Elevated circulating levels of phenylacetylglutamine in stroke patients with T2D are linked to specific gut microbiota

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Research Article

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Abstract

Objective

Type 2 diabetes (T2D) aggravates the injury of ischemic stroke (IS). The alterations of gut microbiota and its metabolite phenylacetylglutamine (PAGln) levels in stroke patients with T2D remain unclear. Therefore, our study aimed to explore the differences in gut microbiota and its metabolite PAGln between IS patients with and without T2D.

Methods

In our study, 35 IS with T2D (IS-T2D group), 50 IS patients without T2D (IS-NT2D group), and 29 healthy controls (HC group) were recruited. Fecal samples were collected and analyzed using high-throughput sequencing of 16S rRNA genes, and plasma samples were subjected to targeted metabolomics to detect metabolite PAGln. Plasma PAGln levels of rats with transplantation of fecal microbes from patients were assessed.

Results

Our results showed that the plasma PAGln levels in IS-T2D patients were significantly higher than those in IS-NT2D patients. Correlation analysis showed that plasma PAGln levels were significantly correlated with the relative abundance of *Enterobacteriaceae*, *Verrucomicrobiota*, and *Klebsiella*, which were enriched in IS-T2D patients. Further studies demonstrated that plasma PAGln levels were positively correlated with the concentration of neutrophil extracellular traps (NETs), and NETs levels were increased in a dose-dependent manner according to PAGln levels. Moreover, the rats transplanted with fecal microbes from IS-T2D patients developed more severe brain injury and higher plasma PAGln levels compared to the rats transplanted with fecal microbes from IS-NT2D patients.

Conclusions

Our results suggest that T2D may contribute to aggravation in stroke patients via NETs, mediated in part by gut microbiota and its metabolite PAGln.

Introduction

Stroke is an important public health problem affecting more than 10 million people every year around the world¹. In China, the mortality rate of stroke is 149.49/100,000, accounting for 22% of the national overall mortality rate². It is widely known that type 2 diabetes (T2D) is an independent risk factor for ischemic stroke (IS)³. Surveys have shown that among IS patients, up to 23.5% of patients had diabetes mellitus,

accompanied by higher risks of stroke recurrence, disability, and mortality^{4, 5}. As the prevalence of IS with T2D (IS-T2D) has seen a steady increase globally, it is increasingly important for finding new pathophysiological mechanisms and therapeutic targets for IS-T2D.

In recent years, increasing evidence suggests that changes in the gut microbiota and its metabolites participate in the pathophysiological mechanism of IS and T2D^{6–13}. A cohort study of more than 5000 people showed that patients with T2D and patients who developed incident adverse cardiovascular events after 3-year follow-up had higher plasma phenylacetylglutamine (PAGln) levels, which were proved to be an independent predictor of adverse cardiovascular events¹⁴. The metabolite, PAGln, is produced by gut microbiota via catabolizing the essential amino acid phenylalanine (PHE) in the distal colon¹⁵. As a gut microbiota-derived metabolite, PAGln is not only related to elevated blood glucose but also closely related to the risk of thrombotic events, such as coronary heart disease (CHD)^{16, 17}. Similarly, IS is also a dreadful thrombotic event. Multiple studies have shown that gut microbiota and its metabolites changed after stroke and then exacerbated cerebral infarction in turn^{18, 19}. Nevertheless, the relationship among gut microbiota, its metabolite PAGln, and stroke with T2D remains unknown.

A study showed that neutrophils (NE) mediated inflammation to amplify cerebral microvascular damage in the early stage of T2D, resulting in more severe brain edema and nerve damage after ischemia²⁰. Furthermore, NE also participated in inflammatory damage by releasing neutrophil extracellular traps (NETs), which are network structures of DNA fibers, composed of histones and active particles^{21, 22}. Extensive research has confirmed the formation of NETs in blood circulation and thrombosis in patients with IS^{23, 24}. Moreover, high glucose levels or hyperglycemia in diabetic patients could increase the release of NETs^{22, 23}. In inflammatory bowel disease, NETs are involved in inflammation after intestinal dysbiosis²⁵. Therefore, we hypothesized that gut microbiota and its metabolites were disrupted in stroke patients with T2D, leading to NET-related immune imbalance.

Here, we evaluated the characteristics of gut microbiota in IS-T2D patients, IS patients without T2D (IS-NT2D), and healthy control people (HC) via 16S ribosomal RNA sequencing and determined the plasma PAGln concentration in those groups by targeted liquid chromatography-mass spectrometry. We also analyzed the relationship between PAGln levels and NETs. In addition, we constructed a prediction model based on the differential relative abundances of microbiota, plasma PAGln levels, and NETs levels for discriminating between IS-T2D patients and IS-NT2D patients. Finally, we validated whether the fecal microbes from IS-T2D patients could exacerbate brain infarction and elevate circulating PAGln levels in IS-T2D patients via fecal microbiota transplantation (FMT) in animal experiments.

Methods

Study population

We recruited 85 patients with IS in the Department of Neurology, Xiangya Hospital, Central South University (Changsha, China) from December 2019 to December 2020. And the patients were divided into 50 patients without T2D (IS-NT2D group) and 35 patients with T2D (IS-T2D group) (T2D was defined as T2D medical history or typical diabetes symptoms with either random blood glucose ≥ 11.1 mmol/L, fasting blood glucose ≥ 7.0 mmol/L, or blood glucose at 2h after glucose load ≥ 11.1 mmol/L²⁶). Meanwhile, 29 healthy controls (HC group) were recruited. Inclusion criteria of patients were as follows: (1) age between 18 and 80 years old; (2) first diagnosed acute IS (stroke was defined as a rapid clinical onset of a neurological impairment lasting more than 24 hours or resulting in death, with no cause other than that of vascular origin²⁷. IS was further confirmed by comprehensive neurological physical examination, head computed tomography, and/or magnetic resonance imaging); (3) admitted within two weeks of IS onset. In addition, patients with T2D treated with metformin or acarbose were excluded. HC matched by age and sex were recruited. Exclusion criteria of patients and HC were as follows: (1) used antibiotics or probiotics before admission or after admission within 1 month; (2) suffered from acute inflammatory disease, severe infective disease and/or cancer, severe liver and kidney failure, hepatic impairment, autoimmune disease, severe mental illness, and gut disease (i.e., inflammatory bowel disease, ulcerative colitis, and Crohn's disease); (3) had a history of intestinal surgery. For all participants, plasma samples were collected within 24h, and fecal samples were obtained within 48h of admission. At the same time, baseline characteristics including demographic and cerebrovascular risk factors (histories of hypertension, dyslipidemia, diabetes mellitus, CHD, and smoking) were collected and recorded at admission. Each participant in this study provided written informed consent, and the study protocol complied with the principles of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Xiangya Hospital, Central South University, China.

