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The effect of acupuncture on depression and its correlation with metabolic alterations
A randomized controlled trial

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Abstract
Background: Electroacupuncture (EA) treatment has antidepressant effect and when patients were treated with EA and antidepressants, the effect could be maintained for a longer time. However, the effect of EA combined with antidepressants based on metabolism is still in the initial observation stage, which requires further research.

Methods: A total of 60 patients with moderate depression were assigned into 2 groups at a ratio of 1:1, the EA group (receiving EA and antidepressants) and the control group (taking antidepressants only) in this randomized controlled pilot trial. The EA treatment was performed 3 times a week for 8 consecutive weeks and then follow up for 4 weeks. The patients' depressive mood was measured by the Hamilton Depression scale (HAMD) at baseline, week 4, week 8 and week 12. Before and after 8-week treatment, morning urine samples from all patients were analyzed by the gas chromatography–mass spectrometry (GC–MS) to find possible metabolic markers of depression and of EA treatment related changes.

Results: Compared with the control group, the EA group showed more significant improvements in depressive symptoms measured by HAMD at week 4 (16.89 ± 5.74 vs 25.58 ± 7.03, $P < .001$), week 8 (9.59 ± 5.13 vs 25.04 ± 7.49, $P < .001$) and week 12 (11.07 ± 6.85 vs 27.25 ± 7.14, $P < .001$). The significant differences in urinary specific metabolites before and after EA treatment were malonic acid (fatty acid biosynthesis), cysteine (glutamate metabolism), glutathione (glutamate metabolism), tryptophan (tryptophan metabolism), proline (glutamate metabolism), and N-acetyl-5-hydroxytryptamine. These metabolites are involved in tryptophan metabolism, glutamate metabolism, and fatty acid biosynthesis.

Conclusion: EA treatment combined with antidepressants is more effective in improving depressive symptoms than antidepressants alone. EA may treat depression by acting on tryptophan metabolism, glutamate metabolism, and fatty acid biosynthesis.

Trial registration: Chinese Clinical Trial Registry: ChiCTR-2000030786.

Abbreviations: AUC = area under the curve, BSTFA = N, O-bis (trimethylsilyl)trifluoroacetamide, CDF = Computable Document Format, CONSORT = Consolidated Standards of Reporting Trials, CRF = case report form, CSV = comma separated values, DNA = deoxyribonucleic acid, DSM-IV = Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, EA = electroacupuncture, GABA = gamma-aminobutyric acid, GC-MA = gas chromatography-mass spectrometry, GSH = glutathione, HAMD = Hamilton depression scale, HMDB = human metabolites database, OPLS-DA = orthogonal partial least squares-discriminate analysis, PCA = principal component analysis, PE = polyethylene, PLS-DA = partial least squares-discriminate analysis, QC = quality control, ROC = receiver operating characteristic, SCFA = short-chain fatty acid, SD = standard deviation, SPSS = Statistic Package for Social Science, TCM = traditional Chinese medicine, TIC = total ion current chromatogram, TMCS = trimethylsilyl chloride, VIP = variable importance of projection.

Keywords: depression, electroacupuncture, GC–MS, urinary metabolite

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1. Introduction

Depression is a common psycho-emotional disease whose marked and persistent symptoms can have a severe negative impact the quality of life of affected patients. The lifetime risk of suffering from depression is as high as from 15% to 17%. A failure to treat depression adequately effects patients both mentally and physically.[1,2] Antidepressants are commonly used in clinic based on the monogramming neurotransmitter hypothesis of depression. Aside from the long-lasting beneficial effects, antidepressants may cause clear unfavorable responses and side effects. These drawbacks can lead to poor treatment compliance. Therefore, it is necessary to find other safe and effective alternatives.

At present, many patients have shown a preference for acupuncture as a primary treatment option for depression. Clinical studies have revealed that electroacupuncture (EA) has a definitive antidepressant effect and can quickly relieve the main symptoms of depression.[3] It can effectively improve systemic physical symptoms such as poor digestion and insomnia through its overall regulatory effect. Acupuncture can effectively improve depressive symptoms compared with control acupuncture,[4] and it may play a unique role in the antidepressant mechanism. Animal experiments also showed that learning, memory, and depressive behaviors of rat samples improved considerably after EA treatment. Thus, the mechanism of acupuncture needs to be studied urgently. In clinical observation, some researchers have noticed that the long-term follow-up investigation effect of acupuncture was similar to that of antidepressants alone, without meaningful improvement for depression patients.[4–6] But when patients were treated with acupuncture and antidepressants, the effect could be maintained for a longer time.[7] Aiming to answer this question, we should consider from a macro perspective, and explore the mechanical differences between the use of acupuncture with antidepressants in the treatment of depression compared with antidepressants alone.

Metabolomics is an enlightening research method in systems biology. Metabolomics refers to the overall spectrum of metabolites in biological samples, such as urine and blood. It is used to study changes to the body's small molecule metabolites when external stimuli (pathological factors and drugs) in the biological system cause stability changes to the body's internal environment. It provides a platform for finding metabolic pathways related to potential biomarkers and therapeutic mechanisms.[8] The researchers selected the body's endogenous small molecule metabolites as the research object and used these to reveal the changes in the overall functional status of the organism and the response to external stimuli. Compared with behavioral research, the conclusions drawn from this are more sensitive and objective.[9] Metabolomics can provide a holistic view of the entire biological system, which is particularly consistent with the overall thinking of traditional Chinese medicine (TCM).[10] It is reported that, urine and blood metabolomics were changed in patients affected with depression.[11] However, the antidepressant effect of acupuncture combined with metabolism targeting drugs is still in the initial observation stage and requires further research. We might be the first group to report the application of the gas chromatography–mass spectrometry (GC–MS) when observing the effect of EA. The impacts of EA on the urine metabolites group were investigated with concurrent study of the improvement of the scores of Hamilton Depression scale (HAMD) and other bio indicators of depression. In this paper, the role of acupuncture in combination with antidepressants is examined through the evaluation of urine metabolomics, which offers a new experimental basis for the study of the mechanism of depression.

2. Material and methods

2.1. Participants

This was a randomized controlled pilot study, aiming to find the effect of EA treatment on depression in a small sample size. A total of 60 patients with moderate depression and unsatisfied with their medication therapy were recruited from the acupuncture department in Shanghai Municipal Hospital of Traditional Chinese Medicine from September 2017 to December 2018 in Shanghai, China.

