



OPEN Association between airway microbiota and systemic inflammation markers in non-small cell lung cancer patients

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Growing evidences have suggested the airway microbiota may participate in lung cancer progression. However, little was known about the relationship between airway microbiota and lung cancer associated systemic inflammation. Here we aimed to explore the association between sputum microbiota and systemic inflammation in lung cancer. The microbiota of spontaneous sputum samples from 51 non-small cell lung cancer (NSCLC) patients and 6 patients with lung benign nodules were sequenced via 16 S rRNA sequencing. Neutrophil-lymphocyte ratio (NLR), platelet-lymphocyte ratio (PLR) and C reactive protein (CRP) were used to represent systemic inflammation. Patients were divided into 2 groups based on level of inflammatory biomarkers respectively (CRP_low versus CRP_high; NLR_low versus NLR_high; PLR_low versus PLR_high). α -diversity was significantly decreased in CRP_high and NLR_high patients. β diversity analysis based on weighted unifracc distance indicated that microbial community structure differed significantly between patients with different inflammation status. Lefse identified genera *Porphyromonas*, *Selenomonas*, *Moryella*, *Megasphaera*, *Corynebacterium* were enriched in CRP_low group. Compared with NLR_high, genera *Veillonella*, *Neisseria*, *Bulleidia*, *Moryella* were enriched in NLR_low group. For patients with different PLR level, genera *Veillonella*, *Prevotella*, *Moryella*, *Selenomonas* were increased in PLR_low patients. Function analysis identified propionate metabolism pathway was significantly enriched in CRP_low and PLR_low groups. Moreover, RDA analysis showed that compared with PLR, NLR and CRP had strongest association with microbial community. Airway microbial structure differed between lung cancer with different systemic inflammation status. Patients with relative high inflammation status were associated with alteration of specific airway genera and microbial metabolic function.

Lung cancer is the second leading malignancy for morbidity and the first for cancer deaths worldwide¹. Non-small cell lung cancer (NSCLC) is the main pathological type of lung cancer. Although with the fast advancement of target therapy and immunotherapy, the 5-year survival rate of lung cancer remain low². Tumor associated systemic inflammation may contribute to the carcinogenesis and development of lung cancer^{3,4}. In order to assess systemic inflammation of cancer patients, numerous hematological inflammation biomarkers were developed. These biomarkers include neutrophil-related markers, such as neutrophil-lymphocyte ratio (NLR), platelet-related markers, such as platelet-lymphocyte ratio (PLR) and C reactive protein (CRP). Numerous studies demonstrated NLR⁵⁻⁷, PLR^{5,7-9} and CRP¹⁰ were associated with prognosis and efficacy of immunotherapy of lung cancer patients, irrespective of gender.

Microorganisms that inhabit in human organ are referred as microbiota, which may play an important role in lung cancer development¹¹. With the advent of 16 S rRNA sequencing, the lower airway microbiota has been clarified recently^{12,13}. As our understanding of the microbial ecology of the lung improves, it is becoming increasingly apparent that certain disease states can disrupt the microbial-host interface and ultimately affect pathogenesis of several lung diseases¹⁴. Accumulating studies have suggested that the lung microbiota of

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lung cancer patients was significantly different from healthy or benign control^{15–17}. A recent meta-analysis included 5 studies suggested that phylum *Actinobacteria* and several genera included *Corynebacterium*, *Lachnoanaerobaculum* and *Halomonas* were significantly decreased in lung tumor tissues of lung cancer patients, regardless of gender, compared with tumor-adjacent normal tissues¹⁸. Additionally, lung microbiota was associated with several clinical parameters of lung cancer. A recent study demonstrated that significantly differential respiratory microbiome taxa, abundance, and diversity in lung cancer of different pathology and some stages¹⁹. Our previous studies showed that airway microbiota was linked with lung cancer histological type, stage and EGFR mutation^{20,21}.

Previous studies in humans have shown that inflammation activation may link lung microbiota and lung cancer development²². Toll-like receptors (TLRs) are members of the pattern recognition receptor family that recognize microorganism-specific molecular patterns²³. Dysbiosis of microbiota can disrupt TLR signaling pathways, resulting in uncontrolled inflammation and promote carcinogenesis²⁴. In addition, microbiota induces MYD88 in myeloid cells, triggering IL-23 signaling to promote tumor progression and the development of a tumoral IL-17 response²⁵. A pilot preclinical study has shown that lung microbiota may create an inflammatory environment and promote lung cancer progression in mouse model via proliferation and activation of lung $\gamma\delta$ T cells and production of effector molecules such as IL-17²⁶. Although the above-mentioned studies indicated that resident bacterial flora may affect lung cancer through induction of the host's inflammatory response, relationship between airway microbiota and lung cancer associated inflammation remained obscure.

In the light of the comprehensive understanding of the interaction between lung microbiota and lung cancer, further studies are needed to clarify the relevance between lung microbiota and lung cancer associated systemic inflammation. In this study, 16s sequencing analysis of the airway microbiota was performed to profile spontaneous sputum samples in relation to lung cancer patients based on the level of different blood systemic inflammation marker.

Materials and methods

Patients and samples

The study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University. Preliminarily diagnosed non-small cell lung cancer (NSCLC) patients were prospectively admitted in this study at NanFang Hospital, Southern Medical University between August 2018 and August 2019. For NSCLC patients, the inclusion criteria were as mentioned before²⁰: pathologically diagnosed of NSCLC; aged 30–80; did not receive any anti-tumor therapy; no evidence of community-acquired pneumonia, acute exacerbation of chronic obstructive pulmonary disease, bronchiectasis with infection, acute bronchitis or asthma; had no fever; no history of antibiotic treatment within 14 days; without a history of other malignant diseases. For patients with lung benign nodules, the inclusion criteria were as follows: pathologically diagnosed or the nodule shrank via antibiotic treatment; aged 30–80; no evidence of community-acquired pneumonia, acute exacerbation of chronic obstructive pulmonary disease, bronchiectasis with infection, acute bronchitis or asthma; had no fever; no history of antibiotic treatment within 14 days; without a history of other malignant diseases. We conducted a questionnaire and reviewed the electronic medical records to obtain demographic and clinical data. NLR was defined as ratio of the absolute neutrophil count to the absolute lymphocyte count. PLR was defined as ratio of the absolute platelet count to the lymphocyte count.

Prior to sampling, participants were instructed to thoroughly rinse their mouths. Participants are then asked to cough deeply and expectorate the first sputum of the day, post tooth brushing in the morning, into a sterile, wide-mouthed container provided by the research team. A minimum of 2 mL of sputum is required for a thorough analysis. Each sample is labeled with a unique identifier to ensure participant confidentiality and to facilitate tracking throughout the study. The sputum sample was transferred into -20°C refrigerators within a strict timeframe of 2 h. Subsequently, within 1 week, the samples were further transferred into -80°C refrigerators for long-term storage. The spontaneous sputum sample was deemed as qualified sputum based on the presence of bronchial cells.

