Original Article
Dysbiosis of gut microbiota is associated with serum lipid profiles in male patients with chronic traumatic cervical spinal cord injury

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Abstract: Neurogenic bowel dysfunction (NBD) and gut dysbiosis frequently occur in patients with traumatic cervical spinal cord injury (TCSCI). We evaluated neurogenic bowel management and changes in the gut microbiota in patients with TCSCI as well as associations between these changes and serum biomarkers. Fresh fecal and clinical data were collected from 20 male patients with TCSCI and 23 healthy males. Microbial diversity and composition were analyzed by sequencing the V3-V4 region of the 16S rRNA gene. Moderate NBD was observed in patients with TCSCI. The diversity of the gut microbiota was lower in patients with TCSCI than in healthy adults. Furthermore, patients with TCSCI showed altered levels of serum biomarkers related to lipid metabolism, indicating unfavorable lipid profiles. Interestingly, Firmicutes had a positive effect and Verrucomicrobia had a negative effect on lipid metabolism (P < 0.05). At the genus level, *Bacteroides* and *Blautia* were significantly more abundant in patients than in healthy subjects and could be associated with lipid metabolism (P < 0.05). *Faecalibacterium, Megamonas,* and *Prevotella,* which were correlated with lipid metabolism markers, may be suitable targets for the treatment of TCSCI. *Lactobacillus* was positively correlated with glucose levels. The dysbiosis of several key gut bacteria was associated with serum biomarkers of lipid metabolism in patients with TCSCI. The observed interdependency of the microbiota and lipid metabolism provides a basis for understanding the mechanisms underlying lipid disorders after cervical SCI.

Keywords: Gut microbiota dysbiosis, lipid metabolism, chronic traumatic cervical SCI, neurogenic bowel dysfunction, firmicutes, serum lipid biomarkers

Introduction

Traumatic cervical spinal cord injury (TCSCI) is a life-threatening condition that not only leads to a loss of motor and sensory function but also to dysfunctions of multiple organs and pathways, including the respiratory, gastrointestinal, urinary, metabolism, and autonomic nervous system [1-3]. Despite a recent increase in survival rates, the quality of life remains poor for patients with TCSCI, as cervical injuries often result in paralysis and bowel dysfunction. Neurogenic bowel dysfunction

(NBD) is a major physical and psychological problem in patients with SCI. The two main manifestations of NBD are constipation (in 40-58% of patients) and fecal incontinence (in 2-61% of patients) [3-5]. Patients with chronic cervical SCI tend to spend more time evacuating their bowels than healthy individuals. An effective bowel program should address issues of fecal incontinence, constipation, and effective evacuation. In the current study, we documented neurogenic bowel management in male patients with chronic TCSCI at our center.

The human intestinal tract is colonized by thousands of bacteria species whose number and genetic content exceed those of the host by factors of 10- and 150-fold, respectively [6]. These taxa are critical for normal digestion, nutrient absorption, metabolism, and cell function [7, 8]. Common causes of gut dysbiosis include antibiotic use, prolonged stress, and gastrointestinal dysfunction [7-10]. Most patients with acute complete SCI have substantial changes in physiological processes after injury, such as the excessive growth of bacteria and the displacement of the intestinal flora, making the intestines the largest "endotoxin pool" in the human body. In addition, cervical SCI often requires antibiotic treatment due to compounding factors, such as pulmonary infection. The use of antibiotics also affects healthy intestinal micro-ecological systems [11-14]. Gut dysbiosis is highly likely in TCSCI. Few clinical studies have evaluated the intestinal microecology after cervical SCI. In a clinical study of intestinal microbes in 30 patients with SCI and 10 healthy controls using 16S rRNA technology, Gungor et al. detected significantly fewer butyrate-producing members in patients with SCI than in healthy individuals [14].

Lipoprotein metabolism is influenced by a wide range of factors, including anthropometric factors, peripheral insulin resistance, catecholamines, physical activity, performance capacity, and sex [15-18]. Patients with SCI have a high incidence of dyslipidemia and cardiovascular disease [15]. The adverse lipid profile in men with SCI compared with control subjects results from physical inactivity with low maximum oxygen uptake levels depending on the injury level [16, 17]. In previous studies, differences were found between the lipoprotein profiles of men with SCI and those of male controls. Among men with SCI, tetraplegia was associated with higher levels of very low-density lipoprotein (VLDL) and triglycerides (TG) and lower levels of high-density lipoproteins (HDL). Similarly, paraplegia was associated with high total cholesterol (TCHO) and LDL [18]. In the current study, we report the lipid profiles of patients with chronic TCSCI at our center.

Therapies to improve quality of life and restore function, even partially, would have a huge impact on patients with TCSCI and their caretakers [19]. Analyses of the gut microbiota in patients with TCSCI might provide insight into the interactions between gut microbes and lipid metabolism and provide a basis for diagnosis and therapy. The aim of this study was to investigate the association between the intestinal microbiota and serum biomarkers by a comparative analysis of male patients with chronic TCSCI and healthy subjects.

Materials and methods

Ethics statement

Approval from the hospital ethics committee was obtained before commencing the study (No. CRRC-IEC-RP-SQ-006-01).

Patients and controls

A total of 20 male patients with cervical chronic TCSCI were enrolled at our center between June 2017 and November 2017. We carried out a face-to-face clinical questionnaire survey and obtained signed informed consent before assessment. In addition, we used the "International Spinal Cord Injury Core Data Set", "International bowel function basic spinal cord injury data set", and "International bowel function extended spinal cord injury data set" to obtain the neurogenic bowel dysfunction (NBD) symptoms data [20, 21]. Patients were included if they met the following criteria: 1) neurologically complete SCI (ASIA grade A) occurring 6 or more months prior to the study, 2) 18-60 years of age, 3) traumatic cervical SCI, and 4) male. The diagnostic criteria for patients with chronic traumatic complete SCI included 1 and 3 of the above criteria. The exclusion criteria were as follows: 1) inability to complete a questionnaire survey; 2) a history of antibiotic or probiotic use in the first month before enrolment; and 3) diabetes, gastrointestinal system diseases, multiple sclerosis, and immune metabolic diseases.

The clinical dates and fresh stool specimens of the subjects were collected. After extracting fecal genomic DNA, the V3-V4 region of 16S rDNA was amplified, and an Illumina MiSeq platform was used to analyze and compare the gut microbiota of healthy males with that of male patients with SCI.

The criteria for the healthy control group were as follows: 1) 18-60 years of age; 2) no history

of antibiotics or probiotics use 1 month prior to study; and 3) no history of diabetes, gastrointestinal system diseases, multiple sclerosis, and immune metabolic diseases. All subjects were selected before sampling and signed informed consent with a full understanding of the sampling process and research options. All patients and healthy subjects were fed standard hospital food 2 weeks before stool collection to exclude the potential effects of diet on the gut microbiota.

Microbial diversity analysis

Stool sampling: A total of 43 fresh specimens were collected, including 23 healthy males and 20 patients with cervical SCI. Fresh fecal samples were collected and transferred to the laboratory. Samples were placed in a new 2-mL sterile centrifuge tube, quickly placed on ice, and transferred to a freezer maintained at -80°C for cryopreservation. The entire sampling process was completed in 30 minutes.

DNA extraction and PCR amplification: Microbial DNA was extracted from stool samples using an E.Z.N.A.® Stool DNA Kit (Omega Biotek, Norcross, GA, U.S.) according to the manufacturer's protocols. The V3-V4 region of the bacterial 16S rRNA gene was amplified by PCR (95°C for 2 minutes, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 minutes) using primers 338F 5'-ACTCCTACGGG-AGGCAGCA-3' and 806R 5'-GGACTACHVGG-GTWTCTAAT-3'. PCRs were performed in triplicate in a 20 μL mixture containing 4 μL 5 × FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL FastPfu Polymerase, and 10 ng template DNA.

