

Metabolomic Characterization of Acute Ischemic Stroke Facilitates Metabolomic Biomarker Discovery

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Metabolomic characterization of acute ischemic stroke facilitates metabolomic biomarker discovery

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Abstract

Acute ischemic stroke (AIS) is characterized by a sudden blockage of one of the main arteries supplying blood to the brain, leading to insufficient oxygen and nutrients for brain cells to function properly. Unfortunately, metabolic alterations in the biofluids with AIS are still not well understood. In this study, we performed high-throughput target metabolic analysis on 44 serum samples, including 22 from AIS patients and 22 from healthy controls. Multiple reaction monitoring analysis of 180 common metabolites revealed a total of 29 metabolites changed significantly ($\text{VIP}>1$, $P<0.05$). Multivariate statistical analysis unraveled a strikingly separation between AIS patients and healthy controls. Comparing AIS with Control group, the contents of argininosuccinic acid, beta-D-glucosamine, glycerophosphocholine, L-abrine, and L-pipeolic acid were down-regulated in AIS patients. 29 out of 112 detected metabolites, enriched in aminoacyl-tRNA biosynthesis, glycerophospholipid metabolism, lysine degradation, phenylalanine, tyrosine and tryptophan biosynthesis metabolic pathways. Collectively, these results will provide a sensitive, feasible diagnostic prospect for AIS patients.

Keywords Acute ischemic stroke, Targeted metabolomics, Metabolite panels, Glycerophospholipid metabolism, Lysine degradation

Introduction

Stroke, a rapid process of focal neurological deficits caused by a disruption of blood supply to the corresponding region of the brain, is the second leading cause of death worldwide and a major contributor to the global disease burden [1]. There are two categories of stroke, ischemic stroke and hemorrhagic stroke. AIS accounts for 87% of all stroke-related incidents [2], caused by abrupt and sustained reduction in regional cerebral blood flow, led to acute loss of neurons, astroglia and oligodendroglia as well as synaptic damage [3]. Due to the blood vessel blockage by a thrombus or emboli, oxygen and glucose supply limited

to the affected brain region, symptoms including aphasia and hemiparesis were occurred [4].

Generally, computed tomographic (CT), CT angiography (CTA), magnetic resonance imaging (MRI), MR angiography (MRA) are used as auxiliary for AIS diagnose [5-7]. However, AIS treatment is racing against time - the therapeutic window for intravenous thrombolysis within 4.5 h or for mechanical thrombectomy within 8 h [8]. Thus, exploring early diagnostic biomarkers will save the brain tissue that hasn't died and achieve optimum efficiency for AIS treatment.

Because the pathogenesis of AIS is heterogeneous and complicated, it is crucial to establish systematic metabolomic approach to facilitate clinical diagnoses and risk predictions via elucidating metabolic biomarkers and pathways for AIS. To date, metabolomics captures a comprehensive set of analytes characterizing the human phenotype and its complex metabolic processes in real-time. In detail, by measuring small molecule qualitatively and quantitatively, metabolomic studies have the great potential to identify specific biomarkers, predict clinical outcome, and improve our understanding of the pathophysiologic basis of disease states [9-11].

Recent advances in metabolomic technologies unravel the unequivocal value of metabolomics tools in systems biology, biomarker discovery and diagnostic platforms [12-14]. Our results provide new insights into the mechanisms underlying AIS progression and the discovery of novel metabolite biomarkers and their related pathways.

Currently, multiple reaction monitoring (MRM) is extensively used to definitely quantify metabolites. MRM is performed on triple quadrupole instruments with liquid chromatography (LC) system to analyze the appropriate fragment ions [15-16]. Due to its high sensitivity, high throughput, broad dynamic range, and good reproducibility, MRM approach is promising in the discovery of disease biomarker [17-18]. Development of mass spectrometry (MS) based metabolites measurement has gain widely application in clinic, and

MRM has the potential to bridge the gap between biomarker discovery and validation. To date, MRM has been applied in newborn mass screening and antenatal diagnosis [19], metabolic disorders like serum 25-Hydroxyvitamin D [20], cancer metabolism [21], cardiovascular disease risk assessment [22], diabetes monitoring [23], drug abuse [24], and microbial metabolites identification [25].

