92 **Introduction**

93 Chronic rhinosinusitis (CRS) is a persistent inflammatory condition of the nasal mucosa and sinus. It
94 is one of the most common upper airway diseases in Western countries and in Asia(Zhang et al. 2017).
95 Indeed, this disease exhibits considerable heterogeneity at the clinical and molecular pathophysiologic
96 levels(Tomassen et al. 2016). Based on this, previous studies have demonstrated that the endotypes of
97 CRS could be characterized based on cytokines(Tomassen et al. 2016), symptoms(Divekar et al.
98 2015), microbiota composition(Cope et al. 2017), and clinical characteristics(Liao et al. 2018).

99 Recently, multi-omic analyses were performed to reveal interrelationships among the microbiome,
100 metabolome, transcriptome, and proteome in association with human diseases(Misra et al. 2018). For
101 example, molecular profiles of host (transcriptomics and genomics) and microbial profiles
102 (metagenomics, metaproteomics, and metatranscriptomics) revealed that profiling of disease-
103 associated microbiomes should be accounted for corresponding molecular changes in the host
104 epithelium(Lloyd-Price et al. 2019). Metagenomic, transcriptomic, and proteomic analyses identified
105 the interactions between the sputum microbiome and host interferon signaling in chronic obstructive
106 pulmonary disease (COPD) patients(Wang et al. 2019). Therefore, integrative analyses of
107 metagenomics and secreted proteomics in CRS could provide a better understanding of heterogeneity
108 based on associated changes in nasal environment (microbiome) and host response (secreted
109 proteome).

110 For the medical treatment of CRS, antibiotics such as penicillins/betalactams and macrolides were
111 prescribed in about 70% of CRS visits(Smith et al. 2013). However, most of previous metagenomic
112 analyses(Aurora et al. 2013; Choi et al. 2014; Biswas et al. 2015; Ramakrishnan et al. 2015; Biswas et
113 al. 2017; Cope et al. 2017; Hoggard et al. 2017; Jain et al. 2017; Lal et al. 2017; Ramakrishnan et al.
114 2017; Chalermwatanachai et al. 2018; Copeland et al. 2018; Koeller et al. 2018; Mahdavinia et al.
115 2018; Biswas et al. 2019; Gan et al. 2019; Paramasivan et al. 2019; Rom et al. 2019) examining CRS
116 excluded patients who had taken antibiotics within approximately one month prior to sampling. Here,
117 we performed metagenomics and proteomics analyses using nasal secretions in healthy control and
118 CRS patients. To investigate the means by which antibiotics affect both the microbiome and the

119 secreted proteome in CRS, the subjects were divided into two groups that included the subjects who
120 had taken antibiotics 3 months prior to sampling (ABX) and those who had not (NABX). Furthermore,
121 we sought to gain insight into any potential interactions between the nasal microbiome and the
122 secreted proteome.
123

Results

Differences in the microbial composition and proteome according to disease status

From our data, we identified 1,329 operational taxonomic units (OTUs) at the genus level in 29 controls, 30 CRSsNP, and 40 CRSwNP patients. To determine if the microbial composition was different according to disease status, we analyzed the alpha and beta diversity in a total of 99 subjects. Shannon and Simpson indices were significantly increased across control to CRSwNP, while Chao1 and the number of observed OTUs were not increased (Fig. 1A and Fig. S2A). There were significant differences in microbial composition in relation to disease status (Fig. 1B).

To investigate which bacterial taxa were different in relation to disease status, we compared the nasal bacterial composition. *Firmicutes* and *Bacteroidetes* were significantly increased from control to CRSwNP, while *Cyanobacteria* levels were significantly decreased from control to CRSwNP (Fig. S2B and C). At the family level, *Staphylococcaceae*, *Propionibacteriaceae*, and *Moraxellaceae* were significantly decreased from control to CRSwNP (Fig. 1C and Fig. S2D). *Prevotellaceae* was significantly increased from control to CRSwNP. Linear discriminant analysis (LDA) effect size (LEfSe) analyses revealed *Propionibacteriaceae* and *Moraxellaceae* were the most abundant in the control samples, and *Entomoplasmataceae* was the most abundant in the CRSsNP samples at the family level (Fig. S2E).

To determine if the secreted proteome was different in relation to disease status, we performed proteomic analysis of the nasal secretions of 69 subjects that were divided into 23 control, 24 CRSsNP, and 22 CRSwNP samples. We quantified a total of 2,162 proteins, and on average we quantified approximately 1,440 proteins. The number of quantified proteins from each subject is represented in Figure S3. When comparing the control and CRSsNP groups, relatively small proteomic changes were observed compared to changes observed when comparing the control and CRSwNP or CRSsNP and CRSwNP groups (Fig. 1D and Table S1).