Illumina MiSeq sequencing: Amplicons were extracted from 2% agarose gels, purified by using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) and quantified by using QuantiFluor™-ST (Promega, U.S.). Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive database (Accession Number: SRP158549).

Processing of sequencing data: Raw fastq files were quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) the reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window. (ii) Sequences whose overlap was longer than 10 bp were merged according to their overlap with no more than 2 mismatched bp. (iii) The sequences of each sample were separated according to barcodes (exactly matching) and primers (allowing 2 mismatching nucleotides), and reads containing ambiguous bases were removed.

Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70% Roche 454 (Roche, Switzerland). High-throughput sequencing of the PCR products was performed by Shanghai Majorbio Biological Technology Co. Ltd., Shanghai, China.

Bioinformatics and statistical analysis

Sequencing reads were processed using QIIME (version 1.9.0), with additional quality trimming, demultiplexing, and taxonomic assignments. Profiling of the predictive urine microbiota was performed using PiCRUSt based on the Greengenes database, accessed August 13, 2013 [22]. The KW rank sum test and pairwise Wilcoxon test were used for the identification of the different markers, and LDA was used to score each feature in LEfSe analysis. The index of alpha diversity was calculated with QIIME based on sequence similarity of 97%. Beta diversity was measured by unweighted UniFrac distance, which was also calculated by QIIME. Hierarchical clustering was performed, and a heatmap was generated using Spearman's rank correlation coefficient as a distance measure and a customized script developed in the R statistical package. The output file was further analyzed using the Statistical Analysis of Metagenomic Profiles software package (version 2.1.3) [23].

Statistical analysis was performed using the SPSS data analysis program (version 21.0) and Statistical Analysis of Metagenomic Profiles software. For continuous variables, independent t-tests, Welch's t-tests, White's nonpara-

Table 1. Descriptive data of the NBD management in chronic cervical traumatic complete SCI participants

	TCSCI n (%)
Course	69.4 ± 52.72 (months)
Segment	C3-C4 12 (60%)
	C5-C8 8 (40%)
NBD Scores	11.17 ± 5.16
Defecation time	41.789 ± 19.29 (minutes)
Pathogenesis	Traffic accident 10 (50%)
	Bruised by heavy object 4 (20%)
	Falling down 3 (15%)
	Other causes 3 (15%)
Frequency of bowel care	Once daily: 5 (25%)
	Not daily but more than twice every week 15 (75%)
Main techniques for fecal evacuation	Suppository 20 (100%)
	Manual evacuation 8 (40%)
	Digital stimulation 6 (30%)
Supplementary interventions	Abdominal massage 10 (50%)
	Digital anus-rectal stimulation 7 (35%)
	Digital evacuation 2 (10%)
	Taking cathartic drug 1 (5%)
Timing of bowel care	Morning 1 (5%)
	Afternoon 14 (70%)
	Evening 5 (25%)
Location during evacuation	Bed 12 (60%)
	Potty chair 6 (30%)
	Toilet seat 2 (10%)
Degree of assistance needed	Need all help 19 (95%)
	Need special help 1 (5%)
Abdominal symptoms	Abdominal discomfort 12 (60%)
	Constipation 14 (70%)
	Bloating symptom 16 (80%)Flatus incontinence 18 (90%)

metric t-tests, and Mann-Whitney U-tests were applied. For categorical variables between groups, we used either the Pearson chi-square or Fisher's exact test, depending on validity of assumption. For taxa among subgroups, ANOVA was applied (Tukey-Kramer post hoc test was used, and the effect size was Eta-squared) with Benjamini-Hochberg FDP false discovery rate correction [23, 24]. All tests of significance were two-sided and $P < 0.05$.

Results

Characteristics and neurogenic bowel management of male patients with chronic TCSCI

In total, 20 patients with chronic cervical SCI fulfilling the enrolment criteria were interviewed

and asked to complete a survey (**Tables 1**, **2**). The causes of injury in descending order of frequency were traffic accidents (50%), bruised by heavy object (20%), and falls from heights (15%). The mean NBD score was 11.17 ± 5.16. The mean defecation time was 41.789 ± 19.29 min. Most patients (75%) engaged in bowel care more than twice every week, and the remaining patients (25%) engaged in bowel care once daily. The main techniques used for fecal evacuation in descending order of frequency were suppository (100%), digital stimulation (40%), and manual evacuation (30%). Supplementary interventions for fecal evacuation included abdominal massage (50%), digital anus-rectal stimulation (35%), digital evacuation (10%), and cathartic drug use (5%).

Table 2. Demographics and serum biomarkers between male healthy and patients with chronic cervical traumatic complete SCI

	Health Male	TCSCI-Male	P
N	23	20	
AGE	40 ± 9.03	39.9 ± 10.57	0.998
BMI	24.8 ± 2.677	23.11 ± 2.876	0.022
ALT	26.791 ± 16.367	26.2 ± 19.303	0.903
AST	23.848 ± 17.097	21 ± 9.8	0.429
UREA	4.416 ± 1.224	4.403 ± 1.14	0.966
CR	64.3 ± 12.701	60.7 ± 11.8	0.265
UA	309 ± 69.81	378.1 ± 64.93	0.001
GLU	4.343 ± 0.528	5.266 ± 1.964	0.033
TG	1.436 ± 1.319	1.928 ± 1.207	0.137
TCHO	3.695 ± 0.794	4.217 ± 1.005	0.038
HDL	0.9152 ± 0.2091	0.917 ± 0.163	0.974
LDL	2.177 ± 0.596	2.617 ± 0.701	0.005
APOA1	1.417 ± 0.11	1.03 ± 0.129	0.001
APOB	0.833 ± 0.11	0.927 ± 0.183	0.045
LP-A	40.465 ± 25.32	94.16 ± 72.16	0.002
NEFA	0.251 ± 0.09	0.353 ± 0.207	0.038

More than half of the bowel care time occurred in the afternoon (70%), and the remaining bowel care time occurred in the evening (25%) and morning (5%). The location of bowel care was a bed (60%), a potty chair (30%) or a toilet seat (10%). Of the patients, 95% needed full assistance during defecation time, while the remaining 5% needed partial help. Further, 60% had abdominal discomfort, 70% had constipation, 80% had bloating, and 90% had flatus incontinence.

There was no significant difference (independent *t*-test, P = 0.998) between the mean age of healthy adult males (40 ± 9.03 years) and patients with cervical SCI (39.9 ± 10.57 years). The body mass index (BMI) of healthy males was significantly higher (24.8 ± 2.677 kg/m²) than that of SCI patients (23.11 ± 2.876 kg/m²) (independent *t*-test, P = 0.022). The serum levels of TG, TC, LDL, apolipoprotein B (APOB), lipoprotein A (LPA), and non-esterified fatty acids (NEFA) in the SCI group were higher than those in the healthy group, while levels of HDL and apolipoprotein A1 (APOA1) were lower than those in the healthy group. The serum biomarkers uric acid (UA), TCHO, LDL, APOA1, APOB, LPA, and NEFA showed a significant difference between the two groups (independent *t*-tests, P < 0.05; **Table 2**).