The inclusion criteria of the participants were as follows:

1. aged 18 to 70 years,
2. met the diagnostic criteria of moderate-severe depression according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV),
3. HAMD scores were between 20 and 35,
4. had no aphasia and intelligence disorder, and can understand the contents of the scale and cooperate with the treatment,
5. did not concurrently participate in any other clinical efficacy evaluation studies of depression,
6. voluntarily agreed and signed a written informed consent form before the trial started.

The exclusion criteria of the participants were as follows:

1. with liver and kidney dysfunction, cardiovascular and cerebrovascular diseases, and any other severe diseases,
2. was previously diagnosed with schizophrenia, bipolar depression, epilepsy, and other psychiatric encephalopathies,
3. with alcoholism and psychoactive substance dependence,
4. currently pregnant or breast-feeding,
5. had taken acupuncture treatment 6 months before the trial.

We followed the Consolidated Standards of Reporting Trials (CONSORT) guideline for designing and reporting the clinical trial part. All participants were recruited after they fully understood the trial, and were asked to sign the consent form to participate in this study. The protocol was approved by the ethics committee of Shanghai Municipal Hospital of Traditional Chinese Medicine (2015SHL-KY-21) and the trial was registered in Chinese Clinical Trial Registry (ChiCTR-2000030786).

2.2. Randomization and blinding

Eligible patients were recruited and assigned into the EA group or the control group at a ratio of 1:1. A random number table was created through operating the statistical software Statistic Package for Social Science (SPSS) 21.0. The random numbers were sealed in opaque envelopes with continuous numbers written outside. The envelopes were arranged in the order of screening. An independent researcher responsible for the screening of participants opened the envelopes and assigned them into either group. Other researchers, including the outcome assessors and the statistician, were all blinded to the group assignments. Patients were asked to wear an eye mask during the treatment, which took place in a closed unit. All researchers received training about the specification of this research method before and were required to strictly adhere to the task separation principle.

2.3. Intervention

The intervention began on the day after the randomization.

Except for the routine antidepressants, patients in the EA group accepted EA treatment 3 times per week (once every other day), 30 min per session, for a period of 8 weeks. Acupoints chosen were fixed as followed: BaiHui (DU20), YinTang (Ex-HN03), ShenTing (GV24), and bilateral ShenMen (HT7), NeiGuan (PC6), ZuSanLi (ST36), and SanYinJiao (SP6).

During each intervention, patients were asked to lie supine while a practitioner disinfected the acupoint sites. Stainless steel, sterile and disposable needles were tapped through a tube and inserted into the skin. After conventional insertion method, a rotating manipulation or lifting-thrusting manipulation was applied for "deqi" sensation. BaiHui (DU20) and YinTang (Ex-HN03) were connected with the EA instrument receiving pulses of 10 Hz of continuous waves.

Patients in the control group continued to take their routine antidepressants in the same amount and frequency as before. Since they would not have acupuncture treatment, 10 free sessions of acupuncture treatment would be given as compensation for waiting after they finished the follow-up period.

Patients in both groups were instructed not change the dosage of their antidepressants optionally during the trial. If the change of the dosage occurred under the psychiatrist's advice, the details should be recorded in the case report form (CRF).

2.4. Outcome measurements

2.4.1. Depressive symptoms.
The HAMD was used to evaluate the severity of patients' depressive symptoms during the trial. The scale includes 24 items, dividing depressive symptoms into 7 categories of factors: anxiety/somatization, weight change, cognitive impairment, diurnal variation, tardiness, sleep disorders, and despair. The total score is the main statistical indicator. Trained evaluators conducted the scales independently in the form of inquiry and conversation. The assessments were made at baseline, 4 weeks and 8 weeks after the beginning of intervention and at the end of 4 weeks' follow-up. Any score higher than 35 points were classified as severe depression; 20 to 35 points were classified as mild to moderate depression; 8 to 20 points were classified as depression without clinical significance; and scores below 8 points did not qualify as depression.

2.4.2. Urinary metabolites.
An 8 mL morning midstream urine sample (7:00–8:00) was collected from all participants at baseline and the end of the 8-week treatment period. The samples were quickly transferred to the sterile tubes before being given to the researchers. Metabolites were discovered as predictive markers in urine samples by using the GC–MS experiments were conducted to elucidate metabolites. By comparing the meaningful metabolites in the control group and the EA group, the differentiated metabolites might give clues of the possible pathways of depression that EA might act upon.

All urinary samples were centrifuged at 15,000 rpm for 10 min at 4°C. After that, the supernatant was put into 1.5 mL polyethylene (PE) tubes, divided with equal aliquots and stored at −80°C in the laboratory for GC–MS analysis. A 100 μL aliquot of urine sample was centrifuged at 15,000 rpm for 10 min at 4°C. A 50 μL aliquot of the supernatant was transferred to a 1.5 mL PE tube, adding 20 μL urease (type C-3, 30 U/10 μL) and incubating for 15 min at 37°C. The supernatant was mixed with 10 μL internal standards (0.3 mg/mL 2-chlorophenylalanine in

methanol). The solution was extracted with 170 μL methanol–acetonitrile (2:1, v/v) and vortexed for 30 s, then cooled at −20°C for 10 min. The samples were centrifuged at 12,000 rpm for 10 min at 4°C. A quality control (QC) sample was prepared by mixing aliquots of all samples to be a pooled sample. An aliquot of the 200 μL supernatant was transferred to a glass sampling vial for vacuum-drying at room temperature. The residue was derivatized using a 2-step procedure. First, 80 μL methoxyamine (15 mg/mL in pyridine) was added to the vial, vortexed for 30 s and kept at 30°C for 90 min followed by 80 μLN, O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) (1% Trimethylsilyl chloride [TMCS]) and 20 μL n-hexane at 70°C for 60 min. The samples were placed at ambient temperature (25–28°C) for 30 min before GC–MS analysis.

2.4.3. GC–MS analysis of urinary samples.
The derivatized serum samples were analyzed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977A MSD system (Agilent, CA). ADB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific, Folsom, CA) was utilized to separate the derivatives. Helium (>99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min through the column. The injector temperature was maintained at 260°C. The injection volume was 1 μL by splitless mode. The initial oven temperature was 50°C, increased to 125°C at a rate of 15°C/min, to 210°C at a rate of 5°C/min, to 270°C at a rate of 10°C/min, to 305°C at a rate of 20°C/min, and finally held at 305°C for 5 min. The temperature of MS quadrupole and ion source (electron impact) was set to 150°C and 230°C, respectively. The collision energy was 70 eV. Mass data were acquired in a full-scan mode (m/z 50–450), and the solvent delay time was set to 5 min.

