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RESEARCH ARTICLE

Untargeted metabolomic analysis of pregnant women exposure to perfluorooctanoic acid at different degrees

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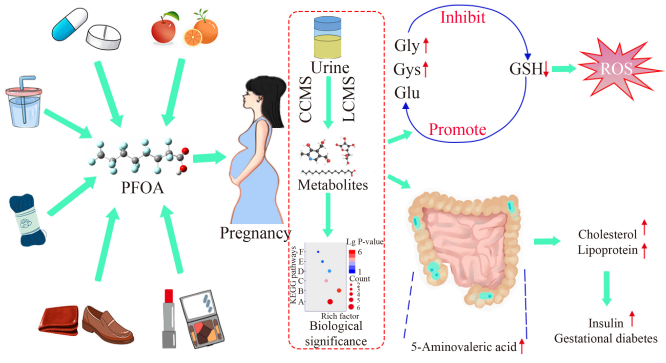
HIGHLIGHTS

- Metabolome can distinguish pregnant women exposure to PFOA at different degrees.
- Metabolome can reveal the metabolic changes of pregnant women exposure to PFOA.
- PFOA exposure degrees could affect the GSH metabolism of pregnant women.
- PFOA exposure degrees could change the microbiota metabolism of pregnant women.

GRAPHIC ABSTRACT



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ABSTRACT

Perfluorooctanoic acid (PFOA) is a novel type of persistent synthetic organic pollutant, and its exposure on pregnant women can cause some adverse effects, such as pregnancy-induced hypertension, gestational diabetes mellitus, and preeclampsia. Therefore, understanding the metabolic changes caused by PFOA exposure is of great significance to protect pregnant women from its adverse effects. In this study, the metabolomes from the urine samples of pregnant women exposure to PFOA at different degrees were analyzed by GC-MS and LC-MS. The samples in different groups were distinguished and the differential metabolites were screened based on the VIP value, FC, and P-value of each comparison group through multivariate statistical analysis. The pathways related to differential metabolites were searched to reveal the effects of PFOA exposure on metabolic changes in pregnant women at different degrees. Finally, the ROC of differential metabolites was performed, and the differential metabolites with large area under the curve (AUC) values were selected and compared to identify the mutually differential metabolites. Meanwhile, these metabolites were fitted with a multivariable to explore if they could be used to distinguish different groups. The quantitative comparison of mutually differential metabolites revealed that the levels of L-cysteine, glycine, and 5-aminovaleric acid were positively correlated with the degree of PFOA exposure, indicating that different degrees of PFOA exposure could affect the synthesis or degradation of GSH and change the metabolism of oral or intestinal microbiota. Additionally, they may cause oxidative stress and abnormal fat metabolism in pregnant women.

1 Introduction

Perfluorooctanoic acid (PFOA) is a type of synthetic organic compound, which has high consumer value and is

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suitable for industrial product applications due to its high thermal stability, chemical inertness and amphiphilicity, good surfactant properties, and high C-F bond energy. The stable chemical properties and a long half-life of PFOA enable it to be ubiquitously present in nature. For instance, the PFOA content in many water bodies' surfaces is several times higher than the safe level recommended by the US Environmental Protection Agency (USEPA), confirming its existence in drinking water as well (Podder et al., 2021). PFOA can also migrate over long distances and accumulate in the food chain, causing high bioaccumulation. This is the reason PFOA is identified as a novel type of persistent organic pollutant posing a serious threat to the environment (Lv et al., 2019; Gebreab et al., 2020). The biological half-life of PFOA significantly varies between species and genders. PFOA can also exist in the human body for a prolonged period due to its long half-life of about 4.37 a, causing toxicity, such as liver toxicity, kidney toxicity, neurotoxicity, and reproductive system toxicity (Kudo et al., 2003). Besides, it affects infant development, immune function, thyroid function, and other metabolic processes (Steenland et al., 2020; Fenton et al., 2021; Podder et al., 2021).

Prenatal and postnatal PFOA exposure can cause pregnancy-induced hypertension, gestational diabetes mellitus, and preeclampsia and affect infants (Savitz et al., 2012; Avanasi et al., 2016; Wang et al., 2022). PFOA can cross the placenta and be excreted in mother's milk, causing the serum level of PFOA in infants to exceed that of their mothers. Therefore, breastfeeding can be a potential excretion route of PFOA in women and a PFOA exposure pathway in infants (Gyllenhammar et al., 2018; VanNoy et al., 2018). Accumulating studies have reported that prenatal PFOA exposure is significantly associated with the changes in indicators in infants at birth and after birth, such as low birth weight (Lam et al., 2014; Wikström et al., 2020), increased risk of obesity (Halldorsson et al., 2012; Wikström et al., 2020) and decreased bone mineral density (Cluett et al., 2019) during development. Additionally, PFOA exposure to pregnant mice causes a significant increase in FAS and FASL expression in the decidual cells in a dose-dependent manner and induces uterine cell apoptosis through the FAS/FASL pathway, leading to embryo loss or damage (Zhang et al., 2021).

Prenatal and postnatal PFOA exposure can be harmful to themselves, as well as their offspring. At the same time, their metabolic, physiological, and pathological processes are changed depending on the levels of PFOA exposure. Therefore, the present study aimed to perform metabolomic analysis of urine samples of pregnant women with different PFOA levels *in vivo* using the GC-MS and LC-MS technologies. Furthermore, the correlation between the PFOA levels *in vivo* and the changes in the metabolic, physiological, and pathological processes in pregnant women was studied to explore the toxicity mechanism of PFOA and formulate more precise treatments and response plans for different individuals.

2 Materials and methods

2.1 Collection and classification of urine samples

A total of 90 urine samples were collected from pregnant women at 12–16 weeks of gestation who went to the Minhang Maternal and Child Health Hospital in Shanghai, China, for their first prenatal care. A single spot urine sample from each participant was collected during recruitment and then frozen at –80 °C before being shipped to the laboratory for analysis. All participants provided written informed consent for themselves and their neonates. This study was approved by the ethical committees at the Shanghai Institute of Planned Parenthood Research.

