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ORIGINAL RESEARCH

Metabolic Disturbances in a Mouse Model of MPTP/Probenecid-Induced Parkinson's Disease: Evaluation Using Liquid Chromatography-Mass Spectrometry

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Purpose: Parkinson's disease (PD) is a common neurodegenerative disease that severely affects patients' daily lives and places a significant burden on the global economy. There are currently no specific biomarkers for distinguishing between the different stages of PD.

Methods: We divided 78 mice into six equal groups, including five model PD groups (W1–W5; based on the PD stage induced by length of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/propofol induction time) and a control group. Then, we used metabolomics technology to detect the serum small-molecule metabolites present in each group. Ultimately, we screened for potential biomarkers using the variable importance in the projection of the orthogonal partial least squares discriminant analysis and the coefficient value of LASSO ordinal logistic regression.

Results: We identified 12 potential biomarkers, including dehydroepiandrosterone sulfate, pipecolic acid, N-acetylleucine, 2-aminoadipic acid, L-tyrosine, uric acid, and 5-hydroxyindoleacetaldehyde. Pathway analysis revealed their involvement in amino acid metabolism, caffeine metabolism, steroid hormone biosynthesis, and purine metabolism. Additionally, the receiver operating characteristic curve indicated that a biomarker panel comprising the 12 biomarkers could differentiate between the different PD stages.

Conclusion: Different PD stages are characterized by different metabolites. The biomarkers identified in this study are helpful to understand the PD process.

Keywords: Parkinson's disease, metabolomics, biomarkers, metabolic disturbances, LC–MS

Introduction

Parkinson's disease (PD), the second most common chronic neurodegenerative disease after Alzheimer's disease, is a multisystem disorder with multiple mechanisms and neurochemical features. It affects 2% of people aged >65 years and 4% of people aged >80 years.[1–3] The number of people with PD is expected to reach 12 million by 2050[4], and this increased incidence may cause high economic and social burdens. PD is a multifactorial and sporadic disease. Its highly complex pathogenesis has not been fully elucidated and may be related to mitochondrial dysfunction, oxidative stress, and the inflammatory response.[5] The diagnostic criteria for PD include the recognition of specific clinical symptoms that are already evident in the patient, usually several years after the neurodegenerative process has occurred. Additionally, even if the diagnostic criteria are correctly applied, the rate of PD misdiagnosis is high for some nonspecific clinical symptoms. Although positron emission tomography and single-photon emission computed tomography imaging are highly sensitive and can be used for imaging diagnosis of PD, they are not specific to PD, are costly, and carry the risk of

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radiation exposure. Therefore, it is crucial to investigate the mechanisms and evolution of PD and to search for PD biomarkers.[6]

Metabolomics is the science of quantitatively measuring the composition of all metabolites of a biological system (cell models, tissues, organs, or whole organisms), usually small-molecule metabolites with relative molecular masses <1000, as well as dynamic alterations in these metabolites in response to internal and external stimuli.[7] This rapidly developing emerging discipline follows the advent of genomics, transcriptomics, and proteomics and has been widely used in various fields, such as nutrition, toxicology, and disease diagnosis.[8] Improvements in high-resolution mass spectrometry technology have led to the increasingly extensive application of metabolomics technology in clinical medicine, especially in screening for disease biomarkers and intrinsic regulatory mechanisms. Metabolites play active regulatory roles in systems biology, and metabolomics tools provide a direct functional readout of an organism's physiological state, which is difficult to obtain using other histological methods. Thus, the resultant metabolic profile is a complete description of the organism's phenotype.[9] In recent years, the development of analytical and bioinformatic tools has resulted in the exponential growth of metabolomics research. Further studies, such as the functional analysis of metabolites, may lead to the discovery of relevant bioinformation, such as proteins and genes upstream of the metabolite, which, in turn, can be linked to other histological studies.

Liquid chromatography-mass spectrometry (LC-MS)-based metabolomics is a powerful tool for analyzing metabolite changes and has been used to decipher metabolic reprogramming in many disease types, including neurodegenerative diseases.[10–12] In this study, we used untargeted metabolomics technology to screen five established groups of PD model mice and a healthy control group for serum metabolites that may be potential biomarkers of early PD and or progression biomarkers that are produced in response to the changes in the different PD stages.

Material and Methods

Construction of the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)/Probenecid Mouse Model

We purchased 78 male 10-week-old C57BL/6 mice from Shanghai SLAC Animal Laboratory Animal Co., Ltd. and housed them under a 12/12 h light/dark cycle (23°C ± 2°C, 45% ± 5% humidity). The experimental treatment of the mice complied with the ethical code of the Second Affiliated Hospital of Soochow University.

