

Objective diagnosis of post-stroke depression using NMR-based plasma metabonomics

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Background: Post-stroke depression (PSD) is a frequent and serious complication of stroke. However, the underlying molecular basis of PSD remains largely unknown, and no empirical laboratory tests were available to diagnose this disorder.

Materials and methods: A proton nuclear magnetic resonance (¹H NMR)-based metabonomic approach was employed to profile plasma samples from 32 PSD, 35 stroke patients and 35 healthy comparison subjects (the training set) in order to identify metabolite biomarkers for PSD. Then, 10 PSD, 11 stroke patients and 11 healthy comparison subjects (test set) were used to validate the diagnostic performance of these biomarkers.

Results: The multivariate statistical analysis demonstrated that PSD group was significantly distinguishable from non-PSD groups (non-depression stroke patients and healthy comparison group). Five plasma metabolites (phenylalanine, tyrosine, 1-methylhistidine, 3-methylhistidine and LDL CH₃-(CH₂)_n-) were identified responsible for distinguishing PSD from non-PSD subjects. These metabolites were mainly involved in neurotransmitter metabolism and oxidative stress. The biomarker panel composing of these metabolites was capable of distinguishing test samples with a sensitivity of 100.0% and a specificity of 95.5%.

Conclusion: Our findings suggest that plasma disturbances of neurotransmitter levels and oxidative stress were implicated in the onset of PSD; these disturbed metabolites biomarkers facilitate to the development of diagnostic tool for PSD.

Keywords: post-stroke depression, stroke, metabonomics, diagnosis, NMR

Introduction

Post-stroke depression (PSD) is a serious complication in a variety of strokes, affecting approximately one-third of stroke survivors.¹ PSD hampers stroke rehabilitation and negatively impacts the quality of life.²⁻⁵ Previously, several hypotheses have attempted to explain the pathogenesis of PSD.⁶⁻⁹ The pathophysiology of PSD is complex and likely involves a combination of biological and psychosocial factors. Proposed biological factors include lesion location, genetic susceptibility, inflammation, neurogenesis in response to ischemia, alterations in neurotrophic factors, disruption of cortico-striato-pallido-thalamic-cortical projections and alterations in serotonergic, noradrenergic and dopaminergic pathways, leading to changes in amine levels.¹⁰ However, the definite molecular mechanisms of PSD remain obscure. PSD is considered to be a mood disorder due to stroke with depressive features, or major depressive-like episode. Stroke patients with a diagnosis of PSD should have either a persistent depressed mood or loss of interest or pleasure. Currently, due to lack of

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objective diagnostic methods, PSD diagnosis still solely relies on subjective identification of clinical symptoms. PSD patients usually suffer from physical and cognitive impairments that may hinder performance during psychiatric interview,^{11,12} resulting in underdiagnosed.¹³ In light of these facts, understanding the molecular basis of PSD and development of objective diagnostic approaches for PSD is of considerable clinical value.

Metabonomics, which focuses on investigation of metabolic perturbations in disease states, has shown promising potential in diagnosis of various diseases.^{14,15} Nuclear magnetic resonance (NMR)-based metabonomic method has proven to be an effective strategy for diagnosing diseases.¹⁶ In the field of neuropsychiatry, this method has been used in the diagnosis of stroke, bipolar disorder and schizophrenia.^{17–19} Deranged blood–brain barrier permeability has been reported in depressive disorder,²⁰ which implies that small molecule exchange may occur between the brain and peripheral bloodstream. Therefore, the plasma metabolic profile may hold pathophysiological information related to PSD. Recently, using this platform, this group found that plasma and urine metabolomics enabled distinguishing between depression and healthy comparison subjects with high accuracy.^{21–23} Given previous studies have demonstrated that both stroke patients and depression display plasma metabolic abnormalities,^{17,21} this group hypothesizes that the metabolic signature of PSD may be significantly disturbed in plasma and this specific changes may be used to diagnose this disorder.

Here, proton nuclear magnetic resonance (¹H NMR) spectra of plasma sampled from 32 PSD subjects, 35 stroke patients and 35 healthy controls were recorded and analyzed by multivariate statistical analysis. The metabolic phenotype homogeneity between ischemic and hemorrhagic subjects, major PSD and minor PSD Subjects were assessed separately by OPLS-DA model. The first purpose of this study was to determine whether the NMR-based plasma metabonomic approach enabled effectively discriminating the PSD group from non-PSD groups (non-depression stroke and healthy control group). The second purpose of this study was to identify metabolite biomarkers responsible for discriminating PSD subjects from non-PSD subjects. The functional enrichment of these metabolites was also performed to uncover the potential molecular basis of PSD. The third purpose of this study was to validate the diagnostic performance of these PSD biomarkers using independent test samples (10 PSD, 11 stroke patients and 11 healthy comparison subjects).

Material and methods

Clinical samples

The study was conducted in accordance with the Declaration of Helsinki. Written informed consents were obtained from all participants. The Ethical Committee of Chongqing Medical University reviewed and approved the protocol of this study and the procedures employed for sample collection. 42 PSD subjects and 46 stroke patients were enrolled in the Department of Neurology of the First Affiliated Hospital at Chongqing Medical University. Candidates with either ischemic or hemorrhagic stroke were recruited. The inclusion criteria were as follows: 1) age 18 years or older, 2) elapsed time of 6 months or less after stroke onset, 3) no history of neurological or psychiatric disorders and antidepressant drug used before stroke, 4) no disorders of consciousness, 5) no severe comprehension deficits, as demonstrated by inability to complete Hamilton Depression Rating Scale (HDRS) or patients with neuropsychological testing who showed impaired decision-making capacity, 6) no life-threatening heart or respiratory failure, renal or hepatic failure, severely disabling musculoskeletal disorder, cancer and neurodegenerative disorders such as Parkinson disease or Alzheimer disease, 7) no alcohol or substance abuse within the past 12 months prior to enrollment. The PSD recruits must have met the DSM-5 criteria; patient with a diagnosis of mood disorder due to stroke with a major depression-like episode must have depressed mood or loss of interest or pleasure along with four other symptoms of depression lasting 2 or more weeks, and have a 17-item HDRS score of greater than 8.²⁴ In addition, 46 healthy comparison subjects were recruited in the medical examination center of the First Affiliated Hospital at Chongqing Medical University. These healthy comparison subjects were required to have no current or previous lifetime history of neurological, DSM-V Axis I/II and/or systemic medical illness. 10 of 32 PSD were administrated antidepressant treatment in training set group, and 4 of 10 PSD were administrated antidepressant treatment in test set group; however, there was no significant relief of depressive symptoms when recruited. There was no history of neurological or psychiatric disorders and antidepressant drug used in both stroke patients and healthy controls.

The recruited subjects were divided into a training set and a test set. The training set, including 32 PSD (21 ischemic stroke and 11 hemorrhagic stroke; 14 Major

depression and 18 Minor depression), 35 stroke patients (25 ischemic stroke, 10 hemorrhagic stroke) and 35 healthy comparison subjects, metabolic phenotype homogeneity between ischemic and hemorrhagic subjects, major PSD and minor PSD subjects were assessed separately by OPLS-DA model; then, the samples were used to identify potential metabolite markers for PSD; the remaining subjects were used to construct the test set to further validate the diagnostic performance of these metabolite markers.

Comparisons of demographic characteristics of recruited subjects

As appropriate, the ANOVA test, the parametric Student's *t*-test or Mann-Whitney test was used to compare continuous variables (eg, HDRS and age). The Chi-square test was applied to analyze categorical data. All the statistical analyses were carried out with SPSS software (version 13.0). A *P*-value of less than 0.05 was deemed to be statistically significant.

