RESEARCH

Metabolomics Study on Improvement of Mild Cognitive Impairment by SCPE: a UPLC-Q/TOF-MS Mass Spectrometric Study

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Abstract

Background: Mild Cognitive Impairment (MCI) is a common and easily neglected neurological disease in clinic. Aging is the main cause of the disease, but its exact pathogenesis is not yet clear. Sagacious Confucius' Pillow Elixir(SCPE) is a classic medicine for the treatment of amnesia in traditional Chinese medicine.The aim of this study was to prove the stability and reliability of SAMP8 mice as MCI model, and to find potential biomarkers for the transformation of mild cognitive impairment to dementia, so as to reveal the pathogenesis of mild cognitive impairment and the mechanism of SCPE at the whole animal level.

Methods: Senescence-accelerated P8 (SAMP8) mice were selected as the model of MCI. Based on UHPLC/Q-TOF-MS platform, the effect of SCPE on MCI mice was evaluated by metabolomics to screen and identify brain tissue biomarkers and pathways of MCI. T test of SPSS 22.0 software and meta-analysis software were used for univariate and multivariate analysis to screen and identify the biomarkers and pathways of brain tissue with mild cognitive impairment; SPSS 22.0 statistical software was used to conduct t-test on the total ionic strength corresponding to the different metabolites in each group, and to clarify the change mechanism of biomarkers and pathways in different doses of SCPE.

Results: From the point of view of brain metabolomics, 84 MCI-related biomarkers and 8 metabolic pathways were identified. It was found that different doses of SCPE could call back 36 same metabolic markers and 3 common metabolic pathways.And with the increase of dose, the number of SCPE callback metabolic markers gradually increased(LD:40; MD:48; HD:54), and the types of main metabolic pathways also changed to a certain extent (the biosynthesis of valine, leucine and isoleucine in low dose changed to cysteine and methionine metabolic pathway in middle and high dose),which may be related to the effect of aminoacyl tRNA.

Conclusions: The stability and reliability of MCI model, and the effectiveness of SCPE in the treatment of MCI were clarfied. Compared three different dose groups, the high dose SCPE group has more advantages.

Keywords:
Metabolomics; Mild Cognitive Impairment; UPLC-Q/TOF-MS mass spectrometry; SCPE; Traditional Chinese medicine

Background

Mild Cognitive Impairment (MCI) refers to a state in which there are subjective and objective cognitive impairment, but the activities of daily living are basically normal [1]. MCI is a state of cognitive impairment between normal aging and Alzheimer's disease. It is an important early stage of AD[2]. The specific pathogenesis of MCI is not clear. At present, some hypotheses and studies have put forward [3], including senile plaque caused by β-amyloid (Aβ), nerve fiber tangles caused by hyperphosphorylation of Tau protein, synaptic dysfunction, chronic inflammatory cascade reaction, oxidative stress damage, etc. are the main pathogenesis of MCI. Most of the drugs used in the treatment of MCI are the drugs used in the treatment of AD, but the curative effect is still uncertain. However, traditional Chinese medicine prescription is a combination of Chinese herbal medicine, which can affect multiple targets and show synergistic therapeutic effect [4]. Therefore, it may be necessary to find a strategy to use traditional Chinese medicine to interfere with the progress of MCI.

SCPE is first published in *Qianjin Yifang* by Sun Simiao of Tang Dynasty. It is composed of tortoise shell (broiled), keel, acorus and thin-leaf milkwort root. The function of tortoise plastron in the prescription is to enrich yin and subdue yang, supplement essence and boost kidney, supplement the heart and nourish the blood, and has the function of supplementing water to restrict fire. The keel has the function of submerging yin fire and converging floating yang. Thin-leaf milkwort root, Acorus , dissolving phlegm to open the orifices, awakening the mind and boosting wisdom.The combination of the four drugs can restore interaction between the heart and the kidney, dissolving phlegm to open the orifices, awakening the mind and boosting wisdom, so as to achieve the effect of treating amnesia. We previously found that SCPE can improve cognitive function by reducing the content of Aβ protein in hippocampus and serum and the degree of phosphorylation of Tau protein [5].

Metabolomics is another part of systems biology after genomics and proteomics. It mainly makes quantitative analysis of all metabolites in local tissues and body fluids of organisms, and looks for the relationship between metabolites and physiological and pathological changes in vivo [6]. The research objects of metabolomics are mostly small molecular substances with relative molecular weight less than 1000 Da. Compared with genomics and proteomics studies, metabolomics is more sensitive to biological changes, and small changes in gene and protein expression levels are magnified in metabolites, making the detection more real and accurate. At present, it is believed that metabolic group is the biological end point of all phenotypic groups and will play an important role in precision medicine. In this study, the mice model of MCI was studied by means of non-targeted metabolomics, in order to analyze the metabolic changes of brain tissue in mice with MCI and after SCPE treatment, to find the potential biomarkers of the transformation from MCI to dementia, and the possible mechanism of SCPE intervention in MCI. So as to reveal the pathogenesis of MCI and the mechanism of SCPE at the overall animal level.

Methods

Drugs and chemical reagents

SCPE was purchased from Beijing Tongrentang Co,Ltd. (Beijing, China). 0.9% saline was purchased from the first affiliated Hospital of Heilongjiang University of traditional Chinese Medicine (China). Formic acid (mass spectrum grade) was purchased from Thermo Fisher Scientific Company (USA). Ultra-pure water was purchased from Thermo Fisher Scientific (USA). Sodium formate was purchased from Thermo Fisher Scientific (USA). Pentobarbital was purchased from Sigma (USA) and methanol (mass spectrum grade) was purchased from Merck (USA). Ammonium acetate was purchased from Sigma Company (USA). Acetonitrile was purchased from Merck (USA).

Animal and Model Construction

Male SAMR1 mice, SAMP8 mice, with a body mass of 20-22g and 7 months old, were provided by the first affiliated Hospital of Tianjin University of traditional Chinese Medicine. (Tianjin, China). Before the formal study, all animals were fed adaptively for 7 days in a control room with a temperature of 20°C to 22°C, a relative humidity of 40% to 60%, and a light / dark cycle of 12 hours, and were free to eat sterile feed and autoclaved water.

All the animals were randomly divided into 5 groups with 6 animals in each group: blank group (SAMR1 mice, given the same volume of distilled water); MCI model group (SAMP8 mice, given the same volume of distilled water); SCPE low dose group (SAMP8 mice, SCPE solution 2.3mg/kg); Medium dose group (SCPE solution 4.6mg/kg); High dose group (SCPE solution 9.2mg/kg).

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Ethics Committee of Heilongjiang University of Traditional Chinese Medicine.

Brain tissue sample processing

Firstly, the brain tissue sample was thawed slowly at 4°C to completely thawed, then the methanol / acetonitrile / water solution precooled by 1mL (at 2:2:1 volume ratio) was slowly added to the sample to fully swirl mixed concussion. After low temperature ultrasonic 30min, the supernatant was dried at-20°C by 10min, 14000g centrifugation at 4°C for 20min. In mass spectrometry analysis, 100μL acetonitrile aqueous solution (acetonitrile:water = 1:1) was re-dissolved and swirled. The protein was precipitated by centrifugation at 14000rpm/min, 4°C and centrifugation time was 15min. The supernatant was taken to the injection bottle for metabolomic analysis.

UPLC-Q/TOF-MS analysis of brain tissue

Chromatographic conditions: the brain tissue samples were separated by ACQUITY UPLC BEH C18 column (100mm to 2.1mm, 1.7μm, Waters, UK) with a column temperature of 50°C and a flow rate of 0.4mL/min. The mobile phase is composed of A and B parts, A mobile phase: water + 0.1% formic acid; B mobile phase: methanol + 0.1% formic acid. The metabolites of brain tissue were eluted with the following gradients: 100% A for 0-2min, 0-100% B for 2-11min, 100% B for

11-13min, 0-100% A for 13-15min. The samples in the whole process are placed in an automatic injection container at 4°C, and the sample volume of each sample is 10μL. The random sequence was used to analyze the samples continuously. Insert QC samples into the sample sequence to evaluate and monitor the stability of the whole system and the reliability of the data.

Mass spectrometry conditions: the small molecular substances eluted from the chromatographic column were collected by high resolution tandem mass spectrometry Xevo G2-XS QTOF (Waters, UK). In the positive ion mode, the taper hole voltage and the tube voltage are 40.0V and 3.0kV, respectively. In anion mode, the taper hole voltage and capillary voltage are 40.0V and 2.0kV, respectively. Using MSE mode to collect CENTROID data, 50-1200 Da is the range of first-level scanning, and the scanning time is 0.2s. All parent ions are fragmented according to the energy of 20 to 40eV, and the information of all fragments is collected, and the scanning time is also 0.2s. In the process of data acquisition, the real-time quality correction of LE signal is carried out every 3 seconds. At the same time, each interval of 10 samples to carry out a mixed quality control sample collection, has reached the evaluation of the stability of the instrument in the process of sample collection.

Data preprocessing

First of all, the original data obtained from the mass spectrometry were imported into the Progenesis QI software (version 2.2, QI) for peak extraction, in order to obtain the mass-charge ratio, ion area and retention time and other information related to the metabolites. The workflow of QI mainly includes: peak alignment, peak extraction and peak identification. Data preprocessing is completed using meta X software, including filling the missing values of the extracted data using the KNN method, removing low-quality ions (removing more than 50% of the missing ions in the QC samples, or removing more than 80% of the missing ions in the actual samples), and using QC-RSC (Quality control-based robust LOESS signal correction) method for correction.

Principal component analysis

In this part of the experiment, log2 conversion and proportion adjustment (scaling) are carried out on the data before PCA analysis. Pareto adjustment method (Pareto scaling) are used. PCA is mainly used to observe the trend of separation between groups in the experimental model, and whether there are outliers, and to reflect the degree of variation between groups and within groups from the original data.

Partial least squares discriminant analysis

In this part of the experiment, the log2 transformation is carried out before making the PLS-DA model, and the Pareto scaling method is used to adjust the scale of the data (scaling). Different from principal component analysis, PLS-DA is a supervised discriminant analysis statistical method, which can reflect the differences between classification groups to the greatest extent. This method uses partial least square regression to establish the relationship model between metabolite expression and sample categories to realize the modeling and prediction of sample categories.

Identification of differential metabolites in brain tissue

Firstly, according to the difference variables obtained from the analysis, the compounds in the brain tissue were identified by matching with the databases such as HMDB, METLIN, KEGG, etc., and the output included the data matrix including the name of the metabolites, HMDB ID, mUniz, RT, the ion form of adducts and so on.

Analysis of metabolic pathway in brain tissue

The identified brain tissue differential metabolites were introduced into MetaboAnalyst (http://www.metaboanalyst.ca) database in the format of HMDB ID for metabolic pathway analysis. The whole process mainly includes data processing, standardization, statistical analysis and advanced functional interpretation, in order to reveal the possible biological mechanisms.