All patients were assessed with the National Institutes of Health Stroke Scale (NIHSS) scores on admission and evaluated by the modified Rankin scale (mRS) scores 3 months after onset. Both NIHSS and mRS scores were rated by two trained clinical staffs who were blinded to the study protocol.

16S rRNA amplification and sequencing of fecal microbiota samples

Fecal samples of all research objects were stored at -80°C within 30 minutes once obtained. Total genome DNA from the samples was extracted using a DNA kit (Magnetic Soil And Stool DNA Kit, TIANGEN, DP712, China) according to the manufacturer's instructions. We chose the V3-V4 region of the 16S rRNA gene to finish polymerase chain reaction (PCR) amplification and used specific primer 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). The mixture of PCR products was purified using Qiagen Gel Extraction Kit (Qiagen, Germany) and sequencing libraries were generated with the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). At last, the library was sequenced on an Illumina NovaSeq platform.

Bioinformatics and Biostatistics

We used Quantitative Insights into Microbial Ecology (QIIME) V1.9.1 software to analyze microbial data. The Effective Tags of all samples were clustered by the Uparse algorithm (Uparse v7.0.1001). Sequences were clustered into operational taxonomic units (OTUs) with 97% identity, and species annotations were made to the OTUs sequence (the threshold was set to 0.8 ~ 1). QIIME was used for species alpha and beta diversity analysis, and R software (2.15.3) was used for drawing the species accumulation curve. The linear discriminant analysis (LDA) effect size (LEfSe) was used to determine the difference among the three groups of bacteria, with a threshold of 4. PICRUSt was used to predict the metagenome function of 16S rRNA biological information. According to the 16S rRNA sequencing data, the function prediction based on the KEGG database was performed.

Laboratory tests

12h fasting blood samples were collected on admission, centrifuged at 3000 RPM for 10 minutes, and stored at -80°C. A routine blood test was analyzed with an automatic biochemical analyzer. Blood biochemistry such as blood urea nitrogen (BUR), serum creatinine (Scr), triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), glucose, glycosylated hemoglobin (HbA1C), homocysteine (Hcy) was analyzed with automated enzymatic analysis.

Plasma PAGIn concentrations were quantified by targeted liquid chromatography-mass spectrometry. The plasma was diluted 10-fold with ddH₂O. 48μL diluted plasma was mixed with 2μL internal standard (1ppm D5-PAGIn), diluted 3-fold with cold methanol, then centrifuged (21,000 x g; 4°C for 15 min), and then transferred to a clean glass bottle for testing in AB SCIEX TripleTOF 6500 System (AB SCIEX, Foster City, CA, USA). Finally, 1 uL supernatant was analyzed by injecting Acquity UPLC BEH C18 column for analysis (50×2.1 mm, 1.7 μm) at a column temperature of 40°C, a flow rate of 0.3 mL/min, mobile phase A containing 0.1% acetic acid, mobile phase B containing 0.1% acetic acid. The concentration of PAGIn was measured by establishing a standard curve based on the known PAGIn concentration.

Quantification of NETs markers

Citrullinated histone H3 (CitH3) is currently considered to be the most specific marker of NETs²³. Therefore, we evaluated the concentration of CitH3 in the plasma to represent the level of NETs. CitH3 was measured with the CitH3 Detection ELISA kit (Cayman Chemical, 501620, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Animals

The experimental protocols were approved by the Experimental Animal Welfare Ethics Committee of Central South University on September 7, 2021 (Approval No. 2019-0004). All experimental procedures were conducted following the Care and Use of Laboratory Animals by the guidelines of the National Institutes of Health (NIH).

Sprague-Dawley (SD) rats (5–6 weeks old) (purchased from Hunan SJA Laboratory Animal Co. Ltd) were placed in a specific pathogen-free (SPF) environment and raised under the conditions of cycles of 12h light/dark, 50%-55% humidity, and 100–200 Lux of light intensity. The animals were randomly divided

into the FMT-IS-NT2D group (transplanted with stool samples from IS-NT2D patients) and the FMT-IS-T2D group (transplanted with stool samples from IS-T2D patients).

Fecal microbiota transplantation (FMT)

Before FMT, rats in both groups were fed with drinking water containing antibiotics (vancomycin 500mg/L, neomycin 1g/L, ampicillin 1g/L, metronidazole 1g/L) for 1 week. The drinking water containing antibiotics was replaced every 1–2 days²⁸. Then, stool samples from 5 IS-T2D patients and 5 IS-NT2D patients were selected. Next, the stool homogenate was centrifuged at 1000 RPM and the supernatant was collected. Fecal supernatant from the two patient groups was gavaged to the two rat groups (2ml/d for each rat), respectively, for 1 week, and then modeling was made²⁹.

Middle cerebral artery occlusion (MCAO) procedure

Before inducing the MCAO model, 1–2 ml orbital blood of anesthetized rats was collected to measure the PAGln concentration. The collected blood was immediately centrifuged at 3000 RPM for 10 minutes, and the plasma was separated and stored at -80°C for testing. After blood collection, rats were subjected to middle cerebral artery occlusion as previously described³⁰. Briefly, rats were placed in a supine position, and a midline incision was made in the neck to expose the external carotid artery (ECA) and common carotid artery (CCA). The ECA was ligated and the internal carotid artery (ICA) was separated. A 4–0 silicon-coated monofilament suture was inserted into ICA after cutting on ECA. After 90min of occlusion, the suture was taken out and the ECA was ligated. The neck wound was sutured and the animals were recovered and reared for 24 hours before being killed.

After anesthesia, the blood of stroke rats was collected through the cardiac puncture, and the plasma was centrifuged as described above. The separated plasma was placed in a refrigerator at -80°C for testing. The plasma PAGln concentration of rats was measured with the rat PAGln ELISA assay kit (Shanghai Jianglai Biotechnology, JL51250, Shanghai, China) according to the manufacturer's instructions.