Sequencing data

16S rRNA gene sequences were generated using the Illumina MiSeq platform. Reads were clustered into OTUs at an identity threshold of 97%. Rarefaction curves showed clear asymptotes, and the Good's coverage for the observed OTUs was 99.8% (**Figure 1A**). These findings indicated a near-complete sampling of the community. A total of 746 OTUs were recognized. No significant differences in OTU abundance (ACE or Chao1 index; Student's *t*-test, P = 0.78 and 0.76, respectively) were observed between healthy males and patients with cervical SCI. At the genus level, the Simpson alpha-diversity index indicated lower diversity in patients with cervical SCI than in healthy subjects (Student's *t*-test, P = 0.017; **Figure 1B**). In total, 495 of the 760 OTUs were shared between the two cohorts (**Figure 1C**). A principle coordinates analysis (PCoA) of phyla and a non-metric multidimensional scaling analysis (NIMDS) of OTUs revealed significant differences in bacterial community composition between the two groups (**Figure 1D**, **1E**). ANOSIM/Adonis revealed significant differences in the structure of the gut microbiota between the two groups (ANOSIM, r^2 = 0.4532, P = 0.001; **Figure 1F**). Based on the dispersion of the sample point distribution in a partial least square-discriminant analysis (PLS-DA) at the OTU, phylum, and genus levels, we found differences in the bacterial community composition between the two groups (Supplementary File 1A-C).
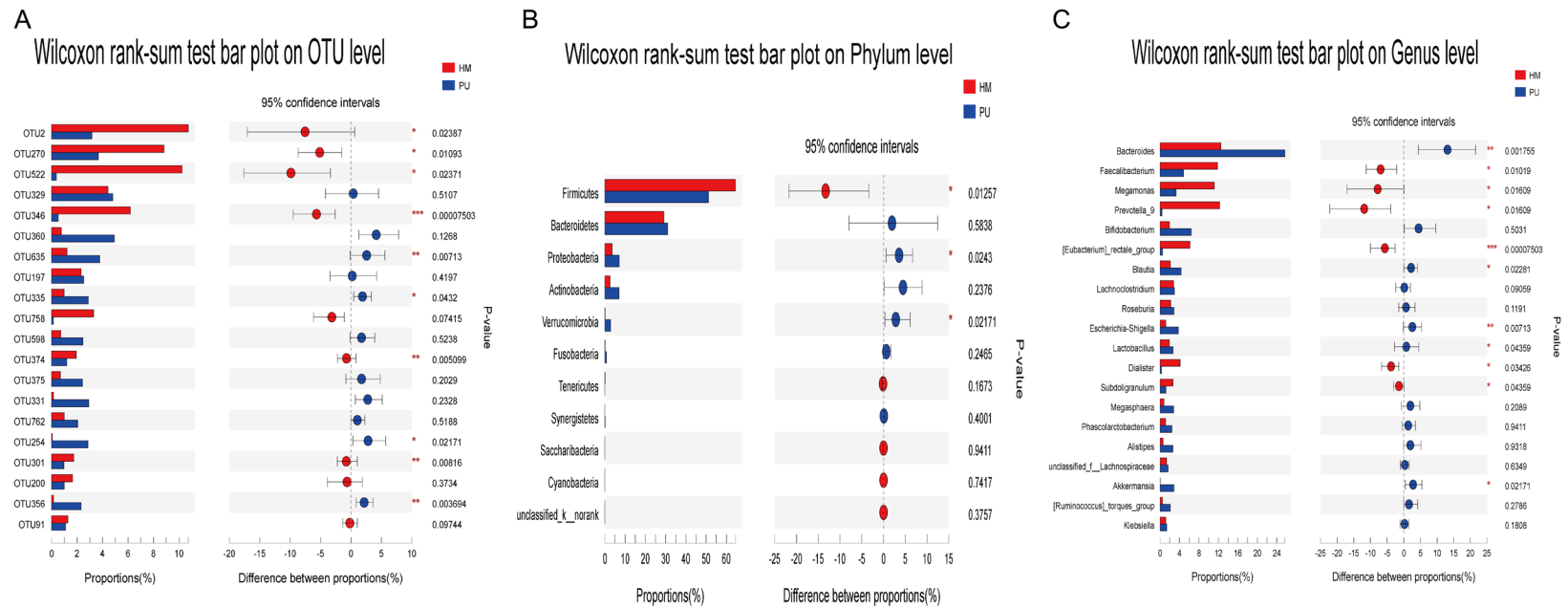
Associations of the gut microbiota and cervical SCI

A STAMP analysis indicated that 10 of the top 20 OTUs showed a significant difference between the two groups (Wilcoxon rank-sum test, P < 0.05; **Figure 2A**). Additionally, 3 phyla and 11 of the top 20 genera showed a significant difference between the two groups (Wilcoxon rank-sum test, P < 0.05; **Figure 2B**, **2C**). Firmicutes, *Faecalibacterium*, *Megamonas*, Prevotella_9, [Eubacterium]_rectale_group, *Dialister*, and *Subdoligranulum* were significantly more abundant in the healthy male group than in the cervical SCI group (Wilcoxon rank-sum test, P < 0.05); Proteobacteria, Verrucomicrobia, *Bacteroides*, *Blautia*, *Escherichia-Shigella*, *Lactobacillus*, and *Akkermansia* were significantly more abundant in the cervical SCI group than in the healthy male group
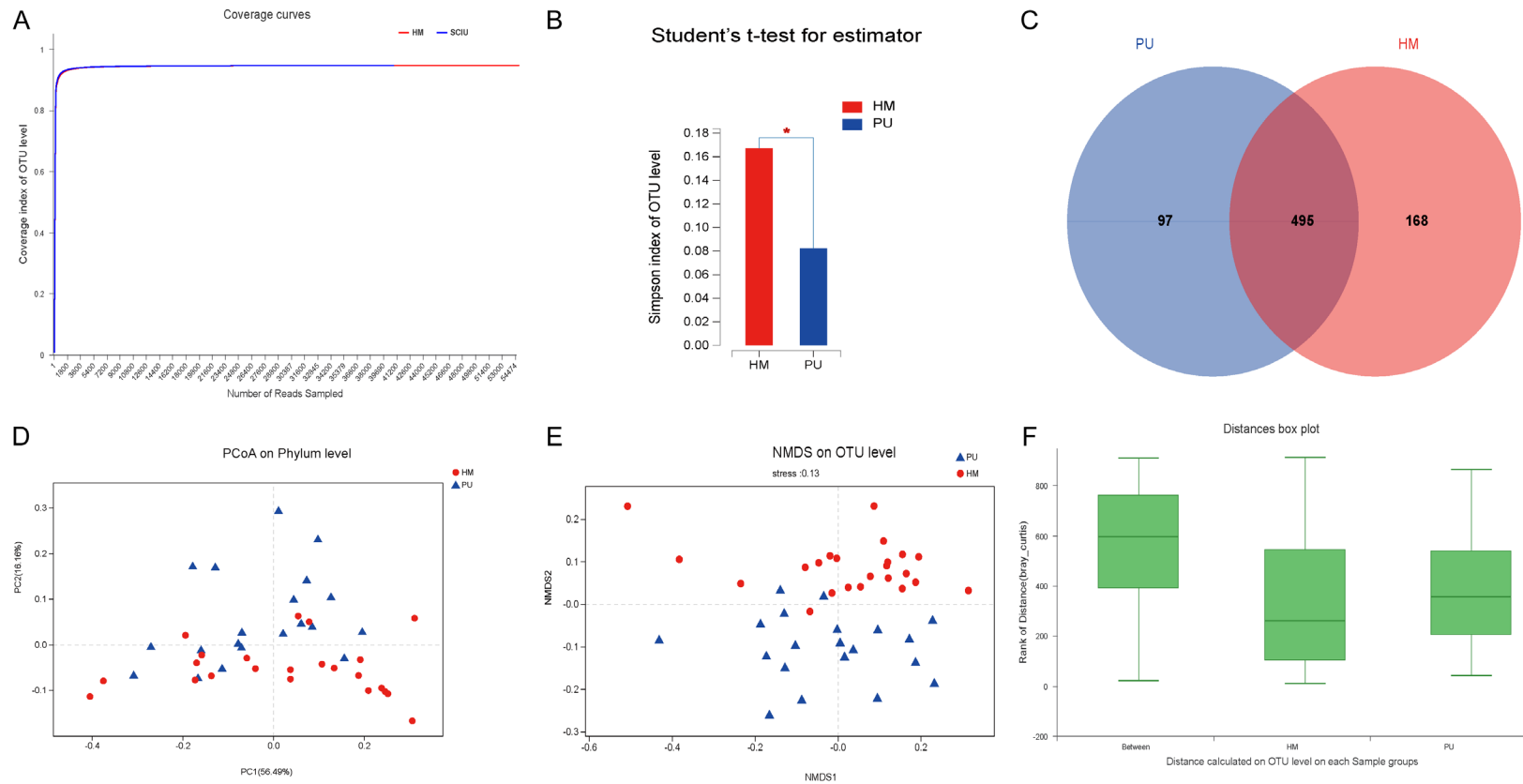
Figure 1. Diversity and taxonomic analysis in the health male and cervical SCI groups. A. The rarefaction curves showed clear asymptotes and the Good's coverage for the observed OTUs was 99.8% in the health male and cervical SCI groups. B. OTU diversity index Simpson showed a significant difference between the health male and cervical SCI groups (Student's t test, P = 0.017). C. A Venn diagram demonstrated 495 of the total 760 OTUs were shared between the health male and cervical SCI groups. D. The PCoA on phylum level of beta-diversity analysis showed there was significant difference in bacterial community composition between two groups (PCoA, PC1 = 56.49%; PC2 = 16.16%). E. The NIMDS on OTU level of beta-diversity analysis showed there was significant difference in bacterial community composition between two groups (NIMDS, stress = 0.13). F. ANOSIM/Adonis revealed significant differences in the structure of gut microbiota among the two groups (ANOSIM, r² = 0.4532, P = 0.001).