Sample preparation and NMR acquisition

After overnight fasting, morning plasma samples were collected in 5-mL vacutainer tubes containing the chelating agent ethylene diamine tetraacetic acid (EDTA). Plasma samples were centrifuged at 1500 g for 15 mins. The resulting supernatant was immediately divided into equal aliquots and stored at -80°C . No plasma sample underwent more than two freeze-thaw cycles prior to undergoing NMR analysis.

Prior to NMR analysis, plasma samples were thawed and centrifuged at 15,000 g for 10 mins to remove precipitation. To ensure pH stabilization, 200 μl of plasma was mixed with 400 μl of phosphate buffer (0.2 M $\text{Na}_2\text{HPO}_4/0.2$ M NaH_2PO_4 , pH 7.4). After centrifugation at 12,000 g for 10 mins, 500 μl samples of supernatant were transferred into 5 mm NMR tubes. The proton spectra of the samples were collected on a Bruker Avance 600 spectrometer operating at a 600.13 MHz ^1H frequency with a standard CPMG (Carr-Purcell-Meiboom-Gill) sequence $\text{D}[-90^{\circ}-137(\tau-180^{\circ}-\tau)\text{n}-\text{ACQ}]$.²⁵ Blood plasma metabolites were assigned by comparison with chemical shift, which is detailed both in our previous work and published literature.^{21,26}

Processing of NMR spectra and multivariate pattern recognition

All spectra were manually phased and baseline referenced to lactate resonance at $\delta 1.33$. The NMR spectra (0.5–8.5

ppm) were segmented into equal widths (0.002 ppm) using the AMIX package (Bruker Biospin, Germany). Spectral regions of $\delta 3.69-3.57$, $\delta 3.26-3.06$ and $\delta 2.72-2.51$ were excluded to eliminate variations caused by imperfect water suppression, EDTA and EDTA metal complexes. The integral values of samples were imported into SIMCA-P + 12.0 software (Umetrics, Umea, Sweden) as variables for multivariable statistical analysis.

Orthogonal partial least-squares discriminant analysis (OPLS-DA) was applied to the unit variance-scaled spectral data to visualize discrimination among groups.^{27,28} The coefficient loading plots of the OPLS-DA model were used to identify the spectral variables contributing to sample discrimination on the scores plot.²⁹ Based on the number of samples (32 PSD, 35 stroke patients, 35 HC [health comparison]) used to construct the OPLS-DA model, the correlation coefficients were considered to be significant when $|r| > 0.35$, which corresponded to the critical value of a correlation coefficient at $P < 0.05$. The hues assigned to spectral signals projected onto the coefficient plot proportionally represent the magnitude of the metabolites responsible for the separation between groups based on *r* values. Red coloring denotes a high correlation, and blue coloring denotes no correlation. The quality of the OPLS-DA model was described by three parameters ($R^2\text{X}$, $R^2\text{Y}$ and $Q^2\text{Y}$), which were calculated by the default leave-one-out procedure. $R^2\text{X}$ and $R^2\text{Y}$ were used to quantify the goodness-of-fit; $Q^2\text{Y}$ was employed to assess model predictability.³⁰

Metabonomic analysis of plasma samples

Initially, the OPLS-DA models were constructed to compare PSD and stroke patients samples with healthy comparison samples. Moreover, the OPLS-DA model was further employed to assess whether this NMR-based metabonomic approach is capable of distinguishing PSD subjects from stroke patients. The reliability of constructed OPLS-DA model was assessed by the original training samples.³¹ Specifically, all original training samples were randomly divided into a training set and a test set. The training set accounted for 80% samples and the test set for the remaining 20% samples. Initially, the OPLS-DA model was regenerated by the samples from the training set. Then, the reconstructed OPLS-DA model was used to predict the class membership of the remaining 20% of samples. Combining the foregoing analysis, the diagnostic accuracy of the OPLS-DA model was calculated. In order to assess the homogeneity of the metabolic phenotypes between ischemic and

hemorrhagic subjects, the OPLS-DA model was initially constructed to compare 21 ischemic PSD samples with 25 ischemic stroke samples. Then, the reconstructed OPLS-DA model was used to predict the class membership of the 11 hemorrhagic PSD subjects and 10 hemorrhagic stroke subjects. The OPLS-DA models were constructed to compare minor PSD subjects with stroke patients or healthy comparison subjects to assess the homogeneity of metabolic phenotype between the minor PSD and major PSD subjects.

The overall flowchart of this metabonomic study was summarized in Figure S1. The OPLS-DA method was used to explore the metabolic differences among the PSD, stroke patients and healthy comparison groups (representative plasma 600 MHz 1D-CPMG ^1H NMR spectra obtained from a PSD subject, a stroke subject and a healthy comparison subject are shown in Figure S2).

Results

Demographic and clinical characteristics of subjects

The detailed characteristics of demographic and clinical data obtained from the PSD and non-PSD stroke groups (non-depression stroke patients and healthy comparison groups) are provided in Tables 1 and S1. The PSD group scored higher on the Hamilton Depression Rating Scale than non-PSD groups in both training set and test set. In addition, the three groups were not differentiable by any key demographic characteristic (age, gender or BMI) in both sets (Table 1).

Metabonomic analysis of plasma samples

OPLS-DA score plots demonstrated that both PSD and stroke subjects were significantly differentiated from healthy comparison subjects (Figure 1A, PSD vs HC, $R^2\text{X}=0.323$, $R^2\text{Y}=0.624$, $Q^2=0.335$; Figure 1B, stroke vs HC, $R^2\text{X}=0.368$, $R^2\text{Y}=0.773$, $Q^2=0.633$). OPLS-DA score plots demonstrated that the PSD group was significantly distinguishable from the stroke group (Figure 2, $R^2\text{X}=0.358$, $R^2\text{Y}=0.604$, $Q^2=0.163$). As the three parameters for assessing the OPLS-DA models ($R^2\text{X}$, $R^2\text{Y}$ and $Q^2\text{Y}$) were all positive, these findings suggest that plasma metabolic perturbation observed in PSD subjects relative to stroke patients and healthy comparison subjects was robust.

To assess the reliability of constructed OPLS-DA model, 80% of the recruited training subjects were randomly selected to construct new OPLS-DA models. Then, these models were used to predict class membership in the remaining 20% of subjects. Initially, using the plasma metabolic profiling from the 25 PSD subjects and 28 stroke patients, a new OPLS-DA model was reconstructed. This analysis resulted in a clear discrimination between the PSD and stroke groups. The T-predicted scatter plots of the OPLS-DA model are shown in Figure 3A and B. 6 of the 7 PSD subjects and 6 of the 7 stroke patients were correctly predicted by the OPLS-DA model, yielding a sensitivity of 85.7%, specificity of 85.7% and accuracy of 85.7%. A similar OPLS-DA model was then reconstructed with data from the 25 PSD subjects and 28 healthy comparison subjects, and a clear discrimination between the two groups

Table 1 Demographic and clinical details of the recruited subjects

	Variables	PSD	Stroke	HC	Statistics	df	P-value ^a
Training set	Sample Size	32	35	35	–	–	–
	Age (Year) ^b	62.1±9.3	60.7±9.0	58.5±4.8	F=1.8	101	0.169
	BMI (kg*m-2) ^b	22.8±2.5	23.2±3.45	21.8±2.7	F=1.9	101	0.150
	HDRS Scores ^b	17.4±4.9	1.9±1.8	0.2±0.5	F=346.1	101	0.000
	Sex (M/F)	17/15	20/15	14/21	$\chi^2=2.2$	2	0.326
	Smoking (Y/N)	11/21	16/19	10/25	$\chi^2=2.3$	2	0.317
	Drinking (Y/N)	8/24	13/22	7/28	$\chi^2=2.7$	2	0.256
Test set	Sample Size	10	11	11	–	–	–
	Age (Year) ^b	62.4±5.2	61.5±7.8	63.8±3.5	F=0.5	31	0.634
	BMI (kg*m-2) ^b	22.3±2.9	22.6±3.7	21.0±2.0	F=0.9	31	0.403
	HDRS Scores ^b	16.8±4.3	1.6±2.8	0.4±0.8	F=98.2	31	0.000
	Sex (M/F)	6/4	6/5	6/5	$\chi^2=0.1$	2	0.959
	Smoking (Y/N)	4/6	4/7	3/8	$\chi^2=0.4$	2	0.816
	Drinking (Y/N)	2/8	6/5	1/10	$\chi^2=6.1$	2	0.047

Notes: ^aANOVA test for continuous variables (age, BMI and HDRS Scores); Chi-square analyses for categorical variables (sex, smoking, drinking). ^bValues expressed as the mean ± SD (range).

