# Analysis of intestinal microbial communities of cerebral infarction and ischemia patients based on high throughput sequencing technology and glucose and lipid metabolism

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**Abstract.** Currently, cerebral infarction (CI) is the leading cause of disability and the second leading cause of mortality in China, seriously affecting patient quality of life. Ischemia (IS) is considered to be the early stage of CI. The present study aims to investigate the variation of intestinal microbial communities in patients with CI and IS using high throughput sequencing technology, and then analyze the results to identify a novel potential pathogenic mechanism of CI and IS. In total, 8 patients with CI, 2 patients with IS and 10 healthy volunteers as a control were selected. Throughput sequencing technology was used to analyze the character and microbial population of the gut. The abundance of *Escherichia*, *Bacteroides*, *Megamonas*, *Parabacteroides*, *Akkermansia*, *Prevotella*, *Faecalibacterium*, *Dialister*, *Bifidobacterium* and *Ruminococcus* was the significant difference in the intestinal microbial communities of the CI and IS patients compared with the healthy group. It was also observed that CI and IS were closely associated with internal glucose metabolism. The intestinal gut disturbance of CI patients may be one of the causes inducing CI by glucose metabolism and maybe considered as a potential method to predict the disease.

## Introduction

Cerebral infarction (CI) and ischemia (IS) are common diseases in elderly people seriously affecting the quality of life. Patients with IS exhibit insufficient blood supply, which is considered an early stage of CI (1,2). It is predicted that by 2030, the Chinese population aged ≥60 will be >300 million, and ~2/3 patients with primary cerebral vascular disease will be aged ≥60 years. In recent years, due to personal habits or environmental factors, the age of patients with primary cerebral vascular disease has gradually reduced (3,4). Cerebrovascular disease and CI are currently the leading cause of disability and the second leading cause of mortality in China (5,6). Patients with cerebrovascular disease who survive are prone to suffer from the disease again or more than once, ~3/4 of them lost their ability to work to varying extents and among them 40% are severely disabled. Expensive treatments for cerebrovascular disease have meant great mental and financial burdens on many patients and families. Although in recent years the treatment level of CI has been improved, treatment of the majority of patients remains unsatisfactory, with high mortality and disability rates and poor prognosis. Therefore, investigating the pathogenesis of CI and preventing it is considered an economical and efficient way to control the disease.

The intestinal microbial ecosystem has been widely studied in the past several years (7). The present study hypothesizes that diet can affect the intestinal gut and that variations in intestinal microbial communities can also affect human glucose and lipid metabolism; and variations in glucose and lipid metabolism have already been suggested to be potential causes of CI (8). Therefore, the present study adopted high throughput sequencing technology, which is the latest method to analyze the microbial communities of the intestinal gut, to investigate the intestinal guts of healthy volunteers, CI patients and IS patients, to explore the relation between CI, IS and the intestinal gut, and to consider the utility of using the intestinal gut as a diagnostic marker of CI and IS.

## Materials and methods

*Patients.* A total of 10 patients were selected; 8 with CI and 2 with IS. They were recruited from Tianjin Huanhu Hospital (Tianjin, China) between May 2015 and October 2015, and had