16S rRNA amplification and sequencing

Sputum samples kept on dry ice were transferred to Sagene Biotechnology Company, GuangZhou. The 16S rRNA amplification and sequencing methods were as mentioned in our previous study²¹. The V3-V4 region of 16S rRNA was amplified using specific primers (16S_341F:5'-CCTAYGGGRBGCASCAG-3'; 16S_806R:5'-GGACTACNNGGGTATCTAAT). PrimeSTAR HS DNA Polymerase was used for PCR reaction. The concentration and length of the PCR products were detected by 1% agarose gel electrophoresis. Samples with a bright main strip were used for further experiments. Sequencing libraries were conducted using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® sequencing (New England Biolabs, United States). The quality of the library was evaluated under a Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Sequencing was conducted to generate 250-bp paired-end reads using an Illumina HiSeq 2500 sequencer according to the manufacturer's instructions.

Microbiota analysis

Raw data was obtained and then further filtered to eliminate reads with adapter pollution and low quality to obtain an amplicon sequence variant (ASV) table by using QIIME2²⁷. The sequences were thereby denoised by using DADA2²⁸. ASVs were taxonomically classified with the SILVA database 138 by using the naïve Bayesian algorithm provided in QIIME2.

We applied ASVs data in online microbiome data analyze platform (MicrobiomeAnalyst) (<https://www.microbiomeanalyst.ca/>) to compare microbiota community structure at both inter-community at α -diversity level and β -diversity level²⁹. For α diversity, we chose Chao1 value, Simpson index and Shannon index to evaluate.

For β diversity, we estimated using unweighted unifrac distance and Bray Curtis distance visualized by principal coordinate analysis (PCoA). Differential taxon was identified by LefSe (Linear discriminant analysis (LDA) effect size) analysis. PICRUSt2 was used to predict the functional profiling of microbial communities based³⁰. Differentially present pathways between groups were analyzed with Welch t test. Inflammation association analysis was performed using redundancy analysis (RDA) to analyze the effects of CRP, NLR and PLR on the microbial structure.

Statistical analysis

The software Graphpad Prism (Version 9) was used for statistical analysis. The continuous variables were compared between two groups by Mann-Whitney U test or independent t test. The categorical variables were compared by chi-square test. Correlation analysis between differential taxa and inflammatory biomarkers was conducted using Spearman analysis.

Results

Baseline characteristics and sputum microbiota of subjects

In this study, spontaneous sputum samples were collected from 51 NSCLC patients and 6 patients with lung benign nodule who met eligible criteria. Clinical characteristics of the 51 NSCLC patients and 6 lung benign disease patients were listed in supplementary table S1 and supplementary table S2. The average age of the 51 NSCLC subjects was near 60y. Adenocarcinoma was the main pathological type (88.2%) and over half of the subjects (56.9%) were at stage IV. Shannon rarefaction curve was constructed to evaluate sequence depth (Supplementary figure S1). The result indicated that sequence depth of sputum samples was sufficient enough to reach a reliable estimate of microbiome structure. To identify ecological diversity changes associated with lung cancer, α diversity (within samples) and β diversity analysis (between samples) were used to compare sputum microbiota between NSCLC patients (LC group) and lung benign nodule patients (BN group). Chao1, Simpson index, and Shannon index were selected to estimate the α diversity. The results revealed significant difference in Chao1 index and Shannon index in the 2 groups (Supplementary figure S2 A-C). Chao1 was 509.31 in LC group and 408.42 in BN groups (Mann-Whitney U test, $P=0.042$). Shannon index was 5.26 in LC group and 4.24 in BN groups (Mann-Whitney U test, $P=0.045$). Simpson index was similar with 0.907 in LC groups and 0.855 in BN group (Mann-Whitney U test, $P=0.177$). The principal coordinates analysis (PCoA) of β diversity based on Weighted unifrac distance and Bray Curtis distance were used to estimate the sputum microbial community structure in different groups (Supplementary figure S2 D, E). Significant difference of sputum microbiota was observed based on Bray Curtis distance (PERMANOVA test, $P=0.014$). Microbial structure was similar based on Weighted unifrac distance (PERMANOVA test, $P=0.228$).

In total, 20 phyla and 153 genera were detected. The top 5 phyla of the 51 NSCLC participants were *Firmicutes* (43.4%), *Actinobacteria* (21.4%), *Bacteroidetes* (16.3%), *Proteobacteria* (8.3%) and *Fusobacteria* (5.9%). The top 10 genera of the 51 participants were *Streptococcus* (24.2%), *Rothia* (9.9%), *Prevotella* (9.7%), *Actinomyces* (6.4%), *Neisseria* (4.4%), *Leptotrichia* (4.2%), *Porphyromonas* (3.6%), *Veillonella* (3.0%), *Granulicatella* (2.6%) and *Atopobium* (2.5%). The top phyla and genera were shown in supplementary figure S3.

Characterization of sputum microbiota among subjects with different CRP level

CRP is one of the most common markers to assess systemic inflammation. To explore airway microbiota alteration associated with CRP level, the 51 NSCLC participants were divided into CRP_high (CRP ≥ 10 mg/L) and CRP_low group (CRP < 10 mg/L). This cut-off value was based on a recent study which identify prognosis role of CRP in advanced non-small cell lung cancer³¹. Baseline characteristics were comparable between the 2 groups (supplementary table S3). For the α diversity of the sputum microbiome community, the results showed that Shannon index and Simpson index differed significantly between the 2 groups (Fig. 1B, C). Shannon index was 4.763 in CRP_high group and 5.337 in CRP_low group (Mann-Whitney U test, $P=0.043$). Simpson index was 0.8746 in CRP_high group and 0.9259 in CRP_low group (Mann-Whitney U test, $P=0.046$). For Chao 1 index, it tended to be lower in CRP_high than CRP_low group, however, the P value did not reach level of statistical significance (731.2 for CRP_high, 903.7 for CRP_low, P value = 0.463) (Fig. 1A). β diversity analysis indicated that taxon composition among the 2 groups differed significantly based on Weighted unifrac distance (PERMANOVA test, $P=0.009$) but not Bray Curtis distance (PERMANOVA test, $P=0.112$) (Fig. 1D, E).