Abbreviations: BMI, Body Mass Index; HDRS, Hamilton Depression Rating Scale; M/F, Male/Female; Y/N, Yes/No. HC, healthy comparison; PSD, post-stroke depression.

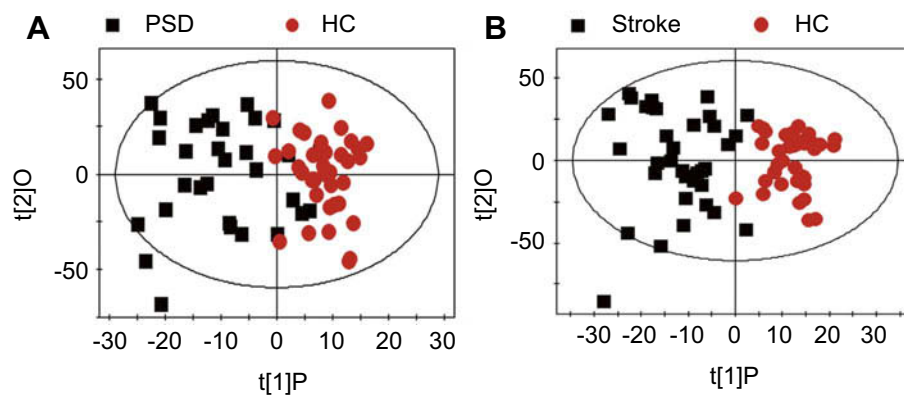


Figure 1 (A) The OPLS-DA model score plots show that PSD subjects were clearly distinguished from healthy comparison subjects; (B) A clear discrimination between and stroke and control group was observed.

Abbreviations: HC, healthy comparison; OPLS-DA, orthogonal partial least-squares discriminant analysis; PSD, post-stroke depression.

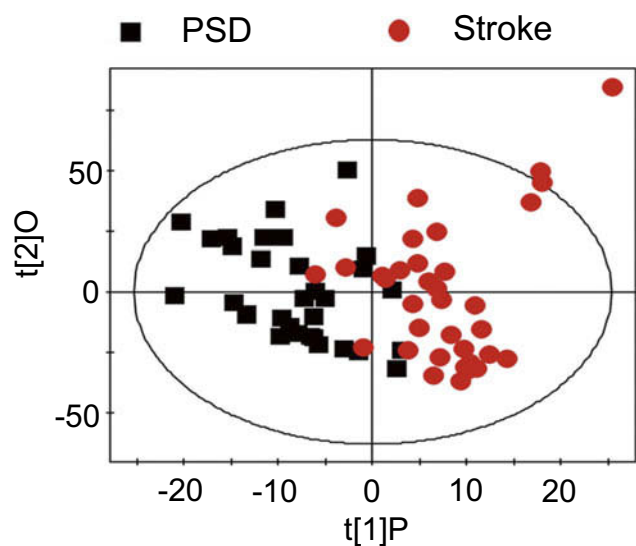


Figure 2 The score plots of the OPLS-DA model showing a clear separation between PSD subjects and stroke subjects.

Abbreviations: OPLS-DA, orthogonal partial least-squares discriminant analysis; PSD, post-stroke depression.

was observed therein. Using this regenerated OPLS-DA model to classify the 7 PSD subjects and 7 healthy comparison subjects, 6 of the 7 PSD subjects and all 7 healthy comparison subjects were correctly classified, resulting in a sensitivity of 85.7%, specificity of 100% and accuracy of 92.8% (Figure 3C and D). The foregoing results confirm the reliability of constructed OPLS-DA model.

Assessment of metabolic phenotype homogeneity between ischemic and hemorrhagic subjects

To assess the homogeneity of the metabolic phenotypes in ischemic and hemorrhagic subjects, the OPLS-DA model was initially constructed to compare 21 ischemic PSD

samples with 25 ischemic stroke samples. Then, the reconstructed OPLS-DA model was used to predict the class membership of the 11 hemorrhagic PSD subjects and 10 hemorrhagic stroke subjects. Consequently, all hemorrhagic PSD samples were mixed with ischemic PSD samples (Figure S3A); 8 of the 10 hemorrhagic stroke samples were mixed with the ischemic stroke samples (Figure S3B). To further validate these findings, an OPLS-DA model using 21 ischemic PSD samples and 35 healthy comparison samples was constructed. This regenerated OPLS-DA model was used to classify the remaining 11 hemorrhagic PSD subjects. 9 of the 11 hemorrhagic PSD subjects were correctly classified (Figure S3C). Identical procedures were also used to classify the 10 hemorrhagic stroke subjects in the OPLS-DA model that was from the 25 ischemic stroke subjects and 35 healthy comparison subjects. This analysis showed that all 10 hemorrhagic stroke subjects were correctly predicted (Figure S3D). These combined findings suggest that the lesion type (ischemic versus hemorrhagic) may not significantly influence the metabolic phenotypes of individuals with stroke or post-stroke depression.

Assessment of metabolic phenotype homogeneity between major PSD and minor PSD subjects

To assess the homogeneity of metabolic phenotype between the minor PSD and major PSD subjects, the OPLS-DA models were initially constructed to compare minor PSD subjects with stroke patients or healthy comparison subjects. The reconstructed OPLS-DA score plots demonstrated that the minor PSD group was significantly distinguishable from the stroke and healthy comparison

