

Salivary microbiota analysis of patients with membranous nephropathy

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Abstract. The oral microbiota are closely related to human health. Nonetheless, to the best of our knowledge, their relationship with membranous nephropathy (MN) remains unstudied. The saliva microbiota collected from 22 patients with MN and 15 healthy controls were analyzed by next-generation sequencing, and bioinformatics analysis of the 16S ribosomal RNA gene was subsequently carried out. The Chaol and Shannon indices in patients with MN were higher than those in healthy controls. Analysis of similarities revealed that the oral microbiota in the patient group were significantly different from those in the healthy controls. At the genus level, the abundance of *Alloprevotella*, *Granulicatella*, *Prevotella*, *Streptococcus* and *Prevotella_7* was markedly higher in patients with MN than in healthy controls. Six operational taxonomic units (OTUs; OTU5, OTU28, OTU9, OTU15, OTU33 and OTU38) were found to be markedly correlated with the clinical factors creatinine, proteinuria in 24 h, estimated glomerular filtration rate and systolic blood pressure. A total of 28 Kyoto Encyclopedia of Genes and Genomes pathways were obtained from the significant OTUs. The oral

microbiota of patients with MN were investigated and it was found that OTU5, OTU28, OTU9, OTU15, OTU33 and OTU38 may be used as biomarkers. The present findings may assist in the diagnosis of patients with MN.

Introduction

Membranous nephropathy (MN) is a common glomerular disease characterized by increased thickness of the diffuse glomerular basement membrane and subepithelial immune deposits (1). A total of one-third of MN cases present primary glomerulonephritis and two-thirds present secondary MN (2). In previous years, the number of patients with MN has increased (3) and ~15% of them develop end-stage renal disease (4). The prognosis of patients with MN is poor, with 15-30% of patients relapsing after remission (5).

The microflora, especially that in the intestine, maintains human health and affects physiological functions (6). A number of studies have reported that patients with cancer, diabetes and chronic kidney disease present an imbalance in the intestinal microbiota, and that dysbiosis further stimulates disease development (7,8). Furthermore, the composition of the intestinal microflora shows different characteristics in different diseases. Yu *et al* (9) found that the composition of the gut microbiome in diabetic kidney disease and MN was markedly different. Furthermore, the oral microbiota is a reflection of health, and the dynamic changes in its diversity affect the balance between disease and health (10). It has also been reported that the oral microbiota is closely related to chronic kidney disease. Duan *et al* (11) found that the diversity of the saliva microbiota was increased, and its abundance and richness were decreased in patients with end-stage renal disease. Our previous study reported an association between the oral microbiota and immunoglobulin A nephropathy (12). Nonetheless, studies on the relationship between the oral microflora and kidney diseases are rare, especially studies investigating the relationship between the diversity and abundance of oral microbiota and MN. In the present study, saliva

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samples from 22 patients with MN and 15 healthy controls were collected for oral microbiota characterization and the possibility of using the microbiota to diagnose patients with MN was explored.

Materials and methods

Sample collection. Saliva was collected from 22 patients with MN (mean age, 41.9 years) and 15 healthy controls (mean age, 42.1 years) between March 1, 2019 and June 31, 2020, at Shenzhen Longhua District Central Hospital (Shenzhen, China). The diagnosis of MN was based on routine light and immunofluorescence microscopy examination. The criteria proposed by Ehrenreich and Churg (13) were used to determine histological staging of MN. Subjects underwent comprehensive dental and periodontal examinations by clinicians who performed clinical status assessments. The subjects brushed their teeth in the morning and 2 h after they were required to spit 4-5 ml of saliva directly into sterile collection containers over 30 min. The collected saliva was naturally produced, without any stimulation. After collection, the samples were immediately mixed with RNA later (cat. no. R0901; MilliporeSigma) and stored at -80°C until use. The patients with MN did not receive hormones, antibiotics, immunosuppressive therapy or alternate therapies when the saliva samples were collected. The serum creatinine (CR), estimated glomerular filtration rate (eGFR), systolic blood pressure (SBP), diastolic blood pressure (DBP) and proteinuria in 24 h (Pro-24) were measured before medication therapy and obtained from the records of the patients, and these clinical characteristics are listed in Table I. The present study was approved by the ethics committee of Shenzhen Longhua District Central Hospital (Shenzhen, China). All participants enrolled voluntarily in the study and signed a written consent form.

Exclusion criteria. Subjects who had diarrhea or other intestinal diseases and had taken antibiotics, probiotics or laxatives in the previous 4 weeks were excluded from the present study.

DNA extraction. A bacterial DNA kit (DP328; Tiangen Biotech Co., Ltd.) was used to extract the microbial DNA from saliva according to the manufacturer's protocols. Agarose gel (1%) electrophoresis was used to check the integrity of the extracted DNA, and the DNA was stored at -20°C for future use.

