

Emerging applications of targeted and non-targeted metabolomics to physiology and pathophysiology

Edited by

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Emerging applications of targeted and non-targeted metabolomics to physiology and pathophysiology

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Editorial: Emerging applications of targeted and non-targeted metabolomics to physiology and pathophysiology

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KEYWORDS

metabolomics, medicine, physiology, pathophysiology, disease

Editorial on the Research Topic

Emerging applications of targeted and non-targeted metabolomics to physiology and pathophysiology

In the ever-changing landscape of biomedical research, metabolomics stands at the forefront of discovery as an “omics” science that captures the dynamic chemical fingerprint of life. By enabling the high-throughput identification and quantification of small molecules (<1500 Da), metabolomics provides a powerful window into the complicated interplay between genetics, environment and physiology. From endogenous compounds like amino acids and lipids to exogenous pollutants and pharmaceuticals, the metabolome reflects both health and disease and leads to generation of new hypotheses.

This Research Topic provides recent contributions to this field which underscores its transformative impact on medicine, physiology and disease modelling.

Zhang et al. explored metabolic disruptions in a rat model of visual fatigue linked to traditional Chinese medicine syndromes, revealing altered sphingolipid pathways. **Passadore et al.** highlighted amino acid signatures in obese children, linking urinary metabolite patterns to early indicators of insulin resistance and metabolic risk. These findings stress metabolomics’ potential in early diagnosis and personalized interventions.

The utility of metabolomics expands into mechanistic insights. **Zhang et al.** offered a comprehensive review of lipid droplet-mitochondria interactions, critical in metabolic syndrome and pointed to pharmacological targets that may reshape future obesity treatment. **Kenéz et al.** connected diet-induced ceramide accumulation to insulin resistance in cattle, an elegant model that reflects similar processes in human metabolic disease, providing a bridge between veterinary science and human health.

In the realm of exercise physiology, **Ou et al.** and **Zhou et al.** examined how ischemic preconditioning and enteric-coated bicarbonate impact metabolomic profiles without

altering performance, revealing that even interventions with no visible performance boost can profoundly shift underlying biochemistry. Landman et al. showed that simple handgrip exercises and remote ischemic preconditioning reduce key inflammatory markers in cerebral small vessel disease, emphasizing how metabolomics can unravel subtle but clinically significant responses to lifestyle interventions.

On the diagnostic Frontier, Wang et al. demonstrated that serum metabolomics can differentiate renal cell carcinoma from benign tumors with exceptional accuracy. Lin et al. provided insights into high-altitude pulmonary edema by identifying arterial and venous metabolite shifts, paving the way for biomarker discovery in acute physiological stress.

Perhaps most compelling is the emerging picture of how parental genetics even without direct gene transmission, shape offspring metabolism. Zhang et al. showed that maternal and paternal eNOS deficiency leads to distinct phenotypes and metabolomic profiles in offspring, suggesting epigenetic and metabolic inheritance as powerful forces shaping health across generations.

As these studies highlight, metabolomics is more than a research tool, it is a language of life, capturing the nuance of disease progression, therapy response, and physiological adaptation. With mass spectrometry and NMR technologies pushing the boundaries of detection and data integration with genomics and proteomics becoming more refined, the metabolome stands as a critical pillar in systems medicine.

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Ceramide metabolism associated with chronic dietary nutrient surplus and diminished insulin sensitivity in the liver, muscle, and adipose tissue of cattle

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High dietary energy and protein supply is common practice in livestock nutrition, aiming to maximize growth and production performance. However, a chronic nutritional surplus induces obesity, promotes insulin insensitivity, and triggers low-grade inflammation. Thirty Holstein bulls were randomly assigned to two groups, low energy and protein (LEP), and high energy and protein (HEP) intake, provided from the 13th to the 20th month of life. Body weight, carcass composition, laminitis score, and circulating insulin and glucose concentrations were assessed. The expression and extent of phosphorylation of insulin signaling proteins were measured in the liver, muscle, and adipose tissue. The sphingolipid metabolome was quantified by a targeted liquid chromatography-mass spectrometry based metabolomics approach. The HEP bulls were obese, had hyperinsulinemia with euglycemia, and expressed clinical signs of chronic laminitis. In the liver, protein kinase B (PKB) phosphorylation was decreased and this was associated with a higher tissue concentration of ceramide 16:0, a sphingolipid that diminishes insulin action by dephosphorylating PKB. In the adipose tissue, insulin receptor expression was lower in HEP bulls, associated with higher concentration of hexosylceramide, which reduces the abundance of functional insulin receptors. Our findings confirm that diet-induced metabolic inflammation triggers ceramide accumulation and disturbs insulin signaling. As insulin insensitivity exacerbates metabolic inflammation, this self-reinforcing cycle could explain the deterioration of metabolic health apparent as chronic laminitis. By demonstrating molecular relationships between insulin signaling and sphingolipid metabolism in three major tissues, our data extend our mechanistic understanding of the role of ceramides in diet-induced metabolic inflammation.

KEYWORDS

sphingolipids, ceramide, insulin resistance, tissue metabolomics, obesity, metabolic inflammation

Introduction

There is a well-documented causal relationship between chronic consumption of hypercaloric diets and insulin resistance demonstrated in humans and various animal models (Deer et al., 2015; Sah et al., 2016). Continuous or recurrent nutrient surplus induces dyslipidemia, triglyceride deposition, and adipocyte hypertrophy, leading to obesity (Gregor and Hotamisligil, 2011). However, due to the complexity of metabolic events initiated, including the highly integrated cellular responses to nutrient excess in various tissues and organ systems, mapping the multifaceted contributing factors of metabolic dysfunction remains challenging (Wisse et al., 2007). There is a chronic low-grade inflammation developing in obesity, mediated by inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and interleukin 8 (IL-8) (Zeyda and Stulnig, 2009). This has been termed metabolic inflammation or “metaflammation,” a common endpoint of converging signaling pathways activated by chronic nutrient excess (Gregor and Hotamisligil, 2011; Hotamisligil, 2017). Multiple interconnected molecular mechanisms have been identified underlying the interference of these inflammatory signals with insulin signaling, including the induction of ceramide synthesis, eventually resulting in insulin resistance (Dali-Youcef et al., 2013). Insulin resistance is determined by impaired sensitivity to insulin in its main target organs, i.e., adipose tissue, liver, and muscle (Zeyda and Stulnig, 2009). Nevertheless, the consequences of such insulin dysregulation also include further metabolic deterioration, triggering an unresolved vicious cycle (Hotamisligil, 2006).

Sphingolipid metabolites, including ceramide, interact with pro-inflammatory pathways and with insulin signaling. Inflammatory cytokines such as TNF- α released from hypertrophic adipocytes and M1 macrophages infiltrating adipose tissues in obesity induce *de novo* ceramide synthesis from palmitate and transformation of sphingomyelin into ceramide by sphingomyelinase activity (Holland and Summers, 2008). Similarly, ceramide accumulation is also induced in response to TLR-4 activation, Fas ligand, and oxidative stress in a variety of cell types (Sokolowska and Blachnio-Zabielska, 2019). Ceramides, particularly long-chain ceramides such as C16:0 and C18:0 antagonize insulin signaling by diminishing Akt (PKB) phosphorylation, a key step for GLUT4 translocation (Meikle and Summers, 2017). In addition, hexosylceramide derivatives, particularly gangliosides, antagonize insulin signaling by displacing the insulin receptor and inhibiting receptor tyrosine phosphorylation (Holland and Summers, 2008). Thereby, ceramides counteract cellular glucose uptake and interfere with nutrient storage, in addition to promoting proinflammatory cytokine production, disrupting hepatic lipid metabolism, and enhancing cell death (Sokolowska and Blachnio-Zabielska, 2019). In addition, the

insulin-sensitizing effect of adiponectin was attributed to its ceramidase activity, further highlighting the central role of ceramides in regulating insulin sensitivity (Reibe-Pal and Febbraio, 2017).