All the 90 urine samples were evenly divided into three groups based on their measured clinical PFOA values: low PFOA concentration group (Exp-L, 5.392–12.357 ng/mL), medium PFOA concentration group (Exp-M, 19.899–27.155 ng/mL), and high PFOA concentration group (Exp-H, 31.085–63.678 ng/mL).

2.2 Metabolite extraction

Each urine sample (100 μL) was centrifuged at 13000 r/min, 4 °C for 10 min, and the supernatant (50 μL) was transferred to a new tube. After the urease solution (20 μL, type C, 30 U/10 μL, Aladdin) was added, the mixture was incubated at 37 °C for 15 min. An internal standard (10 μL, L-2-chloro-phenylalanine, 0.3 mg/mL, dissolved in methanol, Sigma Aldrich) was then added, and the mixture was vortexed for 10 s. Afterward, the methanol-acetonitrile solution (170 μL, v:v = 2:1) was added, and the mixture was vortexed for 30 s and then allowed to stand at –20 °C for 30 min. Subsequently, the mixture was centrifuged at 13000 r/min, 4 °C for 15 min, and the supernatant (200 μL) was transferred into a glass derivatization bottle. At the same time, the extracted supernatants from all samples at equal volume (40 μL) were mixed to prepare a quality control sample (QC). Then, the extracts and several QCs were rotary evaporated to dryness and derivatized using the methoxamine hydrochloride pyridine solution (80 μL, 15 mg/mL, Sigma Aldrich), BSTFA derivatization reagent (80 μL, containing 1 % TMCS, Sigma Aldrich) and fatty acid methyl esters standard mixture (8 mg/mL, dissolved in chloroform, Sigma Aldrich, SMB00937) in a glass derivatization bottle. After vortexing for 5 min, the mixture was incubated at 70 °C for 60 min. After the reaction was completed, the samples were allowed to stand at room temperature for 30 min and then subjected

to GC-MS analysis.

Each urine sample (100 μL) was mixed with the dual internal standard (10 μL, containing of 0.3 mg/mL L-2-chloro-phenylalanine (dissolved in methanol, Sigma Aldrich) and 0.1 mg/mL LPC 17:0 (dissolved in methanol, Avanti)), then 200 μL of methanol-acetonitrile solution (*v*:*v* = 2:1) was added and vortexed for 60 s. The mixture was ultrasonicated in an ice-water bath for 10 min to extract the metabolites and then allowed to stand at –20 °C for 30 min. The mixture was then centrifuged at 13000 r/min, 4 °C for 15 min, and the supernatant (160 μL) was transferred to a sample bottle, and then subjected to LC-MS analysis.

2.3 GC-MS method

The metabolites extracted from the urine samples were analyzed by GC-MS (7890A-5975C, Agilent, USA). The separation of metabolites was performed on an Agilent HP-5MS column (0.25 mm × 30 m, 0.25 μm). The injection volume was set at 1 μL (without split), the inlet temperature was set to 260 °C, helium (99.999 %) was used as the carrier gas, and the total flow rate was 1.0 mL/min. The temperature gradient was set as follows: the initial temperature was 60 °C, rose to 125 °C at 8 °C/min, followed by 210 °C at 5 °C/min, 270 °C at 10 °C/min, 305 °C at 20 °C/min, and finally to 305 °C and held for 5 min. The auxiliary heater temperature was 280 °C. The ion source of the mass spectrometer was an electron impact ionization (EI) source. The ion source temperature, quadrupole temperature, and ionization voltage were set at 150 °C, 230 °C, and 70 eV, respectively. The scan was conducted in full scan mode at a scanning range of the mass-to-charge ratio (*m/z*) 50 to 500. The solvent delay time was set at 6 min.

2.4 LC-MS method

The metabolites extracted from the urine samples were analyzed by the Vanquish UHPLC system consisting of a binary pump, a vacuum degasser, an autosampler, a column oven, and a Q Exactive plus Mass spectrometer (Thermofisher, USA). The metabolites were separated on an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.7 μm, Waters, USA), in which the temperature was set at 45 °C. The mobile phases A and B were composed of 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile, respectively, and the total flow rate was 0.4 mL/min. The gradient elution was performed as follows: 1 % to 90 % mobile phase B for 12 min, followed by 90 % mobile phase B for 1 min. The injection volume was 1 μL.

The ion source of the mass spectrometer was a HESI source, and the scan was performed in the positive and negative modes and DDA mode (1 full scan, followed by 5 MS/MS scans). The normalized collision energy used for ion fragmentation was set at 15 V, and 30 V. Nitrogen (99.999 %) was used as the collision-induced dissociation gas. The spray voltage was set at 3.2 kV for the positive mode and 2.8 kV for the negative mode. The capillary temperature was set at 320°C and the s-lens RF level was set to 50 V. The full mass scan was acquired from m/z 70 to 1000 at a resolution of 70000. The AGC target was set at 1e6, and the maximum ion injection time (MIT) was set to 100 ms. The resolution of dd-MS/MS was 17500, the AGC target was 5e5, and the MIT was 50 ms. The data were acquired by the Xcalibur 3.0 software.

2.5 Data analysis

The raw data obtained from GC-MS were converted into the *abf* format by an Analysis Base File converter and then imported into the MSDIAL software for peak detection, identification and alignment, background subtraction, and missing value interpolation. The identification of metabolites was performed using the LUG database (an untargeted database of GC-MS developed by Shanghai Luming Biological Technology Co., Ltd.). The metabolites with more than 70 % similarity to the database were selected to normalize the internal standards, and the data matrix obtained was subjected to further data analysis.

