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Original Research

Serum Metabolomic Profile in Hypoxia-Induced Pulmonary Hypertension Mice after C75 Treatment

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Abstract

Background: Inhibition of fatty acid synthase (FAS) plays a crucial protective role in pulmonary hypertension (PH). Our aim was to identify novel metabolites in mice with hypoxia-induced PH after treatment with C75 (FAS inhibitor) and to confirm the presence of these metabolites in paediatric patients with PH. **Methods**: The PH mouse model was built by chronic hypoxia and ovalbumin (OVA) assistance. Untargeted metabolomics was used to analyse mouse serum. Six children with PH and six relative controls (patients without lung and heart disease) were selected in Shanghai Children's Hospital and they all performed blood tandem mass spectrometry during hospitalization. **Results**: First, a total of 29 differential metabolites, including lipid metabolites, polyamine, and glutamine were identified as differential metabolites in the hypoxia group compared with the control group. After C75 treatment, symptoms were partially relieved in the PH mouse, and 15 differential metabolites, including lipid metabolites, polyamine, and glutamine were identified in the hypoxia + C75 group compared with the hypoxia group. These differential metabolites were enriched in arginine and glycerolipid metabolism through metabolite set enrichment analyses and were involved in excessive cell proliferation, which was a characteristic of PH. Second, glutamine and caproyl carnitine levels were increased in paediatric patients with PH. **Conclusions**: FAS may be a potential PH therapeutic target. Lipid metabolites, polyamine, and glutamine, are closely related to PH. Putrescine and glutamine might be biomarkers for PH.

Keywords: metabolomics; pulmonary hypertension; C75; biomarkers

1. Introduction

Pulmonary hypertension (PH) occurs in both children and adults. It has a high morbidity and mortality rate [1,2]. PH is characterized by hyperproliferation of pulmonary artery smooth muscle cells (PASMCs) and endothelial cells, resulting in resistant pulmonary arterial obstruction, remodelling of small pulmonary arteries, elevated pulmonary artery pressure; as result, PH can even lead to right ventricular failure and death [3]. There are many PH specific drugs, such as prostanoids, endothelin receptor antagonists, phosphodiesterase inhibitors, and soluble guanylate cyclase stimulators [4]. Although these therapies can improve patients' quality of life to some extent, the long-term prognosis of patients remains poor [4].

Metabolomics analyses have been widely applied to explore biomarkers in PH patients and PH rodent models [5,6]. Previous studies reported that the metabolic profiles were changed in rodent PH model plasma, lung tissue, right ventricle (RV) tissue, and even in PH patients [5,7–10]. A study in the primary Chinese PH patient cohort showed that lipid metabolism, amino acid metabolism, glucose metabolism, and phospholipid metabolism pathways were dysregulated in patients with PH [5]. Hautbergue *et al.* [6] showed that arginine and tryptophan metabolic pathways were the major altered pathways in chronic hypoxia-induced PH rat models, and they thought that acetyl spermidine and putrescine were negatively correlated with RV function [6]. However, there is still no metabolic profile of the PH model after effective treatment.

Metabolites from lipid metabolism and fatty acid oxidation were closely related to PH [5]. Singh *et al.* [11] showed that PH rats' energy metabolism was altered, especially fatty acid synthesis, which was similar that observed for cancer. Fatty acid synthase (FAS) is a key enzyme involved in the production of long-chain fatty acids [12]. Singh *et al.* [11] also noted that modulating de novo fatty acid synthesis via inhibition of FAS was beneficial to PH, and they found that FAS inhibition ameliorated the symptoms and pulmonary vascular remodelling of monocrotaline (MCT)-treated rats. They observed that FAS expression and activity were increased in both hypoxia-exposed PASMCs and the lungs of MCT-treated rats [11]. In our previous study, we demonstrated that C75, a FAS inhibitor, alleviated the symptoms of the hypoxia-induced PH

mouse model [13]. Herein, based on our previous mouse model [13], the serum metabolic profiles of three groups (control, hypoxia, and hypoxia + C75) were detected and analysed via gas chromatography-mass spectrometry (GC-MS) nontargeted metabolomics. In this study, we explored the effect of C75 at the metabolic level and tested whether these metabolites could be serum biomarkers for PH.

2. Materials and Methods

2.1 Chemicals

N, O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), L-norvaline, L-norleucine, methanol, methoxyamine hydrochloride, and anhydrous pyridine, were purchased from Sigma-Aldrich.

2.2 Chronic Hypoxia-Induced Pulmonary Hypertension Mice

Several C57BL/6 mice (8 weeks old) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). The PH mouse model was established through chronic hypoxia and OVA assistance as previously described [13–15]. Briefly, the PH mouse model was as follows: the hypoxia + C75 group received C75 (200 μg/kg/week, dissolved in 0.5% dimethylsulfoxide (DMSO)) for 5 weeks; the hypoxia group was intraperitoneally injected with the same amount of 0.5% DMSO without C75 each week; and the control group received 0.5% DMSO injection and was raised in a normoxic environment [13–15]. All animal experiments were performed with the approval of the Shanghai Jiao Tong University Institutional Animal Care and Use Committee.