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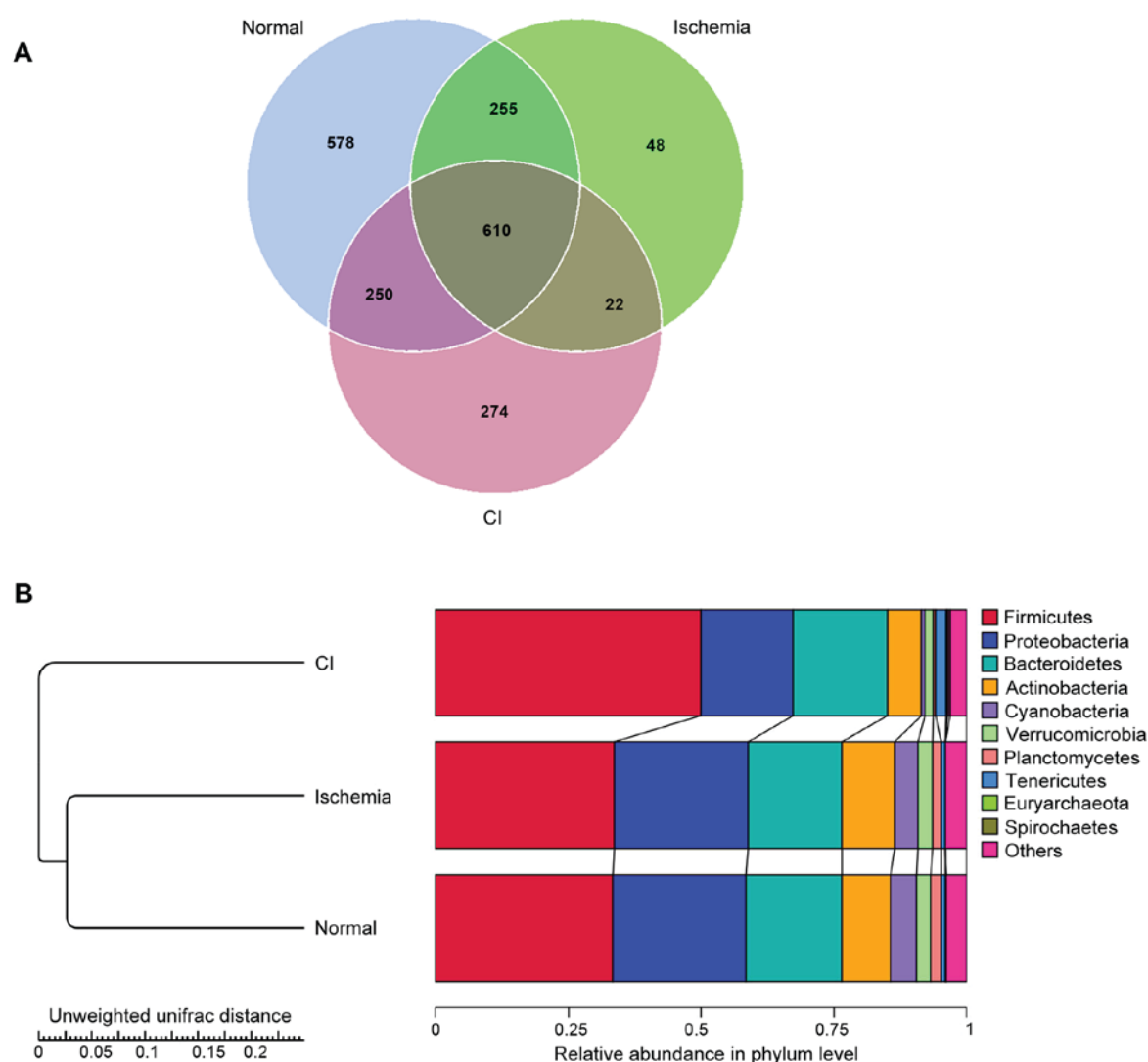E-mail: tjhhhospitallab@126.com

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Figure 1. Analysis of the variation of intestinal gut at the phylum level in healthy subjects and patients with CI and IS. (A) The key operational taxonomic units associated with healthy subjects and patients with CI and IS. (B) The UPGMA analysis of the intestinal gut of healthy subjects and patients with CI and IS. CI, cerebral infarction; IS, ischemia; UPGMA, unweighted pair group method with arithmetic mean.

been diagnosed by skull computed tomography examination. The group comprised6 males and 4 females. Additionally, 10 healthy volunteers, 5 male and 5 female, were selected as the normal group. The age of the individuals was from 53-82 years with a mean age of 64.3. Research subjects did not receive antibiotics in the week prior to the specimen collection. As required by the medical ethics committee, those in the normal group were examined to ensure that they had no metabolic, cardiovascular or cerebrovascular diseases or cancer. All participants signed informed consent prior to the experiment. The study was approved by the Ethics Committee of Tianjin Huanhu Hospital.

