ORIGINAL RESEARCH

Ischemic Stroke and Dysbiosis of Gut Microbiota: Changes to LPS Levels and Effects on Functional Outcomes

Zhiming Jiang, MM; Lili Li, PhD; Lei Liu, MD; Bin Ding, MM; Ying Yang, MM; Fei He, MM; Liao Zhang, MM; Zijian Wu, MD

ABSTRACT

Context • The intestinal microbiota and their metabolites play an important role in acute ischemic stroke (AIS) and modulate brain functions directly or indirectly through immune, endocrine, vagal, and other humoral pathways. However, relatively few investigations have evaluated the gut microbiome and its levels of inflammatory factors or the potential associations of those factors with stroke outcomes in patients who have had acute ischemic stroke (AIS), with different stroke severities.

Objective • The study intended to determine if AIS patients would have different gut microbiota and inflammatory-factor levels than healthy individuals and if those levels would be associated with the stroke's severity and the patient's prognosis.

Design • The research team performed a prospective observational study.

Setting • The study took place in the Department of Rehabilitation at the General Hospital of Wanbei Coal and Electricity Group, which is the Third Affiliated Hospital of Bengbu Medical College in Suzhou, Anhui, China.

Participants • Participants were 90 patients who had received a diagnosis and treatment of AIS within 48 hours of the stroke's onset at the hospital, between October 2021 and March 2022.

Groups • The research team performed multiple comparisons of the baseline demographic and clinical characteristics, the gut microbiota, and levels of inflammatory factors of a number of groups: (1) the AIS patients, the AIS group, to the healthy controls, the control group; (2) the AIS participants who had had a mild or moderate stroke, the mild-moderate group, and those who had had a severe stroke, the severe group; (3) the AIS participants who had had a good primary outcome, the good outcome group, and those who had had a poor primary outcome, the poor outcome group; (4) the mild-moderate and severe groups to the control group; and (5) the good outcome and poor outcome groups to the control group.

Outcome Measures • The research team: (1) obtained participants' fecal samples within 72 hours of admission; (2) collected baseline data for the included AIS patients and controls; (3) used 16S rRNA gene sequencing and an enzyme-linked immunosorbent assay (ELISA) to compare the fecal microbial compositions, lipopolysaccharide (LPS) contents, and inflammatory-factor levels between groups; and (4) evaluated the associations of the fecal microbial compositions with severity of stroke and 90-day functional outcomes, using logistic-regression models.

Results • The gut microflora distinguished AIS patients from healthy controls. The LPS and inflammatory-factor levels were associated with an increased risk of poor functional outcomes at day 90.

Conclusions • Dysbiosis of gut microbiota and LPS and inflammatory-factor levels can increase AIS patients' subsequent risks for poor functional outcomes, indicating that the dysbiosis and levels could be potential prognostic markers and therapeutic targets for stroke. (*Altern Ther Health Med.* 2023;29(5):284-292).

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Acute ischemic stroke (AIS) is a leading cause of acquired adult disability and of death and worldwide has an annual incidence of 258 per 100 000 person years (95% CI 234 to 284) and 88 deaths per 100 000 person years (95% CI 80 to 94).[1,2] Stroke has also become the leading cause of death and disability-adjusted life years (DALYs) in China.[2]

The main causes of AIS are atherosclerosis of the large arteries and thrombosis, and platelet aggregation is a key step in thrombosis.[3,4] Dumitrescu et al and Wang et al have suggested that sterile inflammation of the nervous and vascular systems is a key step in the development and exacerbation of AIS.[5,6] Acute sterile inflammation can disrupt the blood-brain barrier and cause neuronal apoptosis after stroke, which is usually associated with a poor prognosis in AIS.[7-9]

Restoration of cerebral blood flow is the main treatment for AIS patients, but the narrow window for treatment time, a patient's intracranial surgery history, any abnormal coagulation,[10] and other factors can limit its effectiveness, although these restrictions don't apply to most people.

AIS' high morbidity and mortality aren't due only to the disease itself but also to the lack of effective therapeutic treatments.[11,12] Neuroprotective therapies performed in multiple applications during AIS treatment, such as reducing excitotoxicity and oxidative and nitrosative stress and protecting the ischemic penumbra, have attracted attention; however, those treatments have failed in clinical trials.[13-15] Given the complex mechanisms underlying AIS' progression, a method of quickly restoring patients' functions and better integrating them into society through treatment is an important research direction.