Neurological evaluation

Garcia neurofunctional score was used to evaluate the neurological function of rats at 24h after the MCAO procedure³¹. The score includes six tests: spontaneous movement, symmetry of limb movement, symmetry of forelimb extension, climbing, body proprioception, and response to whiskers. The score ranges from 3 to 18, and the lower the score, the more serious the neurological deficit. The score was evaluated by two trained experimenters who were blinded to the study protocol.

Measurement of infarct volume

After anesthesia, rats were sacrificed to extract brain tissue. Brain slices with a thickness of 2mm were taken from a coronal plane, and 5 slices were cut from each brain. Staining was then performed by soaking in a 2% TTC solution at 37°C for 30 min³². Infarct volume was calculated using Image-Pro Plus Image software. The results were expressed as infarct volume percentage: infarct volume percentage = total infarct volume/total brain volume x 100%³³.

Statistical analyses

The data were statistically analyzed and managed by SPSS 26.0 and GraphPad Prism 8.0. For continuous data, it was expressed as mean $\pm$ standard error mean (SEM) if conformed to the normal distribution and was expressed as the median and interquartile range (IQR) if not conformed to the normal distribution. For categorical data, it was expressed as percentages. The mean was analyzed by the t-test and the median was analyzed by the Mann-Whitney u-test in comparison between the two groups. The mean was analyzed by the ANOVA and the median was analyzed by the Kruskal-Wallis test in comparison among the three groups. The percentage was analyzed by the chi-square or Fisher's exact test. Spearman correlation analysis was used to analyze the relationship among PAGln level, gut microbiota, and biochemical indexes. Receiver operator characteristic (ROC) was used to evaluate the diagnostic performance of plasma PAGln levels, gut microbiota, and NETs levels for IS-T2D. A value of $P < 0.05$ was considered significant. Univariate and multivariate logistics regression was used to evaluate whether the plasma PAGln level was a risk factor for IS-T2D and IS-NT2D. The relative risk was shown as the odds ratio (OR) with the 95% confidence interval.

Result

Participant characteristics

A total of 114 subjects were recruited in our study, including 35 patients with IS-T2D, 50 patients with IS-NT2D, and 29 healthy individuals. As showed by the data in Table 1, there were no significant differences in age and sex among the three groups. Risk factors such as hypertension, dyslipidemia, and CHD were not statistically significantly different between the IS-T2D and IS-NT2D groups. The severity of infarction (admission NIHSS score) and short-term prognosis (90-day mRS score) of patients with IS-T2D were worse than those with IS-NT2D ($P < 0.05$). IS-T2D and IS-NT2D groups had significantly higher levels of leukocyte (LEU), NE, blood urea nitrogen (BUN), and serum creatinine (Scr) when compared with the HC group, and the IS-T2D group had the highest levels among the three groups ($P < 0.05$). Moreover, glucose and HbA1c of the IS-T2D group were significantly higher than those of the other two groups ($P < 0.001$).

Table 1
Characteristics of the study participants

Baseline characteristics	HC(n = 29)	IS-NT2D(n = 50)	IS-T2D(n = 35)	P-value
Gender, n (M/F)	12/17	31/19	24/11	0.696
Age (years)	57.79 ± 14.11	57.62 ± 12.08	60.14 ± 11.95	0.629
Risk factors, n (%)				
Hypertension	15(51.7) ^b	36(72.0)	31(88.6)	0.005
Dyslipidemia	8(27.6)	13(26.0)	13(37.1)	0.518
Diabetes	0(0)	0(0)	35(100)	/
CHD	3(10.3)	5(10.0)	6(17.1)	0.574
Current smoking	9(31.0)	26(52.0)	16(45.7)	0.194
Clinical findings				
Admission NIHSS score	/	3(1, 6) ^b	6(4, 8)	/
90-Day mRS score	/	1(0.75, 2) ^b	2(2, 3)	/
Biochemical data				
LEU (x10 ⁹ /μL)	5.8(5.0, 6.6)	6.5(5.3, 8.2)	7.3(6.2, 8.8)	0.002
NE (x10 ⁹ /μL)	3.4(2.9, 4.0)	4.4(3.4, 4.9)	5.0(3.8, 6.0)	< 0.001
LY (x10 ⁹ /μL)	1.5(1.3, 1.8)	1.4(1.1, 2.1)	1.6(1.2, 2.0)	0.883
NLR	2.1(1.8, 3.1)	2.4(2.0, 3.8)	3.3(2.3, 4.4)	0.032
BUN, mmol/L	4.36(3.51, 5.36)	4.64(3.94, 5.93)	5.35(4.45, 6.54)	0.030
Scr(mmol/L)	70.0(62.0, 84.0)	78.0(65.4, 78.6)	80.1(71.0, 93.7)	0.360
TG (mmol/L)	1.51(1.23, 2.01)	1.47(1.06, 2.22)	1.54(1.26, 2.04)	0.730
TC (mmol/L)	4.15(3.61, 4.94)	4.22(3.38, 4.92)	3.55(3.06, 5.02)	0.180
HDL (mmol/L)	2.67(2.30, 3.09)	2.68(1.99, 3.15)	2.23(1.84, 3.00)	0.220
Glu (mmol/L)	4.98(4.81, 5.41)	5.23(4.81, 5.67)	9.20(6.24, 10.82)	< 0.001
HbA1c (%)	5.8(5.4, 5.9)	5.6(5.5, 5.9)	7.8(6.7, 8.4)	< 0.001

M/F, male/female; CHD, coronary heart disease; LEU, leukocytes; NE, neutrophils; LY, lymphocytes; NLR, neutrophil to lymphocyte ratio; BUN, blood urea nitrogen; Scr, serum creatinine; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; Glu, glucose; HbA1c, glycated hemoglobin; Hcy, homocysteine. The three groups were compared by the Kruskal-Wallis test or the ANOVA. ^{b,c} Refers to Mann-Whitney U test; ^b*p* < 0.05 vs IS-T2D, ^c*p* < 0.05 vs IS-NT2D.

Baseline characteristics	HC(n = 29)	IS-NT2D(n = 50)	IS-T2D(n = 35)	P-value
Hcy (μmol/L)	11.7(8.4, 14.7)	12.9(9.8, 17.8)	13.9(10.8, 15.5)	0.335
M/F, male/female; CHD, coronary heart disease; LEU, leukocytes; NE, neutrophils; LY, lymphocytes; NLR, neutrophil to lymphocyte ratio; BUN, blood urea nitrogen; Scr, serum creatinine; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; Glu, glucose; HbA1c, glycated hemoglobin; Hcy, homocysteine. The three groups were compared by the Kruskal-Wallis test or the ANOVA. ^{b,c} Refers to Mann-Whitney U test; ^b $p < 0.05$ vs IS-T2D, ^c $p < 0.05$ vs IS-NT2D.				