Figure 2. STAMP analysis on significant difference test between health male and cervical SCI groups. A. STAMP analysis indicates there were 10 OTUs showed a significant difference (Wilcoxon rank-sum test, P < 0.05) among two groups in top 20 OTUs. B. There were 3 Phylum showed a significant difference (Wilcoxon rank-sum test, P < 0.05) among two groups. C. There were 11 of top 20 Genus showed a significant difference (Wilcoxon rank-sum test, P < 0.05) among two groups.
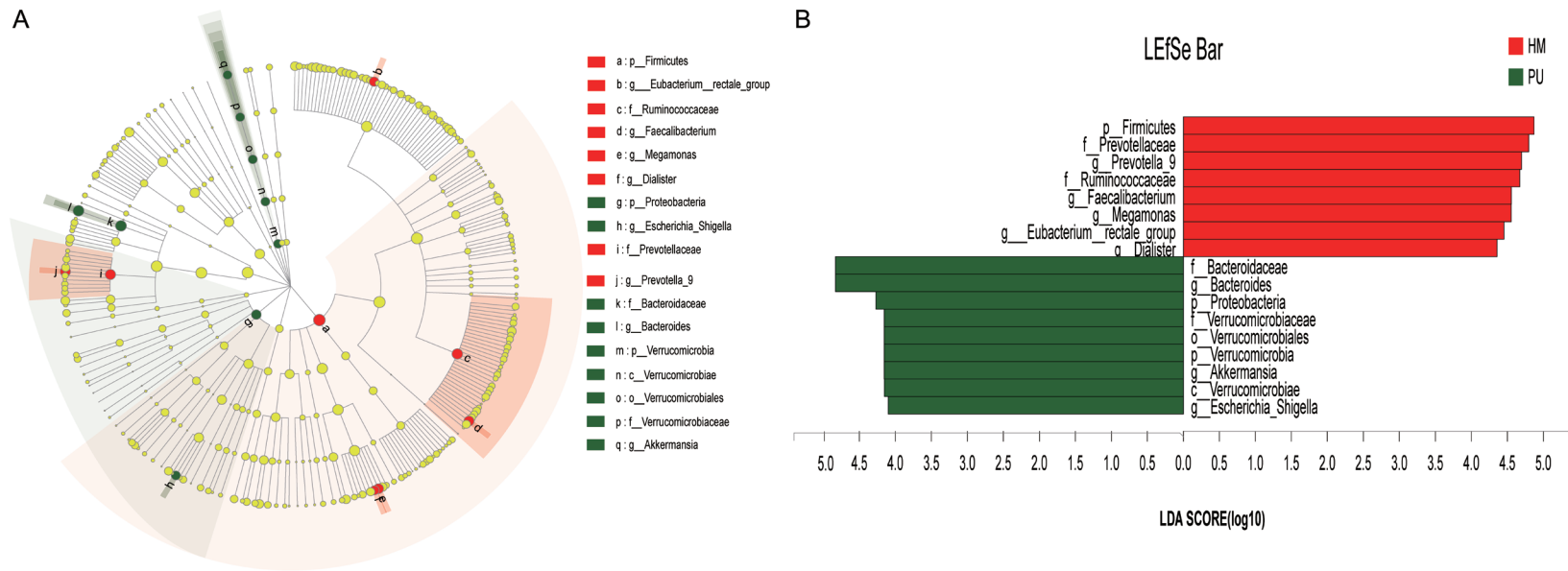
Figure 3. Cladogram showing differentially abundant taxa of microbiota between health male and cervical SCI groups. A. LEfSe cladogram showed the most differentially abundant taxa of 16S sequences between the two groups. B. LEfSe analysis (LDA threshold of 4) indicated that Bacteroidaceae and Bacteroides were significantly enriched in the SCI group compared with the healthy group, which showed enrichment of Firmicutes and Prevotellaceae (Kruskal-Wallis (KW) sum-rank test).

(Wilcoxon rank-sum test, P < 0.05) (**Figure 2B**, **2C**).

Cervical SCI associated biomarkers

An LEfSe analysis (LDA threshold of 4) indicated that Bacteroidaceae and Bacteroides were significantly enriched in the SCI group compared with the healthy group, which was enriched for Firmicutes and Prevotellaceae. A cladogram representative of the structure of the gut microbiota, predominant bacteria, and the greatest taxonomic differences between the two cohorts are displayed in **Figure 3A** and **3B**. These data suggested that dysbiosis was extensive in patients with cervical SCI. Further, Bacteroidaceae, Bacteroides, and Proteobacteria could be used as potential distinguishing biomarkers.

Gut microbiota affected by participants' conditions

We selected the following factors for redundancy analysis (RDA)/correspondence analysis (CCA): age, BMI, alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, UA, creatinine (CR), glucose (GLU), TG, TCHO, HDL, LDL, APOA1, APOB, LPA, and NEFA (Supplementary File 2). RDA/CCA showed that GLU (P = 0.047, r^2 = 0.1475), HDL (P = 0.032, r^2 = 0.1547), APOA1 (P = 0.001, r^2 = 0.5067), and LPA (P = 0.001, r^2 = 0.2143) significantly affected bacterial composition at the phylum level. In the top 20 genera, HDL (P = 0.035, r^2 = 0.1508) and APOA1 (P = 0.002, r^2 = 0.2785) significantly affected bacterial composition. Linear regression of the factors showed association between the serum levels of GLU, LPA on phylum level and HDL, APOA1 on genus level according to PCoA (**Figure 4A-D**). We found that serum biomarkers GLU, HDL, APOA1, and LPA had significant correlations with the microbial community structure (P < 0.05).