Library construction for next-generation sequencing based on 16S ribosomal RNA (16S rRNA). Illumina Bridge PCR-compatible primers, barcode primers and a pair of primers were used to amplify the V4 region of the 16S rRNA gene by DNA Taq polymerase (cat. no. P102-01, Vazyme Biotechnology Co. Ltd.). The PCR conditions were as follows: 95°C for 2 min; 30 cycles at 95°C for 15 sec, 56°C for 30 sec and 72°C for 30 sec; and 72°C for 10 min. The amplicons were purified using DNA binding beads (cat. no. DP705; Tiangen Biotech Co., Ltd.). The sequences of the degenerate primers for amplification of the V4 region were designed by Primer 3 (version 2.4.0; <https://primer3.ut.ee/>) and they were: V4 forward, 5'-GTCCAGCMGCCGCGGTAA-3' and reverse, 5'-GGACTACHVGGGTWTCTAAT-3'. An Agilent

2100 Bioanalyzer (Agilent Technologies, Inc.) was used to assess the size of the amplicons, and Qubit 3.0 (Thermo Fisher Scientific, Inc.) was used to measure the concentration. VAHTS Universal DNA Library Prep Kit for Illumina V3 (cat. no. ND607, Vazyme Biotechnology Co. Ltd.) was used to prepare sequencing library. VAHTS Library Quantification kit for Illumina (cat. no. NQ101, Vazyme Biotechnology Co. Ltd.) was used to quantify the concentration and a loading concentration of 4 pM for sequencing. The Illumina HiSeq X (Illumina, Inc., San Diego, CA, USA) was used to sequence the libraries using read length of 250 bp from each end (paired-end 250).

Quality control for raw data and data assembly. The Trimmomatic (V0.33) software (14) was applied to pair-end raw data for quality control, and all the parameters were set as default to obtain clean reads. The mothur (V1.35.1) software (15) was used to categorize the clean reads for each sample according to their barcodes. The FLASH (V1.2.11) software (16) was used to assemble the paired-end reads to obtain raw tags (original splicing sequence). Clean tags (effective splicing fragments) were obtained after quality control and filtering.

Bioinformatics analysis. All clean tags were clustered by USEARCH (v9.0.2132) (17) and aligned to the operational taxonomic units (OTUs) by UPARSE (V10.0.240) (18) to identify the taxonomy. The parameters for identity were set as the default at 97%. Each sequence was sorted out into OTUs by Quantitative Insights Into Microbial Ecology (QIIME) (V1.9.1) (19) after singleton OTUs were removed by USEARCH and chimeric sequences were removed by UCHIME (V4.1) (20). QIIME was used to select the best representative sequence from optimized QIIME-selected sequences for the final OTU cluster to align with the Human Oral Microbiome Database (21). The species annotation information of OTUs was obtained and the OTUs that were annotated as chloroplast or mitochondrion and those which could not be annotated to any species were deleted. The relative abundance of each OTU was each OTU read normalized to total OTUs reads of each sample (in-house scripts). QIIME was also used to perform α -diversity analysis of all OTU cluster data. The nonparametric Mann-Whitney test (two-tailed; 95% CI) in GraphPad Prism (version 6.0; GraphPad Software, Inc.) was performed to analyze the abundance difference between patients with MN and healthy controls at the genus level. $P < 0.05$ was considered to indicate a statistically significant difference. Analysis of similarities (ANOSIM) was performed between the patients with MN and healthy controls using the Vegan package (V2.4-3) of R language (22). Chao1 and Shannon indices between the patients with MN and healthy controls were analyzed using QIIME. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Lefse analyses were predicted based on the abundance of OTUs using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST; V1.1.4) software (23) and Lefse (V1.1.2) (24).

Statistical analysis. The differences of clinical indicators between patients with MN and healthy controls were analysed by one way ANOVA using Tukey's post hoc. Metastats

Table I. Clinical information of patients with membranous nephropathy and healthy controls.