To find out the specific taxon signatures associated with CRP level and their relative contribution, Lefse analysis was conducted and identified several enriched taxa in CRP_low group, included phylum *Bacteroidetes*, genera *Porphyromonas*, *Gemella*, *Selenomonas*, *Moryella*, *Megasphaera*, *Corynebacterium* and *Schwartzia* (Fig. 2A). These differential taxa were further shown in Cladogram based on various phylogenetic levels (Fig. 2B). Apart from genera *Gemella* and *Schwartzia*, all other differential genera were with relative abundance $\geq 1\%$. Venn Plot showed that there was 3,886 distinctive ASVs only existed in CRP_low group (Fig. 2C). Among these 3,886 ASVs, 854 (22.0%) ASVs belonged to phylum *Bacteroidetes* and 124 ASVs (3.2%) belonged to genus *Porphyromonas*. Genera *Porphyromonas*, *Selenomonas*, *Megasphaera* and *Corynebacterium* were moderately negatively associated with CRP level based on Spearman correlation analysis (Fig. 2D). P value and r value for the correlation between each differential taxa and CRP level were shown in Supplementary table S4.

16 S rRNA gene analysis does not provide information with respect to their functions. Therefore, KEGG pathways analysis by PICRUSt2 analysis was conducted³²⁻³⁴. In total, 32 pathways were significantly different between CRP_high group and CRP_low group (Supplementary table S5). Among them, some anti-inflammatory microbial metabolism pathways, such as propionate metabolism pathway (independent t test, P value = 0.0154) was significantly increased in CRP_low group.

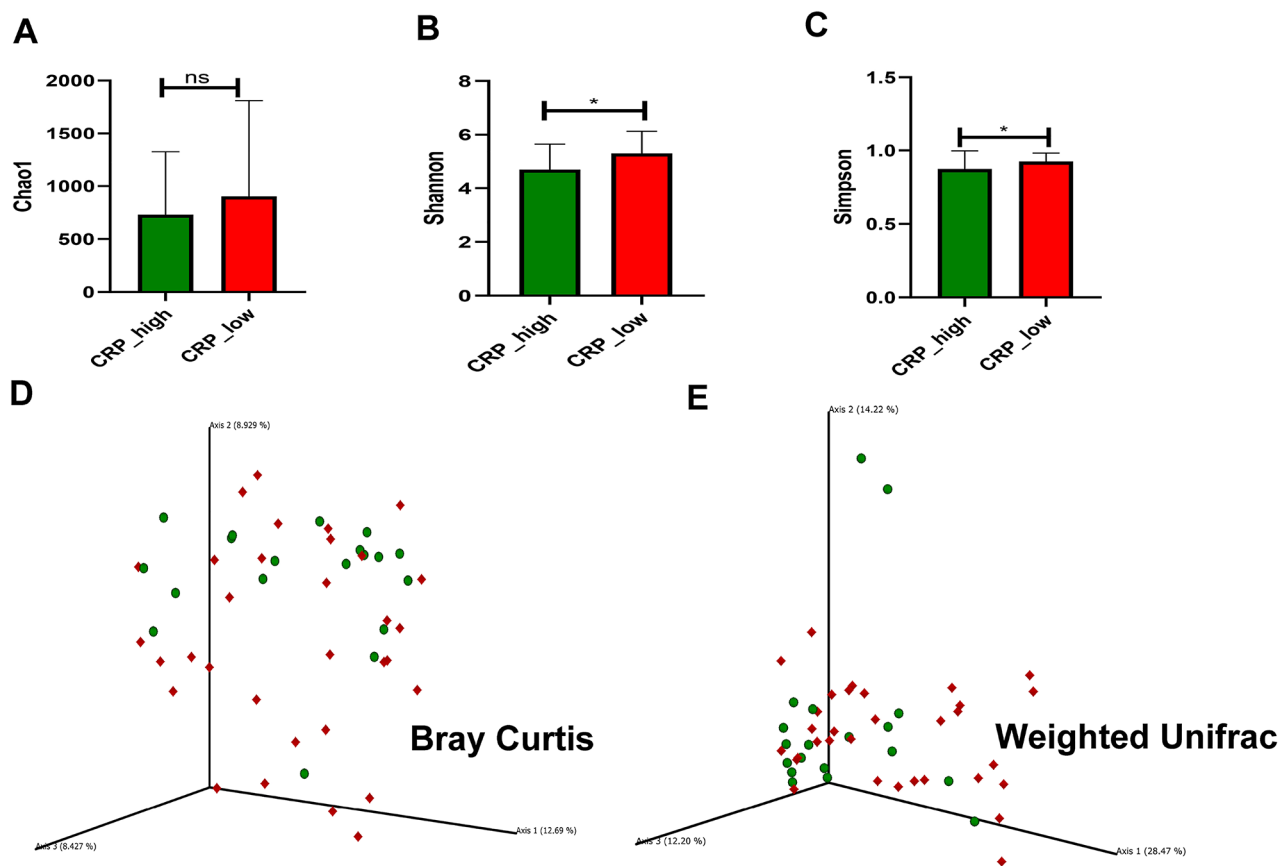


Fig. 1. Difference of sputum microbial structure between CRP_low group and CRP_high group. (A) Chao1 index; (B) Shannon index; (C) Simpson index; (D) PCOA plot based on Bray-Curtis distance; (E) PCOA plot based on Weighted unifrac distance. ns: non-significant. *: $P < 0.05$. P was calculated using Mann-Whitney U test.

Characterization of sputum microbiota among subjects with different NLR level

The cancer-associated systemic inflammatory response is often correlated with an increase in circulating neutrophil counts. Essilfie reported that airway *Haemophilus influenzae* infection drives the development of neutrophilic inflammation³⁵, suggesting airway microbiota may be linked with neutrophilic inflammation. To identify microbiota alteration associated with neutrophil related systemin inflammation, all NSCLC subjects were divided into NLR_high and NLR_low group. Currently, no well-recognized NLR cut-off value exists to estimate lung cancer prognosis. A meta-analysis of 12 studies on NSCLC patients was conducted³⁶. The NLR cut-off values ranged from 2.0 to 5, with the largest sample size study having a value of 2.5³⁷, close to the median of NLR in another study (2.22). Due to the median being less affected by outliers, it was used as cut-off value in our study. Baseline characteristics were comparable between the 2 groups (supplementary table S6). Compared with NLR_low group, a diversity of NLR_high group was significantly decreased (Fig. 3A-C). Shannon index in NLR_high group was significantly lower than that in NLR_low group (4.747 in NLR_high, 5.379 in NLR_low, Mann-Whitney U test, P value=0.011). In consistence, Simpson index in NLR_high group was significantly lower (0.9182 in NLR_high, 0.9462 in NLR_low, Mann-Whitney U test, P value=0.004). Chao1 index was similar between the 2 groups (476.2 in NLR_high, 522.6 in NLR_low, Mann-Whitney U test, P value=0.273). PCOA analysis based on weighted unifrac distance revealed distinctive microbial community between the 2 groups (PERMANOVA test, P value=0.04) (Fig. 3D). For Bray-Curtis distance, microbial community of patients in NLR_high group did not differ significantly from NLR_low group (PERMANOVA test, P value=0.273) (Fig. 3E).

