**Supplementary materials**

**Microbiome-Gut-Brain Axis Contributes to Patients and Bama Miniature Pigs with Acute Large Ischemic Stroke**

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**Materials and methods**

*1H NMR-based serum metabolic profiling of AIS patients and healthy controls*

 Serum samples of AIS patients and healthy controls were prepared by mixing 200 μL serum with 400 μL saline solution containing 10% D2O. Following vortexing and centrifugation (11180g, 10 min and 4 °C), 550 μL samples was transferred into 5 mm NMR tubes. A water-presaturated Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (recycle delay-90°-(τ-180°-τ)n-acquisition) was employed to attenuate NMR signals from macromolecules. 90° pulse length was adjusted to about 10 μs for each sample and water signal was suppressed with a weak continuous wave irradiation during recycle delay (RD). Data points (64 K) were collected for each spectrum with a spectral width of 20 ppm (12 kHz) and RD of 2 s. The spin−spin relaxation delay, 2nτ, was set to 96 ms. Free induction decays so obtained for all samples were multiplied by an exponential function with a line broadening factor of 1 Hz prior to Fourier transformation. Chemical shifts for all spectra were referenced to the anomeric proton signal of α-glucose (δ5.23). For the purposes of signal assignments, a series of two-dimensional NMR (2D NMR) spectra were recorded and processed for selected samples. These spectra included 1H−1H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), 1H−13C heteronuclear single quantum correlation (HSQC) and 1H−13C heteronuclear multiple bond correlation (HMBC) spectra.

All the 1H NMR spectra were corrected for phase and baseline distortions using Topspin (V2.0, Bruker Biospin) and the spectral region δ 0.5−9.5 were divided into buckets with equal width of 0.004 ppm (2.4 Hz) using AMIX software package (V3.8.3, Bruker Biospin). The regions at δ 4.32−5.5 was discarded to eliminate the effects of imperfect water saturation. Multivariate data analysis was conducted with SIMCA-P+ package (V14.0, Umetrics, Sweden) following normalization to the volume of serum samples. Principal component analysis (PCA) was carried out on the mean-centered data to generate an overview and check for the outliers. Partial least-squares discriminant analysis (PLS-DA) and the orthogonal projection to latent structure with discriminant analysis (OPLS-DA) were subsequently performed using the unit-variance scaled data to find metabolites having significant intergroup differences. The OPLS-DA models were built with two components calculated and with 6-fold cross-validation. These models were further evaluated for their validities with CV-ANOVA method. After back-transformation, the loadings were plotted using an inhouse developed Matlab (V7.8, The Mathworks, MA) script with correlation coefficients color-coded for each variable (or the metabolite signals). The color-coded variables indicate the significance of metabolites contributing to the intergroup differentiation with a “hot” colored (e.g., red) metabolite being more significant than a “cold” colored (e.g., blue) ones. Cutoff values for the correlation coefficients were chosen depending on the number of samples used to extract metabolites having significant intergroup differences based on the discrimination significance (p < 0.05) for the Pearson’s product-moment correlation coefficients.

*Quantitative analysis of plasma and brain tryptophan metabolites*

Tryptophan metabolites extraction were performed from plasma and brain tissues of Bama Miniature Pigs. Samples mixed with internal standard (10 μL d5-TRP) were respectively homogenized with 400 μL cooled methanol and 50 μL acetonitrile: water solution (1:1 v/v) containing 0.1% formic acid using the Qiagen Tissue-Lyser (Retsch GmBH, Germany) at 20 Hz for 90 s. After extraction for two times, the combined supernatants were collected and evaporated into dryness following centrifugation. Serum sample (10 μL) was uniformly mixed with 10 μL of internal standard (d5-trp), 150 μL cooled methanol and 10 μL acetonitrile: water solution (1:1 v/v) containing 0.1% formic acid. After centrifugation for 20 min (4 °C), the supernatants were collected and lyophilized for removing methanol in vacuum. Dried extracts were reconstituted in 100 μL of acetonitrile: water solution (1:1 v/v) containing 0.1% formic acid. Qualitative and quantitative analyses of tryptophan metabolites were performed using an ultrahigh performance liquid chromatography (Agilent 1290) coupled with a 6460 triple quadrupole mass spectrometry (UHPLC-QQQ-MS, Agilent Technologies, Inc.). The precursor ions of tryptophan metabolites were pre-scanned through multiple reaction monitoring (MRM) of all sample mixtures and the structures were identified through MS/MS spectra. Quantification of tryptophan metabolites was performed using calibration curves based on MRM and the ratios of the integrated peak areas of tryptophan metabolites and internal standards.