The raw data obtained from LC-MS were processed through peak selection, calibration, and normalization using the Progenesis QI v2.3 software (Nonlinear Dynamics, Newcastle, UK), from which a data matrix consisting of retention time (RT), *m/z*, and peak intensity or peak area were estimated. As for data identification and screening, the samples were first grouped, and the QC was then selected by the Progenesis QI software. The possible adduct ions in the positive mode included M+H, 2M+H, M+2H, M+Na, M+NH_4, and those in the negative mode included M+H–H_2O and M–H, 2M–H, and M+$HCOO^-$. The time range, minimum peak width, and RT deviation were 0.5–12 min, 0.15 min, and 0.1 min, respectively. The mass accuracy deviation of the precursor ion and product ion was 5 ×10^{-6} and 10 ×10^{-6}, respectively, and the mass range was 50–1000 Da. The isotope peaks were removed, and the peak intensity threshold was set to 100. The fragment ion similarity score was set to 35. The metabolites were identified based on accurate mass, product ion fragments, RT, and isotope distribution by searching the HMDB_v2018 and METLIN databases using the Progenesis MetaScope serum/urine available in Progenesis QI. The data matrix consisting of RT, *m/z*, and peak intensity or peak area of the identified metabolites was used for further data analysis.

The metabolites and their relative contents in the urine samples of pregnant women (QC, Exp-L, Exp-M, and Exp-H) determined by GC-MS and LC-MS were first analyzed by the unsupervised principal component

analysis (PCA) to determine whether the samples in different groups could be effectively distinguished. Then, the supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was performed for each pair of the Exp-L, Exp-M, and Exp-H groups, and the variables with larger contribution (determined based on variable importance of the projection, VIP) to the OPLS-DA models were acquired. Afterward, the fold change (FC) and P-value of the identified metabolites between each pair of the Exp-L, Exp-M, and Exp-H groups were calculated by moderate t-statistic using the GraphPad Prism 8 software. The differentially expressed metabolites were screened based on the following criteria: for GC-MS data, VIP > 1, P-value < 0.05, and FC > 1.4 (up-regulated) or < 0.7 (down-regulated); and for LC-MS data, VIP > 1, P-value < 0.05, and FC > 1.1 (up-regulated) or < 0.9 (down-regulated). The Volcano plot of the identified metabolites was constructed using the VIP, FC, and P-value of each pair by GraphPad Prism 8 software. The KEGG pathway was searched using the screened differential metabolites. The obtained pathways were ranked by their $-\log_{10}$P-value, and the top 20 pathways are displayed in bubble charts.

The receiver operating characteristic (ROC) curve of the screened differential metabolites was constructed, and the area under the curve (AUC) was calculated using the IBM SPSS Statistics 26 software. The differential metabolites between each pair of the Exp-L, Exp-M, and Exp-H groups with a large AUC value (AUC > 0.70 in GC-MS or LC-MS data) were selected and compared to identify the mutually differential metabolites. Afterward, the box plots of the relative content of the mutual differential metabolites in the Exp-L, Exp-M, and Exp-H groups were plotted using the GraphPad Prism 8 software. Finally, the mutual differential metabolites were fitted with the comprehensive variable before being subjected to ROC analysis, and the AUC was calculated.

3 Results and discussion

3.1 Multivariate statistical analysis

The urine samples of 90 % women selected for this study were divided into three groups according to the PFOA concentration in the urine samples to study the metabolic effects on pregnant women exposure to PFOA at different degrees (which could be expressed as PFOA intake level or *in vivo* PFOA concentration). Since renal excretion is the dominant elimination mechanism of PFOA and the PFOA level in urine is positively correlated with the PFOA level in blood (Zhang et al., 2013; Feng et al., 2021), the PFOA concentration in the urine sample could be a representative of *in vivo* PFOA concentration, and the *in vivo* PFOA concentration varied in the pregnant women of three groups.

After the metabolome information of the urine samples was obtained by GC-MS and LC-MS, the multivariate statistical analysis was performed to explore the significant differences between the metabolomes of the three groups. The multivariate statistical analysis can simultaneously explore the effects of all metabolites and the relationship between the samples and the metabolites.

According to the metabolomic data of the urine samples of pregnant women in the QC, Exp-L, Exp-M, and Exp-H groups acquired by GC-MS and LC-MS, the PCA and OPLS-DA were performed to determine if these sample groups could be effectively distinguished and if the PFOA concentration *in vivo* could affect the metabolic processes. The PCA score plots of the metabolic profiles (Figs. 1(a) and 1(b)) showed that almost all samples were distributed within a 95 % confidence interval, and the QC samples were well clustered, indicating that the precision and stability of the instruments and methods meet the requirement of the experiments. The errors between the samples in each batch were controllable. However, the samples in the Exp-L, Exp-M, and Exp-H groups were widely distributed, and some of the positions overlapped. This result shows that these samples cannot be distinguished by the PCA models, demanding further statistical analysis.

The supervised OPLS-DA combines both the orthogonal signal correction and the partial least squares. The method concentrates on the primary gap between the predicted principal components, therefore, can better distinguish between different groups. According to the OPLS-DA score plots of the metabolic profile of each pair of the Exp-L, Exp-M, and Exp-H groups acquired by GC-MS (Figs. 1(c), 1(d), and 1(e)) and LC-MS (Figs. 1(f), 1(g), and 1(h)), each group could be well distinguished. Meanwhile, R^2Y (cum) and Q^2 (cum) from 200 sort responses of all OPLS-DA models were less than the original values, and the intercepts of the regression lines of Q^2 (cum) and Y-axis were less than 0, confirming that these models were effective and credible with no overfitting. These results suggest that there is a link between the degree of PFOA exposure to pregnant women and the changes in metabolic processes. Moreover, the variables with a larger contribution (based on VIP value) could be obtained from these OPLS-DA models and used to screen the differential metabolites.

3.2 Screening of differential metabolites

Identifying the differential metabolites in several groups can explore the mechanism underlying the changes in the metabolic, physiological, and pathological processes. Thus, screening differential metabolites, which can be used in subsequent bioinformatics search, is an important step in the metabolomic analysis. A multi-standard evaluation method is generally selected to improve the screening accuracy and reliability in the screening of differential metabolites.

