Richness of sputum microbiome in acute exacerbations of eosinophilic chronic obstructive pulmonary disease

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Abstract

Background: The eosinophilic chronic obstructive pulmonary disease (COPD) is known to be more sensitive to corticosteroid. The sputum microbiome has been shown to affect COPD prognosis, but its role in acute exacerbations of eosinophilic COPD is unclear. This study aimed to investigate the dynamic changes of the airway microbiome in patients with acute exacerbations of eosinophilic COPD.

Methods: Fifty-seven patients with acute exacerbations of COPD from the First Affiliated Hospital of Guangxi Medical University between June 2017 and June 2018 were divided into two groups. Patients with eosinophils ≥300 cells/μL in the peripheral venous blood were assigned to the eosinophilic group (Eos) and the rest to the non-eosinophilic group (Noneos). All patients received similar treatment including inhaled budesonide according to the guidelines. The induced sputum microbiome was analyzed on the 1st and 7th day of treatment using the 16S ribosomal RNA (rRNA) method. The levels of interleukin (IL)-6 and IL-8 were measured in the plasma and the sensitivity to corticosteroids was determined in isolated peripheral blood mononuclear cells. Quantitative data were compared between the two groups using the independent samples *t* test or Mann-Whitney *U* test. Categorical data were evaluated using Chi-squared test or Fisher's exact test.

Results: Twenty-six patients were classified into Eos group and 31 patients were classified into Noneos group. Prior to treatment, the alpha diversity (Shannon index) (2.65 ± 0.63 *vs.* 2.56 ± 0.54, $t = 0.328$, $P = 0.747$) and the structure of the sputum microbiome were similar in the Eos group and the Noneos group. After 7 days of treatment, alpha diversity increased in both groups, while the microbiome richness (Ace index) was significantly lower in the Eos group (561.87 ± 109.13 *vs.* 767.88 ± 148.48, $t = -3.535$, $P = 0.002$). At the same time, IL-6 (12.09 ± 2.85 pg/mL *vs.* 15.54 ± 2.45 pg/mL, $t = -4.913$, $P < 0.001$) and IL-8 (63.64 ± 21.69 pg/mL *vs.* 78.97 ± 17.13 pg/mL, $t = -2.981$, $P = 0.004$) decreased more significantly in the Eos group, and the percentages of inhibition of IL-8 at dexamethasone concentrations 10^{-8} to 10^{-6} mol/L were significantly higher in the Eos group than those in the Noneos group (all $P < 0.05$).

Conclusions: The induced sputum microbiome richness decreased more significantly following treatment in the Eos patients compared to the Noneos patients. The lower plasma inflammatory factor levels and the higher percentage of inhibition of IL-8 might be due to higher corticosteroid sensitivity in Eos patients.

Keywords: Acute exacerbations of chronic obstructive pulmonary disease; Sputum; Microbiome; Eosinophilic; Corticosteroid; Interleukin-8 inhibition

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent respiratory symptoms and irreversible airflow limitation.[1] Despite similar clinical presentations, COPD is a highly heterogeneous disease. Recently, more evidence suggests that eosinophils play an important role in some patients with COPD and eosinophilic COPD is recognized as a distinct phenotype of the disease.[2] High numbers of eosinophils reportedly appear in up to 28% of cases of acute exacerbations of COPD (AECOPD).[3] Compared with COPD cases with low numbers of eosinophils, the eosinophilic phenotype of COPD has several unique features.[2] Patients with eosinophilic inflammation have fewer allergies although exacerbations are more frequent. Most importantly, eosinophilic COPD responds best to corticosteroid treatments.[4-6] While inhaled corticosteroids (ICSs) can increase the risk of pneumonia in non-eosinophilic COPD,[7] patients with eosinophilic COPD show better improvement according to the forced expiratory volume in 1 s (FEV_1)[8] and a shorter hospitalization time after using corticosteroids.[9] The long-term use of ICSs has

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also been shown to significantly decrease exacerbation frequency in eosinophilic stable COPD.[10]

The exact threshold value used to define eosinophilia varies and is dependent on the outcomes studied and the stage of disease. Blood eosinophil concentrations are generally thought to be reasonably good predictors of eosinophil concentrations in the airways. Patients with high blood eosinophilia (defined as ≥300 cells/μL) have also been found to have significantly greater mean concentrations of eosinophil counts in induced sputa compared to patients with mean blood eosinophil counts <300 cells/μL.[11] According to the most recent Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2019 guidelines, combination treatment with ICSs and long-acting beta-agonists (LABA) should be considered when blood eosinophils reach ≥300 cells/μL.[12]

The lung is considered a relatively sterile environment. However, with the development of culture-independent techniques for microbe identification and quantification such as 16S ribosomal RNA (rRNA) high-throughput sequencing of amplicons, broad-range polymerase chain reaction (PCR), and 454 pyrosequencing technology, increasing amount of evidence shows that the lungs contain many different microbes. Imbalance in the lung bacterial community — "bacterial dysbiosis" — has been associated with an increased risk of exacerbations and an accelerated loss of lung function in COPD.[13] Age, smoke, course of disease, drugs, and microanatomy all have been associated with changes in the relative abundance and diversity of the bacterial communities in the lungs.[14-18] Recent studies have demonstrated that the lung microbiota also differ between eosinophilic and non-eosinophilic patients with AECOPD.[19,20] In addition, treatment with ICSs correlated with lung microbiota composition and enrichment in stable COPD.[20,21]

Patients with AECOPD in most previous studies were treated with either ICSs or systematic corticosteroid plus antibiotics, so it is unclear if the observed differences reflect the effects of corticosteroids or of the eosinophils. The present study (using the 16S rRNA method) analyzed the microbiome in induced sputum samples of patients with eosinophilic and non-eosinophilic AECOPD. The levels of inflammatory mediators (interleukin [IL]-8 and IL-6) in the plasma before and after treatment were also analyzed to determine the effects of treatment. In addition, the corticosteroid sensitivity of patients with AECOPD was quantified *in vitro* using isolated peripheral blood mononuclear cells (PBMCs) to study the degree of IL-8 inhibition produced by increasing concentrations of dexamethasone (Dex). We hypothesized that differences in the induced sputum microbiota are associated with the amount of blood eosinophils and responsiveness to ICSs treatment.