AIS metabolite panels were established here by combining standardized methods for extracting metabolites from clinical samples and high-reproducible MRM technology. In summary, we develop a novel quantitative MS approach with antibody-independent method to monitor AIS progression and to assess stroke occurrence risk.

Materials and methods

Patients and clinical specimens.

The study was conducted in accordance with the guidelines of the Declaration of Helsinki. AIS patients and healthy individuals were recruited from a single center, Xiamen Branch, Zhongshan hospital of Fudan University, between March 2018 to February 2020. This study was obtained the approval of the Research Ethics Committee from this hospital of Fudan University.

Inclusion criteria were as follows: an initial National Institutes of Health Stroke Scale (NIHSS) score from 6 to 22, aged of 46 to 75 years, presentation <24 h after stroke onset, and stroke localization in the area of the middle cerebral artery. Patients with diabetes, cardiovascular diseases, or other diseases that would affect the metabolic profiles were excluded from the study. Healthy donors with a stroke history or showing any sign of stroke based on CT or MRI evaluation were excluded from the control group. Metabolomics analyses began with an unbiased search for serum metabolites. Cases were randomly selected from 22 AIS patients, while an age- and gender-matched control group was randomly selected from 22 healthy individuals. Detailed information on these subjects is summarized in [Table S1](#). Written informed

consent was provided by all participants. Immediately after collection via vein blood sampling, blood samples were centrifuged at 3,000 rpm 4°C for 15 min, and the supernatants were collected and stored at -80°C for further uses.

Metabolite extraction for MS analysis

LC-MS grade methanol (MeOH), acetonitrile (ACN), ammonium acetate ($\text{CH}_3\text{COONH}_4$), and ammonium hydroxide (NH_4OH) were purchased from ANPEL Laboratory Technologies Inc (Shanghai, China). Total metabolites were extracted from 100 μL serum using a MeOH:ACN:H₂O (2:2:1, v/v) solvent mixture. To precipitate protein, serum was incubated at -20°C for 1h, then centrifuged at 13,000 rpm 4°C for 15 min. The supernatant was removed and evaporated to dryness at a gentle nitrogen flow. The dry extracts were reconstituted in 100 μL solvent mixture of ACN:H₂O (1:1, v/v), vortexed for 30 s and sonicated for 10 min, then centrifuged at 12,000 rpm 4°C for 15 min to remove the insoluble debris. Finally, 60 μL supernatant was transferred into a new LC/MS glass vial for the UHPLC-QQQ-MS analysis, and pool 15 μL from each sample and mix as quality control (QC) samples.

To monitor the data quality and process variation, QC samples containing aliquots from serum samples of all participating subjects were parallel-processed. Additionally, the orders of sample injection were randomized to avoid systematic biases.

LC-MS analysis

The prepared samples were analyzed by an UPLC system (Agilent 1290 series, USA) couple with a triple quadrupole mass spectrometer system (Agilent 6460 series, USA). In brief, 3 μL of each sample was injected into an UPLC BEH Amide column (1.7 μm , 2.1×100 mm, Waters) at 25°C. The mobile phase composed of water including 25 mM $\text{CH}_3\text{COONH}_4$ and 25 mM NH_4OH (solvent A) and 100% ACN (solvent B). The gradient for metabolites elution solvent B switched from 95% to 65% after 14 min, then declined to 40% for 2 min and kept for 2 min, finally backed to 95% in 0.1 min and maintained for 4.9 min. The flow rate was 0.3 mL/min.

MS conditions were set as followings: scanning method, MRM. ESI source temperature, 100°C. Desolvation temperature, 300°C. Desolvation gas flow, 3.0 L/min.

Data processing

A total of 180 metabolites were analyzed accurately and quantitatively via MRM approach. The raw data were assessed for peak detection and alignment using Profiling Solution software. Multivariate analysis, including principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA), were used to visualize general clustering, trends and outliers of LC-MS/MS data via the software SIMCA-P+ 14.1 (Umetrics, Sweden). A volcano plot was used to filter important features that exhibited large variable significance in $VIP>1$ and statistical adjusted $p<0.05$ between AIS and control groups.