From an evolutionary point of view, a close cross-talk between immune function and metabolic response is a highly desired trait (Hotamisligil, 2006). In general, inflammation is a physiological mechanism to help restore metabolic homeostasis and functional integrity of organs and tissues (Hotamisligil, 2017). Furthermore, the organism needs to coordinate and redistribute nutrients during an inflammatory response, which is why the integration of pathogen- and nutrient-sensing pathways and the control of anabolic pathways are a meaningful biological strategy for survival under challenged conditions (Beutler, 2004). However, in contrast to temporarily diverting energy sources away from synthetic pathways during a pathogen-induced “classical” inflammation, the activation of these evolutionarily conserved systems during “metaflammation” contributes to the unresolved vicious cycle of metabolic deterioration induced by chronic nutrient surplus (Hotamisligil, 2006). Since obesity and the cluster of obesity-related metabolic disorders have reached epidemic proportions in humans, the molecular mechanism of metabolic dysregulation, including sphingolipid signaling during obesity-induced metaflammation have been extensively studied in humans and rodent models (Holland and Summers, 2008; Gregor and Hotamisligil, 2011; Meikle and Summers, 2017; Sokolowska and Blachnio-Zabielska, 2019; Summers et al., 2019). Metaflammation triggered by excessive lipid mobilization after over-conditioning, among other factors, has also been identified as a key component of metabolic disorders commonly occurring in modern-day high-performance dairy cows during the transition from gestation to lactation (Bradford et al., 2015). Presumably owing to the evolutionary conserved regulation, underlying pathophysiological mechanisms of metabolic dysfunction (i.e., inflammatory signaling and inhibition of insulin signaling with an increase of ceramide mediators) largely overlap in these conditions between humans and cattle (Bradford et al., 2015; Rico et al., 2018; McFadden and Rico, 2019; Bradford and Swartz, 2020; McFadden, 2020).

We postulate that studying the relationship between chronic nutrient surplus, ceramide metabolism, and insulin signaling in cattle will extend our global understanding of cellular mechanisms driving metabolic health. We hypothesized that an intensive fattening regimen in bulls based on a high energy and protein diet would reduce insulin sensitivity by inducing a pro-inflammatory shift in the sphingolipid metabolome. Our objectives were to measure the abundance and phosphorylation level of key proteins of insulin signaling and nutrient sensing, as well as to quantify the concentration of various sphingolipids, including ceramides, in a metabolomics approach. By extending these measurements to the liver, skeletal muscle, and adipose

tissue in Holstein fattening bulls, we aimed to map the effect of chronic nutrient surplus on the interplay between organs as well.

Materials and methods

Experimental design

The animal experiment was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle (Muenchweiler a.d. Alsenz, Germany) as reported previously (Bäßler et al., 2021). All experimental procedures were approved by the Animal Ethics Committee of the Department for Animal Welfare Affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany) in agreement with the German Animal Welfare Act (permit number: G-17-20-070). Briefly, Holstein bulls were randomly assigned to a high energy and protein (HEP; $n = 15$) or a low energy and protein (LEP; $n = 15$) fattening regimen at an average age of 13 months and average body weight of 500 kg (HEP 506 ± 35 kg, LEP 499 ± 35 kg; mean $\pm$ SD). The experimental feeding lasted from 13 months of age until the time of slaughtering 7 months later. The sample size was consistent with commonly reported study designs in livestock metabolomics (Goldansaz et al., 2017).

Experimental diet

The diets were formulated according to the high- (HEP) and low-end (LEP) of the range of recommendations of the Bavarian State Research Center for Agriculture (LfL) for Simmental fattening bulls. While the LEP diet consisted of silage only, the HEP diet consisted of silage and concentrate (a blend of ground corn, rapeseed meal, ground wheat, palm kernel meal, wheat bran, molasses, and soybean meal), accounting for an increased total sugar, total starch, and crude protein, as well as a decreased fiber content of the HEP diet, relative to LEP. The diets were fed as total mixed ration (TMR) and the nutrient composition of the TMR was determined as reported previously (Bäßler et al., 2021). The ingredients and chemical composition of the TMR were published previously (Bäßler et al., 2021) and included here in the [Supplementary Table S1](#).

Sample and data collection

At the end of the experimental feeding period, all bulls were slaughtered at an average age of 20 months, and live weight, carcass conformation, and fat class, and laminitis score were recorded upon slaughtering, and serum insulin and plasma glucose were measured as published previously (Bäßler et al., 2021). In addition, tissue samples of the liver (the ventral third of the diaphragmatic surface), muscle (musculus longissimus

dorsi), and retroperitoneal adipose tissue (perirenal region, between the peritoneum and the abdominal muscles) and subcutaneous adipose tissue (at the tailhead region) were collected immediately after slaughtering. Tissue samples were trimmed of any connective tissue, cut into approximately $0.5 \text{ cm} \times 0.5 \text{ cm} \times 0.5 \text{ cm}$ pieces, rinsed in ice-cold physiological saline solution, snap-frozen in liquid nitrogen, and stored at -80°C until analyses.

Western blot analysis

The expression and phosphorylation of key proteins of the insulin signaling pathway were measured in liver, muscle, and retroperitoneal adipose tissue samples by Western blotting following our previously published protocol (Kenéz et al., 2019). The conditions of antibody detection for insulin receptor β (InsR), mechanistic target of rapamycin (mTOR), phosphorylated mTOR, protein kinase B (PKB), phosphorylated PKB, 5' adenosine monophosphate-activated protein kinase α (AMPK), and phosphorylated AMPK are listed in [Table 1](#).

Metabolomics analysis

The sphingolipid metabolome was quantified by a targeted liquid chromatography-mass spectrometry (LC-MS) based metabolomics assay in the liver, muscle, and subcutaneous adipose tissue samples. This analysis was carried out at the UVic Node of The Metabolomics Innovation Centre (TMIC; Genome BC Proteomics Centre, The University of Victoria, Victoria, BC, Canada) following the previously published workflow (Leung et al., 2020). The 77 compounds targeted herein belonged to the following sphingolipid classes: 3-keto-sphinganine (3-KSpha), sphinganine (Spha), sphinganine-1-phosphates (Spha-P), dihydroceramides (dhCer), dihydroceramide-1-phosphates (dhCer-P), ceramides (Cer), ceramide-1-phosphates (Cer-P), galactosyl-ceramides (GalCer), glucosyl-ceramides (GlcCer), lactosyl-ceramides (LacCer), sphingomyelins (SM), dihydrosphingomyelins (dhSM), and sphingosines (Spho). In brief, 200 mg of the tissue samples were homogenized in 200 μl of water and subsequently mixed with methanol-chloroform (5:2, v/v, 19 $\mu\text{l}/\text{mg}$ raw tissue) containing butylated hydroxytoluene (0.1 mg/ml). The sample was then sonicated (5 min) in an ice bath, and centrifuged (30 min, $4,000 \times g$, 10°C). The clear supernatant was collected and the precipitated pellet was extracted with chloroform (1:1, v/v, 10 $\mu\text{l}/\text{mg}$ raw tissue). The clear supernatant was collected again, dried off under a nitrogen gas flow (30°C), and the residue was dissolved in methanol-chloroform (1:1, v/v, 30 $\mu\text{l}/\text{mg}$). The sphingolipid measurement was performed by ultra-performance liquid chromatography-tandem mass spectrometry in multiple reaction monitoring mode (UPLC-MS) using the