Correlation heatmap analysis of the relationship between different factors and the community composition of the two groups showed that Firmicutes was positively correlated with APOA1 (Pearson r = 0.604, P = 0.0001) and HDL (Pearson r = 0.358, P = 0.018), while it was negatively correlated with LPA (Pearson r = -0.447, P = 0.003). Proteobacteria was positively correlated with UA (Pearson r = 0.415, P = 0.006). Verrucomicrobia was positively correlated with LPA (Pearson r = 0.009, P = 0.003)

and LDL (Pearson r = 0.31, P = 0.043), negatively correlated with APOA1 (Pearson r = -0.378, P = 0.012) and UREA (Pearson r = -0.322, P = 0.035) in phylum level ,other correlation analysis were showed in **Figure 5A**. In the top 15 genera, as significant difference genera between healthy and SCI groups, the Bacteroides was negative correlated with APOA1 (Pearson r = -0.414, P = 0.006) and ALT (Pearson r = -0.318, P = 0.038); Faecalibacterium was negative correlated with UA (Pearson r = -0.351, P = 0.021), TG (Pearson r = -0.381, P = 0.012), LDL (Pearson r = -0.357, P = 0.019) and APOB (Pearson r = -0.314, P = 0.04). Prevotella_9 was negatively correlated with NEFA (Pearson r = -0.336, P = 0.028) and positively correlated with APOA1 (Pearson r = 0.425, P = 0.005), APOB (Pearson r = 0.009, P = 0.045) and UREA (Pearson r = 0.426, P = 0.004); Dialister was negatively correlated with LDL (Pearson r = -0.422, P = 0.005), TCHO (Pearson r = -0.314, P = 0.04) and LPA (Pearson r = -0.303, P = 0.048), positively correlated with HDL (Pearson r = 0.349, P = 0.022). [Eubacterium]_rectale group was positively correlated with APOA1 (Pearson r = 0.515, P = 0.0001), negatively correlated with UA (Pearson r = -0.366, P = 0.016) and TG (Pearson r = -0.333, P = 0.029). Lactobacillus was positively correlated with GLU (Pearson r = 0.33, P = 0.031) and negatively correlated with CR (Pearson r = -0.322, P = 0.035). Escherichia-Shigella was positively correlated with UA (Pearson r = 0.35, P = 0.022). Lachnoclostridium was positively correlated with UA (Pearson r = 0.322, P = 0.036). Megamonas was negatively correlated with GLU (Pearson r = -0.605, P < 0.001), NEFA (Pearson r = -0.336, P = 0.028), and positively correlated with APOA1 (Pearson r = 0.314, P = 0.041) (**Figure 5B** and Supplementary Files 3, 4).

Relationship between the gut microbiota and the condition of participants association between the gut microbiota and metabolism

According to a 16S rRNA functional predictive analysis, the abundances of taxa related to carbohydrate, amino acid, nucleotide, and lipid metabolism, transcription, and translation were decreased in patients with cervical SCI (**Figure 6A**). Of these, nucleotide (Wilcoxon rank-sum test, P = 0.027), lipid metabolism (Wilcoxon rank-sum test, P = 0.027), and translation (Wilcoxon rank-sum test, P = 0.019) were sig-
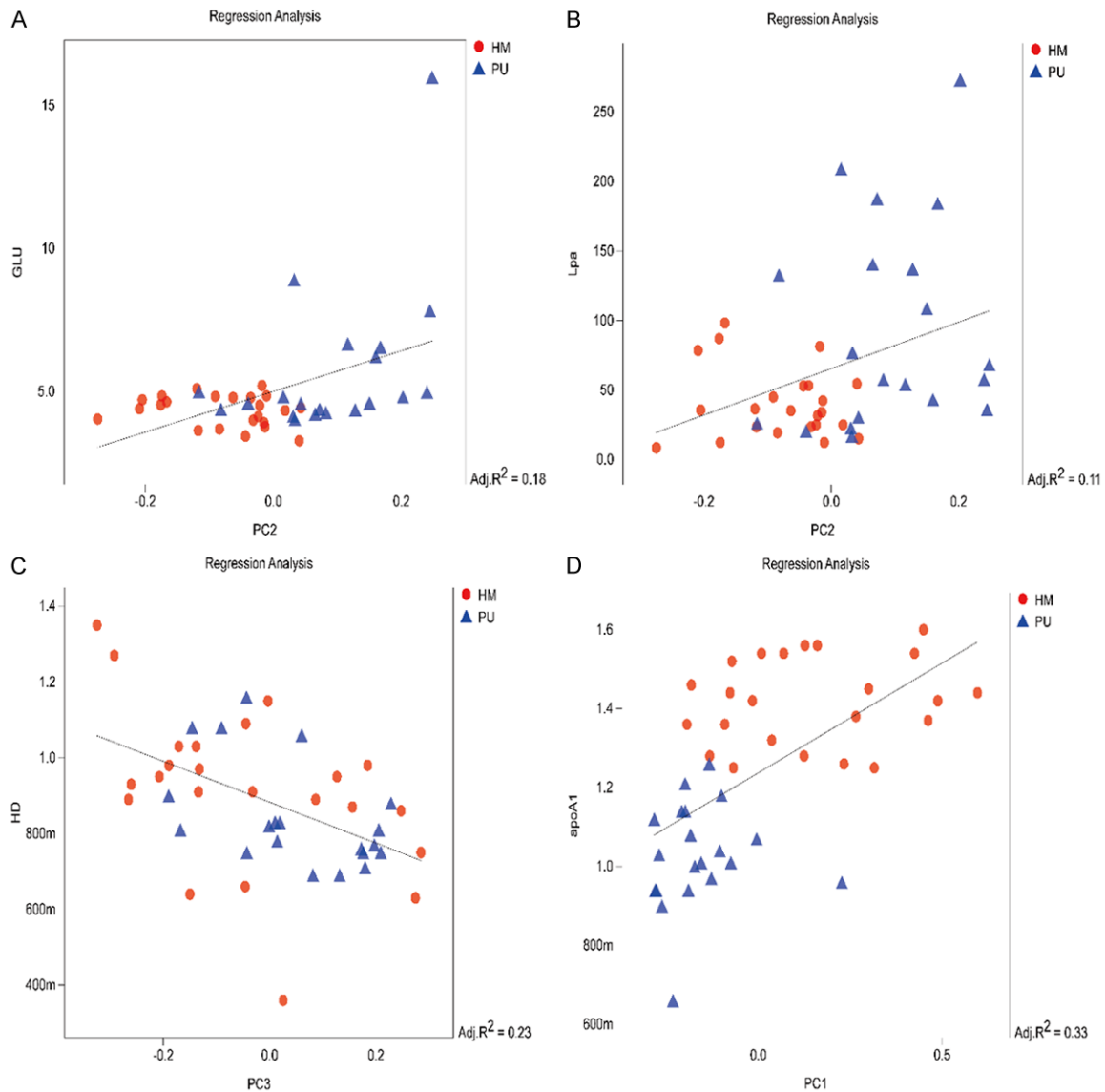
Figure 4. Linear regression of environmental factors between the serum lipid profiles and principal co-ordinates analysis in health male and cervical SCI groups. Linear Regression of the serum level of GLU on phylum (A), LPA on phylum (B), HDL on genus level (C), APOA1 on genus level (D) and principal co-ordinates analysis in health male and cervical SCI groups showed significant correlation with microbial community structure (A, R^2 = 0.18, P < 0.05; B, R^2 = 0.11, P < 0.05; C, R^2 = 0.23, P < 0.05; D, R^2 = 0.33, P < 0.05).
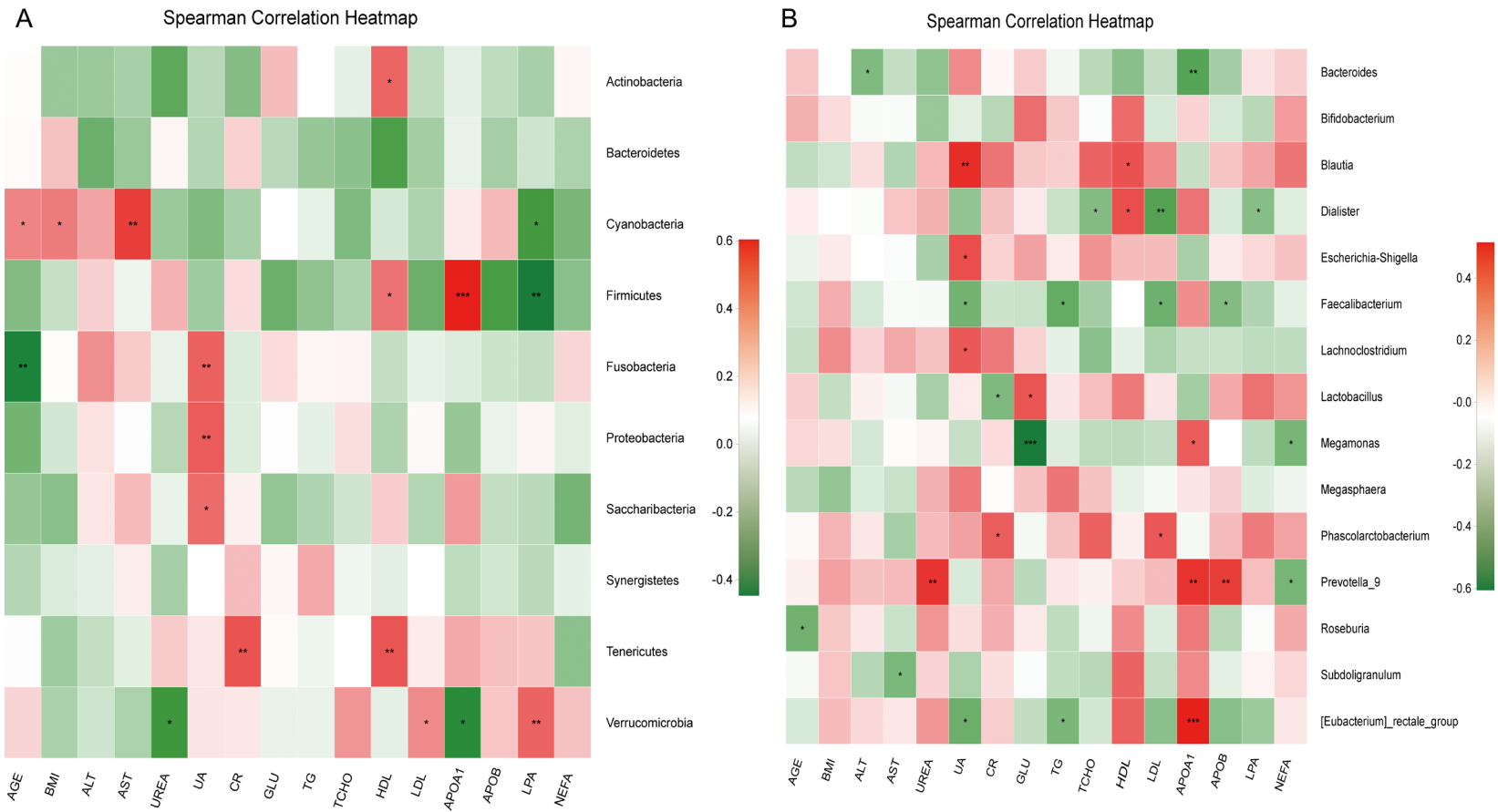
nificantly decreased in the cervical SCI group. Lipid metabolism in level 2 of the of KEGG metabolic pathway hierarchy (Wilcoxon rank-sum test, P = 0.025) and fatty acid metabolism in level 3 (Wilcoxon rank-sum test, P = 0.014) were significantly decreased in the cervical SCI group (**Figure 6B**).