Lefse identified several significantly increased taxa in NLR_low group. Phylum *Proteobacteria*, genera *Neisseria*, *Veillonella*, *Bulleidia*, *Moryella*, *Butyrivibrio* were significantly enriched in NLR_low group; while genera *Serratia* was significantly enriched in NLR_high group (Fig. 4A). Apart from genera *Butyrivibrio* and *Serratia*, the relative abundance of all other differential genera was $\geq 1\%$. All the differential taxa were further shown in Cladogram based on various phylogenetic levels (Fig. 4B). Venn plots showed several distinctive and common ASVs among the 2 groups (Fig. 4C). 3604 specific ASVs only existed in NLR_low group. Among them, 544 ASVs (15.1%) belonged to phylum *Proteobacteria*, 140 ASVs (3.9%) belonged to genus *Neisseria*, 126 ASVs (3.5%) belonged to genus *Veillonella*. Spearman correlation analysis indicated that phylum *Proteobacteria*,

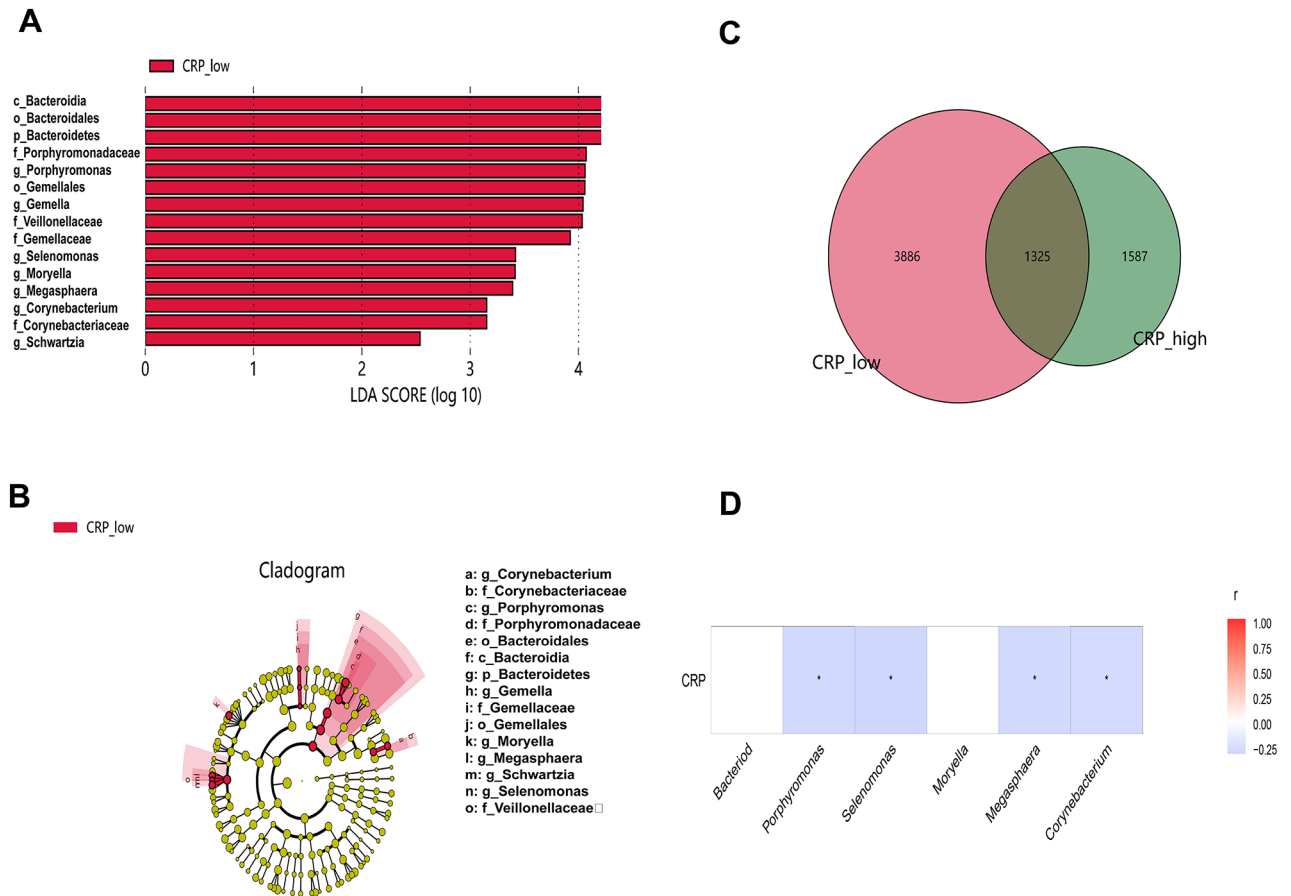


Fig. 2. Sputum taxon signatures in CRP_low group and CRP_high group. **(A)** Differentially abundant taxa between the 2 groups identified by LEfSe analysis; **(B)** Differential taxa were listed based on various phylogenetic levels; **(C)** The number of specific ASVs in the 2 groups; **(D)** Correlation analysis between differential taxa and CRP level. *: $P < 0.05$. P was calculated using Spearman correlation analysis.

genera *Neisseria*, *Bulleidia*, *Moryella* were moderately negatively associated with NLR level (Fig. 4D). P value and r value for the correlation between each differential taxon and NLR level were shown in supplementary table S7.

Results of PICRUST2 analysis showed that 25 differential KEGG pathways were identified (Supplementary table S8). Among them, some inflammation related pathways such as IL-17 signaling (t-test, P value = 0.024) and Th17 cell differentiation pathways (t-test, P value = 0.024) were significantly enriched in NLR_low group.

Characterization of sputum microbiota among subjects with different PLR level

Platelets also contribute to cancer inflammatory response. Microbiota was found to be associated with platelet function³⁸. In aim to explore whether airway microbiota was related with platelet associated systemic inflammation, all NSCLC subjects were categorized into PLR_high and PLR_low group. Like NLR, no well-recognized PLR cut-off value exists for estimating lung cancer prognosis. A meta-analysis of 11 articles on NSCLC patients was conducted, with PLR cut-off values ranging from 106 to 300³⁹. Wu et al. enrolled 366 Chinese NSCLC patients at stage III and IV with a cut-off value of 119.5⁴⁰. As the participants were similar and 119.5 is close to the median (137.35) in this study, the median of PLR was used as the cut-off value in our study. Baseline characteristics were comparable between the 2 groups (supplementary table S9). α diversity between the 2 groups were similar (Mann-Whitney U test, $P = 0.701$ for chao1 comparison; $P = 0.095$ for Simpson index comparison; $P = 0.084$ for Shannon comparison) (Fig. 5A-C). For β diversity, PCOA plot based on Weighted unifracs distance showed that sputum microbiota differed significantly (PERMANOVA test, P value = 0.023) (Fig. 5D). However, β diversity based on Bray Curtis distance did not reach statistical significance (PERMANOVA test, P value = 0.209).