Potential biomarkers were assessed by receiver operating characteristic (ROC) analysis. The area under the ROC curve (AUC) of the proposed metabolite panel is used as a metric to evaluate the sensitivity and specificity of the biomarker performance. List of these differential metabolites were imported into MetaboAnalyst 5.0 (<http://www.metaboanalyst.ca/>) for pathway enrichment analysis [26-27].

Results

AIS patients were differentiated by PCA and OPLS-DA analysis

To test the reproducibility of the sample preparation procedures and to assess the reliability of the LC-MS system, the QC samples were prepared by mixing aliquots of all the biological samples and analyzed between every six clinical samples. PCA analysis was performed to get an overview of the difference on metabolites profiling between both sample dataset and QC injections (Figure S1). The first principal component (PC1) accounted for 53.8% of the total variance. Seven QC samples were clustered tightly in the PCA score plot, which indicated that the precision and repeatability of the experiments

were excellent.

A total of 112 metabolites were acquired from serum samples including 22 AIS patients and 22 healthy donors. Systematic metabolomic changes occurring in different groups were then assessed by two widely used multivariate methods - PCA and OPLS-DA. The PC1 accounted for 54.5% of the total variance and separates AIS group from control group ([Figure 1A](#)). To achieve the maximum distinction and identify differential metabolites that accounted for the separation between groups, OPLS-DA analysis was further conducted ([Figure 1B](#)). Samples were within 95% confidence interval at Hotelling's t-test.

Then, the permutation test for OPLS-DA showed that the Q2 regression line had a negative intercept and all R_{2y} and Q₂ values on the left were lower than the original points on the right ([Figure S2](#)), which demonstrated that the OPLS-DA model in the present study was valid. It shows that Ischemia was clearly separated from the Control group, which holds a higher efficiency to distinguish between groups comparing with PCA score plots.

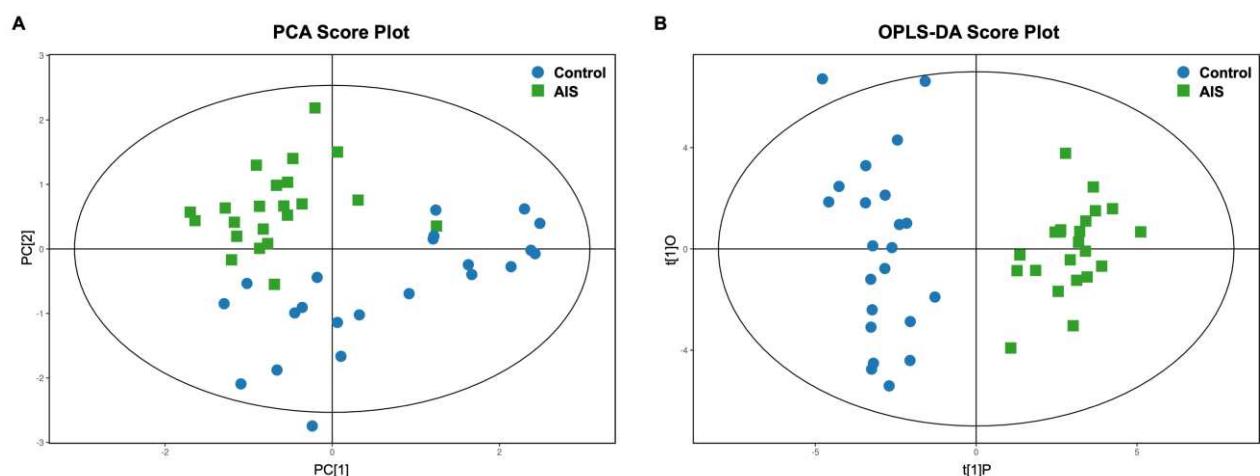


Figure 1. Score scatter plot of multivariate methods for groups between AIS patients and healthy controls. PCA score plot (A) and OPLS-DA score plot (B) of AIS and control groups. The x-axis t[1]P and y-axis t[1]O denotes predictive principal component scores and orthogonal principal component scores for PC1, respectively.