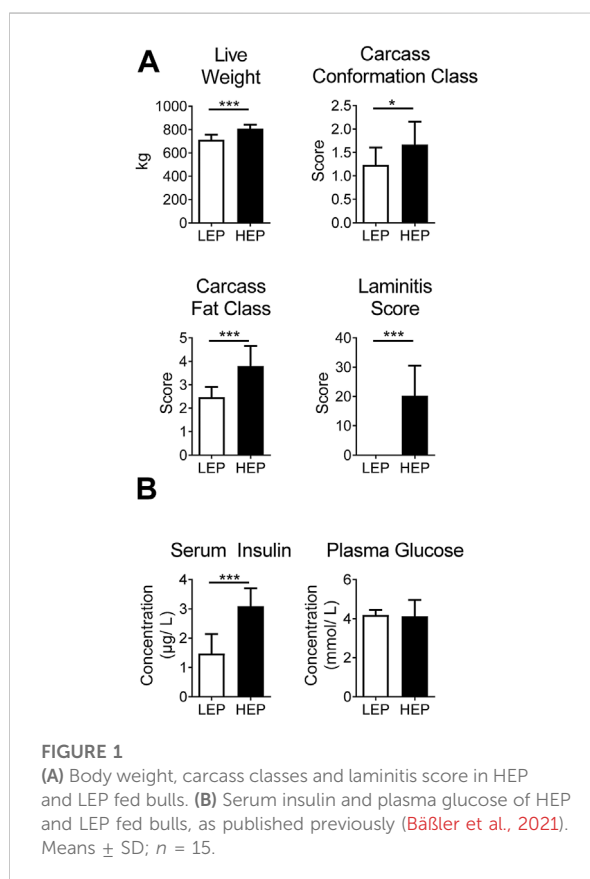
TABLE 1 Primary and secondary antibodies used for western blot analyses.

Target antigen	Antibody	Dilution	Manufacturer	Buffer	Blocking agent
Insulin receptor β (InsR)	Rabbit anti InsR- β (4B8)	1:2,000	Cell Signaling Technology Inc. (Danvers, MA, United States)	5% bovine serum albumin	5% fat-free milk powder
Mechanistic target of rapamycin (mTOR)	Rabbit anti mTOR (7C10)	1:1,000	Cell Signaling Technology Inc.	5% bovine serum albumin	5% fat-free milk powder
Phosphorylated mechanistic target of rapamycin (Ser2448; p-mTOR)	Rabbit anti p-mTOR (Ser2448)	1:500	Cell Signaling Technology Inc.	5% bovine serum albumin	5% bovine serum albumin
Protein kinase b (PKB)	Rabbit anti AKT	1:2,000	Cell Signaling Technology Inc.	5% bovine serum albumin	5% fat-free milk powder
Phosphorylated protein kinase b (Ser473; p-PKB)	Rabbit anti p-AKT (Ser473) (D9E) XP	1:2,000	Cell Signaling Technology Inc.	5% bovine serum albumin	5% fat-free milk powder
5' adenosine monophosphate-activated protein kinase α (AMPK- α)	Rabbit anti AMPK α	1:4,000	Bethyl Laboratories Inc. (Montgomery, TX, United States)	5% fat-free milk powder	5% fat-free milk powder
Phosphorylated 5' adenosine monophosphate-activated protein kinase α (Thr172) (p-AMPK- α)	Rabbit anti p-AMPK α (Thr172) (40H9)	1:1,000	Cell Signaling Technology Inc.	5% fat-free milk powder	5% fat-free milk powder
Rabbit IgG (secondary antibody)	Goat anti-rabbit IgG, HRP-linked	1:2,000	Cell Signaling Technology Inc.	5% fat-free milk powder	5% fat-free milk powder

positive (ESI+) and negative (ESI-) ion modes for sphingolipids and phosphorylated sphingolipids, respectively. Concentrations were calculated by peak area calibration curves with standard dilutions. Sphingolipid metabolites were filtered according to the limit of detection (LOD) of each feature, and only those features were retained that were effectively quantified (concentration > LOD) in at least 80% of the samples of either one of the experimental groups. Missing values were replaced by LOD/2 according to our previously published workflow (Leung et al., 2021).

Statistical analysis

Live weight, carcass class, laminitis score, circulating insulin and glucose, and protein abundance and phosphorylation data were compared between the HEP and LEP treatments by unpaired Student's *t*-test in GraphPad Prism (version 9.3). Sphingolipid metabolome data were analyzed by partial least-squares discriminant analysis (PLS-DA). The PLS-DA models were evaluated by their goodness of fit (R^2) and prediction quality index (Q^2). R^2 and Q^2 values of 1.0 represent the best possible models and the models were considered valid herein if $Q^2 > 0$ for the first component on the first run. Within each PLS-DA model, the metabolites' contribution to the model was assessed by their variable importance in projection (VIP) scores (greater values indicating a more significant contribution). Metabolome data were also subjected to an unpaired Student's *t*-test with false discovery rate (FDR) correction, and heatmap combined with hierarchical cluster analysis in MetaboAnalyst (version 5.0) (Pang et al., 2021). Further, the associations between insulin



signaling protein expression and sphingolipid concentrations were evaluated by principal component analysis (PCA) loading plots according to (Jorge-Smeding et al., 2021).

