Published in "Journal of Chromatography B 1081–1082: 101–108, 2018"
which should be cited to refer to this work.

Potential biomarkers of Parkinson's disease revealed by plasma metabolic profiling

Huiyuan Zhao[a], Cui Wang[b], Nan Zhao[a], Wenxue Li[c], Zhaofei Yang[d], Xinxin Liu[a], Weidong Le[d,*], Xiaozhe Zhang[a,*]

[a] CAS Key Laboratory of Separation Sciences for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, China
[b] Department of Neurology, Dalian Central Hospital, Dalian, China
[c] Department of Medicine, University of Fribourg, 1700 Fribourg, Switzerland
[d] Center for Clinical Research on Neurological Diseases, The First Affiliated Hospital of Dalian Medical University, Dalian, China

Keywords:
Parkinson's disease
Plasma
Metabolomics
Metabolites
Pathway

The plasma of Parkinson's disease (PD) patients may contain various altered metabolites associated with the risk or progression of the disease. Characterization of the abnormal metabolic pattern in PD plasma is therefore critical for the search for potential PD biomarkers. We collected blood plasma samples from PD patients and used an LC-MS based metabolomics approach to identify 17 metabolites with significantly altered levels. Metabolic network analysis was performed to place the metabolites linked to different pathways. The metabolic pathways involved were associated with tyrosine biosynthesis, glycerol phospholipid metabolism, carnitine metabolism and bile acid biosynthesis, within which carnitine and bile acid metabolites as potential biomarkers are first time reported. These abnormal metabolic changes in the plasma of patients with PD were mainly related to lipid metabolism and mitochondrial function.

1. Introduction

Parkinson's disease (PD) is a long-term degenerative disorder of the central nervous system (CNS) characterized by the motor symptoms such as shaking, rigidity, slow movement, and difficulty walking [1]. The cause of the motor symptoms is the death of dopaminergic brain neurons in the brain substantia nigra. The etiology of PD is generally believed to involve multiple factors, both genetic and environmental, while the pathogenic mechanism may be related to protein aggregation, immune inflammation, oxidative stress, and mitochondrial dysfunction [2,3]. The diagnosis of PD is largely based on the presence of motor features. However, even when the clinical criteria for PD diagnosis are strictly applied by movement disorder specialists, they only have a positive predictive value of 85.7% in advanced disease, and much lower at early stages [4]. The accuracy of early (duration < 5 years) PD diagnosis based on neuropathologic findings is a mere 53% [5]. There has been an increased interest in earlier intervention with various forms of exercise in addition to pharmacological treatments [6], such as gene target [7] and neuroinflammatory intervention [8]. The inability to accurately diagnose PD before the motor symptoms develop results in a loss of valuable intervention time. Therefore the development of reliable diagnostic and prognostic markers of PD, including new biochemical markers, is urgently required [9].

Identification of biomarkers for PD is an important step towards improving the current diagnostic criteria. Additionally, biomarkers could provide insights into the disease mechanisms, which in turn could be used to identify aberrant biochemical pathways and therapeutic targets for new efficacious medications [10,11]. Previous studies have used two strategies for biomarker discovery, targeted analysis and unbiased analysis. Targeted analysis was used to identify uric acid, involved in the oxidative stress pathway [12–14], and tryptophan metabolites, related to energy metabolism [15,16], as biomarkers of PD. Unbiased analysis has been increasingly utilized in metabolomics-based studies of disease mechanism since 2008 [17–24]. Not only does this approach study multiple pathways and quantify small molecules and their metabolites at the same time, it also offers high sample resolutions, rapid rates of analysis, and the ability to detect dynamic changes and regular patterns [25,26]. Thus, a metabolic analysis revealed a general alteration in the *N*-acetylation of amino acids in the cerebrospinal fluid (CSF) that was associated with excitotoxicity and oxidative stress in the pathogenesis of PD [27]. This alteration was also found in the serum of the rapidly progressing PD patients [28]. Several

Abbreviations: PD, Parkinson's Disease; UPLC, ultra-high performance liquid chromatography; TOF-MS, time of flight-mass spectrometry; RSD, relative standard deviation; PLS-DA, Partial least squares discriminant analysis; VIP, variable importance in projection; ROC, receiver operating characteristic; QC, quality control

* Corresponding authors.
E-mail addresses: wdle@sibs.ac.cn (W. Le), zhangxz@dicp.ac.cn (X. Zhang).

amino acids (e.g. tryptophan, histidine, tyrosine), fatty acids with 5 to 22 carbons, and redox metabolites levels were found to be altered in the plasma or CSF of patients with PD [29–31]. Among urinary biomarkers, steroidogenesis metabolites other than amino acids were found to be abnormally regulated in PD [17,32]. Biomarkers identified in clinical samples can be validated in animal models [33–35]. However, most of the biomarker findings described above were ultimately based on the currently known pathogenesis of PD with correlations among various altered metabolites needing further study.

Blood is the ideal sample for both biomarker discovery and clinical diagnose because its collection is simple relatively non-invasive, and it's easy to analyze. Entire, detailed metabolic phenotypes of organs beyond the CNS can be obtained from blood plasma that reflect the pathogenesis and pathophysiology of PD [30]. In present study, we sought to find novel PD biomarkers and pathways, and to investigate the correlations among them. We used peripheral blood plasma to screen for metabolic biomarkers in patients with PD to better understand the disease mechanism. We used liquid chromatography-mass spectrometry (LC-MS), a technique characterized by a sample preparation procedure that is simpler than that of gas chromatography-mass spectrometry (GC-MS) and a higher resolution compared to that of nuclear magnetic resonance (NMR). We also used dual statistical criteria to identify significantly altered metabolites level.

2. Material and methods

2.1. Clinical samples

The plasma samples were obtained at the Department of Neurology, Dalian Central Hospital, China. PD patients were diagnosed according to International Movement Disorders Society-sponsored Unified Parkinson's Disease Rating Scale (MDS-UPDRS) criteria. Subjects that served as controls were healthy people or patients free of PD or PD symptoms. Blood from 46 participants was all collected in the morning before patients took food or water, and immediately centrifuged to obtain plasma. Plasma samples were aliquoted and stored at −80 °C until LC-MS analysis. Forty-six plasma samples were collected, 18 of which were controls and 28 PD. According to Hoehn-Yahr (H-Y) staging in 1967 [36], there are 54% (15/28) in stage H-Y2, 39%(11/28) in stage H-Y1, 7%(2/28) in stage H-Y4. The female/male ratios were 7/11 for the controls and 12/16 for PD. The average ages were 64 for the controls and 70 for PD. The gender or age information between the two groups is comparable.