The 78 mice were divided into six groups (n = 13/group), including one control group and five experimental groups with PD at different stages. After acclimating the mice to the laboratory environment, all mice in the experimental groups were subcutaneously injected with 2 mg/mL MPTP (Sigma, cat # MO896) in saline and then intraperitoneally injected with 250 mg/mL probenecid (Aladdin, cat# p129440) 1 h later.[13] The above treatment procedure was performed twice a week (3.5 d intervals) for 1–5 weeks, depending on the PD group. Mice with early, middle-stage, and advanced PD were euthanized at predetermined ages. During weeks 1–5 after the establishment of the PD model, blood samples were taken from each mouse group. The samples were centrifuged at 3500 × *g* for 5 min to obtain the supernatant serum, which was then removed, divided between two Eppendorf tubes, and stored at −80°C for untargeted metabolomics analysis.

Blood Sample Collection and Pre-Treatment

Serum samples stored at −80°C were removed and thawed. Then, 100 μL of serum was placed in a 1.5 mL Eppendorf tube, 400 μL methanol(containing approximately 200 ng/mL of 2-chlorophenylalanine as an internal standard) was added, and the mixture was vortexed for 5 min, followed by centrifugation for 10 min at 13000 rpm. Finally, the supernatant (containing the isolated metabolites) was removed and placed in an injection vial. The entire process was performed on ice. Quality control (QC) samples were made from a mixture of all samples and processed in the same manner as the samples.

UPLC-MS/MS Analysis

Ultra-performance liquid chromatography (UPLC) was performed on an UltiMate 3000 UPLC system (Dionex Corp., USA) in tandem with a high-resolution Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA) using

ACQUITY UPLC HSS T3 columns (2.1 × 150 mm, 1.8 μm; Waters Corp, Milford, MA, USA). The samples were measured randomly using the protocol described by Want et al[14] The column temperature was 40°C, and the flow rate was 0.25 mL/min. The detailed gradient elution conditions and optimal mass spectrometry parameters are presented in the supplementary materials (Table S1 and S2). The loading volume for each sample was 5 μL, and the other liquid phase parameters were the same as previously described.[15] During sample LC-MS analysis, we interspersed one QC sample in every five mouse serum samples for subsequent quality judgment of metabolomics data and data preprocessing.

Metabolite Identification and Differential Metabolite Screening

Compound Discoverer 3.3 software was used for the automatic extraction and preliminary identification of mass spectrometry raw data. A matrix containing information such as the mass-to-charge ratio, substance name, retention time, and peak area was obtained. Firstly, we found the identified internal standard substances and calculated the RSD of the peak area of the internal standard(2-chlorophenylalanine), which was less than 10% in this study, and consequently, we deleted the substances with RSD greater than 30% from the identified substances in QC. Subsequently, we used the Human Metabolome Database (http://www.hmdb.ca/) to perform secondary identification and corroboration of the identified substances based on the sample information. Ultimately, we identified 201 serum metabolites. Detailed substance information is provided in the supplementary materials (Table S3).

Differential Metabolite Analysis

We used R 4.2.2 software for principal component analysis (PCA) and heatmap construction. The orthogonal partial least squares discriminant analysis (OPLS-DA) model was constructed using the ropls package in R.[16] Each group (negative control [NC] and W1–W5) was transformed as an ordinal variable in the OPLS-DA model. The LASSO ordinal logistic regression (LOLR) model was constructed using the glmnetcr package in R.[17] The feature selection in LOLR was performed based on the base peak chromatogram (BPC) criteria. The performance of the LOLR model was evaluated using the receiver operating characteristic (ROC) curve. Pathway analysis was performed using MetaboAnalyst 5.0 software (http://www.metaboanalyst.ca).

Results

Successful Modeling

A total of 78 C57BL/6 mice were used in this study. They were divided into six groups: an NC control group and five PD model groups. These PD groups represented increasingly severe PD stages due to different lengths of MPTP/propofol induction time on the hippocampal tissues of PD mice as follows: W1. early PD; W2, transition from early development to middle-stage PD; W3, middle-stage PD; W4, transition from middle-stage to advanced PD; and W5, advanced PD. Due to the death of two mice during the modeling process, data from 76 mice were analyzed (NC, 12; W1, 13; W2, 13; W3, 13; W4, 12; and W5, 13).