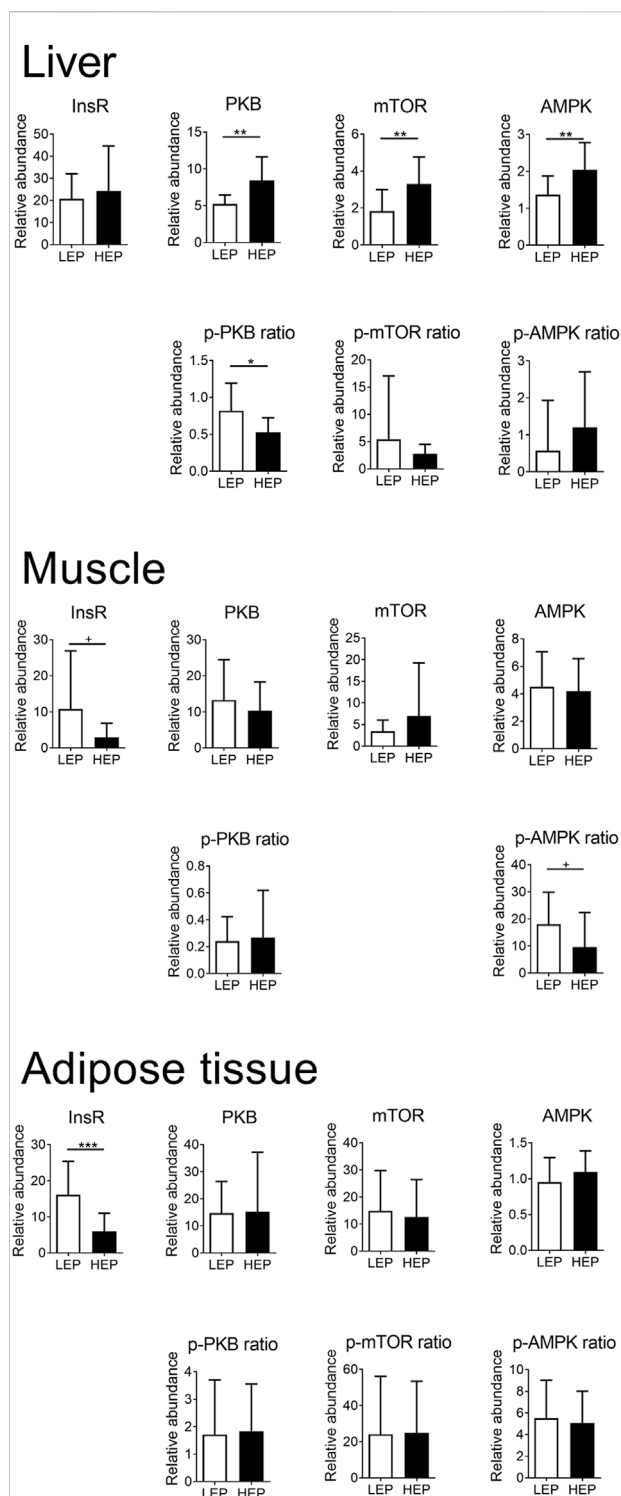


FIGURE 2

Protein expression and phosphorylation of key components of the insulin signaling pathway in liver, muscle, and retroperitoneal adipose tissue. Insulin receptor β (InsR), mechanistic target of rapamycin (mTOR), protein kinase B (PKB), 5' adenosine monophosphate-activated protein kinase α (AMPK), and their phosphorylated forms were detected by Western blotting. Means $\pm$ SD; $n = 15$.

Results

Body condition, laminitis scores, and circulating insulin and glucose concentrations

Bulls in the HEP group had significantly higher final body weight ($p < 0.001$) upon slaughtering, as well as higher carcass conformation ($p = 0.01$) and fat class ($p < 0.001$) than the LEP bulls (Figure 1A), as published previously (Bäßler et al., 2021). Furthermore, HEP bulls had a high laminitis score, while LEP bulls scored zero on the same scale, resulting in a greater average score for the HEP bulls ($p < 0.001$). Bulls of the HEP group had basal hyperinsulinemia with euglycemia, in contrast to LEP (serum insulin $p < 0.001$; plasma glucose $p = 0.79$) (Figure 1B).

Insulin signaling

Figure 2 shows the extent of expression and phosphorylation of the insulin signaling proteins in all tissues, comparing HEP and LEP. The expression of InsR was decreased in the retroperitoneal adipose tissue ($p < 0.001$) and tended to be decreased ($p = 0.07$) in the muscle in the HEP group, compared with LEP. The downstream signaling elements of the insulin pathway were similar between HEP and LEP in the muscle and the retroperitoneal adipose tissue (all $p > 0.1$), however, a tendency for lower AMPK phosphorylation ratio was detected in HEP, compared with LEP ($p = 0.07$). The abundance of InsR protein was not different between HEP and LEP in the liver, however, greater abundances of PKB, mTOR, and AMPK protein ($p = 0.001$, $p = 0.005$, and $p = 0.007$, respectively) were detected, concurrently with lower phosphorylation ratio of PKB ($p = 0.01$) in HEP, compared with LEP. Representative Western blot images are shown in Supplementary Figure S1.

Sphingolipid metabolome

A total of 71 out of the targeted 77 sphingolipid species were effectively quantified (concentrations higher than the lower limit of detection in at least 80% of the samples). These were assigned to three main sphingolipid pathways: *de novo* synthesis (3-KSph, Spha, Spha-P, dhCer, dhCer-P, Cer), sphingomyelinase pathway (SM, dhSM), and salvage pathway (GalCer, GlcCer, LacCer, Cer-P, Spho) (Supplementary Table S2). The sphingolipid metabolome profiles were significantly separated between HEP and LEP in all three tissues, as assessed by PLS-DA (Figure 3), which had valid models with high values for fitness and prediction quality for the three tissues (liver: $R^2 = 0.82$, $Q^2 = 0.74$; muscle: $R^2 = 0.76$, $Q^2 = 0.68$; adipose tissue: $R^2 = 0.93$, $Q^2 = 0.82$). The heatmaps in Supplementary Figure S2 show the relative concentration of individual sphingolipids that were