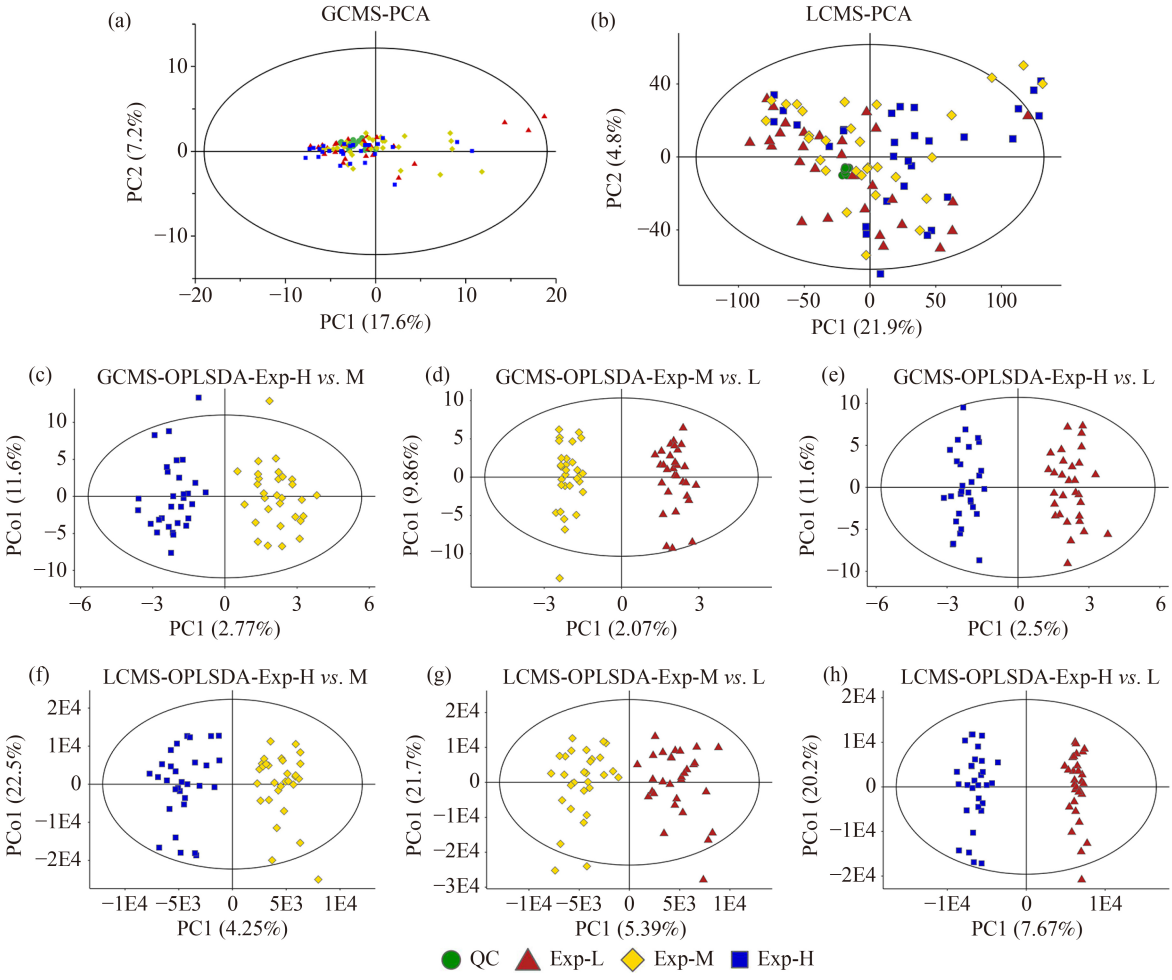

Fig. 1 Multivariate statistical analysis of the metabolomic data of pregnant women's urine samples in the QC, Exp-L, Exp-M, and Exp-H groups acquired by GC-MS and LC-MS. The PCA score plots of metabolomic data of pregnant women's urine samples in the QC, Exp-L, Exp-M, and Exp-H groups acquired by (a) GC-MS and (b) LC-MS. The OPLS-DA score plots of metabolomic data of (c) Exp-H *vs.* Exp-M, (d) Exp-M *vs.* Exp-L, and (e) Exp-H *vs.* Exp-L groups acquired by GC-MS. The OPLS-DA score plots of metabolomic data of (f) Exp-H *vs.* Exp-M, (g) Exp-M *vs.* Exp-L, and (h) Exp-H *vs.* Exp-L groups acquired by LC-MS.

Based on the VIP, FC, and P-value, the differential metabolites in the Exp-H *vs.* Exp-M, Exp-M *vs.* Exp-L, and Exp-H *vs.* Exp-L detected by GC-MS (Figs. 2(a), 2(b), and 2(c)) and LC-MS (Figs. 2(d), 2(e), and 2(f)) were screened, and the obtained differential metabolites are summarized in Tables S1 and S2, respectively. These differential metabolites have important biological significance and can be used to explore the relationship between the PFOA concentration *in vivo* and the changes in the metabolic, physiological, and pathological processes in pregnant women.

3.3 Biological significance of differential metabolites

The obtained differential metabolites were searched against the KEGG database to search for the metabolic pathways that were altered upon PFOA exposure to different degrees. The pathways involved in the differential metabolites of Exp-H *vs.* Exp-M, Exp-M *vs.* Exp-L, and Exp-H *vs.* Exp-L detected by GC-MS (Figs. 3(a), 3(b), and 3(c)) and LC-MS (Figs. 3(d), 3(e), and 3(f)) mainly included amino acid metabolism, purine metabolism, energy metabolism, including citrate cycle and oxidative phosphorylation, glucose metabolism, glutathione metabolism, and digestion and absorption of nutrients, such as carbohydrates, proteins, and vitamins. This result indicates that the degrees of PFOA exposure have a significant impact on pregnant women, and these changes in the metabolic processes might be associated with pregnancy-induced hypertension, gestational diabetes mellitus, and preeclampsia.