Given that the nasal microbiome and secreted proteome were different in relation to disease status, we hypothesized that they could correlate with each other. To confirm this hypothesis, we divided the nasal microbiome into two groups that included families with increased relative abundance from

control to CRSwNP (IFc) and families exhibiting decreased relative abundance from control to CRSwNP (DFc) (Kruskal-Wallis, $p < 0.05$). We also divided the secreted proteome into two groups that included proteins with increased normalized intensity from control to CRSwNP (IPc) and proteins exhibiting decreased normalized intensity from control to CRSwNP (DPc) (ANOVA, $p < 0.05$). A number of significant positive or negative correlations were observed among the groups (IFc, DFc, IPc, and DPc) (Fig. 1E). To confirm the associations among the four groups, we applied adaptive sum of powered correlation (aSPC) tests. These analyses revealed significant global association among the groups (IFc, DFc, IPc and DPc) (aSPC test, $p < 0.01$) (Fig. 1F). In contrast, less significant correlations were detected between each group (IFc, DFc, IPc and DPc) and others in regard to the microbiome and proteome, respectively (aSPC test, $p > 0.01$). These findings suggest a strong association between the nasal microbiome and the secreted proteome according to disease status.

Differential microbiome composition according to the use of antibiotics

Further, we sought to examine variations in the microbial community in relation to disease status in NABX and ABX. The NABX group consisted of 27 control, 22 CRSsNP, and 24 CRSwNP patients, while the ABX group consisted of 2 control, 8 CRSsNP, and 16 CRSwNP patients. In NABX, unlike Chao1 and the number of observed OTUs, the Shannon and Simpson indices were significantly increased from control to CRSwNP (Fig. 2A and Fig. S4A). A clearly identifiable clustering was observed in NABX (Fig. S4C); however, in ABX, there was no significant difference in the alpha and beta diversities (Fig. 2B and Fig. S4B and D).

Next, we compared the nasal bacterial composition in NABX and ABX. In NABX, *Firmicute*, *Cyanobacteria*, and *Bacteroidetes* significantly differed according to disease status (Fig. S5A and C). At the family level, the relative abundance of *Propionibacteriaceae*, *Moraxellaceae*, and *Prevotellaceae* significantly differed according to disease status in NABX (Fig. 2C and Fig. S6A). LEfSe analysis revealed that *Propionibacteriaceae* was significantly decreased in CRSwNP and CRSsNP compared to levels in the control group (Fig. S6B). In the ABX, there was no significant difference in phyla and families that possessed a relative abundance of greater than 3 percent (Fig. 2D and Fig. S5B). When all identified families were analyzed using LEfSe, we observed that

Peptostreptococcaceae, *Desulfovibrionaceae*, and *Alteromonadaceae* were the most dominant families in the control group (Fig. S6C). *Sulfurovaceae* was found to be significantly enriched in CRSsNP. Taken together, these findings indicate that antibiotic use could reduce differences in microbial communities according to disease status.

To determine the differences in the microbial community resulting from antibiotic use, we compared NABX and ABX in regard to each disease status. In a total of 99 subjects, unlike alpha diversities, PERMANOVA revealed that microbial composition significantly differed between NABX and ABX (Fig. 2E and Fig. S7A and B). Among bacterial families with a relative abundance of more than 3%, *Staphylococcaceae*, *Propionibacteriaceae*, and *Streptococcaceae* exhibited significant differences between the two groups (Fig. S7C). In all families that we identified, we found that *Staphylococcaceae*, *Streptococcaceae*, and *Propionibacteriaceae* were significantly decreased in ABX compared to levels in NABX (Fig. S7D). *Entothionellaceae* and *Sandaracinaceae* were significantly enriched in ABX compared to levels in NABX.

Then, we compared the microbial communities between ABX and NABX in control group. There was no significant difference in alpha and beta diversities between the two groups (Fig. 2E and Fig. S8A and B). Additionally, families with a relative abundance of more than 3% were not significantly different between the two groups (Fig. S8C). LEfSe analysis indicated that antibiotic use led to enrichment of families whose relative abundance was lower than 3% (Fig. S8D). In CRSsNP patients, the alpha diversity indices and microbial composition did not significantly differ between NABX and ABX (Fig. 2E and Fig. S9A and B). Among the families with a relative abundance of more than 3%, *Staphylococcaceae* was significantly decreased in ABX compared to levels in NABX (Fig. S9C). LEfSe analysis that was performed on all identified families revealed that *Staphylococcaceae*, *Intrasporangiaceae*, and *Neisseriaceae* were significantly decreased in ABX compared to levels in NABX (Fig. S9D). Additionally, *Prevotellaceae* and *Legionellaceae* were enriched significantly in ABX compared to levels in NABX. Last, we compared the microbial communities between NABX and ABX in CRSwNP patients. Unlike Chao1 and the number of observed OTUs, Shannon and Simpson indices were significantly lower in ABX compared to those in NABX (Fig. 2E and Fig.