2.2. Sample preparation

For LC-MS analysis of small molecules in plasma, the first step of sample preparation is based typically on the removal of proteins via solvent precipitation [37]. We investigated two aspects of sample preparation, analysis mode, protein precipitation regents and, before conducting our analyses. With the positive MS collision mode, we obtained dense peaks and far more ions (7892) than with the negative mode (1408), is also proved by previous study that positive mode gives more adequate, as shown in reference article [28]. Positive mode shows a higher analysis efficiency because most of the plasma metabolites (amino acids, ammonia, glycerophospholipid, and so on) can be better ionized at positive mode, which is consistent with previous studies that applied positive mode for plasma metabolomics [38]. We therefore used the positive mode in all subsequent analyses. We also tested commonly used protein precipitation reagents including acetonitrile (ACN) [28,39,40] and methanol (MeOH) [41], which are organic reagents of low toxicity. ACN as a protein precipitation reagent performed better than MeOH: we did not achieve complete protein precipitation with MeOH, the consistent protein precipitation result was obtained by previous study [42]. Even though we obtained slightly more ions with MeOH than ACN, we chose to use ACN because any remaining soluble protein may interfere with the detection of small molecules. For each analysis included in this study, 400 µL of plasma was added to 800 µL of ACN, and immediately vortexed for 2 min, followed by centrifugation at 16000 ×*g* for 30 min at 4 °C. The supernatant was transferred to a clean tube and lyophilized. The lyophilized powder was redissolved in 80 µL of 80% ACN (0.2% formic acid) and subjected to ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) analysis in the positive mode. The quality control (QC) sample was made by the mixture of 46 redissolved samples, with 5 µL from each.

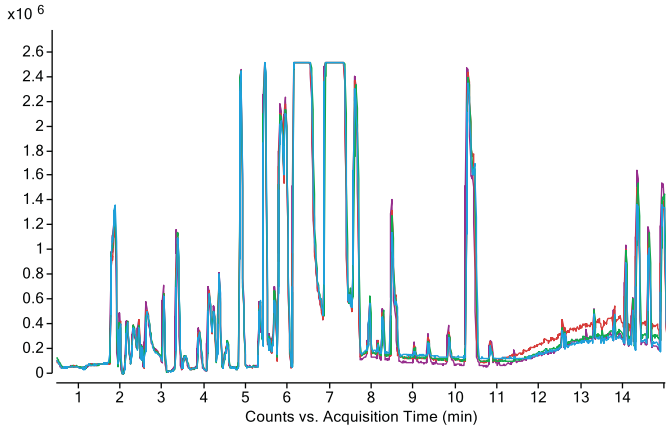

Fig. 1. Typical chromatograms of the LC-MS data for plasma metabolites.
The high repeatability can be observed in the chromatograms of four QC samples. Peaks are highly overlapped (the RT drift of peaks are less than 0.5 min.), which shows the stability of LC-MS system during this analysis procedure and the repeatability of all QC injections. (For interpretation of the references to colour in this figure, the reader is referred to the online version of this chapter.)

2.3. UPLC-Q-TOF-MS data acquisition

Each extracted sample was analyzed using an Agilent 1290 UPLC system coupled with an Agilent 6520 TOF-MS analyzer. For the UPLC-Q-TOF-MS analysis, a Zorbax Eclipse plus C18 column (3.0id ∗ 150 mm, 1.8 µm; Agilent Technologies, USA) with an on-line filter was used. The column temperature was maintained at 60 °C, and the injection volume was 5 µL. The separation was performed using a gradient program with water (solvent A, modified by the addition of 0.5% acetic acid) and ACN (solvent B). The pump flow rate was 0.3 mL/min with an initial solvent composition of 5% B. The gradient was conducted from 5% to 100% B over 15 min (eluting time), held at 100% B for 5 min for column washing, decreased to 5% B within 30 s, and held at 5% B for another 5 min for column balancing. First peak was eluted at 1.8 min, so the dwell volume of the system is 0.54 mL (0.3 mL/min × 1.8 min). To ensure repeatability of the measurements and stability of the instrument, QC sample was analyzed prior to the first sample injection, after every eight injections, and at the end of the experiment. The acceptance criteria of QC data repeatability were that the RT drift of every peak < 0.5 min in different QC injections, otherwise all samples will be re-analyzed when the QC data is outside of the criteria. 46 paticipants' samples were injected randomly; a repeated analysis cycle was conducted. Mass spectra were acquired in positive electrospray ionization mode according to our pilot experiment results. The optimal capillary voltage and the cone voltage were set at 3.5 kV and 40 V, respectively. The nebulization gas flow rate was set at 8 L/min, and the liquid nebulizer was set at 40 psi. The temperature was set at 350 °C. Data were acquired at a rate of 1 spectrum per second for MS and 3 spectra per second for MS/MS (centroid mode). The collision energy for MS/MS was 25 eV. The mass scan range was from 100 to 2000 for MS and 20 to 1000 for MS/MS. Chromatographic separation followed by full-scan MS was carried out to record the RT (retention time) and *m/z* of all