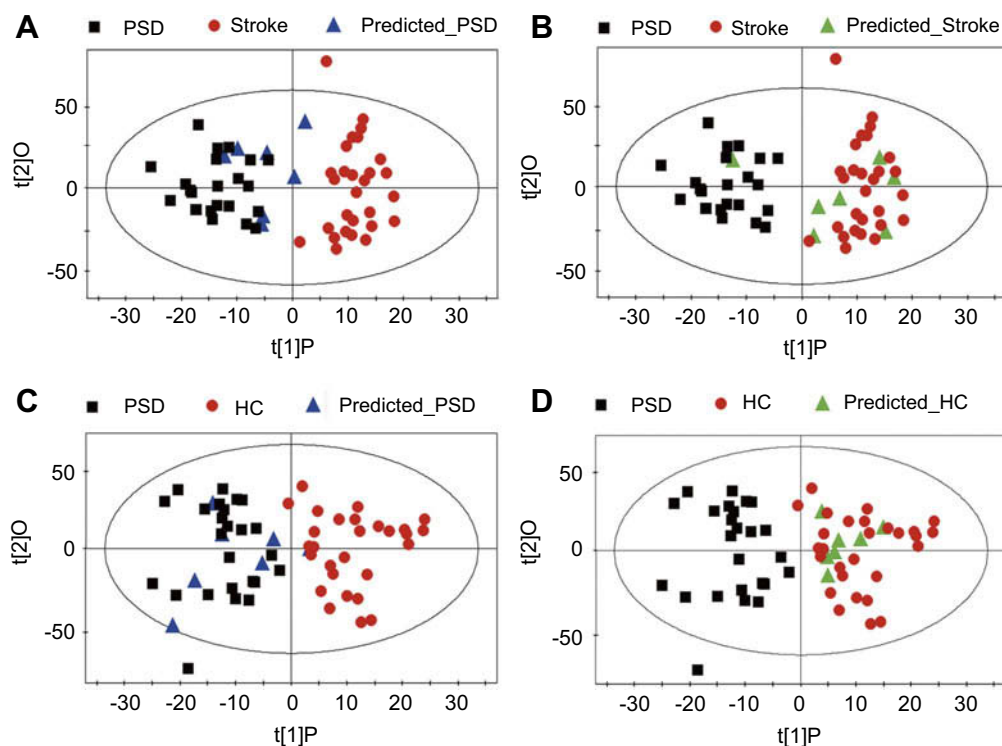


Figure 3 Assessing reliability of constructed OPLS-DA model. The new OPLS-DA models constructed with 80% of the recruited subjects were used to predict class membership in the remaining 20% of subjects. (A and B) 6 of the 7 PSD subjects and 6 of the 7 stroke subjects were correctly predicted, yielding an accuracy of 85.7%; (C and D) 6 of the 7 PSD subjects and all 7 healthy comparison subjects were correctly classified, resulting in an accuracy of 92.8%.

Abbreviations: HC, healthy comparison; OPLS-DA, orthogonal partial least-squares discriminant analysis; PSD, post-stroke depression.

group (Figure S4A, minor PSD vs HC, $R^2X=0.348$, $R^2Y=0.683$, $Q^2=0.380$; Figure S4C, minor PSD vs stroke, $R^2X=0.382$, $R^2Y=0.667$, $Q^2=0.242$). Then, the reconstructed OPLS-DA models were used to predict the class membership of the remaining 14 major PSD subjects. This analysis showed that 10 of 14 major PSD subjects were correctly predicted by the OPLS-DA model constructed with minor PSD and healthy comparison subjects (Figure S4B). Moreover, 12 of 14 major PSD subjects were correctly predicted by the OPLS-DA model generated from minor PSD subjects and stroke subjects (Figure S4D). These findings demonstrated that the type of depression may not significantly influence the metabolic phenotypes of individuals with episode PSD.

Identification for metabolite biomarkers discriminating PSD patients from non-PSD patients

To identify the differential plasma metabolites responsible for discriminating the PSD subjects from non-PSD subjects, the corresponding coefficient loading plots of the OPLS-DA models were analyzed. By analyzing the coefficient loading plots

of the OPLS-DA model derived from the PSD and healthy comparison groups, 12 metabolites contributing to the discrimination of PSD subjects from healthy comparison subjects were identified (Figure 4A). Compared to healthy comparison subjects, the plasma of the PSD subjects were characterized by higher levels of lactate, phenylalanine, tyrosine and glucose combined with lower levels of 1-methylhistidine, 3-methylhistidine, LDL CH₃-(CH₂)_n- lipid -CH₂-CH=CH-, lipid -CH₂-C=O and N-acetyl glycoprotein. Moreover, using the same analysis, 14 metabolites contributing to the discrimination of stroke subjects from healthy comparison subjects were also identified (Figure 4B). Interestingly, 12 of the 14 perturbed plasma metabolites in stroke subjects were consistently changed in PSD subjects (Table 2), suggesting the evolution of metabolic profiling in stroke potentially associated with the development of PSD.

To specify the PSD-specific metabolites, the OPLS-DA loading coefficient plots (PSD group versus non-depression stroke group) were analyzed. This analysis resulted in identification of five differential plasma metabolites contributing to the discrimination between these two groups (Figure 4C). Compared to stroke subjects, the plasma of PSD subjects were characterized by higher levels of

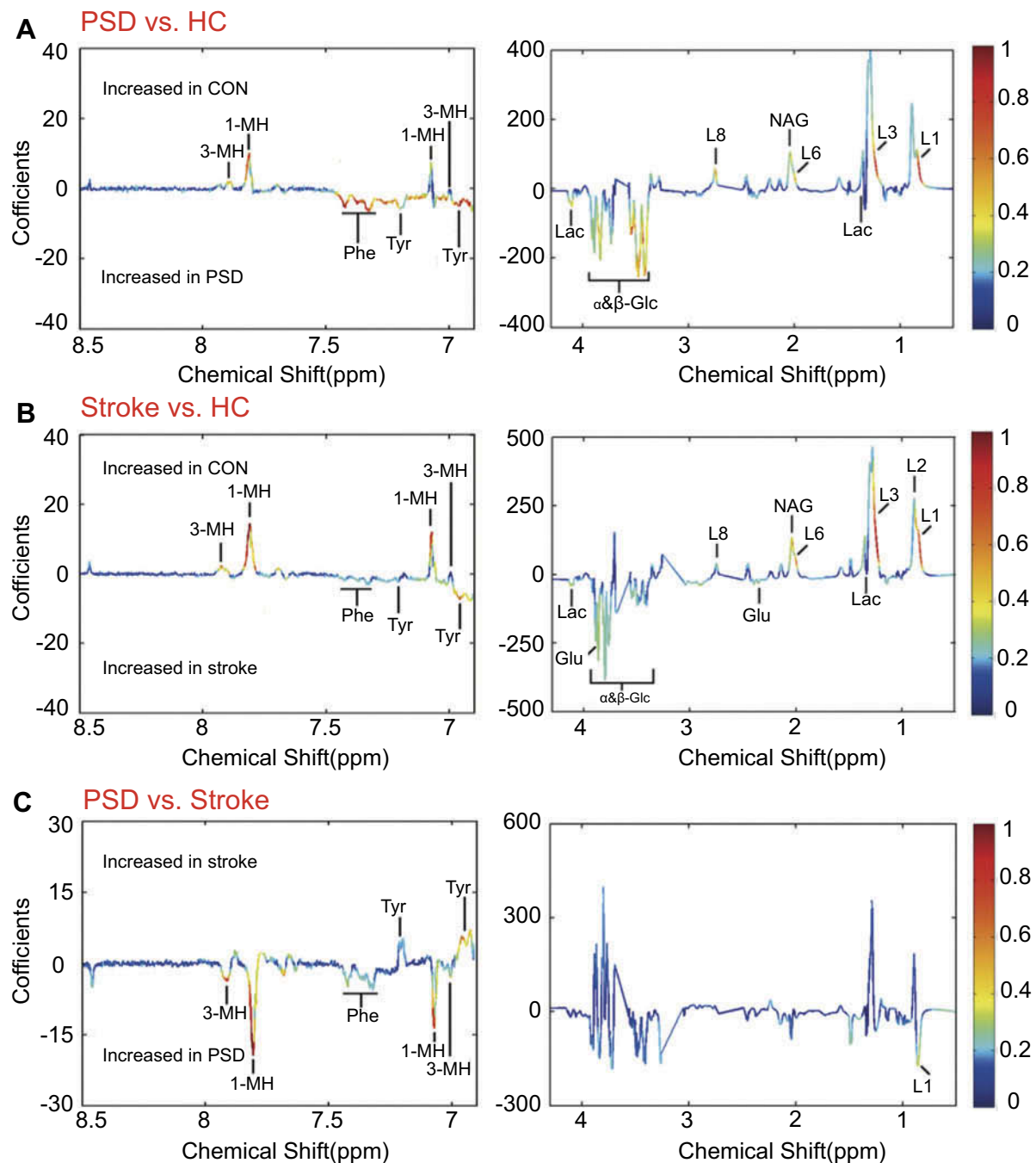


Figure 4 Identification of differential metabolites responsible for discrimination among PSD, stroke and healthy comparison groups. **(A and B)** The loading coefficient plots of the OPLS-DA models show metabolite variations attributable to distinguishing the PSD group from the stroke and healthy comparison groups; **(C)** The plasma metabolites responsible for discrimination between stroke and healthy comparison subjects.