PAGln concentration was an independent risk factor of IS-T2D

We measured the plasma PAGln concentration of the subjects by the targeted liquid chromatography-mass spectrometry. The plasma PAGln levels of the patients with IS-T2D were significantly higher than IS-NT2D patients and HC group (median, 2.77 vs. 1.77 μmol/L, $P = 0.001$; median, 2.77 vs. 1.04 μmol/L, $P < 0.001$) (Fig. 1A). Furthermore, the PAGln levels of the IS-NT2D group were also higher than the HC group (median, 1.77 vs. 1.04 μmol/L, $P = 0.027$) (Fig. 1A).

Next, we sought to explore the associations among IS, T2D, and the PAGln levels. The univariate logistic regression analysis showed that PAGln levels (OR 1.68, 95%CI 1.04 to 2.72, $P = 0.04$) were a risk factor for patients with IS (Fig. 1B). This significance still existed after adjusting for gender, age, hypertension, CHD, smoking, LEU, NE, BUN, Scr, TG, TC, Hcy (OR 2.34, 95%CI 1.08 to 5.09, $P = 0.03$) (Fig. 1B). That could translate to an increase of 2.34-times in the risk of IS for every 1 μmol/L increase in plasma PAGln levels. More importantly, elevated PAGln levels increased the risk of T2D for stroke patients by both univariate (OR 1.56, 95%CI 1.13 to 2.17, $P = 0.01$) and multivariate logistic regression analyses (adjustment for gender, age, hypertension, CHD, smoking, LEU, NE, BUN, Scr, TG, TC, Hcy) (OR 1.53, 95% CI 1.01 to 2.30, $P = 0.044$) (Fig. 1C). This also means an increase of 1.53-times in the risk of T2D in stroke patients for every 1 μmol/L increase in plasma PAGln levels. Therefore, increased plasma PAGln levels were an independent risk factor for IS patients and stroke patients with T2D.

The gut microbiota was disturbed in stroke patients with T2D

Previous studies have demonstrated that PAGln was a metabolite of gut microbiota. Moreover, PAGln was elevated in the plasma of patients with T2D^{34,35}. Therefore, to investigate whether PAGln-producing bacteria were enriched in the gut microbiota of stroke patients with T2D, we conducted the 16S rRNA gene sequencing.

The species accumulation boxplot showed that our fecal samples were sufficient and the species were abundant (Fig. 2A). The Shannon index and the Simpson index were calculated to evaluate the richness and evenness of intestinal microorganisms of each group. However, we found that there were no significant differences in α diversity among the three groups (Fig. 2B – 2C). Next, the β diversity of the three groups was analyzed by principal coordinate analysis (PCoA) based on the unweighted UniFrac

distance. The results showed that the microbiome structures of both IS-T2D and IS-NT2D patients were separated from the HC group, but community structure was similar between IS-NT2D and IS-T2D groups (IS-T2D vs. HC, $R^2 = 0.050$, $P = 0.001$; IS-NT2D vs. HC, $R^2 = 0.034$, $P = 0.002$; IS-T2D vs. IS-NT2D, $R^2 = 0.017$, $P = 0.059$, Adonis test) (Fig. 2D).

A total of 5360 OTUs were obtained from 114 fecal samples by 16S rRNA gene sequencing and classified into 63 bacterial phyla, 455 bacterial families, and 778 bacterial genera. The gut microbiota differed among the three groups at the phylum, family, and genus levels. At the phylum level, the gut microbiota was mainly composed of *Firmicutes* (51.0%), *Proteobacteria* (18.7%), *Bacteroidota* (21.3%), *Actinobacteriota* (4.2%), *Fusobacteriota* (1.7%), and *Verrucomicrobiota* (3.1%) (Fig. 2E). The relative abundance of *Firmicutes* in patients with IS-T2D was significantly lower than that in the IS-NT2D and HC groups (43% vs. 52%, $P = 0.027$; 43% vs. 55%, $P = 0.005$) (Fig. 2F). The relative abundance of *Proteobacteria* and *Verrucomicrobiota* in the IS-T2D group was higher than that in the IS-NT2D group (23% vs. 17%, $P = 0.024$; 4.3% vs. 1.4%, $P = 0.05$) (Fig. 2F). At the family level, the harmful bacteria *Enterobacteriaceae* were enriched in IS-T2D patients with significant differences compared with IS-NT2D and HC groups (21.6% vs. 16.5%, $P = 0.027$; 21.6% vs. 13.0%, $P = 0.019$) (Fig. 2G). The relative abundance of *Akkermansiaceae* in the IS-T2D group was the highest among the three groups, but there was a significant difference only between the IS-NT2D and HC groups (1.4% vs. 3.3%, $P = 0.046$) (Fig. 2G). At the genus level, the beneficial bacteria *Faecalibacterium*, *Dialister*, and *Roseburia* were all significantly reduced in IS-T2D and IS-NT2D groups when compared with the HC group (*Faecalibacterium*, 7.4% vs. 15.4%, $P < 0.001$; 9.6% vs. 15.4%, $P = 0.003$; *Dialister*, 3.0% vs. 3.7%, $P < 0.001$; 2.0% vs. 3.7%, $P = 0.005$; *Roseburia*, 0.9% vs 3.4%, $P < 0.001$, 1.4% vs 3.4%, $P = 0.001$) (Fig. 2H). And the relative abundance of harmful bacteria *Klebsiella* significantly increased in the IS-T2D group compared with the HC and IS-NT2D groups (4.5 vs. 1.8, $P = 0.002$; 4.5 vs. 2.5, $P = 0.01$) (Fig. 2H). In summary, the diversity of gut microbiota in patients with IS-T2D decreased, with decreased beneficial bacteria and increased harmful bacteria.

In addition, to better identify the microbial markers among the three groups, we used the LEfSe tool. The results showed that there was a total of 20 gut microbiota taxa from phylum to species, which were differentially abundant bacterial taxa (LDA score > 4) in three groups (Fig. 2I). Specifically, there were 6 increased abundant taxa in the IS-T2D group, including *o_Enterobacterales*, *f_Enterobacteriaceae*, *p_Verrucomicrobiota*, *c_Verrucomicrobiota*, *s_Klebsiella_pneumoniae*, and *g_Klebsiella*.