Discussion

In this study, we evaluated the bowel management of NBD in male patients with chronic

TCSCI at our center. In a comparison of the gut microbiomes of healthy adult males and male patients with chronic TCSCI, the diversity of gut microbiota was lower in the TCSCI group than in healthy adult males and the structural composition was different. The dysbiosis of the gut microbiota involving several key bacteria was associated with serum biomarkers of lipid metabolism in participants with SCI. The lipid metabolism in the COG functional database and lipid metabolism (level 2) and fatty acid metabolism (level 3) in the KEGG metabolic

Figure 5. Correlation heatmap analysis of different environmental factors on the community composition of the healthy male and cervical SCI groups in phylum level (A) and genus level (B).
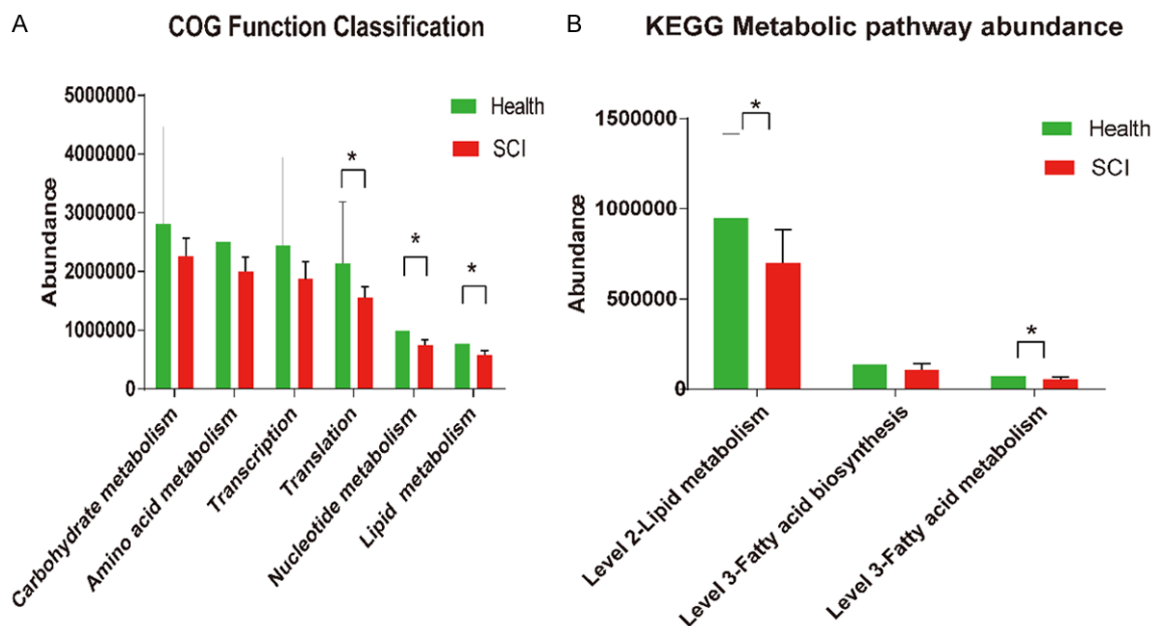
Figure 6. 16S functional prediction of gut microbiota in healthy male and cervical SCI groups. A. The abundance of nucleotide (Wilcoxon rank-sum test, P = 0.027), lipid metabolism (Wilcoxon rank-sum test, P = 0.027) and translation (Wilcoxon rank-sum test, P = 0.019) had significant decreased in SCI group. B. The lipid metabolism in level 2 (Wilcoxon rank-sum test, P = 0.025) and fat acid metabolism in level 3 (Wilcoxon rank-sum test, P = 0.014) of KEGG metabolic pathway abundance had significant decreased in SCI group.

pathways were significantly decreased in the cervical SCI group when compared with healthy males. To our knowledge, microbiome studies targeting lipid profiles in patients with cervical SCI are non-existent and the results of this study suggest that gut microbiome dysbiosis ha implications in cervical SCI.