The QC samples were injected at regular intervals (every 10 samples) throughout the analytical run to provide a set of data from which repeatability can be assessed.

2.4.4. Identification of differential metabolites.
In the ion flow chromatogram collected by GC–MS, we selected the chromatographic peaks on basis of the commercially available standards in the signal-to-noise ratio as objects. The retention time of each substance combined with the characteristic fragment ions were compared with the NIST library 2005 and online databases such as human metabolites database (HMDB) (http://www.hmdb.ca/) to identify the compounds.

2.4.5. Data analysis and multivariate analysis of samples.
In this study, the acquired raw GC–MS data were transformed into Computable Document format (CDF) using ChemStation (version e.02.02.1431, Agilent, CA). Then, Chroma TOF (version 4.34, LECO, St Joseph, MI) software was imported for data preprocessing, including peak extraction, denoising, deconvolution, etc. Finally, peak alignment was carried out to export files in Comma Separated Values (CSV) format. The file was imported into Excel for **multivariate** statistical analysis.

The data obtained above was imported into SIMCA P13.0 software. After scale conversion of all data, the natural separations within samples taken before and after treatment in both groups were displayed in Principal Component Analysis (PCA) plot in a visual inspection of score plots. Subsequently, orthogonal partial least squares-discriminate analysis (OPLS-DA), partial least squares-discriminate analysis (PLS-DA) and supervised multivariable statistical methods were used to sharpen an already established weak separation between groups of observations plotted in PCA. By incorporating the known

classification and Variable Importance of Projection (VIP) statistics, novel potential biomarker ions were discovered in the PLS-DA and OPLS-DA model. The PLS-DA model was validated by performing permutation tests (n=200) to check the validity of the model with R^2X and Q^2. R^2X showed the explanatory variables of the model, while Q^2 represented the predictability. Both were obtained by changing the order of classification variables Y.

The differential metabolites were selected based on the combination of a statistically significant threshold of VIP values obtained from the OPLS-DA model and P values from two-tailed Student's t test. VIP is a weighted measure that classifies the contribution of each metabolite. Metabolites with VIP values larger than 1.5 and P values <.05 were considered as differential metabolites.[12] Potential biomarkers and metabolic pathways were analyzed using HMDB and resources from National Institute of Standards and Technology (https://www.nist.gov/).

Thereafter, to identify potential diagnostic metabolites of EA in the treatment of depression, we also conducted a receiver operating characteristic (ROC) curve analysis. The OPLS predicted Y value was used for ROC analysis with the outcome indicators being the area under the curve (AUC). Graph Pad Prism 6 was used to generate the ROC curve.

In addition, the MetPA tool was used to analyze the metabolism of depressed patients receiving EA. The Metaboanalyst 3.0 web application (www.metaboanalyst.ca/) provided a fairly comprehensive metabolic pathway map.

2.5. Statistical analysis

Statistical analysis of the data in this study was conducted with SPSS21.0 software. The results of the statistical analysis were expressed as mean±SD (standard deviation). A two-tailed Student's t test was used for baseline age comparison, and a chi-square test was used for gender, education, marital status, medication history, and other data. In evaluation index analysis, measurement data comparison was calculated by repeated measurement analysis of variance. A 5% significance level was used for statistical analysis with two-tailed testing.

3. Results

3.1. Demographic and clinical characteristics

Figure 1 was the CONSORT flowchart of the clinical trial. Table 1 showed the baseline characteristics of all patients. There were no significant differences in age, gender, marital status,
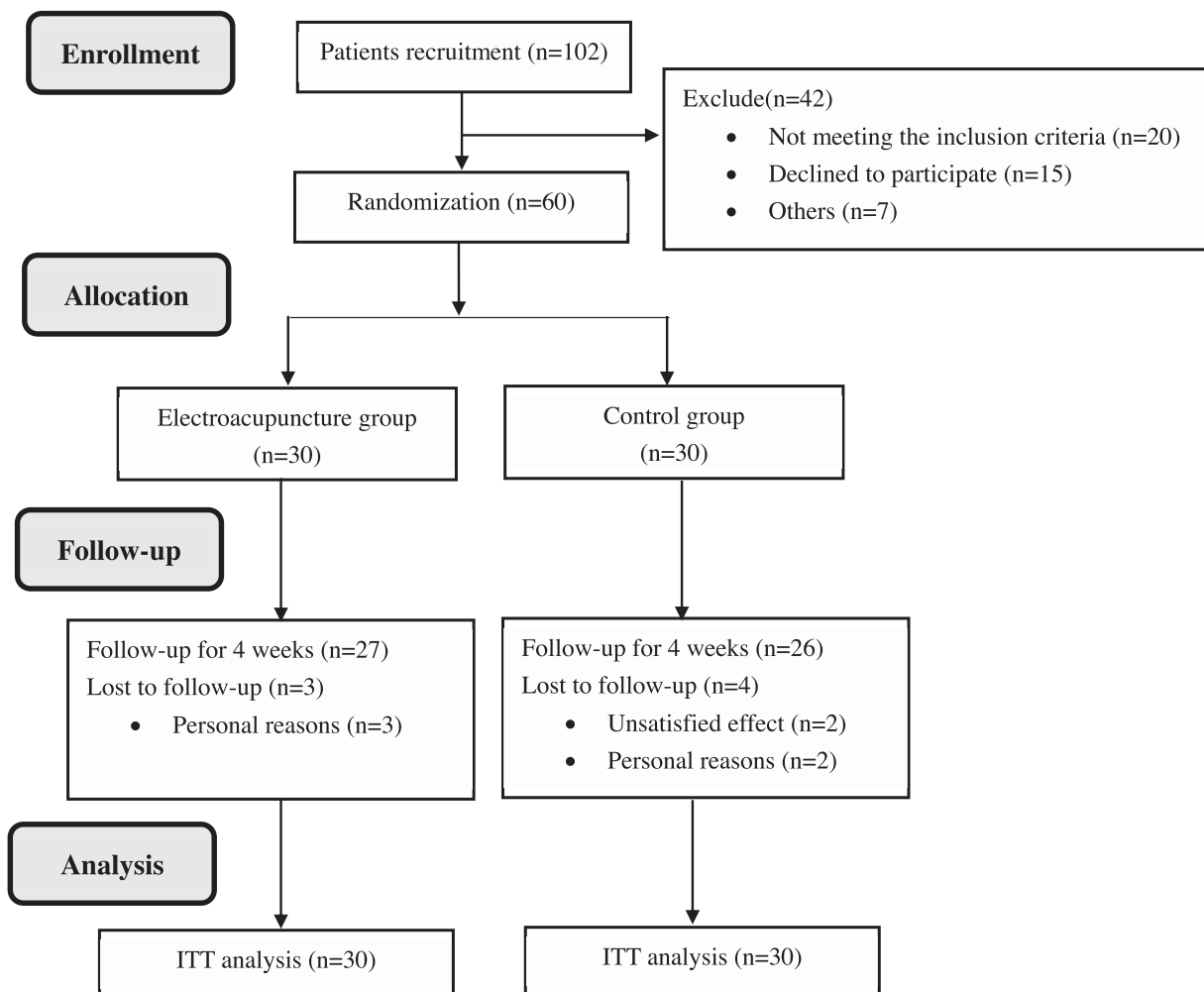