2.3 Sample Collection and Preparation

The serum was collected and frozen at –80 ℃ until gaschromatography-mass spectrometry (GC/MS) analyses. Blood samples from four mice were randomly selected from each group. Serum samples were thawed and vortexed, 20 μL of which was mixed with 80 μL of cold methanol and an internal standard (5 μg/mL L-norvaline). The mixture was vortexed and maintained at –20 ℃ overnight. Following centrifugation, 70 μL of supernatant and 10 μL of L-norleucine (50 μg/mL) in a glass vial were evaporated to dryness under a nitrogen stream. Methoxyamine hydrochloride (50 μL, 20 mg/mL) in pyridine was subsequently added to the residue. 50 μL of BSTFA (with 1% TMCS) was added to the mixture and then derivatized at 70 °C prior to GC-MS metabolomics analyses. At the same time, a blank derivatization sample needs to be prepared in the process of sample preparation and GC/MS analyses. Quality control (QC) samples were pooled from all samples and they were prepared and analysed with the same procedure as the experimental samples.

2.4 GC/MS Analyses

Agilent gas chromatography-mass spectrometry (7890A/5975C) and an OPTIMA® 5 MS Accent fused silica capillary column (30 m × 0.25 mm × 0.25 μm, MACHEREY-NAGEL, Düren, German) were used to perform metabolomics on the derivatives. Helium passed through the column as the carrier gas and the constant flow rate was 1 mL/min. The gradient heating procedure was 60 °C for 1 min, 60 ℃ to 240 °C at a rate of 12 °C/min, 240 °C to 320 °C at a rate of 40 °C/min and finally maintained at 320 °C for 4 min. The solvent delay time was set to 5.4 min. The injection volume was 1 μL in nonshunt mode. The temperatures of the injector, transfer line, and electron impaction source were set to 250 °C, 260 °C, and 230 °C, respectively. The electron ionization energy was set at 70 eV. The data was collected in full-scan mode (m/z 50–600). The samples were annalysed in a random order.

2.5 Data Preprocessing

The original GC-MS data were processed with reference to previously published protocols [16]. The data were normalized against total peak abundance and the normalized data were imported into a SIMCA-P (version 14.1, Umetrics, Umea, Sweden), in which the data were preprocessed by unit variance [17] scaling and mean centering before performing principal component analyses (PCA), partial least-squares discriminant analyses (PLS-DA), and orthogonal partial least-squares discriminant analyses (OPLS-DA). The potential differential metabolites were identified by combining variable influence on projection (VIP) values of the OPLS-DA model (VIP >1) and p values of Student's t test ($p < 0.05$) on the normalized peak areas. The false discovery rate (FDR) was calculated by the Benjamini-Hochberg method. Fold change was calculated as a binary logarithm of the average mass response (normalized peak area) ratio between the two groups.

2.6 Structural Identification of Metabolites

Structural identification of differential metabolites was performed as follows. The AMDIS software (version 2.70, NIST, Gaithersburg, MD, USA) was applied to deconvolute mass spectra from raw GC-MS data, and the purified mass spectra were automatically matched with an author-constructed standard library including retention time and mass spectra, Golm Metabolome Database, and Agilent Fiehn GC/MS Metabolomics RTL Library, by which the metabolites were structurally confirmed.

2.7 Pathway Analyses

The online software MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) was used to analyse the pathways of differential metabolites. Biosynthesis of essential amino acids was included as mouse metabolism is involved in the intestinal flora.