Next, we performed PICRUSt analysis to predict potential functional pathways of the microbiome communities and compared the differences between IS-T2D and IS-NT2D groups (Fig. 2J). In total, there were 300 differential KEGG pathways (level 3), of which 49 were significantly different with the average relative abundance of one of the two groups no less than 0.1%. There were 29 pathways enriched, but 20 pathways were poor in the IS-T2D group. Comparing with the IS-NT2D group, we found that amino acid metabolism (such as glycine, serine, and threonine metabolism, $P = 0.033$; tyrosine metabolism, $P = 0.014$; beta-alanine metabolism, $P = 0.020$; tryptophan metabolism, $P = 0.002$) was significantly more abundant in the IS-T2D group (Fig. 2J).

PAGln levels were associated with characteristic microbiota related to IS-T2D, poor prognosis, and NETs-related inflammation

To investigate the association between PAGln levels and IS-T2D, we performed Spearman correlation analysis. As showed in Table 2, the PAGln levels were positively correlated with age, BUN, Scr, Glu, HbA1c, and Hcy in all subjects, and the correlations between age and PAGln were especially pronounced in stroke patients with T2D. Furthermore, it is worth noting that the PAGln levels were positively correlated with mRS score of 90 days after the onset in all stroke patients. In addition, we found that the PAGln levels were positively correlated with NE in stroke patients with T2D, suggesting an association between inflammation and PAGln levels in stroke patients with T2D.

Table 2
Correlations between clinical indexes, biochemical indexes, and PAGln

	Total population (n = 114)		All IS patients (n = 85)		IS-T2D group (n = 35)	
	PAGln		PAGln		PAGln	
	r	P	r	P	r	P
NIHSS	/	/	0.095	0.386	0.012	0.238
90d-mRS	/	/	0.314	0.003	0.947	0.169
Age	0.469	< 0.001	0.477	< 0.001	0.624	< 0.001
LEU	0.136	0.149	0.042	0.704	0.186	0.284
NE	0.181	0.054	0.147	0.181	0.345	0.042
BUN	0.448	< 0.001	0.438	< 0.001	0.503	0.002
Scr	0.259	0.005	0.25	0.021	0.184	0.289
Glu	0.437	< 0.001	0.437	< 0.001	0.117	0.505
HbA1c	0.319	0.001	0.289	0.007	0.125	0.473
Hcy	0.267	0.004	0.167	0.127	0.194	0.264
LEU, leukocytes; NE, neutrophils; BUN, blood urea nitrogen; Scr, serum creatinine; Glu, glucose; HbA1c, glycated hemoglobin; Hcy, homocysteine.						

NETs are bactericidal substances, released extracellularly after NE activation, and their dysregulation can lead to inflammation³⁶. Therefore, we measured the plasma NETs concentration of all subjects. We evaluated the concentration of CitH3 in the plasma to represent the level of NETs³⁷. Our results showed that the plasma CitH3 levels in IS-T2D patients were significantly higher than those in IS-NT2D and HC groups (6.34 vs. 5.57ng/ml, $P < 0.001$; 6.34 vs. 4.73ng/ml, $P < 0.001$). The plasma CitH3 levels in the IS-

NT2D group were also significantly higher than those in the HC group (5.57 vs. 4.73ng/ml, $P < 0.001$) (Fig. 3A). Moreover, Spearman correlation analysis revealed a significant correlation between PAGln and CitH3 levels ($r = 0.41$, $P < 0.001$) (Fig. 3B). We further analyzed the distribution of CitH3 levels based on PAGln levels as assessed by quartiles. The concentration of plasma CitH3 showed a dose-dependent increase according to PAGln levels with the highest levels being observed in subjects with the highest PAGln concentrations (Q4: 6.15 vs Q1: 5.27ng/ml, $P < 0.01$) (Fig. 3C).

Next, we explored the relationships between PAGln, clinical indicators, and gut microbiota. We performed Spearman correlation analysis on the data of all stroke patients. Spearman correlation analysis showed that PAGln level was significantly positively correlated with 6 microbial markers (*o_Enterobacterales*, *f_Enterobacteriaceae*, *p_Verrucomicrobiota*, *c_Verrucomicrobiota*, *s_Klebsiella_pneumoniae*, and *g_Klebsiella*) related to IS-T2D. Interestingly, *o_Enterobacterales*, *f_Enterobacteriaceae*, *p_Verrucomicrobiota*, and *c_Verrucomicrobiota* were also positively correlated with NE. Meanwhile, *o_Enterobacterales* and *f_Enterobacteriaceae* were positively correlated with Glu and Hcy; *s_Klebsiella_pneumoniae* and *g_Klebsiella* were positively correlated with HbA1c (Fig. 3D).

PAGln levels, NETs levels, and gut microbiota were diagnostic indexes for IS-T2D.

We next explored whether the plasma PAGln, gut microbiota, and NETs could be used as biomarkers of stroke with T2D. Based on the plasma PAGln levels, differential microbiota (defined as the relative abundance of bacteria in gut microbiota with LDA score > 4), and NETs levels, we conducted ROC analysis. As shown in Fig. 4, plasma PAGln levels (AUC: 0.7160, 95%CI: 0.6074–0.8246; $P = 0.0007$), different gut microbiota (AUC: 0.8757, 95%CI: 0.8016–0.9462; $P < 0.0001$) and NETs levels (AUC: 0.8229, 95%CI: 0.7353–0.9105; $P < 0.0001$) could well distinguish patients with IS-T2D from stroke patients (Fig. 4). Notably, when plasma PAGln levels and the differential microbiota were incorporated into the model construction, the AUC increased to $88.7 \pm 3.54\%$ ($P < 0.0001$). And when the PAGln and NETs levels were incorporated into the model construction, the AUC increased to $83.4 \pm 4.38\%$ ($P < 0.0001$). Finally, we included PAGln levels, NETs levels, and differential microbiota into the model construction, the area under the ROC curve was up to $94.7 \pm 2.26\%$ ($P < 0.0001$). In summary, the intestinal metabolite PAGln, differential microbiota, and inflammatory indicator NET could all be used as diagnostic indicators for stroke with T2D. Moreover, the combination of these biomarkers might improve diagnostic efficiency.