A, Patients with membranous nephropathy								
ID	Sex	Age, years	CR, $\mu\text{mol/l}$	eGFR, ml/min/1.73 m^2	SBP, mmHg	DBP, mmHg	Pro-24, g/day	Pathological results
160801	M	51	304.4	20.1	137	69	5.55	MN, stage III
160901	M	30	68	126.2	122	83	4.36	MN, stage II
161004	M	46	90	83.8	112	67	7.26	MN, stage II
161005	F	43	48	130.2	126	72	1.92	MN, stage II
161007	F	44	61	98.2	123	83	3.66	MN, stage II
170102	F	39	67	90.3	122	75	4.36	MN, stage II
170103	M	53	82	90.6	139	82	8.90	MN, stage II
170201	M	42	93	82.2	117	77	2.20	MN, stage II
170302	F	45	83	68.5	139	79	1.87	MN, stage I
170402	M	50	90	82.4	161	100	3.63	MN, stage II
170403	M	30	95	85.8	149	73	7.50	MN, stage II
170407	M	34	64	132.0	113	81	5.45	MN, stage II
170502	M	44	80	96.8	162	106	9.33	MN, stage II
170903	M	50	220	29.4	130	74	6.33	MN, stage III
180102	M	37	79	101.8	119	61	0.78	MN, stage II
180304	F	43	71	82.8	139	81	1.80	MN, stage III
180305	F	24	64	105.1	105	70	2.94	MN, stage II
180602	M	46	77	100.3	137	92	5.96	MN, stage II
180702	M	44	78	99.7	105	56	3.26	MN, stage II
180801	M	35	88	90.9	121	81	2.93	MN, stage II
180902	F	47	54	111.6	135	85	3.93	MN, stage I
181003	M	44	124	58.4	108	73	10.36	MN, stage II
B, Healthy controls								
ID	Sex	Age, years	CR, $\mu\text{mol/l}$	eGFR, ml/min/1.73 m^2	SBP, mmHg	DBP, mmHg	Pro-24, g/day	Pathological results
HE1	M	40	69	117.1	121	56	0.04	Healthy
HE2	M	55	80	92.5	98	73	0.03	Healthy
HE3	M	34	68	123.0	105	79	0.10	Healthy
HE4	M	33	78	105.7	123	55	0.034	Healthy
HE5	M	42	70	114.0	130	85	0.06	Healthy
HE6	M	34	73	113.4	116	79	0.05	Healthy
HE7	M	40	75	106.4	108	88	0.08	Healthy
HE8	M	45	67	118.3	123	72	0.12	Healthy
HE9	M	50	82	91.7	113	59	0.09	Healthy
HE10	M	43	54	119.9	125	86	0.08	Healthy
HE11	F	58	58	98.5	108	84	0.10	Healthy
HE12	F	43	54	113.6	107	76	0.08	Healthy
HE13	F	38	67	90.8	98	82	0.12	Healthy
HE14	F	34	50	130.2	106	75	0.03	Healthy
HE15	F	42	57	107.3	113	83	0.04	Healthy

Clinical indicators of each patient and healthy controls are listed. Pathological results of patients with membranous nephropathy are also provided. The normal range of CR is 57-97 $\mu\text{mol/l}$. SBP and DBP were measured before medication therapy. CR, creatinine; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; Pro-24, proteinuria at 24 h; M, male; F, female; MN, membranous nephropathy.