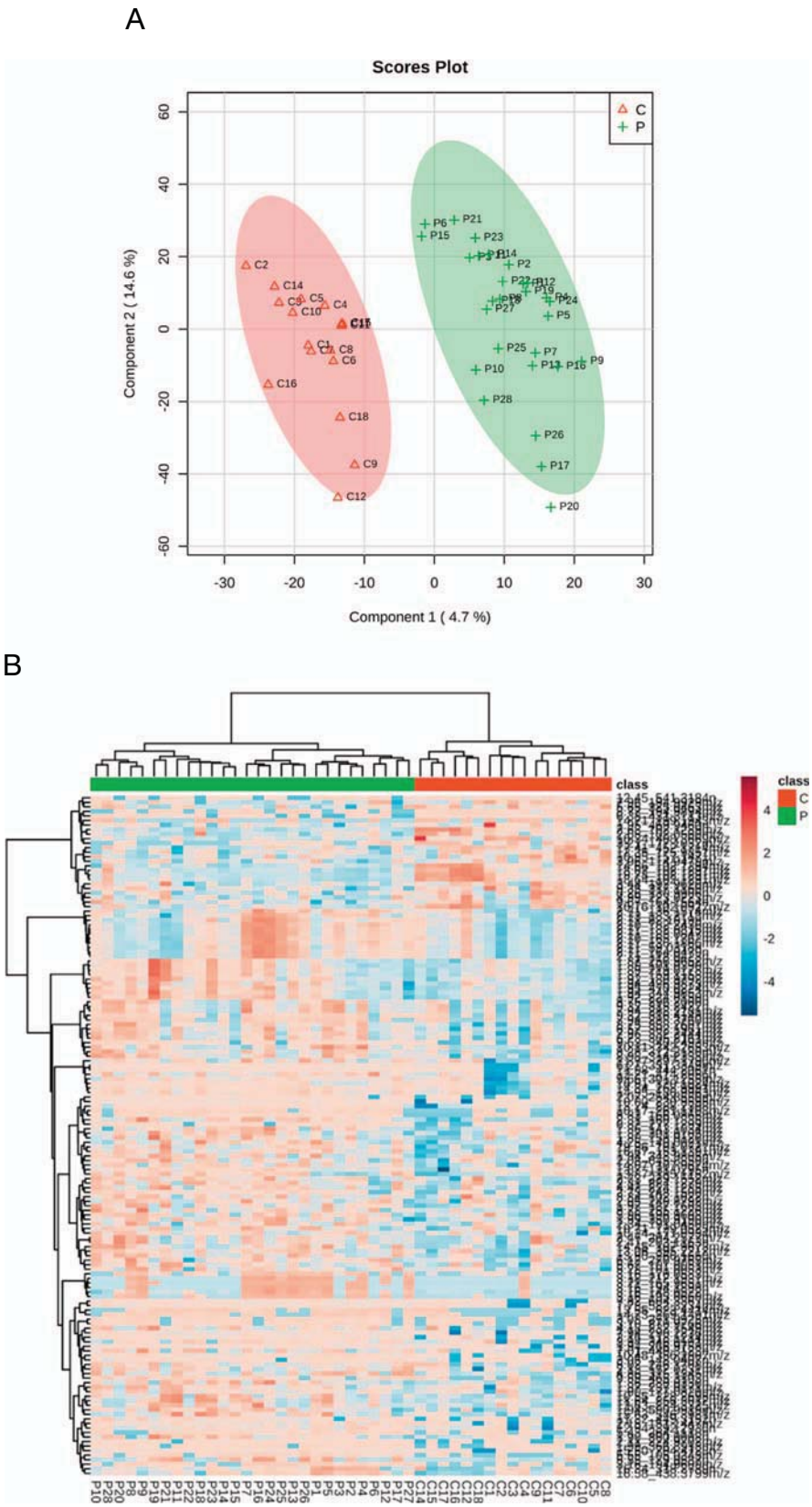

Fig. 2. Metabolomics analysis of samples that contain PD and control.
PLS-DA analysis shows that the imported ions grouped clinical samples into two groups PD (green) or control (red) (Fig. 1A) very well, which indicates that there are metabolites changed or disordered in PD plasma compared to control. Heat map (Fig. 1B) get the validated result with PLS-DA. The cell colour represent the ion abundance or the metabolite level in a sample, it is high abundance to low abundance from red to blue. And the group colour at the top of the figure represents the sample belong to PD (green) or control (red). The set of t-test ions entirely separates PD (green) and control (red), which indicates the metabolic model is successful, thus confirming that there are metabolites changed or disordered in PD plasma compared to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the online version of this chapter.)

detectable ions presented in the samples.

2.4. Data analysis and compounds' identification

Peaks in the raw UPLC-Q-TOF-MS data were aligned and extracted with the ProgenesisQI software (Nonlinear Dynamics, Newcastle, UK). The raw UPLC-Q-TOF-MS data file was imported with the filter strength set at 0.3. The ions with absolute abundance lower than 1000 were discarded, as were any ion before 0.5 min and after 15 min. An excel file containing LC and MS information for each sample was exported after

completing a section. Ions in samples were also controlled by QC before conducting metabolomics analysis. We calculated the RSD value of the intensity of each ion in all QC data, only ions with relative standard deviation (RSD) value < 50% in QC samples were considered to be stable in the whole analysis procedure. Otherwise ions whose RSD in QC were larger than 50% were discarded in all experimental data. Then the ions were browsed into MetaboAnalyst 3.0 [43] after conversion to the comma-separated values (CSV) file format. Statistical analysis was conducted after preliminary processing consisting of three steps in MetaboAnalyst 3.0 software. The first step was missing value estimation and filtration. The assumption of this approach is that most missing values are caused by low abundance metabolites, while too many missing values will cause difficulties for downstream analysis. Our setting is removing the features with missing values in > 80% of the samples. And the remaining missing values will be replaced by a small value (half of the minimum positive value in the original data). Next, data was filtered by its RSD with a default set of the software system. Finally, samples were normalized by Pareto scaling to make features more comparable with log transformation. Pareto-scaling is a classical data normalization mode for metabolomics analysis [44], which is calculated using mean-centred and divided by the square root of standard deviation of each variable. Partial least squares discriminant analysis (PLS-DA) and variable importance in projection (VIP) scores were computed to determine how well the PD and control groups were classified by the principal components.

In a search for abnormal metabolites, any ion that passed the *t*-test and had a VIP value larger than 1 was determined to be a candidate ion. A heat-map was calculated to distinguish the PD and control groups as a model. The pathway analysis tool was applied to obtain the pathway weight distribution picture and scores. Identification of the biochemical was achieved through comparison of the ionic features with those in the Metlin reference library of metabolic standards. These comparisons were made with data that including RT, *m/z*, preferred adducts, in-source fragments, and associated MS/MS spectra. A metabolic network of the abnormal changed metabolites was constructed according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) pathway database and the Human Metabolome Database (HMDB [45] http://www.hmdb.ca/).

3. Results and discussion

3.1. Identification of altered ions in the plasma of patients with PD

To investigate the difference in plasma metabolite profiles between the PD and control groups, the data was subjected to a metabolomics analysis with MetaboAnalyst 3.0. From the raw LC-MS data, 4013 ions were extracted, 2637 of which were removed with RSD filtering, leaving 1376 ions to be browsed into MetaboAnalyst 3.0. PLS-DA refers to grouping a collection of abstract objects into two groups that consist of similar objects. As shown in Fig. 1A, the imported ions grouped clinical samples into two distinct groups, PD (green) and control (red), indicating altered or dysregulated metabolites in the plasma of patients with PD compared to control. To confirm the difference in metabolic profiles between the PD and control groups, we conducted a heat map analysis. A heat map represents the levels of expression of many ions across a number of comparable samples. The cell color represented the ion abundance or metabolite level in a sample, with high abundance in red and low abundance in blue. The color at the top of the figure represented the PD (green) or control (red) sample group. The ions with *t*-test *p* value lower than 0.05 were used to construct a high-confidence metabolic classification model. These ions entirely separated PD (green) and control (red) groups (Fig. 1B), indicating that the model was accurate and confirming the presence of altered or dysregulated metabolites in the plasma of patients with PD. So we can use the data to search abnormal metabolites.