Results

The model is established according to the grouping. And the reliability of the model is verified through the analysis. The differential metabolites are then determined. Then the metabolites of different dose groups are analyzed to study the effect of SCPE on MCI.

The establishment of models in each group

Principal component analysis

In order to more intuitively reflect the clustering between groups and within groups, the ion strength information of each sample in each group is imported into SIMCA-P 14.1 software, and the specific results of principal component analysis (PCA) in three-dimensional mode are shown in Figure 1.

As can be seen from Figure 1, the intra-group clustering of blank group (Cont), model group (Mod), low (LD) medium (MD) and high (HD) dose group is good, and the clustering between groups has a tendency of separation to some extent, especially the (Cont) of blank group is obviously separated from that of model group. Although each dose of SCPE did not completely coincide with the intra-group clustering of the blank group, it also showed a trend towards the blank group, in which the tendency under the positive ion mode (pos) was more obvious, indicating that SCPE has the callback function of MCI biomarkers.

PLS-DA analysis

In this part, metaX software was used to establish the PLS-DA model among blank group (Cont), model group (Mod), low (LD) medium (MD) and high (HD) dose groups. It was found that the parameters (R2 and Q2) were higher, indicating that the current PLS-DA model was more reliable. At the same time, the parameters R2 and Q2 of the model were tested 200 times, and the PLS-DA models of each group in positive and negative ion mode were obtained (Figure 2).

As can be seen from Figure 2, the left side is the PLS-DA score map of each comparison group in the positive ion mode, and the right side is the PLS-DA score map of each comparison group in the negative ion mode, and the Abscissa represents the first principal component PC1, ordinate represents the second principal

component PC2. Each point in the graph represents a sample, and the discreteness of the two color symbols represents the distribution trend of the two groups of samples on the PC1 and PC2 axes, respectively. The values of Q2 and R2 in the positive and negative ion mode were higher than 0.5 and almost close to 1, and the trend of each dose group was similar to that of the blank group, that is, it was obviously separated from the model group, indicating that SCPE has a better positive intervention effect.

Screening and identification of differential metabolites

The ions detected by mass spectrometry conditions were analyzed by SPSS22.0 software T test and metaX analysis software for univariate and multivariate analysis, so as to screen the differential metabolites between groups.

Univariate analysis

In this part, T-test and multiple of variation analysis (Fold change analysis, FC analysis) were used to make statistics, and the p-value generated by statistical test was corrected by FDR to get Qmurvalue. the final result showed two indexes of difference multiple (Fold Change, FC) and q-value in the form of volcanic map (Volcano plot). The condition for screening differential metabolites was (FC) ≥ 1.2 or ≤ 0.8333, Q value ¡ 0.05. the results are shown in Table 1 and Figure 3.

Table 1 contains the differential ions between the two groups, and shows the general situation of the down-regulation of the differential ions of the model group (Mod) relative to the blank group (Cont) (for example, the group is ContvsMod, indicating that the Cont group is relative to the Mod group), as well as the identification of the first and second levels.

Log2 (fold change) is Abscissa, and the negative logarithm of q-value-log10 (q-value) is ordinate. The dots with fold change ≤ 0.8333 or ≥ 1.2, q-value ¡ 0.05 were marked red, and the other dots were purple. The red dots were the differential ions screened by univariate analysis.

Multivariate analysis

Because simple univariate analysis can not dig out reliable metabolic markers. Therefore, on the basis of univariate analysis, we used multivariate statistical analysis of the VIP values of the first two principal components of the PLS-DA model, combined with univariate analysis of the multiple of difference (fold-change) and q-value values in the volcano map to screen differentially expressed metabolites. That is, the screening conditions of this part are as follows: 1) VIP ≥ 1; 2) fold change ≥ 1.2 or ≤ 0.8333; 3) q-value ¡ 0.05, the three intersect, and the common ions, namely differential ions, are identified by ProgenesisQI (version2.2) software. The differential ions and the identification results are shown in Table 2 and Figure 4.

The preliminary screened differential ions are analyzed by cluster analysis, which is presented in the form of VIP diagram and heat map, as shown in Figure 4.

Each row in the picture represents a differential ion, each column represents a sample, and different colors represent different intensities, ranging from green to red, indicating that the intensity is from low to high.

Combined with Table 2 and Figure 4, after multivariate statistical analysis (VIP $\geq$ 1), the range of differential metabolites was further reduced, in which the differential ions in positive ion mode were screened from 313 to 302 and the differential ions in negative ion mode were screened from 356 to 169 in univariate statistical analysis. It provides a more clear candidate differential ion set for further identification of differential metabolites.

Identification of differential metabolites

Through the above differential metabolite screening process, 471 differential ions were obtained, including 302 in positive ion mode and 169 in negative ion mode. Compared with the blank group(Cont), the differential ions in the model group(Mod) showed obvious up-down relationship. To further identify potential metabolic markers of MCI, we combined the matching degree in mass spectrometry and used related mass spectrometry websites such as: Human Metabolome Database[1]; Lipidomics Gateway[2]; METLIN Metabolite Database[3]. Differential metabolites were identified by SDBS[4] and related literature reports. Results A total of 84 biomarkers were identified, including 45 in positive ion mode (32 up-regulated and 13 down-regulated). It mainly includes angiotensin, leukotriene D4, cetane diacid, (3z,6z) 3-nonadienal, isoprenaline, D-ornithine, methoxamine, citrulline, coriander aldehyde, isopropylmalic acid, etc. Thirty-nine (31 up-regulated and 8 down-regulated) in negative ion mode, including L-rhamnose, N-acetyl-L-histidine, coriander glycoside, arginine, ornithine, 2-isopropylmalic acid, L-leucine, γ-glutamyl-selenomethylselenocysteine, xylobiose, L-positive leucine, etc. the specific results are shown in Table 3, the content information is shown in Figure 5 and 6, and the cluster analysis is shown in Figure 7 and 8.

The specific contents of 84 differential metabolic markers identified were analyzed, that is, the total ionic strength of mass spectrometry and differential metabolites in each group was tested by SPSS22.0 software, and the results were expressed in the form of mean ±standard deviation (mean±SD). If the metabolite screened in the previous step is not statistically significant between the model group and the blank group (P ¿ 0.05), it means that the metabolite has no significant correlation with MCI, but when the comparison between the two groups is P ¡ 0.05, it is considered to be statistically significant, and the metabolite is the metabolic marker of MCI (Figure 5 and 6).

From Figure 5 and 6, it can be seen that the 84 metabolic markers in the model group changed significantly compared with the blank group, and the difference was statistically significant (P-value ¡ 0.01). It is suggested that the stability of MCI model and the reliability of metabolite screening. In order to better reflect the changes of differential metabolites, we carried out cluster heat map analysis of 84 metabolic markers. Each row in Figure 7 and 8 represents the ions corresponding to a biomarker, and each column represents a sample. Different colors represent different intensity, from blue to red, indicating that the intensity is from low to high.

[1]http://www.hmdb.ca/
[2]http://www.lipidmaps.org/
[3]http://metlin.scripps.edu/
[4]http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgiSp

Biological interpretation of metabolic markers

After the identification and verification of metabolic markers, we found a total of 84 metabolic markers, which were sorted according to FC and VIP values. In the positive ion mode (pos), the top 10 metabolites are angiotensin, leukotriene D4, cetane dicarboxylic acid, (3z,6z) 3,6-nonadienal, isoprenaline, D-ornithine, methoxyamine, citrulline, coriander aldehyde, isopropylmalic acid, etc; In the anion mode(neg), the top 10 metabolites are: L-rhamnose, N-acetyl-L-histidine, coriander glycoside, arginine, ornithine, 2-isopropylmalic acid, L-leucine, γ-glutamylselenomethylselenocysteine, xylobiose, L-n-leucine and so on.

Angiotensin is a peptide with strong vasoconstrictive effect, which is mainly involved in the regulation of human blood pressure[7]. Leukotriene D4[8] is an important inflammatory mediator in human body, which participates in the process of chronic inflammation and oxidative stress injury in the brain. Hexadecanedioic acid[9] is an intermediate substance involved in fatty acid metabolism. (3z,6z) 3,r6-nonadienal is a metabolic intermediate with aromatic odor. At present, there is mainly research in the field of botany, but there is little research in the field of animal and medicine[10]. Isoproterenol[11] is a common β-adrenoceptor agonist, which mainly promotes myocardial contraction, increases cardiac output, dilates blood vessels and accelerates heart rate. D-ornithine, arginine and ornithine are all intermediates in the metabolism of urea, which are mainly involved in the production of urea in human body[12]. The main function of methoxamine is to stabilize the arterial pressure of the body, which can effectively prevent low perfusion of brain tissue caused by the decrease of blood pressure[13]. Citrulline is an α-amino acid produced from ornithine in the urea cycle, which is mainly involved in the chronic inflammatory response of the body[14]. Pine and cypress aldehyde has anti-inflammatory, sedative, bacteriostatic and analgesic effects[15]. L-rhamnus gum sugar has good intestinal permeability and has a strong correlation with the metabolism of bacteria, viruses and other microorganisms [16]. N-acetyl-L-histidine can inhibit neuronal apoptosis and HIV virus replication[17]. Cilantro is a neuroprotective agent, which mainly inhibits neuroinflammation and improves the formation of neuronal edema in brain tissue. The substance has the advantages of clear chemical structure, stable content and clear metabolic mechanism in the body[18]. γ-glutamylselenomethylselenocysteine is a selenium-rich metabolite, which is mainly studied in the field of fortified food nutrition[19]. Xylobiose can enhance the proliferation of intestinal bifidobacteria and improve gastrointestinal function [20]. L-leucine and L-leucine are the main products of amino acid metabolism and participate in the pathogenesis of neuroinflammation and mental disorders[21].

Pathway Analysis of Biological Metabolic markers

In order to more intuitively reflect the potential mechanism of 84 biomarkers identified, this part analyzed their pathways, mainly based on KEGG and HMDB database to annotate metabolite metabolic pathways. Using MetaboAnalyst[5] online software analysis tool, the differential metabolites identified in the previous step were used for pathway analysis. The input format of metabolites was Compound-name/HMDBID/KEGGID, and Impact was set to greater than 0.1 as the screening

[5]https://www.metaboanalyst.ca/

criteria for potential main metabolic pathways. After analysis, 8 main metabolic pathways related to MCI were obtained, which were as follows: D-Arginine and D-ornithine metabolism; Alanine, aspartate and glutamate metabolism; Phenylalanine metabolism; Arginine and proline metabolism; Valine, leucine and isoleucine biosynthesis; Cysteine and methionine metabolism; Aminoacyl-tRNA biosynthesis; D-Glutamine and D-glutamate metabolism.

Figure 9 shows the metabolic pathway analysis of MCI biomarkers. Y axis represents p value (from path enrichment analysis), X axis represents pathway impact value (from path topology analysis). The color of each node is made based on the p value, from light to deep represents the p value from large to small, and the radius of the node represents the impact value from small to large based on its corresponding pathway impact value. This part marks the first 15 metabolic pathways in the diagram, and the sequence number and the content represented in the diagram are consistent with the information in Table 4. Among them, 1-8 is the potential metabolic pathway obtained after screening.

