



Metabonomic analysis of cerebrospinal fluid in epilepsy

Di Niu^{1#}, Pin Sun^{2#}, Fenghua Zhang^{1,3}, Fan Song⁴

¹College of Laboratory Medicine, Dalian Medical University, Dalian, China; ²Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China; ³Department of Laboratory Medicine, Zhoupu Hospital Affiliated of Shanghai University of Medicine and Health Sciences, Shanghai, China; ⁴Department of Neurology, The First Affiliated Hospital of Dalian Medical University, Dalian, China

Contributions: (I) Conception and design: D Niu, F Zhang; (II) Administrative support: F Song, F Zhang; (III) Provision of study materials or patients: D Niu, F Zhang; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: D Niu, P Sun, F Zhang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Fenghua Zhang. Department of Laboratory Medicine, Zhoupu Hospital Affiliated of Shanghai University of Medicine and Health Sciences, 1500 Zhouyuan Road, Shanghai 201318, China. Email: zpyzfh@163.com.

Background: We sought to explore the relationship between epilepsy and cerebrospinal fluid metabolomics and identify biomarkers for the diagnosis, treatment, and prognosis of epilepsy.

Methods: In total, 23 epileptic patients treated at The First Affiliated Hospital of Dalian Medical University from April 2019 to September 2019 were selected for the disease group and 13 non-epileptic patients were selected for the control group. Cerebrospinal fluid samples were collected from both groups, and the metabolites were analyzed by gas chromatography–mass spectrometry. The metabolites differentially expressed in the cerebrospinal fluid samples were identified. A differential metabolite enrichment analysis was performed to determine the metabolic pathways.

Results: Using a variable importance in the projection value >1 and a P value <0.05 as the screening criteria, we found that 3 metabolites (i.e., alpha-ketoisocaproic acid 1, xylose 1, and glycine 2) were differentially expressed in the cerebrospinal fluid of the 23 epileptic patients compared to the 13 non-epileptic patients. Alpha-ketoisocaproic acid 1 and xylose 1 were highly expressed in the epileptic cerebrospinal fluid samples, while glycine 2 was lowly expressed in the epileptic cerebrospinal fluid samples. Additionally, the 3 metabolites were significantly enriched in the 5 metabolic pathways of primary bile acid biosynthesis, valine, leucine, and isoleucine degradation, glutathione metabolism, glyoxylate and dicarboxylate metabolism, and glycine, serine, and threonine metabolism.

Conclusions: The present study examined the metabolites of the cerebrospinal fluid of epileptic patients and non-epileptic patients. Our findings provide insights that may inform the discovery of therapeutic targets and diagnostic markers for epilepsy.

Keywords: Epilepsy; cerebrospinal fluid; metabolites

Submitted Feb 15, 2022. Accepted for publication Apr 02, 2022.

doi: 10.21037/atm-22-1219

View this article at: <https://dx.doi.org/10.21037/atm-22-1219>

Introduction

Pathogenesis is complex and involves many factors (1). At present, the key factors leading to seizures are not apparent (2,3). Epilepsy is an uncontrollable and unpredictable condition (4). Due to the lack of accurate and effective biomarkers, the misdiagnosis rate of epilepsy is high, and

many patients are still unable to get effective treatment (5,6). More than 20 kinds of antiepileptic drugs are used in clinical settings; however, drug treatments are still ineffective in about 1/3 of epileptic patients (7). Thus, it is crucial that new key pathogenic factors and therapeutic targets for epilepsy be identified. No specific biological markers

of epilepsy have been found due to the pathological and clinical heterogeneity of epilepsy. It is of great significance to find specific therapeutic targets. A previous study has suggested that a group of molecular markers, rather than a single molecular marker, may be needed to characterize the clinical typing of epilepsy, construct the pathogenic mechanism network or serve as a therapeutic target (8).

Few studies have looked for epilepsy markers in brain tissue because the opportunity to obtain brain tissue of patients with epilepsy is rare and challenging to operate. A more direct method is to look for markers from cerebrospinal fluid samples (9). Therefore, metabolomics has significant advantages over genomics since the research object of metabolomics is small molecular metabolites that can enter body fluid freely. Moreover, metabolomics can quantitatively analyze all metabolites in organisms, thus potentially investigating the relationship between metabolites and physiological and pathological changes. Cerebrospinal fluid is the preferred biological sample for neuropathological research. The abnormal metabolic components of cerebrospinal fluid can directly reflect the pathophysiological changes of the brain.