Metabolomics Profiling

We assessed the quality of various aspects of the metabolomics data prior to data analysis. We obtained BPC plots with good peak shapes, and the classical BPC plots for each group are shown in Figure 1A and B. Additionally, the percentage of variables with an RSD greater than 30% in the QC sample was less than 20% of the total variables. Finally, we evaluated QC clustering using PCA plots. In metabolomics studies based on LC–MS technology, reliable and high-quality data can only be obtained if analytical errors do not affect the results of multivariate analysis. Thus, the stability of the LC–MS system needs to be evaluated, and this is achieved by running QC samples throughout the sample analysis process.[18] The PCA model showed QC samples clustered together and near the far point of the coordinate axis, indicating that there were few systematic errors during sample processing and detection and that the metabolomics method design was stable Additionally, there was a definite trend of separation between the normal group and the various PD developmental stages (Figure 1C).

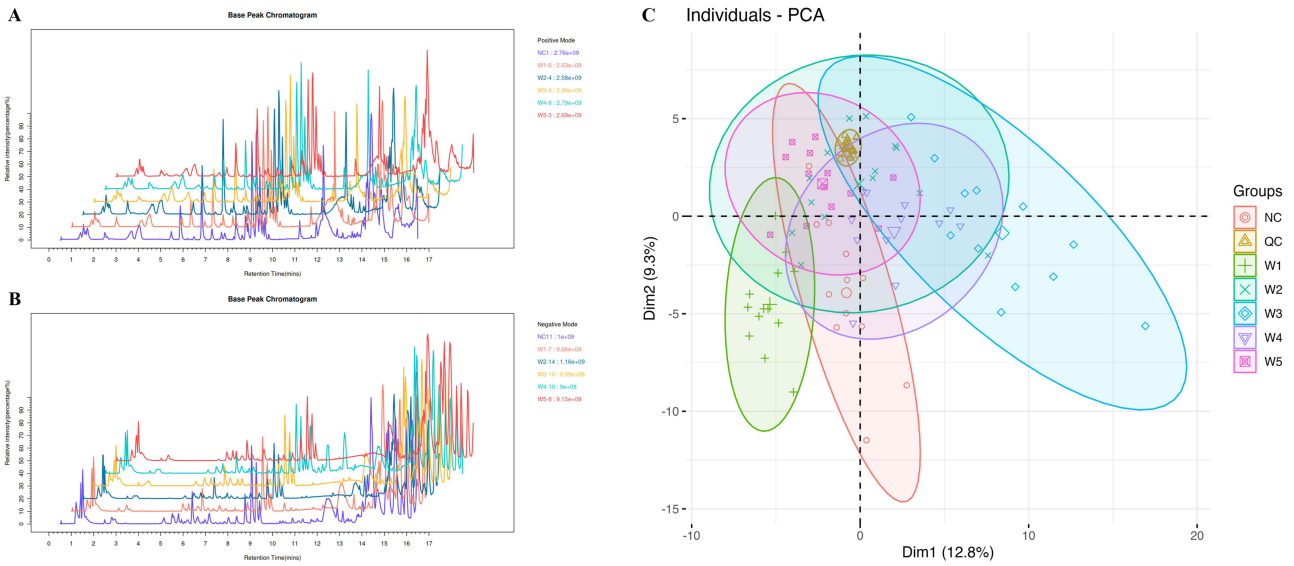

Figure 1 Representative BPC plots and PCA plots for the six groups. (**A**) Representative BPC plots for each group in positive ion mode. (**B**) Representative BPC plots for each group in negative ion mode. (**C**) PCA plots for six groups and QC samples.

The OPLS-DA model is a supervised model that can be used for screening for differential metabolites. As seen in the OPLS-DA model of this study, the distinction between the NC group and the PD model groups was clearly evident, and there was a definite trend in the horizontal coordinate from W1 to W5 (Figure 2A).

Serum Biomarker Screening

We defined the developmental progression of the PD model mice on a scale of 0–5, where 0 represented the healthy control group, and 1–5 represented the progressive severity of PD. The biomarkers were screened as variable importance in the