84 major metabolic markers related to mild cognitive impairment were identified after further analysis of metabolic markers and 8 major metabolic pathways were obtained by MetaboAnalyst and Enrichment analysis. The relevant data were imported into Cytoscape3.7.1 software to visualize the results.

As can be seen from Figures 11 and Table 4, the metabolites involved in D-Arginine and D-ornithine metabolism are: D-ornithine and ornithine, Account for 2/8 of the total; The metabolites involved in Alanine,aspartate and glutamate metabolism are: N-Acetyl-L-aspartic acid, L-Aspartic acid, L-Asparagine, L-Glutamic acid, Account for 4/24 of the total; The metabolites involved in Phenylalanine metabolism are: L-Phenylalanine, D-Phenylalanine, 2-Phenylacetamide, trans-Cinnamic acid, 4-Hydroxycinnamic acid, Enol-phenylpyruvate, Phenylacetaldehyde, Account for 7/45 of the total; The metabolites involved in Arginine and proline metabolism are: L-Glutamic acid, L-Aspartic acid, Citrulline, Ornithine, Account for 4/77 of the total; The metabolites involved inValine, leucine and isoleucine biosynthesis metabolism are: 2-Isopropylmalic acid, L-Valine, Isopropylmaleate, L-Leucine, L-Isoleucine, Account for 5/27 of the total; The metabolites involved in Cysteine and methionine metabolism are: L-Aspartic acid, L-Cystathionine, 2-Aminoacrylic acid, Account for 3/56 of the total; The metabolites involved in Aminoacyl-tRNA biosynthesis are: L-Asparagine, L-Phenylalanine, L-Valine, L-Isoleucine, L-Threonine, L-Aspartic acid, L-Glutamic acid, Account for 7/75 of the total; The metabolites involved in D-Glutamine and D-glutamate metabolism are: L-Glutamic acid, Account for 1/11 of the total.

Regulatory effect of SCPE on metabolic markers of MCI

In order to clarify the regulatory mechanism of SCPE on 84 related biomarkers identified in the previous step, we used SPSS22.0 statistical software to test the total ionic strength of different metabolites in each group, and the results were expressed in the form of mean ±standard deviation (mean ±SD). Compared with the model group, the P ¡ 0.05 of each dose group was considered to be statistically significant, and P ¡ 0.01 represented a significant difference. It is suggested that different doses of SCPE can regulate the biomarker.

Screening and pathway analysis of biomarkers of low dose SCPE callback MCI
Through T-test and consulting the basic information of each metabolite in the literature, it is concluded that low dose SCPE can call back 40 biomarkers of MCI. In this part, the 40 biomarkers screened are shown in Table 5, metabolite intensity histogram (Figure 12-A) and heat map (Figure 12-B), which can directly reflect the changes of effective biomarkers of low dose SCPE intervention in MCI.

In order to clarify the action mechanism of the 40 biomarkers obtained in the previous step, this part analyzed their pathways, mainly based on KEGG and HMDB database to annotate metabolite metabolic pathways. Using MetaboAnalyst[6] online software analysis tool, the pathway analysis, metabolite input format of the differential metabolites identified in the previous step is Compoundname/HMDBID/KEGG ID, and the Impact value is set to greater than 0.1 as the screening criteria for potential main metabolic pathways. After analysis, four main metabolic pathways were obtained, which were as follows: Phenylalanine metabolism; Alanine, aspartate and glutamate metabolism; Valine, leucine and isoleucine biosynthesis; D-Glutamine and D-glutamate metabolism.

Taking Impact as a factor, the enrichment analysis of each metabolic pathway of low dose SCPE intervention MCI is shown in figure 14, in which light red represents the main metabolic pathway and blue is the related metabolic pathway.

After further analysis of 40 biomarkers of low dose SCPE callback MCI, it was found that 11 metabolites were significantly call back (P-value ¡ 0.01): Hexadecanedioic acid, Eicosadienoic acid, L-Isoleucine, Leukotriene D4, 11Z-Eicosenoic acid, D-Phenylalanine, Isopropylmaleate, trans-Cinnamic acid, N1-Acetylspermine, L-Phenylalanine, Gibberellin A15 open lactone. Four main metabolic pathways and their participating metabolites were obtained by MetaboAnalyst and Enrichment-analysis analysis: (1) Phenylalanine metabolism: L-Phenylalanine, D-Phenylalanine, trans-Cinnamic acid, Account for 3/45 of the total; (2) Alanine, aspartate and glutamate metabolism: L-Glutamic acid, Account for 1/24 of the total; (3) Valine, leucine and isoleucine biosynthesis: 2-Isopropylmalic acid, Isopropylmaleate, L-Leucine, L-Isoleucine, Account for 4/27 of the total; (4) D-Glutamine and D-glutamate metabolism: L-Glutamic acid, Account for 1/11 of the total.

Screening and pathway analysis of biomarkers of medium dose SCPE callback MCI
After T-test and consulting the basic information of each metabolite in the literature, it was determined that the medium dose SCPE could call back 48 biomarkers of MCI. The 48 biomarkers screened were shown in Table 7, metabolite intensity histogram (Figure 15-A) and heat map (Figure 15-B), which could directly reflect the changes of effective biomarkers of MCI intervened by medium dose SCPE.

The pathways of 48 metabolites from the medium dose SCPE callback obtained in the previous step were analyzed, and the metabolic pathways were annotated mainly based on KEGG and HMDB database. Using MetaboAnalyst[7] online software analysis tool, the input format of metabolites was Compoundname/HMDBID/KEGG ID, and Impact was set to greater than 0.1 as the screening criteria for potential

[6]https://www.metaboanalyst.ca/
[7]https://www.metaboanalyst.ca/

main metabolic pathways. After analysis, four main metabolic pathways were obtained (Figure 16 and Table 8): Phenylalanine metabolism; Alanine, aspartate and glutamate metabolism; Cysteine and methionine metabolism; D-Glutamine and D-glutamate metabolism.

Taking Impact as a factor, the enrichment analysis of each metabolic pathway of medium dose SCPE intervention MCI is shown in figure 17, in which red represents the main metabolic pathway and blue is the related metabolic pathway.

After further analysis of 48 biomarkers of medium dose SCPE callback MCI, it was found that there were 17 significant call back metabolites (P-value ¡ 0 01), of which 9 metabolites were the same as those of low dose callback, namely: Hexadecanedioic acid, Eicosadienoic acid, L-Isoleucine, Leukotriene D4, 11Z-Eicosenoic acid, trans-Cinnamic acid, N1-Acetylspermine, L-Phenylalanine, Gibberellin A15 open lactone. The 8 different significant callback metabolic markers are: L-Carnitine, 4-Hydroxyifosfamide, Angiotensin, 16-Hydroxy hexadecanoic acid, Ricinoleic acid, 5-Hydroxyindoleacetate, Methdilazine, L-Leucine. Four main metabolic pathways and their participating metabolites were obtained by MetaboAnalyst and Enrichment analysis: (1) Phenylalanine metabolism: L-Phenylalanine, D-Phenylalanine, trans-Cinnamic acid, 4-Hydroxycinnamic acid, Account for 4/45 of the total; (2) Alanine, aspartate and glutamate metabolism: L-Glutamic acid, Account for 1/24 of the total; (3) Cysteine and methionine metabolism: L-Cystathionine, Account for 1/56 of the total; (4) D-Glutamine and D-glutamate metabolism: L-Glutamic acid, Account for 1/11.

Screening and pathway analysis of biomarkers of high dose SCPE callback MCI

After T-test and consulting the known basic information of each metabolite in the literature, it was determined that high-dose SCPE could call back 54 biomarkers of MCI. The 54 biomarkers screened were shown in Table 9, metabolite intensity histogram (Figure 18-A) and heat map (Figure 18-B), which could directly reflect the changes of effective biomarkers of high dose SCPE intervention in MCI.

The pathways of 54 metabolites from the high dose SCPE callback obtained in the previous step were analyzed. Firstly, the metabolic pathways were annotated based on KEGG and HMDB database, and then the MetaboAnalyst[8] online software analysis tool was used to set the input format of metabolites to Compound-name/HMDBID/KEGG ID, and set Impact greater than 0.1 as the screening criteria for the main metabolic pathways. After analysis, four main metabolic pathways were obtained (Figure 19 and Table 10): Phenylalanine metabolism; Alanine, aspartate and glutamate metabolism; Cysteine and methionine metabolism; D-Glutamine and D-glutamate metabolism.

Taking Impact as a factor, the enrichment analysis of each metabolic pathway of high dose SCPE in MCI is shown in figure 20, in which crimson represents the main candidate metabolic pathway and blue is the related metabolic pathway.

After further analysis of 54 biomarkers of high-dose SCPE callback MCI, it was found that there were 23 significant callback metabolites (P-value ¡ 0.01), including 9 metabolic markers of low dose significant callback: Hexadecanedioic acid, L-Isoleucine, Leukotriene D4, 11Z-Eicosenoic acid, Isopropylmaleate, trans-Cinnamic acid, N1-Acetylspermine, L-Phenylalanine, Gibberellin

[8]https://www.metaboanalyst.ca/

A15 open lactone; Metabolites with significant pullback in medium dose: L-Carnitine, 4-Hydroxyifosfamide, Methdilazine; Metabolites of their own significant callbacks: Piperidine, 1-Phenyl-1, 2-propanedione, o-Cresol, LysoSM(d18:1), (3Z,6Z)-3,6-Nonadienal, Alpha-Tocotrienol, Mannitol 1-phosphate, beta-Tyrosine, L-Norleucine, 2-Isopropylmalic acid, (S)-2-(Hydroxymethyl)glutarate. Four main metabolic pathways and their participating metabolites were obtained by MetaboAnalyst and Enrichment analysis analysis: (1) Phenylalanine metabolism: L-Phenylalanine, D-Phenylalanine, trans-Cinnamic acid, 4-Hydroxycinnamic acid, Account for 4/45 of the total; (2) Alanine, aspartate and glutamate metabolism: L-Glutamic acid, Account for 1/24 of the total; (3) Cysteine and methionine metabolism: L-Cystathionine, 2-Aminoacrylic acid, Account for 2/56 of the total; (4) D-Glutamine and D-glutamate metabolism: L-Glutamic acid, Account for 1/11 of the total.

Discussion

Venn analysis of metabolic markers

The metabolic markers of the model and the callback of low, medium and high dose SCPE were intersected by Venn map to obtain the common metabolic markers and unique metabolic markers of different doses of SCPE in MCI. The effects of SCPE on brain metabolic markers with doses were analyzed, the possible mechanism of different doses was revealed and the best dose of MCI was determined (Figure 21).