During seizures, various metabolites and metabolic pathways become dysregulated, such as changes in the abundance of essential products in the three acid cycles, energy metabolism, amino acid metabolism, phospholipid metabolism and purine metabolic pathways (9,10). At present, studies have found different metabolites in the cerebrospinal fluid of patients with epilepsy caused by traumatic brain injury, including glutamate, norepinephrine, homovanillic acid, hydroxyindole (8,11). A metabolomics study had pointed out that the amino acid metabolic pathway plays a leading role in the occurrence and development of epilepsy (11). A study had also suggested that the pathogenesis of epilepsy may be related to citrate and the intermediates of the citric acid cycle pathway (12).

It should be emphasized that most previous studies have been limited to the effects of particular metabolites on epilepsy. This study screened the differentially expressed metabolites in cerebrospinal fluid of patients with epilepsy and healthy controls by high-throughput sequencing, explored the correlation between the differentially expressed metabolites, and enriched the metabolic pathway of the differentially expressed metabolites. This study comprehensively expounds on the relationship between epilepsy and cerebrospinal fluid metabolomic changes. By combining differential metabolites, this study provides a basis for the clinical classification of epilepsy and the

network research of epilepsy pathogenesis to screen the biomarkers of epilepsy diagnosis, treatment, and prognosis. We present the following article in accordance with the MDAR reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-1219/rc>).

Methods

Research objects

In this study, 23 epileptic patients treated at The First Affiliated Hospital of Dalian Medical University from April 2019 to September 2019 were allocated to the disease group and 13 non-epileptic patients were allocated to the control group. To be eligible for inclusion in the study, epileptic patients in the disease group had to meet the epilepsy diagnostic criteria of the international anti-epilepsy Alliance [2014], while non-epileptic patients in the control group had to have no history of seizures and no family history of epilepsy. Patients were excluded from the study if they met any of the following exclusion criteria (which were the same for both the disease and control groups): (I) had a metabolic disease; (II) had definite brain organic lesions; (III) had an abnormal quantification of leukocytes or proteins in the cerebrospinal fluid; and/or (IV) had participated in other clinical studies. There were 14 males and 9 females in the epilepsy group, with an average age of 49.87 ± 18.98 years. There were 7 males and 6 females in the control group, with an average age of 56.15 ± 19.58 years. No significant difference in age and gender was observed between the epilepsy and control group ($P > 0.05$). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The First Affiliated Hospital of Dalian Medical University (No. PJ-KS-KY-2020-18), and informed consent was provided by all the patients.

Research methods

Cerebrospinal fluid retention

In this study, a lumbar puncture was performed on each participant, and 2 mL of cerebrospinal fluid was collected in a sterile test tube. The tube containing the cerebrospinal fluid was sealed immediately after it was taken, and stored in a -80 °C refrigerator awaiting analysis. A total of 36 cerebrospinal fluid samples were collected. After routine examinations of the cerebrospinal fluid, the quality of the cerebrospinal fluid samples was confirmed, and all the

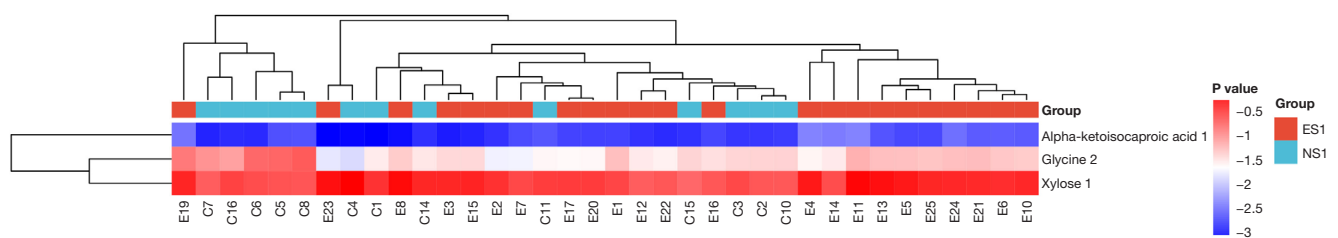


Figure 1 Using a P value <0.05 as the screening standard, the heat map shows the metabolites that were differentially expressed in the cerebrospinal fluid of the non-epileptic and epileptic patients. ES, epilepsy sample; NS, normal sample.

samples were retained.