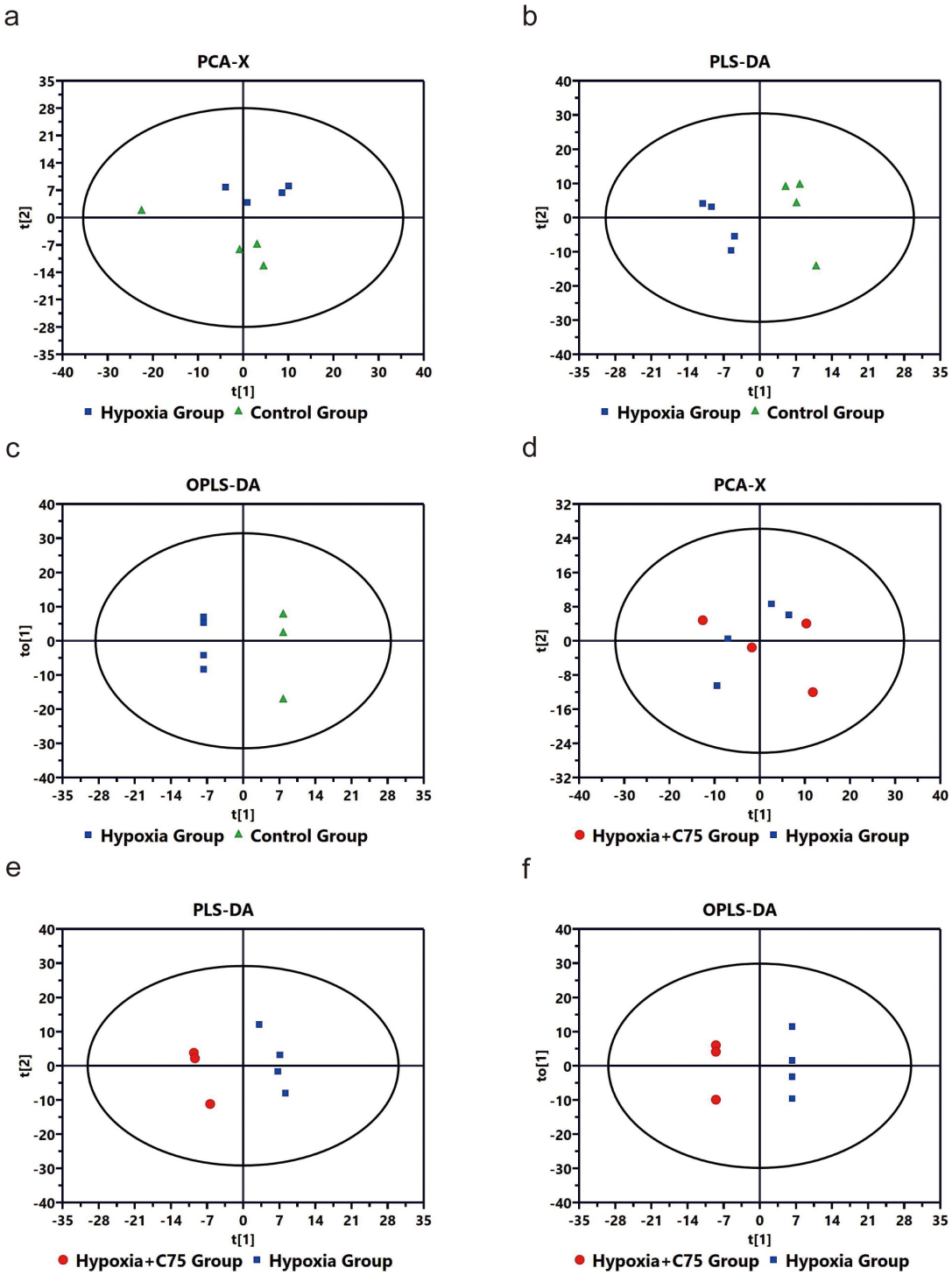

Fig. 1. The metabolic differences in hypoxia-induced PH mice serum. (a–c) The PCA, PLS-DA, OPLS-DA plots between the control and hypoxia group. (d) The PCA plots between the hypoxia and hypoxia + C75 group. (e,f) The PLS-DA, OPLS-DA plots between the hypoxia and hypoxia + C75 group after discarding an outlier of hypoxia + C75 group. PCA, principle component analyses; PLS-DA, partial least-squares discriminant analyses; OPLS-DA orthogonal partial least-squares discriminant analyses.

2.8 The Serum Levels of Metabolites in Pulmonary Hypertension Patients

The data were analysed retrospectively and were obtained from six patients with PH (PH group) and six relative controls (patients without PH and heart disease) of Shanghai Children's Hospital, Shanghai, China, between 2018 and 2021. All patients underwent blood tandem mass spectrometry during hospitalization. Recruited patients in the PH group were diagnosed with PH according to Doppler echocardiography and clinical presentation [18]. Echocar-