Methods

Ethical approval

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University in 2017 (No. 2017KY-E-024). Written informed consent was obtained from all participants prior to enrollment, and all participants could withdraw from the study at any time.

Study design

Fifty-seven patients admitted to the First Affiliated Hospital of Guangxi Medical University for AECOPD from June 2017 to June 2018 were enrolled in the present study. The inclusion criteria were as follows: (1) symptoms of recurrent cough, expectoration, and a history of smoking or smoke exposure; (2) FEV_1/forced vital capacity (FVC) <70% after bronchodilation; and (3) currently experiencing an acute exacerbation that requires additional treatment. The exclusion criteria were as follows: (1) history of asthma, interstitial pulmonary disease, pulmonary embolism, cancer, or tuberculosis; (2) serious respiratory failure and acidosis; (3) serious heart, liver, kidney, or gastrointestinal comorbidities; and (4) unable to perform lung function test or receive venipuncture.

Enrolled patients were divided into two groups according to the number of eosinophils in their peripheral blood upon admission. If the peripheral blood eosinophil numbers were ≥300 cells/μL, then they were assigned to the eosinophilic group (Eos). If the peripheral blood eosinophil numbers were <300 cells/μL, then they were assigned to the non-eosinophilic group (Noneos). Ten of 26 patients with AECOPD enrolled into the Eos group obtained qualified induced sputum; and for the subjects of the Noneos group, ten of 31 patients received qualified induced sputum.

All enrolled patients received ICS treatment with inhaled budesonide 2 mg three times a day. Other therapies included long-acting muscarinic antagonist (LAMA), LABA, broad-spectrum antibiotics, and low flow oxygen according to local treatment guidelines for AECOPD.[22] Induced sputum and peripheral venous blood were collected on the 1st day of treatment before using antibiotics and inhaled budesonide, and again on the morning of the 7th day of treatment. Induced sputum microbiome, inflammatory mediators in the plasma, lung function, blood gas, and COPD assessment test (CAT) scores were also evaluated.

Inflammatory cytokines and C-reactive protein assay

Five milliliters of peripheral venous blood were collected on both the 1st and 7th day of treatment. Plasma was separated after centrifugation at 3000 rpm for 10 min and stored at −80°C for subsequent analysis. The levels of IL-8 and IL-6 were determined by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Cusabio, Wuhan, Hubei, China). The levels of C-reactive protein (CRP) were detected by an automatic biochemical analyzer (Siemens Healthcare Diagnostics Inc., Tokyo, Japan).

Determination of corticosteroid sensitivity in PBMCs

An additional 10 mL of peripheral venous blood collected on both the 1st and 7th day of treatment from each patient was used to isolate PBMCs by Ficoll-Hypaque density gradient centrifugation as previously described.[23] Isolated PBMCs were cultured in RPMI-1640 medium (Thermo

Fisher, Waltham, MA, USA), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher), at 37°C with 5% CO_2. They were treated with increasing concentrations of Dex (Chenxin Pharmaceutical, Jining, Shandong, China) (10^{-12}–10^{-6} mol/L) for 2 h and then stimulated overnight with 0.5 μL (1 mg/L) tumor necrosis factor-α (TNF-α) (Solarbio Pharmaceutical, Beijing, China). The supernatant was collected and stored at −80°C for subsequent analysis. The level of IL-8 in the supernatant was quantified with an ELISA (Cusabio) according to the manufacturer's protocol. The percentage of inhibition of IL-8 by Dex (The percentage of inhibition of IL-8 [%]= [(IL-8 at Dex concentration 0 mol/L–IL-8 at each Dex concentration)/IL-8 at Dex concentration 0 mol/L]×100%) and subsequently the 50% inhibitory concentration of Dex (IC50-Dex) were calculated using Microsoft Excel (version 2013; Microsoft, Washington, DC, USA).

Collection and processing of the induced sputum samples

Induced sputum was collected following inhalation of a nebulized solution of 3% saline over a 15-min period, as previously described.[24,25] The subjects were asked to spit a saliva sample into a separate cup. The mouth was rinsed with saline water before sputum induction to minimize oral contamination. A small amount of sputum was mixed with a four-fold volume of 0.1% dithiothreitol (DTT) solution (Solebao, Beijing, China). And then they were warmed at 37°C for 1 h. An equal volume of phosphate buffer saline (PBS) buffer (Solebao) with DTT was then added into the sputum. The mixture was filtered by a nylon gauze (74 μm). The filtered samples were centrifuged at 1500 rpm for 10 min. Then the cells were resuspended in PBS buffer and were made into the sputum smear. They were stained by the Wright's Giemsa method (Reagan, Beijing, China). Finally, total and differential counts of inflammatory cells were performed under an optical microscope, and the percentages of neutrophils, eosinophils, macrophages, and lymphocytes were calculated. The quality of induced sputum was assessed by sputum smear. Samples were considered unqualified if the squamous epithelial cells were >20%. The remaining sputum samples were stored at −80°C within 1 h of collection until further processing.

DNA extraction, amplification, and sequencing

Total bacterial DNA was extracted from the induced sputum samples using the Power Soil DNA isolation kit (MO BIO Laboratories, San Diego, CA, USA) according to the manufacturer's protocol. DNA quality and quantity were assessed by the 260 nm/280 nm and 260 nm/230 nm ratios, respectively. The extracted DNA was stored at −80°C until further processing.