Extraction and analysis of metabolites

The 36 cerebrospinal fluid samples were derivatized to reduce sample polarity and improve volatility and thermal stability. The metabolites in the cerebrospinal fluid samples were analyzed by gas chromatography-mass spectrometry (GC-MS). The peak signals of the cerebrospinal fluid samples were collected and standardized. Metabolite information was obtained after alignment database.

Data analysis

One-dimensional difference analysis

The fold change was calculated as follows: metabolite expression in the epilepsy sample/metabolite expression in the normal sample. The differences between the two groups were compared and analyzed by *t*-tests. A P value <0.05 was set as the screening standard.

Multi-dimensional difference analysis

An orthogonal partial least squares discriminant analysis (OPLS-DA) eliminates any noise information irrelevant to the classification and obtains the relevant metabolite information, and any significant difference between two groups are found by filtering out the signals irrelevant to the model classification, including the orthogonal signals. The quality of the model was examined. After establishing the model, the variable importance in the projection (VIP) value of the sample was calculated, and a VIP value >1 was set as the screening standard. The fold difference calculation was the same as the 1-dimensional analysis.

Correlation analysis of differential metabolites

The Spearman correlation analysis method was used to analyze differences in the metabolite data between the groups.

Metabolic pathway enrichment analysis

First, the differential metabolites were mapped to authoritative metabolite databases, such as Kyoto Encyclopedia of Genes and Genomes (KEGG), The Small Molecule Pathway Database (SMPDB), and PubChem. The metabolic pathway analysis was carried out using the pathway database of the species according to the matching information.

Statistical analysis

SPSS 24.0 software was used for the statistical analysis of the data. A P value <0.05 was considered statistically significant.

Results

One-dimensional test difference analysis

Compared to the cerebrospinal fluid of the 13 non-epileptic patients, 3 metabolites were differentially expressed in the cerebrospinal fluid of the 23 epileptic patients; that is, alpha-ketoisocaproic acid 1, xylose 1, and glycine 2 (P<0.05). Alpha-ketoisocaproic acid 1 and xylose 1 were highly expressed in the epileptic cerebrospinal fluid samples, and glycine 2 was lowly expressed in the epileptic cerebrospinal fluid samples. A heat map of the differentially expressed metabolites is shown in *Figure 1*.

Multi-dimensional test difference analysis

A VIP value calculated by OPLS-DA was used to identify the differential metabolites. Compared to the cerebrospinal fluid of the 13 non-epileptic patients, 56 metabolites were differentially expressed in the cerebrospinal fluid samples of the 23 epileptic patients, including 36 upregulated metabolites and 20 downregulated metabolites (all with

a VIP >1). The heat map of the differentially expressed metabolites is shown in *Figure 2*. The correlations between the 56 metabolites are shown in *Figure 3*.

One-dimensional and multi-dimensional difference cross analyses

The 1-dimensional and multi-dimensional differentially expressed metabolites were cross analyzed using a VIP value >1 and a P value <0.05 as the screening conditions. The cross overlap of the 1-dimensional and multi-dimensional differentially expressed metabolites is shown in *Figure 4*. The two differentially expressed metabolites all met the screening conditions (i.e., each had a VIP value >1 and a P value <0.05). A differential expression diagram is shown in *Figure 5*. A box diagram is shown in *Figure 6*. Alpha-ketoisocaproic acid 1 and xylose 1 were highly expressed in the epileptic cerebrospinal fluid samples, and glycine 2 was lowly expressed in the epileptic cerebrospinal fluid samples.

Enrichment analysis of the differential metabolite metabolic pathways

The 3 differential metabolites were significantly enriched in the 5 metabolic pathways of primary bile acid biosynthesis, valine, leucine, and isoleucine degradation, glutathione metabolism, glyoxylate and dicarboxylate metabolism, and glycine, serine, and threonine metabolism (see *Figure 7*).