Elevated PAGln levels in IS-T2D patients could be transmitted through the gut microbiota

To investigate whether the gut microbiota of stroke patients with T2D contributed to the elevation of plasma PAGln levels, we treated antibiotic-treated rats with a fecal transplant from IS-T2D and IS-NT2D patients (Fig. 5A). Results demonstrated that compared with the rats receiving fecal microbes from patients with IS-NT2D (preFMT-IS-NT2D group), the PAGln concentration of the rats receiving fecal microbes from patients with IS-T2D (preFMT-IS-T2D group) was significantly increased and nearly doubled (preFMT-IS-T2D vs. preFMT-IS-NT2D, $P = 0.02$) (Fig. 5D). MCAO model was established in rats

after FMT. 24 hours after stroke, the rats receiving fecal microbes from IS-T2D patients had more severe stroke than the rats receiving fecal bacteria from IS-NT2D patients, with decreased neurological function scores (FMT-IS-T2D vs. FMT-IS-NT2D, $P=0.042$) (Fig. 5B) and increased infarct volume (FMT-IS-T2D vs. FMT-IS-NT2D, $P=0.042$) (Fig. 5C). Meanwhile, after stroke, PAGln levels in rats receiving fecal microbes from patients with IS-T2D were also nearly twice as high as those receiving fecal microbes from patients with IS-NT2D (FMT-IS-T2D vs. FMT-IS-NT2D, $P=0.03$) (Fig. 5D). Notably, PAGln levels in rats receiving fecal microbes from patients with IS-T2D were further elevated after stroke (preFMT-IS-T2D vs. FMT-IS-T2D, $P=0.021$), while the PAGln levels in rats receiving fecal microbes from IS-NT2D patients were in an increasing tendency with no significant differences (preFMT-IS-NT2D vs. FMT-IS-NT2D, $P=0.31$).

Discussion

This study is the first time for exploring the relationship between gut microbiota and its metabolite PAGln in stroke patients with T2D. Our data suggest that: (i) in the IS-T2D group, the gut microbiota was significantly imbalanced and the plasma PAGln levels increased partly caused by the disorder of gut microbiota, which was further confirmed by animal studies (Fig. 6); (ii) elevated PAGln levels were associated with poor functional outcomes and plasma NETs levels; (iii) PAGln levels, gut microbiota, and NETs levels could be used as combined diagnostic indicators for IS-T2D.

Previous research has established that PAGln was associated with CHD, peripheral artery disease, heart failure, and other cardiovascular diseases^{17,38–40}. In addition, PAGln was also closely related to obesity, diabetes, prediabetes, and other metabolic diseases^{16,34,41–43}. Research shows that metabolic disorders such as insulin resistance, dyslipidemia, and fatty can increase platelet activity and aggregation via dysregulation of the NO-mediated signaling pathway, leading to thrombosis and atherosclerotic lesion formation⁴⁴. And PAGln also has the effect of driving platelet invasiveness¹⁴. A metabolic disorder like diabetes mellitus may promote vascular injury via PAGln-mediated molecular mechanisms. Our results showed that IS-NT2D caused an increase in plasma PAGln levels. More importantly, PAGln levels were higher in IS patients with the complication of T2D. Although the plasma clearance of PAGln is closely related to renal function, that is, if renal function is impaired, the plasma PAGln clearance will be severely reduced, there was no significant difference in BUN or Scr levels between IS-T2D and IS-NT2D groups, indicating that the elevated PAGln levels in IS-T2D patients were mainly influenced by T2D^{38,45}. Except for stroke, Tang et al. found that plasma PAGln levels were significantly higher in patients with heart failure and diabetes mellitus than in those with heart failure alone, which suggested the elevation of plasma PAGln is associated with diabetes mellitus⁴⁰. Our results also revealed that elevated PAGln was an independent risk factor for the patient with stroke and T2D, indicating that PAGln may be an important molecule of T2D in contributing to an exacerbation in stroke injury, of which the mechanism remains obscure and needs to be further explored.

PAGln is a metabolite of phenylalanine degradation by gut microbiota. Previous studies have revealed that, except for abnormal metabolism, people with diabetes mellitus had severely disturbed gut

microbiota^{11, 46}. Therefore, increased plasma PAGln levels in IS-T2D patients indicate that they might not only use more amino acids as energy sources, but have more gut microbiota to degrade phenylalanine compared with stroke patients, which was confirmed by our results. We found that the gut microbiota of IS-T2D patients was disordered, and there was more PAGln-related gut microbiota in these patients, including *o_Enterobacterales*, *f_Enterobacteriaceae*, *p_Verrucomicrobiota*, *c_Verrucomicrobiota*, *s_Klebsiella_pneumoniae*, and *g_Klebsiella*. *Enterobacteriaceae* is recognized as harmful gut microorganisms and showed a growth advantage in both stroke and T2D^{18, 47}. A study has reported that *Enterobacteriaceae* was closely related to the mortality of stroke patients, increasing inflammatory factors such as TNF- α and IL-1 β through the LPS-TLR4 pathway, thus accelerating systemic inflammation and exacerbating cerebral infarction¹⁸. In addition, studies have shown that *Escherichia coli* belonging to *Enterobacteriaceae* can catabolize aromatic compounds such as phenylalanine and phenylacetic acid^{48–50}. The phenylacetic acid is the middle product that PHE is metabolized to PAGln. Our results showed that *Enterobacteriaceae* was one of the major differential bacteria in the IS-T2D group, which was positively correlated with PAGln, indicating that *Enterobacteriaceae* might cause the aggravation of brain injury by diabetes mellitus via metabolites PAGln. Consistent with Ottosson et al., a positive correlation was also found between plasma PAGln levels and *Verrucomicrobiota* in a CHD risk cohort¹⁷. In our study, *c_Verrucomicrobiota* was dominated by *g_Akkermansia* (99.4%). It is generally believed that *Akkermansia* is a kind of beneficial bacteria to maintain the health of the intestinal epithelium, which can enhance intestinal barrier function, produce short-chain fatty acids (SCFAs), improve metabolic disorders, and it has even been considered the new probiotics which could be developed and utilized^{51, 52}. However, its role in the development of stroke and diabetes mellitus remains elusive. Studies have reported that *Akkermansia* was elevated in patients with prediabetes and diabetes as well as in diabetic animal models^{53, 54}. In addition, it has been suggested that a significant increase in *Akkermansia* could contribute to the erosion of the mucous layer and enhancement of pathogen filtration in the intestinal epithelial layer when the dietary fiber was lacking in the host gut⁵⁵. The latest study found that *Akkermansia* strains could be divided into five distinct candidate species with different host preferences and functions, of which only *Akkermansia mucinophilus* had the health-associated properties referring to being associated with low body mass index in the host⁵⁶. In our study, *Akkermansia* was significantly decreased in IS-NT2D patients but increased in IS-T2D patients, and *c_Verrucomicrobiota*, dominated by *Akkermansia*, was significantly associated with PAGln. We speculated that T2D could increase a harmful species of *Akkermansia*, which is linked with PAGln metabolism. Therefore, it is necessary to further explore the relationship between each candidate species of *Akkermansia* and PAGln. Consistent with the study of Li et al., our results suggested that PAGln was significantly correlated with *Klebsiella*⁵³. *Klebsiella* is widely believed to be a pathogenic bacterium and a strain with the TMA gene^{57–59}. TMA is an essential precursor of the gut microbiota metabolite TMAO, which has been found to promote thrombosis via increasing platelet reactivity⁶⁰. And TMAO is closely associated with atherosclerosis and poor prognosis in IS^{61, 62}. Therefore, it is reasonable to hypothesize that *Klebsiella* is a strain with the characteristics of promoting thrombosis. In addition to the above bacteria, previous research also reported that PAGln was associated with *Enterococcus*, *Streptococcus*, *Escherichia*