The V3-V4 region of the bacterial 16S rRNA gene was amplified with a common primer pair (forward primer, 5′-ACTCCTACGGGAGGCAGCA-3′; reverse primer, 5′-GGACTACHVGGGTWTCTAAT-3′) by combining adapter and barcode sequences. PCR amplification was performed in a total reaction volume of 50 μL, containing 10 μL buffer, 0.2 μL Q5 high-fidelity DNA polymerase, 10 μL high GC enhancer, 1 μL deoxy-ribonucleoside triphosphate (dNTP; Biolabs LTD, Beijing, China), 10 μmol/L of each primer, and 60 ng genomic DNA. The thermal cycling conditions were: an initial denaturation at 95°C for 5 min, followed by 15 cycles at 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min.

The products from the first step of the PCR were purified through VAHTS™ DNA Clean Beads (Vazyme, Nanning, Guangxi, China). A second round of PCR was then performed in a 40 μL reaction that contained 20 μL 2 × Phusion High-Fidelity Master Mix (Biolabs LTD), 8 μL double-distilled water (ddH_2O; TianGen, Beijing, China), 10 μmol/L of each primer, and 10 μL PCR products from the first step. The thermal cycling conditions were: an initial denaturation at 98°C for 30 s, followed by ten cycles at 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min.

Finally, all PCR products were quantified by Quant-iT™ double-stranded DNA (dsDNA) HS Reagent (Vazyme) and pooled together. High-throughput sequencing analysis of the bacterial rRNA genes was performed on the purified, pooled sample using an Illumina HiSeq 2500 platform (2 × 250 paired ends) at Biomarker Technologies Corporation, Beijing, China.

Microbiome profiling

Raw reads were joined by FLASH (version 1.2.11, Oxford, UK).[26] Paired-end reads were trimmed to the V3-V4 region by using Trimmomatic (version 0.33, Oxford, UK).[27] Then, the chimera was removed by UCHIME (version 8.1, Oxford, UK) to obtain a quality tag sequence.[28] The final quality reads were assembled into operational taxonomic units (OTUs) at the 97% similarity level by USEARCH (version 10.0, Oxford, UK).[29] Bacterial OTUs were taxonomically identified by using the SILVA taxonomy outlines (Release 128, http://www.arb-silva.de).[30] Diversity indices were analyzed by Mothur (http://www.mothur.org/).[31] The Ribosomal Database Project (RDP) version 2.2 classifier (http://gordonlab.wustl.edu/SuppData.html) was used to assign taxonomy and OTUs (http://sourceforge.net/projects/rdpclassifier/).

Statistical analysis

Statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Continuous data with normal distribution were expressed as the mean ± standard deviation (SD) and those with non-normal distribution as median (interquartile ranges). Comparisons between the Eos and Noneos groups were performed by using the independent samples *t* test or Mann-Whitney *U* test as appropriate. Categorical data were expressed as counts (percentages) and analyzed by Chi-squared test or Fisher's exact test. *P* values less than 0.05 were considered statistically significant.

Results

Clinical characteristics

The baseline clinical characteristics were similar between the Eos and Noneos groups. Indeed, there were no

statistically significant differences in age, sex, smoking history, body mass index (BMI), Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage, or medications between the two groups. The induced sputum samples were qualified in ten patients in each group. The mean eosinophil counts in both the blood ($t = 13.983$, $P < 0.001$) and sputum ($Z = -5.626$, $P < 0.001$) were significantly higher in the Eos group than the Noneos group. The data are presented in Table 1.

Alpha diversity of the microbiome

A total of 7,474,913 raw reads were obtained from all samples. After removing the low-quality reads, 6,448,748 sequence clean reads were obtained, with an average of 161,219 total reads per sample. The clean reads were used for subsequent analyses, resulting in 1515 OTUs. The rarefaction curves of samples [Figure 1A] and groups [Figure 1B] were used to reflect whether the high-throughput sequencing depth of the induced sputum DNA samples met the requirements of this study. The curve tends to be flat on the X-axis, suggesting that the amount of sequencing is sufficient.

The Shannon indexes were similar between the Eos and Noneos groups prior to treatment (2.65 ± 0.63 *vs.* 2.56 ± 0.54, $t = 0.328$, $P = 0.747$). On the 7th day of treatment, the Shannon indexes were significantly higher in both groups compared to the 1st day (Eos group: 3.39 ± 0.45 *vs.* 2.65 ± 0.62, $t = 3.074$, $P = 0.007$; Noneos group: 3.34 ± 0.59 *vs.* 2.56 ± 0.54, $t = 3.078$, $P = 0.006$) [Figure 2A]. The Ace index decreased significantly in the Eos group on the 7th day of treatment compared to the 1st day (561.87 ± 109.13 *vs.* 693.60 ± 133.79, $t = -2.413$, $P = 0.027$) [Figure 2B], while there were no significant differences in the Ace index of the Noneos group before and after treatment (767.88 ± 148.48 *vs.* 615.08 ± 204.97, $t = 1.909$, $P = 0.072$). The Ace index was also significantly lower in the Eos group than in the Noneos group on the 7th day of treatment ($t = -3.535$, $P = 0.002$) [Figure 2B].