Table S1. The primer sequences of qPCR used in this study.

|  |  |  |
| --- | --- | --- |
| Name | Forward primer (5’ -3’) | Reverse primer (5’-3’) |
| IL-1β  | GGCCATAGTACCTGAACCCG | TTGGGTGCAGCACTTCATCT |
| TNF-α | GGCCCAAGGACTCAGATCAT | GGCATACCCACTCTGCCATT |
| E-cadherin | CGACGGTGTGGTTACAGTCA | AGAGGGAGAGTCCTGATGGC |
| ZO-1 | GATGTTTATGCGGACGGTGG | CATTGCTGTGCTCTTAGCGG |
| β-actin | TCAGCAAGCAGGAGTACGAC | GGAATGCAACTAACAGTCCGC |
| GAPDH | GTCGGAGTGAACGGATTTGGC | GGAGGTCAATGAAGGGGTCA |

Table S2. NMR assignments for the metabolites in human serum.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Key | metabolites | Moieties | δ 1H (ppm) and multiplicity a | δ 13C (ppm) |
| 1 | HDL | CH3 | 0.82(m) | b |
| 2 | LDL | CH3 | 0.85(m) | b |
| 3 | VLDL | CH3 | 0.88(m) | b |
| 4 | Leucine | αCHβCH2γCHδCH3δ′CH3 | 3.74(t)1.69(m)1.72(m)0.97(d)0.96(d) | 56.027.242.824.424.4 |
| 5 | Isoleucine | αCHβCHγCH2γ′CH3δCH3 | 3.67(d)1.98(m)1.27(m),1.47(m)1.01(d)0.94 (t) | 62.1138.427.717.414.2 |
| 6 | Valine | αCHβCHγCH3 | 3.62(d)2.28(m)1.05(d) | 63.231.920.8 |
| 7 | D-3-hydroxybutyrate | CHCH2γCH3CH2 | 4.16(dt)2.41(dd)1.20(d)2.31(dd) | 68.849.524.449.5 |
| 8 | Lactate | αCHβCH3COOH | 4.12(q)1.33(d) | 71.222.4184.9 |
| 9 | Alanine | αCHβCH3COOH | 3.78(q)1.48(d) | 53.518.9178.8 |
| 10 | Lysine | αCHεCH2βCHγCH2δCH2COOH | 3.76(t)3.03(t)1.92(m)1.72(m)1.45(m) | 64.142.233.429.923.5177.0 |
| 11 | Arginine | γCH2βCH2δCH2αCH | 1.69(m)1.92(m)3.25(t)3.76(t) | b |
| 12 | Acetate | CH3 | 1.91(s) | 26.5/184.4 |
| 13 | N-acetyl-glycoproteins(NAG) | CH3 | 2.04(s) | b |
| 14 | Glutamate | αCHβCH2γCH2COOHCOOH | 3.76(m)2.06(m)2.12(m)2.35(m) | 57.429.836.2177.4184.0 |
| 15 | Glutamine | αCHβCH2γCH2COOHCO | 3.77(t)2.14(m)2.46(m) | 57.229.034.0b180.3 |
| 16 | Citrate | CH2CH2C-OHCOOHCOOH | 2.55(d)2.69(d) | 48.147.978.2181.7184.4 |
| 17 | Unsaturated fatty acids | CH | 6.53(s) | 137.8 |
| 18 | Lipid | CH3(CH2)nCH2-CH=CHCH2-CO=C-CH2-C=CH=CH | 0.89(m)1.27(m)2.0(m)2.3(m),2.78(m)5.3(m) | b |
| 19 | Choline | N(CH3)3NCH2OCH2 | 3.21(s)3.53(m)4.07(m) | 56.770.358.2 |
| 20 | Phosphoryl-choline(PC) | N(CH3)3NCH2OCH2 | 3.22(s)3.60(m)4.17(m) | 56.469.360.8 |
| 21 | Glycerophospho-choline(GPC) | N(CH3)3NCH2OCH2 | 3.24(s)3.69(m)4.33(m) | 56.4bb |
| 22 | Glucose/amino acids | α-CH resonances | 3.3-3.9 | b |
| 23 | Scyllitol | CH | 3.36 | b |
| 24 | Glycine | CH2COOH | 3.56(s) | 44.3175.3 |
| 25 | β-glucose | 1-CH | 4.66(d) | 98.6 |
| 26 | α-glucose | 1-CH | 5.23(d) | 94.8 |
| 27 | Tyrosine | βCH2αCH3 or 5-CH2 or 6-CH | 3.06,3.14(dd)3.94(t)6.90 (d)7.20(d) | 38.357.4119.1134.0 |
| 28 | Histidine | CHCH | 7.09(s)7.89(s) | 120.2139.1 |
| 29 | Phenylalanine | CH2CH2′N-CH2 or 6-CH3 or 5-CH4-CH | 3.14(dd)3.28(dd)3.99(dd)7.33(m)7.40(m)7.38 (m) | bbb132.4132.2131.1 |
| 30 | Formate | CH | 8.46(s) | 174.3 |
| 31 | α-mannose | 1. CH
2. CH
 | 5.18(d)3.93(m) | 97.175.3 |
| 32 | Acetylcarnitine | CH3C=Oα-CHα-CH′γ-CH2 | 2.13(s)2.46(m)2.63(m)3.90 (m) | b |
| 33 | Myo-inositol | 1,3-CH 2-CH 4,6-CH | 3.65(m)3.29(m)3.57(m) | b |
| 34 | Triglycerides | CHOCH2OCH2'O | 5.21(m)4.07(m)4.28(m) | 69.962.862.8 |

a Key: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublet.

b The signals or the multiplicities were not determined.