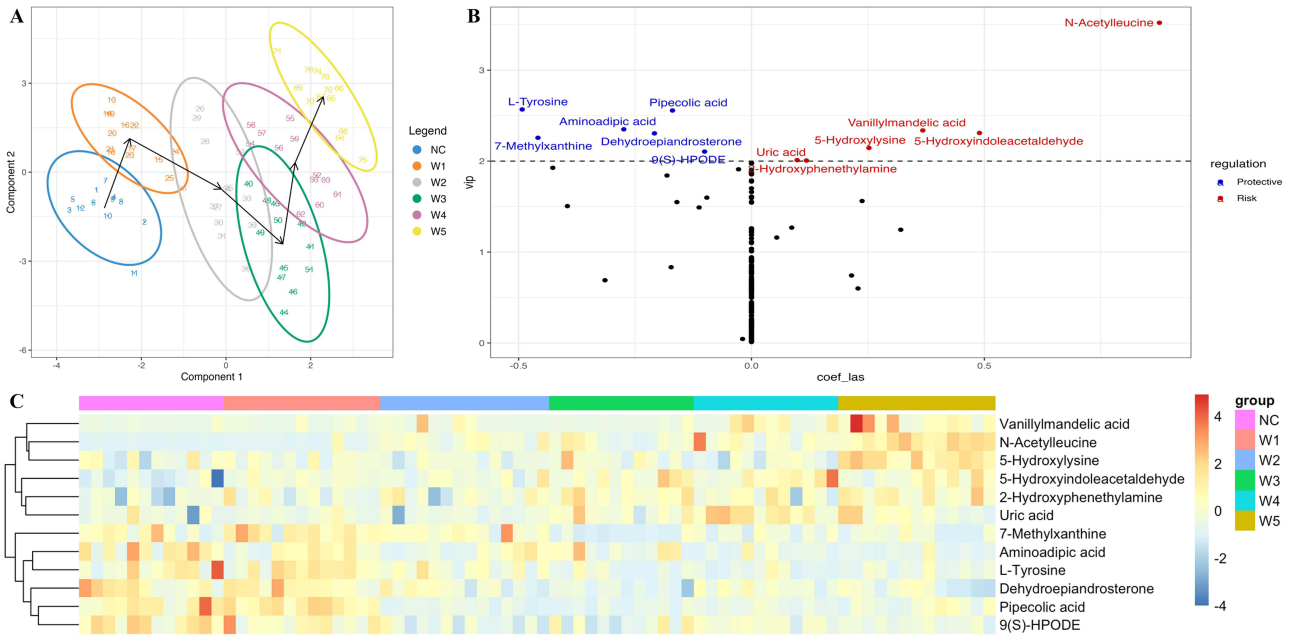

Figure 2 Different PD stages have different metabolic characteristics. (**A**) OPLS-DA plot of the six mouse groups. (**B**) Scatterplot of variable importance in the projection (VIP) values in the OPLS-DA model plotted against the coefficients of LOLR. Biomarker screening was based on the principles of VIP values >2 and coefficient values ≠ 0. Red represents risk factors, and blue represents protective factors. (**C**) Heatmap of the 12 serum biomarkers for PD in the six groups. Blue represents decreasing levels, and red represents increasing levels.

projection >2 in OPLS-DA and |coefficient|>0 in LOLR, and the visualization diagram is shown in Figure 2B, where red represents risk factors and blue represents protective factors. We ultimately identified 12 differential metabolites, including pipecolic acid (PA), L-tyrosine, uric acid, vanillylmandelic acid (VMA), 5-hydroxylysine, 2-aminoadipic acid (2-AAA), dehydroepiandrosterone sulfate (DHEAS), 2-hydroxyphenethylamine, 7-methylxanthine, 5-hydroxyindoleacetaldehyde (5-HIAL), 9(S)-HPODE, and N-acetylleucine (NALL) (Table 1).

Temporal Analysis of Biomarker Trends

We used MetaboAnalyst 5.0 to plot the heatmap representing the different stages of PD. The results revealed that some of the serum biomarkers showed an upward trend and some showed a downward trend from the early to advanced PD stages (Figure 2C). We plotted the trend of change for the 12 PD biomarkers to understand their trends in the different PD stages (Figure 3). 2-Hydroxyphenethylamine, NALL, 5-HIAL, and VMA showed a clear increasing trend during PD progression, whereas 2-AAA and L-tyrosine showed a clear decreasing trend during PD progression. Interestingly, DHEAS and uric acid showed an increasing and then decreasing trend, whereas PA, 7-methylxanthine, and 9(S)-HPODE showed a decreasing and then increasing trend.

ROC Curve Analysis

Through metabolomics analysis combined with machine learning, we identified potential biomarkers that changed according to the PD stage. ROC curves are widely used to evaluate the sensitivity and specificity of biomarkers and to visualize the relationship between sensitivity and specificity.[19] The area under the curve corresponding to each ROC curve is a determination of the diagnostic efficiency of the biomarker, and the larger the area, the higher the value.[20] To understand the processes of PD, we constructed ROC curves using the 12 PD biomarkers. We found this biomarker panel helped us to understand the PD processes (Figure 4).