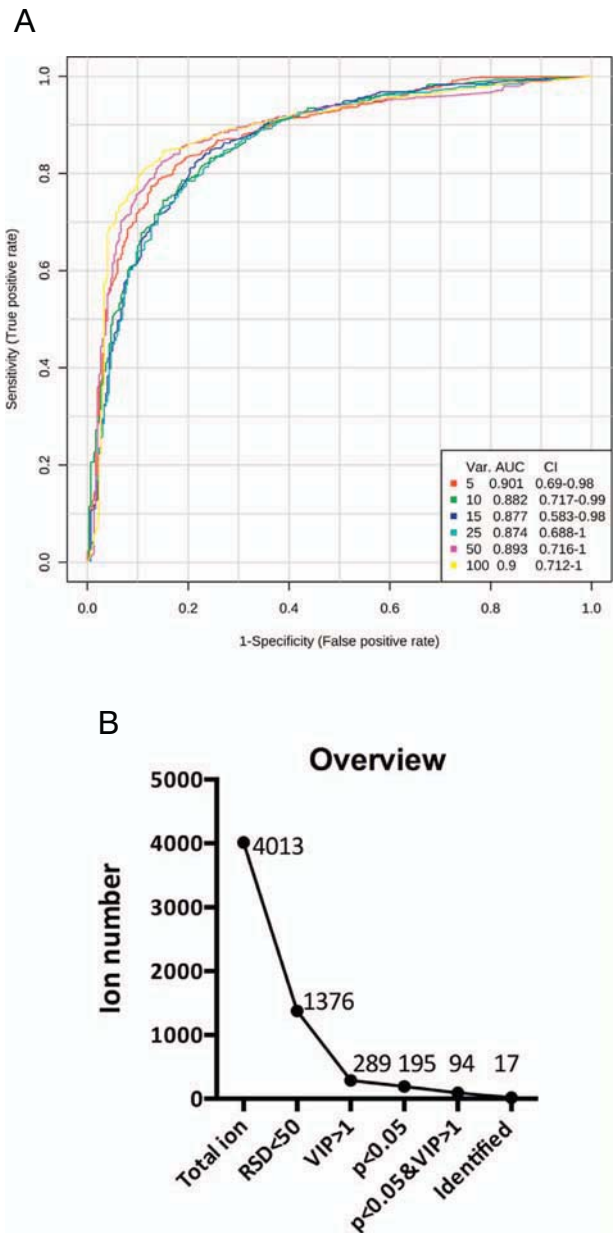

Fig. 3. Biomarker potentiality of altered ions.
ROC curve was performed using *t*-test p value lower than 0.05 ions. As shown in Fig. 2A, the AUC values were all above 0.87 no matter we use the top 5 or 10, 15, 25, 50, 100 *t*-test ions to do the biomarker analysis. This result suggested these groups of ions can be a good predictor to differ PD and control.

3.2. Altered ions as potential PD biomarkers

We conducted biomarker analysis to test the PD-predictive power of altered ions using receiver operating characteristic (ROC) curves, which is a method of biomarker identification and performance evaluation. The area under the curve (AUC) was above 0.87 when the top 5, 10, 15, 25, 50, or 100 ions identified as significant in the *t*-test were used (Fig. 2A). This result suggested that ions with t-test *p* values lower than 0.05 as a model was a good predictor of PD. As shown in Fig. 2B, a total of 195 ions were found with *t*-test p values lower 0.05. For getting higher confidence in the discovery of biomarkers, we used an additional standard with PLS-DA analysis to define significantly changed ions at the same time with the t-test. A total of 289 ions were found with VIP values higher than 1. After integration, the overlapping set of 94 ions that were significant both in the *t*-test and PLS-DA was used to do

Table 1
Identified endogenousabnormal metabolites in PD plasma (both $p < 0.05$ and VIP > 1).

Metabolite	VIP value	t-test p value	Fold Change	Formula	Practical *m/z*	Theatrical *m/z*	△Mass (ppm)	HMDB ID
Amino acids metabolites								
Phenylalanine	4.2	0.0016	+4.6	$C_9H_{11}NO_2 + H^+$	166.0865	166.0863	1	HMDB0000159
Vanillactic acid	5.3	0.0006	+24.3	$C_{10}H_{12}O_5\text{-}H_2O + H^+$	195.0660	195.0664	2	HMDB0000913
Bile acids metabolites								
3b–Hydroxy-5-cholenoic acid	2.4	0.0199	+2.7	$C_{24}H_{38}O_3\text{-}H_2O + H^+$	357.2797	357.2788	2	HMDB0000308
Glycoursodeoxycholic acid	2.7	0.0315	−11.8	$C_{26}H_{43}NO_5 + H^+$	450.3224	450.3220	2	HMDB0000708
Glycerol phospholipid metabolites								
LysoPC(18:2)	3.0	0.0250	+2.8	$C_{26}H_{50}NO_7P + Na^+$	542.3257	542.3217	7	HMDB0010386
PA(18:2/15:0)	3.7	0.0106	+2.6	$C_{31}H_{60}NO_8P\text{-}2H_2O + H^+$	623.4414	623.4446	5	HMDB0114948
Fatty acids metabolites								
Valeric acid	1.4	0.0108	−1.3	$C_5H_{10}O_2 + NH_4^+$	120.1024	120.1019	3	HMDB0000892
2-Octenoic acid	1.5	0.0001	+2.3	$C_8H_{14}O_2 + NH_4^+$	160.1335	160.1332	1	HMDB0000392
Docosene	3.1	0.0043	−1.8	$C_{22}H_{44} + Na^+$	331.3348	331.3335	4	HMDB0062602
Carnitine metabolites								
Carnitine	1.2	0.0351	−4.5	$C_7H_{15}NO_3\text{-}H_2O + H^+$	144.1021	144.1019	1	HMDB0000062
2-Methylbutyroylcarnitine	1.3	0.0046	−1.6	$C_{12}H_{23}NO_4 + H^+$	246.1708	246.1700	3	HMDB0000378
Other metabolites								
4-Hydroxybenzaldehyde	4.6	0.0007	+10.1	$C_7H_6O_2\text{-}H_2O + H^+$	105.0343	105.0340	2	HMDB0011718
Adrenochrome	1.5	0.0196	+16.2	$C_9H_9NO_3 + H^+$	180.0658	180.0655	1	HMDB0012884
Leukotriene B3	3.7	0.0018	+1.7	$C_{20}H_{34}O_4 + NH_4^+$	356.2807	356.2795	3	–
Cytidine 2′,3′-cyclic phosphate	2.6	0.0441	+3.8	$C_9H_{12}N_3O_7P + H^+$	306.0479	306.0486	2	HMDB0011691
3-Methylene-indolenine	1.1	0.0474	+3.3	$C_9H_7N + H^+$	130.0654	130.0651	2	HMDB0011664
Heptanoylcholine	3.2	0.0227	−3.3	$C_{12}H_{26}NO_2\text{-}H_2O + H^+$	199.1943	199.1936	3	HMDB0013239

VIP: Variable Importance in Projection; *m/z*: ratio of mass to charge. In “Fold change” column, “+” means up-regulated, while “−” means down-regulated; In “HMDB ID” column, ‘−’ means metabolite that is identified only in “Metlin” database.

identification for abnormal metabolites (Fig. 3).