Discussion

A body-fluid composition analysis is an indispensable indicator in disease diagnosis and pathogenesis exploration. To date, little research has been conducted on the application of cerebrospinal fluid in modern medicine. Cerebrospinal fluid performs many critical physiological functions in the brain and spinal cord, including the exchange of metabolites between blood and the brain (13). Compounds with different structures can penetrate the blood-brain barrier from the blood, enter the cerebrospinal fluid, and then enter the brain cells, thus affecting the function of the central nervous system.

The abnormal expression of metabolites in cerebrospinal fluid is closely related to epilepsy. Thus, abnormally expressed metabolites in cerebrospinal fluid are potential biomarkers of epileptic brain injury, and have good diagnostic efficacy or may become effective therapeutic targets. A previous study has confirmed that compared

to the non-epileptic patients, the Zin- α 2-glycoprotein (ZAG) and total tau (T-Tau) in the cerebrospinal fluid of patients with temporal-lobe epilepsy was decreased, but no significant difference in (tau phosphorylated at threonine 181) P-Tau 181 was found among the patients. The P-Tau 181/T-Tau ratio in patients with temporal-lobe epilepsy was significantly higher than that of patients in the control group. A Spearman correlation analysis showed that T-Tau and the P-Tau 181/T-Tau ratio were positively correlated with the white matter injury score ($P < 0.01$). Cerebrospinal fluid ZAG, T-Tau, and the P-Tau 181/T-Tau ratio may be potential biomarkers of brain injury in temporal-lobe epilepsy, which may also be involved in the potential mechanism of brain injury in temporal-lobe epilepsy (14).

With the rise of GC-MS technology, cerebrospinal fluid metabolomics has been extensively researched. We used GC-MS to analyze the cerebrospinal fluid of epileptic and non-epileptic patients. The differentially expressed metabolites in the cerebrospinal fluid samples of the epileptic and non-epileptic patients were screened using a VIP value of >1 and a P value <0.05. In this study, 3 differentially expressed metabolites were identified; that is, alpha-ketoisocaproic acid 1, xylose 1, and glycine 2.

Among the 3 differentially expressed metabolic species, we found that the relationship between alpha-ketoisocaproic acid 1 and xylose 1 and neurological diseases was unclear. We reviewed many previous studies and only found evidence that xylose 1 may cause convulsions by affecting sugar transport in the brain (15-17). As a classical inhibitory neurotransmitter, glycine has an apparent pathogenic effect on epilepsy (18). Abnormal glycine metabolism or a transport disorder may be related to the pathogenesis and cognitive impairment of patients with epilepsy. Glycine receptor blockers have been shown to reduce epileptic symptoms in patients (19). These results suggest that an increase of glycine in the cerebrospinal fluid may be an essential link in the pathogenesis of epilepsy, which is consistent with our results. Glycine and aspartic acid are both natural ligands of the N-methyl-D-aspartate (NMDA) receptor. Aspartic acid is the endogenous agonist of the NMDA receptor, and glycine is its necessary synergistic agonist. The dynamic balance regulation mechanism between them guarantees the normal function of the NMDA receptor in the physiological state. The disorder of its regulation mechanism is a leading cause of epilepsy. Alpha-ketoisocaproic acid 1, xylose 1 and glycine 2 are differentially expressed in cerebrospinal fluid of patients with epilepsy and are related to convulsive symptoms,