As can be seen from figure 21, LD, MD and HD can call back 36 common metabolic markers; LD and MD can call back 39 common metabolic markers, including 3 unique metabolic markers; LD and HD can call back 37 common metabolic markers, including one unique metabolic marker; MD and HD can call back 45 common metabolic markers, including 9 unique metabolic markers. In addition, HD can call back 8 unique metabolic markers. The basic information of callback metabolic markers in each dose group is shown in Table 11.

As can be seen from figure 21 and Table 11, the metabolic markers of MCI in each dose group of SCPE account for 68.9% of the total, indicating that SCPE, a classic prescription for the treatment of amnesia in traditional Chinese medicine, has a better effect on improving cognitive function. Among them, the metabolic markers of MCI47.7 can be recalled in the low dose group, the metabolic markers of MCI57.2% can be recalled in the middle dose group, and 64.3% of the metabolic markers can be recalled in the high dose group, while there are a small number of unique metabolic markers in different doses of SCPE. The results showed that with the change of dose, the types of MCI metabolic markers changed to a certain extent, and the effect of high dose was the most obvious. The analysis of the reason may be related to the effect of drug concentration on the upstream and downstream relationship of metabolites in the metabolic pathway.

Analysis of metabolic pathway

The common and unique metabolic markers of the three groups were enriched and analyzed to make clear the action pathway of the common metabolic markers of each dose of SCPE callback MCI and the significance of the specific metabolic markers under different doses. The metabolic mechanism of different doses was analyzed to explore the relationship between metabolic pathways.

As can be seen from Figure 22-A, compared with the model group, the metabolic markers of each dose group were significantly adjusted. It is suggested that common metabolic markers play an important role in the process of SCPE intervention in MCI.

As can be seen from Figure 22-B, red represents positive correlation and blue represents negative correlation. Comprehensive analysis showed that there was a strong positive correlation between Hexadecanedioic acid and (S)-2-(Hydroxymethyl)glutarate, Angiotensin, 5-Hydroxyindoleacetate, L-Glutamic acid, D-Phenylalanine, Toluene, L-Leucine, Isoproterenol, Leukotriene D4, Methdilazine, o-Cresol, trans-Cinnamic acid, L-Isoleucine, L-Phenylalanine (R ¿ 0.8); There was a strong negative correlation between 16-Hydroxy hexadecanoic acid and 11Z-Eicosenoic acid, o-Cresol, Coniferyl aldehyde, L-Rhamnulose, N-Acetyl-L-histidine, D-Phenylalanine, trans-Cinnamic acid, Toluene, Hexadecanedioic acid, L-Isoleucine, L-Phenylalanine (R¡-0.80). It is suggested that cetyldicarboxylic acid and 16-hydroxyhexadecanoic acid play an important role in the pathogenesis of MCI and the improvement of cognitive impairment by SCPE. It is found that[22], hexadecanoic acid and 16-hydroxyhexadecanoic acid are important substances involved in fatty acid metabolism. However, abnormal fatty acid metabolism is gradually regarded as an important factor leading to cognitive impairment, but the research on fatty acid spectrum of cognitive disorders is not clear at present[23]. This part of the study provides two important metabolic markers to reveal the pathogenesis of MCI and the improvement of SCPE from the perspective of fatty acid metabolism.

As can be seen from Figure 22-C, three candidate pathways with Impact greater than 0.1 were obtained by MetPA analysis of 36 metabolic markers, which are: Phenylalanine metabolism; Alanine, aspartate and glutamate metabolism; D-Glutamine and D-glutamate metabolism.

As can be seen from Figure 22-D, 4 candidate pathways with Impact greater than 0.1 were obtained by MetPA analysis of 40 metabolic markers of LD callback: Phenylalanine metabolism; Alanine, aspartate and glutamate metabolism; Valine, leucine and isoleucine biosynthesis; D-Glutamine and D-glutamate metabolism.

As can be seen from Figure 22-E, 4 candidate pathways with Impact greater than 0.1 were obtained by MetPA analysis of 48 metabolic markers of MD callback: Phenylalanine metabolism; Alanine, aspartate and glutamate metabolism; Cysteine and methionine metabolism; D-Glutamine and D-glutamate metabolism.

As can be seen from Figure 22-F, 4 candidate pathways with Impact greater than 0.1 were obtained by MetPA analysis of 54 metabolic markers of HD callback: Phenylalanine metabolism; Alanine, aspartate and glutamate metabolism; Cysteine and methionine metabolism; D-Glutamine and D-glutamate metabolism.

It can be seen from Figure 22-C∼F that 3 of the four main metabolic pathways did not change significantly with the increase of dose, suggesting that phenylalanine, alanine, aspartic acid and glutamate did not change significantly, and the main metabolites of D-glutamine and D-glutamate did not change significantly, indicating that the above three metabolic pathways are the main metabolic pathways of MCI and SCPE intervention in MCI.

Phenylalanine metabolism is a metabolic pathway closely related to cognitive impairment. Phenylalanine is an essential amino acid in the human body, which

metabolizes to tyrosine in the liver tissue, and then participates in the synthesis of brain neurotransmitters such as dopamine(DA), 5-hydroxytryptamine (5-HT) and norepinephrine (NE)[24]. A variety of factors can lead to abnormal metabolism of phenylalanine, including cerebrovascular diseases, neuroimmune diseases, genetic diseases and other factors, leading to the disturbance of tyrosine production and the increase of phenylalanine / tyrosine ratio in blood[25]. Studies have found that high levels of phenylalanine or phenylalanine / tyrosine will destroy the activity of Na+-K+-ATP on the synaptic serosa, resulting in cognitive impairment, mental retardation, depression and other diseases[26].

Alanine, aspartic acid and glutamic acid are non-essential amino acids in human body, and this metabolic pathway is mainly involved in aerobic glycolysis and tricarboxylic acid cycle[27]. Among them, alanine metabolism is closely related to sugar metabolism. Glycolysis provides pyruvate for the biosynthesis of alanine, which is then formed by combined deamination or transamination. In addition, as another source of non-essential amino acids, alanine is the product of tryptophan metabolism. Aspartic acid is the precursor of α-ketoglutaric acid, and glutamic acid is the precursor of oxaloacetic acid, which is mainly involved in aerobic glycolysis and tricarboxylic acid cycle, and is closely related to the uptake of free glucose by brain tissue to maintain normal brain metabolism[28, 29, 30].

The metabolism of D-glutamine and D-glutamate is closely related to aging, and the metabolic level of D-glutamine and D-glutamate will be disordered in the aging body[31]. Among them, D-glutamate is an important neurotransmitter in the nervous system, which is mainly involved in the regulation of human memory, emotion and intelligence. However, the study found that[32], high levels of glutamate can induce neurotoxicity, brain tissue degeneration, intellectual and emotional disorders and so on. Glutamine can form glutamic acid with perdeamination.

As can be seen from Figure 22 and 23, the metabolic pathway with Impact greater than 0.1 changed from the biosynthesis of valine, leucine and isoleucine in low dose to the metabolic pathway of cysteine and methionine in middle and high dose. The reason may be related to the biosynthesis of aminoacyl tRNA enriched by the common metabolites.

Aminoacyl refers to the univalent group left after the hydroxyl group of amino acid molecule is removed. tRNA mainly performs the translation function from DNA to protein. The process of tRNA carrying certain amino acids to ribosomes needs to be completed by a variety of aminoacyl-tRNA synthetases[33, 34, 35]. Aminoacyl-tRNA synthesis mainly affects the synthesis of a variety of proteins, thus affecting the secretion of neurotransmitters in brain tissue, the synthesis of related metabolic enzymes, the synthesis of iconic proteins and neuronal regeneration. It has been found that the metabolism of valine, leucine and isoleucine is closely related to aminoacyl tRNA, which further affects the biological metabolism of related amino acids and proteins in human body. at present, this metabolic pathway has been proved to be related to the pathogenesis of hypertension and coronary heart disease[36, 37, 38]. Aminoacyl-tRNA is also an important participant in the metabolism of cysteine and methionine, which mainly affects the production of blood homocysteine. It has been found that homocysteine is the main risk factor affecting the occurrence and development of nervous system diseases such

as atherosclerosis, hypertension, dementia and so on[39]. And homocysteine can be catalyzed by cystathionine synthetase, using vitamin B6 as coenzyme, homocysteine condenses with serine to form L-cystathionine, and then produces cysteine and α-ketobutyric acid. α-ketobutyric acid is then metabolized by tricarboxylic acid to produce glucose for brain metabolism, affecting cognitive function[40].

Conclusions

In this study, 84 MCI-related biomarkers and 8 metabolic pathways (D-arginine and D-ornithine metabolism; alanine, aspartic acid and glutamate metabolism; phenylalanine metabolism; arginine and proline metabolism; valine, leucine and isoleucine biosynthesis; cysteine and methionine metabolism; aminoacyl tRNA biosynthesis; D-glutamine and D-glutamate metabolism). The mechanism of SCPE intervention on brain metabolism of MCI was discussed with low, middle and high doses. It was found that different doses of SCPE could call back 36 same metabolic markers and 3 common metabolic pathways (phenylalanine metabolism; alanine, aspartic acid and glutamate metabolism; D-glutamine and D-glutamate metabolism). And with the increase of dose, the number of SCPE callback metabolic markers gradually increased (LD:40; MD:48; HD:54), and the types of main metabolic pathways also changed to a certain extent (the biosynthesis of valine, leucine and isoleucine in low dose changed to cysteine and methionine metabolic pathway in middle and high dose) which may be related to the effect of aminoacyl tRNA. The results above indicate the stability and reliability of 7-month-old SAMP8 mice as a MCI model, and demonstrate the effectiveness of SCPE in the treatment of MCI. Compared with the MD and LD groups, the results showed that the high dose SCPE group has more advantages.

To sum up, this study is of great significance for in-depth understanding of brain metabolomics in mice with MCI and exploring the metabolic mechanism of SCPE in the intervention of MCI. It also provides a new strategy for further understanding the therapeutic mechanism of traditional Chinese medicine.

Abbreviations

The following abbreviations are used in this manuscript:

Aβ	β-amyloid
Aβ	β-amyloid
AD	Alzheimer's disease
Cont	Blank group
FC	Fold change
FC analysis	Fold change analysis
HD	High dose group
LD	Low dose group
MCI	Mild cognitive impairment
MD	Medium dose group
Mean±SD	Mean ±standard deviation
Mod	Model group
PCA	Principal Component Analysis
QC-RSC	Quality control–based robust LOESS signal correction
SAMP8	Senescence-accelerated P8
SCPE	Sagacious confucius' pillow elixir

Ethics approval and consent to participate
The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Ethics Committee of Heilongjiang University of Traditional Chinese Medicine.

Consent for publication
Not applicable.

Availability of data and materials
The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Author's contributions
Designed the experiments: JC, ZS and HS. Performed the experiments: ZH, MW, TM, YZ, LS, and XS. Analyzed the data and revised the manuscript: CH, QW, YL, AS and ZH. All authors discussed the results and commented on the manuscript. All authors approved the final version of manuscript for submission.