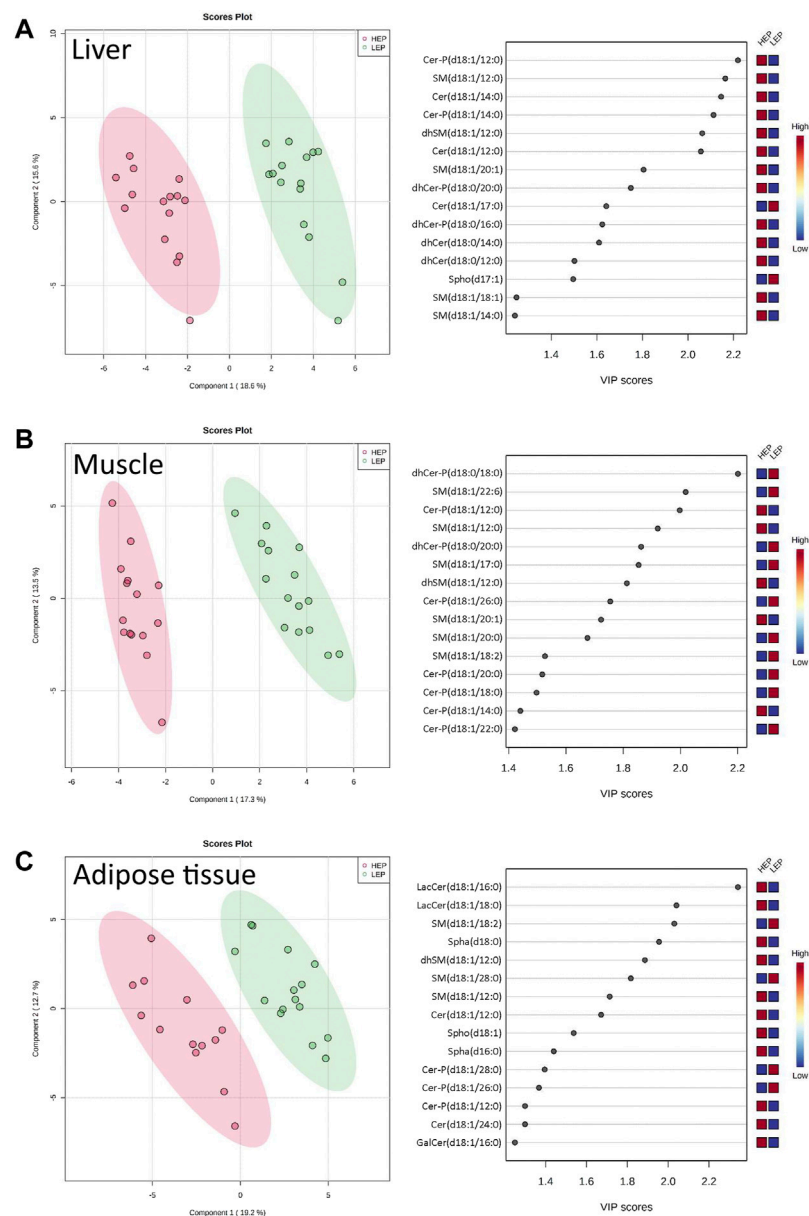


FIGURE 3

Partial least squares-discriminant analysis (PLS-DA) scores plots with variable importance in projection (VIP) scores of sphingolipid metabolome profiles of (A) liver, (B) muscle, and (C) subcutaneous adipose tissue of bulls on HEP and LEP dietary regimens ($n = 15$). Spha, sphinganine; dhCer, dihydroceramides; dhCer-P, dihydroceramide-1-phosphates; Cer, ceramides; Cer-P, ceramide-1-phosphates; GalCer, galactosyl-ceramides; LacCer, lactosyl-ceramides; SM, sphingomyelins; dhSM, dihydrosphingomyelins; Spho: sphingosines.

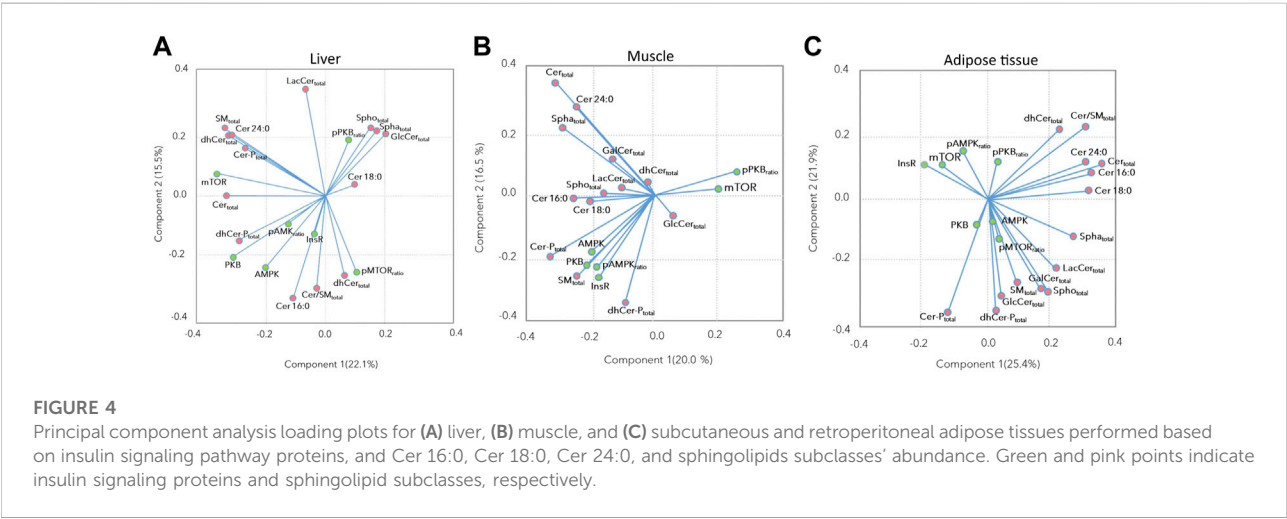
significantly different between HEP and LEP (t -test, FDR corrected $p < 0.05$). A total of 20, 21, and 11 sphingolipid species had different tissue concentrations in the liver, muscle, and adipose tissue, respectively (Supplementary Figure S2). When categorizing and summing the individual sphingolipid species according to their chemical characteristics, several differences were detected between LEP and HEP bulls in the three tissues (Table 2). Specifically, Cer C16:0 ($p = 0.009$) and

total dhCer-P ($p < 0.001$) had greater concentrations in HEP than LEP in the liver. In the muscle, a greater ratio of Cer:SM ($p = 0.007$) and lower concentrations of total dhCer ($p = 0.037$), total dhCer-P ($p = 0.001$), and total SM ($p = 0.001$) were found in HEP than LEP. In the subcutaneous adipose tissue, greater concentrations of Cer 24:0 ($p = 0.006$), total hexosylceramide ($p = 0.003$), total Spha ($p = 0.001$), and Spho ($p = 0.005$) were observed in HEP, compared with LEP.

TABLE 2 Insulin signaling protein expression and phosphorylation and abundance of ceramides C16:0, C18:0, and C24:0 and summative sphingolipid classes in the liver, skeletal muscle, and subcutaneous adipose tissue (sphingolipids) or retroperitoneal adipose tissue (insulin signaling proteins) of bulls fed either the low energy and protein (LEP) or the high energy and protein (HEP) diet. All concentrations all expressed as nmol/g tissue.

Feature	Liver					Muscle					Adipose tissue				
	LEP	±SD	HEP	±SD	<i>p</i> -value	LEP	±SD	HEP	±SD	<i>p</i> -value	LEP	±SD	HEP	±SD	<i>p</i> -value
Cer 16:0	1.02	0.08	1.17	0.18	0.009	0.016	0.005	0.014	0.005	0.344	0.462	0.120	0.562	0.305	0.251
Cer 18:0	0.028	0.008	0.025	0.005	0.056	0.143	0.022	0.136	0.027	0.476	0.044	0.014	0.078	0.094	0.171
Cer 24:0	0.369	0.161	0.309	0.116	0.251	0.174	0.059	0.208	0.077	0.184	0.181	0.035	0.260	0.095	0.006
Total Cer	2.21	0.32	2.29	0.35	0.530	0.211	0.049	0.239	0.060	0.202	0.985	0.189	1.226	0.492	0.092
Total Cer-P	78.4	6.2	82.6	6.6	0.083	56.0	4.2	53.5	2.7	0.065	63.3	9.7	64.7	8.8	0.696
Total dhCer	1.27	0.36	1.28	0.27	0.959	0.057	0.040	0.032	0.015	0.037	0.203	0.150	0.252	0.135	0.376
Total dhCer-P	4.30	0.62	5.33	0.78	< 0.001	23.6	6.0	17.4	2.6	0.001	7.98	2.39	8.80	1.77	0.319
Total HexCer	0.75	0.20	0.65	0.17	0.403	0.21	0.06	0.26	0.08	0.087	0.41	0.13	0.63	0.22	0.003
Total SM	129.8	19.8	126.1	13.4	0.554	67.7	5.5	59.2	7.5	0.001	86.8	9.6	91.9	16.1	0.309
Total sphinganes	0.206	0.222	0.106	0.080	0.108	0.066	0.018	0.058	0.011	0.186	0.057	0.016	0.095	0.034	0.001
Total sphingosines	0.960	0.928	0.486	0.299	0.070	0.431	0.103	0.452	0.083	0.537	0.167	0.061	0.282	0.131	0.005
Total Cer:SM	0.017	0.002	0.018	0.002	0.153	0.0031	0.0008	0.0040	0.0008	0.007	0.011	0.003	0.014	0.007	0.238

Cer, ceramides; Cer-P, ceramide-phosphates; dhCer, dihydroceramides; dhCer-P, dihydroceramide-1-phosphates; HexCer, hexosylceramides (sum of glucosyl-, galactosyl-, and lactosylceramides); SM, sphingomyelins.