Furthermore, the ROC analysis was performed, and the AUC values were calculated to further study the clinical diagnosis values of these differential metabolites. The AUC values were then used as the parameters to select the key differential metabolites, which could potentially be used as biomarkers to distinguish the different degrees of PFOA exposure to pregnant women and explore the

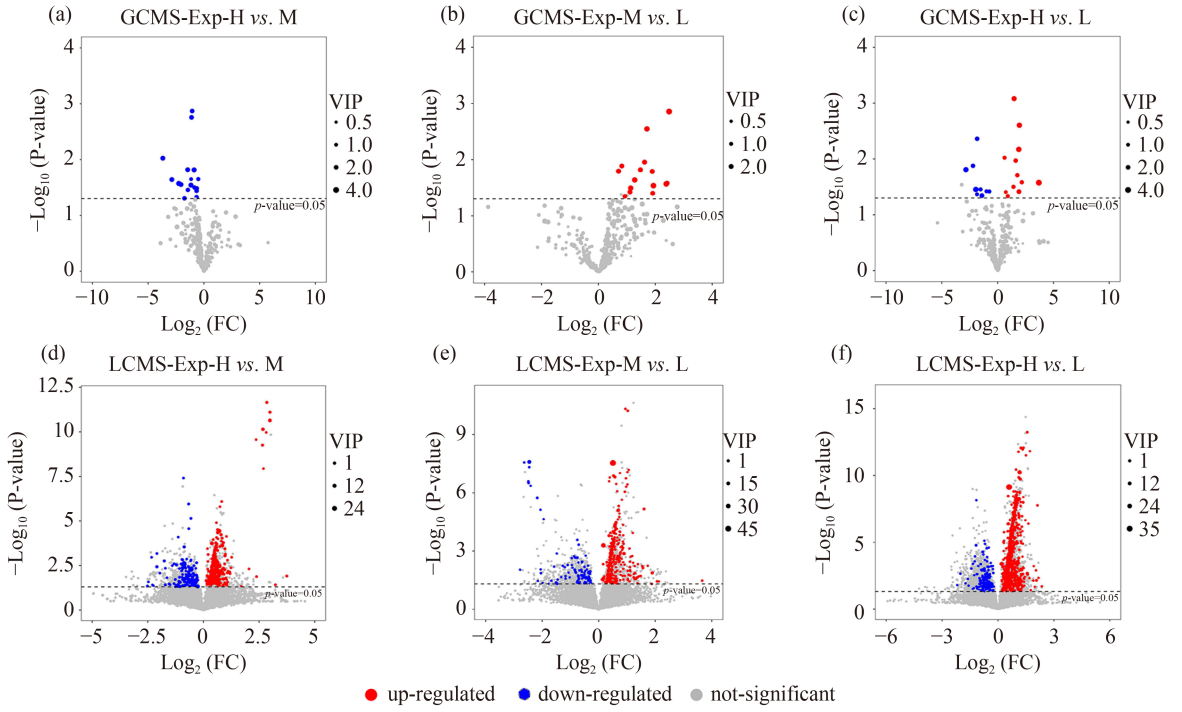

Fig. 2 Volcano plots of metabolites in the urine samples of pregnant women in the Exp-H, Exp-M, and Exp-L groups acquired by GC-MS and LC-MS. The volcano plots of metabolites in the urine samples of (a) Exp-H *vs.* Exp-M, (b) Exp-M *vs.* Exp-L, and (c) Exp-H *vs.* Exp-L groups acquired by GC-MS. The volcano plots of metabolites in the urine samples in (d) Exp-H *vs.* Exp-M, (e) Exp-M *vs.* Exp-L, and (f) Exp-H *vs.* Exp-L groups acquired by LC-MS.

toxicity mechanism of PFOA in pregnant women. The AUC values of the obtained differential metabolites are summarized in Tables S1 and S2. Later, the differential metabolites obtained from the comparison of each pair of samples in the Exp-L, Exp-M, and Exp-H groups with large AUC values (AUC > 0.70 in both GC-MS and LC-MS data) were selected and compared to find the mutually differential metabolites. Based on the data acquired by GC-MS, L-cysteine and dehydroabietic acid were found in at least two comparison groups (Exp-H *vs.* Exp-M, Exp-M *vs.* Exp-L, and Exp-H *vs.* Exp-L groups). Based on the data acquired by LC-MS, glycine and 5-aminovaleric acid were found in all comparison groups. According to the box plots of the content of the above metabolites depicted in Fig. 4, the contents of L-cysteine and dehydroabietic acid analyzed by GC-MS in the Exp-M group significantly increased than that of the Exp-L group, and those in the Exp-M and Exp-H groups were not significantly different. Additionally, the contents of glycine and 5-aminovaleric acid obtained from LC-MS increased with increasing PFOA content in pregnant women. These results indicate that there is a correlation between the changes of L-cysteine, dehydroabietic acid, glycine, and 5-aminovaleric acid contents in the urine samples and the degree of PFOA exposure on pregnant women.