Gut Dysbiosis

The gut-brain axis may play an important role in stroke. Gut microbiota directly or indirectly regulate brain function through the immune-function, endocrine, vagal, and other humoral pathways and also regulate intestinal and blood-brain barrier (BBB) permeability, thus playing an essential role in brain injury, inflammation, and brain disease.[16-20]

Previous studies have shown that significant gut microflora dysbiosis occurs in AIS patients, and cerebral ischemia can lead to intestinal dysbiosis and disruption of the intestinal barrier.[21] Remodeling the gut microbiota is beneficial in protecting the BBB.[22,23] Benakis et al found that both platelet aggregation and aseptic inflammation were closely associated with gut microbiota and microbial metabolites.[24]

Another study by Benakis et al as well as Chidambaram et al's study found that bidirectional communication between the brain and gut along the brain-gut axis involve gut microbiota and that disturbances of microbial metabolites, especially lipopolysaccharides (LPS), can exacerbate sterile inflammation.[24,25] In addition, Yuan et al found that gut-microbiota dysregulation can be a driving force for stroke development and progression.[24,26] Battaglini et al found that structural changes could occur in the intestinal microbiota of AIS patients through 16S ribosomal RNA (rRNA) sequencing, and demonstrated that the dysbiosis index of intestinal microbiota in stroke was related to cranial nerve injury and prognosis.[21] Bonsack et al, using quantitative reverse transcription polymerase chain reaction (RT-PCR), also demonstrated that intestinal dysbiosis can occur in AIS patients, and found that it was associated with host metabolism and inflammation.[27] Xu et al confirmed that remodeling the gut microbiota can provide an effective treatment for cerebral ischemic stroke.[28]

Lipopolysaccharides

LPS are an important microbial metabolite that increases the levels of proinflammatory cytokines, such as tumor necrosis factor α (TNF- α), nuclear factor-kappa B (NF- κB), interleukin-6 (IL-6), and interleukin-1 β (IL-1 β).[29] Subsequently, the systemic release of proinflammatory cytokines can act on microglia to induce neuroinflammation, thereby aggravating atherosclerosis, leading to a poor prognosis for stroke.[30]

In addition, LPS directly interacts with platelets to stimulate platelet secretion and can significantly increase aggregation, with low concentrations of platelet agonists.[31] Therefore, regulating intestinal flora and reducing the inflammatory response may become a new therapeutic target for ischemic stroke.

AIS and LPS

Tan et al and Varanoske et al found that Actinobacteriota, Bacteroides, Parabacteroides, and Faecalibacterium were the most abundant in patients with severe ischemic stroke at the phylum, genus and species levels and that Escherich coli, Shigella, and other pathogenic bacteria of Enterobacteriaceae were overgrowth.[32,33]

Peh et al and Wei et al found that the enrichment of Enterobacteriaceae and other pathogenic bacteria in the intestine of patients with ischemic stroke can produce a large number of LPS and can enter the body through the damaged intestinal barrier and combine with lipopolysaccharide binding protein (LBP), further inducing the body's inflammatory response through a series of biological effects and affecting the functional recovery of patients with ischemic stroke.[34,35]

Inflammatory Factors

Camara-Lemarroy et al found that the integrity and permeability of the intestinal barrier was strongly correlated with the plasma concentrations of D-lactate (D-LA), intestinal fatty acid binding protein (iFABP), and lipopolysaccharide binding protein (LBP).[36] Clinicians widely use D-Lactate and iFABP as biomarkers to evaluate intestinal barrier function and intestinal integrity in clinical practice.

Mark H Sundman et al found that three inflammatory cytokines, TNFα, IL-1β and IL-6, are the main factors that destroy the BBB and induce neurodegeneration.[31] When neuroinflammation is uncontrolled, it can change the permeability of the BBB and worsen neurological diseases or injuries.[37] Ghelani et al and Chen et al found that high levels of inflammatory cytokines can weaken the barrier function

of intestinal epithelial cells and aggravate the body's inflammatory response and can directly act on acute stroke lesions through the circulation, affecting patients' prognosis and rehabilitation.[38,39] Therefore, improving the homeostasis and abundance of gut microbiota may be a therapeutic target for improving prognosis for stroke patients.

Current Study

Relatively few investigations have evaluated the gut microbiome and its levels of inflammatory factors or the potential associations of those factors with stroke outcomes in patients who have had acute ischemic stroke (AIS), with different stroke severities.

The current study intended to determine if AIS patients would have different gut microbiota and inflammatory-factor levels than healthy individuals and if those levels would be associated with the stroke's severity and the patient's prognosis.