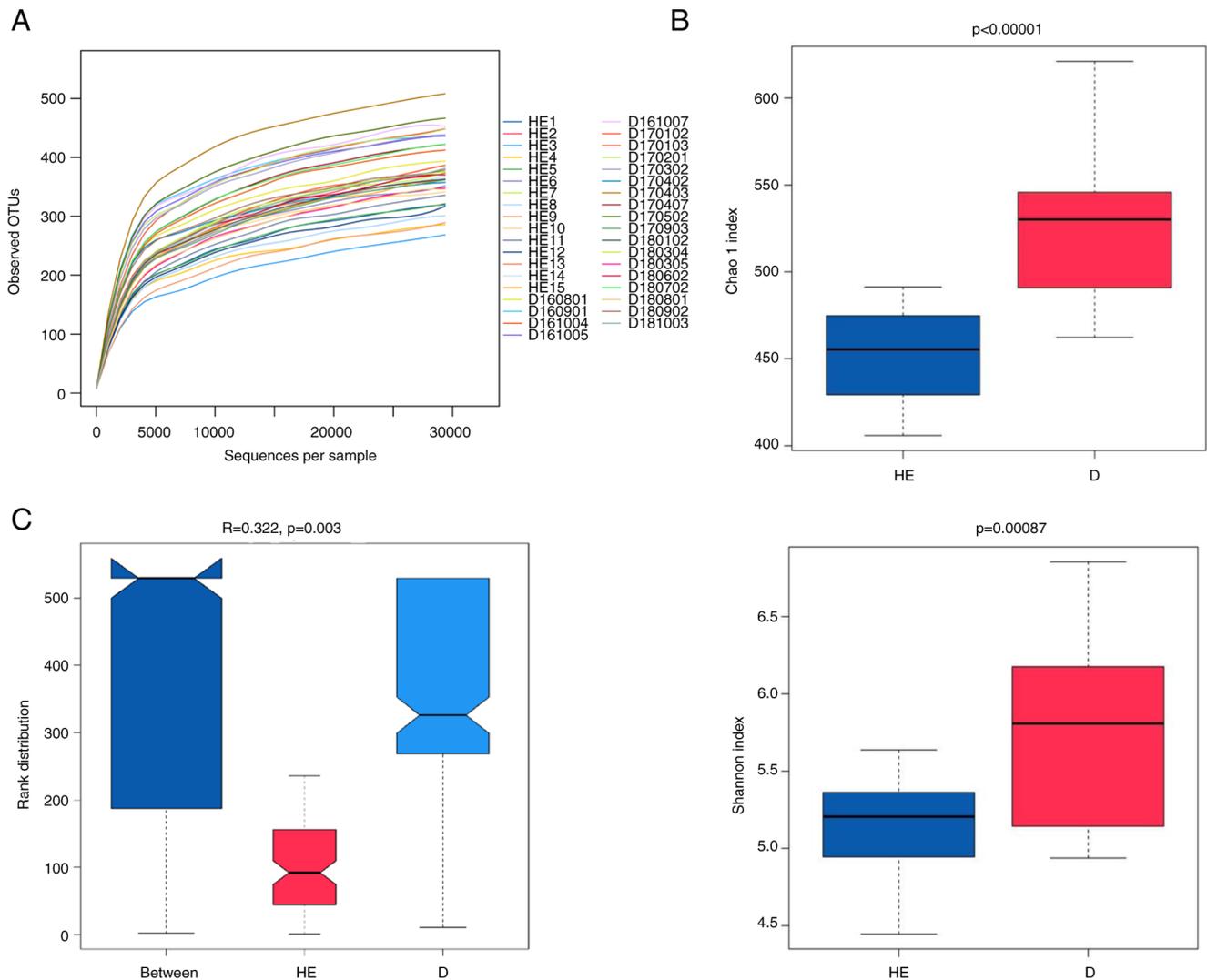


Figure 1. (A) Rarefaction curves of the samples collected from patients with membranous nephropathy and healthy controls. (B) Chao and Shannon index value distribution of samples. (C) ANOSIM results. The y-axis represents the Bray-Curtis rank. OTU, operational taxonomic unit; ANOSIM, analysis of similarities; PC, principal component; HE, healthy control samples; D, membranous nephropathy samples.

analysis allows a comparison of metagenomic samples (represented as counts of individual features such as organisms, genes and functional groups) from two treatment populations (for example, healthy vs. disease) and identifies those features that statistically distinguish the two populations. Spearman was used to analyse the correlation between OTUs and clinical indicators using Stats package of R language (22).

Results

Characterization of the sequencing results. The salivary microbiota of patients with MN and healthy controls were analyzed by sequencing of the 16S rRNA gene using Illumina platform. After quality control, the total number of salivary microbial sequences was 1,534,154 reads at an average of 69,734 reads per sample for the 22 patients with MN, and 1,414,400 at an average of 88,400 reads per sample for the 15 healthy controls. The rarefaction curve plateaued, indicating that the sequencing depth could be used to analyze the composition of the salivary microbiota (Fig. 1A).

Taxonomic analysis of the microbiota. Through the OTU clustering of sequencing reads, a total of 1,362 OTUs were found in the 22 samples of the MN group. The number of OTUs in each sample ranged between 391 and 559, with an average value of 462. The healthy group had fewer OTUs than the group of patients with MN. The number of OTUs in each sample was 344-453, with an average of 401. Bacterial species richness (Chao1 index) and diversity (Shannon index) of the patients with MN were significantly higher than those of the healthy controls (Fig. 1B). The Chao1 index (a value of 521) of patients with MN was higher than that of the healthy controls (which presented a Chao1 index of 452). The P-values for the Chao1 index and Shannon index were <math>< 0.00001</math> and 0.00087, respectively. ANOSIM is a non-parametric statistical test widely used to assess the similarities between groups. According to ANOSIM analysis, the microbial composition in the MN group was statistically different from that in the healthy group ($P = 0.003$; Fig. 1C).

Heatmap analysis. The OTU heatmap showed that the MN group and healthy control group were mostly clustered