Figure 1. CONSORT flowchart of the trial.

Table 1

Baseline characteristics of participants in two groups.

Characteristic	EA group	Control group	F/x^2	P
Age				
Years	40.30 ± 10.99	38.75 ± 11.45	0.191	.665
Gender				
Male	11	11	0	1
Female	19	19		
Marriage				
Divorced	3	0	3.985	.228
Death of a spouse	1	0		
Unmarried	5	7		
Married	21	23		
Highest educational attainment				
Graduate	1	6	4.175	.142
Undergraduate	13	9		
Senior high	16	15		
Junior high	0	0		
HAMD scores	28.63 ± 5.72	28.47 ± 4.70	0.015	.902

EA = electroacupuncture, HAMD = Hamilton Depression scale.

education level and HAMD scores between the 2 groups (all $P > .05$).

3.2. Outcomes of mental health

Table 2 presented the HAMD scores of patients in 2 groups from baseline to follow-up. There were significant differences in the EA group before and after 8-week EA treatment (all $P < .001$); no statistical differences were found in the control group with routine antidepressants (all $P > .05$). Compared with those in the control group, patients receiving EA treatment had more significant improvement in HAMD scores after 4 weeks (respectively 16.89 ± 5.74 vs 25.58 ± 7.032, $P < .001$) and 8 weeks (respectively 9.59 ± 5.13 vs 25.04 ± 7.49, $P < .001$) of treatment, and the effects lasted for at least 1 month after the treatment finished (respectively 11.07 ± 6.85 vs 27.25 ± 7.14, $P < .001$).

3.3. GC–MS-based metabolomic results

Under the GC–MS conditions mentioned below, the typical total ion current chromatograms (TICs) of all samples were detected. The data displayed strong signals for analysis as well as a large peak capacity and good retention time reproducibility. After data reduction, variables between the treatment groups were detected from the chromatograms.

As were seen in the PCA plot (Fig. 2A and B), each point represents a urine metabolite. The distribution of the control group in PCA plot (Fig. 2B) was not so precisely separated, indicating that the urine metabolic pattern did not change

significantly after the maintenance of the original drug. But the PCA models in Figure 2A revealed that urine metabolite profiles of being treated by additional electropuncture were clearly altered and became clustered. ($R^2X = 0.637$, $Q^2 = 0.235$; Fig. 2A, $R^2X = 0.637$, $Q^2 = 0.235$; Fig. 2B). Previous literature has already verified that acupuncture and drugs have different metabolic pathways.[11]

Considering the small number of sample size, the data were also analyzed in the PLS-DA plot. The patterns in the Figure 3A and B show that the EA group changed more significantly due to the EA. The results shown in Figure 3C and D confirmed us that the constructed PLS-DA models were robust and not overfit.

With the random differences eliminated, the pattern drawn by OPLS-DA in Figure 3E and F exhibited that both treatments had an obvious influence on patients.

3.4. Potential biomarker identification

After effective screening, metabolites with VIP values > 1.5 and P values < .05 were defined as metabolically meaningful. A total of 20 metabolites were identified in the EA group (Table 3); while 10 metabolites were in the control group (Table 4). While 12 metabolites were found to exclusively present in the EA group, just 6 of them met our standards and would be analyzed later (Table 5), listed as cysteinyl glycine, glutamine, glutathione, N-acetyl-5-hydroxytryptamine, dehydroascorbic acid, and malonic acid.

3.5. Diagnostic performance assessment

To detect the clinical diagnostic sensitivity of these differential metabolites, the ROC curve was performed and the area under the AUC value of all significant differential metabolites was calculated. The sevens of all the 12 unique metabolites were displayed as their AUCs were higher than 0.7. All we can see was that the all the six metabolites (Fig. 4) we need except glutamine exhibited certain value in the diagnostic test, listed as cysteinyl glycine, glutathione, N-acetyl-5-hydroxytryptamine, dehydroascorbic acid, and malonic acid.

4. Discussion

The results showed that EA markedly reduced the HAMD scores, suggesting that the depressive symptoms could be observably improved by the 8-week EA treatment. Besides, the duration of the improvement was prolonged through follow-up observations. The effect of acupuncture treatment for depression has been questioned for a long time, with the lack of proof and clear mechanisms as the principal problem. Sometimes, even if patients present positive feedback, it is difficult to secure a convincing evaluation of acupunctures effectiveness owing to the lack of

Table 2

Changes in HAMD from baseline to follow-up (mean ± SD).