S10A). Additionally, PERMANOVA revealed significant differences in microbial composition between the two groups (Fig. S10B). According to these results, it appeared that antibiotics exerted stronger effects on the microbial community in CRSwNP patients (NP) compared to those in the control and CRSsNP patients (Non-NP). Among the bacterial families with more than 3% relative abundance, *Streptococcaceae* and *Lachnospiraceae* were significantly decreased in the ABX compared to levels in the NABX group (Fig. S10C). In all identified families, LEfSe analysis revealed that *Streptococcaceae*, *Lachnospiraceae*, and *Neisseriaceae* were significantly decreased in ABX compared to levels in NABX (Fig. 2F).

Differences in secreted proteome according to the use of antibiotics

As we determined, antibiotics may exert stronger effects on the microbial community in NP compared to that in Non-NP groups, and based on this, we also compared host responses to antibiotics in the NP and Non-NP groups using proteomic analysis. The NABX group consisted of 22 control, 19 CRSsNP, and 11 CRSwNP patients, while the ABX group consisted of 1 control, 5 CRSsNP, and 11 CRSwNP patients. In the ABX group, relatively small proteomic changes were observed between NP and Non-NP patients compared to those in the NABX group (Fig. 3A). This was consistent with our results indicating that the use of antibiotics reduced differences in microbial communities according to disease status. As shown in Tables S2 and S3, proteins with a fold change ≥ 2.0 at a p value < 0.05 in the NABX group were considerably different compared to those in the ABX group. Furthermore, to determine the differences in the secreted proteome based on antibiotic use, we compared NABX and ABX in 69 patients, Non-NP and NP (Fig. 3B and Table S4-6). Based on previous results obtained using metagenomics, antibiotics may exert stronger effects on the secreted proteome in NP patients compared to that in total and Non-NP patients. To identify the canonical pathways that were most significantly involved with the proteins that exhibited a fold change ≥ 2.0 at a p value < 0.05 in each group, we performed ingenuity pathway analysis (IPA). When examining all subjects, the pathways were completely different from those identified in NP, as the NP pathways were associated with innate immunity and production NO and ROS (Fig. 3C and D). There was no significant pathway found in the Non-NP group ($p > 0.001$). As LXL/RXR activation was the most significant pathway according

to IPA analysis, proteins associated this pathway were labeled in a volcano plot derived from the NP group (Fig. 3B). These analyses indicated that the use of antibiotics may result in stronger effects on the nasal microbiome and the secreted proteome in NP compared to those in Non-NP.

Antibiotic-dependent relationships between nasal microbiome and secreted proteome

We next sought to investigate if the associations between the microbiome and the secreted proteome may differ according to antibiotic use. If associations with a high number of significant correlations were altered by exposure to antibiotics, this would be meaningful. Therefore, we arranged the microbiome and secreted proteome in descending order from the highest to the lowest number of significant correlations with each other (Fig. S11A and B). Among these, we clustered the top 25 percent of the microbiome and secreted proteome in NABX and ABX (Fig. 4A and B). The average *R*-squared value in ABX was higher than that in NABX. From these analyses, we confirmed that the microbiome and secreted proteome in ABX correlated strongly with one another compared to the correlation observed in NABX. Furthermore, among these top 25 percent microbiome and secreted proteome components in NABX and ABX, there was little overlap between NABX and ABX (Fig. 4C and D). Likewise, the use of antibiotics altered the associations between the microbiome and secreted proteome. Moreover, their correlations were strengthened in subjects who had taken antibiotics.

Antibiotic-dependent relationships between the nasal microbiome and the secreted proteome in Non-NP and NP

Finally, given that antibiotics may exert different effects on the nasal microbiome, secreted proteome, and their association according to disease status, we hypothesized that the associations that were altered by antibiotics could differ according to disease status. To confirm this hypothesis, we divided the nasal microbiome into two groups that included families with increased relative abundance in ABX (IFa) and families with decreased relative abundance in ABX (DFa). We also divided the secreted proteome into two groups that included proteins with increased normalized intensity in ABX (IPa) and proteins with decreased normalized intensity in ABX (DPa). We could not detect the IFa group in the NP patients. A number of significant positive or negative correlations were observed among the four groups (IFa, DFa, IPa and DPa) in both the Non-NP and NP patient groups

(Fig. 5A and B). We confirmed that NP patients showed stronger correlations with each other than those in the Non-NP patients. To confirm the associations among the four groups, we performed aSPC tests (Fig. 5C). The associations between DFa and IPa or DFa and DPa in the NP patients were more significant than those observed in the Non-NP patients; however, we found no correlation between each group (IFa, DFa, IPa and DPa) and others in regard to the microbiome and the proteome. From these analyses, in NP it was apparent that the associations between the microbiome and the proteome that were altered by antibiotics were different compared to those in the Non-NP patients. Moreover, the associations among the four groups in the NP patients were more significant than those in the Non-NP patients.