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Figures

Figure 1 PCA score plots in the mode of positive and negative ions.

Figure 2 PLS-DA score plots of model analysing.

Figure 3 Volcano plot of differential ion.

Figure 4 VIP map and Heat map of differential ion clustering analysis.

Figure 5 Changes of metabolic markers in the control group and the model group under the positive ion mode. Compared with the blank group, **P-value ¡ 0.01.

Tables

Figure 6 Changes of metabolic markers in the control group and the model group under the negitive ion mode. Compared with the blank group, **P-value ¡ 0.01.

Figure 7 Heat map of differential metabolites cluster analysis under the positive ion mode.

Figure 8 Heat map of differential metabolites cluster analysis under the negitive ion mode.

Figure 9 Metabolic pathway analysis of MCI biomarkers.

Figure 10 Metabolic pathway enrichment analysis of MCI biomarkers.

Figure 11 Metabolic pathway network diagram of MCI mice.

Figure 12 Metabolic biomarkers of MCI intervented by low dose of SCPE. (A) low dose (LD) SCPE intervention MCI biomarker intensity histogram, compared with the model group (Mod), *P-value ¡ 0.05; **P-value ¡ 0.01; (B) biomarker heat map. The color is proportional to the difference in metabolites (red: up-regulated; blue: down-regulated).

Figure 13 Pathway analysis of biomarkers for MCI intervention with low dose of SCPE.

Figure 14 Metabolic pathway enrichment analysis of MCI biomarkers intervened by SCPE.

Figure 15 Metabolic biomarkers of MCI intervented by medium dose of SCPE. (A) Histogram of biomarker intensity of medium dose (MD) intervention in MCI, compared with the model group (Mod) *P-value ¡ 0.05; **P-value ¡ 0.01; (B)Heat map of biomarkers (red: up-regulated; blue: down-regulated).

Figure 16 Pathway analysis of biomarkers for MCI intervention with medium dose of SCPE.

Figure 17 Metabolic pathway enrichment analysis of MCI biomarkers intervened by SCPE.

Figure 18 Metabolic biomarkers of MCI intervented by high dose of SCPE. (A) Bar chart of metabolic intensity of biomarkers of MCI treated with high dose (HD), compared with the model group (Mod), *P-value ¡ 0.05; **P-value ¡ 0.01; (B) Heat map of biomarkers (red: up-regulated; blue: down-regulated).

Figure 19 Pathway analysis of biomarkers for MCI intervention with high dose of SCPE.

Figure 20 Metabolic pathway enrichment analysis of MCI biomarkers intervened by SCPE.

Figure 21 Venn diagram of metabolic markers in different doses of SCPE. (A) Model group (Model), Low dose group (LD), Medium dose group (MD), High dose group (HD) Venn diagram; (B) Low dose group (LD), Medium dose group (MD), High dose group (HD) Venn diagram.

Figure 22 Combination diagram of metabolites analysis. (A) Thermograms of 36 metabolic markers in common callback of low, medium and high dose SCPE; (B) Pearson correlation analysis of 36 metabolic markers in the common callback of low, middle and high dose SCPE; (C) The pathway map of 36 metabolic markers for the common callback of low, medium and high dose SCPE; (D) Pathway map of 40 metabolic markers in low dose SCPE callback; (E) The pathway map of 48 metabolic markers for medium dose SCPE callback; (F) The pathway map of 54 metabolic markers for high dose SCPE callback.

Figure 23 Metabolic pathway analysis.

Table 1 Results of differential ions screening.

Mode	Group	Diff ion number	Up	Down
pos	Cont vs Mod	313	151	162
neg	Cont vs Mod	356	151	205

Table 2 Identification table of differential ions.

Mode	Group	Diff ion number	Up (MS)	Down (MS)	Up (MS2)	Down (MS2)
pos	Cont vs Mod	302	114	77	64	35
neg	Cont vs Mod	169	88	21	47	13

Table 3: 84 major metabolic markers associated with mild cognitive impairment identified by UHPLC-Q-TOF/MS (positive/negative ion mode).

Mode	No.	Metabolite	Formula	m/z	RT.min.	Adducts	ratio	VIP	ID.1	KEGG.ID	Trend
pos	1	Angiotensin	$C_{30}H_{48}N_8O_9$	647.4	5.26775	M+H-H2O	61.112	5.5206	C15852	C15852	↑
	2	Leukotriene D4	$C_{25}H_{40}N_2O_6S$	514.3	5.645633	M+NH4	28.983	5.0035	HMDB0003080	C05951	↑
	3	Hexadecanedioic acid	$C_{16}H_{30}O_4$	309.2	5.53135	M+Na	14.281	4.2154	HMDB0000672	C19615	↑
	4	(3Z,6Z)-3,6-Nonadienal	$C_9H_{14}O$	139.1	6.216	M+H	12.832	4.1182	HMDB0031152	C16323	↑
	5	Isoproterenol	$C_{11}H_{17}NO_3$	229.2	3.67795	M+NH4	10.563	3.6591	HMDB0015197	C07056	↑
	6	D-Ornithine	$C_5H_{12}N_2O_2$	115.1	0.64785	M+H-H2O	9.8904	3.6995	C00515	C00515	↑
	7	Methoxamine	$C_{11}H_{17}NO_3$	229.2	4.0559	M+NH4	8.4959	3.4573	HMDB0014861	C07513	↑
	8	Citrulline	$C_6H_{13}N_3O_3$	214.1	0.64785	M+K	8.3406	3.6848	HMDB0000904	C00327	↑
	9	Coniferyl aldehyde	$C_{10}H_{10}O_3$	201.1	0.740017	M+Na	8.207	3.2201	C02666	C02666	↑
	10	Isopropylmaleate	$C_7H_{10}O_4$	176.1	0.825733	M+NH4	6.6125	3.1223	HMDB0012241	C02631	↑
	11	Porphobilinogen	$C_{10}H_{14}N_2O_4$	244.1	0.725733	M+NH4	4.9492	3.1194	C00931	C00931	↑
	12	11Z-Eicosenoic acid	$C_{20}H_{38}O_2$	311.3	10.8873	M+H	4.33	2.8575	HMDB0002231	C16526	↑
	13	Piperidine	$C_5H_{11}N$	86.1	0.825733	M+H	4.1153	2.8547	HMDB0034301	C01746	↑
	14	L-Isoleucine	$C_6H_{13}NO_2$	132.1	2.180333	M+H	4.0919	2.7451	HMDB0000172	C00407	↑
	15	D-Phenylalanine	$C_9H_{11}NO_2$	148.1	3.9559	M+H-H2O	4.022	2.5231	C02265	C02265	↑
	16	Topotecan	$C_{23}H_{23}N_3O_5$	444.2	0.840017	M+Na	3.9278	2.538	HMDB0015164	C11158	↑
	17	Toluene	C_7H_8	93.07	3.6923	M+H	3.6764	2.5385	HMDB0034168	C01455	↑
	18	2-Phenylacetamide	C_8H_9NO	136.1	0.825733	M+H	3.5094	2.5669	HMDB0010715	C02505	↑
	19	L-Glutamic acid	$C_5H_9NO_4$	148.1	2.308917	M+H	3.465	2.3271	HMDB0000148	C00025	↑
	20	4-Hydroxycinnamic acid	$C_9H_8O_3$	182.1	1.931017	M+NH4	3.2223	2.3325	HMDB0002035	C00811	↑
	21	Erucic acid	$C_{22}H_{42}O_2$	356.4	8.41255	M+NH4	3.0437	2.6268	HMDB0002068	C08316	↑
	22	Aminocaproic acid	$C_6H_{13}NO_2$	132.1	0.825733	M+H	2.8944	2.3466	HMDB0001901	C02378	↑
	23	1-Phenyl-1,2-propanedione	$C_9H_8O_2$	166.1	1.3322	M+NH4	2.8934	2.2699	C17268	C17268	↑
	24	trans-Cinnamic acid	$C_9H_8O_2$	166.1	3.67795	M+NH4	2.7338	2.2063	C00423	C00423	↑
	25	Eicosadienoic acid	$C_{20}H_{36}O_2$	331.3	9.264467	M+Na	2.6525	2.284	HMDB0005060	C16525	↑
	26	Norepinephrine	$C_8H_{11}NO_3$	187.1	0.711433	M+NH4	2.428	1.9473	HMDB0000216	C00547	↑
	27	Enol-phenylpyruvate	$C_9H_8NO_3$	182.1	0.825733	M+NH4	2.2705	1.8435	HMDB0012225	C02763	↑
	28	Phenylacetaldehyde	C_8H_8O	121.1	0.825733	M+H	1.9547	1.6372	HMDB0006236	C00601	↑
	29	o-Cresol	C_7H_8O	91.06	3.6923	M+H-H2O	1.7744	1.483	HMDB0002055	C01542	↑
	30	L-Cystathionine	$C_7H_{14}N_2O_4S$	205.1	0.825733	M+H-H2O	1.69	1.0319	HMDB0000099	C02291	↑
	31	5-Hydroxy-L-tryptophan	$C_{11}H_{12}N_2O_3$	203.1	0.825733	M+H-H2O	1.6491	1.4414	HMDB0000472	C00643	↑
	32	L-Carnitine	$C_7H_{15}NO_3$	162.1	0.633567	M+H	1.4357	1.389	HMDB0000062	C00318	↑
	33	4-Hydroxyifosfamide	$C_7H_{15}Cl_2N_2O_3P$	259	5.559917	M+H-H2O	0.7513	1.1344	C16553	C16553	↓
	34	Alpha-Tocotrienol	$C_{29}H_{44}O_2$	425.3	11.16025	M+H	0.6676	1.1892	HMDB0006327	C14153	↓
	35	Allyl isothiocyanate	C_4H_5NS	100	0.825733	M+H	0.6049	1.4158	HMDB0005843	C19317	↓
	36	L-Aspartic acid	$C_4H_7NO_4$	134	0.81145	M+H	0.582	1.814	HMDB0000191	C00049	↓