	EA group		Control group		t	P
		Paired *t*-test *P* value		Paired *t*-test *P* value		
Week 0	28.44 ± 5.78		27.46 ± 6.72		0.494	.623
Week 4	16.89 ± 5.74	<.001	25.58 ± 7.03	.273	−4.950	<.001
Week 8	9.59 ± 5.13	<.001	25.04 ± 7.49	.09	−8.677	<.001
Week 12	11.07 ± 6.85	<.001	27.25 ± 7.14	.921	−8.252	<.001

EA = electroacupuncture, HAMD = Hamilton Depression scale, SD = standard deviation.
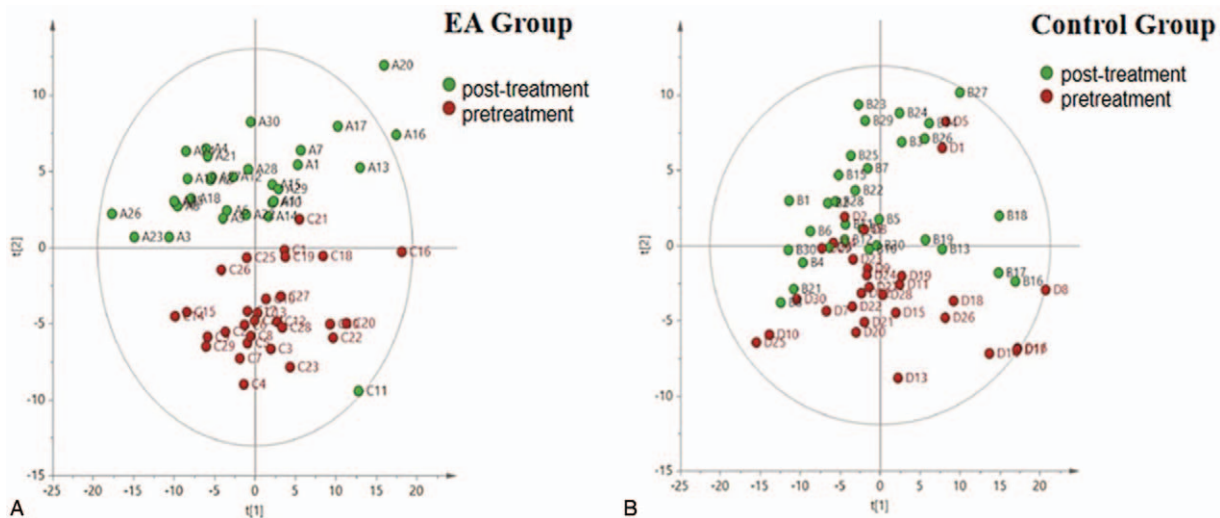
Figure 2. PCA plots of EA group and control group. The green dots presented the post-treatment situations, and the red ones presented the pretreatment situations. PCA plots showed the distribution of metabolic components before and after being treated in (A) the EA group and (B) control group. Similar ingredients will show a certain degree of aggregation. The clearer the dot boundaries of different colors were, the greater the differences in composition were.

specific clinical indicators and the challenge of distinguishing targets.

Metabolomics has become an active approach to detect disease markers, which can reveal the mechanism of disease metabolism and find therapeutic targets. Urine is a sterile, clear liquid produced by the kidneys. It contains a high concentration of inorganic urea Salt, ammonia, various water-soluble toxins, creatinine and urobilin. Urine, on the other hand, is largely considered water, While the trace amounts of metabolites has a high diagnostic value of biological fluid and it can be used to search for biomarkers of central nervous system diseases.[13,14] So far, many researchers have used metabolomics successful identification of potential diagnostic biomarkers neuropsychiatric disorders, such as bipolar disorder and schizophrenia Autism.[15,16] Studies also found that differences in urine metabolites can be used to Distinguish between healthy and depressed patients.[17,18] However, few articles have applied the method of metabolomics to explore the mechanism of acupuncture in treating depression. Therefore, we designed this experiment to fill this gap. Nowadays, many studies of metabolomics are conducted to observe the changes of metabolites in different groups at the same time. Even under the assumption of well consistency in patients' fundamental conditions, there performs still huge individual variations among each patient.[19,20] These differences may result from diverse eating habits, educational background, living environment, etc, which may demonstrate why different studies utilizing metabolomics for the same disease tend to reach various outcomes. Considering that the metabolome is sensitive to external factors, especially diet and sleeping schedules, we adopted the design of the patient before and after a control experiment to manage this uncontrollable factor. This situation can effectively eliminate the result deviation caused by some individual differences and more clearly illustrate the effect of different treatment methods.

4.1. Analyzing metabolist's pathway and function

When coming to the multivariate approach, people settle the interpretation of the metabolic on this assumption that a metabolic pathway may have biological or metabolic significance if random changes occur in the metabolites involved. Based on the model that AUC values >0.75, we may consider cysteine, glutathione, tryptophan, malonic acid, proline, and N-acetyl-5-hydroxytryptamine as sufficient evidence of the effectiveness of the combined use of acupuncture and medicine when healing depression. The metabolic pathway of MetPA analysis indicates that we can speculate that acupuncture may play a therapeutic role by adjusting the metabolism of tryptophan, glutamate, and fatty acids. The pathogenesis of depression is very complex and there is still no consensus. Up to now, there is no clear evidence to indicate whether there are differences in the metabolite of different degree of depression. However, in the pharmacological treatment studies of depression, the rapid improvement of major depressive disorder by ketamine and esketamine is related to tryptophan metabolism and glutamate metabolism, which are consistent with our results.[21] Our result may bring the possibility for reverse reasoning on the mechanism of the effect of acupuncture. Next, we will examine the links between the different symptoms of depression and the metabolic pathways we have found that acupuncture may affect.