The mutually differential metabolites can be used to identify the degree of PFOA exposure on pregnant women. The data on L-cysteine and dehydroabietic acid acquired by GC-MS, and the data on glycine and 5-aminovaleric acid acquired by LC-MS were fitted with the multivariable model through logistic regression. As depicted in Figs. 5(a), 5(b), and 5(c), the ROC of the multivariable model fitted well with the data on L-cysteine and dehydroabietic acid acquired by GC-MS in the comparison pairs of Exp-H *vs.* Exp-M, Exp-M *vs.* Exp-L, and Exp-H *vs.* Exp-L, while the AUC/sensitivity/specificity were 0.509/0.767/0.367, 0.800/0.633/0.933, and 0.872/0.733/0.867, respectively. According to Figs. 5(d), 5(e), and 5(f), the ROC of the multivariable model fitted well with the data on glycine. The 5-aminovaleric acid acquired by LC-MS in the comparison pairs Exp-H *vs.* Exp-M, Exp-M *vs.* Exp-L, and Exp-H *vs.* Exp-L, and the AUC/sensitivity/specificity were 0.748/0.633/0.833, 0.809/0.867/0.633, and 0.912/0.867/0.833, respectively. It can be seen that the AUC values of the two multivariable in the Exp-H *vs.* Exp-M and Exp-M *vs.* Exp-L pairs were less than 0.85, while those in the Exp-H *vs.* Exp-L pair were higher than 0.85. These results indicate that the multivariable is unable to distinguish between groups with continuous PFOA concentration *in vivo* but could distinguish between groups with largely different PFOA concentrations *in vivo*. The results also confirmed that the changes in the metabolic processes in pregnant women were significantly correlated with the degree of PFOA exposure. Moreover, we may be able to approximate the

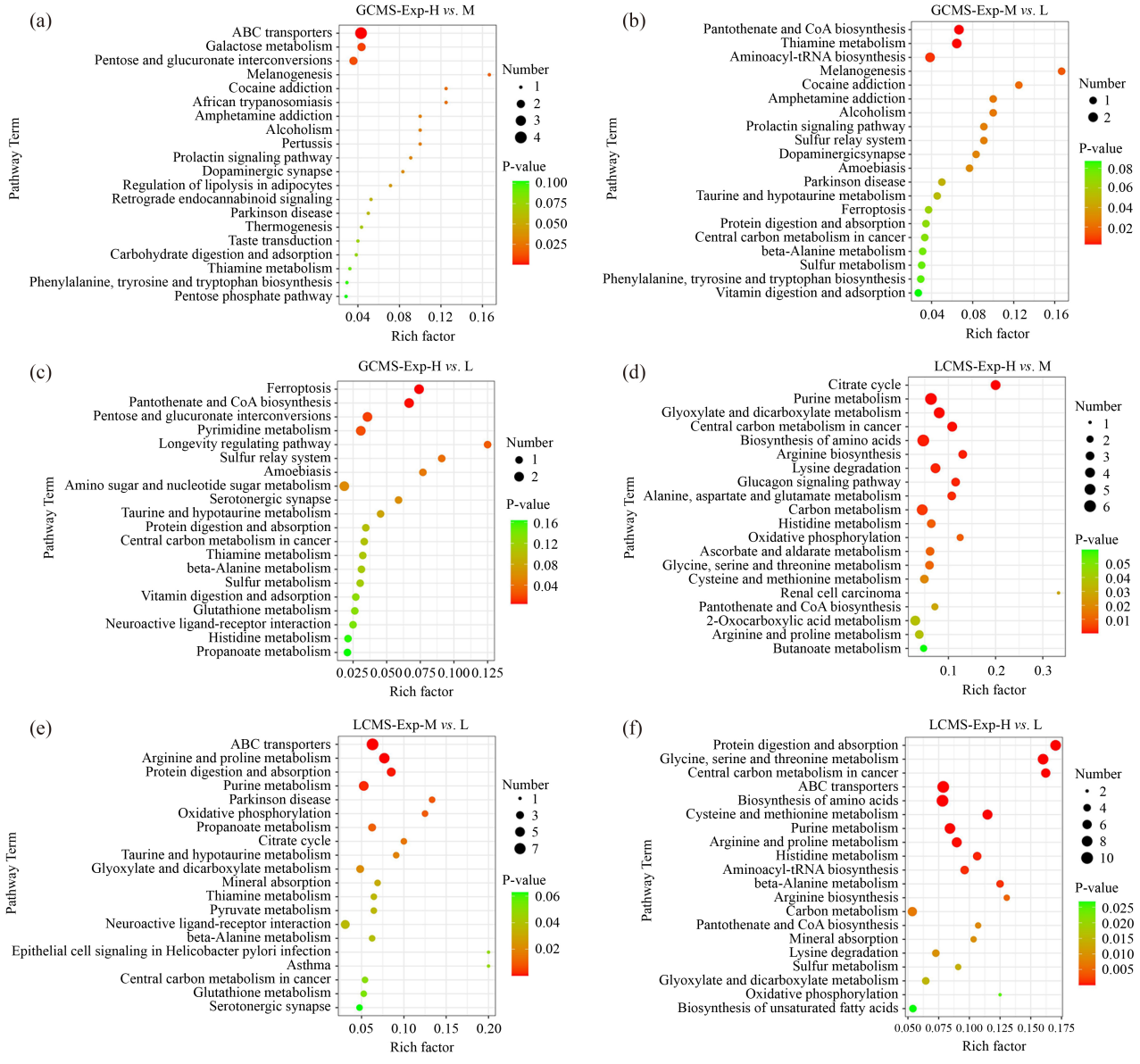

Fig. 3 Bubble diagrams of the KEGG pathways of differential metabolites in Exp-H, Exp-M, and Exp-L groups acquired by GC-MS and LC-MS. Bubble diagrams of the KEGG pathways of differential metabolites observed in (a) Exp-H *vs.* Exp-M, (b) Exp-M *vs.* Exp-L, and (c) Exp-H *vs.* Exp-L by GC-MS. Bubble diagrams of the KEGG pathways of differential metabolites detected in (d) Exp-H *vs.* Exp-M, (e) Exp-M *vs.* Exp-L, and (f) Exp-H *vs.* Exp-L by LC-MS.

degrees of PFOA exposure on the pregnant women based on these mutually differential metabolites and their fitted multivariable (except for those obtained from the Exp-H *vs.* Exp-M pair). This information can be helpful for formulating countermeasures and relieving the toxicity of PFOA in pregnant women.