3.3. Identification of abnormal PD metabolites

Out of the 94 ions that showed dual statistical significance, we were able to identify 17 endothelial metabolites by matching their LC-MS and MS2 characteristics with those found in the HMDB (Fig. 3). These metabolites were classified in Table 1 according to their structures and biological functions. For each metabolite, we provided not only the formula and MS characteristics but also the trend of change in PD plasma relative to control plasma. The lower the mass error of a metabolite, the higher its statistical confidence.

The abnormal metabolites we found in PD can be divided into the following main categories: phenolic amino acids (phenylalanine and tyrosine), fatty acids, glycerol phospholipids, and bile acids. And with these metabolites, we can better understand the mechanism of PD only if we know the correlations among them. Phenylalanine is an essential amino acid and the precursor for tyrosine. Phenylalanine and tyrosine are both the precursors of catecholamines (tyramine, dopamine, epinephrine, and norepinephrine), which are adrenalin-like substances. Phenylalanine is highly concentrated in the human brain and plasma [17]. In our study, the levels of phenylalanine and Vanillactic acid, a tyrosine metabolite, were increased in the plasma of patients with PD. One study, reported another catecholamine, homovanillic acid, as a biomarker of PD [15]. As one review article has reported, the main amino acids link to PD thus far include tryptophan, tyrosine, and phenylalanine [46], all of which are involved in mitochondrial metabolism.

Glycerol phospholipids, bile acids, and fatty acids are subunits of lipids. Among them, bile acids are the most upstream molecules in lipid metabolism. The unique detergent properties of bile acids are essential for the digestion and intestinal absorption of hydrophobic nutrients, dietary fats and vitamins. They also modulate bile flow and lipid secretion, and have been implicated in the regulation of the key enzymes involved in cholesterol homeostasis [47]. Recent studies have found that PD patients have abnormal cholesterol levels in their urine [17]. In the present study, we found that the plasma levels of two bile acids were altered in patients with PD, suggesting that steroid dysregulation in PD may be caused by abnormal bile acids production. It is generally believed that cholesterol is associated with atherosclerosis, which has inseparable relationships with body weight and obesity. One study reported most PD patients losing weight during the evolution of their disease [48]. Dysregulation of the hypothalamus, considered to be the regulatory center of satiety and energy metabolism, could play a major role in this phenomenon. Our data provide a link between weight loss and abnormal bile acid metabolism in PD. We identified both elevated and suppressed levels of lipid metabolites in patients with PD, indicating that the disturbance of lipid metabolism as a whole may play a key role in PD pathogenesis.

Fatty acid and carnitine metabolism takes place in mitochondria. Fatty acids have been reported playing a role in PD. For example, one study emphasized that the supplementation of omega-3 polyunsaturated fatty acids presented a potential neuroprotective action in hemiparkinsonism model [49]. Here we found another Valeric acid and Docosene's down regulation and 2-Octenoic acid's up-regulation, the disturbance of fatty acids gives a direction of future PD mechanism study. Numerous disorders have been described that lead to disturbances in energy production and in intermediary metabolism in the organism which are characterized by the production and excretion of unusual acyl carnitines [50]. Both carnitine and 2-methylbutyroylcarnitine were found to be down-regulated in the plasma of patients with PD in our study. Energy production from long-chain fatty acids (LCFAs) requires LCFA transport into the mitochondrial matrix. This transport is carnitine-dependent and involves active translocation machinery. Therefore, fatty acid metabolism dysregulation may be directly related to the carnitine metabolic abnormalities in PD patients. Consistent with this hypothesis, others have found decreased levels of serum LCFAs in individuals with PD [30].

3.4. The abnormal metabolic profile and mechanism of PD

To understand the relationships between abnormal metabolites and the mechanism of PD, one needs to know the relevant biochemical pathways and their biological functions. Therefore, we performed

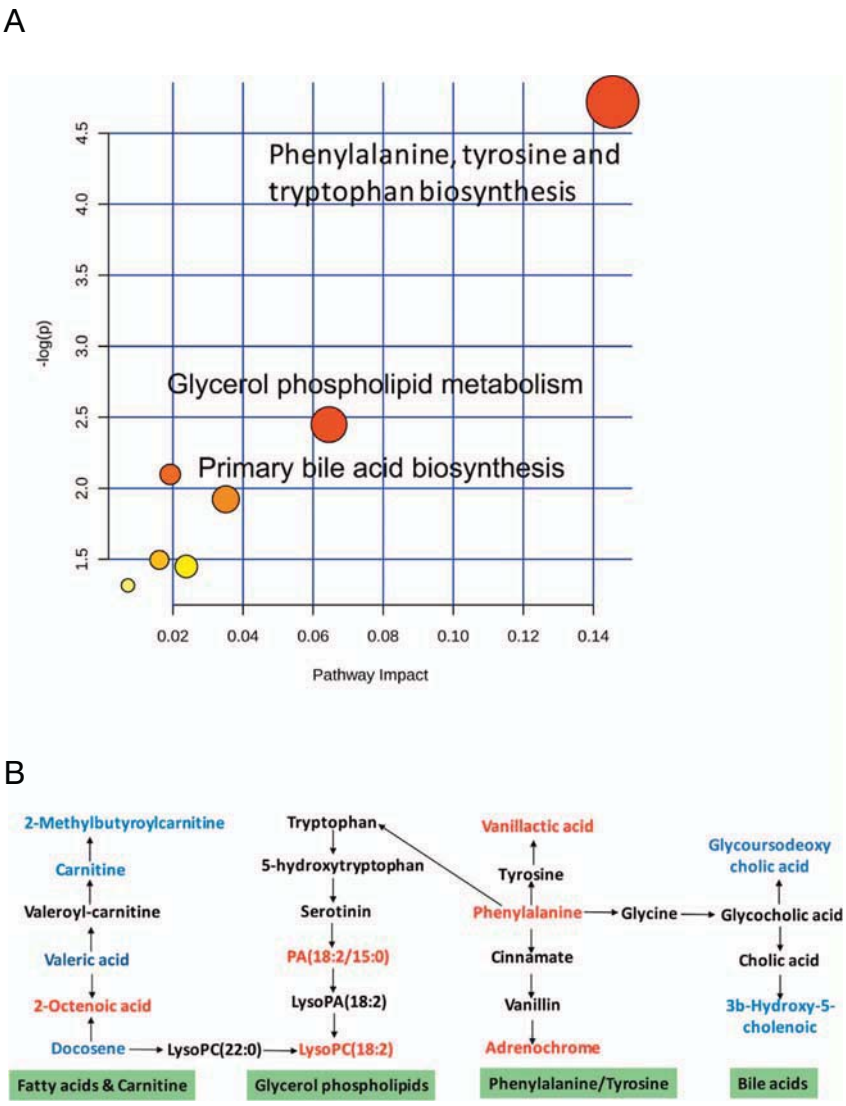

Fig. 4. Metabolic mechanism among abnormal metabolites in plasma of patients with PD.
Pathway analysis (A) are conducted using 17 of identified abnormal metabolites, and the heavier colour of a pathway means the more relevant to PD. From a summary way, it shows the primary metabolic pathways closely related to PD are phenylalanine and tyrosine biosynthesis, glycerol phospholipid metabolism and bile acids biosynthesis. The metabolic network (B) show the changing of these metabolites from an overall view, and the arrow represents their subordinate relationship. Red-labeled metabolites indicate up-regulation in PD plasma compared to control, while blue-labeled metabolites indicate down-regulation in PD plasma. From the network we can observe that except for basic amino acids disorder, there are metabolic changing among bile acids, carnitine, fatty acids and so on. (For interpretation of the references to colour in this figure legend, the reader is referred to the online version of this chapter.)