METHODS

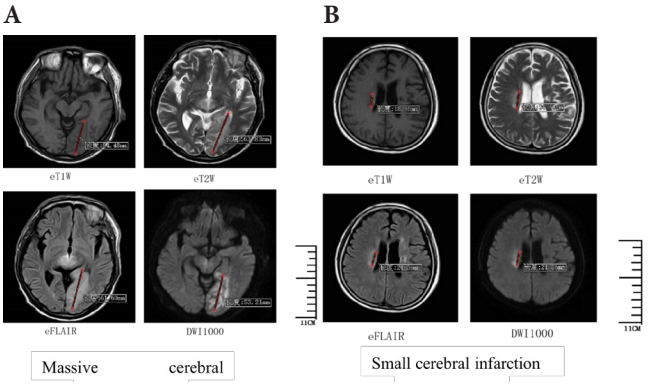
Participants

The research team performed a prospective observational study, which took place at Anhui Wanbei Coal-Electricity Group General Hospital in Suzhou, Anhui, China. Prospective participants were patients who had received a diagnosis and treatment of AIS at the hospital within 48 hours of the stroke, between October 2021 and March 2022 and 60 healthy individuals who had no previous history of stroke. The study included prospective AIS participants if they: (1) were aged >18 years and (2) had been admitted to the hospital within 48 hours of the stroke's onset. The study included prospective healthy controls if they had no prior history of myocardial infarction, stroke, or gut diseases. All patient information in this study was obtained from the General Hospital Hospital of Wanbei Coal and Electricity Group, AIS patients were hospitalized in the Department of Neurology, and the prognosis of AIS patients was evaluated in the rehabilitation department. The study excluded prospective AIS participants and healthy controls if they: (1) had taken antibiotics, prebiotics, or probiotics in the 3 months prior to the study; (2) had had gastrointestinal symptoms in the 3 months prior to the study; (3) had other concomitant neurological diseases; (4) had gut diseases; (5) suffered from advanced cancer; (6) had other serious internal diseases; or (7) had failed to provide stool samples within 72 hours of admission.

Of the 127 potential participants, the research team excluded 37 participants for not meeting the criteria above: (1) three—antibiotics, prebiotics, or probiotics; (2) two—gastrointestinal symptoms; (3) three—concomitant neurological diseases; (4) three—gut diseases; (5) three—advanced cancer; (6) two—serious internal diseases; and (7) 20—no stool samples within 72 hours of admission.

All participants or their legal representatives signed written informed consents in accordance with the Declaration of Helsinki. The ethics committee of the hospital approved the study's protocols.

Figure 1. Participant's Imaging Examination After Admission. The red line marks the infarct's diameter. Figure 1A shows a massive cerebral infarction, and Figure 1B shows a small cerebral infarction.



Procedures

Data collection. The health control group data are from the Anhui Wanbei Coal-Electricity Group General Hospital health examination population in the same period. The research team: (1) consecutively recruited patients diagnosed with AIS for the study; (2) recorded their demographic and clinical characteristics at baseline; and (3) used the National Institutes of Health Stroke Scale (NIHSS) to evaluate participants' stroke severity, with scores of ≤15 points defined as mild or moderate stroke, and those >15 points defined as severe stroke.

Based on the diameter of participants' infarcts from the imaging examination after admission, the team designated those with a diameter of >4 cm as large infarcts (Figure 1A) and those with a diameter of ≤4 cm as mall and medium infarcts (Figure 1B). Two staff members trained on the study's design assessed participants face-to-face using the Modified Rankin Scale (mRS) to evaluate 90-day poor functional outcomes (mRS score ≥3).

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing. The research team: (1) stored all participants' stool samples at -80C for 3 h after the participants' voiding and used 0.2 g per sample for all DNA extraction; and (2) extracted bacterial DNA using a PowerSoil DNA Extraction 88 Kit (Shenzhen Bioeasy Biotechnology, Shanghai, China).

The team used the bar-coded primers V4F (5'-GTGTGYCAGCMGCCGCGGTAA-3') and V4R (5'-CCGGACTACNVGGGTWTCTAAT-3') to amplify the V3-V4 region of the bacterial 16S rRNA gene, using a LightCycler 480 II, real-time, fluorescence, PCR system (Roche Diagnostics, Basel, Switzerland).

Using GeneTools analysis software, version 4.03.05.0 (SynGene, Shanghai, China) and an EZNA Gel Extraction Kit (Omega, Omega Bio-tek, Norcross, GA, USA), the team: (1) mixed the PCR products in equimolar ratios and purified them; and (2) sequenced the PCR amplicons on an Illumina HiSeq 2500 platform (Illumina, San Diego, USA).

The team: (1) incorporated the paired-end sequences based on the overlap between two paired-end sequences,

using the Illumina SeqPrep; (2) assessed the quality of the results using the open-source software Quantitative Insights Into Microbial Ecology (QIIME), version 1.9.1,[40] for quantitative insights into the microbial ecology; (3) trimmed the sequences >200 bp to 200 bp and removed those that were <200 bp; (4) used QIIME workflow scripts to pick closed-reference otus.py; remove the chimera, cluster-based, reference-operant taxonomy; and generate a Biological Observation Matrix (BIOM) file; (5) normalized all samples to the same level to avoid possible errors due to the use of different sequencing depths; and (6) normalized each sample to 3500 sequences to retain as many sequences as possible.

Quantification of intestinal fatty acid binding protein (iFABP), D-lactate (D-LA), LPS, lipopolysaccharide-binding protein (LBP), TNF-α, IL-6, and IL-1β.

The research team: (1) isolated all participants' serum by centrifugation at 3000 rpm for 10 minutes and stored samples in a refrigerator at -80°C until testing; (2) determined the levels of iFABP, D-LA, LPS, LBP, TNF-α, IL-1β, and IL-6 using commercially available, enzyme-linked immunosorbent assay (ELISA) kits (Bioswamp, Myhalic Biotechnology, Wuhan, China).

An experienced staff member who was blinded to the study's design performed all measurements. The standard curves were all within the expected range, indicating good linearity and a high precision for the dilution.