Phyla distribution

All samples contained five major bacterial phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria*) which accounted for over 95% of the total sequences in both the Eos and Noneos groups [Figure 3A]. The composition of the phyla was similar between the Eos and Noneos groups prior to treatment [Figure 3B]. In the Eos group, the relative abundances of *Bacteroidetes* ($t = 2.565$, $P = 0.019$) and *Fusobacteria* ($t = 2.430$, $P = 0.026$) were both significantly increased after treatment; while the relative abundances of *Acidobacteria* ($Z = -2.560$, $P = 0.007$) and *Chloroflexi* ($Z = -2.742$, $P = 0.005$) both significantly decreased. In the Noneos group, the relative abundance of *Fusobacteria* increased significantly after 7 days of treatment ($t = 2.961$, $P = 0.008$). The relative abundances of *Acidobacteria* ($Z = -2.571$, $P = 0.009$) and *Chloroflexi* ($Z = -2.883$, $P = 0.003$) were significantly higher in the Noneos group

Table 1: Baseline characteristics of the patients in the Eos and Noneos groups ($N = 57$).

Characteristics	Eos ($n = 26$)	Noneos ($n = 31$)	Statistics	P
Age, years	68.2 ± 10.5	63.9 ± 12.1	1.442*	0.155
Males, *n* (%)	19 (73.1)	22 (71.0)	0.031†	0.860
Current smokers, *n* (%)	7 (26.9)	9 (29.0)	0.031†	0.860
Smoking history, pack-years	40.5 ± 8.1	36.3 ± 9.8	1.719*	0.091
BMI, kg/m^2	21.3 ± 3.1	21.1 ± 2.9	0.302*	0.764
GOLD stage, *n* (%)			0.076†	0.962
I	0 (0)	0 (0)		
II	4 (15.4)	5 (16.1)		
III	16 (61.5)	18 (58.1)		
IV	6 (23.1)	8 (25.8)		
White blood cell count, ×10^9/L	11.52 ± 2.19	12.55 ± 3.42	−1.319*	0.193
Blood neutrophil count, ×10^9/L	9.58 ± 1.86	9.85 ± 2.99	−0.417*	0.690
Blood eosinophil count, ×10^9/L	3.49 ± 0.85	0.69 ± 0.66	13.983*	<0.001
Medications, *n* (%)				
ICS	11 (42.3)	15 (48.4)	0.211†	0.646
LABA	18 (69.2)	21 (67.7)	0.015†	0.904
LAMA	14 (53.8)	15 (48.4)	0.169†	0.681
Theophylline	13 (50)	17 (54.8)	0.133†	0.716
Sputum cell counts‡, ×10^6/mL				
Total cells	3.55 (3.20, 4.10)	3.70 (3.45, 4.05)	−0.313§	0.755
Neutrophils	2.33 (2.18, 2.73)	2.48 (2.44, 2.71)	−0.508§	0.612
Macrophages	0.78 (0.48, 0.98)	0.81 (0.46, 1.11)	−0.290§	0.772
Lymphocytes	0.35 (0.32, 0.41)	0.39 (0.34, 0.55)	−0.910§	0.363
Eosinophils	0.09 (0.06, 0.13)	0.02 (0.00, 0.03)	−5.626§	<0.001

Data are expressed as mean ± standard deviation, *n* (%), or median (interquartile ranges). * *t* values. † χ^2 values. ‡ The induced sputum samples were qualified in ten patients in each group. § Z values. Eos: Eosinophilic group; Noneos: Non-eosinophilic group; BMI: Body mass index; GOLD: Global Initiative for Chronic Obstructive Lung Disease; ICS: Inhaled corticosteroids; LABA: Long-acting β-agonist; LAMA: Long-acting muscarinic antagonist.