TCSCI is most prevalent in adults and is often caused by falls from heights (49.7%) and traffic accidents (36.4%) [25]. In the current study, traffic accidents (50%), bruises by heavy objects (20%), and falls from heights (15%) were the main causes of injury. The average age of patients with cervical SCI in this study was 39.9 years old, which is considered the prime of life and the period when individuals also particularly susceptible to accidental injuries. Wu et al. reported that the male/female ratio for TCSCI is 5/1 [25]. Consistent with this, most of the patients with chronic traumatic complete cervical SCI admitted to our center were male; thus, we chose male patients as our research subjects.

The prevalence of NBD in patients with SCI is approximately 80-97.3% according to previous studies [3, 26, 27]; similarly, in our study, all patients with cervical SCI (100%) had at least one gastrointestinal problem. In previous studies, no significant correlation was detected between the prevalence of gastro-intestinal symptoms and injury level in SCI [28-30]. However, our study included only hospitalized patients with complete cervical SCI, and the prevalence of NBD was therefore higher owing to the limited physical activity. The average NBD score was 11.17, which is considered moderate [31]; the average defecation time was 41 min, which was similar to the 31-45 min reported in 72-80% patients in a previous study [26, 27]. The main technique for fecal evacuation was suppository and supplementary interventions were abdominal massage and digital anus-rectal stimulation; 60% of patients were in bed during evacuation and 95% patients needed help to complete evacuation; constipation and bloating were common symptoms in those patients. Due to the absence of nervous control in patients with cervical SCI, NBD results in constipation, incontinence, and discoordination of defecation [3]. Patients with cervical SCI who participated in this study had serious symptoms of NBD, severely affecting the quality of life. Despite bowel management measures, but gastrointestinal problems were

not completely addressed. The gut microbiota provides a potential target to address this problem.

Cardiovascular disease is among the leading causes of death among patients with SCI. The risk factors for cardiovascular disease include unfavorable lipid profiles, such as high concentrations of TC, LDL, and TG and low concentrations of HDL in individuals with tetraplegic SCI [18, 32-36]. We found that differences in lipid profiles between groups; the serum biomarkers TG, TCHO, LDL, APOB, LPA, and NEFA were higher while HDL and APOA1 were lower in the SCI group than in healthy males. The serum biomarkers TCHO, LDL, APOA1, APOB, LPA, and NEFA showed a significant difference between SCI and healthy male groups, indicating that the patients with SCI in our study exhibited dyslipidemia. This may be explained by inactivity and dysfunction of the autonomic nervous system of patients with cervical SCI. Inactivity is associated with higher insulin resistance, increased occurrence of glucose intolerance and diabetes mellitus, and reduced activity of the lipoprotein lipase. This could be related to decreased body fat, increased cardiovascular fitness, and associated metabolic changes [37, 38]. Different serum catecholamine levels resulting from the impairment of the sympathetic nervous system have been observed in patients with SCI. Schmid et al. found that the lower the noradrenaline values, the lower the HDL [18]. LDL is also correlated with levels of catecholamines and particularly with levels of adrenaline [18]. After SCI, sympathetic tone diminishes, reducing the resting metabolic rate, which could decrease lipid metabolism [39].

We found lower intestinal microbial diversity in patients with cervical SCI than in healthy males and differences in the bacterial community composition. Multiple linear regression and PCoA showed associations between the serum levels of GLU and LPA and taxa at the level of phyla and between HDL and APOA1 and taxa at the genus level. Additionally, the serum biomarkers GLU, HDL, APOA1, and LPA were significantly correlated with the microbial community structure. In the SCI group, the phylum Firmicutes decreased significantly, consistent with the results of Gungor et al. [14], who showed that butyrate-producing taxa are spe-

cifically reduced in patients with SCI when compared with healthy subjects. Short-chain fatty acids, particularly butyrate, have potent anti-inflammatory effects on macrophages [40], which contributes to secondary tissue damage and axonal retraction following SCI [41, 42]. In our study, the abundance of fatty acid metabolism in level 3 of the KEGG metabolic pathway hierarchy in the 16S rRNA functional prediction was significantly decreased in the SCI group, suggesting that reduced levels of butyrate may contribute to macrophage-mediated neurotoxicity in these patients. The low butyrate levels may have an impact on long-term recovery after cervical SCI. Moreover, we found that the prevalence of [Eubacterium]_rectale, a butyrate-producing bacterial genus [43], in the SCI group was positively correlated with APOA1 and negatively correlated with TG. The decreased APOA1 and increased TG indicate that [Eubacterium]_rectale may affect lipid metabolism via these factors.

When the cervical spinal cord is injured, most or all brainstem circuitry that provides tonic executive control over sympathetic preganglionic neurons (SPNs) is lost. Injury to these pre-sympathetic axons unplugs SPNs from the brain and brainstem, eliminating the feedback regulation of autonomic reflexes that are initiated below the level of injury by, for example, visceral or somatic sensory nerves that relay information to the spinal cord from the bowel. After SCI, the loss of descending control over SPNs causes autonomic reflex circuitry to become dysfunctional, leading to dysfunctions, such as autonomic dysreflexia and SCI-immune depression syndrome.

SCI also causes an autonomic imbalance in the gastrointestinal tract, which leads to deficits in colonic motility, mucosal secretions, and vascular tone [44, 45]. The autonomic nervous system and gastrointestinal dysfunction may be causes of gut microbiota dysbiosis in patients with cervical SCI at our center.

The changes in bacterial taxa were associated with changes in the lipid profiles of participants. At the phylum level, the decreased Firmicutes was positively correlated with APOA1 and HDL, and negatively correlated with LPA. The phyla Proteobacteria and Verrucomicrobia were significantly increased in the cervical SCI group,

and the increase in Verrucomicrobia was positively correlated with LPA and LDL and negatively correlated with APOA1. Verrucomicrobia is increased in rats with non-alcoholic fatty liver disease [46]. Dramatic colonization of the human gut microbiota by Verrucomicrobia following a broad-spectrum antibiotic regimen occurred without significant gastrointestinal manifestations [47], suggesting that the phylum negatively regulates lipid metabolism.

ApoA1, a major structural protein among HDLs, is mainly synthesized in the liver [48]. There is a very close correlation between ApoA1 and HDL cholesterol (HDLC), and ApoA1 is a protective factor for atherosclerosis [49]. ApoA1 activates key enzymes in cholesterol metabolism. Thus, the reduced serum levels of APOA1 and HDL might lead to a blood lipid metabolism disorder and have a negative effect on SCI recovery.

At the genus level, *Bacteroides* and *Blautia* were significantly more abundant, while *Faecalibacterium*, *Megamonas*, Prevotella_9, *Lactobacillus*, and *Dialister* were significantly less abundant in the cervical SCI group than in healthy males. These key bacteria were associated with serum biomarkers of lipid metabolism. *Bacteroides* is a gram-negative bacterium, accounting for more than 1/4 of the adult intestinal flora with important roles in the health of hosts. When immunity is low, bacterial proliferation and translocation can cause endogenous infections [50, 51]. In our study, *Bacteroides* was negatively correlated with APOA1, indicating that increased *Bacteroides* may have adverse effects in immune-compromised patients with cervical SCI. *Blautia* produces short-chain fatty acids [50]. *Blautia* was positively correlated with UA and HDL, suggesting that increased *Blautia* in the SCI group may improve lipid metabolism in these patients.