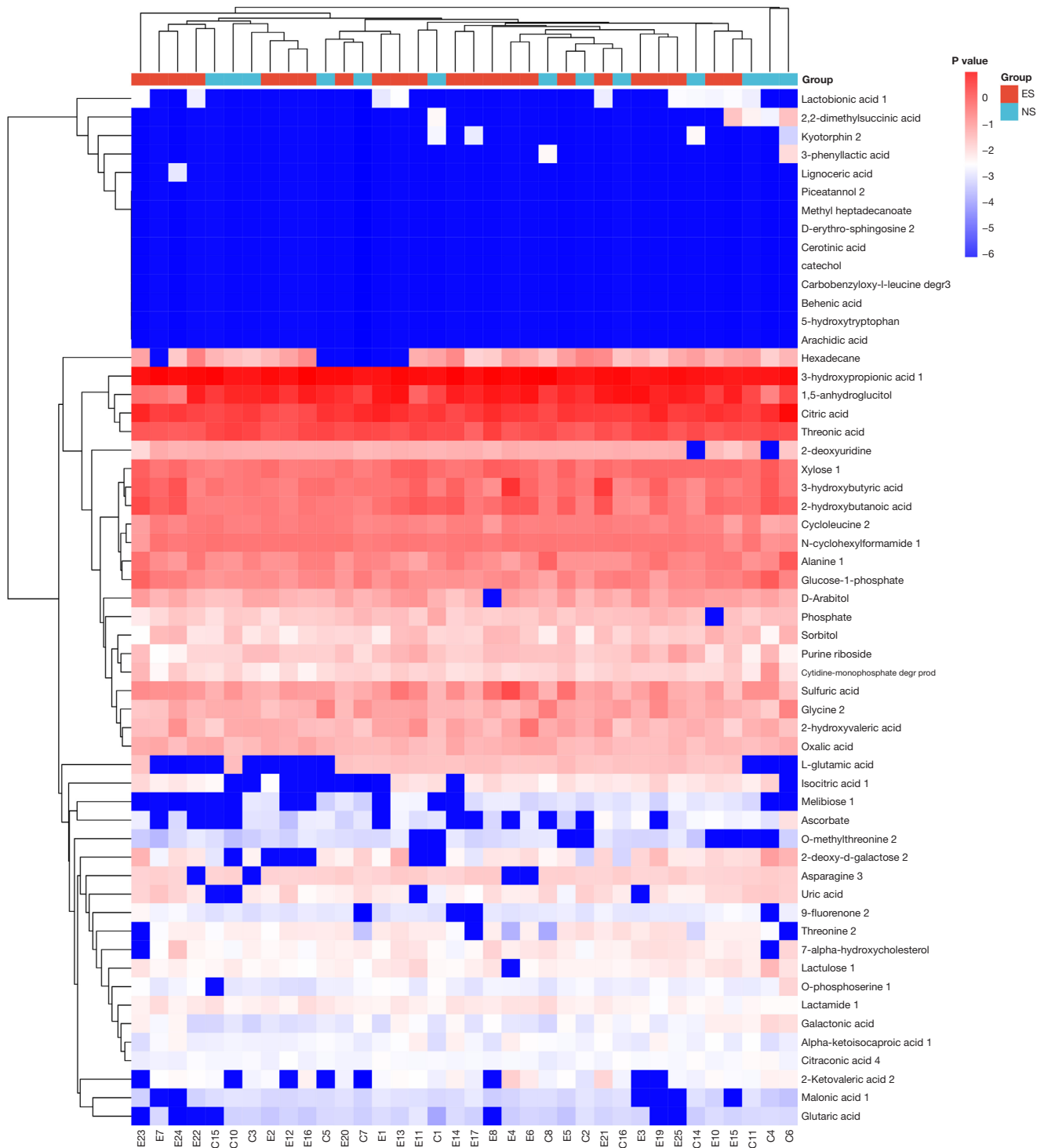


Figure 2 Using a VIP value >1 as screening criteria, the heat map shows the metabolites that were differentially expressed in the cerebrospinal fluid of the non-epileptic and epileptic patients. VIP, variable importance in the project. ES, epilepsy sample; NS, normal sample.