Abbreviations: HC, healthy comparison; OPLS-DA, orthogonal partial least-squares discriminant analysis; PSD, post-stroke depression.

1-methylhistidine, 3-methylhistidine, LDL CH₃-(CH₂)_n- and phenylalanine combined with lower levels of tyrosine (Table 2). Functional enrichment of these differential metabolites suggests that disturbance of neurotransmitter metabolism (phenylalanine and tyrosine) and oxidative stress (1-methylhistidine, 3-methylhistidine and LDL CH₃-(CH₂)_n-) were implicated in the onset of PSD. All

the differentially expressed metabolites in PSD subjects relative to stroke subjects were also changed in PSD subjects or stroke patients relative to healthy comparison subjects. These combined findings demonstrate that different levels of those metabolites account for discriminating PSD patients from stroke patients and healthy comparison subjects.

Table 2 Key differential metabolites among PSD, stroke and healthy comparison groups

Metabolites	Chemical shift/multiplicity ^a	r ^b		
		PSD/Stroke ^c	PSD/HC ^d	Stroke/HC ^e
1-Methylhistidine	7.06(s), 7.77(s)	-0.746	0.516	0.759
3-Methylhistidine	7.00(s), 7.91(s)	-0.680	0.548	0.553
Glutamate	2.12(m), 2.35(m), 3.78(t)	-	-	-0.413
L1: LDL, CH ₃ -(CH ₂) _n -(L1)	0.85(br)	-0.439	0.559	0.641
L2: VLDL, CH ₃ -(CH ₂) _n -	0.89(br)	-	-	0.544
L3: LDL, CH ₃ -(CH ₂) _n -	1.28(br)	-	0.433	0.663
L6: Lipid, -CH ₂ -CH=CH-	2.02(br)	-	0.446	0.588
L8: Lipid, -CH ₂ -C=O	2.78(br)	-	0.466	0.398
Lactate	1.33(d), 4.11(q)	-	-0.429	-0.464
N-Acetyl glycoprotein	2.04(s)	-	0.414	0.560
Phenylalanine	7.32(d), 7.37(t), 7.42(dd)	-0.419	-0.618	-0.355
Tyrosine	6.90(d), 7.19(d)	0.445	-0.450	-0.443
α-Glucose	3.42(t), 3.54(dd), 3.71(t), 3.73(m), 3.84(m), 5.23(d)	-	-0.563	-0.441
β-Glucose	3.25(dd), 3.41(t), 3.46(m), 3.49(t), 3.90(dd), 4.65(d)	-	-0.531	-0.358

Notes: ^aMultiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet; br, broad resonance. ^bA correlation coefficient of $|r| > 0.350$ was used as the cutoff value for statistical significance based on the discrimination significance at $p=0.05$. "-" indicates a correlation coefficient $|r|$ of less than 0.350. ^cPositive correlation coefficients indicate significantly lower levels in PSD subjects relative to stroke subjects; negative values indicate significantly higher levels in PSD subjects relative to stroke subjects. ^dPositive correlation coefficients indicate significantly lower levels in PSD subjects relative to healthy comparison subjects; negative values indicate significantly higher levels in PSD subjects relative to healthy comparison subjects. ^ePositive correlation coefficients indicate significantly lower levels in stroke subjects relative to healthy comparison subjects; negative values indicate significantly higher levels in stroke subjects relative to healthy comparison subjects.

Abbreviations: PSD, post-stroke depression; HC, healthy comparison.

Independent validation of diagnostic performance of PSD-biomarkers

To independently validate the diagnostic performance of these PSD-specific metabolites, new OPLS-DA models were reconstructed with plasma NMR spectra obtained from five metabolites to compare PSD with non-PSD samples. These analyses resulted in a clear discrimination between PSD and non-PSD groups (Figure 5A, PSD vs stroke, $R^2X=0.482$, $R^2Y=0.485$, $Q^2=0.354$; Figure 5D, PSD vs HC, $R^2X=0.447$, $R^2Y=0.537$, $Q^2=0.397$). T-predicted scatter plots of these OPLS-DA models were then used to predict class membership of test samples. 10 of the 11 healthy comparison subjects, all PSD and stroke subjects were correctly predicted by the reconstructed OPLS-DA models (Figure 5B, C, E and F). These findings suggest that the PSD-specific metabolite panel may serve as an effective diagnostic approach for PSD, achieving a sensitivity of 100.0%, specificity of 95.5% and accuracy of 96.9%.

Discussion

Current diagnosis of PSD remains subjective, and the underlying molecular mechanisms remain largely unknown. Here, we showed that NMR-based plasma metabolomics provide excellent performance in distinguishing

PSD subjects from the non-PSD subjects in both training set and test set. The satisfactory predictability of this method suggests that it may serve as a useful tool in diagnosing PSD. In addition, a PSD-specific metabolic signature was identified, suggesting disturbance of neurotransmitter metabolism (phenylalanine and tyrosine) and oxidative stress (1-methylhistidine, 3-methylhistidine and LDL CH₃-(CH₂)_n-) were implicated in the onset of PSD.

The key characteristic of any diagnostic method is accuracy of detection. Here, PSD-biomarkers identified by NMR-based metabolomics approach enabled distinguishing PSD subjects from non-PSD subjects with a sensitivity of 100.0%, specificity of 95.5% and accuracy of 96.9%. The diagnostic performance of this method is significantly higher than the currently available psychiatric interview-based method.¹³ To develop deeper insight into the underlying molecular mechanisms of PSD, the functional analysis of PSD-specific differential metabolites was undertaken. These five metabolites are involved in 1) monoamine neurotransmitter metabolism (phenylalanine (Phe) and tyrosine (Tyr)), and 2) oxidative stress (1-methylhistidine, 3-methylhistidine and LDL CH₃-(CH₂)_n-). This finding is not surprising, as there is ample reported evidence indicating that the two processes are implicated in the pathophysiology of PSD.^{7,32}