4.1.1. Fatigue glutathione, and glutamate metabolism.
Depression and fatigue are highly comorbid phenotypes with overlapping symptoms.[22] Our results suggested that glutathione, r-glutamyl cysteine, glutathione (GSH) and their metabolism might be therapeutic targets for the comorbidities of both fatigue and depression. GSH is a tripeptide consisting of glutamic acid, cysteine, and glycine, which contains thioamide bonds and sulfhydryl groups.[23,24] It enjoys high biological activity, which can play the role of antioxidant defense, protein folding, and signal transduction. Besides this, it is involved in regulating cell proliferation, apoptosis, immune function, and liver fibrosis. GSH consumption is associated with fatigue. During the mice experiment, we found that post-exercise GSH supplementation reduced the pH rate between muscles and the amount of non-esterified fatty acids in the plasma. After GSH supplementation, the content of peroxidase and mitochondrial
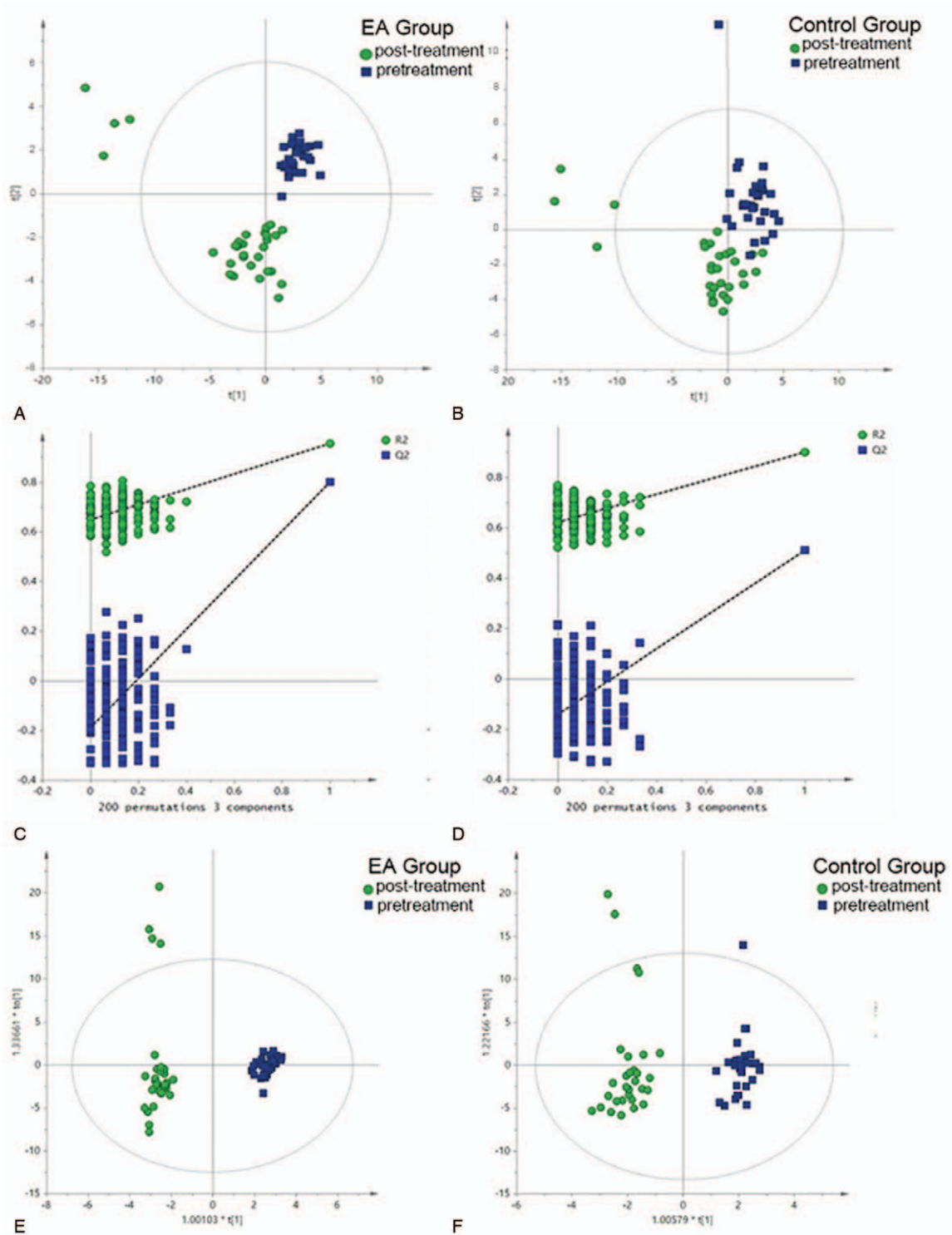
Figure 3. PLS-DA plots, permutation tests, OPLS-DA plots of EA group and control group. PLS-DA plots were used when the sample size was in a small number. It amplificated the separation between pre-therapy group and post-therapy group. Furthermore, to minimize the effect of intra-group differences, the pairwise PLS-DA score plots were used to demonstrate farther statistical differences between the pre-therapy group and post-therapy group of (A) the EA group ($R^2X=0.508$, $R^2Y=0.786$, $Q^2=0.568$), and of (B) the control group ($R^2X=0.769$, $R^2Y=0.989$, $Q^2=0.94$). Permutation tests were thus conducted to validate the quality of the PLS-DA models of (C) the EA group and (D) the control group. Under the premise that the PLS-DA model is valid, this study further removed the information irrelevant to the experiment by using OPLS-DA. Each point is distributed according to variable importance in the projection value (VIP). Metabolites with VIP values > 1.5 and P values < .05 in (E) the EA group and (F) the control group were deemed to be statistically significant according to the OPLS-DA model.

Table 3

Differential metabolites related to EA group.

Metabolite name	Average Rt (min)	VIP	P	log2 (FC)	Metabolic pathway
1-Methyladenosine	10.32	2.22	<.001	1.71	Adenosine metabolism
2-Keto-L-gulonic acid	17.76	3.63	<.001	−26.44	Glycine and serine metabolism
3-Hydroxypropionic acid	10.25	1.51	.024	−0.52	Phosphatidylethanolamine biosynthesis
5-Methoxyindole-3-acetic acid	14.30	1.26	<.001	2.72	Tryptophan metabolism
Cysteinylglycine	13.42	1.70	<.001	0.43	Glutathione metabolism
Dehydroascorbic acid	5.19	2.56	<.001	2.12	Fatty acid biosynthesis
d-Glucoheptose	27.04	1.94	.003	−0.99	Nucleotide sugars metabolism
Glucoheptonic acid	19.38	3.63	<.001	−5.17	Fatty acid biosynthesis
Glutamine	28.12	1.26	.020	0.61	Glutathione metabolism
Glutathione	10.04	2.02	<.001	0.64	Glutathione metabolism
Histidine	20.45	1.01	.020	0.47	Arginine and proline metabolism
Malonic acid	5.29	2.64	<.001	−0.60	Fatty acid biosynthesis
N-Acetyl-5-hydroxytryptamine	24.07	2.08	<.001	1.63	Tryptophan metabolism
N-Acetyl-D-galactosamine	23.31	1.82	.001	23.39	Amino sugar metabolism
Orotic acid	11.08	1.32	.004	0.58	Pyrimidine metabolism
Proline	8.31	2.53	<.001	2.19	Glutathione metabolism
Serine	9.31	1.17	.029	0.24	Phosphatidylethanolamine biosynthesis
Tryptophan	25.16	1.17	.018	0.35	Tryptophan metabolism
Xanthine	22.30	1.26	.021	0.46	Purine metabolism
Xanthurenic acid	17.82	1.19	<.001	−0.81	Tryptophan metabolism

Meaningful differences were defined as VIP values > 1.5 and P values < .01.
EA = electroacupuncture, FC = fold change, VIP = variable importance of projection.

deoxyribonucleic acid (DNA) increased significantly in mice that did not exercise.