The associations between the insulin signaling pathway and the sphingolipid concentrations, as assessed by the loading plots of the PCA showed that most of the insulin signaling proteins were positively associated with Cer 16:0, Cer:SM ratio, total dhCer, and Cer-P, and negatively associated with Cer 18:0, total Spho, total Spha, and total GlcCer in the liver (Figure 4A). In the muscle, most of the insulin signaling proteins were positively associated with total SM, total dhCer-P, Cer16:0, and Cer 18:0, while the p-PKB ratio and mTOR showed a negative association with these compounds (Figure 4B). In the adipose tissue, while the p-mTOR ratio and AMPK showed positive associations with each other, InsR, mTOR,

and p-AMPK ratio showed negative associations with several sphingolipid subclasses including total SM, total GlcCer, total LacCer, total Spho, and total Spha (Figure 4C).

Discussion

These findings contribute to our understanding of the pathogenesis of metabolic dysregulation, including an unresolved metabolic inflammation, associated with the chronic surplus of dietary energy and protein intake. Although the experiment was done in fattening bulls, our

findings may also be relevant for other species, including humans, regarding the proposed molecular level associations of insulin resistance and metabolic inflammation. Intensively fed obese bulls expressed a compensated disturbance of the insulin-glucose homeostasis reflected by euglycemic hyperinsulinemia. This systemic insulin insensitivity was based on reduced insulin signaling in insulin-sensitive tissues with tissue-specific patterns. Concomitantly, ceramide metabolism was stimulated by high dietary energy and protein intake resulting in intermediates, which are known to interact with the cellular insulin signaling cascade. As a major clinical outcome, this metabolic condition was associated with chronic inflammatory insults of the claw's corium causing chronic intermittent phases of laminitis. In the following sections, interactions between the diet, insulin signaling, and ceramide metabolism are discussed. Further, underlying pathomechanisms of unresolved metabolic inflammation are proposed as a working hypothesis for future research about metabolic inflammation in humans and animals.

Hepatic metabolic responses to high dietary energy and protein intake

Hepatic insulin signaling was interrupted at an early step of the signaling cascade; PKB phosphorylation was decreased by high dietary energy and protein intake, despite its higher total protein abundance. Consequently, downstream targets were less stimulated. However, the basal protein expression of PKB, mTOR, and AMPK was higher, most likely expressing a compensatory response to reduced PKB phosphorylation. Among the sphingolipids, Cer 16:0 and total dhCer-P had increased levels in the liver of HEP bulls. These metabolites are derived from *de novo* sphingolipid metabolism and their flux rate depends on free fatty acid availability (Holland and Summers, 2008). Dihydroceramide synthases produce dhCer from sphinganine (Pewzner-Jung et al., 2006), which can be phosphorylated to dhCer-P. Further, dhCer is a precursor for ceramide synthesis. Among the ceramides, Cer16:0 and Cer 18:0 are intermediates with proven detrimental effects on insulin signaling (Meikle and Summers, 2017). Ceramides are phosphorylated by ceramide kinase, and Cer-Ps are then involved in the eicosanoid synthesis (Lamour and Chalfant, 2005). Ceramides are known to decrease PKB phosphorylation by mainly targeting protein phosphatase 2A (PP2A) in different types of cells; however, the modulation of insulin signaling by ceramides is more complex than only through PP2A activation (Holland and Summers, 2008). A reduced PKB phosphorylation was also observed in the liver of fattening bulls, most likely due to higher hepatic ceramide concentrations, especially higher Cer 16:0 concentrations. Considering all bulls, the loading plot (Figure 4A) demonstrated a negative association between pPKB and Cer 16:0, but also with total dhCer, total dhCer-P, and the total Cer:SM ratio. These observations confirm the

relationship between ceramide metabolism and insulin signaling in ruminants (McFadden and Rico, 2019). Thus, the hepatic metabolism of these bulls contributed to the systemic insulin insensitivity, presumably by causing an increased pancreatic insulin secretion or a reduced insulin turnover as a compensatory response. In humans, a higher rate of Cer production and Cer accumulation are associated with the development of insulin resistance and type 2 diabetes; however, the exact dysregulatory processes of lipid metabolism are not yet fully deciphered (Meikle and Summers, 2017).

The serine/threonine kinase mTOR is known to play a vital role in protein, glucose, and lipid metabolism (Mao and Zhang, 2018). Furthermore, mTOR function is strongly interrelated with AMPK function (Mao and Zhang, 2018). Both proteins, mTOR and AMPK were increased in their amount, however, their ratios of phosphorylated to total protein were equal in the LEP and HEP bulls. Thus, despite reduced PKB phosphorylation, the higher amount of total protein with maintained phosphorylation ratios suggested that these downstream targets were still activated at a higher level in HEP. Since mTOR stimulates glucose uptake and glycolysis by modulating the transcription factor hypoxia-inducible factor (HIF) 1 alpha (Düvel et al., 2010), that stimulation might contribute to maintaining euglycemia in HEP bulls. The lipogenic effect of mTOR activation leading to hepatosteatosis might be less biologically relevant in ruminants than in humans, since *de novo* lipogenesis is a feature of the adipose tissues in ruminants, and it is based on acetate utilization instead of glucose (Chilliard, 1993).

Muscle and adipose metabolic responses to high dietary energy and protein intake

The expression of insulin receptor protein was not significantly affected in the muscle of HEP bulls; however, a significant decrease in insulin receptor expression was observed in the retroperitoneal adipose tissue of HEP bulls, compared with LEP. Associated changes in the sphingolipidome revealed that the HEP muscle had lower total SM, total dhCer, and total dhCer-P concentrations. While the association between sphingolipid metabolism and insulin resistance in skeletal muscle is well documented (Bandet et al., 2019), previous studies found that fatty acid-evoked myotubular ceramide accumulation and insulin insensitivity were rather correlated but independent events (Pillon et al., 2018). Accordingly, insulin signaling protein expression and their extent of phosphorylation were not significantly affected in the muscle of the HEP bulls, despite the differences in ceramide concentrations compared to LEP bulls.

The HEP bulls had higher Cer 24:0, total hexosylceramides, total sphinganine, and total sphingosine concentrations in their

subcutaneous adipose tissue. Technically, insulin signaling protein expression was not measured in the subcutaneous but in the retroperitoneal adipose tissue due to the lack of sufficient sample material. However, we consider that the findings of both subcutaneous and retroperitoneal depots can be combined because the protein abundance of insulin signaling components, especially basal expression of the insulin receptor, were found to be equal in both depots in Holstein dairy cows (Kinoshita et al., 2016; Kenéz et al., 2019).