16S rRNA amplicon data processing. The research team: (1) analyzed the 16S rRNA sequencing data using QIIME 1.80[40]; (2) analyzed beta-diversity—the differences between microbial communities—based on unweighted UniFrac distances, a distance metric for comparing biological communities.

Principal coordinate analysis (PCoA) is a dimensionality reduction method to illustrate the relationship between samples based on a distance matrix. The research team used the R software package ade4[41] to highlight the differences between the groups.

The research team: (1) used the Adonis test for microbial analysis; (2) constructed the relative abundance of dominant groups using the R software package ggplot2[41]; (3) performed linear discriminant analysis (LDA) and effect size measurement (LEfSe) to identify and differentiate metagenomic biomarkers using the online Galaxy program[42] (http://huttenhower.sph.harvard.edu/galaxy) at Harvard University.

The research team plotted significantly different bacteria with an LDA score of ≥2.5 on a classification bar chart and used a box graph to show the relative abundance of microorganisms.

The team assessed each bacteria's association with the AIS participants' 90-day functional outcome using 16SrRNA gene sequencing and ELISA determination to obtain fecal bacterial populations as well as LPS and serum inflammatory-factors levels.

Groups. The research team performed multiple comparisons of the baseline demographic and clinical characteristics, the gut microbiota, and levels of inflammatory factors of a number of groups: (1) the AIS patients, the AIS group, to the healthy controls, the control group; (2) the AIS participants who had had a mild or moderate stroke, the mild-moderate group, and those who had had a severe stroke, the severe group; (3) the AIS participants who had had a good primary outcome, the good outcome group, and those who had had a poor primary outcome, the poor outcome group; (4) the mild-moderate and severe groups to the control group; and (5) the good outcome and poor outcome groups to the control group.

Outcome Measures. The research team: (1) obtained participants' fecal samples within 72 hours of admission; (2) collected baseline data for the included AIS patients and controls; (3) used 16S rRNA gene sequencing and an enzyme-linked immunosorbent assay (ELISA) to compare the fecal microbial compositions, lipopolysaccharide (LPS) contents, and inflammatory-factor levels between groups; and (4) evaluated the associations of the fecal microbial compositions with severity of stroke and 90-day functional outcomes, using logistic-regression models.

Outcome Measures

Baseline characteristics. The research team compared the characteristics: (1) of the AIS group and the control group, (2) of the mild-moderate group and the severe group; and (3) of the poor outcome group and good outcome group. The compared characteristics were age, gender, body mass index (BMI), histories of hypertension or diabetes, levels of neutrophils (NEUs), levels of N-terminal B-type natriuretic peptides (NTproBNPs) as well as infarct size for the good outcome and poor outcome groups.

Gut microbiota. The research team assessed whether specific differences existed in gut the microbiota of the two sets of AIS groups—the mild-moderate and severe groups and the poor outcome and good outcome groups—and the control group by analyzing stool samples from each participant.

Inflammatory factors. The research team measured the levels of iFABP, D-Lactate, LPS, LBP, TNF-α, IL-6, and IL-1β in the serum of participants.

Statistical Analysis

The research team analyzed the data using the SPSS 22.0 software (SPSS, Inc, Chicago, IL, USA). The team: (1) expressed measurement data that conformed to a normal distribution as means ± standard deviations (SDs) and used the independent sample *t* test for comparisons between groups, (2) expressed counting data as numbers (n) and percentages (%) and used the Chi-square (χ^2) test to compare groups, (3) used the Pearson test for correlation analysis, (4) analyzed the factors influencing prognosis using multifactor logistic regression analysis, and (5) analyzed the predictive value of the prognosis using the receiver operating characteristic (ROC) curve. $P < .05$ indicated a statistically significant result.