LEfse analysis showed that compared with PLR_high patients, genera *Prevotella*, *Veillonella*, *Moryella*, *Desulfovibrio*, *Selenomonas*, *Desulfobulbus*, *Butyrivibrio* were significantly enriched in PLR_low patients, while genera *Pseudomonas* and *Enterococcus* were significantly decreased (Fig. 6A). Except for genera *Desulfovibrio*, *Desulfobulbus*, *Butyrivibrio*, *Pseudomonas* and *Enterococcus*, other differential genera were with relative abundance $\geq 1\%$. All the differential taxa were further shown in Cladogram (Fig. 6B). Venn Plot showed that 3518 specific ASVs belonged to PLR_low group (Fig. 6C). Among them, 410 (11.7%) ASVs belonged to genus *Prevotella*, 130 (3.7%) ASVs belonged to genus *Veillonella*. Correlation analysis revealed that genus *Prevotella*

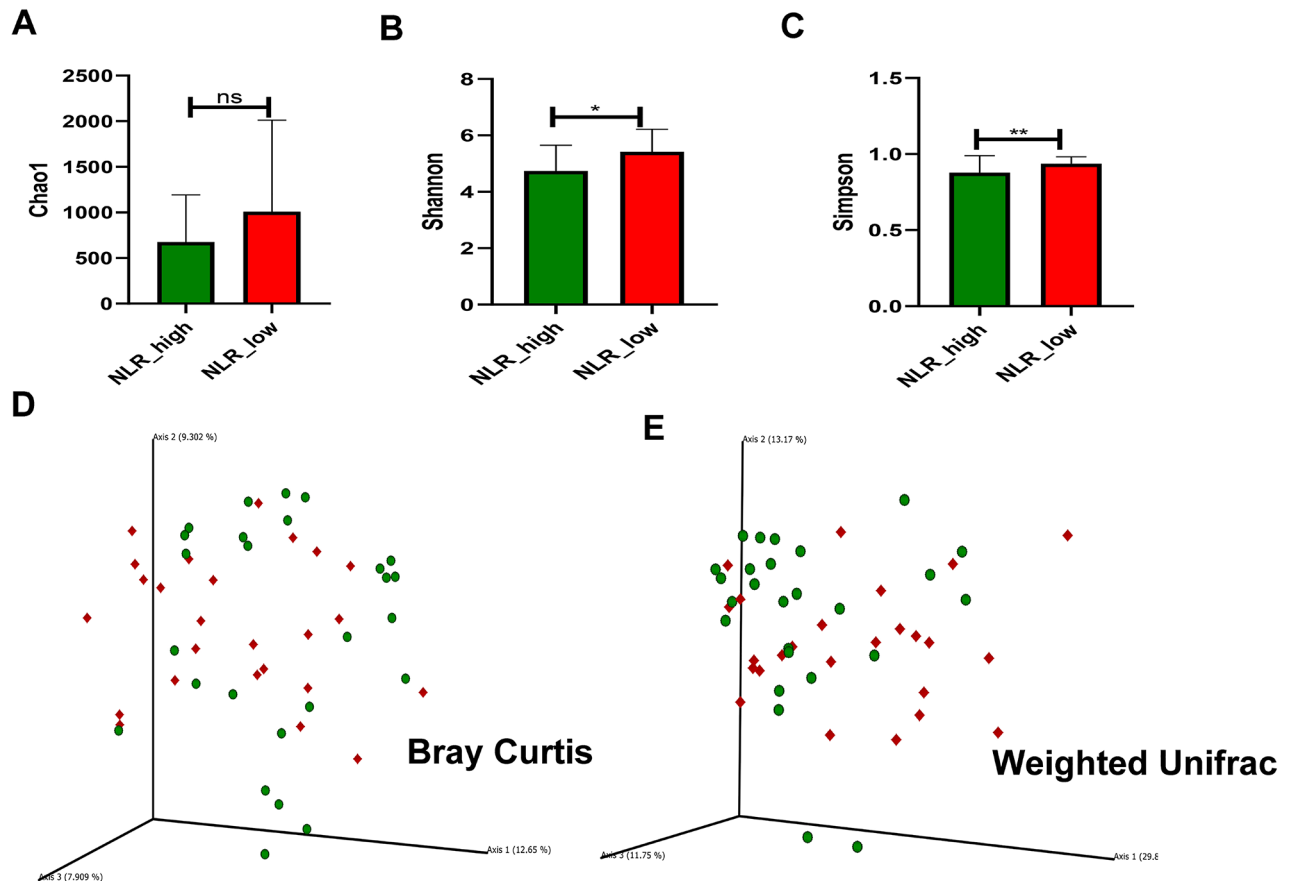


Fig. 3. Difference of sputum microbial structure between NLR_low group and NLR_high group. (A) Chao1 index; (B) Shannon index; (C) Simpson index; (D) PCOA plot based on Bray-Curtis distance; (E) PCOA plot based on Weighted unifrac distance. ns: non-significant. *: $P < 0.05$, **: $P < 0.01$. P value was calculated using Mann-Whitney U test.

was moderately negatively associated with PLR level (Fig. 6D). P value and r value for the correlation between each differential taxa and PLR level were shown in supplementary table S10.

PICRUSt2 analysis revealed 31 differential KEGG pathways (Supplementary table S11). In consistent with the differential pathways in CPR_low group, propionate metabolism pathway (independent t test, P value = 0.0288) was also significantly increased in PLR_low group.

Differential effect of systemic inflammatory biomarkers on sputum taxon structure

We next investigated differential effect of the 3 systemic inflammatory biomarkers on sputum flora structure. Here, we used RDA analysis based on genus level to visualize the association between CRP, NLR, PLR and sputum microbiota (Supplementary figure S4 A). The inflammatory factors were plotted with black arrows, and each factor's weight was proportional to its arrow length. The result indicated that the strongest association with sputum microbial communities was NLR and CRP, while PLR had relative weak linkage with sputum flora. Spearman analysis was used to investigate the association between the 3 systemic inflammatory biomarkers and α diversity (Supplementary figure S4 B). NLR was the only inflammatory biomarker associated with a diversity (Shannon index and Simpson index). Taken together, the results suggested that NLR may has closer relationship with sputum microbiota.