Discussion

To our knowledge, this is the first report to analyze the effects of antibiotics on the microbiome, secreted proteome, and their associations in CRS using multi-omics. The relatively large number of subjects used for this study enabled us to more fully understand the associations between CRS and antibiotics. As expected, antibiotic use could reduce differences in the microbial community and the secreted proteome according to disease status. Interestingly, antibiotics may exert strong effects on not only the nasal microbiome and secreted proteome, but also their associations in NP patients compared to that in Non-NP patients. Additionally, their correlations were strengthened in subjects who had taken antibiotics.

Previous studies examining the nasal microbiome resulted in inconsistent outcomes for alpha diversity indices in CRS patients compared to those in the control group. Some studies reported that there was no significant difference in alpha diversity between the control and the CRS patients (Koeller et al. 2018; Mahdavinia et al. 2018; Paramasivan et al. 2019). Meanwhile, other studies reported that the alpha diversity indices significantly decreased in CRS patients compared to those in the control group (Choi et al. 2014; Biswas et al. 2015; Cope et al. 2017). However, Aurora *et al.* (Aurora et al. 2013) reported that bacterial diversity was increased in CRS compared to that in the control. Additionally, Lal *et al.* (Lal et al. 2017) identified that control and CRSwNP patients exhibited significantly higher faith phylogenic and Shannon diversity compared to those values in CRSsNP patients. Copeland *et al.* (Copeland et al. 2018) reported that Shannon diversity was significantly increased in CRSsNP patients compared to that in control and CRSwNP patients. Moreover, a number of previous studies (Biswas et al. 2017; Hoggard et al. 2017; Chalermwatanachai et al. 2018; Gan et al. 2019; Rom et al. 2019) indicated that alpha diversity was increased, decreased, or unchanged in CRS compared to that in control, and this was dependent upon the alpha diversity index.

To date, the existence of any association between antibiotic use and nasal microbiome in the context of CRS remains unknown. Palleja *et al.* (Palleja et al. 2018) demonstrated that microbial richness within the gut was significantly decreased at approximately 6 months following antibiotic treatment. As the nasal microbiome studies in CRS generally excluded patients who had taken antibiotics within

approximately 1 month prior to sampling, the differences in alpha diversity according disease status could not be clearly identified. In our study that included subjects who had taken antibiotics within three months, Shannon and Simpson indices were significantly increased from control to CRSwNP patients, and we are confident that these results reflect real-world criteria. Based on this, antibiotic use should be taken into account when comparing diversity according to disease status.

We also found that antibiotics could exert stronger effects in NP compared to that in Non-NP patients. Additionally, antibiotics could alter the nasal secreted proteome and the associations between the microbiome and the secreted proteome. However, the mechanisms by which antibiotics could affect the microbiome according to disease status and could alter the secreted proteome remain unknown. Shaw *et al.*(Shaw et al. 2019) reported a simple quantitative model based on a stability landscape framework. They demonstrated that the microbiome existed in multiple stable equilibria of landscape that could be influenced by sufficiently strong perturbations such as antibiotics that could alter the microbiome from its normal equilibrium to other states. We speculated that perturbation by antibiotics could shift the microbiome towards equilibrium possessing a different value of alpha diversity (lower diversity) in only NP patients. On the other hands, treatment with antibiotics disrupted the intestinal tight junction(Tulstrup et al. 2015; Feng et al. 2019). Kohanski *et al.*(Kohanski et al. 2017) reported that levofloxacin, a commonly used antibiotic for upper airway infections, simulated reactive oxygen species (ROS) and caspase-3 activity in cultured human sinonasal epithelial cells. Thus, we speculated that antibiotic use could affect the nasal epithelial cells and alter the proteome secreted by these cells.

The IPA analysis revealed that B cell-associated pathways and anti-microbial pathways were more highly activated in ABX patients compared to levels in NABX patients in the total patient population and the NP group, respectively. PI3K regulated B cell receptor-mediated antigen presentation and airway remodeling(Yoo et al. 2017). Activated B cells utilized mTORC1 to activate p70 ribosomal S6 kinase (p70S6K), and this preceded antibody synthesis(Gaudette et al. 2020). In contrast, macrophages that released proinflammatory cytokines played an important role in clearing bacteria, and this was accompanied by the production of nitric oxide (NO) and ROS upon contact with

pathogens(Tan et al. 2016; Finney et al. 2019). Several studies have reported that LXR activation exerted anti-inflammatory functions(Higham et al. 2013) and inhibited bacterial infection of host macrophages(Pascual-Garcia et al. 2013; Matalonga et al. 2017).