Differential metabolites in AIS patients were visualized by volcano plot.

Metabolites were inspected carefully before selection as biomarker candidates to reduce the risk of misreading and to ensure an accurate correlation with AIS occurrence risk.

Relative quantifications were applied to all the identified metabolites in these two groups, and 29 significantly changed metabolites were presented in [Table S2](#). Significant differences for the variables between AIS group and control group were depicted as volcano plot, including 13 up-regulated and 16 down-regulated ([Figure 2](#)). Collectively, our metabolomic data unraveled a strikingly consistent separation between AIS patients and healthy donors.

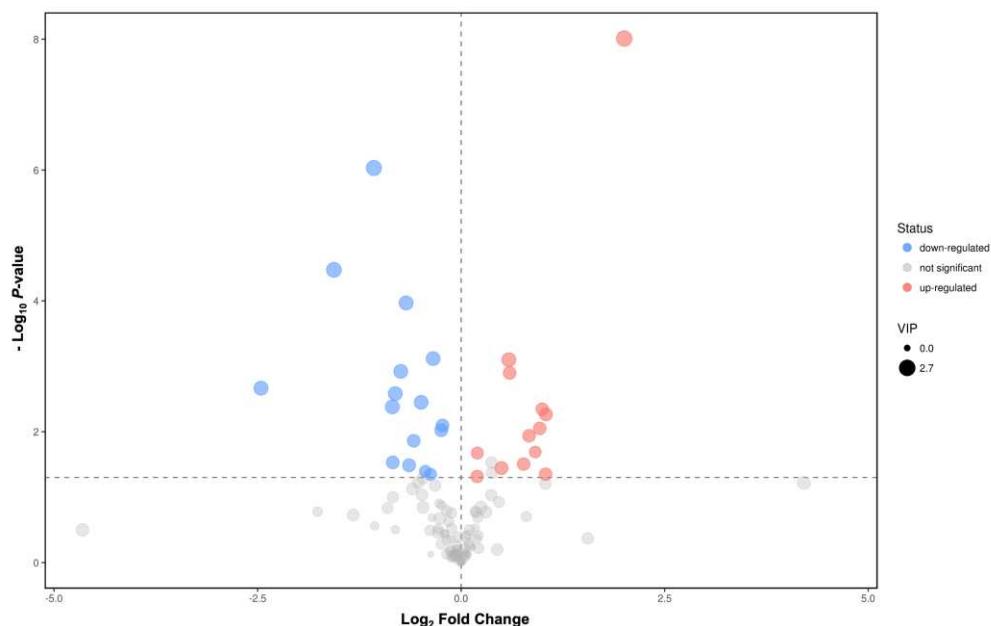


Figure 2. Volcano plot for AIS group vs control group. The x-axis represents fold changes (log2) and the y-axis represents adjusted p values ($-\log_{10} p < 0.05$).

AIS biomarker candidates were identified by metabolites panel screening

The metabolite panel was developed based on a logistic regression model. ROC analyses were performed for 29 significantly changed metabolites, and ROC curves were shown in [Figure 3](#). With the criterion of ROC area >0.8 , 5 metabolites were screened as potential biomarkers for AIS diagnosis, including argininosuccinic acid (ROC area = 0.897), beta-D-glucosamine (ROC area = 0.909), glycerolphosphocholine (ROC area = 0.816), L-abrine (ROC area = 0.841), and L-pipecolic acid (ROC area = 0.804). Surprisingly, all these five

metabolites were down-regulated in AIS patients compared with healthy controls.