Metabolic Pathway Analysis

We used MetaboAnalyst 5.0 to analyze the potential metabolite data obtained above by searching for metabolic pathways in which the metabolites may be involved. Pathway analysis of potential markers revealed that three of the ten pathways were differential metabolic pathways, namely lysine degradation, phenylalanine, tyrosine and tryptophan biosynthesis,

Table 1 Detailed Information on the Potential Biomarkers

HMDB ID	Biomarker	Chemical formula	VIP value	Coefficient value
HMDB0000070	PA	$C_6H_{11}NO_2$	2.57	−0.49
HMDB0000158	L-Tyrosine	$C_9H_{11}NO_3$	2.57	−0.49
HMDB0000289	Uric acid	$C_5H_4N_4O_3$	2.01	0.10
HMDB0000291	VMA	$C_9H_{10}O_5$	2.34	0.37
HMDB0000450	5-Hydroxylysine	$C_6H_{14}N_2O_3$	2.14	0.25
HMDB0000510	2-AAA	$C_6H_{11}NO_4$	2.35	−0.27
HMDB0001032	DHEAS	$C_{19}H_{28}O_5S$	2.31	−0.21
HMDB0001065	2-Hydroxyphenethylamine	$C_8H_{11}NO$	2.01	0.12
HMDB0001991	7-Methylxanthine	$C_6H_6N_4O_2$	2.26	−0.46
HMDB0004073	5-HIAL	$C_{10}H_9NO_2$	2.31	0.49
HMDB0006940	9(S)-HPODE	$C_{18}H_{32}O_4$	2.1	−0.10
HMDB0011756	NALL	$C_8H_{15}NO_3$	3.52	0.88

Notes: For convenience of viewing, substances are numerically sorted according to their HMDB ID.

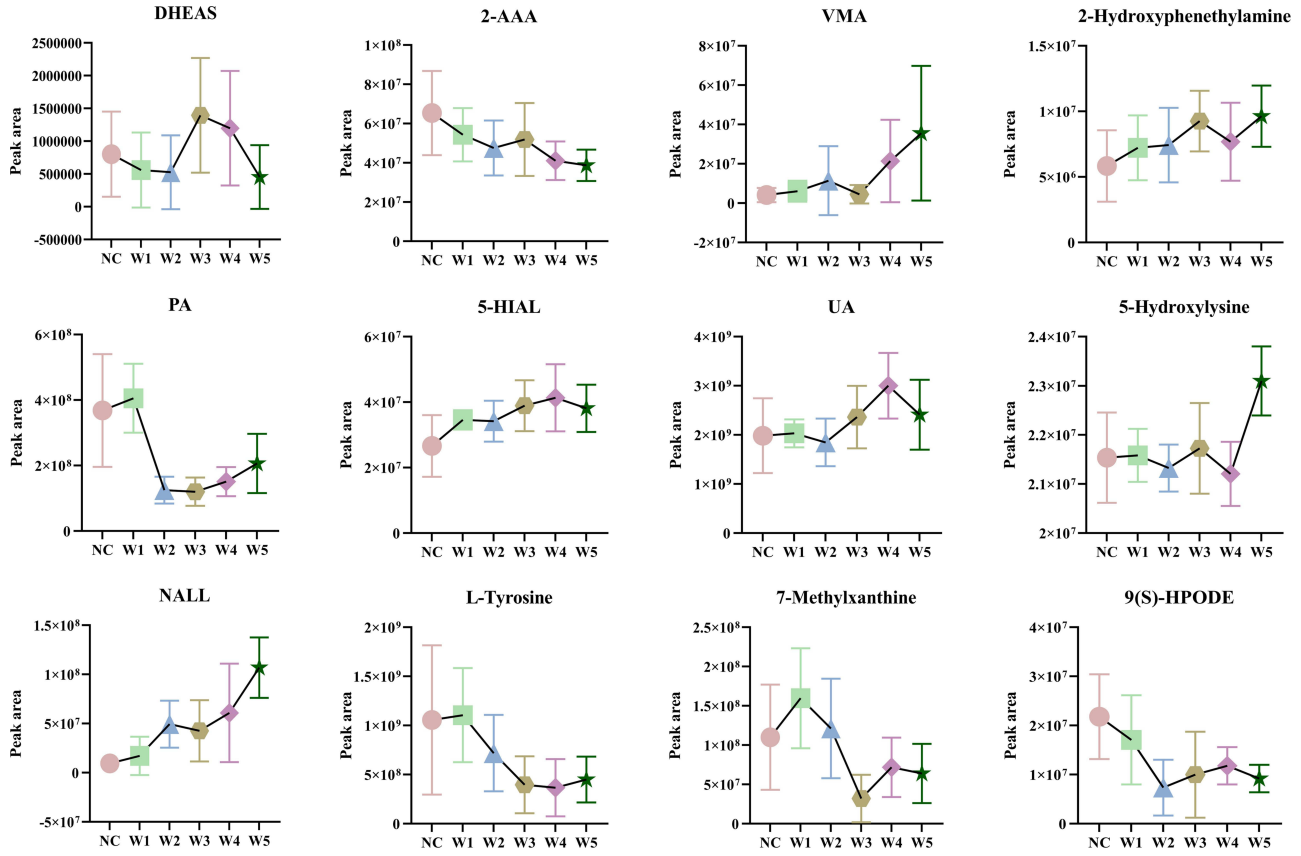

Figure 3 Trends of the 12 PD biomarkers identified in the six groups.