*Intestinal gut DNA extraction.* A 300 mg middle segment feces sample was collected in the morning and the genomic DNA was extracted using the Stool DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). Bacteria cells were split at 95°C for 10 min and 4 $\mu$l RNase A was added and incubated at 70°C for 30 min. DNA concentrations were determined using a microplate reader (Multiskan FC, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and stored at -80°C.

*Polymerase chain reaction (PCR) amplification and Illumina sequencing.* The bacterial genomic DNA was amplified with primers specific for the V4 hypervariable regions of the 16SrDNA gene. The sequences were forward, 5'-GTGCCA GCMGCCGCGGTAA-3' and reverse, 5'-GGACTACHV GGGTWTCTAAT-3', and the reaction was performed using Phusion High-Fidelity PCR master mix with GC buffer (New England Biolabs, Inc., Ipswich, MA, USA). Samples were sequenced by Illumina MiSeq platform provided by Illumina, Inc. (San Diego, CA, USA) with the following conditions: Denaturation 1 min at 98°C, 10 sec at 98°C, 30 sec at 50°C and 30 sec at 72°Cfor 30 cycles, and 72°C at 5 min.

*Serum glucose and lipid detection.* A total of 5 ml venous blood was collected from the elbow of the subjects, early in the morning and before they had eaten, and placed in a coagulation-promoting tube then centrifuged at 300 x g for 10 min at room temperature. The serum was collected and the blood glucose and lipid concentrations ascertained using a Beckman AU5800 biochemical detector (Beckman Coulter, Inc., Brea, CA, USA).
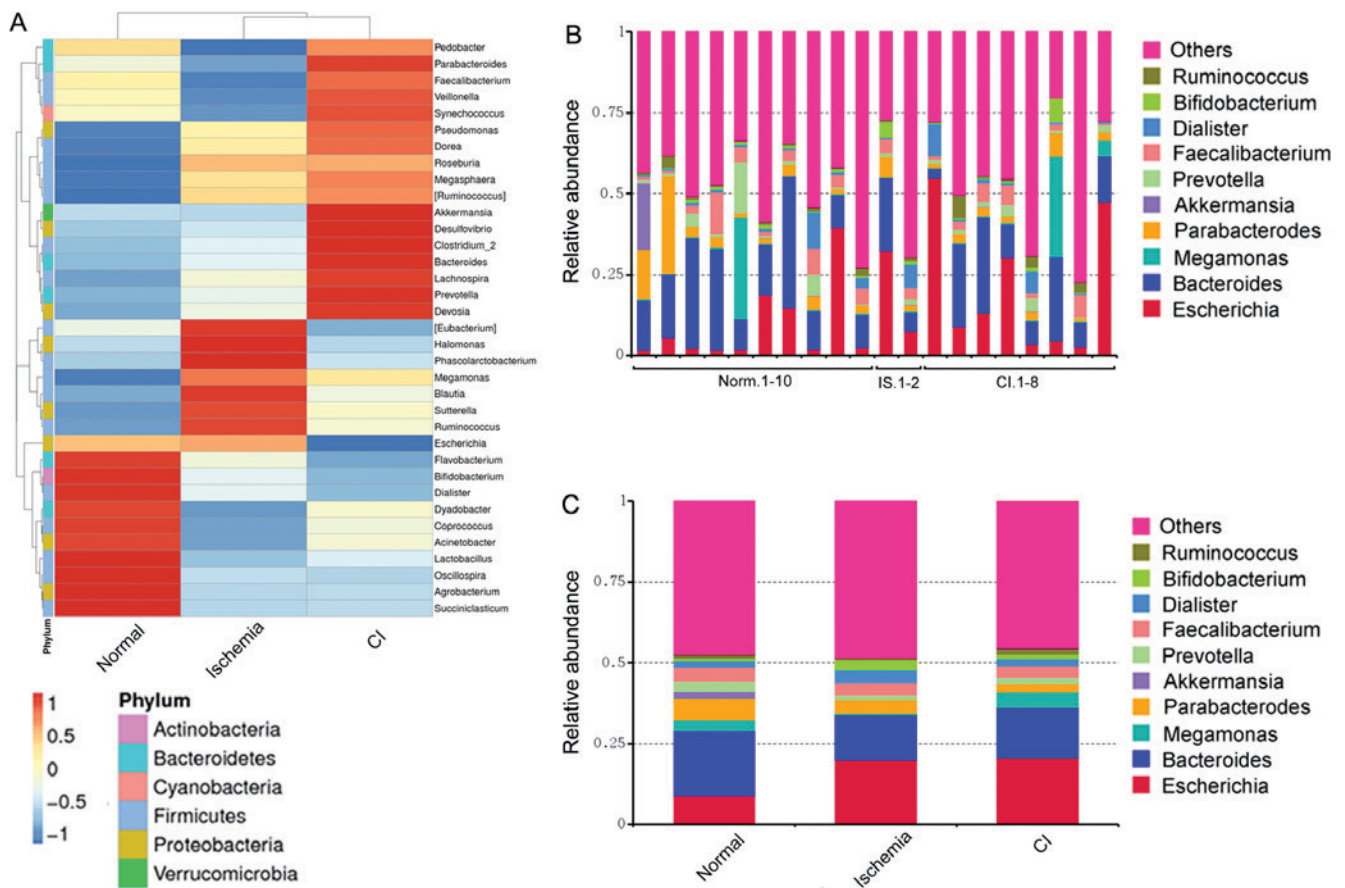
Figure 2. Analysis of the variation of intestinal gut in genus level in healthy subjects and CI and IS patients. (A) The heatmap of microorganisms in the intestinal gut at the genus level in healthy subjects and CI and IS patients. Red signifies an increased level and blue signifies a decreased level. (B) The relative abundance of each genus in the intestinal gut in each sample from healthy subjects, and CI and IS patients. (C) The relative abundance of each genus in intestinal gut samples from the healthy subjects (n=10) and patients with CI (n=8) and IS (n=2). CI, cerebral infarction; IS, ischemia.