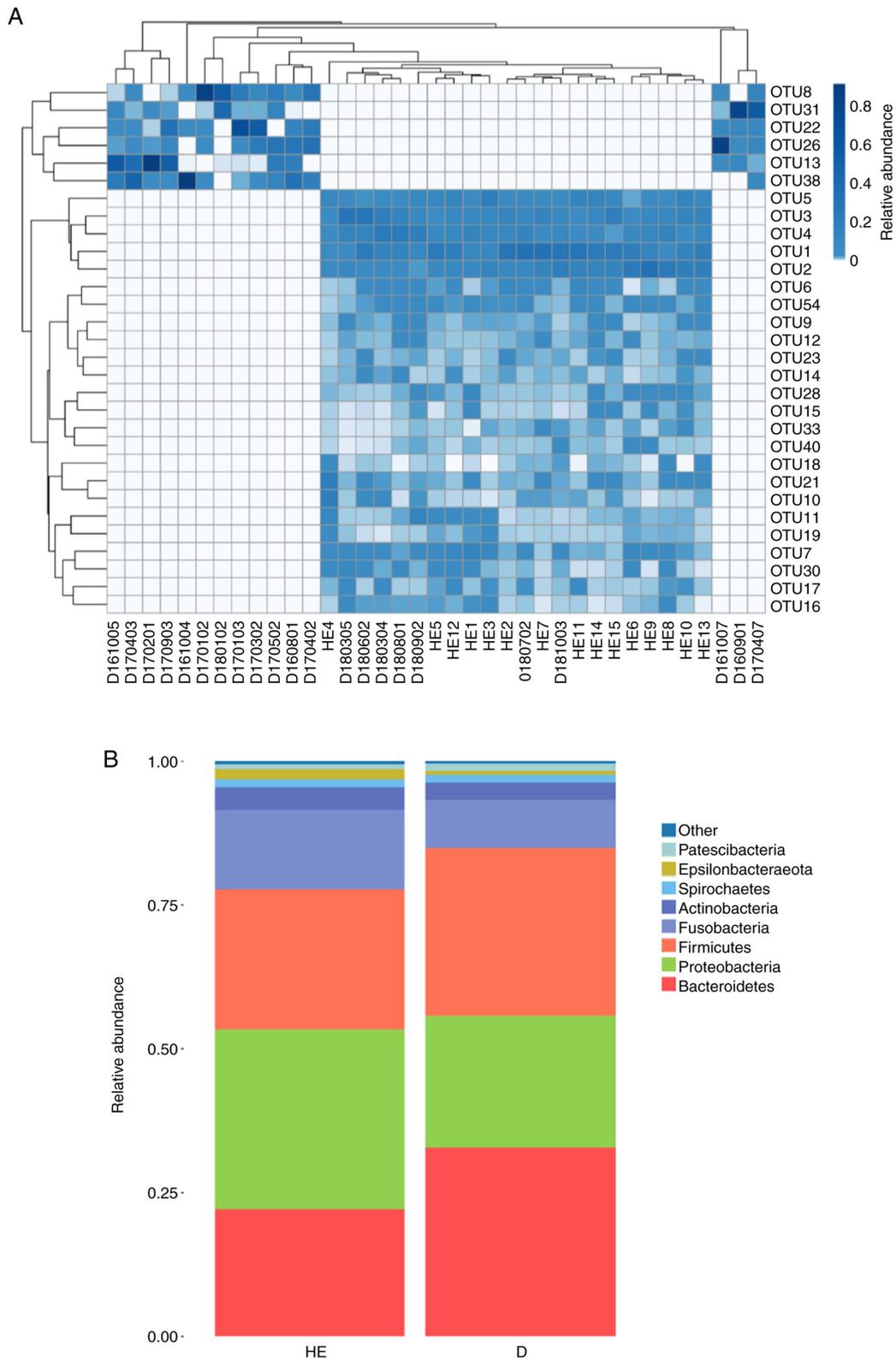


Figure 2. (A) Heatmap analysis based on OTU abundance. Different colors indicate relative abundance. The relative abundance increases from white to blue. In the heatmap, the x-axis indicates the patient ID number and the y-axis indicates the OTU number. (B) Comparison of the relative abundance at the phyla level between patients with membranous nephropathy and healthy controls. OTU, operational taxonomic unit; HE, healthy control samples; D, membranous nephropathy samples.

separately (Fig. 2A; Table SI). In the microbial classification, eight phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Fusobacteria*, *Actinobacteria*, *Spirochaetes*, *Patescibacteria* and *Epsilonbacteraeota*) accounted for >99% of the microbiota

in the MN group and >99% in the healthy control group (Fig. 2B). Metastat analysis was conducted at the genus level and revealed that there were 47 different genera between the MN group and the healthy control group ($P < 0.05$; Table SII).

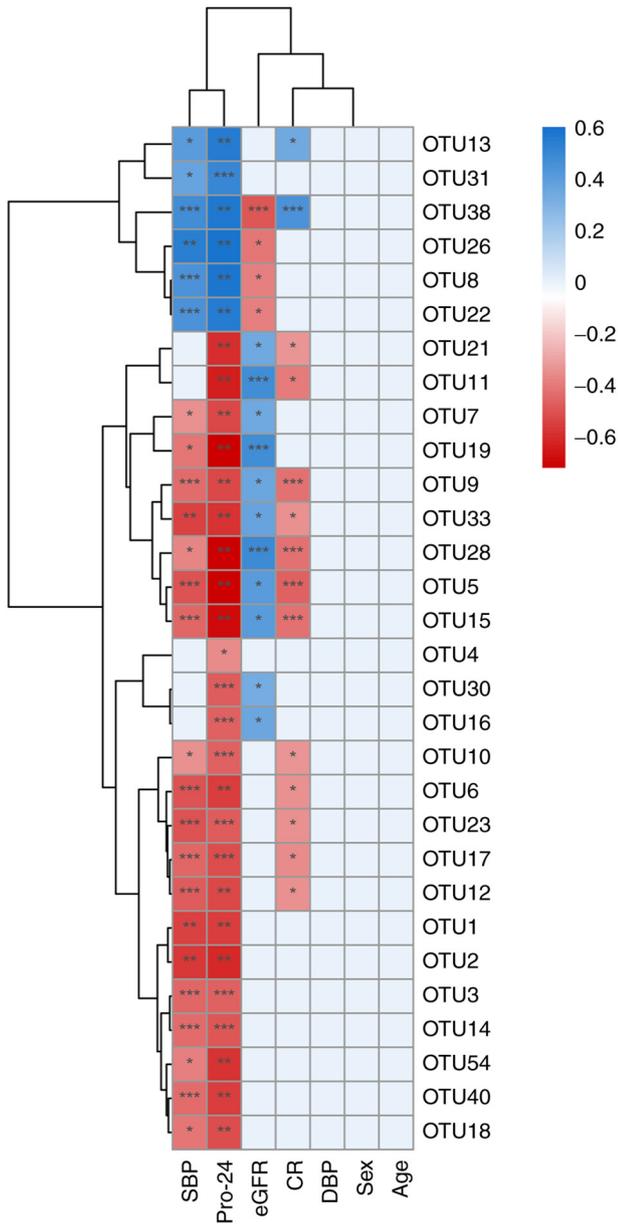


Figure 3. Correlation between OTUs and the clinical indicators. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. Blue indicates a positive correlation and red indicates a negative correlation. OTU, operational taxonomic unit; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; Pro-24, proteinuria at 24 h; CR, creatinine; DBP, diastolic blood pressure.