Combining the mutually differential metabolites acquired by GC-MS and LC-MS, except for dehydroabietic acid, an exogenous substance, L-cysteine, glycine, and 5-aminovaleric acid were endogenous metabolites. These metabolites play significant roles in the changes in the metabolic processes in pregnant women exposed to PFOA. These changes might be dependent on the exposure degree.

Dehydroabietic acid, one of the primary components of rosin and is widely used in the food and pharmaceutical, paint, soap, and rubber industries. Therefore, dehydroabietic acid could be found in the paper mill wastewater, river and can be detected in a wide range of consumer products, such as medicaments, cosmetics, and food packaging materials (Qiu et al., 2020). As a result, there is a high risk of human exposure to dehydroabietic acid, which can be detected in human urine (Baldwin et al., 2007) and blood (Xu et al., 2021) samples. In our study, the reason for detecting dehydroabietic acid might be related to their living environment, living habits, and

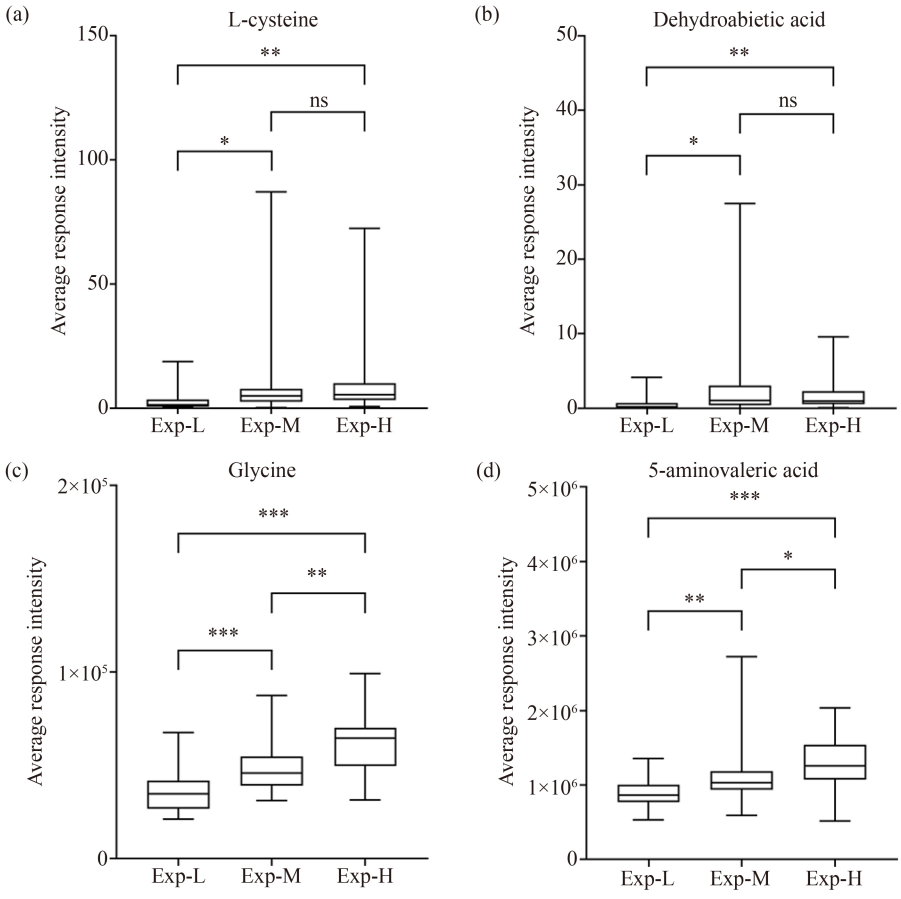

Fig. 4 Box plots of L-cysteine, dehydroabietic acid, glycine, and 5-aminovaleric acid contents in pregnant women's urine samples in the Exp-L, Exp-M, and Exp-H groups: (a) L-cysteine and (b) dehydroabietic acid analyzed by GC-MS; and (c) glycine and (d) 5-aminovaleric acid analyzed by LC-MS. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

eating habits, such as the preference for some types of foods and cosmetics or unqualified drinking water in the place of residence.

L-cysteine and glycine are essential precursor amino acids for the synthesis of glutathione (GSH), an important antioxidant in the body that plays a vital role in the elimination of reactive oxygen species (ROS) (Arazi et al., 2021). One of the major sources of ROS is oxidative phosphorylation (Asantewaa and Harris, 2021), which was altered in pregnant women exposed to PFOA, as shown in Fig. 3. The imbalance between the production and elimination of ROS can lead to oxidative stress, which could cause oxidative damage to the tissues and degradation of biomolecules, such as DNA, lipids, and proteins (Arazi et al., 2021). The imbalance might cause liver toxicity, nephrotoxicity, neurotoxicity, and reproductive system toxicity, as well as poor development of infants (Steenland et al., 2020; Fenton et al., 2021; Podder et al., 2021). In this study, the contents of L-cysteine and glycine increased with the increase of the PFOA content in the pregnant women increased, indicating that the PFOA exposure might interfere with the synthesis of GSH or promote the degradation of GSH. The synthesis or degradation of GSH caused by PFOA exposure can reduce the content of GSH, turn affecting the elimination of ROS, resulting in oxidative stress.