There were limitations in this present research study. First, the proportion of NABX and ABX in the control group was smaller than that in the CRSsNP and CRSwNP groups. Second, larger number of subjects should be analyzed to improve reliability. Thus, a similar proportion of subjects who had taken antibiotics and a larger sample size for each disease status will be required to verify the association between CRS and antibiotics.

In summary, we determined that antibiotics could exert strong effects on the microbial diversity and the secreted proteome in NP patients compared to those in Non-NP patients. Integrative analyses revealed that the associations and correlations between the microbiome and the secreted proteome could be altered and strengthened in subjects who had recently used antibiotics. These findings provide new insight into the effects of antibiotics on the nasal environment and the host response in CRS.

337 **Methods**

338 *Study design and collection of nasal secretions*

339 The Internal Review Board of Seoul National University Hospital (SNUH) (no. C-1308-099-515)
 340 approved this study. All subjects were informed of the purpose of the study, and they all signed
 341 written informed consent forms. The diagnosis of CRS was based on patient history, physical
 342 examination, nasal endoscopy, and sinus computed tomography in accordance with the 2012
 343 European position paper on rhinosinusitis and nasal polyps (EPOS) guidelines(Fokkens et al. 2012).
 344 Patients possessing deviated nasal septa but lacking any sinonasal disease were considered as the
 345 control. Subjects that were less than 14 years of age and who were diagnosed with unilateral
 346 rhinosinusitis, antrochoanal polyps, allergic fungal sinusitis, cystic fibrosis, or immotile ciliary disease
 347 were excluded from the study. The demographic characteristics of the subjects are summarized in
 348 Table 1. Nasal secretions were obtained from both sides of the nose as previously described(Kim et al.
 349 2019). Sterilized strips of filter paper (7 × 30 mm; Whatman No. 42, Whatman, Clifton, NJ, USA)
 350 were placed on the middle meatus for 10 minutes. Two filter papers from each subject were
 351 transferred into a tube. Then, 1 mL of nuclease-free water was added to each tube, and the tubes
 352 were rotated for 1 h at room temperature. The nasal secretions were stored in aliquots at -70 °C.
 353 The workflow is shown in Figure S1.

354

355 *DNA extraction and sequencing*

356 After preparation of nasal secretions, 16S rDNA was extracted using the PowerSoil DNA Isolation
 357 Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The manufacturer's instructions for the kit were
 358 followed for all subsequent procedures. The isolated DNA was sent to Macrogen Corporation
 359 (Macrogen Inc., Seoul, South Korea) for amplification and sequenced on a Miseq instrument
 360 (Illumina, USA). A sequencing library was prepared by amplifying the V3-4 region of 16S rDNA. For
 361 the amplification, primers 341F (5' -TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG
 362 CCT ACG GGN GGC WGC A-3') and 805R (5' -GTC TCG TGG GCT CGG AGA TGT GTA TAA
 363 GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3') were used. The library was quantified using

TapeStation DNA ScreenTape D1000 (Agilent, USA) and Picogreen assay. The 16S rDNA libraries were sequenced using the MiSeq platform for 2 × 300 cycles.

Preparation of nasal secretions for proteomics

A 100 µL aliquot of each nasal secretion was prepared as previously described(Kim et al. 2019). The samples were centrifuged at 15,000 rpm for 10 minutes at 4 °C. We measured the protein concentration in the supernatant by tryptophan fluorescence emission at 350 nm using an excitation wavelength of 295 nm(Wisniewski and Gaugaz 2015). For the analysis, 50 µg of nasal secreted proteins were used per sample. Proteins were digested using the 2-step FASP procedure with some modifications(Han et al. 2014; Woo et al. 2015). Pellets were resuspended in SDT buffer (2% SDS, 10mM TCEP, and 50mM CAA in 0.1M Tris pH 8.0). Then, the solution loaded onto a 10K Amicon filter (Milipore). The buffer was exchanged to UA solution (8M urea in 0.1M Tris pH 8.5) using centrifugation at 14,000 g. Following the exchange of 40mM ammonium bicarbonate (ABC) buffer, proteins were digested at 37 °C overnight using a trypsin/LysC mixture (protein-to-protease ratio of 100:1). The peptides were isolated using centrifugation. After the filter units were rinsed with 40 mM ABC, we performed second digestion at 37 °C for 2 hours using trypsin (enzyme-to-substrate ratio [w/w] of 1:1000). The digested peptides were acidified using 10% TFA and desalted using homemade C18-StageTips as described(Han et al. 2014; Woo et al. 2015). Finally, we used a vacuum dryer to dry it and stored at -80 °C.