pathway analyses of the 17 metabolites dysregulated in PD. Fig. 4A shows the identified pathways, with more intense colours indicating higher relevance of a pathway for PD. In general, the primary metabolic pathways involved in PD were tyrosine biosynthesis, glycerol phospholipid metabolism, and bile acid biosynthesis. The metabolic network shown in Fig. 4B was constructed using the 17 identified abnormal metabolites according to the KEGG metabolic pathway database and the HMDB to show the connections of these metabolites to one another. From the network, one can see that, besides basic amino acid metabolism dysregulation, there are some metabolic changes involving bile acids, one of whose biological functions is regulating lipid metabolism. Lipids include fatty acids and glycerol phospholipids, both of which are also dysregulated in the plasma of patients with PD. Together, these observations suggest that the lipid perturbations in PD maybe caused by abnormal bile acid metabolism.

The carnitine metabolic disturbance we found is evidence of mitochondrial dysfunction in PD. Carnitine-dependent oxidation of fatty acids is an alternative way of energy production in mitochondria. Currently, although the pathogenesis of PD is still obscure, overwhelming evidence demonstrates that oxidative stress plays a role in the progress of PD [51]. Therefore, the oxidation dysregulation of fatty acid metabolism dysregulation may be further evidence of mitochondrial dysfunction in PD. Because both lipid metabolism and mitochondrial metabolism are direct sources of energy, most of the abnormal metabolite levels in the plasma of patients with PD appear to be related to energy production. Perturbed energy production may therefore play a critical role in PD pathogenesis.

3.5. *Summary of abnormal metabolites closely related to PD*

Our overall search strategy for potential PD biomarkers is shown in Fig. 2B. By LC-MS analysis of clinical plasma samples, we obtained characteristics of 4013 ions representing the condition of the sample. To make sure the ions used for metabolism analyses were comparable, only 1376 ions were selected based on the QC sample RSD lower than 50%. To ensure biological significance, we used the *t*-test to select ions with abnormal levels and found 195 such ions. Within those 195 ions, PLS-DA identified 94 ions with VIP values larger than 1. These were considered high-significance because of their satisfying dual statistical criteria. After identifying the chemical structures of these 94 ions, we obtained 17 compounds that were consistently altered in the plasma of patients with PD.

Compared with the urine and CSF metabolites found in literature, it is found that the plasma metabolites are upstream, and urine metabolites are downstream [23,24,32,52–54]. Our findings support the existence of at least three distinct metabolic mechanisms of PD pathogenesis. First, bile acid dysregulation may directly cause lipid metabolism dysfunction. Second, perturbations of carnitine metabolism

may directly lead to lower LCFA levels. Third, the abnormal metabolite levels found in this study may collectively disrupt energy production.

4. Conclusion

Biomarkers could be used for the early diagnosis, tracking disease progression, and selection of disease-modifying treatments of PD. Up to date, no reliable biomarkers of early neurodegeneration in PD have been identified, making discovery of promising PD biomarker candidates critically important. Our study used LC-MS-based metabolism analyses to find potential PD biomarkers in clinical patients' peripheral blood plasma samples. We found multiple abnormal changes in metabolite levels in the plasma of patients with PD. Seventeen significantly dysregulated metabolites were identified to be mainly related to lipid metabolism and mitochondrial function, both important aspects of energy metabolism. Our findings indicate that abnormal changes in energy metabolism might have a direct correlation with PD progression.

Acknowledgements

This work is supported by the Ministry of Science and Technology of the People's Republic of China [2015DFG42460]; National Science Foundation [21475128]; and Chinese Academy of Science [100Talent Project].