Shigella, *G_f_o_SHA.98c_clostridia*, and *Eggerthella lenta*^{17, 53, 63}. And finally, we transplanted the fecal microbiota from patients with IS-T2D into rodents, ultimately resulting in increased plasma PAGln levels, which provided direct evidence that elevated PAGln levels in IS-T2D patients were partly caused by intestinal microorganism disorders. It is noteworthy that the PAGln level of rats receiving the fecal microbes from IS-T2D patients further increased after stroke. A possible explanation for this might be that under the acute stress of stroke, this part of microbiota further grew and multiplied, resulting in an increase of PAGln levels. Additionally, our study indicated that gut bacteria functions such as amino acid metabolism were enhanced and abnormal in patients with IS-T2D. Further work should be undertaken to search for strains related to PAGln metabolism and improve the prognosis of IS-T2D by targeting PAGln-producing bacteria.

The formation of NETs is originally identified as an important antimicrobial phenomenon, including resisting microbial invasion, stopping the microbial spread, and killing pathogens⁶⁴. However, mounting evidence indicates that NETs play a negative role in many diseases such as endocrine diseases, nervous system diseases, and respiratory diseases^{65–67}. Consistent with previous research, our results showed an increase in NETs levels in stroke patients and a further increase in stroke patients with T2D. Platelet-neutrophil interactions are a critical pathophysiological process of thromboinflammation⁶⁸. In the arterial microenvironment of thrombosis, activated platelets trigger NE to form and release NETs containing thrombotic tissue factor (TF) through several mechanisms, including NE autophagy induced by the presentation of high mobility group frame 1 (HMGB1) protein, release of the NETs inducer–inorganic polyphosphate (polyP), and activation of the platelet Toll-like receptor 4 (TLR 4)^{69–71}. In addition, NETs, in turn, can further aggravate thromboinflammation via promoting thrombin and fibrin formation and binding to platelet-derived microparticles (PMPs) and coagulation factors⁷². Recently, a study had indicated the effects of the gut microbiota metabolite PAGln in driving platelet invasiveness and thrombosis via β adrenergic receptors¹⁴. According to our data, plasma PAGln levels were positively correlated with NETs, and NETs showed a dose-dependent increase according to PAGln levels. Therefore, we speculated that the gut microbiota metabolite PAGln might contribute to platelet-neutrophil interactions via enhancing platelet reactivity, which ultimately promotes the formation of NETs. Meanwhile, our data showed a positive correlation between PAGln and IS-T2D-associated gut microbiota, of which were positively correlated with NE. Dysregulation of gut microbiota in patients with arteriosclerotic cerebral small vessel disease has been reported to independently enhance the proinflammatory property of NE⁷³. We thus inferred that IS-T2D-associated gut microbiota could exacerbate inflammation status of NE, and then combine with elevated PAGln in plasma to promote the formation of NETs, which may ultimately contribute to the aggravation of thromboinflammation. In the meantime, our data suggest that elevated plasma PAGln levels were correlated with a 90-day poor prognosis in stroke patients, further supporting that the PAGln plays a negative role in the development of stroke. Above all, our study revealed that the presence of T2D could increase the inflammatory reaction in IS patients, mediated in part by the altered composition of gut microbiota and the effects of bacterial metabolite PAGln.

In this study, we not only confirmed the dysbiosis of gut microbiota and the increase of harmful molecular PAGln in IS-T2D patients, but also found that the gut microbiota dysbiosis, the increased plasma PAGln, and NETs could serve as important diagnostic markers for stroke with T2D.

Limitations of the study: Firstly, the stroke patients included in our study were within 2 weeks of onset, which was enough to cause significant differences in microbiota between samples in group¹⁸. In addition, the NIHSS scores of the patients included were low, limiting the representativeness of the study. Secondly, our sample size was relatively small, which needs to be further expanded. Meanwhile, we need to exclude confounding factors like dietary habits, lifestyle, and fecal status, and increase the T2D group in the future, to enhance persuasion of the experimental results. Finally, in the animal experiment, we only conducted a simple FMT experiment, and the transplanted fecal samples included bacteria, fungi, and other microorganisms, which is hard to determine which strain plays a role. Further experimental studies need to be carried out in the future.

Conclusions

Collectively, we found that stroke patients with T2D had gut microbiota disorders and increased plasma PAGln levels, a microbiota metabolite, which was positively correlated with NETs. Our findings contribute to the understanding of the role of gut microbial metabolite-related mechanisms in the progression of stroke with T2D and provide a new therapeutic target — PAGln for the treatment of stroke with T2D. The causal relationship between PAGln and IS with diabetes mellitus and its mechanism should be further explored.