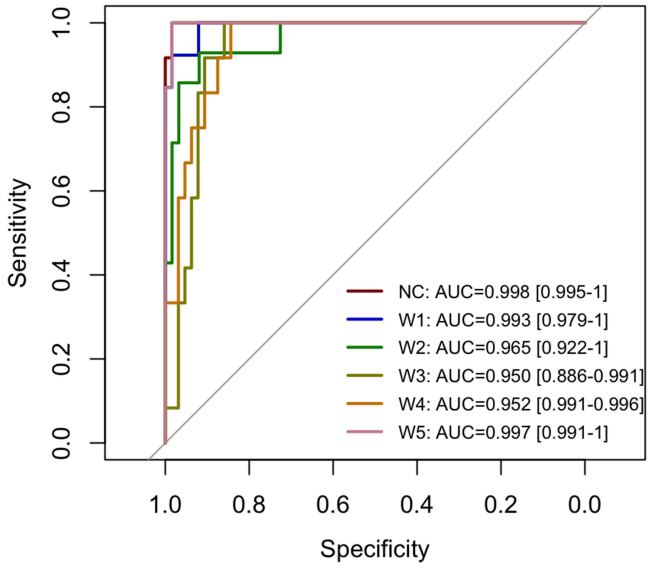

Figure 4 ROC curve of a biomarker panel composed of the 12 PD biomarkers.

and tyrosine metabolism (Figure 5). The detailed results and parameters of the pathway analysis are presented in the supplementary materials (Table S4).

Discussion

Despite the advances in elucidating PD pathogenesis and pathophysiology, no clinical markers have been identified that are 100% reliable for PD detection in clinical practice, and neurologists continue to rely on clinical expertise to diagnose

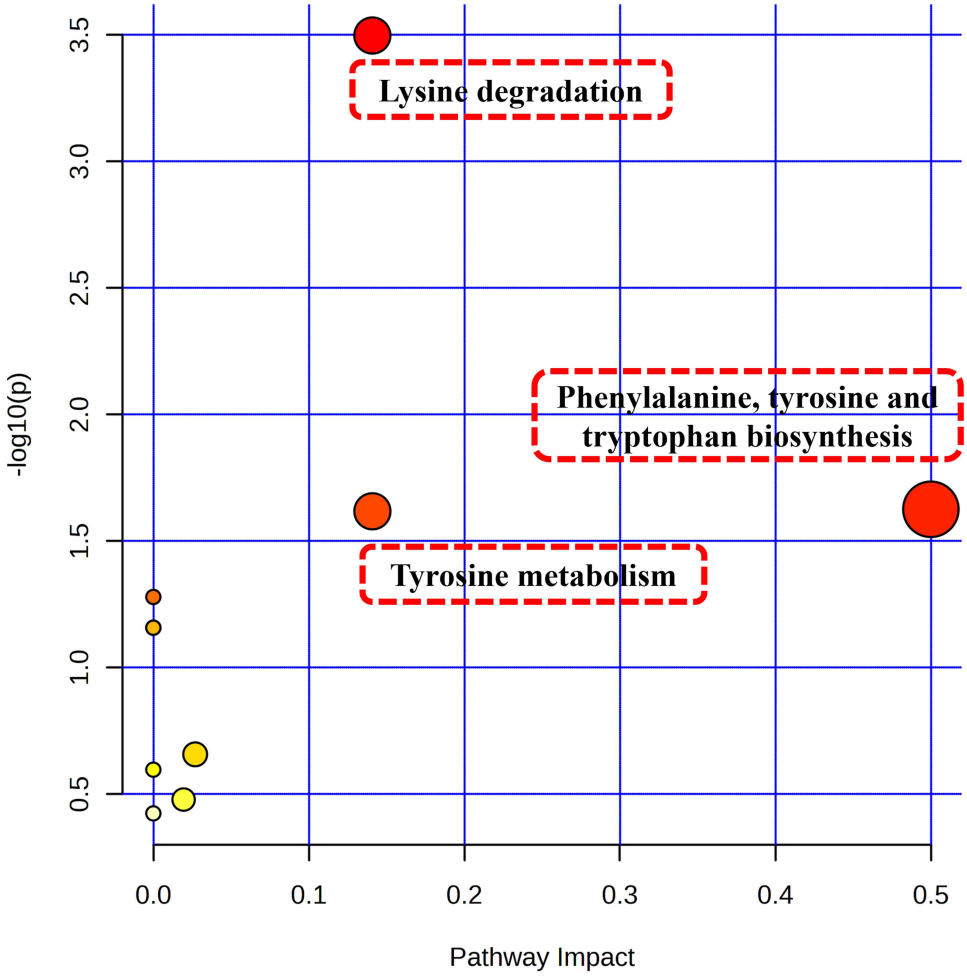

Figure 5 Plot of metabolic pathway for the 12 PD biomarkers.