Correlation analysis of biochemical indicators and OTUs. OTUs of each sample were used to analyze the correlation with the biochemical indicators CR, Pro-24, eGFR, SBP and DBP, which are used clinically (Table SIII). Pro-24, SBP and eGFR were found to be significantly different between the patients with MN and healthy controls ($P<0.05$; Table SIV). The abundance of OTUs was considered to be related to clinical indicators. Serum CR was negatively correlated with 12 OTUs, of which OTU12, OTU17, OTU23, OTU6, OTU10, OTU33, OTU21 and OTU11 had a P -value <0.05 , and OTU5, OTU9, OTU15 and OTU28 had a P -value <0.001 , and it was positively correlated with OTU13 ($P<0.05$) and OTU38 ($P<0.001$). Pro-24 was negatively correlated with 24 OTUs (OTU4 with $P<0.05$; OTU1, OTU2, OTU5, OTU6, OTU7, OTU9, OTU11, OTU12,

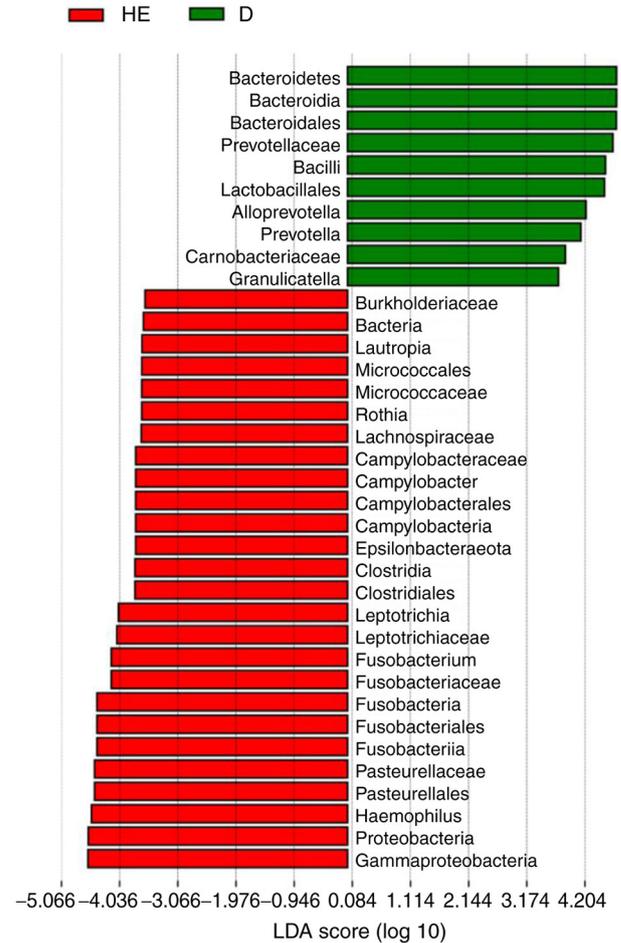


Figure 4. Lefse analysis. Green indicates biomarkers for patients with membranous nephropathy; red indicates biomarkers for healthy controls. HE, healthy control samples; D, membranous nephropathy samples; LDA, linear discriminant analysis.

OTU15, OTU18, OTU19, OTU21, OTU28, OTU33, OTU40 and OTU54 with $P<0.01$; OTU3, OTU10, OTU14, OTU16, OTU17, OTU23 and OTU30 with $P<0.001$) and positively correlated with 6 OTUs (OTU8, OTU13, OTU22, OTU26 and OTU38 with $P<0.05$; and OTU31 with $P<0.001$). eGFR was negatively correlated with 4 OTUs (OTU8, OTU22 and OTU26 with $P<0.05$; OTU38 with $P<0.001$) and positively correlated with 11 OTUs (OTU5, OTU7, OTU9, OTU15, OTU16, OTU21, OTU30 and OTU33 with $P<0.05$; OTU11, OTU19 and OTU28 with $P<0.001$). The SBP was negatively correlated with 19 OTUs and positively correlated with 6 OTUs. No OTUs were associated with DBP, age or sex (Fig. 3). Among all OTUs, OTU5, OTU9, OTU15, OTU28 and OTU33 were significantly correlated with SBP, Pro-24, eGFR and CR.