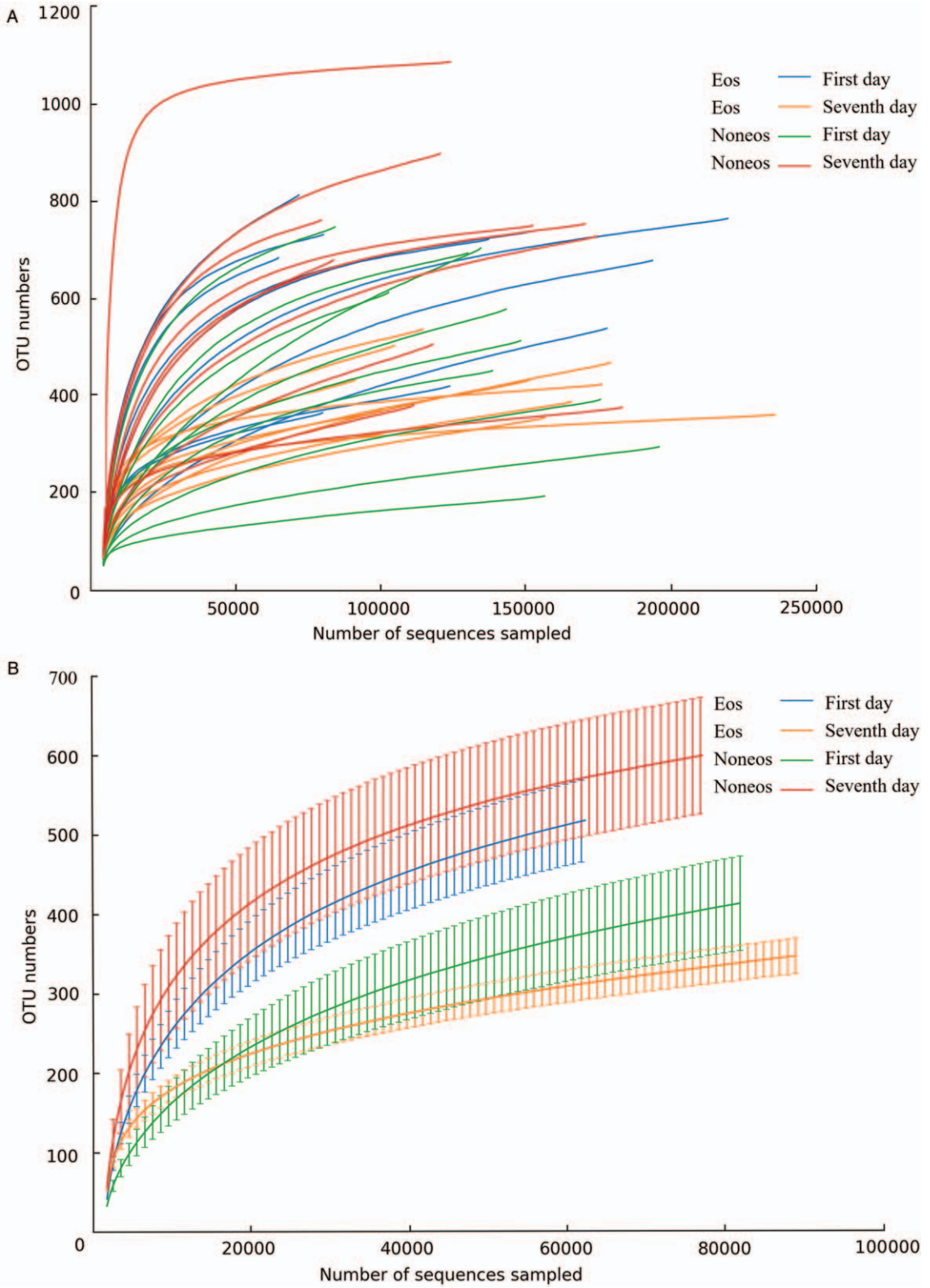

Figure 1: Rarefaction curve at OTU levels. (A) Individual samples. (B) Groups. The curve tends to be flat on the X-axis, suggesting that the amount of sequencing is sufficient. Eos: Eosinophilic group; Noneos: Non-eosinophilic group; OTU: Operational taxonomic units.

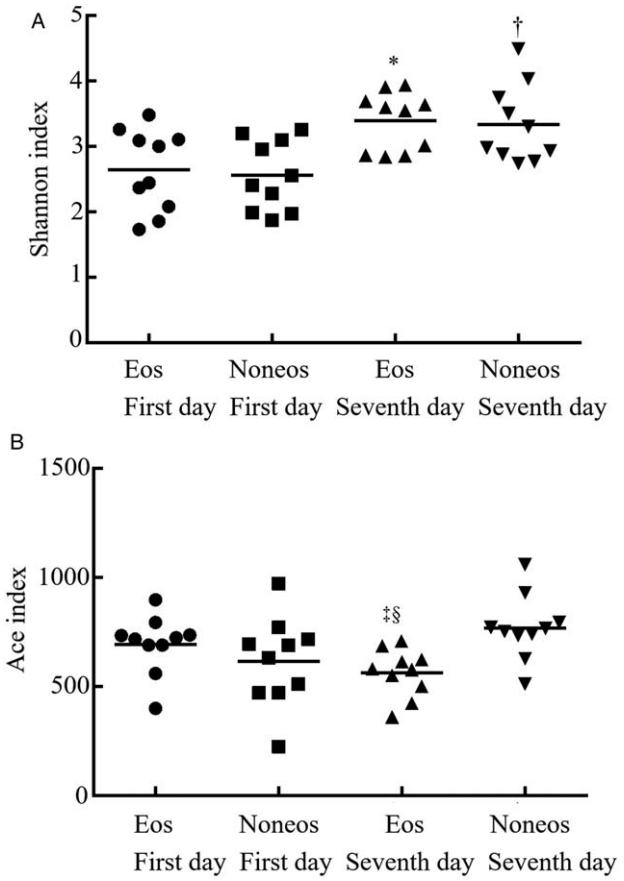

Figure 2: Shannon index (A) and Ace index (B) of the microbiome in induced sputum of the Eos ($n = 10$) and Noneos groups ($n = 10$) on the 1st and 7th day of treatment. $^{*}P < 0.01$ compared with Eos group on the 1st day of treatment. $^{\dagger}P < 0.01$ compared with Noneos group on the 1st day of treatment. $^{\ddagger}P < 0.05$ compared with Eos group on the 1st day of treatment. $^{\S}P < 0.01$ compared with Noneos group on the 7th day of treatment. Eos: Eosinophilic group; Noneos: Non-eosinophilic group.

than in the Eos group on the 7th day of treatment [Figure 3C].

Levels of inflammatory mediators in the plasma

The concentrations of inflammatory cytokines IL-6, IL-8, and CRP were similar between the two groups prior to treatment ($P > 0.05$). On the 7th day of treatment, they all decreased significantly in both groups compared to the 1st day ($P < 0.001$). Levels of IL-6 ($t = -4.913$, $P < 0.001$) and IL-8 ($t = -2.981$, $P = 0.004$) were significantly lower in the Eos group compared to the Noneos group after 7 days of treatment [Table 2].

Levels of blood gas, lung function, and CAT scores

The levels of arterial partial pressure of oxygen (PaO_2), FEV_1% predicted, and FEV_1/FVC were significantly higher in both groups after treatment compared to those before treatment (all $P < 0.001$), and the arterial partial pressure of carbon dioxide ($PaCO_2$) levels and CAT scores significantly decreased (both $P < 0.001$). There was no significant difference in the levels of blood gas, lung function, and CAT scores between Eos and Noneos group before and after treatment (all $P > 0.05$) [Table 3].

IC50-Dex in PBMCs and the inhibition of IL-8

There was no significant difference in the IC50-Dex and the percentage of inhibition of IL-8 between Eos and Noneos group prior to treatment (both $P > 0.05$). After 7 days of treatment, the IC50-Dex significantly decreased in both groups (both $P < 0.001$) and the IC50-Dex was significantly lower in the Eos group compared to the Noneos group ($t = -2.418$, $P = 0.006$) [Figure 4A]. The percentage of inhibition of IL-8 at Dex concentrations 10^{-8}–10^{-6} mol/L were also significantly higher in the Eos group than the Noneos group after 7 days of treatment (all $P < 0.05$) [Figure 4B].