Mode	No.	Metabolite	Formula	m/z	RT.min.	Adducts	ratio	VIP	ID.1	KEGG.ID	Trend
	37	2-Aminoacrylic acid	$C_3H_5NO_2$	88.04	0.825733	M+H	0.5131	2.0989	HMDB0003609	C02218	↓
	38	Mannitol 1-phosphate	$C_6H_{15}O_9P$	245	0.66215	M+H-H2O	0.4915	1.6516	HMDB0001530	C00644	↓
	39	Boldione	$C_{19}H_{24}O_2$	307.2	5.46775	M+Na	0.4217	1.7867	HMDB0003422	C20144	↓
	40	N-Acetyl-L-aspartic acid	$C_6H_9NO_5$	158	0.81145	M+H-H2O	0.3781	2.3555	HMDB0000812	C01042	↓
	41	Episterol	$C_{28}H_{46}O$	381.4	11.68417	M+H-H2O	0.3379	2.0687	HMDB0006847	C15777	↓
	42	Cytosine	$C_4H_5N_3O$	112.1	0.725733	M+H	0.3143	2.7065	C00380	C00380	↓
	43	2-Arachidonylglycerol	$C_{23}H_{38}O_4$	396.3	7.627967	M+NH4	0.1923	2.7597	HMDB0004666	C13856	↓
	44	LysoSM(d18:1)	$C_{23}H_{50}N_2O_5P$	466.4	7.93465	M+H	0.0376	4.7106	HMDB0006482	C03640	↓
	45	Taurochenodesoxycholic acid	$C_{26}H_{45}NO_6S$	517.3	6.565317	M+NH4	0.035	4.4026	HMDB0000951	C05465	↓
neg	46	L-Rhamnulose	$C_6H_{12}O_5$	199	0.725683	M+Cl	50.831	5.8453	C00861	C00861	↑
	47	N-Acetyl-L-histidine	$C_8H_{11}N_3O_3$	196.1	0.725683	M-H	31.585	5.2531	C02997	C02997	↑
	48	Diosmin	$C_{28}H_{32}O_{15}$	607.2	0.8257	M-H	13.039	4.806	C10039	C10039	↑
	49	Argininic acid	$C_6H_{13}N_3O_3$	174.1	0.647817	M-H	6.585	3.6599	HMDB0003148	null	↑
	50	Ornithine	$C_5H_{12}N_2O_2$	131.1	0.647817	M-H	5.2992	3.4788	C01602	C01602	↑
	51	2-Isopropylmalic acid	$C_7H_{12}O_5$	211	0.61925	M+Cl	5.1237	3.422	HMDB0000402	C02504	↑
	52	L-Leucine	$C_6H_{13}NO_2$	130.1	2.108883	M-H	4.9642	3.2231	HMDB0000687	C00123	↑
	53	gamma-Glutamyl-Se-methyl- selenocysteine	$C_9H_{16}N_2O_5Se$	311	3.692283	M-H	4.3956	3.126	C05695	C05695	↑
	54	Xylobiose	$C_{10}H_{18}O_9$	281.1	0.576383	M-H	4.3586	3.1162	C01630	C01630	↑
	55	L-Norleucine	$C_6H_{13}NO_2$	130.1	0.839983	M-H	4.2301	3.2557	HMDB0001645	C01933	↑
	56	beta-Tyrosine	$C_9H_{11}NO_3$	180.1	0.839983	M-H	3.9327	2.4783	C04368	C04368	↑
	57	3-Hydroxy-4-hydroxymethyl-2-methyl- pyridine-5-carboxylate	$C_8H_9NO_4$	182	0.8257	M-H	3.5571	2.6123	C04773	C04773	↑
	58	Phenoxybenzamine	$C_{18}H_{22}ClNO$	302.1	0.839983	M-H	3.5439	2.6334	HMDB0015061	C07435	↑
	59	4-Hydroxy-4-(3-pyridyl)-butanoic acid	$C_9H_{11}O_3$	180.1	1.945267	M-H	3.5409	2.7072	HMDB0001119	C19579	↑
	60	L-Phenylalanine	$C_9H_{11}NO_2$	164.1	3.678	M-H	3.4942	2.8079	HMDB0000159	C00079	↑
	61	Benzocaine	$C_9H_{11}NO_2$	164.1	1.9167	M-H	3.4863	2.1073	C07527	C07527	↑
	62	L-Valine	$C_5H_{11}NO_2$	116.1	0.76855	M-H	3.4154	2.9604	C00183	C00183	↑
	63	N6-Acetyl-L-lysine	$C_8H_{16}N_2O_3$	223.1	0.8257	M+Cl	3.378	2.6149	HMDB0000206	C02727	↑
	64	5-Hydroxyindoleacetate	$C_{10}H_9NO_3$	190.1	4.319617	M-H	3.2636	2.9245	C05635	C05635	↑
	65	L-Asparagine	$C_4H_8N_2O_3$	131	0.590667	M-H	2.5767	2.5534	HMDB0000168	C00152	↑
	66	(S)-2-(Hydroxymethyl)glutarate	$C_6H_{10}O_5$	161	0.839983	M-H	2.4021	2.6044	C16390	C16390	↑
	67	Paraquat dichloride	$C_{12}H_{14}Cl_2N_2$	255	0.8257	M-H	2.0974	2.3268	C00225	C00225	↑
	68	Oxypurinol	$C_5H_4N_4O_2$	151	0.8257	M-H	2.0833	1.4448	HMDB0000786	C07599	↑
	69	Methdilazine	$C_{18}H_{20}N_2S$	331.1	7.278317	M+Cl	1.9345	1.5244	C07175	C07175	↑
	70	5-(4-Acetoxy-1-butynyl)-2,2'-bithiophene	$C_{14}H_{12}O_2S_2$	275	0.725683	M-H	1.8312	1.4485	HMDB0034454	C04485	↑
	71	Gibberellin A15 open lactone	$C_{20}H_{28}O_5$	347.2	8.062867	M-H	1.7504	1.3783	C11860	C11860	↑
	72	N1-Acetylspermine	$C_{12}H_{28}N_4O$	279.2	8.062867	M+Cl	1.7265	1.2824	HMDB0001186	C02567	↑
	73	Thorium-232	Th	231	0.811417	M-H	1.6498	1.4663	C19157	C19157	↑

Mode	No.	Metabolite	Formula	m/z	RT.min.	Adducts	ratio	VIP	ID.1	KEGG.ID	Trend
	74	L-Malic acid	$C_4H_6O_5$	133	0.7114	M-H	1.5318	1.266	HMDB0000156	C00149	↑
	75	Triamcinolone diacetate	$C_{25}H_{31}FO_8$	477.2	10.08828	M-H	1.4132	1.5431	C08184	C08184	↑
	76	Telmisartan	$C_{33}H_{30}N_4O_2$	513.2	10.08828	M-H	1.4056	1.5119	HMDB0015101	C07710	↑
	77	16-Hydroxy hexadecanoic acid	$C_{16}H_{32}O_3$	271.2	9.317567	M-H	0.7498	1.0048	HMDB0006294	C18218	↓
	78	Ricinoleic acid	$C_{18}H_{34}O_3$	297.2	8.733233	M-H	0.7007	1.2711	C08365	C08365	↓
	79	L-Arabinose	$C_5H_{10}O_5$	185	0.7114	M+Cl	0.6415	1.4298	HMDB0000646	C00259	↓
	80	Panaxytriol	$C_{17}H_{26}O_3$	277.2	7.962867	M-H	0.5289	1.735	C16792	C16792	↓
	81	3-Deoxy-D-manno-octulosonate 8-phosphate	$C_8H_{15}O_{11}P$	353	0.7114	M+Cl	0.4685	2.4592	C04478	C04478	↓
	82	(2'E,4'Z,8E)-Colneleic acid	$C_{18}H_{30}O_3$	293.2	8.790383	M-H	0.2308	4.0805	HMDB0030995	C19827	↓
	83	GDP-glucose	$C_{16}H_{25}N_5O_{16}P_2$	604.1	0.839983	M-H	0.2206	2.9012	C00394	C00394	↓
	84	Juvenile hormone III	$C_{16}H_{26}O_3$	265.2	7.92	M-H	0.1109	4.5665	C09694	C09694	↓

Note: ↑ represents up-regulation; ↓ represents down-regulation.

Table 4: Information of metabolic pathways.

No.	Pathway Name	Total	Hits	Expected	Raw p	-log(p)	Holm adjust	FDR	Impact
1	D-Arginine and D-ornithine metabolism	8	2	0.26921	0.02746	3.5952	1	0.36607	0.50000
2	Alanine, aspartate and glutamate metabolism	24	4	0.80764	0.00757	4.8837	0.59035	0.17868	0.48718
3	Phenylalanine metabolism	45	7	1.5143	0.00061	7.4062	0.048597	0.04860	0.35177
4	Arginine and proline metabolism	77	4	2.5912	0.25891	1.3513	1	1	0.18535
5	Valine, leucine and isoleucine biosynthesis	27	5	0.9086	0.00172	6.3662	0.13577	0.06875	0.15898
6	Cysteine and methionine metabolism	56	3	1.8845	0.29104	1.2343	1	1	0.13505
7	Aminoacyl-tRNA biosynthesis	75	7	2.5239	0.01177	4.4418	0.89485	0.18839	0.11268
8	D-Glutamine and D-glutamate metabolism	11	1	0.37017	0.31432	1.1574	1	1	0.11230
9	Drug metabolism - cytochrome P450	86	1	2.8941	0.95012	0.05117	1	1	0.04839
10	Nicotinate and nicotinamide metabolism	44	2	1.4807	0.43987	0.82128	1	1	0.04454
11	Citrate cycle (TCA cycle)	20	1	0.67304	0.49711	0.69894	1	1	0.04361
12	Fructose and mannose metabolism	48	2	1.6153	0.48529	0.723	1	1	0.03942
13	Tyrosine metabolism	76	2	2.5575	0.73446	0.30861	1	1	0.03812
14	Ubiquinone and other terpenoid-quinone biosynthesis	36	1	1.2115	0.71106	0.341	1	1	0.03370
15	Vitamin B6 metabolism	32	1	1.0769	0.66801	0.40346	1	1	0.02697
16	Arachidonic acid metabolism	62	1	2.0864	0.88354	0.12382	1	1	0.02550
17	Glyoxylate and dicarboxylate metabolism	50	1	1.6826	0.82263	0.19525	1	1	0.02420
18	Valine, leucine and isoleucine degradation	40	3	1.3461	0.14956	1.9001	1	1	0.02232
19	Pyrimidine metabolism	60	1	2.0191	0.87506	0.13346	1	1	0.02127
20	Tryptophan metabolism	79	1	2.6585	0.93606	0.06607	1	1	0.01143
21	Glutathione metabolism	38	2	1.2788	0.36803	0.9996	1	1	0.01095
22	Primary bile acid biosynthesis	47	1	1.5816	0.80303	0.21936	1	1	0.00992
23	Porphyrin and chlorophyll metabolism	104	2	3.4998	0.87425	0.13439	1	1	0.00954
24	Nitrogen metabolism	39	5	1.3124	0.00893	4.7179	0.68793	0.17868	0.00830
25	Sphingolipid metabolism	25	1	0.8413	0.5769	0.55009	1	1	0.00191
26	Starch and sucrose metabolism	50	1	1.6826	0.82263	0.19525	1	1	0.00149
27	Phenylalanine, tyrosine and tryptophan biosynthesis	27	1	0.9086	0.60519	0.50221	1	1	0.00062
28	Histidine metabolism	44	2	1.4807	0.43987	0.82128	1	1	0.00051
29	Amino sugar and nucleotide sugar metabolism	88	2	2.9614	0.80562	0.21614	1	1	0.00007
30	Cyanoamino acid metabolism	16	2	0.53843	0.099	2.3127	1	1	0
31	Pantothenate and CoA biosynthesis	27	2	0.9086	0.22978	1.4706	1	1	0
32	Pyruvate metabolism	32	2	1.0769	0.29305	1.2274	1	1	0
33	Glycine, serine and threonine metabolism	48	2	1.6153	0.48529	0.723	1	1	0
34	Selenoamino acid metabolism	22	1	0.74034	0.53067	0.63362	1	1	0
35	beta-Alanine metabolism	28	1	0.94225	0.61863	0.48025	1	1	0
36	alpha-Linolenic acid metabolism	29	1	0.9759	0.63161	0.45948	1	1	0
37	Lysine biosynthesis	32	1	1.0769	0.66801	0.40346	1	1	0