When taking human beings as examples, GSH intake was positively associated with a significant decrease in psychological factors associated with fatigue, which can describe why patients tend to feel more energetic after acupuncture treatment. Besides, emerging evidence implies that glutamate and glutamine levels are low and inactive in the cerebral cortex of depressed patients.[25] When the glutamine shuttle is inhibited, the glutathione cycle can compensate for the reduced excitatory neurotransmission to achieve antidepressant effects.[26]

4.1.2. Sleep, melatonin, and tryptophan metabolism. Currently, polysomnography has confirmed that people with depression often experience sleep disturbances.[27] Even insomnia, to some extent, heralds the possibility of future depression. In this regard, melatonin (5-methoxy-N-acetyltryptamine), an indoleamine synthesized by the pineal gland at night, appears to be a target for comorbid depression and insomnia. It is well known that melatonin helps to synchronize the body's rhythm with the ambient light/dark cycle.[28] Melatonin can improve the sleep quality of adults and children while reducing the use of benzodiazepine drugs.[29,30] The sedative and hypnotic effects of melatonin may be achieved by binding to the brain melatonin MT1 receptor through the enhanced g-coupled protein pathway in which gamma-aminobutyric acid (GABA) binds to the GABAA receptor.[31] We even discovered that melatonin has other positive effects, including combating ageing, slowing cognitive decline, maintaining sleep quality, maintaining mental health, and preventing cardiovascular and cerebrovascular disease, diabetes and cancer.[32] Melatonin is a new type of clinically applied antidepressant suitable for healing various depressions, such as bipolar disorder, anxiety, alcohol dependence, migraine, etc.[33,34] It is particularly effective in improving significant depression.[35] However, due to its short half-life, melatonin is hard to guarantee the longevity of its therapeutic value. Therefore, there is a desire to develop more effective melatonin analogues with long-term effects or to design slow-release melatonin formulations. In our

Table 4

Differential metabolites related to control group.

Metabolite name	Average Rt (min)	VIP	P	log2 (FC)	Metabolic pathway
1-Methyladenosine	10.32	2.27	<.001	1.29	Adenosine metabolism
2-Keto-L-gulonic acid	17.76	2.10	<.001	−24.60	Glycine and serine metabolism
3-Hydroxypropionic acid	10.25	4.50	<.001	−25.17	Phosphatidylethanolamine biosynthesis
5-Methoxyindole-3-acetic acid	14.30	2.58	<.001	4.23	Tryptophan metabolism
Carnitine	17.90	2.14	<.001	−20.31	Fatty acid metabolism
Citrulline	14.64	3.20	<.001	−0.62	Aspartate metabolism
d-Glucoheptose	27.04	3.41	<.001	−20.91	Nucleotide sugars metabolism
Glucoheptonic acid	19.38	2.50	<.001	−24.10	Fatty acid biosynthesis
N-Acetyl-D-galactosamine	23.31	3.61	<.001	24.65	Amino sugar metabolism
Xanthurenic acid	17.82	1.65	.007	−0.59	Tryptophan metabolism

Meaningful differences were defined as VIP values > 1.5 and P values < .01.
FC = fold change, VIP = variable importance of projection.

Table 5

Differential metabolites related to EA only.

Metabolite name	Quant mass	Average Rt (min)	VIP	P
Glutamine	291	28.12	1.26	.02
Glutathione	213	10.04	2.02	<.001
Cysteinylglycine	185	13.42	1.70	<.001
N-Acetyl-5-hydroxytryptamine	209	24.07	2.08	<.001
Dehydroascorbic acid	157	5.19	2.56	<.001
Malonic acid	221	5.29	2.64	<.001

Meaningful differences were defined as VIP values > 1.5 and P values < .01
EA = electroacupuncture, VIP = variable importance of projection.

experiments, tryptophan and N-acetyl-5-hydroxytryptamine showed a significant increase. It converted into N-acetyl-5-hydroxytryptamine after tryptophan acetylation, which was a precursor of melatonin.[36] We speculated that the tryptophan metabolism pathway of patients who experienced acupuncture and drug treatment would therefore change. This shift was tilted towards the path to melatonin synthesis. However, whether these changes mean that the combination of acupuncture and antidepressants can replace the use of exogenous melatonin to a certain extent or solve the melatonin's weak efficacy, both of which require further studies and improvements.

4.1.3. Cognitive ability, malonic acid, and fatty acid biosynthesis. Regarding malonic acid, we have not found relevant evidence to support its connection with depression. However, we