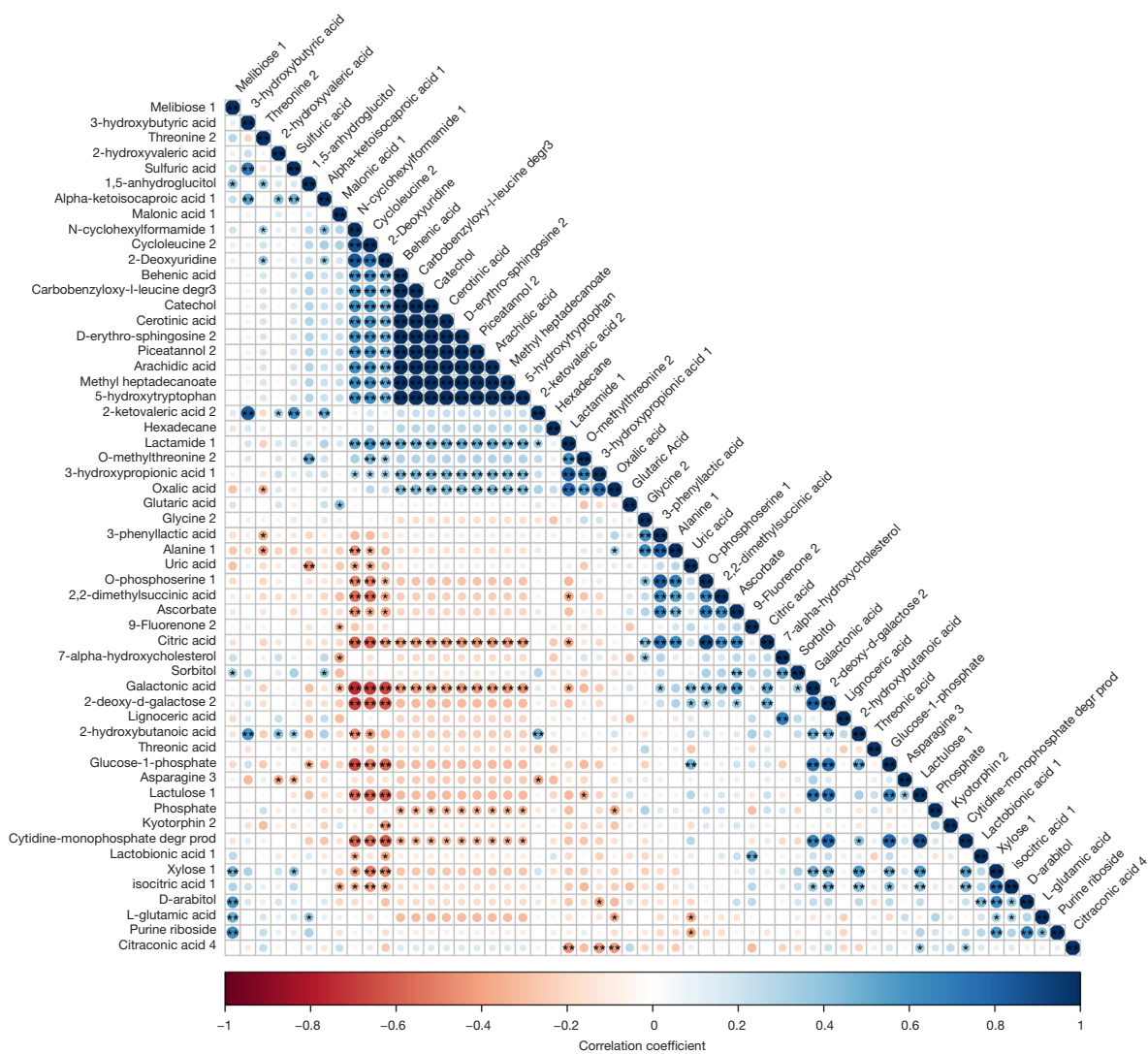


Figure 3 Using a VIP value >1 as the screening standard, a correlation heat map of 56 differentially expressed metabolites was obtained. VIP, variable importance in the project. *, $P < 0.05$; **, $P < 0.01$.

seizures and cognitive impairment. Reverse regulation of these metabolites may reduce the clinical symptoms of patients with epilepsy. These metabolites are potential therapeutic targets.

This study screened the metabolic pathways related to epilepsy based on differential metabolite enrichment. The metabolic pathways are primary bile acid biosynthesis, valine, leucine, and isoleucine degradation, glutathione metabolism, glyoxylic acid and dicarboxylic acid metabolism, and glycine, serine, and threonine metabolism. Important products in these metabolic pathways have been confirmed to be related to the pathogenesis and prognosis

of epilepsy (20,21).