High-pH StageTip-based peptide fractionation

For peptide spectrum library, we performed StageTip-based, high-pH peptide fractionation as described with some modifications(Han et al. 2014). Peptides obtained from pooled samples were dissolved in 200 µL of loading solution (10 mM ammonium hydroxide solution, pH 10 and 2% acetonitrile) and separated on the reversed-phase tip columns, prepared by packing POROS 20 R2

(Invitrogen, Carlsbad, CA) into a 200 μ L yellow tip with C18 Empore disk membranes (3M, Bracknell, UK) at the bottom. After conditioning of microcolumns with methanol, acetonitrile, and loading buffer, peptides were loaded at pH 10, and 20 fractions were subsequently eluted with buffer solutions, pH 10, containing 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 60%, and 80% acetonitrile. To improve the orthogonal fractionation of the RP-RP separation, 20 fractions were combined into six fractions in a noncontiguous manner. The six fractions were dried in a vacuum centrifuge and stored at -80 °C until LC-MS/MS analysis.

LC-MS/MS analysis

Liquid chromatography (LC)-mass spectrometry (MS)/MS analysis was performed using a Q-exactive plus (Thermo Fisher Scientific, Waltham, MA) coupled to an Ultimate 3000 RSLC system (Dionex) via a nano electrospray source as described with some modifications (Lee et al. 2018; Kim et al. 2019). Peptides in samples were separated using the 2-column setup (a trap column [300 μ m I.D. x 5 mm, C18 3 μ m, 100 Å] and an analytical column [50 μ m I.D. x 50 cm, C18 1.9 μ m, 100 Å]). Prior to injection of each sample, we resuspended the dried peptides in solvent A (2% acetonitrile and 0.1% formic acid). After the samples were loaded onto the nano LC, the samples were run with a 90 minute gradient from 8% to 30% solvent B (80% acetonitrile and 0.1% formic acid). The spray voltage was set to 2.0 kV in the positive ion mode, and the temperature of the heated capillary was 320 °C. The MS method consisted of a survey scan at 35,000 resolution from 400 to 1,220 m/z. Automatic gain control (AGC) target of 3×10^6 at injection time of 60ms. Then, 19 DIA windows were acquired at 35,000 resolution with AGC target 3×10^6 and auto for injection time (Bruderer et al. 2015). Stepped collision energy (SCE) was 10% at 27%.

Data processing for spectral library construction

First, we processed MS raw files that obtained from 18 data-dependent acquisition (DDA)

measurements of the pooled samples using MaxQuant (version 1.6.1.0)(Tyanova et al. 2016a). MS/MS spectra were searched against the Human Uniprot database (December 2014, 88,657 entries) and the Biognosys iRT peptides fasta database using the Andromeda(Cox et al. 2011). Data was searched with 6 ppm precursor ion tolerance for total protein level analysis and 20 ppm MS/MS ion tolerance. We used variable modifications (N-acetylation of protein and oxidation of methionine) and a fixed modification (cysteine carbamido-methylation). Enzyme specificity was set as full tryptic digestion. Peptides with a minimum length of six amino-acids and up to two missed-cleavages were considered. The required false discovery rate (FDR) was set to 1% at the peptide, protein, and modification level. To maximize the number of quantification events across samples, we enabled the ‘Match between Runs’ option on the MaxQuant platform. MaxQuant search results were imported as spectral libraries into Spectronaut Pulsar X with default settings.

In addition to the typical DDA-based spectral library, we generated a spectral library from the DIA data using the Spectronaut software (Biognosys, Schlieren, Switzerland). The Pulsar search engine integrated in Spectronaut Pulsar X was used to identify peptides and proteins using only independent DIA dataset with the same search engine parameters (fasta database, modifications, peptide length, miss-cleavage, peptide, and protein FDR) as listed above for MaxQuant.

Data processing for the DIA MS

The DIA data of individual samples were analyzed with Spectronaut Pulsar X (Biognosys, Schlieren, Switzerland). First, we converted the DIA raw files into an htrm format by using the GTRMS Converter provided by the Spectranaut. The FDR was estimated with the mProphet(Reiter et al. 2011) approach and set to 1% at peptide precursor level and at 1% at protein level. The proteins were inferred by the software, and the quantification information was acquired at the protein level by using the q-value < 0.01 criteria, which was used for subsequent analyses.