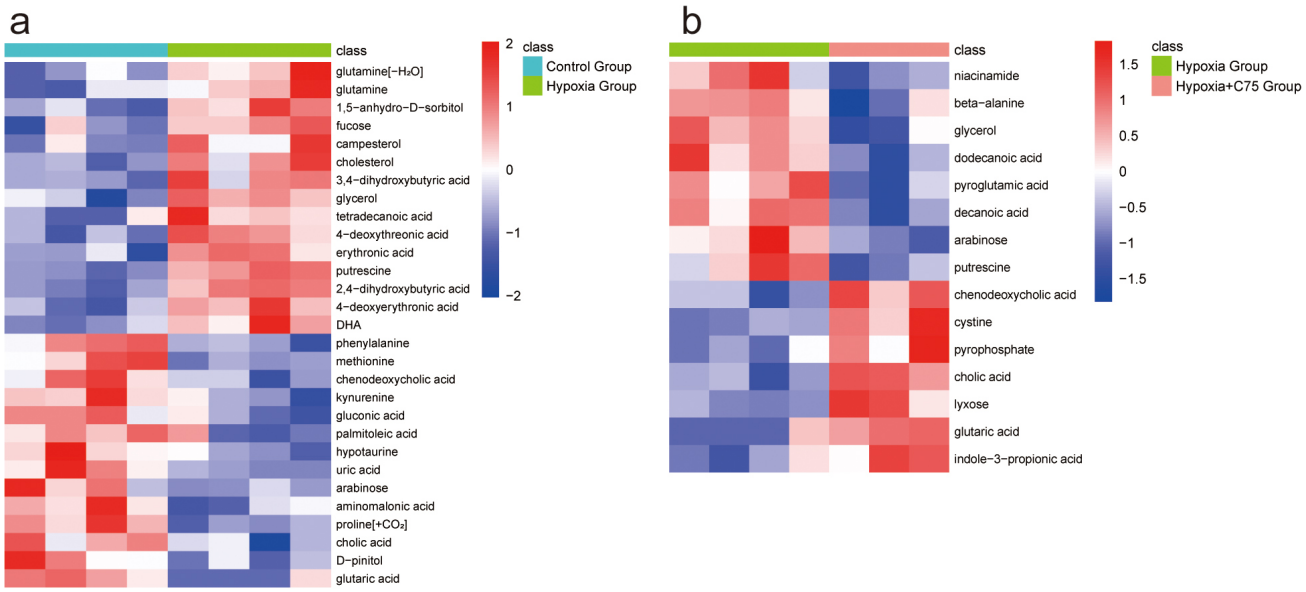

Fig. 2. Heatmap of the potential differential metabolites. (a) Representative image of the comparison between the control and hypoxia groups. (b) Representative image of the comparison between the hypoxia and hypoxia + C75 groups. Red represents the high relative level of each metabolite, and purple represents the low relative level of each metabolite.

diographic signs suggesting right atrial enlargement, right ventricular hypertrophy, pulmonary artery diameter expansion, or congenital heart disease were used to assess the probability of PH in addition to clinical manifestations. Patients with other potential causes of lung disease, such as pneumonia and connective tissue disease were excluded from this study.

2.9 Statistical Analyses

The results of clinical data are represented as the median (interquartile ranges, IQRs). Comparisons among groups were evaluated by a nonparametric method. Statistical analyses were performed using SPSS software, version 21.0 (IBM Corp. Inc., Armonk, NY, USA) and graphed with GraphPad Prism version 7 (GraphPad Software, Inc., San Diego, CA, USA). For univariate statistical analyses, the normalized data derived from GC/MS analyses were calculated by a two-tailed Student's *t* test in Excel 2007 (Microsoft, Redmond, WA, USA). $p < 0.05$ was considered a significant difference.

3. Results

3.1 Metabolomic Profiles

GC/MS-based metabolomics was used to profile the serum metabolome of C57BL mice from 3 groups. PCA was established to show the metabolic difference between the control and hypoxia groups. The PCA results showed that there was a significant difference between the control and hypoxia groups (Fig. 1a) and the current model was reliable ($R^2X = 0.569$). Then, we confirmed the results by a PLS-DA ($R^2X = 0.645$, $R^2Y = 0.999$, $Q^2 = 0.868$) assay (Fig. 1b). An OPLS-DA assay ($R^2Y = 1$, $Q^2 = 0.887$) was used to analyse the differential metabolites. The OPLS-DA results showed a significant separation between the control and hypoxia groups (Fig. 1c). However, the PCA score plots (Fig. 1d) showed that there was no tendency to separate the hypoxia group from the hypoxia + C75 group ($R^2X = 0.476$). When we discarded an outlier of the hypoxia + C75 group, the effective models of PLS-DA ($R^2X = 0.452$, $R^2Y = 0.994$, $Q^2 = 0.739$) (Fig. 1e) and OPLS-DA ($R^2X = 0.867$, $R^2Y = 1$, $Q^2 = 0.999$) (Fig. 1f) were built and the two models were clearly reliable. Both PLS-DA and OPLS-DA score plots showed that the hypoxia and hypoxia + C75 groups were distinctly separated. The two models revealed a significant metabolic difference between the hypoxia and hypoxia + C75 groups.

3.2 Differential Metabolites Including Fatty Acids, Polyamine, and Glutamine

As described above, differential metabolites were identified through the combination of VIP values (VIP value >1) acquired from the OPLS-DA model and *p* values (*p* value < 0.05) from Student's *t* test on the normalized peak. There were 29 differential metabolites identified in the hypoxia group compared with the control group (**Supplementary Table 1**). The visualized heatmap between the control and hypoxia groups was shown in Fig. 2a. Fifteen metabolites were upregulated and 14 were downregulated in the hypoxia group. Among them, glycerol, cholesterol, docosahexaenoic acid, putrescine, glutamine, and other metabolites were significantly increased in the hypoxia group compared with the control group, while cholic acid, chenodeoxycholic acid and other metabolites were decreased.