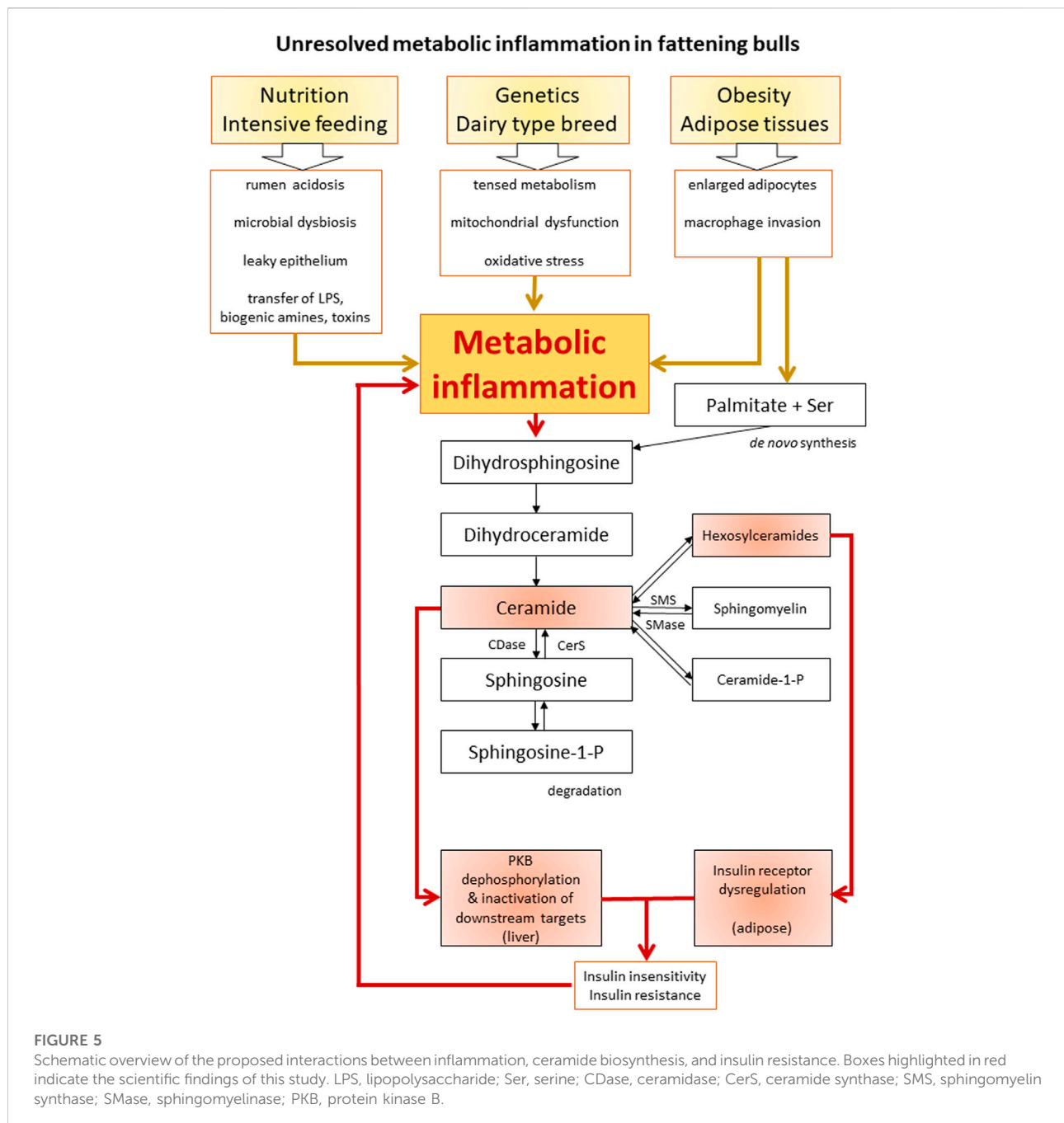
Thus, assuming an equal expression of the insulin receptor in these adipose depots in male Holstein cattle too, alterations in hexosylceramide levels appeared to affect insulin receptor expression. In 3T3-L1 fibroblast cultures, an increase in glucosylceramides inhibited insulin signaling by reduction of PKB phosphorylation after differentiation into mature adipocytes (Chavez et al., 2014). While PKB phosphorylation was not affected in the bulls herein, the insulin receptor expression was reduced in association with higher hexosylceramides. This effect was likely based on an interaction of hexosylceramides and the downstream GM3 gangliosides with the insulin receptor. Hexosylceramides were able to bind non-covalently to a lysine residue (Lys944) of the insulin receptor, just above the transmembrane domain, resulting in a disruption of the connection between membrane anchor proteins (adipocyte-specific caveolins) and the insulin receptor (Inokuchi, 2011). This was discussed as a membrane microdomain (lipid raft) disorder concept underlying metabolic disorders such as insulin resistance, resulting in a lower abundance of functioning insulin receptors in adipocytes (Inokuchi, 2011). Hexosylceramide metabolism was likely stimulated by enhanced inflammatory cytokine production, which was presumably associated with the chronic laminitis in the HEP bulls. In support of this conclusion, several inflammatory cytokines such as IL6 and TNF- α were increased in the plasma of cattle suffering from pasture-associated subclinical laminitis (Zhang et al., 2020). Higher sphinganine levels might reflect the higher availability of long-chain fatty acids for the *de novo* adipose ceramide metabolism, thereby stimulating ceramide synthesis and accumulation (Rico et al., 2016). In human subcutaneous adipose tissue, obese and diabetic individuals expressed lower sphinganine and total Cer concentrations, but sphingosines were not affected (Błachnio-Zabielska et al., 2012). These species-specific differences might be explained by metabolic differences between obese and insulin-resistant humans and bulls, respectively, which was also indicated by higher free fatty acid concentrations in the blood and enhanced dhCer concentrations in the adipose tissue of humans, but not in bulls. Thus, the potential role of lipotoxicity in the pathogenesis of diet-induced metabolic inflammation and insulin insensitivity, as stated for the human chronic over-eating syndrome (Engin and Engin, 2017), was not confirmed in

bulls. Nevertheless, high Cer 24:0 concentration in plasma was associated with obesity and diabetes in humans (Jiang et al., 2013). Thus, in HEP bulls, high Cer 24:0 concentrations in adipose tissue may also signal the obese and insulin insensitive status.

Unresolved metabolic inflammation in bulls in response to nutritional overload—a hypothesis

Metabolic inflammation was characterized as a chronic low-grade inflammatory stage in dairy cattle (Bradford and Swartz, 2020). In the following section, a novel hypothesis about the pathogenesis of an unresolved metabolic inflammation in cattle is discussed based on the findings of the Holstein bulls herein and literature data (Figure 5).

Intensive feeding for high milk performance of dairy cows, as well as for enhanced growth and fattening of bulls, is one of three suggested origins of metabolic inflammation. Starch- and protein-rich diets create challenging conditions for ruminal fermentation. Subclinical rumen acidosis is a common disease in dairy and beef cattle, resulting in disturbed composition and metabolism of the residing microbiota (Owens et al., 1998; Petri et al., 2013; Fiore et al., 2020). As a consequence, the barrier function of rumen epithelium is affected, and biogenic amines, exo-, and endotoxins such as lipopolysaccharides (LPS) can enter the circulation, promoting systemic inflammation (Owens et al., 1998; Gozho et al., 2006). Particularly LPS causes inflammatory responses *via* Toll-like receptor 4 and activation of the nuclear factor kappa B pathway (Chaurasia et al., 2020), promoting metabolic inflammation. A second potential origin for metabolic inflammation is the genetic background of high-performing cattle. Holstein dairy cows are bred for high milk yield; thus, they are highly active in the synthesis and secretion of proteins, fats, and carbohydrates. Accordingly, male Holstein cattle are less genetically determined to gain protein (kg/d) for increasing body mass compared to beef breeds such as Angus and Angus $\times$ Simmental cross-breeds (Perry et al., 1991). Intensive feeding was not only a burden for a healthy rumen function, but also for the whole body metabolism due to an overload of nutrients. Since energy and amino acids could not be efficiently stored in the form of protein, fat accretion increased instead. Furthermore, the lack of physical activity reduced mitochondrial activity to produce ATP despite the high availability of nutrients, leading to inefficient mitochondrial function and generation of reactive oxygen species (ROS) in humans (Liepinsh et al., 2020). Oxidative stress due to intensive production promoted metabolic inflammation in farm animals (Lauridsen, 2019). This condition is similar to the setting in humans affected by a sedentary lifestyle and over-nutrition (Engin and Engin, 2017). The basic genetic design of modern-day humans is still