Additionally, using research methods similar to those used in this study, some other studies have verified our research results to a certain extent. Akiyama *et al.* (22) analyzed the cerebrospinal fluid samples of 64 patients by GC-MS. The study clustered patients according to their age, and a multivariate analysis was conducted for 2 age groups (i.e., a 0–5-year-old group and a 6–17-year-old group) to clarify the effects of epilepsy and antiepileptic drugs on metabolites. In the patients aged 0–5 years (22 with epilepsy and 13 without epilepsy), 2-ketoglutarate was more decreased, and pyridoxamine and tyrosine were

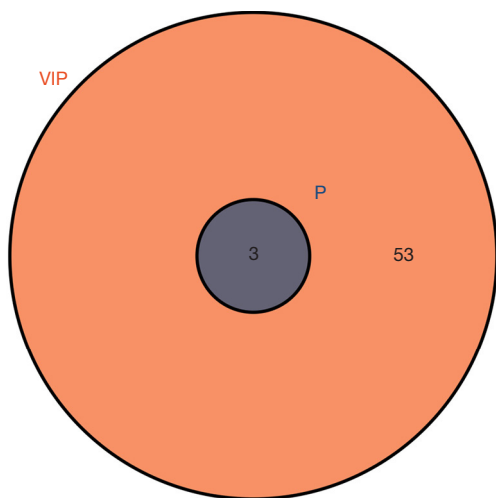


Figure 4 Using a VIP value >1 and a P value <0.05 as the screening conditions, a Wayne diagram of the differentially expressed metabolites was obtained. VIP, variable importance in the project.

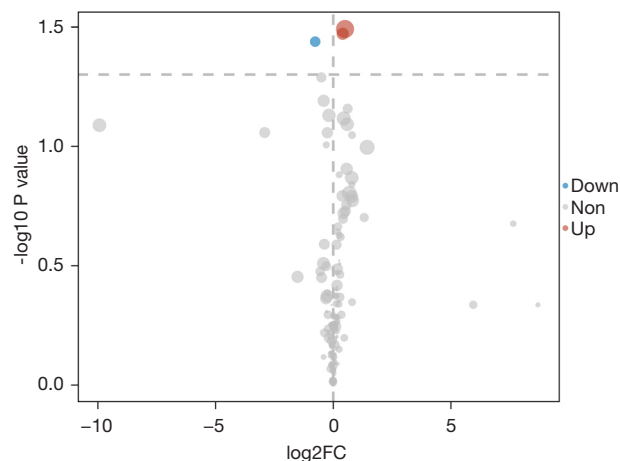


Figure 5 Using a VIP value >1 and a P value <0.05 as the screening conditions, a volcano map of the differentially expressed metabolites was obtained. VIP, variable importance in the project.

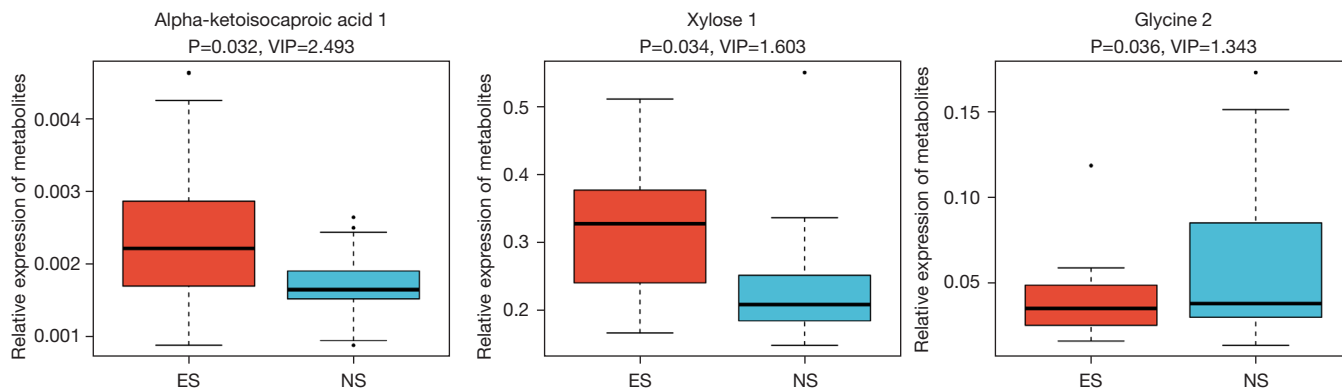


Figure 6 Differential expression metabolite box line diagram. VIP, variable importance in the project; ES, epilepsy sample; NS, normal sample.