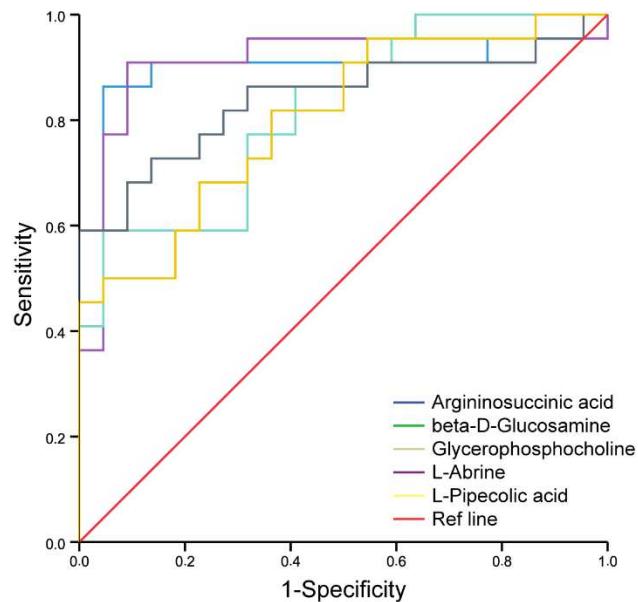


Figure 3. ROC curves for argininosuccinic acid, beta-D-glucosamine, glycerolphosphocholine, L-abrine, and L-pipecolic acid.

Metabolic Pathways are identified in AIS

Bubble plot of KEGG pathway enrichment analysis of 29 significantly changed metabolites compared AIS patients with healthy controls. The tendency of red circles shows the importance of metabolism pathway. 29 out of 112 detected metabolites, enriched in four metabolic pathways, were found significantly affected in AIS metabolome, including aminoacyl-tRNA biosynthesis, glycerophospholipid metabolism, lysine degradation, phenylalanine, tyrosine and tryptophan biosynthesis (Figure 4A). The color of each bubble reflected significance (red indicated a low p -values), while the size of bubble indicated the numbers of differential metabolites. The detailed information of top 8 enriched pathways were presented in Figure 4B.

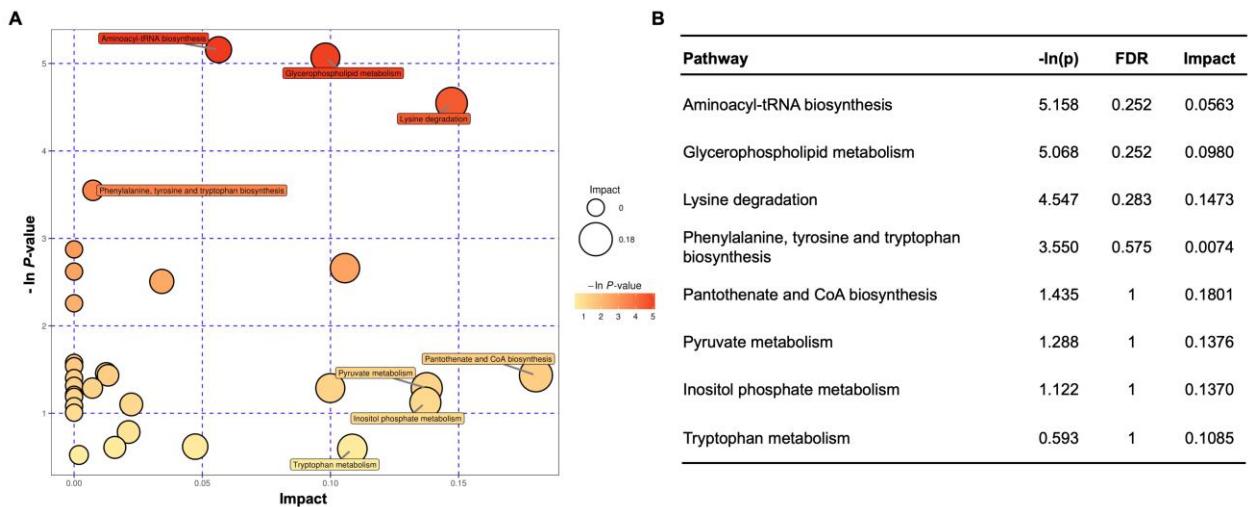


Figure 4. Bubble plot of KEGG pathway enrichment analysis of differential metabolites. (A) Metabolic pathways organized by pathway enrichment analysis (*p*-value) and pathway topology analysis (pathway impact). (B) Table of top 8 enriched pathways, including *p*-value and pathway impact information.