References

[1] J. Wang, J.G. Hoekstra, C. Zuo, T.J. Cook, J. Zhang, Biomarkers of Parkinson's disease: current status and future perspectives, Drug Discov. Today 18 (2013) 155–162.
[2] P. Jenner, H.R. Morris, T.W. Robbins, M. Goedert, J. Hardy, Y. Ben-Shlomo, P. Bolam, D. Burn, J.V. Hindle, D. Brooks, Parkinson's disease–the debate on the clinical phenomenology, aetiology, pathology and pathogenesis, J. Parkinson's Dis. 3 (2013) 1–11.
[3] R. Ren, Y. Sun, X. Zhao, X. Pu, et al., Clin. Chem. Lab. Med. 53 (2015) 1495–1506.
[4] A.J. Hughes, S.E. Daniel, Y. Ben-Shlomo, A.J. Lees, The accuracy of diagnosis of parkinsonian syndromes in a specialist movement disorder service, Brain J. Neurol. 125 (2002) 861–870.
[5] C.H. Adler, T.G. Beach, J.G. Hentz, H.A. Shill, J.N. Caviness, E. Driver-Dunckley, M.N. Sabbagh, L.I. Sue, S.A. Jacobson, C.M. Belden, B.N. Dugger, Low clinical diagnostic accuracy of early vs advanced Parkinson disease: clinicopathologic study, Neurology 83 (2014) 406–412.
[6] C.L. Tomlinson, S. Patel, C. Meek, C.P. Herd, C.E. Clarke, R. Stowe, L. Shah, C.M. Sackley, K.H. Deane, K. Wheatley, N. Ives, Physiotherapy versus placebo or no intervention in Parkinson's disease, Cochrane Database Syst. Rev., (DOI 10.1002/14651858.CD002817.pub4(2013) Cd002817)
[7] B. Zheng, Z. Liao, J.J. Locascio, K.A. Lesniak, S.S. Roderick, M.L. Watt, A.C. Eklund, Y. Zhang-James, P.D. Kim, M.A. Hauser, E. Grünblatt, L.B. Moran, S.A. Mandel, P. Riederer, R.M. Miller, H.J. Federoff, U. Wüllner, S. Papapetropoulos, M.B. Youdim, I. Cantuti-Castelvetri, A.B. Young, J.M. Vance, R.L. Davis, J.C. Hedreen, C.H. Adler, T.G. Beach, M.B. Graeber, F.A. Middleton, J.-C. Rochet, C.R. Scherzer, PGC-1α, a potential therapeutic target for early intervention in Parkinson's disease, Sci. Transl. Med. 2 (2010) (52ra73-52ra73).
[8] M.G. Tansey, M.K. McCoy, T.C. Frank-Cannon, Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention, Exp. Neurol. 208 (2007) 1–25.
[9] B. Mollenhauer, J. Zhang, Biochemical premotor biomarkers for Parkinson's disease, Mov. Disord. 27 (2012) 644–650.
[10] T.M. Marques, H.B. Kuiperij, I.B. Bruinsma, A. van Rumund, M.B. Aerts, R.A.J. Esselink, B.R. Bloem, M.M. Verbeek, MicroRNAs in cerebrospinal fluid as potential biomarkers for Parkinson's disease and multiple system atrophy, Mol. Neurobiol. 54 (2017) 7736–7745.
[11] J.K. Lu, Y. Xu, Z.Z. Quan, Z.X. Chen, Z.Z. Sun, H. Qing, Dysregulated micrornas in neural system: implication in pathogenesis and biomarker development In Parkinson's disease, Neuroscience 365 (2017) 70–82.
[12] W. Maetzler, A.K. Stapf, C. Schulte, A.K. Hauser, S. Lerche, I. Wurster, E. Schleicher, A. Melms, D. Berg, Serum and cerebrospinal fluid uric acid levels in lewy body disorders: associations with disease occurrence and amyloid-beta pathway, J. Alzheimers Dis. 27 (2011) 119–126.
[13] M.Q. Pan, H.M. Gao, L. Long, Y.Q. Xu, M. Liu, J. Zou, A.M. Wu, X.B. Wei, X.H. Chen, B.S. Tang, Q. Wang, Serum uric acid in patients with Parkinson's disease and vascular parkinsonism: a cross-sectional study, Neuroimmunomodulation 20 (2013) 19–28.
[14] I. Schlesinger, N. Schlesinger, Uric acid in Parkinson's disease, Mov. Disord. Off. J. Mov. Disord. Soc. 23 (2008) 1653–1657.
[15] P. LeWitt, L. Schultz, P. Auinger, M. Lu, D.I. Parkinson Study Group, CSF xanthine, homovanillic acid, and their ratio as biomarkers of Parkinson's disease, Brain Res. 1408 (2011) 88–97.
[16] T. Ogawa, W.R. Matson, M.F. Beal, R.H. Myers, E.D. Bird, P. Milbury, S. Saso, Kynurenine pathway abnormalities in Parkinsons-disease, Neurology 42 (1992) 1702–1706.
[17] H. Luan, L.-F. Liu, N. Meng, Z. Tang, K.-K. Chua, L.-L. Chen, J.-X. Song, V.C.T. Mok, L.-X. Xie, M. Li, Z. Cai, L.C. MS-Based Urinary, Metabolite signatures in idiopathic Parkinson's disease, J. Proteome Res. 14 (2015) 467–478.
[18] A.W. Michell, D. Mosedale, D.J. Grainger, R.A. Barker, Metabolomic analysis of urine and serum in Parkinson's disease, Metabolomics 4 (2008) 191–201.
[19] A.K. Shukla, C. Ratnasekhar, P. Pragya, H.S. Chaouhan, D.K. Patel, D.K. Chowdhuri, M.K.R. Mudiam, Metabolomic analysis provides insights on Paraquat-induced Parkinson-like symptoms in *Drosophila melanogaster*, Mol. Neurobiol. 53 (2016) 254–269.
[20] K.K. Johansen, L. Wang, J.O. Aasly, L.R. White, W.R. Matson, C. Henchcliffe, M.F. Beal, M. Bogdanov, Metabolomic profiling in LRRK2-related Parkinson's disease, PLoS One 4 (2009).
[21] M. Bogdanov, W.R. Matson, L. Wang, T. Matson, R. Saunders-Pullman, S.S. Bressman, M.F. Beal, Metabolomic profiling to develop blood biomarkers for Parkinson's disease, Brain J. Neurol. 131 (2008) 389–396.
[22] A.W. Amara, D.G. Standaert, Metabolomics and the search for biomarkers in Parkinson's disease, Mov. Disord. 28 (2013) 1620–1621.
[23] S. Lei, R. Powers, NMR metabolomics analysis of Parkinson's disease, Curr. Metabol. 1 (2013) 191–209.
[24] M.P. Stoop, L. Coulier, T. Rosenling, S. Shi, A.M. Smolinska, L. Buydens, K. Ampt, C. Stingl, A. Dane, B. Muilwijk, R.L. Luitwieler, P. Smitt, R.Q. Hintzen, R. Bischoff, S.S. Wijmenga, T. Hankemeier, A.J. van Gool, T.M. Luider, Quantitative proteomics and metabolomics analysis of normal human cerebrospinal fluid samples, Mol. Cell. Proteomics 9 (2010) 2063–2075.
[25] S.N. Reinke, D.I. Broadhurst, B.D. Sykes, G.B. Baker, I. Catz, K.G. Warren, C. Power, Metabolomic profiling in multiple sclerosis: insights into biomarkers and pathogenesis, Mult. Scler. J. 20 (2014) 1396–1400.
[26] M.P. Quinones, R. Kaddurah-Daouk, Metabolomics tools for identifying biomarkers for neuropsychiatric diseases, Neurobiol. Dis. 35 (2009) 165–176.
[27] P.A. Lewitt, J. Li, M. Lu, T.G. Beach, C.H. Adler, L. Guo, C. Arizona, Parkinson's disease, 3-hydroxykynurenine and other Parkinson's disease biomarkers discovered by metabolomic analysis, Mov. Disord. Off. J. Mov. Disord. Soc. 28 (2013) 1653–1660.
[28] J.R. Roede, K. Uppal, Y. Park, K. Lee, V. Tran, D. Walker, F.H. Strobel, S.L. Rhodes, B. Ritz, D.P. Jones, Serum metabolomics of slow vs. rapid motor progression Parkinson's disease: a pilot study, PLoS One 8 (2013).
[29] T. Hatano, S. Saiki, A. Okuzumi, R.P. Mohney, N. Hattori, Identification of novel biomarkers for Parkinson's disease by metabolomic technologies, J. Neurol. Neurosurg. Psychiatry 87 (2016) 295–301.
[30] E.C. Schulte, E. Altmaier, H.S. Berger, D. Kieu Trinh, G. Kastenmueller, S. Wahl, J. Adamski, A. Peters, J. Krumsiek, K. Suhre, B. Haslinger, A. Ceballos-Baumann, C. Gieger, J. Winkelmann, Alterations in lipid and inositol metabolisms in two dopaminergic disorders, PLoS One 11 (2016).
[31] J. Wu, A. Wuolikainen, M. Trupp, P. Jonsson, S.L. Marklund, P.M. Andersen, L. Forsgren, A. Ohman, NMR analysis of the CSF and plasma metabolome of rigorously matched amyotrophic lateral sclerosis, Parkinson's disease and control subjects, Metabolomics 12 (2016).
[32] H.M. Luan, L.F. Liu, Z. Tang, M.W. Zhang, K.K. Chua, J.X. Song, V.C.T. Mok, M. Li, Z.W. Cai, Comprehensive urinary metabolomic profiling and identification of potential noninvasive marker for idiopathic Parkinson's disease, Sci. Rep. 5 (2015).
[33] J.L. Liu, H.L. Wang, L.F. Zhang, Y.F. Xu, W. Deng, H. Liu, C. Qin, Metabonomics study of brain-specific human S100B transgenic mice by using high-performance liquid chromatography coupled with quadrupole time of flight mass spectrometry, Biol. Pharm. Bull. 34 (2011) 871–876.
[34] X. Chen, C.S. Xie, L.X. Sun, J.H. Ding, H.B. Cai, Longitudinal metabolomics profiling of Parkinson's disease-related alpha-synuclein A53T transgenic mice, PLoS One 10 (2015).
[35] R.E. Musgrove, J. Horne, R. Wilson, A.E. King, L.M. Edwards, T.C. Dickson, The metabolomics of alpha-synuclein (SNCA) gene deletion and mutation in mouse brain, Metabolomics 10 (2014) 114–122.
[36] M.M. Hoehn, M.D. Yahr, Parkinsonism, Onset, Progression, and Mortality, 17 (1967), p. 427.
[37] G. Theodoridis, H.G. Gika, I.D. Wilson, LC-MS-based methodology for global metabolite profiling in metabonomics/metabolomics, TrAC Trends Anal. Chem. 27 (2008) 251–260.
[38] L. Li, W. Ren, H. Kong, C. Zhao, X. Zhao, X. Lin, X. Lu, G. Xu, An alignment algorithm for LC-MS-based metabolomics dataset assisted by MS/MS information, Anal. Chim. Acta 990 (2017) 96–102.
[39] K.P. Law, X. Mao, T.L. Han, H. Zhang, Unsaturated plasma phospholipids are consistently lower in the patients diagnosed with gestational diabetes mellitus throughout pregnancy: a longitudinal metabolomics study of Chinese pregnant women part 1, Clin. Chim. Acta 465 (2017) 53–71.
[40] O. Deda, H.G. Gika, I. Taitzoglou, N. Raikos, G. Theodoridis, Impact of exercise and aging on rat urine and blood metabolome. An LC-MS based metabolomics longitudinal study, Meta 7 (2017).
[41] D. Feng, Z. Xia, J. Zhou, H. Lu, C. Zhang, R. Fan, X. Xiong, H. Cui, P. Gan, W. Huang, W. Peng, F. He, Z. Wang, Y. Wang, T. Tang, Metabolomics reveals the effect of Xuefu Zhuyu decoction on plasma metabolism in rats with acute traumatic brain injury, Oncotarget 8 (2017) 94692–94710.
[42] S.J. Bruce, I. Tavazzi, V. Parisod, S. Rezzi, S. Kochhar, P.A. Guy, Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry, Anal. Chem. 81