more increased in patients with epilepsy than those without epilepsy. In patients aged 6–17 years (12 with epilepsy and 17 without epilepsy), 1,5-dehydrated glucosinol was decreased, but 2-aminobutyric acid, 2-ketoisohexanoic acid, 4-hydroxyproline, acetylglycine, methionine, n-acetylserine, and serine were increased. This study indicated that decreased energy metabolism and changes in vitamin B6 metabolism may play a role in childhood epilepsy (22).

This study has some limitations. First, this study failed

to explain the correlation between differential metabolites and the clinical characteristics of seizures due to the small sample size. This study did not include *in vivo* and *in vitro* experimental validation and functional research, so differential metabolites' role in the pathogenesis of epilepsy cannot be clarified.

In conclusion, this study provided a cerebrospinal-fluid metabolite map of epileptic and non-epileptic patients. Our findings provide insights that may inform the discovery of

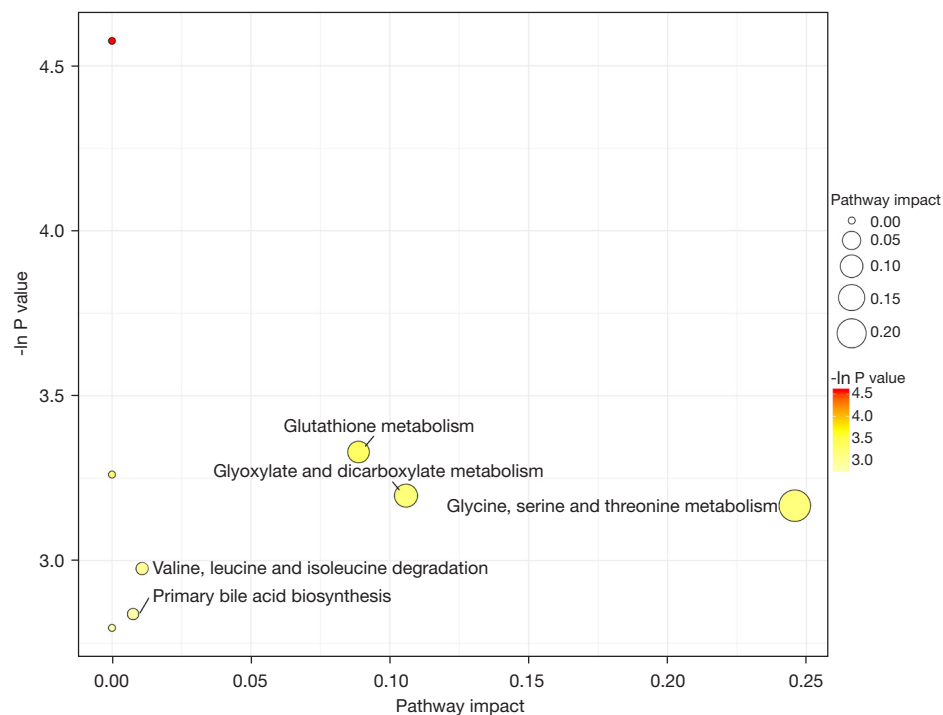


Figure 7 Bubble diagram showing the enrichment analysis results of the differential metabolite metabolic pathway.

therapeutic targets and diagnostic markers for epilepsy.

Acknowledgments

Funding: This study was supported by the 2018 Dalian Science and Technology Innovation Fund Plan (No. 2018J13SN100) and National Key R&D Program of China (No. 2018AAA0100300).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-1219/rc>

Data Sharing Statement: Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-1219/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-1219/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work, including ensuring that any questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The First Affiliated Hospital of Dalian Medical University (No. PJ-KS-KY-2020-18), and informed consent was provided by all the patients.

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- (English Language Editor: L. Huleatt)

Cite this article as: Niu D, Sun P, Zhang F, Song F. Metabonomic analysis of cerebrospinal fluid in epilepsy. *Ann Transl Med* 2022;10(8):449. doi: 10.21037/atm-22-1219