No.	Pathway Name	Total	Hits	Expected	Raw p	-log(p)	Holm adjust	FDR	Impact
38	Propanoate metabolism	35	1	1.1778	0.70084	0.35547	1	1	0
39	Butanoate metabolism	40	1	1.3461	0.74859	0.28957	1	1	0
40	Ascorbate and aldarate metabolism	45	1	1.5143	0.78879	0.23725	1	1	0
41	Lysine degradation	47	1	1.5816	0.80303	0.21936	1	1	0
42	Pentose and glucuronate interconversions	53	1	1.7835	0.8403	0.17399	1	1	0

Note: the analysis results of pathway analysis are shown in detail in the Table 4. Total/Hits is the number of metabolites enriched / the number of all metabolites in the pathway, Holmadjust is the p value corrected by HB method, FDR is the p value corrected by False Discovery Rate, and Impact is the pathway impact value obtained by pathway topology analysis. In order to more intuitively reflect the importance of participating in MCI metabolic pathway, this part uses Impact as a factor to enrich and analyze each pathway, in which blue represents the main candidate metabolic pathway, green is the related metabolic pathway, the specific enrichment situation is shown in Figure 10.

Table 5 List of biomarkers of metabolism for MCI intervened by low dose of SCPE.

Mode	No.	Metabolite	P-value
pos	1	Hexadecanedioic acid	9.98554E-05
	2	Eicosadienoic acid	0.000206941
	3	L-Isoleucine	0.002584405
	4	Leukotriene D4	0.002641808
	5	11Z-Eicosenoic acid	0.002793092
	6	D-Phenylalanine	0.003602312
	7	Isopropylmaleate	0.003611113
	8	trans-Cinnamic acid	0.006786435
	9	Methoxamine	0.010427954
	10	(3Z,6Z)-3,6-Nonadienal	0.011802975
	11	Isoproterenol	0.012203182
	12	Toluene	0.01258429
	13	o-Cresol	0.013207123
	14	Angiotensin	0.014740774
	15	Boldione	0.017072285
	16	2-Arachidonylglycerol	0.020932654
	17	Coniferyl aldehyde	0.022031139
	18	4-Hydroxyifosfamide	0.025223664
	19	L-Glutamic acid	0.025331093
	20	LysoSM(d18:1)	0.033718971
	21	Episterol	0.037604253
	22	Taurochenodesoxycholic acid	0.049833646
neg	23	N1-Acetylspermine	0.004099225
	24	L-Phenylalanine	0.004299337
	25	Gibberellin A15 open lactone	0.006128052
	26	L-Leucine	0.010258797
	27	16-Hydroxy hexadecanoic acid	0.011032448
	28	Telmisartan	0.016892508
	29	5-Hydroxyindoleacetate	0.017305416
	30	L-Rhamnulose	0.019094889
	31	Panaxytriol	0.020138285
	32	2-Isopropylmalic acid	0.022017423
	33	gamma-Glutamyl-Se-methylselenocysteine	0.025697097
	34	N-Acetyl-L-histidine	0.026415795
	35	Paraquat dichloride	0.029581984
	36	(S)-2-(Hydroxymethyl)glutarate	0.033449195
	37	(2'E,4'Z,8E)-Colneleic acid	0.041817992
	38	Juvenile hormone III	0.04439326
	39	Ricinoleic acid	0.044670352
	40	Methdilazine	0.046513563

Table 6: Information of metabolic pathways.

No.	Pathway Name	Total	Hits	Expected	Raw p	-log(p)	Holm adjust	FDR	Impact
1	Phenylalanine metabolism	45	3	0.74782	0.03727	3.2896	1	0.99387	0.29159
2	Alanine, aspartate and glutamate metabolism	24	1	0.39884	0.33245	1.1013	1	1	0.17664
3	Valine, leucine and isoleucine biosynthesis	27	4	0.44869	0.0008717	7.0451	0.06974	0.06974	0.14573
4	D-Glutamine and D-glutamate metabolism	11	1	0.1828	0.16867	1.7798	1	1	0.1123
5	Aminoacyl-tRNA biosynthesis	75	4	1.2464	0.034037	3.3803	1	0.99387	0.05634
6	Drug metabolism - cytochrome P450	86	1	1.4292	0.76949	0.26203	1	1	0.04839
7	Nicotinate and nicotinamide metabolism	44	1	0.7312	0.52482	0.64469	1	1	0.04454
8	Arginine and proline metabolism	77	1	1.2796	0.73054	0.31397	1	1	0.03582
9	Fructose and mannose metabolism	48	1	0.79767	0.55621	0.58661	1	1	0.03029
10	Arachidonic acid metabolism	62	1	1.0303	0.65093	0.42935	1	1	0.0255
11	Valine, leucine and isoleucine degradation	40	2	0.66473	0.14164	1.9545	1	1	0.02232
12	Tryptophan metabolism	79	1	1.3128	0.73972	0.30149	1	1	0.01143
13	Glutathione metabolism	38	1	0.63149	0.47365	0.74729	1	1	0.01095
14	Primary bile acid biosynthesis	47	1	0.78106	0.54856	0.60046	1	1	0.00992
15	Sphingolipid metabolism	25	1	0.41545	0.34365	1.0681	1	1	0.00191
16	Phenylalanine, tyrosine and tryptophan biosynthesis	27	1	0.44869	0.36552	1.0064	1	1	0.00062
17	Histidine metabolism	44	1	0.7312	0.52482	0.64469	1	1	0.00051
18	Nitrogen metabolism	39	2	0.64811	0.13593	1.9956	1	1	0
19	Selenoamino acid metabolism	22	1	0.3656	0.30948	1.1729	1	1	0
20	alpha-Linolenic acid metabolism	29	1	0.48193	0.38667	0.95019	1	1	0
21	Pyruvate metabolism	32	1	0.53178	0.41711	0.8744	1	1	0
22	Butanoate metabolism	40	1	0.66473	0.49128	0.71075	1	1	0
23	Porphyrin and chlorophyll metabolism	104	1	1.7283	0.83162	0.18438	1	1	0

Table 7 List of biomarkers of metabolism for MCI intervened by medium dose of SCPE.

Mode	No.	Metabolite	P-value
pos	1	Hexadecanedioic acid	0.000364007
	2	Eicosadienoic acid	0.002506244
	3	L-Isoleucine	0.002960788
	4	L-Carnitine	0.002974809
	5	Leukotriene D4	0.003270639
	6	4-Hydroxyifosfamide	0.00355378
	7	11Z-Eicosenoic acid	0.005266836
	8	Angiotensin	0.007228939
	9	trans-Cinnamic acid	0.007688179
	10	Toluene	0.013619825
	11	Methoxamine	0.015654557
	12	D-Phenylalanine	0.019709825
	13	Episterol	0.019730763
	14	Isoproterenol	0.020552676
	15	4-Hydroxycinnamic acid	0.021222921
	16	2-Arachidonylglycerol	0.023795703
	17	L-Cystathionine	0.025987653
	18	LysoSM(d18:1)	0.026740733
	19	Isopropylmaleate	0.026761377
	20	o-Cresol	0.028705104
	21	Coniferyl aldehyde	0.029970338
	22	(3Z,6Z)-3,6-Nonadienal	0.030424965
	23	L-Glutamic acid	0.034601098
	24	Boldione	0.037633349
	25	Alpha-Tocotrienol	0.043909143
	26	Taurochenodesoxycholic acid	0.046249079
neg	27	L-Phenylalanine	0.000411359
	28	16-Hydroxy hexadecanoic acid	0.002608362
	29	Ricinoleic acid	0.002881789
	30	Gibberellin A15 open lactone	0.004733181
	31	5-Hydroxyindoleacetate	0.00737662
	32	N1-Acetylspermine	0.008810405
	33	Methdilazine	0.009052052
	34	L-Leucine	0.009499488
	35	beta-Tyrosine	0.017598872
	36	Panaxytriol	0.018113519
	37	Triamcinolone diacetate	0.018457432
	38	Benzocaine	0.022313529
	39	L-Norleucine	0.022543089
	40	L-Rhamnulose	0.023579355
	41	4-Hydroxy-4-(3-pyridyl)-butanoic acid	0.025276493
	42	N-Acetyl-L-histidine	0.026863662
	43	Juvenile hormone III	0.031969851
	44	gamma-Glutamyl-Se-methylselenocysteine	0.032963008
	45	(2'E,4'Z,8E)-Colneleic acid	0.039224291
	46	(S)-2-(Hydroxymethyl)glutarate	0.04129192
	47	Paraquat dichloride	0.043638928
	48	Telmisartan	0.045101423

Table 8: Information of metabolic pathways.

No.	Pathway Name	Total	Hits	Expected	Raw p	-log(p)	Holm adjust	FDR	Impact
1	Phenylalanine metabolism	45	4	0.85999	0.009816	4.6238	0.78527	0.55454	0.30352
2	Alanine, aspartate and glutamate metabolism	24	1	0.45866	0.37208	0.98863	1	1	0.17664
3	Cysteine and methionine metabolism	56	1	1.0702	0.66487	0.40817	1	1	0.13277
4	D-Glutamine and D-glutamate metabolism	11	1	0.21022	0.1916	1.6523	1	1	0.1123
5	Valine, leucine and isoleucine biosynthesis	27	3	0.516	0.013864	4.2785	1	0.55454	0.08214
6	Aminoacyl-tRNA biosynthesis	75	4	1.4333	0.052886	2.9396	1	1	0.05634
7	Drug metabolism - cytochrome P450	86	1	1.6435	0.81544	0.20403	1	1	0.04839
8	Nicotinate and nicotinamide metabolism	44	1	0.84088	0.57547	0.55257	1	1	0.04454
9	Arginine and proline metabolism	77	1	1.4715	0.77909	0.24963	1	1	0.03582
10	Ubiquinone and other terpenoid-quinone biosynthesis	36	1	0.68799	0.50332	0.68654	1	1	0.0337
11	Fructose and mannose metabolism	48	1	0.91732	0.60759	0.49825	1	1	0.03029
12	Arachidonic acid metabolism	62	1	1.1849	0.70237	0.35329	1	1	0.0255
13	Valine, leucine and isoleucine degradation	40	2	0.76444	0.17688	1.7323	1	1	0.02232
14	Tryptophan metabolism	79	1	1.5098	0.78773	0.23861	1	1	0.01143
15	Glutathione metabolism	38	1	0.72622	0.52241	0.64931	1	1	0.01095
16	Primary bile acid biosynthesis	47	1	0.89821	0.59979	0.51117	1	1	0.00992
17	Sphingolipid metabolism	25	1	0.47777	0.38421	0.95658	1	1	0.00191
18	Phenylalanine, tyrosine and tryptophan biosynthesis	27	1	0.516	0.40776	0.89706	1	1	0.00062
19	Histidine metabolism	44	1	0.84088	0.57547	0.55257	1	1	0.00051
20	Nitrogen metabolism	39	3	0.74533	0.036948	3.2983	1	0.98527	0
21	Selenoamino acid metabolism	22	1	0.42044	0.34714	1.058	1	1	0
22	alpha-Linolenic acid metabolism	29	1	0.55422	0.43044	0.84294	1	1	0
23	Butanoate metabolism	40	1	0.76444	0.54078	0.61475	1	1	0
24	Glycine, serine and threonine metabolism	48	1	0.91732	0.60759	0.49825	1	1	0
25	Tyrosine metabolism	76	1	1.4524	0.77464	0.25536	1	1	0
26	Porphyrin and chlorophyll metabolism	104	1	1.9875	0.87145	0.1376	1	1	0

Table 9 List of biomarkers of metabolism for MCI intervened by high dose of SCPE.