Lefse and PICRUST analyses. Lefse analysis was conducted to explore possible biomarkers and PICRUST analysis was conducted to explore pathways. Lefse analysis showed that 10 bacteria could be possibly used as biomarkers for patients with MN (Fig. 4), including *Bacteroidales* (order, $P=6.59\times 10^{-5}$), *Prevotellaceae* (family, $P=2.62\times 10^{-4}$), *Lactobacillales* (order, $P=0.0412$), *Alloprevotella* (genus, $P=7.81\times 10^{-3}$), *Prevotella* (family, $P=0.0145$), *Carnobacteriaceae* (family, $P=0.0172$) and *Granulicatella* (genus, $P=0.0328$).

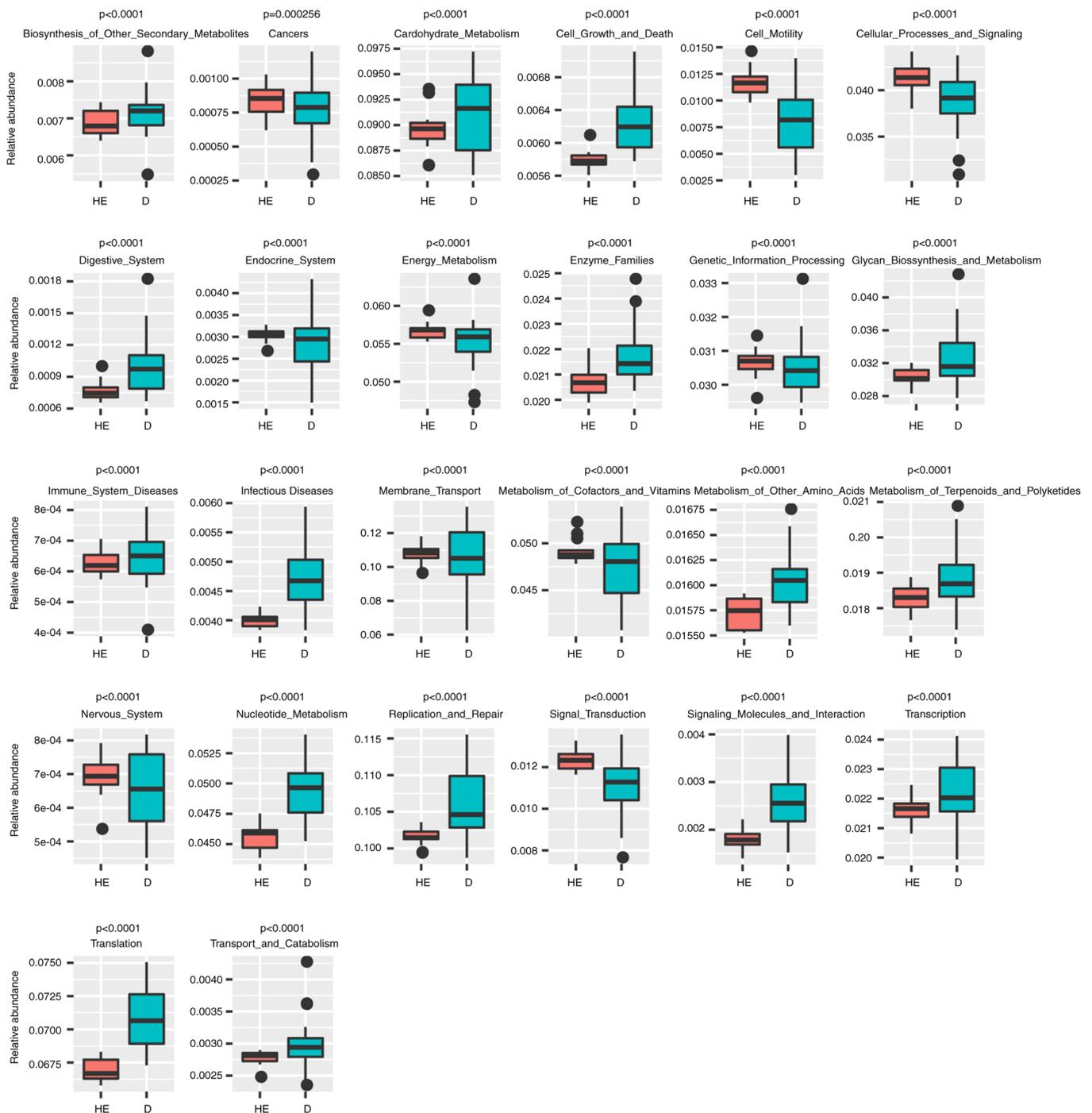


Figure 5. Kyoto Encyclopedia of Genes and Genomes pathways predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States software. The number on the Y axis is the relative abundance. HE, healthy control samples; D, membranous nephropathy samples.

A total of 26 KEGG pathways were obtained from significant OTUs using PICRUST (Fig. 5; Table SV), including 'Immune_System_Diseases' ($P=1.81 \times 10^{-6}$), 'Glycan_Biosynthesis_and_Metabolism' ($P=1.91 \times 10^{-6}$), 'Energy_Metabolism' ($P=7.76 \times 10^{-7}$), 'Endocrine_System' ($P=4.05 \times 10^{-7}$) and 'Digestive_System' ($P=1.31 \times 10^{-4}$).