439 *Bioinformatic analysis*

440 Raw sequences were analyzed and quality-filtered using Quantitative Insights Into Microbial
441 Ecology (QIIME) (version 1.9.1)(Caporaso et al. 2010). An operational taxonomic unit (OTU) was
442 defined at 97% sequence identity using UCLUST against the SILVA reference sequence database
443 (version 132)(Edgar 2010; Quast et al. 2013). Singletons were removed from the OTU table to reduce
444 the noise and finally, 12,634,938 sequences remained. Alpha diversity (Chao1, the number of
445 observed OTUs, Shannon, and Simpson) was calculated in QIIME. Beta diversity was calculated
446 using a Bray–Curtis distance matrix with the vegan package in R software. In addition, principal
447 coordinates analysis (PCoA) was performed using the Phyloseq package in R software(McMurdie and
448 Holmes 2013). Linear discriminant analysis (LDA) effect size (LEfSe) was performed to determine
449 bacterial features most likely to describe differences between groups among all of the identified
450 bacterial families(Segata et al. 2011). The relative abundance of each OTU was calculated by dividing
451 the read counts of an OTU by the total read counts of all OTUs in each subject, except for read counts
452 of *Archaea*, *Chloroplast* and *Mitochondria*. Proteomic data were normalized using width adjustment.
453 Ingenuity Pathway Analysis (IPA; QUAGEN, Hilden, Germany) software was used to predict
454 canonical pathways. Missing values in proteomic analysis were imputed by normal distribution (width
455 = 0.15, downshift = 1.8) using Perseus(Tyanova et al. 2016b). Graphical outputs were visualized using
456 R software (version 3.5.1), Perseus (version 1.6.0), and GraphPad Prism (version 8.1.1).

457

458 *Statistical analysis*

459 Student's t-test or ANOVA was performed to compare the data under normal distribution, while
460 Mann-Whitney *U* or Kruskal-Wallis tests were used for non-parametric analysis. A Chi-square test
461 was performed to examine the associations between categorical variables. The significant differences
462 between groups were calculated using permutational multivariate analysis of variance
463 (PERMANOVA) via Adonis function in vegan package in R software. LEfSe analysis used the
464 Wilcoxon rank-sum test or Kruskal-Wallis rank-sum test. In IPA analysis, *p*-value of Fisher's exact
465 test < 0.001 was considered significant pathways. Spearman correlation was used to assess the

466 correlation and adaptive sum of powered correlation test (aSPC)(Xu et al. 2017) was performed to
467 calculate global association between microbiome and proteome. The statistical analyses were
468 conducted using SPSS ver. 25.0 (SPSS Inc., Chicago, IL, USA), Perseus (version 1.6.0), and R
469 software (version 3.5.1).
470

Author's contributions

Conception and design: Yi-Sook Kim, Hyun-Woo Shin; Acquisition of data: Yi-Sook Kim, Dohyun Han, Ji-Hun Mo, Yong-Min Kim, Dae Woo Kim, Hyo-Guen Choi; Analysis and interpretation of data: Yi-Sook Kim, Dohyun Han, Hyun-Woo Shin; Drafting the article or revising it critically for important intellectual content: Yi-Sook Kim, Jong-Wan Park, Hyun-Woo Shin; Supervision: Hyun-Woo Shin

Competing interests

The authors declared no conflict of interest exists.

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Availability of data and materials

The raw sequence data for the metagenomics are available in the NCBI Sequence Read Archive at study accession number PRJNA557492.

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Figures

Figure 1. Nasal microbiome and secreted proteome profiles

(A) Alpha diversity according to disease status (horizontal line = median and whiskers = min/max range). (B) A PCoA plot based on Bray–Curtis distance matrix. (C) Distribution of bacterial families. The composition of each family exhibiting a relative abundance of greater than 3 percent is illustrated. The parenthesis represents phylum within the family. (D) Volcano plots of the proteome. The horizontal dashed line indicates p values of 0.05 (Student's t-test), and the vertical dashed lines indicate fold changes of 2.0. (E) Spearman correlation heatmap of secreted proteomes (ANOVA, $p < 0.05$) and the nasal microbiome (Kruskal-Wallis, $p < 0.05$). The proteome and microbiome were arranged from top to bottom and from left to right in order of the lowest to the highest p value, respectively. Orange and green boxes indicated increased and decreased families and proteins from control to CRSwNP, respectively. Redundant microbiomes and proteomes were excluded from the heatmap. (F) Association between families and proteins. Orange and green circles represent the same groups previously described in (E). Gray circles represent other microbiomes and proteomes that were identified in this study. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Figure 2. Differential microbial composition according to the use of antibiotics

(A and B) Comparison of Shannon and Simpson indices according to disease status in NABX and ABX. (C and D) Distribution of bacterial families according to disease status in NABX and ABX. The composition of each family exhibiting a relative abundance of greater than 3 percent is illustrated. The parenthesis indicates phylum within the family. (E) Comparison of Shannon and Simpson indices between NABX and ABX using a total of 99 subjects, control, CRSsNP, and CRSwNP patients, respectively (horizontal line = median and whiskers = min/max range). (F) LEfSe analysis identified NABX-enriched families (colored in blue) and ABX-enriched families (colored in red) in CRSwNP patients. The plot indicates taxa with an LDA score > 3.0 and a $p < 0.05$ in all-against-all comparisons (more stringent). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Figure 3. Differences in the secreted proteome according to the use of antibiotics