*Statistical analysis.* Sequencing results analysis was performed by the QIIME software package 1.7.0 (qiime.org) (9) and UPARSE pipeline (10). Weighted/unweighted UniFrac analysis was performed by QIIME. Cluster samples analysis was performed by principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA). Operational taxonomic units (OTUs) analysis was performed by partial least square discriminate analysis via Simca-p+11.5, values higher than 1.5 were considered as key OTUs. The heatmap was constructed with HemI version 1.0 (The CUCKOO Workgroup, Wuhan, Hubei, China). Data analysis was performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA) and one-way analysis of variance with Tukey's test was applied for the intergroup analysis. Values are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Microbiota of intestinal gut in CI and IS patients.* The sequencing results demonstrated that there were 274 (48key) OTUs that were significantly associated with CI and IS (Fig. 1A). A significant difference in the abundance of Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, Verrucomicrobia, Planctomycetes, Tenericutes, Euryarchaeota and Spirochaetesat the phylum level in the CI

group compared with the normal group was observed using UPGMA analysis ($P<0.05$), however, no significant difference was observed in the abundance of the above phyla in the IS group compared with the normal group (Fig. 1B).

The heatmap demonstrates that there was a change in abundance at the genus level among the groups (Fig. 2A). Particularly, the results demonstrated that *Escherichia*, *Megamonas*, *Dialister*, *Bifidobacterium* and *Ruminococcus* were more abundant in the IS and CI groups. The abundance of *Bacteroides*, *Parabacteroides*, *Akkermansia*, *Prevotella* and *Faecalibacterium* was decreased in the CI group compared with the normal group ($P<0.05$). There was a greater abundance of *Escherichia*, *Dialister* and *Bifidobacterium* in the IS group compared with the normal group ($P<0.05$), although the abundance of *Bacteroides*, *Megamonas*, *Parabacteroides*, *Akkermansia*, *Prevotella*, *Faecalibacterium* and *Ruminococcus* was less. There was a greater abundance of *Escherichia*, *Bacteroides*, *Megamonas*, *Prevotella* and *Ruminococcus* in the CI compared with the IS group; although the abundance of *Parabacteroides*, *Akkermansia*, *Faecalibacterium*, *Dialister* and *Bifidobacterium* was less ($P<0.05$; Fig. 2B and C).