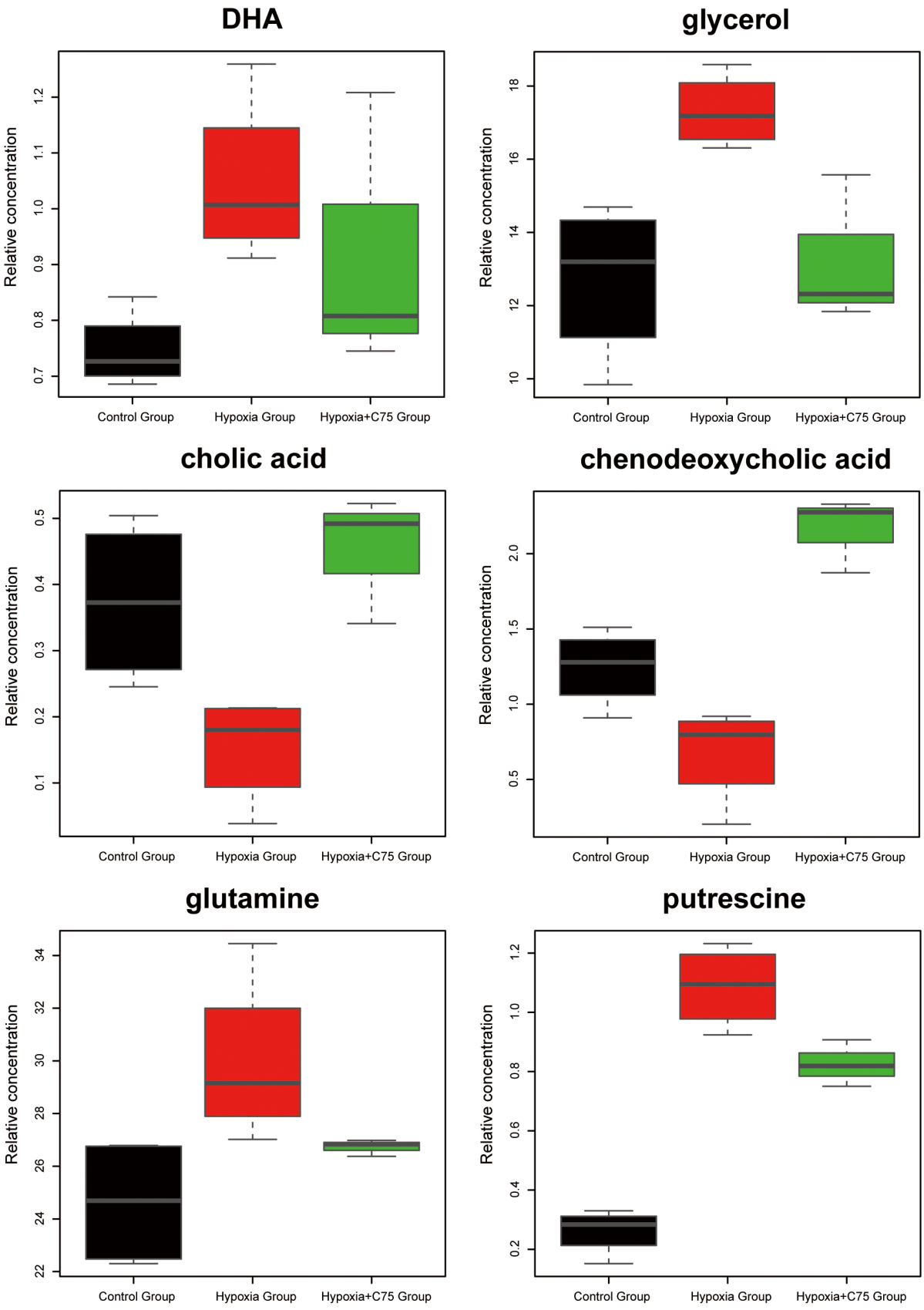

Fig. 3. The statistical diagram of serum differential metabolites including DHA, glycerol, cholic acid, chenodeoxycholic acid, glutamine, and putrescine shared by the three groups of mice. Values in the box plots are presented as the normalized peak areas of the metabolites in the three groups. DHA, Docosahexaenoic acid.