Figure 2. Flowchart of Enrollment

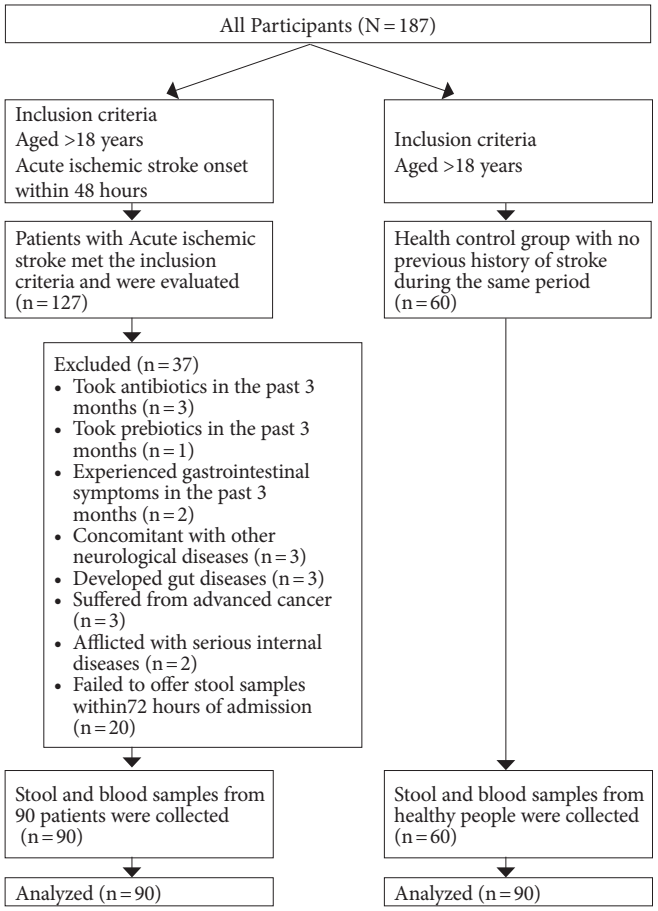


Table 1. Comparison of the Demographic and Clinical Characteristics at Baseline of the Healthy Controls and AIS Participants at Baseline (N = 150)

Characteristics	Control Group n = 60 n (%) Mean ± SD	AIS Group n = 90 n (%) Mean ± SD	*P* value
Gender			
Male	41 (68.33)	60 (66.67)	
Female	19 (31.67)	30 (33.33)	
Age	60.75±8.72	59.77±11.36	>.05
BMI	24.14 ± 1.96	24.63 ± 2.96	>.05
Medical Co-morbidities[a]			
Hypertension	14 (23.33)	81 (90.00)	<.001[b]
Diabetes	2 (3.33)	61 (67.78)	<.001[b]
Laboratory Findings			
NEU, $\times 10^9$/L	3.46 ± 1.54	5.64 ± 3.71	<.001[b]
NT-proBNP, pg/mL	0.00 ± 0.00	126.80 ± 425.92	<.001[b]

[a]Participants could have no comorbidities and more than one co-morbidity, so the groups' totals for that characteristic aren't equal to 60 in the control group or 90 in the AIS group
[b]$P < .001$, indicating that the AIS group was significantly more likely to have a history of hypertension or diabetes and had significantly higher levels of neutrophils (NEU)s and N-terminal B-type natriuretic peptides (NTproBNPs) than the control group did

Abbreviations: AIS, acute ischemic stroke; BMI, body mass index; NEU, neutrophils; NT-proBNP, N-terminal pro-atrial natriuretic peptide.

RESULTS

Participants

Figure 2 shows the flowchart of enrollment of participants. The study included and analyzed the data of 150 participants, 90 AIS patients and 60 healthy controls (Table 1).

The AIS group's mean age was 60.75±8.72 years, and 60 participants were male (66.67%) and 30 were female (33.33%). The control group's mean age of the healthy was 59.77 ± 11.36 years, and 41 participants were male (68.33%) and 19 were female (31.67%). No significant differences existed in age, gender, or BMI at baseline between the AIS participants and the healthy controls.

At baseline, the AIS group was significantly more likely to have a history of hypertension ($P < .001$) or diabetes ($P < .001$) than was the control group. The AIS group also had significantly higher levels of NEUs, with $P < .001$, and NTproBNPs, with $P < .001$, than the control group did.

Stroke Comparison

Stroke severity. Among the 90 AIS participants, the research team identified 74 with mild or moderate strokes (82.22%) and 16 with severe strokes (17.78%).

The mean age of the mild-moderate group was 59.40 ± 11.33 years, and 56 participants were male (75.68%) and 18 were female (24.32%). The mean age of the severe group was 62.22±10.89 years, and nine participants were male (56.25%) and seven were female (43.75%). No significant differences existed in age, gender, or BMI at baseline between AIS participants with mild or moderate ischemic strokes and those with severe strokes.

The severe group was significantly more likely to have a history of hypertension ($P < .001$) or diabetes ($P < .001$) than the mild-moderate group was. The severe group also had significantly higher levels of NEUs, with $P < .001$, and NTproBNPs, with $P < .001$, than those in the mild-moderate group did.

Gut microbiota. Figures 3A, 3B, and 3C show the levels of microbiota for the phylum, genus, and species, respectively, in the control group and the two AIS groups, the mild-moderate and severe groups. Compared to the control group, the severe group had: (1) more abundant Actinobacteriota and less abundant Spirochaetes at the phylum level (Figure 3A); (2) more abundant Bacteroides and Parabacteroides and less abundant *Faecallbacterium*, *Prevotella*, *Roseburia*, and *Lachnospira* (Figure 3B); and (3) the most abundant *Faecalibacterium* (Figure 3C).