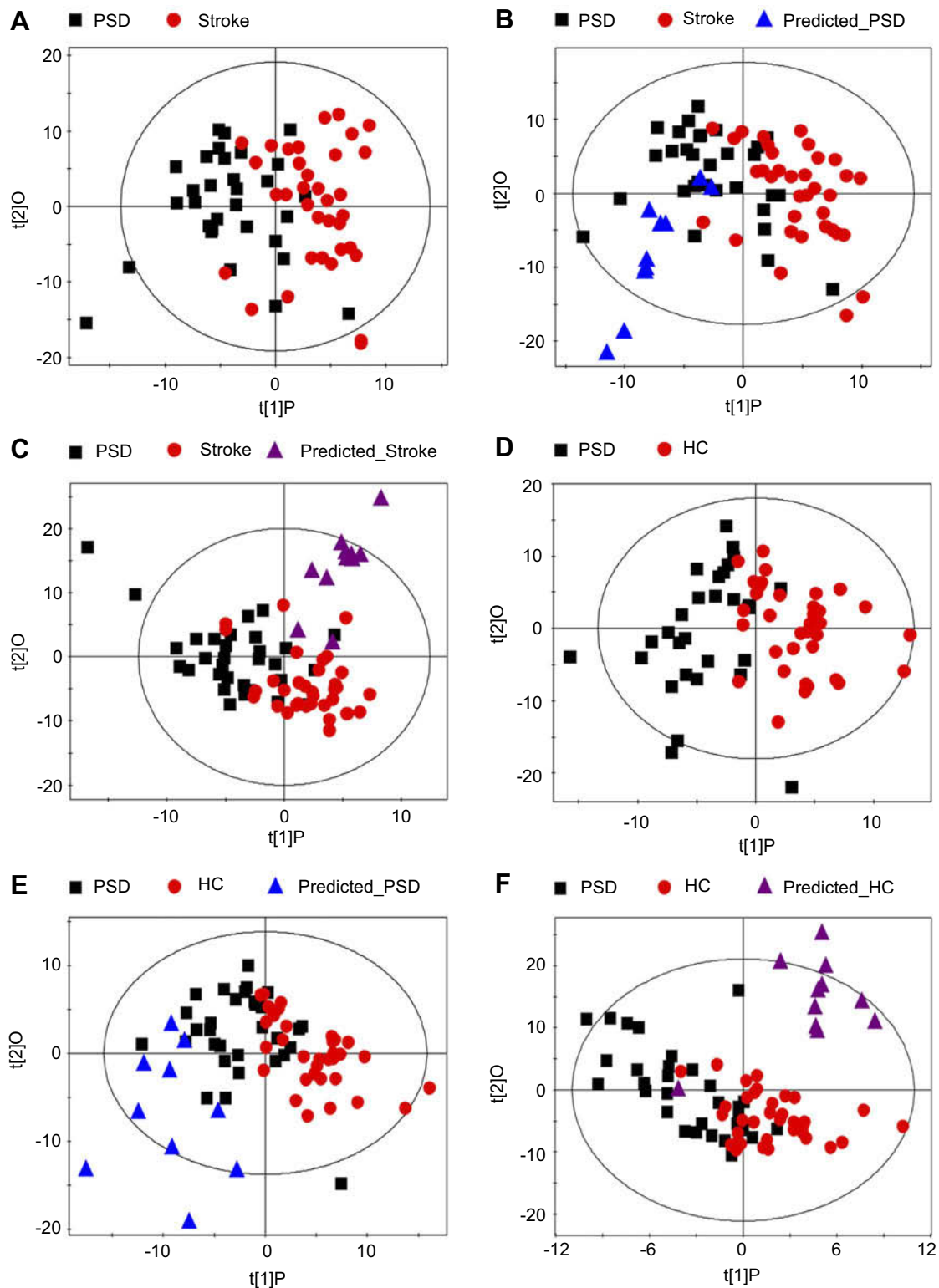


Figure 5 Independent validation of diagnostic performance of PSD-biomarkers. (A–C) The OPLS-DA model constructed with PSD-specific metabolites showing a clear discrimination between PSD and stroke subjects in the training set; The reconstructed OPLS-DA models correctly predicted all PSD and stroke subjects in test set. (D and E) The OPLS-DA model constructed with PSD-specific metabolites showing a clear separation between PSD subjects and healthy comparison subjects. 10 of the 11 healthy comparison subjects and all PSD subjects were correctly predicted by the regenerated OPLS-DA model.

Abbreviations: HC, healthy comparison; OPLS-DA, orthogonal partial least-squares discriminant analysis; PSD, post-stroke depression.

Monoamine neurotransmitter metabolism

The aromatic amino acids Phe and Tyr are the biosynthetic precursors of the monoamine neurotransmitters (dopamine (DA) and noradrenaline (NA)). The rates of these neurotransmitter synthesis and release are directly affected by the CNS levels of their amino acid precursors, namely Phe and Tyr, which in turn are driven by their plasma bioavailability.³³ Therefore, any pathophysiologic factor such as PSD that perturbs plasma levels of these amino acids and/or the branched-chain amino acids that compete for the common large neutral amino acids (LNAA) transporter at the blood–brain barrier (BBB) will change the CNS levels of the foregoing aromatic amino acids, the formation and release of monoamine neurotransmitters, and therefore brain function. More specifically, as the LNAA transporter is competitive, increases in the plasma levels of LNAA (such as Phe) can reduce the uptake of other LNAA and thus lower their CNS levels. Besides, tyrosine hydroxylase which catalyzes the conversion of tyrosine to dopa could be inhibited by high levels of phenylalanine. Therefore, in the present case, the increased plasma levels of Phe in PSD subjects relative to stroke patients result in decreased CNS levels of both Tyr and its downstream catecholamines (DA, NA), since the initial Tyr hydroxylation step in the catecholamine synthesis pathway is rate-limiting and thus controls the rate of catecholamine synthesis. This deduction is consistent with previous work that has found evidence of reduced brain catecholamine levels in the pathophysiology of PSD.^{10,34}

Oxidative stress

In this study, we observed that the levels of 1-methylhistidine and 3-methylhistidine were increased in PSD group relative to the stroke group. 1-methylhistidine and 3-methylhistidine are the metabolic byproducts of two carnosine (beta-alanyl-L-histidine) analogs: anserine (beta-alanyl-L-1-methyl-histidine) and balenine (ophidine, beta-alanyl-L-3-methylhistidine), respectively. Carnosine and its analogs are antioxidant molecules found in the brain, and have been thoroughly investigated in vivo and in vitro as free-radical scavengers and inducers of antioxidant systems in oxidative stress-related conditions, such as neurodegenerative disorders and hypoxic–ischemic injuries.^{35,36} Interestingly, the level of another antioxidant marker, LDL, was also elevated in the PSD patients, which has been linked to increased production of oxygen-derived free radicals, contributing

to the oxidative stress burden.³⁷ Taken together, the upregulation of the foregoing antioxidant stress markers observed here may be indicative of excess oxidative damage in PSD patients relative to stroke patients. In agreement with this speculation, increased oxidative stress was implicated in the development of PSD in previous study.³²

The results of this study should be cautiously interpreted on account of the following limitations. The sample size used in this study was relatively small. This was limited by the fact that the PSD always affected the aged people and was always comorbid with other systemic medical illness. Moreover, all subjects were of the same ethnicity and were recruited from the same site. Thus, ethno- and site-specific biases cannot be ruled out. Further studies recruiting heterogeneous populations conducted across different sites should be performed. Lastly, the detailed mechanisms of disturbed plasma neurotransmitter levels and oxidative stress were required to validate in the animal studies.

Conclusion

To our knowledge, this is the first metabolomic-based study investigating plasma molecular disturbances for PSD. Here, we provided evidence that NMR-based plasma metabolomics may serve as a useful tool in the clinical diagnosis of PSD. Moreover, peripheral disturbances of neurotransmitter levels and oxidative stress were implicated in the onset of PSD.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Clinical details of the recruited PSD and stroke subjects

	Variables	PSD	Stroke	Statistics	df	P-value ^a
Training set	Depression subtype (major/minor)	14/18	—	—	—	—
	Antidepressants treatment (Y/N)	10/22	—	—	—	—
	Stroke subtype (ischemia/hemorrhage)	21/11	25/10	—	—	—
	Time poststroke (days) ^c	53 (30, 150)	33 (21, 33)	Z=-2.3	1	0.023
	Modified Rankin Scale ^b	2.8±1.4	2.6±1.4	t=0.5	65	0.594
	NASID treatment (Y/N)	15/17	20/15	χ ² =0.7	1	0.401
	Sleep meds treatment (Y/N)	2/30	N	χ ² =2.3	1	0.133
	Hypertention (Y/N)	30/2	30/5	χ ² =1.2	1	0.283
	Hyperlipidaemia (Y/N)	8/24	8/27	χ ² =0.04	1	0.837
	Diabetes (Y/N)	10/22	12/23	χ ² =0.07	1	0.792
Test set	Depression subtype (major/minor)	4/6	—	—	—	—
	Antidepressants treatment (Y/N)	4/6	—	—	—	—
	Stroke subtype (ischemia/hemorrhage)	5/5	0/11	—	—	—
	Time poststroke (days) ^b	35.5±16.4	33.3±15.2	t=0.3	19	0.750
	Modified Rankin Scale ^b	2.2±0.9	2.5±1.5	t=-0.5	19	0.650
	NASID treatment (Y/N)	4/6	10/1	χ ² =6.1	1	0.013
	Sleep meds treatment (Y/N)	2/8	N	χ ² =10.8	1	0.001
	Hypertention (Y/N)	7/3	9/2	χ ² =0.4	1	0.525
	Hyperlipidaemia (Y/N)	3/7	3/8	χ ² =0.02	1	0.890
	Diabetes (Y/N)	4/6	5/6	χ ² =0.1	1	0.801

Notes: ^aTwo-tailed student test for continuous variables (Modified Rankin Scale); Chi-square analyses for categorical variables (NASID treatment, sleep meds treatment, hypertension, hyperlipidaemia, diabetes). Time poststroke was analyzed by Mann-Whitney test in training set, and analyzed by two-tailed student test in test set. ^bValues expressed as the mean ± SD (range). ^cValues expressed as median (25%, 75%) quartiles.