Discussion

The present study compared the sputum microbiota and the inflammatory mediator levels in the plasma of eosinophilic and non-eosinophilic patients with AECOPD before and after 7 days of conventional treatment (including ICS) against AECOPD. Results showed that the diversity of the sputum microbiome (Shannon index) increased in both groups after treatment, while the species richness (Ace index) only significantly decreased in the Eos group after treatment but not the Noneos group. The levels of the inflammatory mediators IL-8 and IL-6 also decreased more significantly in the Eos group than the Noneos group. According to an *in vitro* study of the isolated PBMCs, IC50-Dex was significantly lower in the Eos group than in the Noneos group after 7 days of treatment, which suggested higher corticosteroid sensitivity in the Eos group.

COPD is a chronic lung disease with high morbidity and mortality characterized by irreversible airflow obstruction.[32] AECOPD is the sudden worsening of COPD symptoms, which often presents as increased airway inflammation and decreased lung function.[33] One of the most important goals for COPD treatment is to decrease the frequency of AECOPD. Bacterial and viral infections are common triggers for AECOPD. Reportedly over 50% of acute exacerbations are caused by bacterial pathogens and around 25% are caused by viral infections.[34] The bacterial population in the lung is found to be closely related to the use of antibiotics and corticosteroids, as well as clinical factors and eosinophilic airway inflammation.[14-16,21] However, most prior studies have relied on culture-based methods to identify the pathogenic bacteria.[35] Modern sequencing techniques allow more detailed studies on the microbiome diversity and composition.

Results in the present study using the 16s rRNA method shows the five main bacteria species found in the induced sputum of patients with AECOPD — *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria* — were from a phylum consistent with previous research results.[16] The microbiome diversity was similar between the Eos and Noneos group and the diversity increased in both groups after 7 days of treatment. This was similar to previous studies showing that microbiome diversity decreased during acute exacerbations and increased during stable periods.[16,20] The decrease in diversity during acute exacerbations may be related to the accumulation of