(A and B) Volcano plots of the secreted proteome where $\log_2$ of fold changes was plotted versus the $-\log_{10}$ of the p -values from the Student's t -test. The horizontal dashed line indicates p -values of 0.05, and the vertical dashed lines indicate fold changes of 2.0. Red squares represent proteins with a fold change ≥ 2.0 at p value < 0.05 . In NP, the labeled squares indicate proteins involved in LXR/RXR activation. (C and D) Canonical pathway analysis representing significantly up- or down-regulated pathways in ABX compared to those in NABX. The pathways with p -values smaller than 0.001 were arranged from top to bottom in order of the lowest to the highest p -value. The parenthesis represented the $-\log_{10} p$ -value.

Figure 4. Antibiotic-dependent relationships between the nasal microbiome and the secreted proteome

(A and B) Hierarchical clustering of the top 25 percent of the microbiome and proteome with a high number of significant correlations in NABX and ABX. The means of squared coefficients were calculated using all values presented in (A and B). Among the microbiomes and proteomes identified in (A and B), a Venn diagram reveals the number of families (C) and proteins (D) in NABX and ABX. The top 5 proteins and families (non-overlapping and non-redundant) are represented.

Figure 5. Antibiotic-dependent relationships between the nasal microbiome and the secreted proteome in control, CRSsNP, and CRSwNP patients

(A and B) Spearman correlation heatmaps of the secreted proteomes (rows) and the nasal microbiomes (columns) that exhibit significant differences between NABX and ABX in Non-NP and NP groups. The proteome (Student's t -test, $p < 0.05$) and microbiome (Mann-Whitney U test, $p < 0.05$) were arranged from top to bottom and from left to right in order of the lowest to the highest p -value. Orange and green boxes indicate families and proteins enriched in ABX and NABX. Redundant microbiomes and proteomes were excluded from the heatmap. (C) Associations between the microbiome and the proteome. Orange and green circles represent the same groups as described in (A

703 and B), respectively. Gray circles indicate other microbiomes and proteomes that were identified in
704 this study (adaptive sum of powered correlation [aSPC] test, $p < 0.05$).

705

706

Fig.1

Fig.2

Fig.3**A** **B** **NP** **C** **D**

Fig.4

A

Non-NP

B

NP

Fig.5

C

1 Tables

2 Table 1. Demographic characteristics of subjects

Characteristic	Control (n=29)	CRSsNP (n=30)	CRSwNP (n=40)	P-value ^a
Male, n (%)	24 (82.8)	16 (53.3)	27 (67.5)	0.054
Age (y), mean $\pm$ SD	32.4 $\pm$ 14.8	41.6 $\pm$ 15.1 [*]	48.8 $\pm$ 14.4 ^{***}	< 0.001
BMI (kg/m ²), mean $\pm$ SD	23.3 $\pm$ 3.0	23.2 $\pm$ 3.4	25.1 $\pm$ 4.7	0.171
Smoking, n (%)	7 (24.1)	4 (13.3)	15 (37.5)	0.072
Atopy, n (%)	11 (37.9)	7 (24.1)	13 (33.3)	0.515
Polyps, n (%)	0 (0)	0 (0)	40 (100.0)	< 0.001
Lund-Mackay CT score, mean $\pm$ SD	0	14.2 $\pm$ 6.4 ^{***}	17.3 $\pm$ 4.4 ^{***}	< 0.001
Antibiotics ^b , n (%)	2 (6.9)	8 (26.7)	16 (40.0)	0.009
Dental problem, n (%)	1 (3.4)	2 (6.7)	3 (7.5)	0.774
Asthma, n (%)	0 (0)	2 (6.7)	2 (5.0)	0.397
Allergic rhinitis, n (%)	13 (44.8)	12 (40.0)	8 (20.0)	0.063
Methodology				
Metagenomic analysis, n	29	30	40	
Proteomic analysis, n	23	24	22	

^aP-values based on Chi-Square or Kruskal-Wallis test (categorical or continuous variables, respectively)

^bUse of antibiotics in 3 months preceding sampling

^{*}Difference between 2 groups tested using Mann-Whitney U test compared to control group

[†]Difference between 2 groups tested using Mann-Whitney U test compared to CRSsNP group

^{*,†} $p < 0.05$, ^{**,††} $p < 0.01$, ^{***,†††} $p < 0.001$

BMI, body mass index.