*Diversities between CI and IS patients' intestinal gut.* It can be seen from the principal component analysis (PCA) that the diversities were assessed by a net separation of the three
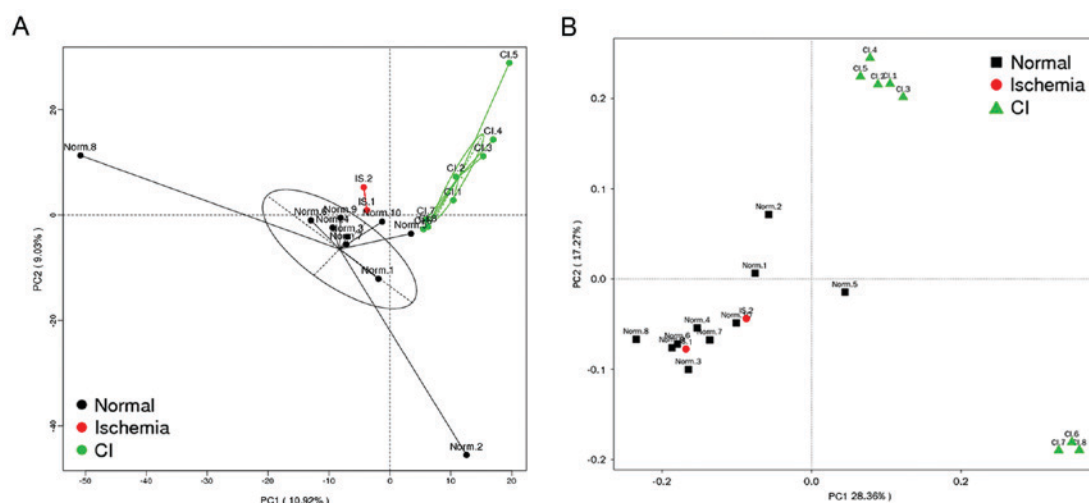
Figure 3. Differences in intestinal gut among healthy subjects and CI and IS patients. (A) Principal component analysis of intestinal gut in healthy subjects, and patients with CI and IS. (B) Principal coordinate analysis of intestinal gut in healthy subjects, and patients with CI and IS. CI, cerebral infarction; IS, ischemia.
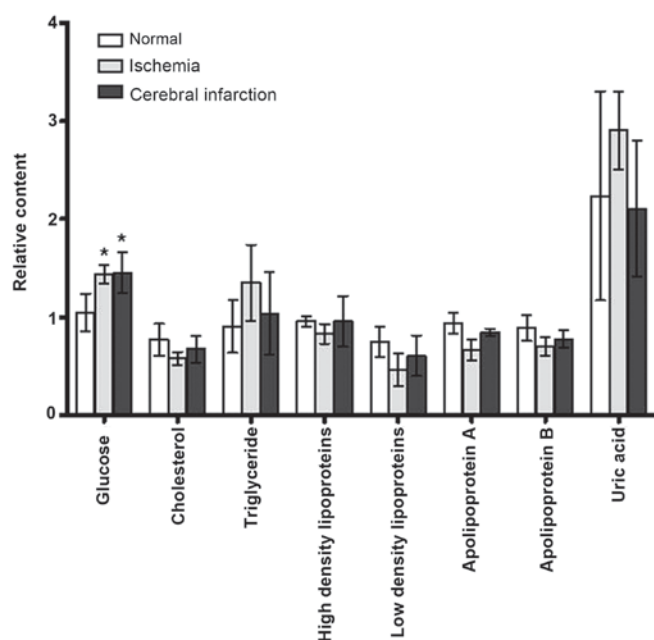


Figure 4. Serum glucose level and lipid metabolism of healthy subjects, and patients with cerebral infarction and ischemia. The graph presents the level of glucose, triglyceride, total cholesterol, high-density lipoprotein and low-density lipoprotein, apolipoprotein A, apolipoprotein B and uric acid. Values are presented as the mean ± standard deviation. *P<0.05 vs. healthy group.

groups produced by the intestinal gut between the healthy group and the CI and IS patients (P<0.05), which indicated less similarity in the microbial structure and composition of the feces between CI and IS patients and the healthy group (Fig. 3A). Notably, the same pattern was observed in the PCoA analysis and that there are also samples that were grouped into two distinct clusters in the intestinal microbial communities of CI patients (P<0.05); this may be associated with the process of CI and requires further investigation (Fig. 3B).