Figures 3D and 3E show the PCoA analysis of beta diversity and alpha diversity, respectively. The PCoA showed that the gut flora distinguished the mild-moderate and severe groups from the control group, and that the severe group significantly had greater distances from the control

Table 2. Comparison of the Demographic and Clinical Characteristics at Baseline of the Mild-Moderate and Severe Groups of AIS Participants (n = 90)

Characteristics	Mild-Moderate Group 74 (82.22) n (%) Mean ± SD	Severe Group 16 (17.78) n (%) Mean ± SD	*P* value
Gender			
Male	56 (75.68)	9 (56.25)	
Female	18 (24.32)	7 (43.75)	
Age	59.40±11.33	62.22±10.89	>.05
BMI	24.57 ± 2.68	25.61 ± 2.96	>.05
Medical Co-morbidities[a]			
Hypertension	50 (67.56)	14 (87.50)	<.001[b]
Diabetes	21 (28.38)	9 (56.25)	<.001[b]
Laboratory Findings			
NEU, $\times 10^9$/L	5.77 ± 3.07	8.14 ± 5.22	<.001[b]
NT-proBNP, pg/mL	111.72 ± 311.25	1399.00 ± 1922.20	<.001[b]
90-day Good Primary Outcome (mRS<3)	52 (70.2)	4 (25)	

[a]Participants could have no comorbidities and more than one co-morbidity, so the groups' totals for that characteristic aren't equal to 74 in the mild-moderate group or 16 in the severe group

[b]$P < .001$, indicating that the severe group was significantly more likely to have hypertension or diabetes and had significantly higher levels of neutrophils (NEU)s and N-terminal B-type natriuretic peptides (NTproBNPs) than the mild-moderate group did

Abbreviations: BMI, body mass index; mRS, Modified Rankin Scale; NEU, neutrophils; NT-proBNP, N-terminal pro-atrial natriuretic peptide.

Table 3. Comparison of the Demographic and Clinical Characteristics at Baseline of the AIS Participants According to their 90-Day Functional Outcomes (n = 90)

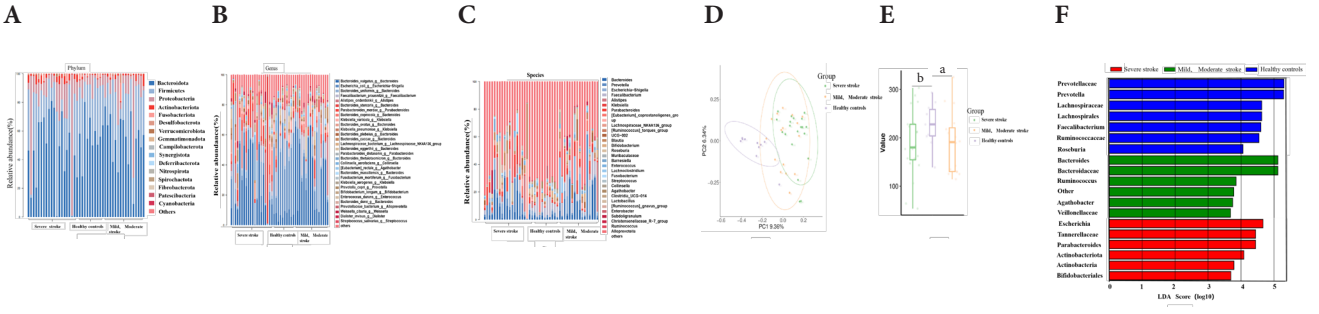
Characteristics	Good Outcome Group 56 (62.22) n (%) Mean ± SD	Poor Outcome Group 34 (37.78) n (%) Mean ± SD	*P* value
Gender			
Male	36 (64.29)	21 (61.76)	
Female	20 (35.71)	13 (38.24)	
Age	62.37± 11.91	58.69 ± 13.61	>.05
Medical Co-morbidities[a]			
Hypertension	47 (83.93)	33 (97.06)	<.001[b]
Diabetes	21 (37.50)	26 (76.47)	<.001[b]
Laboratory Findings			
NEU, $\times 10^9$/L	5.41 ± 3.42	6.14 ± 4.10	<.001[b]
NT-proBNP, pg/mL	88.47 ± 257.68	218.50 ± 1517.82	<.001[b]
Infarct Size	3.69 ± 0.78	4.37 ± 0.34	<.001[b]

[a]Participants could have no comorbidities and more than one co-morbidity, so the groups' totals for that characteristic aren't equal to 56 in the good outcome group or 16 in the poor outcome group

[b]$P < .001$, indicating that the good outcome group was significantly less likely to have hypertension or diabetes, had significantly lower levels of neutrophils (NEU)s and N-terminal B-type natriuretic peptides (NTproBNPs), and had a significantly smaller infarct size than the poor outcome group did

Abbreviations: NEU, neutrophils; NT-proBNP, N-terminal pro-atrial natriuretic peptide.

Figure 3. Comparisons of the Gut Microbiota Between Healthy Controls and Participants With Mild or Moderate Ischemic Stroke or Severe Ischemic Stroke. Figures 3A, B, and C show the taxonomic summary of the gut microbiota at the phylum, genus, and species level, respectively. Figures 3D shows the PCoA analysis of beta diversity in each group. Figure 3E shows an analysis of alpha diversity in each group. Figure 3F shows that the discriminative taxa at the family level based on linear discriminant analysis.