Discussion

Increasing number of clinical studies revealed imbalance of airway microbiota in lung cancer patients¹¹. However, little was known about the association between airway microbiota and lung cancer associated inflammation. In this study, 16 S rRNA sequencing was used to analyze bacterial community of spontaneous sputum and CRP, NLR and PLR were used to represent systemic inflammation of lung cancer. We found airway microbiota diversity among patient with different inflammation status differed significantly. More precisely, a loss of sputum microbiota α diversity was detected in patients with relatively high inflammation status (CRP_high and NLR_high group). β diversity analysis based on Weighted unifrac distance indicated significant distinctive microbial structure between patients with relative high inflammation status (CRP_high, NLR_high group, PLR_high) and patients with low inflammation status (CRP_low, NLR_low group, PLR_low). α diversity represents the number (richness) and distribution (evenness) of taxa expected within a sample. Reduction of lower airway α

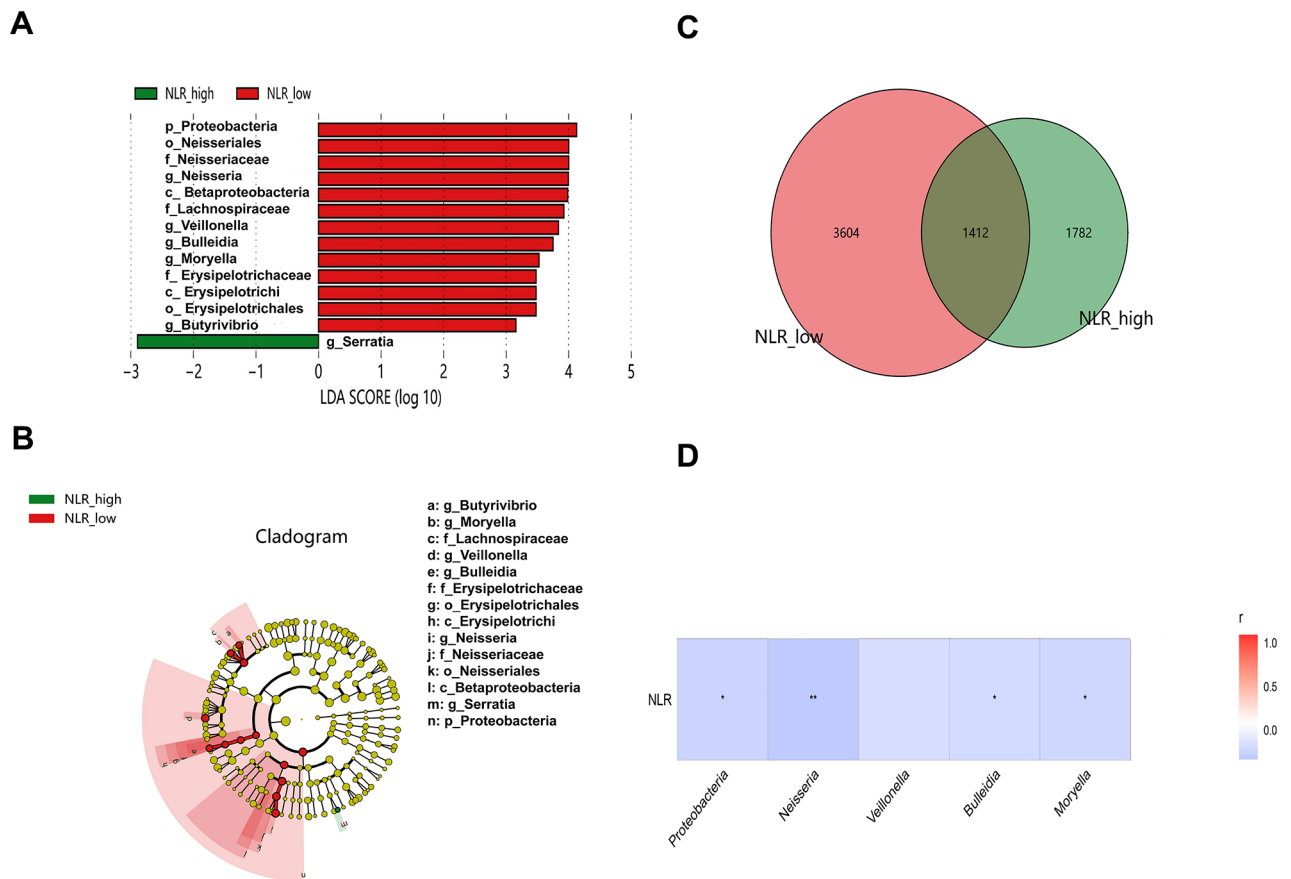


Fig. 4. Sputum taxon signatures in NLR_low group and NLR_high group. **(A)** Differentially abundant taxa between the 2 groups identified by LEFse analysis; **(B)** Differential taxa were listed based on various phylogenetic levels; **(C)** The number of specific ASVs in the 2 groups; **(D)** Correlation analysis between differential taxa and NLR level. *: $P < 0.05$. **: $P < 0.01$. P was calculated using Spearman correlation analysis.

diversity was common in respiratory diseases. Patients with chronic obstructive lung disease or asthma exhibit microbiota enriched with phyla *Proteobacteria*, and *Firmicutes*⁴¹. In term of lung cancer, loss of a diversity was seen in sputum samples⁴², bronchoalveolar fluid¹⁵ and tumor tissue⁴³ in lung cancer patients. Microbiota is crucial in maintaining balance between inflammation and anti-tumor immunity, and microbiota dysbiosis can promote tumors in the lung⁴⁴. Loss of microbial diversity can lead to suboptimal priming of antigen presenting cells⁴⁵. Once microbe is detected by TLRs, downstream NF- κ B and STAT3 are activated⁴⁶, further leading to activation of inflammatory cells that promote inflammatory and carcinogenic pathways⁴⁷.