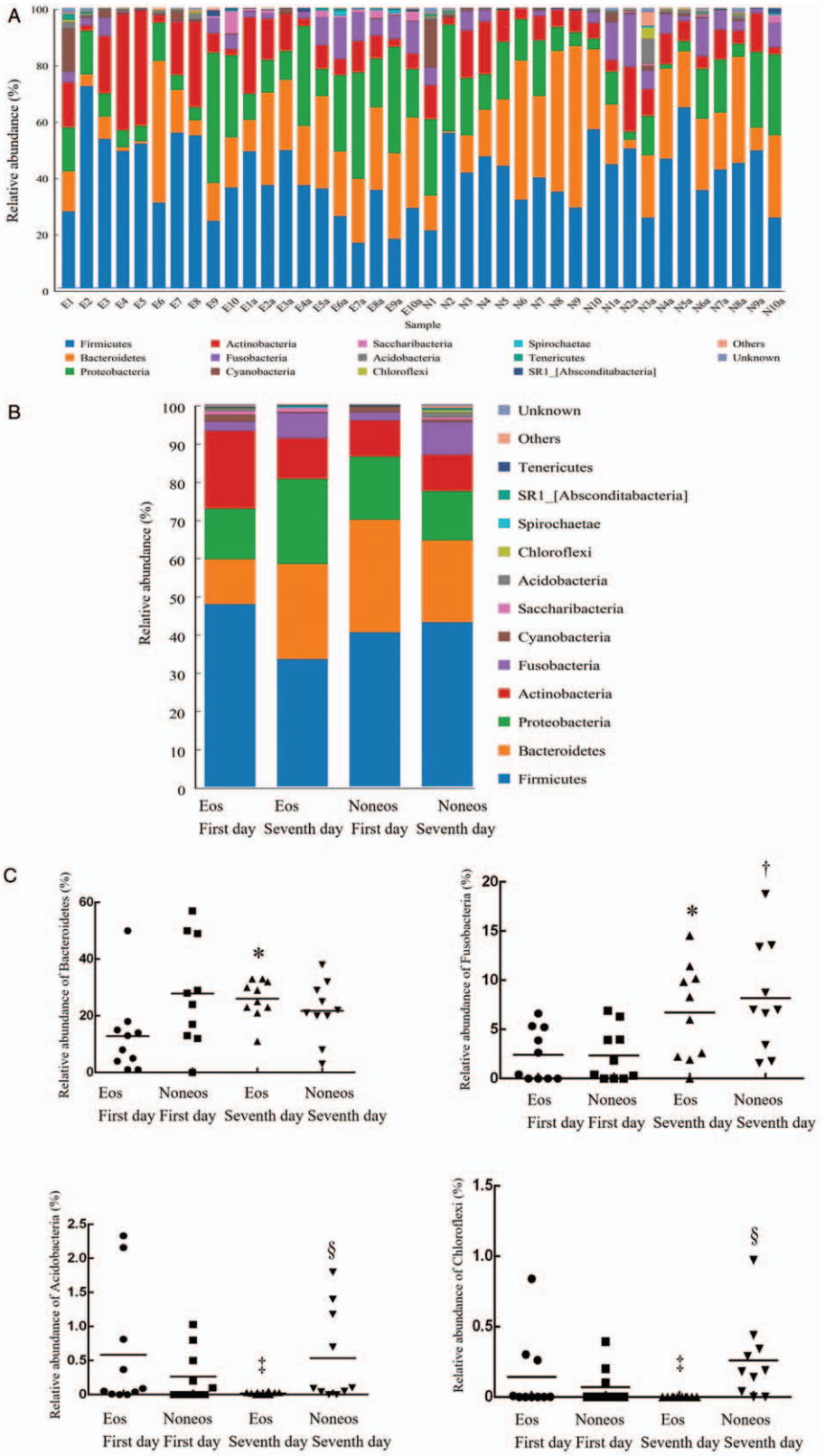

Figure 3: Relative abundance of microbiota at the phylum levels. (A) Individual samples. (B) Groups. (C) Differences in relative abundance of specific phyla between the Eos ($n = 10$) and Noneos group ($n = 10$) before and after treatment. $^{*}P < 0.05$ compared with Eos group on the 1st day of treatment. $^{\dagger}P < 0.01$ compared with Noneos group on the 1st day of treatment. $^{\ddagger}P < 0.01$ compared with Eos group on the 1st day of treatment. $^{\S}P < 0.01$ compared with Eos group on the 7th day of treatment. Eos: Eosinophilic group; Noneos: Non-eosinophilic group.

Table 2: Comparisons of IL-6, IL-8, and CRP between the Eos and Noneos groups on the 1st and 7th day of treatment ($N = 57$).

Parameters	Eos ($n = 26$)		Noneos ($n = 31$)	
	First day	Seventh day	First day	Seventh day
IL-6, pg/mL	22.54 ± 2.70	12.09 ± 2.85*,†	24.24 ± 4.24	15.54 ± 2.45‡
IL-8, pg/mL	131.46 ± 22.21	63.64 ± 21.69*,§	143.76 ± 26.91	78.97 ± 17.13‡
CRP, mg/L	19.07 (7.80, 31.00)	7.18 (3.40, 10.50)*	14.87 (6.00, 21.00)	6.93 (5.00,11.00)‡

Data are expressed as mean ± standard deviation or median (interquartile ranges). * $P < 0.001$ *vs.* Eos group on the first day. † $P < 0.001$ *vs.* Noneos group on the seventh day. ‡ $P < 0.001$ *vs.* Noneos group on the first day. § $P < 0.01$ *vs.* Noneos group on the seventh day. IL-6: Interleukin-6; IL-8: Interleukin-8; CRP: C-reactive protein; Eos: Eosinophilic group; Noneos: Non-eosinophilic group.

Table 3: Comparisons of blood gas, lung function, and CAT scores between the Eos and Noneos groups on the 1st and 7th day of treatment ($N = 57$).

Parameters	Eos ($n = 26$)		Noneos ($n = 31$)	
	First day	Seventh day	First day	Seventh day
PaO_2, mmHg	60.84 ± 5.66	72.13 ± 7.31*	60.50 ± 5.47	69.87 ± 7.11†
$PaCO_2$, mmHg	58.62 ± 7.35	45.77 ± 4.97*	59.54 ± 6.44	48.19 ± 5.64†
FEV_1% pred, %	36.00 (30.29, 39.43)	42.23 (39.87, 44.84)*	36.62 (32.33, 39.81)	40.87 (38.67, 42.15)†
FEV_1/FVC, %	43.13 ± 6.76	50.09 ± 7.26*	43.78 ± 6.63	50.11 ± 4.80†
CAT scores	20.7 (17.0, 24.0)	11.2 (9.0, 13.0)*	18.9 (17.0, 22.0)	11.90 (10.0, 14.0)†

Data are expressed as mean ± standard deviation or median (interquartile ranges). * $P < 0.001$ *vs.* Eos group on the first day. † $P < 0.001$ *vs.* Noneos group on the first day. CAT: Chronic obstructive pulmonary disease assessment test; Eos: Eosinophilic group; Noneos: Non-eosinophilic group; FEV_1: Forced expiratory volume in 1 s; PaO_2: Arterial partial pressure of oxygen; $PaCO_2$: Arterial partial pressure of carbon dioxide; FEV_1% pred: Predicted percentage of forced expiratory volume in 1 s; FVC: Forced vital capacity.

certain dominant pathogenic bacteria, which inhibit the growth of other bacteria. Treatment increased microbiome diversity by decreasing the amount of pathogenic bacteria and restoring the lung microbiome to a stable state.

Previous studies have found that treatment with oral glucocorticoid alone can lead to an increase in the abundance of the bacteria, whereas the use of antibiotics alone has the opposite effect.[20] Results from the present study showed that the microbial richness decreased significantly after 7 days of treatment in eosinophilic patients, but not in non-eosinophilic patients, despite similar treatment regimens being given to both groups: broad-spectrum antibiotics and ICS. A decrease in the microbial richness reflects a decrease in the total bacterial load. The microbiome composition also changed in both groups. In the Eos group, the relative abundances of *Bacteroidetes* and *Fusobacteria* increased after treatment whereas *Acidobateria* and *Chloroflexi* decreased. In the Noneos group, only *Fusobacteria* increased, and the relative abundances of *Acidobateria* and *Chloroflexi* were higher than those in the Eos group. These changes in eosinophilic patients may be relevant to disease recovery.

An investigation of patients with asthma and COPD, consistent with the results of our study, showed a significantly increased amount of *Bacteroidetes* compared with controls.[36] *Fusobacteria* are anaerobes and oral bacteria associated with infections of the respiratory tract.[37] Oral flora may become established after treatment in patients with COPD. The different patterns of airway microbiota and their reaction to treatment between the Eos and Noneos group further suggest the implication of these mechanisms in the pathogenesis of the disease. In the present study, the microbial diversity increased in both eosinophilic and non-eosinophilic patients following treatment, while the microbial richness and relative abundance of *Bacteroidetes* increased only in the eosinophilic patients. This suggests that the eosinophilic patients were more responsive to treatment and may recover more rapidly than patients with non-eosinophilic AECOPD.