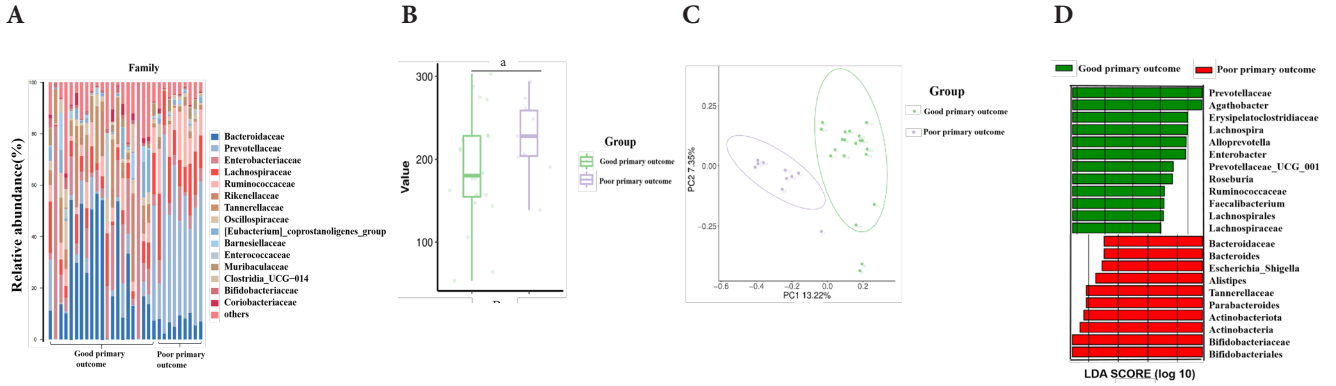


[a]$P < .05$, indicating that the mild-moderate groups' alpha diversity was significantly lower than that of the control group

[b]$P < .01$, indicating that the alpha diversity of the severe group was significantly lower than that of the control group

Abbreviations: LDA, linear discriminant analysis; PCoA, Pharmacy Curriculum Outcomes Assessment.

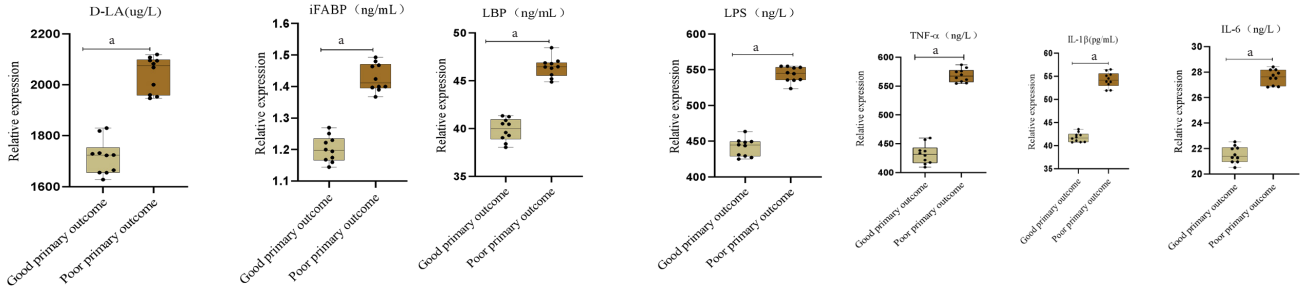
Figure 4. The Taxonomic Summary of the Gut Microbiota of Participants With Good and Poor Primary Outcomes at the Family Level. Figure 4 A shows the taxonomic summary of the gut microbiota at the family level. Figure 4B shows the PCoA analysis of alpha diversity in each group. Figure 4C shows the analysis of beta diversity in each group. Figure 4D shows the discriminative taxa at the family level based on the linear discriminant analysis.



[a]$P < .01$, indicating that the good outcome group's alpha diversity was significantly lower than that of the poor outcome group

Abbreviations: LDA**,** linear discriminant analysis**;** PCoA, Pharmacy Curriculum Outcomes Assessment.

Figure 5. Serum Levels of LPS, LBP, TNF-α, IL-6, and IL-1β of Participants With Different Degrees of Ischemic-stroke Prognosis



[a]$P < .001$, indicating that D-LA, iFABP, LBP, LPS, TNF-α, IL-1β, and IL-6 were significantly higher for the poor outcome group than for the good outcome group

Abbreviations: D-LA, D-lactate; iFABP, intestinal fatty acid binding protein; IL-1β, interleukin-1 beta; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharides; TNF-α, tumor necrosis factor alpha.

group. The mild-moderate groups' alpha diversity was significantly lower than that of the control group ($P < .05$), and the severe group's alpha diversity was significantly lower than that of the control group ($P < .01$).

Figure 3F shows that the abundance of Escherich coli and Shigella in Enterobacteriaceae were positively correlated with the degree of ischemic stroke.

Outcome Comparison

Functional outcomes. Among the 90 AIS participants, the research team identified 56 who had good primary outcomes (62.22%) and 34 (37.78%) who had poor primary outcomes at day 90.

The mean age of the good outcome group was 62.37± 11.91 years, and 36 participants were male (64.29%) and 20 were female (35.71%). The mean age of the participants with a severe stroke was 58.69 ± 13.61 years, and 21 participants were male (61.76%) and 13 were female (38.24%). No significant differences existed in age, gender, or BMI at baseline between participants with good primary outcomes and those with poor primary outcomes.