In lung cancer with relative high inflammation status, the loss of sputum microbial diversity was manifested with reduction of several taxa. In detail, genera *Porphyromonas*, *Selenomonas*, *Moryella*, *Megasphaera*, *Corynebacterium* were found to be significantly enriched in CRP_low group. Genera *Veillonella*, *Neisseria*, *Bulleidia*, *Moryella* were significantly enriched in NLR_low group. Genera *Veillonella*, *Prevotella*, *Moryella*, *Selenomonas* were significantly enriched in PLR_low patients. Genus *Moryella* was enriched in both CRP_low, NLR_low and PLR_low groups and was negatively associated with NLR level. Genus *Moryella* belongs to phylum *Firmicute* and is obligate anaerobic gram-positive bacteria. Little information is available since its first isolation from clinical abscess samples in 2007⁴⁸. Decreased oral *Moryella* was associated with liver cancer⁴⁹ and esophageal Squamous Cell Carcinoma⁵⁰, suggesting it may play a protective role in carcinogenesis. Some oral commensal taxa were found to be signatures in patients with relatively low systemic inflammation. Precisely, genus *Veillonella* was significantly increased in both NLR_low and PLR_low groups and genus *Prevotella* was increased in PLR_low patients. Genera *Veillonella* and *Prevotella* are usually detected in bronchoalveolar lavage fluid of healthy individuals⁵¹. A recent study found that enrichment of oral taxa (such as genus *Veillonella* and *Prevotella*) in lung microbiota of healthy individuals was associated with a blunting of the TLR4-driven immune responses and increased number of Th17 lymphocytes, indicating a role for genera *Veillonella* and *Prevotella* in regulating the basal inflammatory status at the pulmonary mucosal surface⁵². TLR4 is involved in innate immunity and mediates inflammatory responses by recognizing lipopolysaccharide or bacterial endotoxins⁵³. Neutrophil responses are regulated by TLR expression and TLR4 was the principal regulator of neutrophil survival⁵⁴. Moreover, TLR4 is the main stress-inducing members and play an integral role in regulating platelet production and function⁵⁵. Thus, it is plausible that genera *Veillonella* and *Prevotella* may affect production and function of neutrophil and platelet via regulation of TLR4 signaling, further suppress inflammation activation

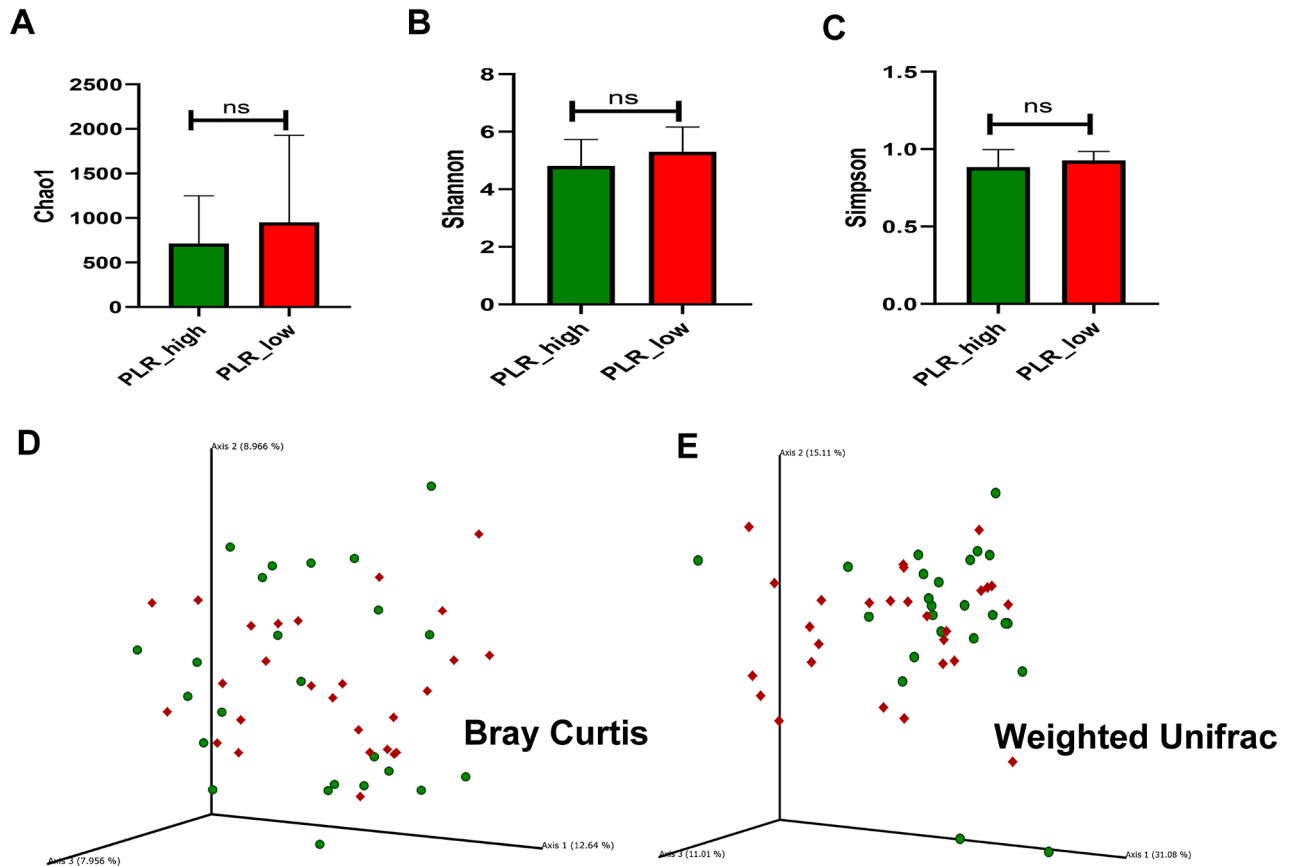


Fig. 5. Difference of sputum microbial structure between PLR_low group and PLR_high group. (A) Chao1 index; (B) Shannon index; (C) Simpson index; (D) PCOA plot based on Bray-Curtis distance; (E) PCOA plot based on Weighted unifrac distance. ns: non-significant. P value was calculated using Mann-Whitney U test.

and inhibit tumor growth. However, the role of genera *Veillonella* and *Prevotella* in lung cancer is controversial. A recent study found that genera *Veillonella* and *Prevotella* was more likely to be found in lower airway lung cancer patients with worse prognosis and was associated with upregulation of several carcinogenesis pathways, such as IL17, PI3K and ERK pathways⁵⁶. Future studies based on reciprocal interaction between these genera, tumor cell and different cell types in tumor microenvironment should be conducted.

For the first time we reported genus *Megasphaera* and *Corynebacterium* were associated with lung cancer inflammation. Previous study found that altered genus *Megasphaera* was found in bronchoalveolar lavage fluids of lung cancer patients¹⁵. Moreover, a recent study showed that decrease of micronuclei frequency in peripheral blood lymphocytes in lung cancer patients was associated with increased sputum genus *Megasphaera*⁵⁷. Significant increment in the frequency of micronuclei frequency was detected in the blood lymphocytes of lung cancer⁵⁸, suggesting a protective role of airway *Megasphaera* on lung cancer development via modulation of host inflammatory cell. A recent meta-analysis reported respiratory *Corynebacterium* was found significantly decreased in lung cancer¹⁸. In addition, studies indicated that genus *Corynebacterium* might have a negative correlation with development of oral squamous cell carcinomas^{59,60}. All these evidences suggested an anti-tumor effect of genus *Corynebacterium*.