Abbreviations: PSD, post-stroke depression; Y/N, Yes/No.

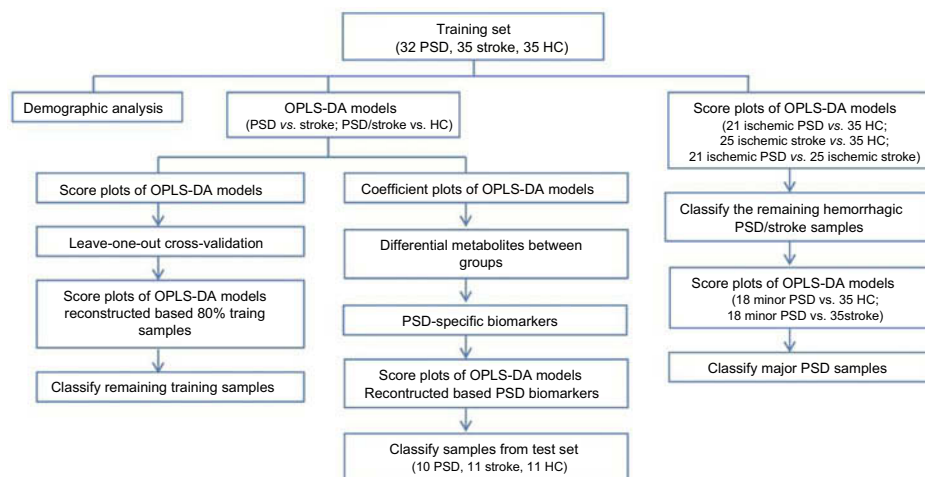
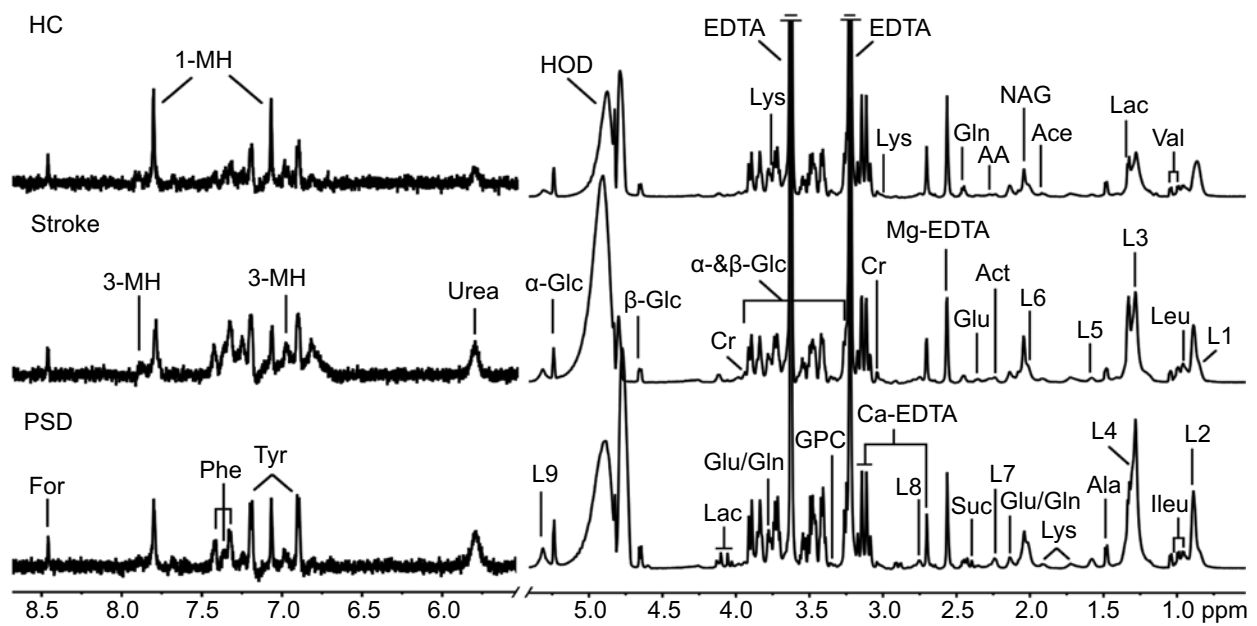
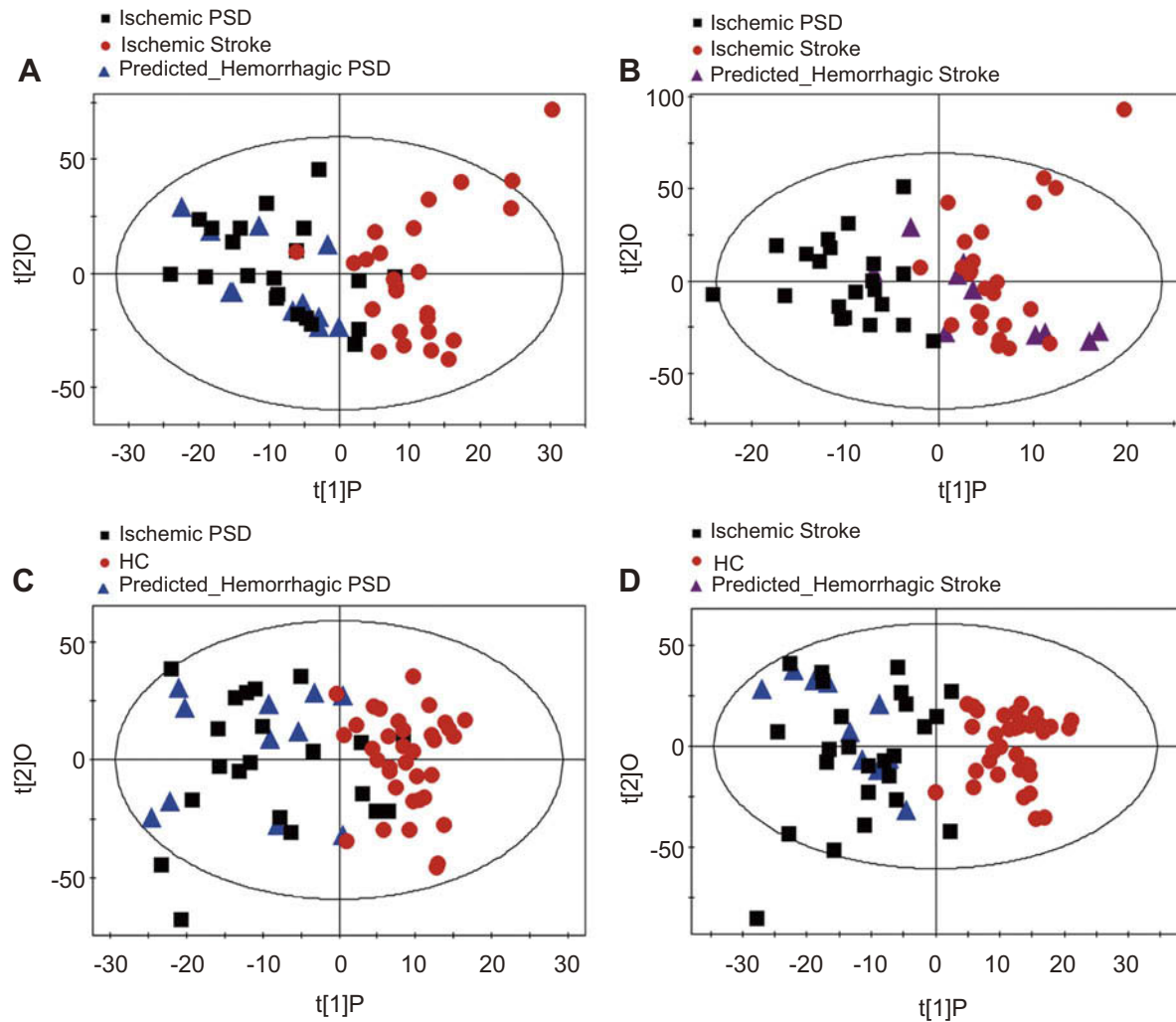