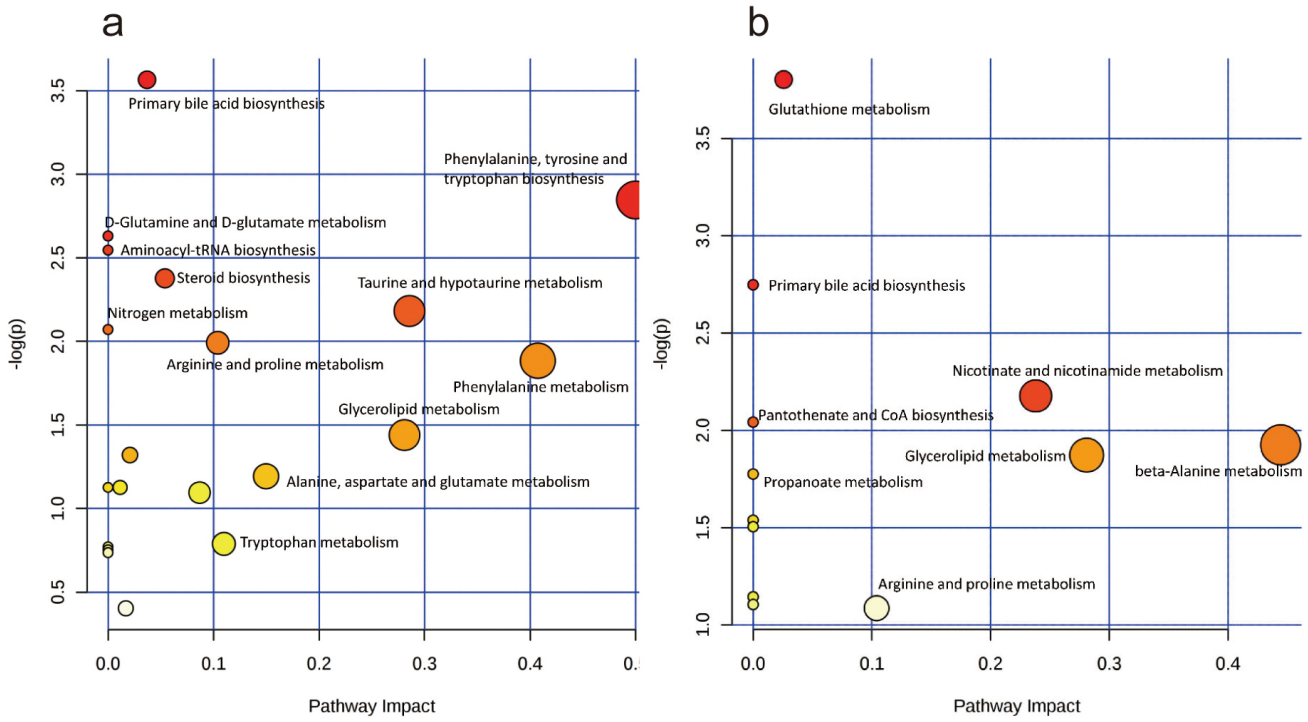

Fig. 4. Metabolic pathway analysis shows the pathways of significantly altered metabolites. (a) The results of metabolite set enrichment analyses of the control and hypoxia group. (b) The results of metabolite set enrichment analyses of the hypoxia and hypoxia + C75 group. The spot size represents the contribution of the metabolic pathway to the difference between the two groups, and the color represents the correlation between the metabolic pathway and difference between the two groups.

Fifteen metabolites were identified in the hypoxia + C75 group compared with the hypoxia group (**Supplementary Table 2**). The visualized heatmap between the hypoxia and hypoxia + C75 groups were shown in Fig. 2b. Seven metabolites were upregulated and 8 were downregulated in the hypoxia + C75 group. We observed that cholic acid and chenodeoxycholic acid levels were increased in the hypoxia + C75 group compared to the hypoxia group, whereas the levels of glycerol, putrescine, and pyroglutamine were decreased.

Based on the above analyses, we found that fatty acids, polyamine, and glutamine were common differential metabolites. In addition, other common differential metabolites including Docosahexaenoic Acid (DHA), glycerol, cholic acid, chenodeoxycholic acid, glutamine, and putrescine were increased in the hypoxia group compared with the control group, and C75 treatment was partially rescued (Fig. 3).

3.3 Metabolic Pathway Analyses

Metabolomics comprehensive treatment software MetaboAnalyst 4.0 (http://www.metaboanalyst.ca, McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada) was used to analyse potential metabolic pathways. Pathway impact $\geq$0.10 was identified as the potential target pathway criterion. Phenylalanine, tyrosine, tryptophan biosynthesis, taurine, hypotaurine, arginine, proline, phenylalanine, glycerolipid, alanine, aspartate, glutamate, and tryptophan metabolism were identified and involved in PH metabolic disorder resulting from chronic hypoxia intervention (Fig. 4a). Nicotinate, nicotinamide, beta-alanine, glycerolipid, arginine, and proline metabolism were identified as changed metabolic pathways in the hypoxia + C75 group compared with the hypoxia group (Fig. 4b). Based on the above, we hypothesized that arginine, proline, and glycerolipid metabolism were related to PH.

3.4 Retrospective Analysis of Serum Metabolite Levels in Paediatric Pulmonary Hypertension Patients

Next, we examined the levels of the differential metabolites mentioned above and other PH related metabolites [5,8] in paediatric PH patients. We selected six PH children and six relative controls (patients without lung and heart disease) and examined the serum metabolite levels. Clinical information of the recruited patients was shown in **Supplementary Table 3**. We found that glutamine and caproyl carnitine levels were increased in PH paediatric patients. Palmitoyl carnitine was also increased in PH paediatric patients; however, the difference was not significant (Fig. 5).