*Level of serum glucose and lipid metabolism in patients with CI and IS, and the healthy group.* The present study demonstrated that the blood glucose of patients with CI and

IS was significantly higher compared with the healthy group; however, no significant changes were observed in triglyceride, total cholesterol, high-density lipoprotein and low-density lipoprotein, apolipoprotein A, apolipoprotein B and uric acid (P<0.05; Fig. 4). Therefore, it is hypothesized that variations in the intestinal microbial communities in CI and IS patients may lead to a disorder of the blood glucose metabolism and thus a potential dangerous factor causing CI.

**Discussion**

CI refers to focal cerebral ischemic necrosis or encephaloma-lacia caused by the disturbance of the cerebral circulation, IS and hypoxia. It has high morbidity, disability and mortality, and seriously endangers human life (11). Therefore, exploring its mechanism with the aim of predicting and preventing CI has become an important area of study.

The present study demonstrated that the intestinal gut serves an important function in cardiovascular and cerebro-vascular diseases and a previous study considered its role in cardiovascular disease (12). The intestinal gut can affect the intestinal permeability through glucose metabolism, increase the quantity of harmful substances in the blood and stimulate the inflammatory response, thus promoting the occurrence and development of the diseases (13,14). However, at present little is known about the variations in the intestinal gut of patients with cerebral arterial thrombosis. The development of the new-generation high throughput sequencing technology offers the opportunity to rapidly acquire and analyze the information on the intestinal microbial community composition, function discovery, metabolic function and other aspects, due to its digitized signal, high data flux, deep sequencing depth, high accuracy and other features, leading to an improved under-standing of the intestinal gut.

Glycolysis breaks down glucose and is essential for human biological function. *Escherichia* are considered to serve an important function in the mechanism of glycolytic pathways for regulating glucose catabolism (15). Previous studies have demonstrated that decreased relative abundance of

*Akkermansia* and increased relative abundance of *Bacteroides*, *Ruminococcus* and *Parabacteroides* in the gut microbiome can improve glucose homeostasis in obese mice and provide a catabolic pathway through a novel mannosylglucose phosphorylase, suggesting that *Akkermansia*, *Bacteroides*, *Ruminococcus* and *Parabacteroides* may serve an important function in the mechanism of glycometabolism (16-19).There appears to be a significant enrichment of *Megamonas* in Chinese people compared with other ethnicities, and it may serve a potential glycometabolism role (20). *Prevotella* has been demonstrated to serve a role in glycometabolism and in promoting increased glycogen storage (21). The abundance of *Faecalibacterium* and *Dialister*, and the abundance of *Bifidobacterium* in patients with type 2 diabetes, was increased by altering lipid and glucose metabolism, glucose tolerance and the inflammatory immune responses (22-25).

By exploring the intestinal microbial communities of patients with CI, the present study identified that the abundance of *Escherichia*, *Bacteroides*, *Megamonas*, *Parabacteroides*, *Akkermansia*, *Prevotella*, *Faecalibacterium*, *Dialister*, *Bifidobacterium* and *Ruminococcus* in intestinal microbial communities was significantly different inpatients with CI and IS compared with healthy subjects, and there were also significant differences in the intestinal microbial profile among different patients with CI by PCA and PCoA analysis; this may be associated with the process of CI and may provide a new basis for diagnosing and classifying CI patients and understanding CI pathogenesis.

In conclusion, we hypothesized that microbial genus profile may be markedly changed prior to the onset of CI and IS and searching for the characteristic intestinal gut of CI patients may provide a new basis for the diagnosis and a possible indicator for prevention and provide a major social and economic benefit. However, there were also certain limitations of the present study, for example, the patient samples sizes for CI and IS were small, and a study on a much larger number will be required to validate these results.

## Acknowledgements

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