5-Aminovaleric acid is a lysine degradation product, and endogenous 5-aminovaleric acid is usually considered a microbial metabolite as it is mainly produced by the oral or intestinal microbiota (Zeng et al., 2020). Studies have reported that the content of 5-aminovaleric acid in the saliva of metabolically unhealthy obese individuals is significantly up-regulated than that of metabolically healthy obese individuals. The up-regulated 5-aminovaleric acid indicates that obesity might be caused by the changes in metabolic processes (Cialiè Rosso et al., 2021). Additionally, the change in 5-aminovaleric acid might be related to cholesterol metabolism (Bisht et al., 2021). Therefore, the degree of PFOA exposure might lead to changes in the oral or intestinal microbiota, causing abnormal fat metabolism in pregnant women, such as lipid production, bile acid metabolism and synthesis, and fatty acid transport. This result was consistent with several previous study results

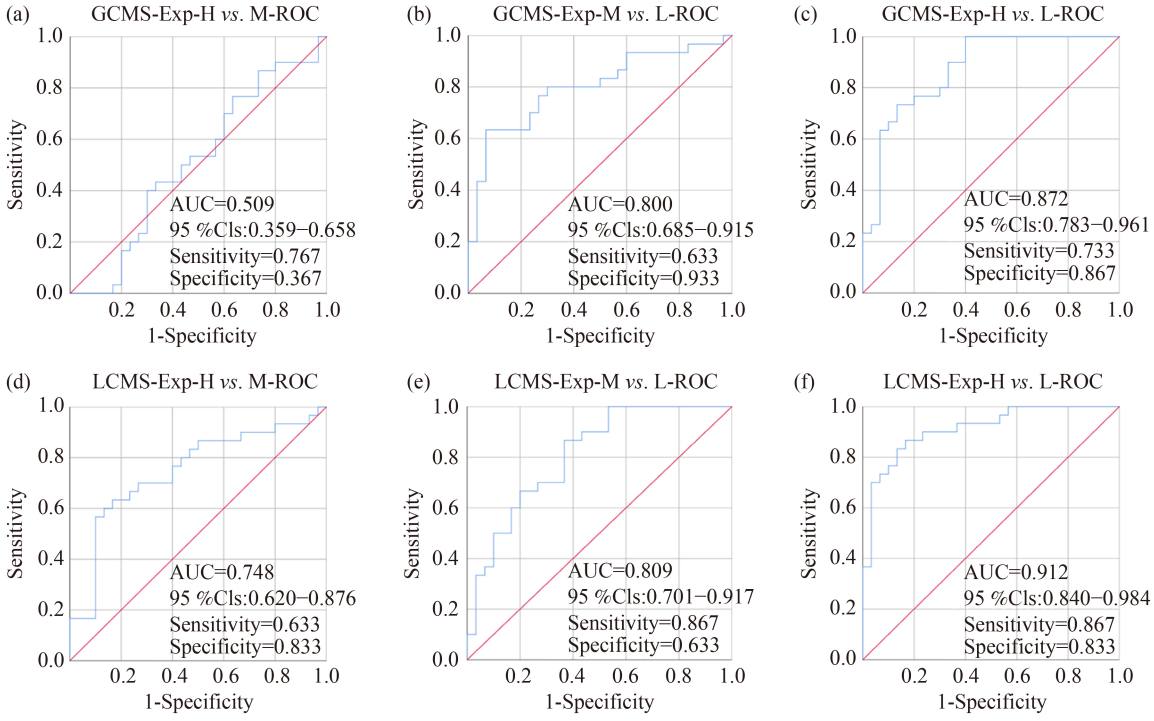

Fig. 5 ROC of fitted multivariable from the comparison pairs Exp-H *vs*. Exp-M, Exp-M *vs*. Exp-L, and Exp-H *vs*. Exp-L. ROC of the multivariable was fitted with the data on L-cysteine and dehydroabietic acid acquired by GC-MS from the comparison pairs (a) Exp-H *vs*. Exp-M, (b) Exp-M *vs*. Exp-L, and (c) Exp-H *vs*. Exp-L. ROC of multivariable fitted using the data on glycine and 5-aminovaleric acid acquired by LC-MS from the comparison pairs (d) Exp-H *vs*. Exp-M, (e) Exp-M *vs*. Exp-L, and (f) Exp-H *vs*. Exp-L.

(Salihovic et al., 2019; Zhang et al., 2019; Behr et al., 2020) reporting that abnormal lipid metabolism has a significant association with PFOA exposure. The dysregulation of lipogenesis could increase insulin resistance in the body and then cause diabetes (Alderete et al., 2019; Lin et al., 2019). Overall, investigating the mechanism by which different degrees of PFOA exposure affect the 5-aminovaleric acid content is of great significance.

4 Conclusions

In this study, the metabolome of urine samples from pregnant women exposed to PFOA at different degrees was detected by GC-MS and LC-MS. Based on the detected metabolites and their relative contents, the samples in different groups were distinguished through OPLS-DA. Based on the VIP, FC, and P-value, the differential metabolites between each comparison group were screened, and the metabolic pathways related to the degree of PFOA exposure of pregnant women were searched in the KEGG database. The ROC analysis of the differential metabolites was performed, and the differential metabolites from each comparison group with a high AUC value were selected and compared to identify the mutually differential metabolites. The mutually differential metabolites were subsequently fitted with a multivariable to determine if they could distinguish between the pregnant women exposed to PFOA at different degrees. Finally, based on the mutual differential metabolites L-cysteine, glycine, and 5-aminovaleric acid, we found that the PFOA exposure and the degree of exposure could affect the synthesis or degradation of GSH and change the metabolic processes of oral or intestinal microbiota, thereby causing abnormal fat metabolism in the pregnant women.

The findings in this study indicated that the exposure to PFOA and the degrees of exposure have an impact on the metabolic, physiological, and pathological processes of pregnant women. However, the non-targeted metabolomic approach using GC-MS and LC-MS showed low accuracy in determining the metabolites. Therefore, a more accurate, targeted metabolomic approach should be further performed to obtain more accurate contents of metabolites. Additionally, it is necessary to perform other biological experiments to explore and verify the toxicity mechanism of PFOA. The data presented in this study provides direction on PFOA toxicity for future research so that we can better avoid and treat the adverse effects of PFOA.

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Author Contributions Kaige Yang: Methodology, Visualization, Formal analysis. Zhouyi Zhang: Formal analysis, Validation. Kangdie Hu: Investigation. Bo Peng: Conceptualization, Methodology. Weiwei Wang: Investigation. Hong Liang: Resources, Supervision. Mingyuan Wu: Methodology. Yan Wang: Conceptualization, Data Curation. Chao Yan: Conceptualization.

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