Mode	No.	Metabolite	P-value
pos	1	Hexadecanedioic acid	0.000229493
	2	L-Carnitine	0.000281269
	3	trans-Cinnamic acid	0.000647719
	4	Piperidine	0.001290075
	5	1-Phenyl-1,2-propanedione	0.001360602
	6	L-Isoleucine	0.001798272
	7	Leukotriene D4	0.0019856
	8	o-Cresol	0.002559176
	9	4-Hydroxycinnamic acid	0.002625843
	10	Isoproterenol	0.004984994
	11	LysoSM(d18:1)	0.005815594
	12	11Z-Eicosenoic acid	0.006075564
	13	(3Z,6Z)-3,6-Nonadienal	0.006386328
	14	Alpha-Tocotrienol	0.006882449
	15	Mannitol 1-phosphate	0.009552884
	16	D-Phenylalanine	0.012434668
	17	Angiotensin	0.013908496
	18	L-Cystathionine	0.014867897
	19	4-Hydroxyifosfamide	0.017142243
	20	Methoxamine	0.022526855
	21	2-Arachidonylglycerol	0.023020948
	22	Toluene	0.024859699
	23	Episterol	0.033378326
	24	Taurochenodesoxycholic acid	0.034556685
	25	Topotecan	0.035930445
	26	Boldione	0.036067574
	27	L-Glutamic acid	0.036760782
	28	2-Aminoacrylic acid	0.044543712
	29	Coniferyl aldehyde	0.048985708
neg	30	L-Phenylalanine	0.001067238
	31	beta-Tyrosine	0.002178563
	32	N1-Acetylspermine	0.003093681
	33	L-Norleucine	0.00402759
	34	2-Isopropylmalic acid	0.005715303
	35	Methdilazine	0.006166321
	36	Gibberellin A15 open lactone	0.006629754
	37	(S)-2-(Hydroxymethyl)glutarate	0.009445376
	38	5-Hydroxyindoleacetate	0.010836668
	39	Benzocaine	0.018071091
	40	Ricinoleic acid	0.025977163
	41	(2'E,4'Z,8E)-Colneleic acid	0.026284967
	42	Phenoxybenzamine	0.026475414
	43	N-Acetyl-L-histidine	0.027232436
	44	Panaxytriol	0.027730829
	45	3-Hydroxy-4-hydroxymethyl-2-methylpyridine-5-carboxylate	0.029693081
	46	Juvenile hormone III	0.036518942
	47	16-Hydroxy hexadecanoic acid	0.037668296
	48	L-Leucine	0.038551263
	49	Triamcinolone diacetate	0.03924337
	50	L-Rhamnulose	0.041703342
	51	GDP-glucose	0.042371144
	52	gamma-Glutamyl-Se-methylselenocysteine	0.04400059
	53	4-Hydroxy-4-(3-pyridyl)-butanoic acid	0.044712702
	54	Telmisartan	0.047739669

Table 10: Information of metabolic pathways.

No.	Pathway Name	Total	Hits	Expected	Raw p	-log(p)	Holm adjust	FDR	Impact
1	Phenylalanine metabolism	45	4	0.99086	0.01602	4.134	1	0.81232	0.30352
2	Alanine, aspartate and glutamate metabolism	24	1	0.52846	0.41548	0.87832	1	1	0.17664
3	Cysteine and methionine metabolism	56	2	1.2331	0.35111	1.0467	1	1	0.13505
4	D-Glutamine and D-glutamate metabolism	11	1	0.24221	0.21763	1.5249	1	1	0.1123
5	Valine, leucine and isoleucine biosynthesis	27	3	0.59452	0.02031	3.8967	1	0.81232	0.09009
6	Aminoacyl-tRNA biosynthesis	75	4	1.6514	0.08078	2.5161	1	1	0.05634
7	Drug metabolism - cytochrome P450	86	1	1.8936	0.8577	0.1535	1	1	0.04839
8	Nicotinate and nicotinamide metabolism	44	1	0.96884	0.62791	0.46536	1	1	0.04454
9	Fructose and mannose metabolism	48	2	1.0569	0.28551	1.2535	1	1	0.03942
10	Arginine and proline metabolism	77	1	1.6955	0.8249	0.1925	1	1	0.03582
11	Ubiquinone and other terpenoid-quinone biosynthesis	36	1	0.79269	0.55403	0.59054	1	1	0.0337
12	Vitamin B6 metabolism	32	1	0.70461	0.51187	0.66969	1	1	0.02697
13	Arachidonic acid metabolism	62	1	1.3652	0.75302	0.28367	1	1	0.0255
14	Valine, leucine and isoleucine degradation	40	2	0.88076	0.21954	1.5162	1	1	0.02232
15	Tryptophan metabolism	79	1	1.7395	0.83278	0.18299	1	1	0.01143
16	Glutathione metabolism	38	1	0.83673	0.57375	0.55557	1	1	0.01095
17	Primary bile acid biosynthesis	47	1	1.0349	0.6524	0.4271	1	1	0.00992
18	Sphingolipid metabolism	25	1	0.55048	0.42848	0.84751	1	1	0.00191
19	Starch and sucrose metabolism	50	1	1.101	0.67531	0.39259	1	1	0.00149
20	Phenylalanine, tyrosine and tryptophan biosynthesis	27	1	0.59452	0.45363	0.79046	1	1	0.00062
21	Histidine metabolism	44	1	0.96884	0.62791	0.46536	1	1	0.00051
22	Nitrogen metabolism	39	3	0.85875	0.05278	2.9416	1	1	0
23	Selenoamino acid metabolism	22	1	0.48442	0.3886	0.9452	1	1	0
24	alpha-Linolenic acid metabolism	29	1	0.63855	0.4777	0.73877	1	1	0
25	Pyruvate metabolism	32	1	0.70461	0.51187	0.66969	1	1	0
26	Butanoate metabolism	40	1	0.88076	0.59261	0.52322	1	1	0
27	Glycine, serine and threonine metabolism	48	1	1.0569	0.66021	0.4152	1	1	0
28	Tyrosine metabolism	76	1	1.6735	0.82082	0.19745	1	1	0
29	Amino sugar and nucleotide sugar metabolism	88	1	1.9377	0.86413	0.14603	1	1	0
30	Porphyrin and chlorophyll metabolism	104	1	2.29	0.90625	0.098435	1	1	0

Table 11 Information of metabolic markers in different doses of SCPE. ∩ stands for intersection.

No.	Metabolite	Existing group	No.	Metabolite	Existing group
1	Hexadecanedioic acid	LD∩MD∩HD	30	gamma-Glutamyl-Se- methylselenocysteine	LD∩MD∩HD
2	L-Isoleucine	LD∩MD∩HD	31	N-Acetyl-L-histidine	LD∩MD∩HD
3	Leukotriene D4	LD∩MD∩HD	32	(S)-2-(Hydroxymethyl) glutarate	LD∩MD∩HD
4	11Z-Eicosenoic acid	LD∩MD∩HD	33	(2'E,4'Z,8E)-Colneleic acid	LD∩MD∩HD
5	D-Phenylalanine	LD∩MD∩HD	34	Juvenile hormone III	LD∩MD∩HD
6	trans-Cinnamic acid	LD∩MD∩HD	35	Ricinoleic acid	LD∩MD∩HD
7	Methoxamine	LD∩MD∩HD	36	Methdilazine	LD∩MD∩HD
8	(3Z,6Z)-3,6-Nonadienal	LD∩MD∩HD	37	Eicosadienoic acid	LD∩MD
9	Isoproterenol	LD∩MD∩HD	38	Isopropylmaleate	LD∩MD
10	Toluene	LD∩MD∩HD	39	Paraquat dichloride	LD∩MD
11	o-Cresol	LD∩MD∩HD	40	2-Isopropylmalic acid	LD∩HD
12	Angiotensin	LD∩MD∩HD	41	L-Carnitine	MD∩HD
13	Boldione	LD∩MD∩HD	42	4-Hydroxycinnamic acid	MD∩HD
14	2-Arachidonylglycerol	LD∩MD∩HD	43	L-Cystathionine	MD∩HD
15	Coniferyl aldehyde	LD∩MD∩HD	44	Alpha-Tocotrienol	MD∩HD
16	4-Hydroxyifosfamide	LD∩MD∩HD	45	beta-Tyrosine	MD∩HD
17	L-Glutamic acid	LD∩MD∩HD	46	Triamcinolone diacetate	MD∩HD
18	LysoSM(d18:1)	LD∩MD∩HD	47	Benzocaine	MD∩HD
19	Episterol	LD∩MD∩HD	48	L-Norleucine	MD∩HD
20	Taurochenodesoxycholic acid	LD∩MD∩HD	49	4-Hydroxy-4-(3-pyridyl) -butanoic acid	MD∩HD
21	N1-Acetylspermine	LD∩MD∩HD	50	Piperidine	HD
22	L-Phenylalanine	LD∩MD∩HD	51	1-Phenyl-1,2-propanedione	HD
23	Gibberellin A15 open lactone	LD∩MD∩HD	52	Mannitol 1-phosphate	HD
24	L-Leucine	LD∩MD∩HD	53	Topotecan	HD
25	16-Hydroxy hexadecanoic acid	LD∩MD∩HD	54	2-Aminoacrylic acid	HD
26	Telmisartan	LD∩MD∩HD	55	Phenoxybenzamine	HD
27	5-Hydroxyindoleacetate	LD∩MD∩HD	56	3-Hydroxy-4-hydroxymethyl-2-methylpyridine-5-carboxylate	HD
28	L-Rhamnulose	LD∩MD∩HD	57	GDP-glucose	HD
29	Panaxytriol	LD∩MD∩HD			