PD. This is an urgent issue that must be addressed by biomarker researchers in the coming years.[21] Emerging evidence indicates that peripheral alterations, including metabolic dysregulation, may precede and contribute to neurodegeneration.[10,22–24] Thus, distinguishing patients with PD from healthy individuals and distinguishing molecular networks at various stages of PD development may lead to new insights into PD pathogenesis and the identification of key biomarkers. In this study, we constructed mouse models of PD at different stages, and with the help of metabolomics and machine learning technology, screening revealed 12 potential biomarkers capable of distinguishing between the different PD stages. Additionally, pathway analysis of the biomarkers revealed several probable metabolic pathways that might lay the foundation for subsequent investigations of PD mechanisms.

Aging is the most important risk factor for the development of neurodegenerative diseases, most of which typically manifest in the elderly.[25] PD is the second most common age-related neurodegenerative condition, and its incidence increases with age.[26–28] DHEAS is an endogenously produced sex steroid with reported antiaging effects.[29] It has been reported in the literature that DHEAS not only modulates astrocyte function, reduces inflammation, and activates cell signaling survival pathways, but also activates the sigma 1 receptor and agonists of this receptor have neuroprotective effects in animal models of PD.[30] DHEAS levels are higher in men than in women, which may be due to the association between testosterone and the maintenance of DHEAS concentrations.[31] Moreover, DHEAS concentrations are associated with cognitive levels in older men but are unlikely to play a functional role in cognitive decline.[32] Our results revealed that DHEAS showed a trend of increasing and then decreasing during PD progression, suggesting that DHEAS exhibited compensatory dysregulation during early-stage PD. However, our study only used male mice, so the gender difference

could not be compared. Thus, future clinical patient population studies on PD should focus on the influence of gender on changes in DHEAS levels.

Metabolic disorders of amino acids are key in PD.[33–35] Potential mechanisms of tryptophan metabolism-mediated neurodegeneration include proteotoxicity via a tryptophan-dependent mechanism, excitotoxicity due to the accumulation of neurotoxic tryptophan metabolites, and energy imbalance resulting from NAD^+ depletion.[36] In this study, we screened a variety of biomarkers related to amino acid metabolism, including PA, 2-AAA, NALL, 5-HIAL, and L-tyrosine, and found that these biomarkers were involved in lysine degradation and the metabolism of tyrosine, phenylalanine, and tryptophan. These phenomena suggest, to some extent, the disturbance of amino acid metabolism in PD.

Several studies have revealed the association of amino acid metabolism with disease. PA is an important biomarker for the diagnosis of peroxisomal diseases, a contributing factor to hepatic encephalopathy, and a possible biomarker of pyridoxine-dependent epileptic seizures.[37] Furthermore, plasma PA levels were elevated in a mouse model of malaria.[38] It has also been proposed that PA, a biological byproduct, slows the progression of diabetic retinopathy by inhibiting the YAP–GPX4 signaling pathway.[39]

2-AAA is produced by lysine degradation,[40] and plasma levels of 2-AAA may be partly regulated by common variants in genes related to mitochondrial and macrophage function. Additionally, elevated plasma levels of 2-AAA have been associated with reduced levels of high-density lipoprotein cholesterol.[41] Our results showed a gradual decrease in serum levels of 2-AAA in mice during PD progression, which is in contrast to the high plasma levels of 2-AAA in PD patients in the study of PD by Molina et al[42] We hypothesize that this may be due to the fact that this literature deals with a small population and does not stratify patients with PD into early, intermediate, and late stages.

Tyrosine is involved in the tyrosine hydroxylase-dopamine (TH-DA) pathway, which has been reported to play an important role in PD pathogenesis. Zhou et al found that LRRK2 mutations up-regulate TH expression and DA levels at the early stage of disease and this leads to DA toxicity and facilitated DA neuron degeneration.[43] In subsequent years, they found that α-methyl-L-tyrosine (α-MT), a tyrosine hydroxylase (TH) inhibitor, was able to reverse the pathologies in human neurons and TG Drosophila models.[44] Those findings provide support for potential clinical trials using the TH–DA pathway inhibitors in early or prodromic PD.