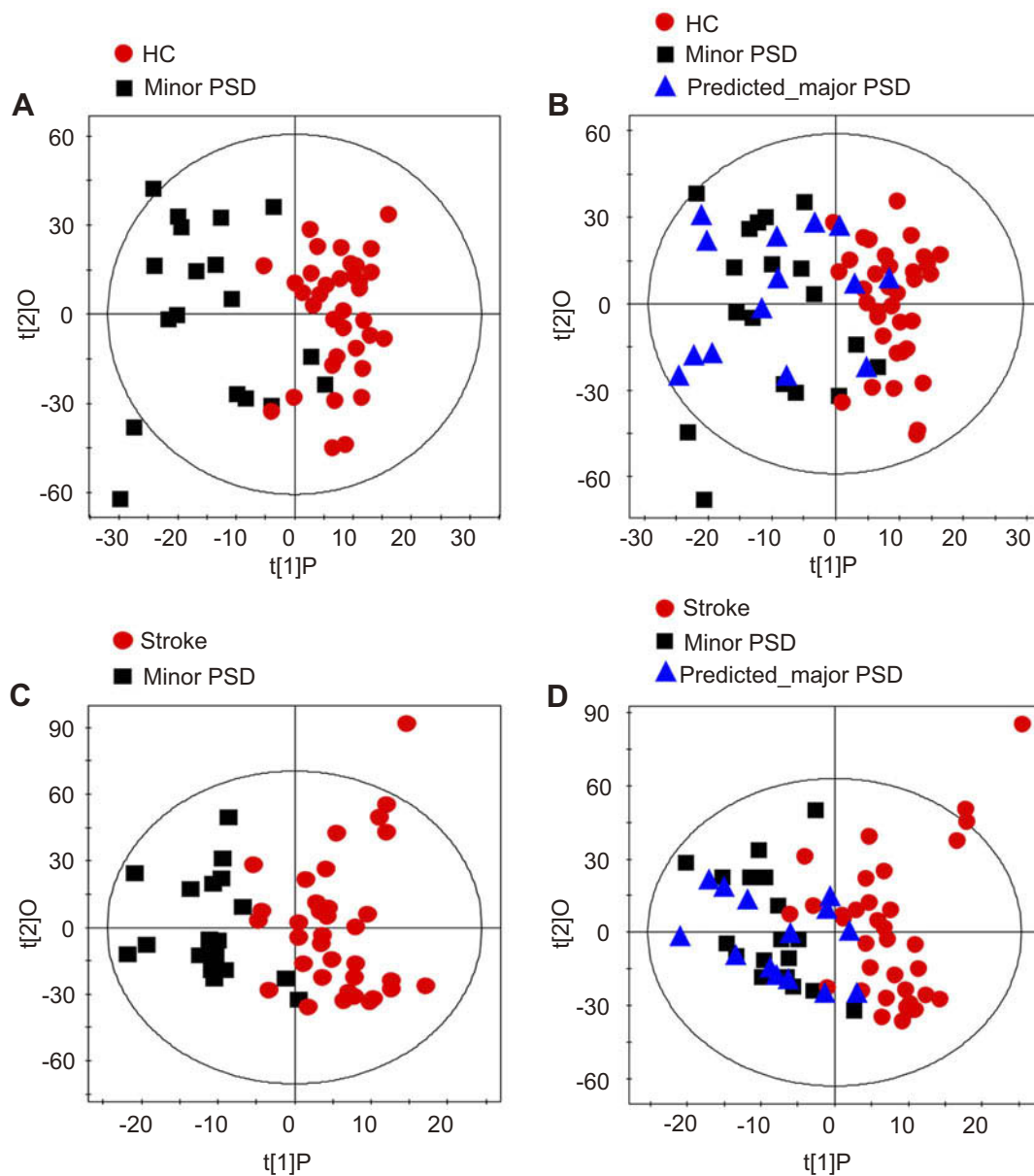
Figure S1 The overall flowchart of this metabolomic study.



Figures S2 Representative plasma 600 MHz one-dimensional Carr-Purcell- Meiboom-Gill (1D-CPMG) ¹H NMR spectra obtained from a PSD subject, a stroke subject, and a healthy control subject. The spectrum resonances assigned to the metabolites including 1-methylhistidine, 3-hydroxybutyrate, 3-methylhistidine, acetoacetate, acetate, acetone, alanine, creatine, formate, glucose, glutamine, glutamate, glycerolphosphocholine, isoleucine, LDL CH₃-(CH₂)_n-, VLDL CH₃-(CH₂)_n-, lipid -CH₂-CH=CH-, lipid -CH₂-C=O, lipid, -CH=CH-, lactate, leucine, lysine, N-acetyl glycoprotein, phenylalanine, succinate, tyrosine, and valine are noted.



Figures S3 Assessment of Metabolic Phenotype Homogeneity in Ischemic and Hemorrhagic Subjects. (A-B) A new generated OPLS-DA model constructed with 21 ischemic PSD samples and 25 ischemic stroke samples was used to predict the class membership of the 11 hemorrhagic PSD subjects and 10 hemorrhagic stroke subjects. All hemorrhagic PSD samples were mixed with ischemic PSD samples; 8 of the 10 hemorrhagic stroke samples were mixed with the ischemic stroke samples; (C) An OPLS-DA model using 21 ischemic PSD samples and 35 healthy control samples was constructed. This regenerated OPLS-DA model was used to classify the remaining 11 hemorrhagic PSD subjects. 9 of the 11 hemorrhagic PSD subjects were correctly classified; (D) Identical procedures were also used to classify the 10 hemorrhagic stroke subjects in the OPLS-DA model that was from the 25 hemorrhagic stroke subjects and 35 healthy controls. This analysis showed that all 10 hemorrhagic stroke subjects were correctly predicted.



Figures S4 Assessment of Metabolic Phenotype Homogeneity between Major PSD and Minor PSD Subjects. (A) The new OPLS-DA models were constructed to compare minor PSD subjects with stroke or healthy comparison subjects. (B) The reconstructed OPLS-DA models were used to predict the class membership of the remaining 14 major PSD subjects. 10 of 14 major PSD subjects were correctly predicted. (C-D) 12 of 14 major PSD subjects were correctly predicted by the OPLS-DA model generated from minor PSD subjects and stroke subjects.

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