Abbreviations

T2D	type 2 diabetes
IS	ischemic stroke
IS-T2D	IS with T2D
IS-NT2D	IS without T2D
PAGln	phenylacetylglutamine
PHE	acid-phenylalanine
CHD	coronary heart disease
NE	neutrophils
NETs	neutrophil extracellular traps
HC	healthy control
NIHSS	National Institutes of Health Stroke Scale
mRS	modified Rankin scale
PCR	polymerase chain reaction
OTUs	operational taxonomic units
LDA	linear discriminant analysis
LEfSe	linear discriminant analysis (LDA) effect size
BUN	blood urea nitrogen
Scr	serum creatinine
TG	triglyceride
TC	total cholesterol
HDL	high-density lipoprotein
HbA1C	glycosylated hemoglobin
Hcy	homocysteine
CitH3	citrullinated histone H3
NIH	National Institutes of Health
SD	Sprague-Dawley
SPF	specific pathogen-free
FMT	fecal microbiota transplantation
MCAO	middle cerebral artery occlusion

ECA	external carotid artery
CCA	common carotid artery
ICA	internal carotid artery
SEM	standard error mean
IQR	interquartile range
ROC	receiver operator characteristic
LEU	leukocyte
OR	odds ratio
PCoA	principal coordinate analysis
AUC	the area under the curve
SCFAs	short-chain fatty acids
TF	tissue factor
HMGB1	high mobility group box 1
Polyp	polyphosphoric
TLR 4	Toll-like receptor 4
PMPs	platelet-derived microparticles

Declarations

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Ethics Approval Each participant in this study provided written informed consent, and the study protocol complied with the principles of the 1975 Declaration of Helsinki and approved by the Ethics Committee of Xiangya Hospital, Central South University, China. The animal experimental protocols were approved by the Experimental Animal Welfare Ethics Committee of Central South University.

Consent to Participate Not applicable

Consent to Publish All the authors verify that they concur with the present submission and that the material submitted has not been previously reported in any other journal.

Competing Interests All authors declare that they have no conflict of interest.

Availability of data and materials

FASTQ files of the 16S rRNA gene sequencing are available under SRA accession number PRJNA 820272 (<https://dataview.ncbi.nlm.nih.gov/object/PRJNA820272>).

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Author Contributions

Experimental design, overall data analysis, and writing original draft: JX and MPW; patients' blood and fecal samples collection and testing: MPW, YFL, and TTZ; animal experiments: MPW and DL; technical support, data collection, and data interpretation: QH, FY, XJF, JF, and QH; manuscript revision: QH and YLY. All authors have read and agreed to the published version of the manuscript.

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Preprint

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Figures

Figure 1

Comparison of plasma PAGln levels in three groups (A) Plasma PAGln levels were increased in IS-T2D and IS-NT2D groups and the highest in the IS-T2D group. **(B)** Logistic regression analysis of risk factors for stroke patients (black curve and black characters represent multivariate analysis; red curve and red characters represent univariate analysis). **(C)** Logistic regression analysis of risk factors for T2D of stroke patients (black curve, black characters, red curve, and red characters are the same as Fig. B). (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; (A) by Mann-Whitney u-test)

Figure 2

The composition and differences of gut microbiota in three study groups (A) The species accumulation boxplot. **(B)** Shannon index (By Mann-Whitney u-test). **(C)** Simpson index (By Mann-Whitney u-test). **(D)** The β -diversity via PCoA based on the Bray-Curtis dissimilarity index (IS-T2D vs HC, $R^2 = 0.050$, $P = 0.001$; IS-NT2D vs HC, $R^2 = 0.034$, $P = 0.002$; IS-T2D vs IS-NT2D, $R^2 = 0.017$, $P = 0.059$, Adonis test). **(E)** Constitution of bacterial phyla in three study groups. **(F)** Comparison of the relative abundance of gut microbiota at phylum-level among three study groups. **(G)** Comparison of the relative abundance of gut microbiota at family-level among three study groups. **(H)** Comparison of the relative abundance of gut microbiota at genus-level among three study groups. **(I)** Characteristics of gut microbiota in three study groups were evaluated with LEfSe (LDA score > 4). **(J)** Prediction of significantly changed metabolic pathways in the IS-T2D group compared to the IS-NT2D group (KEGG pathway annotations in level 3). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # $P = 0.05$; (A-C) by Mann-Whitney u-test).

Figure 3

Comparison of NETs levels in three groups, and the correlation analysis (A) Plasma NETs levels were increased in IS-T2D and IS-NT2D groups and the highest in the IS-T2D group. (B) Positive correlation between plasma PAGIn level and plasma NETs level ($r=0.41$, $P<0.001$). (C) Comparison of the NETs levels between patients with increasing quartiles of PAGIn levels (** indicate $P<0.01$ as compared to NETs in patients from the first quartiles, # indicate $P<0.05$ as compared to NETs in patients from the second quartiles). (D) Association of the altered bacterial composition (LDA score >4) and the clinical and biochemical information was evaluated with Spearman correlation analysis (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$; (A) (C) by Mann-Whitney u-test).

IS-NT2D vs IS-T2D

Figure 4

ROC analysis of predicting efficiency of PAGIn levels, altered bacterial composition, and NETs levels for IS-T2D Orange curve, orange characters, AUC: 0.7160 ± 0.0554 , $P=0.0007$; purple curve, purple characters, AUC: 0.8757 ± 0.0377 , $P<0.0001$; green curve, green characters, AUC: 0.8229 ± 0.0447 , $P<0.0001$; blue curve, blue characters, AUC: 0.8337 ± 0.0438 , $P<0.0001$; red curve, red characters, AUC: 0.8869 ± 0.0354 , $P<0.0001$; black curve, black characters, AUC: 0.9466 ± 0.0226 , $P<0.0001$.

Figure 5

Fecal transplantation from IS-T2D patients resulted in increased neurological impairment and plasma PAGln levels in rats (A) Animal experimental design. (B) Rats receiving fecal microbes from IS-T2D patients had decreased neurological function scores 24h after cerebral ischemia-reperfusion. (C) Rats receiving fecal microbes from IS-T2D patients had increased infarct volume 24h after cerebral ischemia-reperfusion. (D) Rats receiving fecal microbes from IS-T2D patients had increased plasma PAGln levels regardless of whether they had experienced an ischemic stroke. Shown is the mean ($\pm$ SEM) of PAGln levels of rats. $n = 5$ to 8 rats per group. ($*P < 0.05$; by Mann-Whitney u-test) .

Figure 6

Metabolite PAGln may involve in exacerbation of injury of IS-T2D via gut microbiota Gut microbiota disordered and plasma PAGln levels increased in IS-T2D patients. Increased plasma PAGln levels were positively correlated with NETs. The neurological dysfunctions and infarct volume of the rats receiving fecal microbes from IS-T2D patients increased significantly under the action of the gut-brain axis. In addition, plasma PAGln levels were significantly increased in rats with or without stroke.