have noticed malonic acid because malonyl-CoA decarboxylase deficiency is a genetic lipid metabolism disorder. Early literature has documented that abnormal lipid metabolism may increase malonic acid levels in urine.[37,38] A low-fat diet is beneficial for reducing malonic acid in urine, which is due to malonyl-CoA and acetyl-CoA. Both elements are indispensable intermediate metabolites in fatty acid biosynthetic pathways and can reversibly convert into malonic acid and acetic acid in the cell. Malonic acid is a reversible inhibitor of mitochondrial enzyme complex-II. Its abnormal accumulation in the body can cause changes in antioxidant enzyme levels, complex activities of mitochondrial enzymes, mitochondrial redox rate and proinflammatory cytokines, tumor necrosis and an increase in interleukins. These changes eventually lead to neurotoxicity and cognitive impairment.[39,40] There has been a consensus on the possible correlations between depression and cognitive impairment. Many articles have explored the mechanism of their relationship as well.[41,42] With the increase in the explorations of the intestinal flora in recent years, more evidence indicates that the intestinal flora affects inflammatory compounds as well as emotional state and cognition by participating in short-chain fatty acid (SCFA) metabolism (non-digestible polysaccharides produced by SCFAs during fermentation). Disorders of short-chain fatty acid metabolism are also playing an increasingly important role in depression.[43] EA could modulate the intestinal flora of depressive rats in our other unpublished experiments. We are boldly suggesting whether acupoints such as Zusanli (ST36), may change the lipid
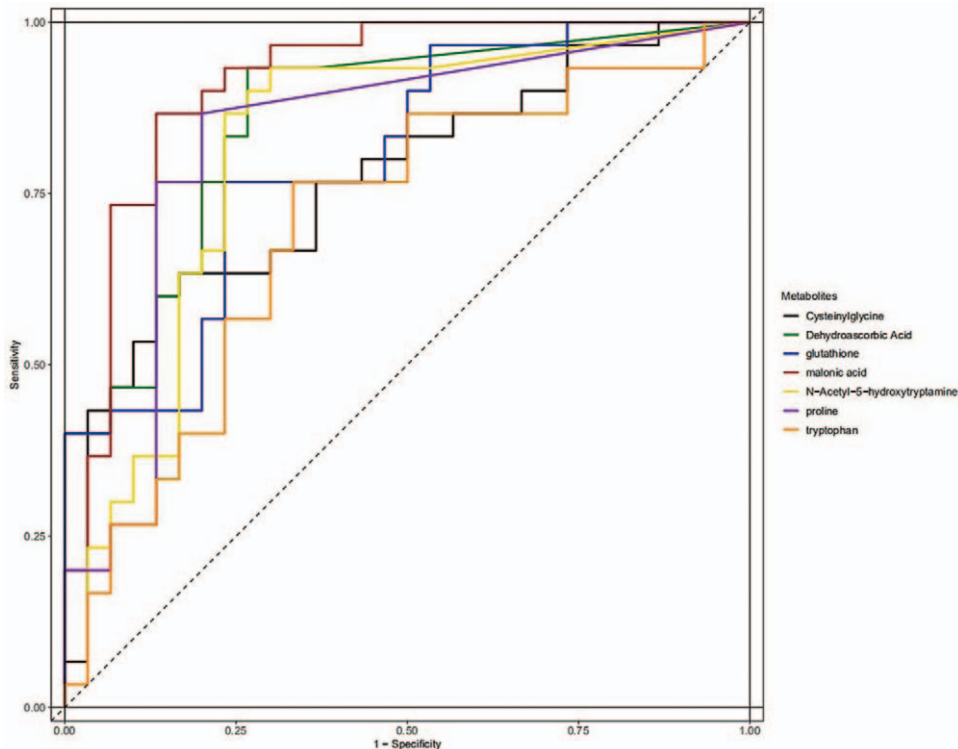

Figure 4. ROC curves and AUCs of the potential biomarkers. The data were statistically significant when the AUCs were higher than 0.7, demonstrating significance in clinical diagnostics. ROC curves and AUCs of the potential biomarkers as cysteinylglycine, dehydroascorbic acid, glutathione, malonic acid, N-acetyl-5-hydroxytryptamine, proline, and tryptophan of urine samples was as followed. AUC (malonic acid) = 0.913, AUC (dehydroascorbic Acid) = 0.858, AUC (N-acetyl-5-hydroxytryptamine) = 0.814, AUC (cysteinylglycine) = 0.768, AUC (glutathione) = 0.8, AUC (proline) = 0.829, AUC (tryptophan) = 0.706. The rest metabolites whose AUCs were below 0.7 would be presented in the appendix.

metabolism by adjusting the intestinal flora, thereby improving the symptoms of depression and the cognitive ability of patients. In this experiment, the combined use of acupuncture and antidepressants for patients with depression reduced the malonic acid in the urine, but the complete mechanism behind it is still worth exploring.

4.2. Challenges facing research on acupuncture for depression

One of the limitations in this trial is that the patients' expectation of EA treatment cannot be ruled out in this trial. The beneficial effects of EA treatment partly result from the possible placebo effects because patient in EA group may expect positive results. Considering the patients' mental state in both groups during this trial, most of them need to take the routine antidepressants and the dosage differs. It is hard to add another positive control, which might affect the results of the metabolomics, and this may be another limitation. A sham EA treatment can be set up in further study to control the placebo effects, to observe whether acupuncture was effective for treating depression through the results of between-group comparisons, and as well as to explore the possible mechanism of EA for treating depression through the results of urine metabolomics.

In this experiment, we applied non-targeted urine metabolomics to examine the metabolic changes of patients after acupuncture treatment. The results have initially discovered the possible metabolic targets of acupuncture and predicted the metabolic pathways involved in it, which provided helpful suggestions for the clarification of acupuncture mechanisms. However, it is unclear whether these changes are the result of an improvement in depressive symptoms or whether these changes lead to an improvement in depressive symptoms, which requires further mechanistic research.

Compared with transcriptomics and proteomics, metabolomics specializes in studying the changes of metabolites caused by gene modification or changes in the external environment of an organism, which is the closest to the phenotype of omics and the ultimate embodiment of the overall function or state of an organism system. Effective minor changes in gene and protein expression can be amplified during protein cascade reactions to produce metabolites, making detection easier. The control group also showed improvement in depressive symptoms after a period of treatment, and there were changes in the metabolic group, but neither change was as significant as that in the EA group. Therefore, it can be objectively reflected through differential urinary metabolites that acupuncture combined with antidepressants is superior to antidepressant only.

In a further study, we hope to answer why there are individual differences in the efficacy of acupuncture. First of all, we should continue to apply targeted metabolomics for further investigation. Secondly, given the detailed records of individual treatments, we consider sampling and analysis of individual patients at different periods before and after treatment, which may support systematically following the dynamic changes of different metabolic pathways in a single patient after treatment. By accumulating cases, combined with factors such as acupoint selection and patient symptom differences, we may be able to answer questions about the specificity of acupoints and the targets of acupuncture in more detail.

5. Conclusions

In this trial, we found that acupuncture in addition to antidepressants had a better effect on improving depressive symptoms than pure antidepressant intervention. With EA, remission lasted for a longer time as well. What's more, several highly correlated hallmark metabolites (cysteine, glutathione, tryptophan, malonic acid, and proline) and possible metabolic pathways (tryptophan metabolism, glutamate metabolism, and fatty acid biosynthesis) were discovered to be related to EA. The regulation of these metabolite levels and dysfunction might explain the reinforced antidepressant effect of EA in combination of antidepressants.

Author contributions

W Li and SF Xu conceived and designed the study, MQ Sun and X Yin interpreted the data, and LX Lao monitored the process and the manuscript. Li W and ZY Kuang drafted the manuscript. All authors read the manuscript and approved the final manuscript.

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