The genus *Faecalibacterium* is an important component of a healthy human gut microbiome. Species in the genus can utilize acetate CoA-transferase to affect butyrate synthesis [52]. Butyrate has histone deacetylase activity and signals via GRP43 and GRP109a, thereby influencing brain function or structure [53]. In this study, *Faecalibacterium* was negatively correlated with UA, TG, LDL, and APOB. The protein APOB is synthesized in the liver and accounts for 97% of the total protein content of LDL. APOB can directly reflect the level of LDL [35]. Decreased *Faecalibacterium* was correlated with increased levels of TG, LDL, and APOB, which is harmful for lipid metabolism, suggesting that this genus is beneficial for lipid metabolism.

Liao et al. reported a positive association between bean consumption and the genus *Megamonas*, suggesting that *Megamonas* is a beneficial microbe [54]. The abundance of *Megamonas* was decreased in the cervical SCI group and was negatively correlated with GLU and positively correlated with APOA1, suggesting that the genus has a positive effect in terms of carbohydrate and lipid metabolism. This may be due to the fact that some carbohydrates in food cannot be digested and absorbed by intestinal digestive enzymes, but they can be metabolized by *Megamonas*.

Although *Prevotella* is considered a beneficial microbe, it is also linked to chronic inflammatory conditions [55, 56]. In this study, decreased *Prevotella* in the cervical SCI group was negatively correlated with NEFA and positively correlated with APOA1, APOB, and UREA. This suggests that *Prevotella* has a positive effect on the body by regulating lipid metabolism.

Lactobacillus is a genus of probiotic bacteria capable of producing organic acids and hydrogen peroxide, creating an inhospitable environment for pathogenic bacteria. In addition, an increase in the proportion of *Lactobacillus* can increase insulin secretion [57]. Therefore, the increased *Lactobacillus* may have a protective effect in patients with cervical SCI by the regulation of carbohydrate and lipid metabolism. Vogt et al. reported that a high abundance of *Dialister* is associated with less Alzheimer's disease (AD) pathology, suggesting that these bacterial taxa have protective effects against the development or progression of AD [58]. In the current study, *Dialister* was negatively correlated with LDL, TCHO, and LPA and positively correlated with HDL. Elevations of these serum markers indicate high blood lipids, which are harmful to health. Together, our observations revealed that carbohydrate, lipid, and fatty acid metabolism were damaged in patients with cervical SCI. This suggests that improvements in carbohydrate and lipid metabolism in patients can correspond with an improvement in the composition of the gut microbiota. Moreover, therapies targeting the gut microbiota can be

used to evaluate and modulate patient metabolism.

To exclude the confounding effects of gender and thoracic splanchnic nerves on gut functions, we only included male patients with complete SCI in this study. However, individual differences in diet-associated flora could not be determined; this remains a major weakness of our study. We found that gut microbiota dysbiosis is associated with serum lipid profiles in male patients with cervical complete SCI and used 16S rRNA sequencing to predict the dysfunctional components of lipid metabolism in these patients. This is the first comprehensive investigation of the correlations between gut dysbiosis and serum biomarkers in patients with TCSCI. This study could contribute to the development of novel strategies for improving conditions of patients with TCSCI. However, further genomic and metagenomic analyses are required to determine the precise functional changes caused by the SCI-mediated dysbiosis of the gut microbiota and the effects of those changes on serum lipid profiles. Further work, including animal experiments and longitudinal human studies, are needed to determine the causal relationship between the gut microbiota and cervical SCI. Determining the role of the gut microbiota in the progression or maintenance of SCI may lead to novel interventional approaches that can alter or restore a healthy gut bacterial composition or to the identification of microbial metabolites that are protective against cervical SCI.

Overall, we demonstrated that microbiota dysbiosis might be related to cervical SCI. The relative abundances of some key bacteria in patients with cervical SCI were different compared with those in healthy males. Moreover, these relative abundances were correlated with serum lipid profiles. Further, there was an interdependency between the gut microbiota and carbohydrate and lipid metabolism. Our study provides a basis for future investigations of the dysbiosis of the gut microbiota and the functional implications of these changes.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

SCI, spinal cord injury; TCSCI, traumatic cervical spinal cord injury; NBD, neurogenic bowel dysfunction; GLU, glucose; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; UA, uric acid; CR, creatinine; CPR, C-reactive protein; OTUs, operational taxonomic units; PLS-DA, partial least squares discrimination analysis; BMI, body mass index; APOA1, apolipoprotein A1; APOB, apolipoprotein B; ALT, alanine transaminase; AST, aspartate transaminase; TG, triglyceride; TCHO, total cholesterol; LPA, lipoprotein A; NEFA, non-esterifed fatty acid; HCY, homocysteine; ASCI, acute spinal cord injury; LPS, lipopolysaccharide; PCoA, Principle coordinates analysis; NMDS, non-metric multidimensional scaling analysis; PLS-DA, partial least square-discriminant analysis; AD, Alzheimer's disease; HM, healthy male; PU, patient with quadriplegia and SCI.

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Interdependency of gut microbiota and serum lipid profiles in patients with TCSCI
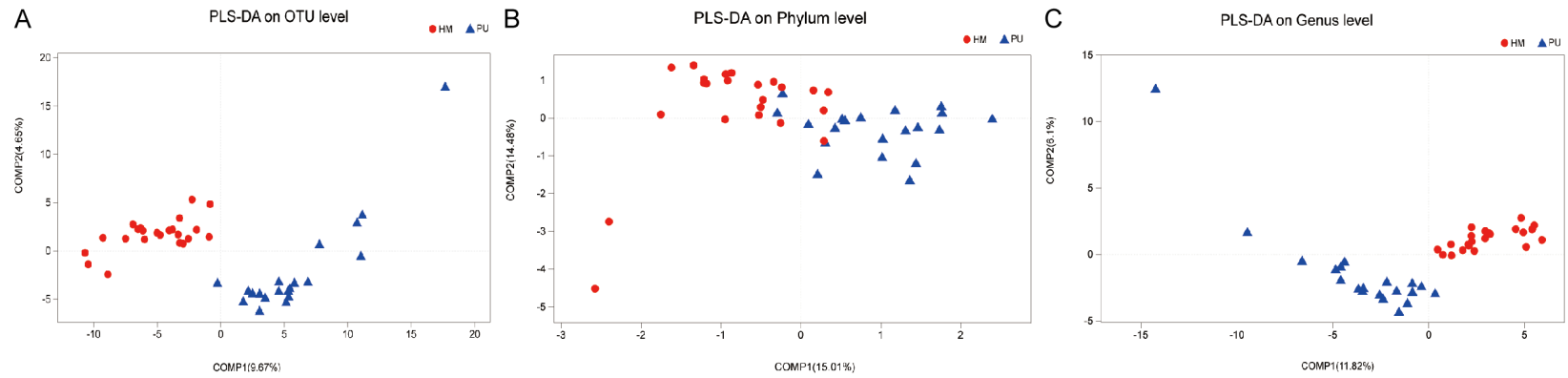
Supplementary File 1. The dispersion of the sample point distribution in the PLS-DA analysis revealed that there were significant differences in bacterial community composition between health males and cervical SCI groups on OTUs, phylum and genus level.

Supplementary File 2. VIF of selected environmental factors by the functions of envfit (permu =999) and vif.cca among the health males and cervical SCI groups.

Supplementary File 3. Correlation heatmap analysis chart of different environmental factors on the community composition of health males and cervical SCI groups in phylum and genus levels (correlation).

Supplementary File 4. Correlation heatmap analysis chart of different environmental factors on the community composition of health males and cervical SCI groups in phylum and genus levels (*P* value).

1