COPD involves not only airway inflammation but also systemic inflammation. Increased airway and systemic inflammation with increased neutrophils, lymphocytes, and eosinophils are characteristics of AECOPD.[33] Inflammatory mediators such as IL-8, IL-6, and TNF-α are also significantly increased in AECOPD. IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells, and endothelial cells. High levels of IL-8 can lead to excessive inflammation and tissue damage. A study by Zhang and Bai[38] found that the level of IL-8 increase in patients with AECOPD was closely associated with increase in blood eosinophils. In the present study, levels of inflammatory mediators, including IL-8 and IL-6, were found to decrease more significantly in eosinophilic patients than non-eosinophilic patients under similar treatment according to the AECOPD treatment guideline. It appears that the degree of inflammation is more easily controlled in eosinophilic patients than non-eosinophilic ones following similar treatments.

Glucocorticoids play an important inflammatory role in the treatment of AECOPD. The use of ICSs for exacerbated COPD treatment is greatly discussed and controversial. Unlike other inflammatory diseases such as asthma, glucocorticoid resistance is common in patients with COPD.[39,40] Therefore, ICSs used in stable COPD are still

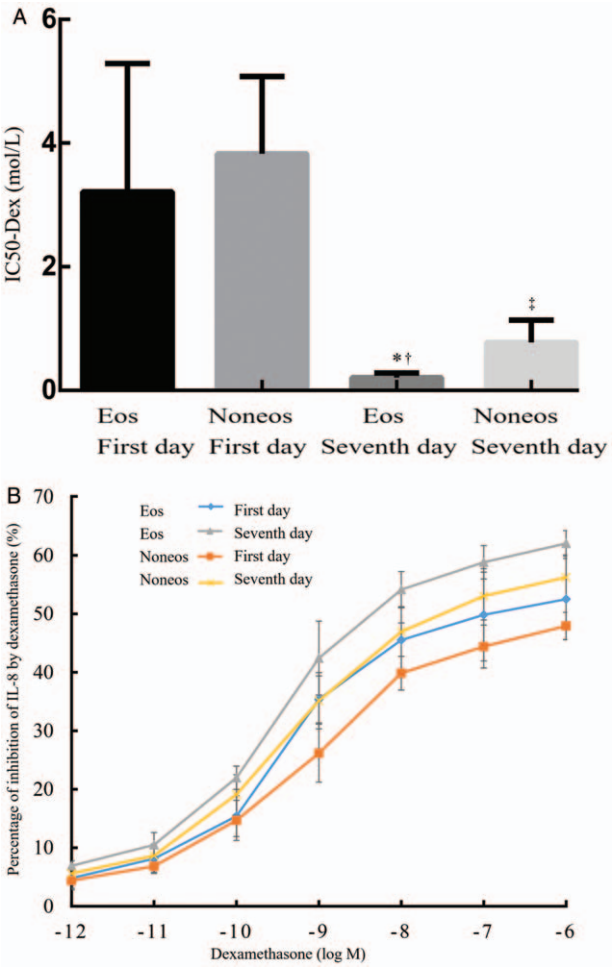

Figure 4: IC50-Dex (A) and the percentage of inhibition of IL-8 by dexamethasone (B) in PBMCs. $^{*}P < 0.001$ compared with Eos group on the 1st day of treatment. $^{\dagger}P < 0.01$ compared with Noneos group on the 7th day of treatment. $^{\ddagger}P < 0.001$ compared with Noneos group on the 1st day of treatment. Eos: Eosinophilic group; Noneos: Non-eosinophilic group; IC50-Dex: 50% inhibitory concentration of dexamethasone; IL-8: Interleukin-8; PBMCs: Peripheral blood mononuclear cells.

highly controversial as they can increase the risk of airway infections.[41] However, eosinophil levels reportedly could be used as a potential biomarker for predicting the clinical response to glucocorticoid therapy.[4,42,43] Therefore, the role of eosinophils in AECOPD is worthy of further elucidation.

Approximately 10% to 40% of patients with COPD are shown to have a relatively elevated level of eosinophils.[44] It has recently been shown that patients with high eosinophil counts show good response to glucocorticoids,[6] and patients with eosinophilic COPD can benefit from ICSs and oral corticosteroid treatment[42]; however, the exact mechanisms responsible for the higher corticosteroid sensitivity seen in the eosinophilic patients remain unclear. Studies have found that glucocorticoids can produce an inhibitory effect on neutrophil apoptosis, while accelerating eosinophil apoptosis under *in vitro* conditions,[45] thereby reducing eosinophil-associated inflammation in the airways. In the present study, PBMCs were isolated from patients with AECOPD and their IC50-Dex for IL-8 inhibition was determined as a marker for corticosteroid sensitivity *in vitro*. The percentage of inhibition of IL-8 following Dex treatment was found to be significantly higher in the Eos group compared to the Noneos group, which confirms the results from previous studies showing that eosinophilic patients are more sensitive to glucocorticoids.[4-6]

In summary, the present study has shown that eosinophilic and non-eosinophilic patients responded differently to conventional AECOPD treatment with broad-spectrum antibiotics and ICS. Eosinophilic patients responded with significantly lower sputum microbial richness and lower levels of inflammatory mediators IL-8 and IL-6. The microbiome composition also changed after treatment. The relative abundances of *Bacteroidetes* and *Fusobacteria* increased after treatment in the eosinophilic patients whereas *Acidobateria* and *Chloroflexi* decreased. Only *Fusobacteria* increased in the non-eosinophillic patients. This might be due to higher sensitivity to corticosteroids among eosinophilic patients as shown by *in vitro* inhibition studies using isolated PBMCs.

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Conflicts of interest

None.

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