Oligomerized alpha-synuclein is thought to be pathogenic in PD, Jinsmaa et al reported that 5-HIAL had the same ability to induce polyribonuclein oligomerization as 5-hydroxytryptophan in PC12 cells overexpressing polyribonuclein.[45] Our results showed that 5-HIAL levels showed a gradual increase during the progression of PD, which to some extent suggests a relationship between the in vivo levels of 5-HIAL and the development of PD.

The abundance of Desulfobacterota in the gut and inflammasomes (NLRP3, ASC, caspase-1, and IL-1β) in the brains of depressed rats significantly decreased after receiving fecal microbiota transplantation from healthy rats.[46] These suggest that Parkinson's disease may be associated with dysbiosis of the microbiota and that Desulfovibrio phylum may be the causative agent leading to its development. Xu et al found that NALL, which is negatively correlated with Desulfovibrio, can effectively inhibit pro-inflammatory factors, and the number of DA neurons in mice significantly increased and motor deficits significantly improved after oral administration of NALL, thus speculating that NALL may inhibit Desulfovibrio through the gut-brain axis pathway, thereby promoting the repair of DA neuron.[47] In addition, NALL has been reported to improve functional recovery and attenuate cortical cell death and neuroinflammation after traumatic brain injury in mice.[48] These findings seem to indicate that elevated NALL appears to be a protective factor, but our stratified study of PD found that NALL was higher in the serum of mice with advanced PD, which appears to be a risk factor, and this result, which contradicts previous studies, deserves to be further explored.

Our screening identified uric acid as a PD biomarker. Due to its antioxidant properties, uric acid has been hypothesized to exert neuroprotective effects,[49] and results from a prospective study revealed an association between low levels of serum uric acid and motor function deterioration in patients with early-stage PD.[50] However, a recent randomized trial did not support this association.[51] Furthermore, we found that uric acid levels in our PD model mice did not show a simple upward or downward trend in the developmental stages of PD. Thus, further studies are necessary to clarify the role of uric acid in PD.

Analysis of the 12 PD biomarkers' ROC curves helped us understand the processes involved in early, middle, and advanced PD stages. Temporal analysis of trends in these biomarkers revealed different metabolic disturbances in

metabolites during different PD stages. Through the pathway analysis of 12 serum markers, the 12 markers that we identified are involved in caffeine metabolism, steroid hormone biosynthesis,[33] and purine metabolism,[52] These metabolic pathways provide new ideas for future mechanistic studies. However, we have not yet found any study proposing a clear relationship between the whole pathway and PD, which deserves further and deeper studies by researchers to connect metabolites with pathway analysis. This is the next step we will take in the clinical study of PD.

Conclusion

In summary, we established chronic MPTP/propofol-induced early, intermediate, and late PD mouse models, and used metabolomics technology to detect small molecule metabolites in mouse serum, and found that different metabolite profiles existed at different PD stages. Subsequently, we established the OPLS-DA model and finally screened 12 potential serum markers in PD mice, including DHEAS, 2-AAA, 5-HIAL, and NALL. We found that these markers have different content levels at different stages of PD and that markers have different trends in different stages of PD and can be used for diagnostic staging of PD. Thus a panel consisting of these 12 markers helps to understand the developmental process of PD. In addition, we found that these markers are involved in amino acid metabolism, caffeine metabolism, steroid hormone biosynthesis, and purine metabolism. All of these findings lay the foundation for our future mechanistic and clinical studies of Parkinson's disease.

Abbreviations

2-AAA, 2-aminoadipic acid; 5-HIAL, 5-hydroxyindoleacetaldehyde; BPC, Base peak chromatogram; DHEAS, dehydroepiandrosterone sulfate; LASSO, Least absolute shrinkage and selection operator; LOLR, LASSO ordinal logistic regression; HMDB, Human Metabolome Database; NALL, N-acetylleucine; NC, Negative control; NAD, Nicotinamide adenine dinucleotide; PA, Pipecolic acid; PCA, Principal component analysis; PD, Parkinson's disease; QC, Quality control; ROC, Receiver operating characteristic; RSD, Relative standard deviation; UPLC, Ultra performance liquid chromatography; VMA, vanillylmandelic acid; VIP, Variable importance in the projection.

Ethical Approval and Informed Consent

The research has the approval of the Local Ethics Committee of The Second Affiliated Hospital of Soochow University.

Consent for Publication

We further confirm that the content has not been published or submitted for publication elsewhere.

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Disclosure

The authors report no conflicts of interest in this work.

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