The microbiota has been shown to contribute to regulate host metabolism. Our studies revealed sputum microbial short chain fatty acids (propionate) metabolism pathways was associated with lung cancer associated systemin inflammation. Various enriched genera in lung cancer patients with relative low inflammation, such as genera *Veillonella*⁶¹, *Prevotella*⁶², *Porphyromonas*⁶³, *Megasphaera*⁶⁴ may produce propionate under certain circumstance. A study revealed that propionate contributed to modulate regulatory T cell production⁶⁵. Moreover, propionate has also been shown to inhibit the expression of lipopolysaccharide induced IL-6 in human mature dendritic cells⁶⁶. All these evidences suggested propionate an important factor in the cross-talk between the microbiota and the immune system. Thus, it is plausible that airway microbiota may influence lung cancer associated inflammation via production of some anti-inflammatory metabolites such as propionate.

Our study provided novel insight into the association between sputum microbiota and systemic inflammation in lung cancer patients. However, there are some limitations in our study. Firstly, the number of patients enrolled in this study is not large enough, so there may be heterogeneity considering the variability of airway microbiota. Secondly, the study is a cross-sectional study and only illustrates the phenomenon at one time point. The mechanism of the airway microbiota and their interaction between lung cancer and inflammatory cells needed further exploration. Thirdly, although studies suggested that spontaneous sputum can partly reflected the taxon

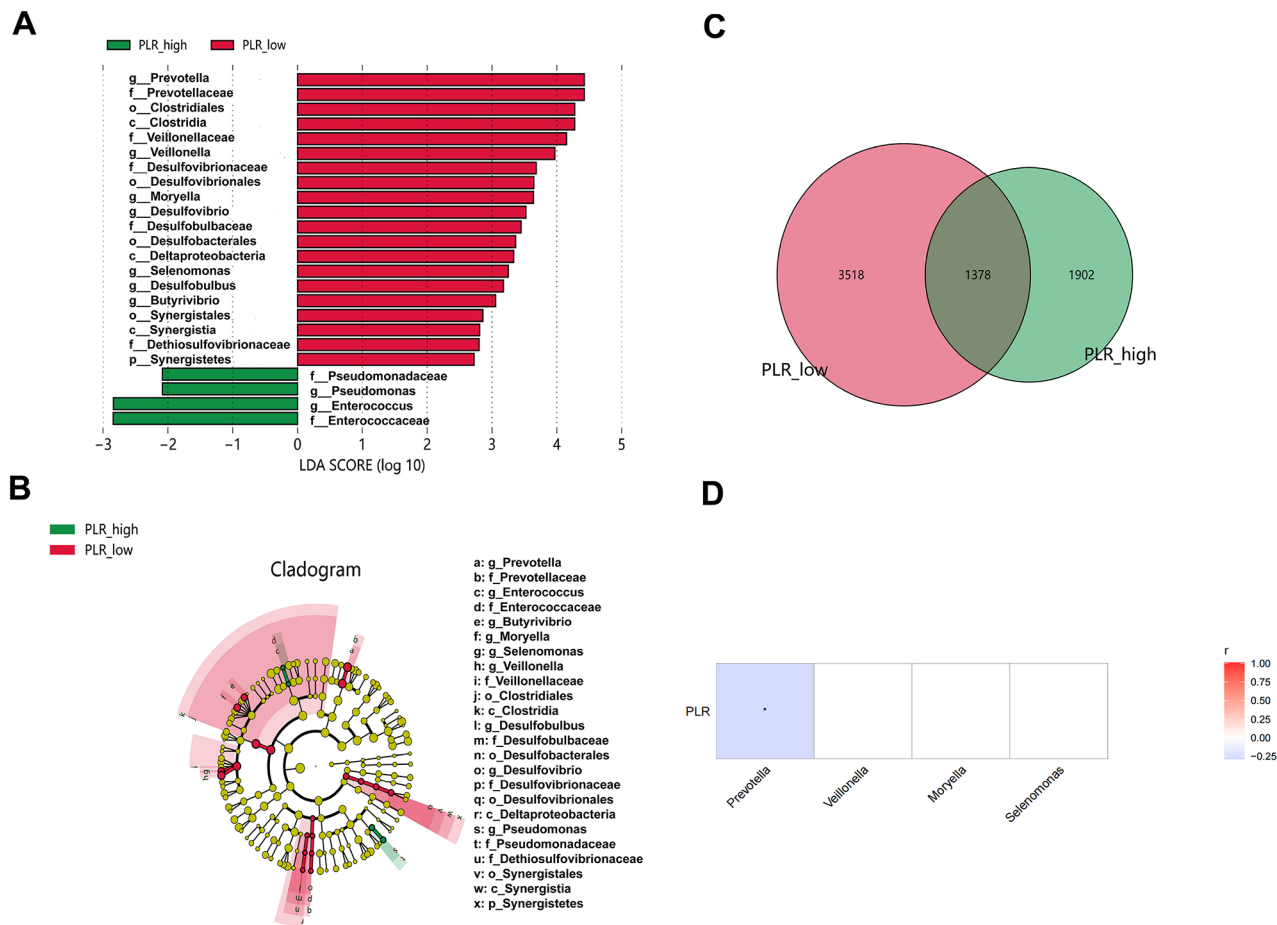


Fig. 6. Sputum taxon signatures in PLR_low group and PLR_high group. **(A)** Differentially abundant taxa between the 2 groups identified by LEFse analysis; **(B)** Differential taxa were listed based on various phylogenetic levels; **(C)** The number of specific ASVs in the 2 groups; **(D)** Correlation analysis between differential taxa and PLR level. *: $P < 0.05$. P was calculated using Spearman correlation analysis.

composition of lower airway⁶⁷, the use of sputum cannot totally surrogate lung cancer tissue and lower airway. It should be caution to interpret lower airway or intratumor microbiota using our results.

Conclusions

Collectively, the present data showed association between systemic inflammation of lung cancer and airway microbiota. The microbial structure differed between patients with different inflammation level. Patients with relative high CRP, NLR and PLR level were associated with alteration of specific airway genera and several predicted function of sputum microbiota. Our study shed light that airway microbiota might participate in inflammation modulation processes that was importantly related to lung cancer development. Further studies with large scale and multi-omics are needed to achieve a better understanding of the reciprocal interaction between microbiota, lung cancer and inflammatory cells could pave a new way for exploring new therapeutic options and biomarkers of lung cancer.

Data availability

Public database with 16 S rRNA sequencing data could be obtained online at the Sequence Read Archive (SRA). The BioProject number is PRJNA741774.

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Declarations

Ethics approval and consent for publication closure

Approval of the research protocol by an Institutional Reviewer Board: The study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University (NFEC-2022-313) and all analysis were performed was performed in accordance with the Declaration of Helsinki. Patients included in the study have provided signed informed consent.

Competing interests

The authors declare no competing interests.

Additional information

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