Discussion

Stroke, especially AIS, is a high mortality disease that caused by blood vessel blockage. In this study, argininosuccinic acid, beta-D-glucosamine, glycerophosphocholine, L-abrine, L-pipecolic acid were down-regulated in AIS patients comparing with healthy volunteers. Through metabolomic analysis, we found that Aminoacyl-tRNA biosynthesis, glycerophospholipid metabolism, lysine degradation, phenylalanine, tyrosine and tryptophan biosynthesis were associated with AIS.

Glycerophospholipid is the main lipid type in cell membranes especially in neural membrane. Glycerophosphocholine (GPC) could provide stability, fluidity, and permeability in neural membranes [28]. Brain fatty acids could be released from the degradation of glycerophospholipids and produce several lipid mediators based on its oxidation. Moreover, those lipid mediators are concerned about neuronal pathways, revealing that an interaction among lipids occurs in brain tissues. GPC is formed via the deacylation of the phospholipid phosphatidylcholine [29]. Accordingly, the alteration of glycerophospholipid

metabolism could be the reason for explain the cerebral damage from AIS.

We also found abnormal L-lysine degradation. L-pipecolic acid is a metabolic intermediate of L-lysine, synthesized in the brain [30-31]. However, reduced levels of lysine were indicated to lead to mental and physical disorders. Lysine is one of the basic amino acids, which involved in protection against brain injury. In our study, the down-regulated L-pipecolic acid might be attributed to decreasing lysine level, then caused brain dysfunction in AIS patients [32].

Other abnormal metabolic pathways, including phenylalanine pathway as well as tyrosine and tryptophan biosynthesis pathway, were related with biomarkers of L-abrine. L-abrine belongs to Indoles. It had been reported that various endogenous indoles might provide an antioxidant defense in the brain, and might participate in the scavenging process of radicals [33]. As a component of Indoles, the content of L-abrine was down-regulated in AIS patients. L-abrine deficient might cause the accumulation of radicals. Consequently, the overproduction of radicals might trigger necrosis or apoptosis in the brain [34].

In conclusion, this study developed a high-throughput metabolomic assay and manifested its utility for metabolite profiling of large scale of biological samples. By using a quantitative MS with antibody-independent approach, a flux of 180 metabolites was established via MRM detection method. Among which 112 metabolites were successfully identified in serum samples, indicated that many serum metabolites associated AIS are affected, including 29 metabolites changed markedly and 5 metabolites are down-regulated. Finally, these metabolites are found to be mainly enriched in 4 metabolic pathways. Our work represented the significant distinct metabolites in plasma samples from Ischemic, demonstrating that metabolites profiling could possibly provide a sensitive, feasible diagnostic prospect for Ischemic patients.

Abbreviations

AIS, Acute ischemic stroke; CT, Computed tomography; CTA, Computed

tomography angiography; MRI, Magnetic resonance imaging; MRA, Magnetic resonance angiography; MRM, Multiple reaction monitoring; LC, Liquid chromatography; MS, Mass spectrometry; NIHSS, National institutes of health stroke scale; QC, quality control; PCA, Principal component analysis; OPLS-DA, Orthogonal projections to latent structures- discriminant analysis; ROC, Receiver operator characteristic; GPC, Glycerophosphocholine

Supplementary Material

Additional supporting information may be found in the Supporting Information section containing supplementary tables 1-2 and supplementary figures 1-2.

Author's Contributions

All authors contributed to the study conception and design. Methodology was performed by Biao Qi and Yanyu Zhang. Data analysis and investigation were performed by Ling Lin and Guoqiang Fei. The original manuscript was written by Biao Qi and Yanyu Zhang, while the manuscript review and editing by Ling Lin and Qiuping Li. Funding acquisition was gained by Biao Qi and Qiuping Li. Resources were prepared by Yuhao Zhang. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding authors on reasonable request.

Compliance with Ethical Standards

Competing Interests

The authors declare no competing interests.

Ethical Approval

This article contains metabolomic study with human subjects. Ethical approval from the Research Ethics Committee from Xiamen Branch, Zhongshan hospital of Fudan University was obtained.

Consent to Participate

Patient written informed consent were obtained.

Consent to Publish

Not applicable.

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