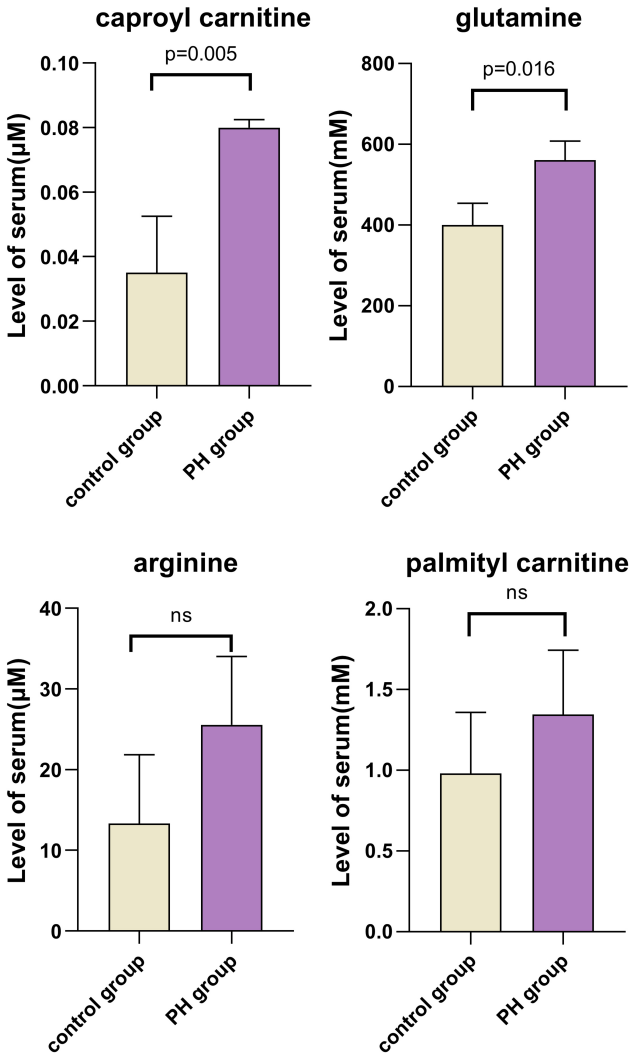

Fig. 5. The serum levels of caproyl carnitine, glutamine, palmityl carnitine, and arginine in PH pediatric patients and their relative controls. Differences between groups were assessed by non-parametric statistical method. Bars represent 25 and 75 percentiles and ns means non-significance. (n = 6). PH group, pulmonary hypertension (PH) patients.

4. Discussion

In this study, we investigated the metabolic profiles in hypoxia- induced PH mice after C75 treatment and validated them in paediatric PH patients. We found that lipid metabolites, putrescine, and glutamine were increased significantly in hypoxia-induced PH mice, and C75 treatment partially reversed these increasing metabolites, which indicated C75 affected the metabolism of hypoxia-induced PH mice. Our previous study also demonstrated that C75 treatment has a protective role in hypoxia-induced PH mice cardiac function [13]. Thus, FAS may be a therapeutic target for PH. Lipid metabolism was associated with polyamine and glutamine, both of which were closely related to PH. Putrescine and glutamine might be biomarkers for PH.

4.1 Lipid Metabolism and Pulmonary Hypertension

Excessive proliferation of PASMCs in PH needs to mobilize lipid metabolism [3,19]. FAS is a key enzyme that produces long-chain fatty acids [12]. FAS inhibitors inhibited proliferation, increased apoptosis in hypoxic human PASMCs, and ameliorated the symptoms and pulmonary vascular remodelling of MCT-treated rats [11]. In this study, we observed that fat synthesis products, such as glycerol, cholesterol, and DHA, were significantly elevated; in contrast, fat breakdown products, such as cholic acid, chenodeoxycholic acid, and uric acid, were decreased in the hypoxia group. C75 treatment partially reversed these effects (Fig. 3), which was consistent with the above findings and showed that C75 treatment played a protective role in hypoxia-induced PH mice by inhibiting the synthesis of fatty acids.

4.2 Polyamines and Pulmonary Hypertension

Polyamines are involved in numerous physiological functions, such as cell growth, gene regulation, differentiation, and development [20]. Polyamines, including spermidine, spermine, and putrescine, are essential factors for eukaryotic cell growth [21,22]. These polyamines were upregulated in cancer transformation and tumour progression [23]. Based on the above information, PH pathogenesis is similar to cancer [3,11,19]. In this study, we found that the putrescine level was elevated in the hypoxia group compared with its relative control, and C75 treatment mitigated the hypoxia-induced elevation (Fig. 3). Our results were in line with the following PH rat model and idiopathic pulmonary arterial hypertension (IPAH) patients [6,24]. Hautbergue *et al*. [6] showed that putrescine levels were upregulated in the PH rat model, whereas arginine (which is the precursor of putrescine) was decreased. Moreover, He *et al*. [24] also reported that plasma spermine levels were higher in patients with IPAH than in healthy controls, and they validated this finding in an experimental rodent model. They also validated spermine function *in vitro*, and spermine promoted human PASMC proliferation and migration, which aggravated pulmonary vascular remodelling [24].