(2009) 3285–3296.
[43] J. Xia, R. Mandal, I.V. Sinelnikov, D. Broadhurst, D.S. Wishart, MetaboAnalyst 2.0–a comprehensive server for metabolomic data analysis, Nucleic Acids Res. 40 (2012) W127–133.
[44] D. Luo, T. Deng, W. Yuan, H. Deng, M. Jin, Plasma metabolomic study in Chinese patients with wet age-related macular degeneration, BMC Ophthalmol. 17 (2017) 165.
[45] D.S. Wishart, T. Jewison, A.C. Guo, M. Wilson, C. Knox, Y. Liu, Y. Djoumbou, R. Mandal, F. Aziat, E. Dong, S. Bouatra, I. Sinelnikov, D. Arndt, J. Xia, P. Liu, F. Yallou, T. Bjorndahl, R. Perez-Pineiro, R. Eisner, F. Allen, V. Neveu, R. Greiner, A. Scalbert, HMDB 3.0–the human metabolome database in 2013, Nucleic Acids Res. 41 (2013) D801–807.
[46] J.F. Havelund, N.H.H. Heegaard, N.J.K. Faergeman, J.B. Gramsbergen, Biomarker research in Parkinson's disease using metabolite profiling, Meta 7 (2017).
[47] T. Claudel, B. Staels, F. Kuipers, The Farnesoid X receptor - a molecular link between bile acid and lipid and glucose metabolism, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 2020–2031.
[48] I. Rieu, Y. Boirie, B. Morio, P. Derost, M. Ulla, A. Marques, B. Debilly, S. Bannier, F. Durif, The idiopathic Parkinson's disease: a metabolic disease? Rev. Neurol. (Paris) 166 (2010) 822–828.
[49] A.S. Barros, R.Y.G. Crispim, J.U. Cavalcanti, R.B. Souza, J.C. Lemos, G. Cristino, M.M. Bezerra, T.F.M. Pinheiro, S.M.M. de Vasconcelos, D.S. Macedo, G.S.D. Viana, L.M.V. Aguiar, Impact of the chronic omega-3 fatty acids supplementation in Hemiparkinsonism model induced by 6-Hydroxydopamine in rats, Basic Clin. Pharmacol. Toxicol. 120 (2017) 523–531.
[50] G. Pierre, A. Macdonald, G. Gray, C. Hendriksz, M.A. Preece, A. Chakrapani, Prospective treatment in carnitine–acylcarnitine translocase deficiency, J. Inherit. Metab. Dis. 30 (2007) 815.
[51] J. Zhao, S. Yu, Y. Zheng, H. Yang, J. Zhang, Oxidative modification and its implications for the neurodegeneration of Parkinson's disease, Mol. Neurobiol. 54 (2017) 1404–1418.
[52] A.D. Andersen, M. Binzer, E. Stenager, J.B. Gramsbergen, Cerebrospinal fluid biomarkers for Parkinson's disease - a systematic review, Acta Neurol. Scand. 135 (2017) 34–56.
[53] D.S. Goldstein, C. Holmes, Y. Sharabi, Cerebrospinal fluid biomarkers of central catecholamine deficiency in Parkinson's disease and other synucleinopathies, Brain J. Neurol. 135 (2012) 1900–1913.
[54] H.M. Luan, X.M. Chen, S.L. Zhong, X.N. Yuan, N. Meng, J.F. Zhang, J. Fu, R. Xu, C. Lee, S.Y. Song, H. Jiang, X. Xu, Serum metabolomics reveals lipid metabolism variation between coronary artery disease and congestive heart failure: a pilot study, Biomarkers 18 (2013) 314–321.