Discussion

MN is a common glomerular disease; however, there are few studies on the oral microbiota of patients with MN. Through

high-throughput sequencing of the 16S rRNA gene of oral microbiota and the subsequent bioinformatics analysis, the composition and characterization of oral microbiota in patients with MN were thoroughly investigated and the associations between clinically used biochemical indicators and OTUs were evaluated. ANOSIM analysis revealed that there was a significant difference in the composition of microbial species between the patients with MN and healthy controls ($P=0.003$). α -diversity analysis showed that The Chao1 index of patients with MN was higher than that of the healthy controls, indicating that the total number of oral microbes

in the patients with MN was higher than that in the healthy controls. Although α -diversity is normally lower in patients than in controls, some reports have indicated that α -diversity is higher in patients than in the control group and might be associated with clinical benefits (25-27). The exact meaning of the observation that α -diversity in patients with MN was higher than in the controls needs further exploration. Additionally, the Shannon index of the patients with MN was higher than that of the healthy controls (5.72 vs. 5.13), indicating that the microbial diversity of the patients with MN was also higher than that of the healthy controls.

According to Lefse analysis, there were 10 significantly different bacteria in patients with MN. At the phylum and class level, *Bacteroidetes*, *Bacteroidia* and *Bacilli* may be candidate biomarkers. *Lactobacillales* and *Bacteroidales* at the order level, *Prevotellaceae*, *Carnobacteriaceae* and *Prevotella* at the family level, and *Alloprevotella* and *Granulicatella* at the genus level may serve as potential biomarkers for patients with MN. *Streptococcus* and *Granulicatella* produce antimicrobial compounds that inhibit bacterial growth, and thus, are beneficial to the oral cavity (28). The abundance of *Streptococcus* and *Granulicatella* was significantly higher in patients with MN than in the control group, which was also observed in the case of immunoglobulin A nephropathy (12). However, the present findings were different from those reported by Piccolo *et al.* (29), and this may be due to the difference in ethnicities. Meanwhile, the phylum *Bacteroidetes* normally implies poor health in the host (9).

The potential KEGG pathway function of microorganisms was predicted using PICRUST (30). The abundance of the pathway 'Immune_System_Diseases' in the MN group was significantly higher than that in the healthy individuals (Fig. 5), indicating that the microbial environment of patients with MN is likely to change according to the immune system condition.

Regarding clinical indicators, CR is a product of muscle metabolism in the human body and is mainly excreted from the body by glomerular filtration. When acute or chronic glomerulonephritis causes the glomerular filtration function to decrease, serum CR levels can increase. eGFR is an indicator of kidney function. It mainly refers to the excretion capacity of the kidneys per unit of time and is used to assess kidney function. Pro-24 is the 24 h urine protein content. Increased urine protein is often present in various glomerular diseases, such as acute nephritis, chronic nephritis, nephrotic syndrome and lupus nephritis (31). Patients with nephritis also often present high blood pressure (32,33). The correlations between the OTU abundance of the 22 patients with MN and these clinical indicators were analyzed to explore the relationship between the microbes and the clinical indicators and the possibility of using them to diagnose and assess the status of the patients with MN. Six OTUs (OTU5, OTU28, OTU9, OTU15, OTU33 and OTU38) were found to be significantly correlated with CR, Pro-24, eGFR and SBP, implying that they could be used as biomarkers for the diagnosis of patients with MN.

The present study has certain limitations. First, the sample size was relatively small. This was a preliminary proof-of-concept study that indicated that the salivary microbiota was associated with MN. The sample size will be increased in subsequent research. Second, it was a retrospective study. For the biomarkers to be used in clinical settings, a prospective

study is necessary. Third, the present study enrolled patients from southern China. Their diet is different from patients from northern China (34). Whether and how the different diets affect our conclusions requires further investigation.

In conclusion, to the best of our knowledge, there are no previous reports on the relationship between oral microbiota and MN. The present study was the first to investigate the oral microbiota in patients with MN. The present results revealed that there were significant differences between the microbiota of the patients with MN and that of healthy controls and that certain microbial strains and OTUs (OTU5, OTU28, OTU9, OTU15, OTU33 and OTU38) can be used as biomarkers to facilitate the diagnosis of patients with MN in clinical settings.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the National Center for Biotechnology Information repository (no. PRJNA698736; <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA698736>).

Authors' contributions

BH and HG conceived and supervised the study. SL, SZ, LP and WH wrote the draft of the manuscript and designed the experiments. HC and XWe analyzed the data. RL, CL, PZ, XWa and WL collected the samples and clinical information and analyzed the data. SL, ZX and YZ designed the experiments, revised the manuscript and provided helpful advice. SL and HG confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by The Ethics Committee of Shenzhen Longhua District Central Hospital (Shenzhen, China). Informed written consent was obtained from each patient and healthy subject enrolled in the present study.

Patient consent for publication

Consent for publication was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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