However, the arginine level in the PH group was higher than that in the control group, although there was no significant difference between the two groups; this result was inconsistent with the change in putrescine in this animal study and previous studies [6], which may be caused by a few clinical studies and sample differences between individuals from the two groups. Selection bias caused by the enrolment requirement could occur. The above results indicated that C75 treatment might play a protective role in hypoxia-induced PH mice by inhibiting the synthesis of putrescine.

4.3 Glutamine and Pulmonary Hypertension

It is well known that glutamine participates in the bioenergetics of tumour cell proliferation and supports cel-

lular resistance to oxidative stress [25,26]. The carbon and nitrogen provided by glutamine are vital for the homeostasis of fatty acids, amino acids, and nucleotides [27]. Glutamine is converted to glutamate by glutaminase (GLS) [27]. GLS is related to tumour progression and growth rate in rodent models, and tumour proliferation is inhibited by gene knockout or GLS inhibitors [28–30], suggesting that glutamine is involved in tumour cell proliferation. Previous studies reported that glutamine was significantly downregulated in PH patients [31,32] and played an important role in scavenging reactive oxygen species (ROS) by producing glutathione, which was involved in tumour progression and PH severity [27,33]. In this study, we noticed that glutamine levels were elevated in the hypoxia group compared with its relative control, and C75 treatment partially reversed this increase (Fig. 3). Change of glutamine level was not consistent with previous studies [34,35], which may have resulted from the small sample size and the different models of PH. Additionally, previous study reported glutamine levels decreased in the RV tissue of model with PH. There may be some differences in the metabolomics of serum and RV tissue.

Furthermore, the changes in glutamine in the PH patients also do not correspond with those of the previous study [31]. The small number of clinical studies and sample differences between individuals might be potential reasons. Additionally, previous studies reported that glutamine levels decreased in the lung tissue of patients with PH [31], and there may be some differences between the metabolomics of serum and lung tissue.

5. Limitations

C57BL/6 mice could develop to PH under various stimuli such as chronic hypoxia or MCT injections, and exhibits several advantages, including a well-defined genetic background, reproducibility, and genetic tools that can be used to manipulate gene expression. Hypoxia/OVA-induced C57BL/6 mice provide a useful model to study PH. Animal models provide potential for revealing the nature of the disease and clinical treatment due to the study of the aetiology, pathogenesis and treatment of PH in animal models. However, it is important to recognize that animal models have no clear clinical relevance. First, metabolites condition and other condition may vary across species. Second, animal experiments are carried out under given conditions, and the results and conclusions obtained can only be limited to such conditions and cannot be blindly expanded. In animal experiments, rather than considering the whole animal, only the influence of some factors needs to be observed. Due to the inherent limitations of animal testing mentioned above, it is challenging to translate the findings in animal models to the human situation and animal experiment result should be applied to the clinic with great caution. After studying a large volume of literature, including that of animals, cells, and humans, it was determined that most findings in this study, including lipid metabolism and putrescine, were supported by the literature [6,11,24,36]. The number of PH patients enrolled was small. Factors led to the small sample size were as following. First, this was a retrospective study. Second, patients with PH who had undergone blood tandem mass spectrometry could be enrolled. In addition, clinical diagnosis was mainly based on the tricuspid regurgitation pressure gradient. So, we couldn't collect that much data on PH patients. We will further collect more data on metabolites from PH patients to expand the sample size for clinical research and collect more parameters in PH patients, including tricuspid annular plane systolic excursion (TAPSE) and right ventricular volume.

6. Conclusions

Lipid metabolism, polyamine, and glutamine play crucial roles in PH through cell proliferation regulation, the level of which were upregulated during the development of PH. Treatment with C75, an inhibitor of FAS, partially relieved PH symptoms and decreased putrescine and glutamine levels, indicating that C75 played a protective role in the metabolic regulation of hypoxia-induced PH mice. FAS may be a therapeutic target for PH. The levels of putrescine and glutamine are convenient to detect and may be potential biomarkers for PH.

Availability of Data and Materials

Raw data will be made available on request and all data generated or analysed during this study are included in this published article and its supplementary information files.

Author Contributions

LX, CH and TX designed the research study. SC performed the research. SL and WL provided help and advice on the experiments. QL,YY,QQ and YZ collected and analysed the clinical data. All authors contributed to the editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The authors retrospectively collected medical records of the recruited children in Shanghai Children's Hospital. This study was approved by the Ethics Review Committee of Shanghai Children's Hospital, Shanghai Jiao Tong University School of Medicine (no. 2019R002-E03) and was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. Parents of the children understood the aim of the study and provided informed consent. The animal study was reviewed and approved by the Ethics Committee of Experimental Research of Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University. This

study was carried out in compliance with the Animal Research: Reporting of *in vivo* experiments (ARRIVE) guidelines.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2810251.

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