The poor outcome group was significantly more likely to have a history of hypertension ($P < .001$) or diabetes ($P < .001$) than the good outcome group was. The poor outcome group also had significantly higher levels of NEUs, with $P < .001$, and NNTproBNPs, with $P < .001$, than those in the good outcome group did. Also, the infarct size for the poor outcome group was significantly larger than that of the good outcome group.

Gut microbiota. Significant differences existed in the composition of gut microbiota between the poor outcome and good outcome groups of AIS participants. The taxonomic classification at the family level showed that *Prevotellaceae*,

Enterobacteriaceae, *Ruminococcaceae*, *Faecalibacterium*, and *Lachnospiraceae* were more abundant in the intestines of participants in the poor outcome group than they were in the good outcome group (Figure 4A).

Figures 4B and 4C show the PCoA analysis of alpha diversity and beta diversity, respectively. Figure 4D shows that the *Bacteroidaceae*, *Tannerellaceae*, and *Bifidobacteriaceae* were more abundant in the intestines of patients in the poor outcome group than they were in good outcome group. The abundance of those microbiota were positively correlated with the outcome of ischemic stroke.

Inflammatory Factors

Figure 5 shows that the levels of iFABP, D-Lactate, LPS, LBP, TNF-α, IL-6, and IL-1β in the serum of participants in the poor outcome group were significantly higher than those of participants in the good outcome group (all $P < .001$).

DISCUSSION

The current study investigated the gut microbiota of AIS patients with different severity levels. Enterobacteriaceae were positively correlated with the degree of ischemic stroke. The increased abundance of Enterobacteriaceae in the gut would have induced changes in the permeability of the intestinal barrier, which rapidly induced systemic inflammation by producing large amounts of LPS. Thus, the relative abundance of Enterobacteriaceae can be an independent risk factor for evaluating the possibility of an early recovery for patients with ischemic stroke. That clinical outcome provides evidence that gut microbiota can be an indicator for the assessment of AIS.

The current research team conducted a 90-day follow-up study to investigate whether that disruption of gut microbiota could affect stroke outcome. The study found that 70.2% of patients with a mild or moderate AIS had good functional outcome, and neutrophil levels were lower than those of the severe group.

The current study found that the levels of iFABP, D-Lactate, LPS, LBP, TNF-α, IL-6, and IL-1β in the serum of participants in the poor outcome group were significantly higher than those of participants in the good outcome group, and these findings are consistent with those of Camara-Lemarroy et al.[36]

The current study found that the good outcome group's lactic acid bacteria, belonging to the genus Lactobacillus, were higher than those of the poor outcome group. Two other studies have reported that ischemic stroke was associated with an increase in Lactobacillus ruminis and a decrease in the Lactobacillus sakei subgroup.[34,43]

The current study also found that Bacteroidaceae, including Bacteroides was positively correlated with systemic inflammation in patients with ischemic stroke, which further suggested that dysbiosis of gut microbiota may lead to changes in gut barrier integrity and permeability.

The current study revealed dysbiosis of gut microbiota in AIS, characterized by decreased abundance and diversity. The reduction in the abundance and diversity of the intestinal microbiota can affect the integrity of the intestinal barrier, leading to the production of a large number of LPS in the body and inducing a further increase in inflammatory factors, which can affect the functional prognosis of patients at 90 days. This suggests that the reduced abundance and diversity of gut microbiota and LPS levels may be useful biomarkers for identifying a poor prognosis. Dietary fiber supplements can increase the abundance and diversity of gut microbiota and may produce a large number of beneficial bacteria. Further studies with large sample size and high quality need to be conducted to confirm the current findings through clinical or experimental dietary interventions.

The current study had some limitations. First, the prevalence of hypertension and diabetes was lower in healthy controls than in patients with ischemic stroke. However, hypertension and diabetes and other diseases are risk factors for ischemic stroke. In future studies, the current research team should focus on the time periods before ischemic stroke onset and acute stroke to determine whether stroke itself affects gut microbiota and its metabolites.

In addition, this investigation didn't address interventions. It's not known whether gut-microbiota supplementation can improve stroke outcomes.

CONCLUSIONS

Dysbiosis of gut microbiota and LPS and inflammatory-factor levels can increase AIS patients' subsequent risks for poor functional outcomes, indicating that the dysbiosis and levels could be potential prognostic markers and therapeutic targets for stroke.

DATA AVAILABILITY STATEMENT

All relevant data are within the manuscript. Additional supporting information may be found online in the Supporting Information section at the end of the article..

AUTHORS' DISCLOSURE STATEMENT

The authors declare that they have no conflicts of interest related to the study. The Provincial Natural Science Foundation of Anhui (2108085MH307), and the Science and Technology Program of Suzhou city, Anhui Province (SZSKJJZC043,SZZCZJ202210); and the Scientific Research Project of Suzhou Health Commission, Anhui Province(SZWJ2022a053) supported the study

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