considered to suit an ancient hunter and gatherer lifestyle, in which metabolism can easily cope with fasting but less potential is available to deal with a surplus of nutrients (Hotamisligil, 2006; Crittenden and Schnorr, 2017). As the third origin of metabolic inflammation, enlarged fat depots are known to secrete pro-inflammatory adipokines contributing to insulin resistance and metabolic inflammation (Sadri et al., 2010). While high non-esterified fatty acid (NEFA) load on the liver due to excessive adipose lipolysis was also responsible for metabolic inflammation

in dairy cows, no increase in circulating NEFA was observed in the bulls suffering from chronic laminitis (Bäßler et al., 2021). However, even in the absence of high lipolytic activity, the secretory activity of large adipocytes that are filled with saturated fatty acids in intensively fed bull could still contribute to metabolic inflammation. So far, the secretory profile of pro-inflammatory signaling molecules that originate from hypertrophic adipose tissues of fattening bulls has not yet been characterized.

To summarize, a high amount of available saturated fatty acids, oxidative stress by inefficient mitochondria, and proinflammatory factors from rumen and adipocytes can be the main factors driving ceramide accumulation and metabolic inflammation. The enhanced synthesis of Cer decreases mitochondrial efficiency, blocks lipolysis, and reduces insulin signaling tissue-specifically. Further downstream in the sphingolipid pathways, hexosylceramides, SM, and Cer-P are also involved in metabolic disturbances (Summers et al., 2019). In the bulls, hexosylceramides were associated with decreased insulin receptor amount in adipose tissue, while Cer was linked with reduced PKB phosphorylation in the liver. The resulting systemic insulin insensitivity was compensated by hyperinsulinemia. Tissue level insulin resistance is known to be closely associated with metabolic inflammation expressing mitochondrial dysfunction, oxidative stress, and disturbed lipid metabolism (Hotamisligil, 2006); the latter two are factors that also promote metabolic inflammation. Thereby, ceramide synthesis was further enhanced in the bulls as a consequence of a positive but detrimental feedback mechanism. Adipose tissues may play an important role in connecting ceramide metabolism, insulin resistance, and inflammation in diet-induced obesity in humans (Chaurasia et al., 2020), as well as in intensively fed bulls exposed to a chronic nutrient surplus. Ceramides further promote insulin insensitivity and mitochondrial dysfunction, thereby strengthening the inflammatory state. This condition can represent a self-reinforcing cycle of unresolved metabolic inflammation in the bulls of this study (Figure 5). Under this condition of unresolved metabolic inflammation, homeostasis cannot be maintained, and the risk of metabolic diseases is increased (Bradford et al., 2015). In the intensively fed bulls, this risk manifested in chronic laminitis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee of the Department for Animal Welfare Affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany).

Author contributions

Study conceptualization and design: ÁK and KH; data collection: SB; data analysis and interpretation of results: EJ-S, SB, ÁK, and KH; writing—original draft preparation: ÁK and KH; writing—review and editing: ÁK, SB, EJ-S, and KH; supervision: ÁK and KH.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2022.958837/full#supplementary-material>

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Acute enteric-coated sodium bicarbonate has negligible effect on anaerobic performance but affects metabolomics and attenuates the gastrointestinal response

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Sodium bicarbonate ingestion before exercise has a performance-enhancing effect on high-intensity exercise. However, gastrointestinal symptoms can be a problematic side-effect. Enteric-coated sodium bicarbonate can attenuate gastrointestinal symptoms following acute bicarbonate loading. In addition, the subsequent effects on exercise performance and metabolomics have not been investigated. The purpose of this study was to investigate the acute effect of enteric-coated sodium bicarbonate supplementation on the anaerobic performance, physiological profile, and symptoms of gastrointestinal discomfort after severe-intensity intermittent exercise. At the same time, targeted metabolomics was used to study the changes in urine metabolism after ingestion of enteric-coated sodium bicarbonate and to explore the characteristics of biological metabolism. In a randomized crossover design, twelve male college students completed four Wingate anaerobic 30-s cycling tests (WACT) after consuming a placebo (PL) and two experimental conditions: 0.2 g/kg body mass in enteric-coated sodium bicarbonate pills (ES) or general sodium bicarbonate pills (GS). Blood lactate (BLA), heart rate (HR), ratings of perceived exertion (RPE), and gastrointestinal-symptoms assessment questionnaire (GSAQ) were measured pre-exercise and post-exercise. In contrast, mean power (MP) and peak power (PP) were recorded immediately post-exercise. Urine samples were collected before formal tests and 50 min after the third WACT. Our findings indicate the following: 1) mean power and peak power showed no significant difference among conditions (MP: $F_{2,0,33} = 0.541$, $p = 0.587$, $\eta^2 = 0.032$; PP: $F_{2,0,33} = 0.526$, $p = 0.596$, $\eta^2 = 0.031$). The PP decline of the ES and GS after the third WACT was lower than that of the PL; 2) There were no significant differences in physiological responses, such as BLA ($F_{2,0,33,0} = 0.191$, $p = 0.827$, $\eta^2 = 0.011$) and heart rate ($F_{2,33} = 0.418$, $p = 0.662$, $\eta^2 = 0.025$), between the three conditions. Although blood lactate concentration after 10 min of the third WACT was lower with ES and GS than

with placebo; 3) Fewer participants experienced gastrointestinal symptoms with enteric-coated than with general sodium bicarbonate; 4) The metabolites with differences among the three conditions 50 min after exercise were 3-phospho-D-glycerate, D-Glucose 6-phosphate, pyruvate, cis-aconitate, oxaloacetate, and citrate. ES had higher levels of 3-phospho-D-glycerate, D-Glucose 6-phosphate, pyruvate, and cis-aconitate than GS. The 3-phospho-D-glycerate, D-Glucose 6-phosphate, pyruvate, and cis-aconitate levels in GS were significantly lower than in PL. In contrast, the citrate level in GS was significantly higher than that in other experimental conditions. Compared to PL, the level of oxaloacetate was higher after exercise in ES. This data suggests that supplementation of enteric-coated and general sodium bicarbonate before exercise can alter energy metabolism following anaerobic exercise, involving the metabolism of 3-phospho-D-glycerate, D-Glucose 6-phosphate, pyruvate, cis-aconitate, oxaloacetate, citrate, and lactate. However, they do not affect anaerobic performance and blood lactate. The supplementation of acute enteric-coated sodium bicarbonate and general sodium bicarbonate can enhance some of the weak effects of blood lactate clearance during anaerobic exercise, which may be beneficial for glycolytic energy supply. In addition, enteric-coated sodium bicarbonate intake mitigates gastrointestinal symptoms compared to general sodium bicarbonate.

KEYWORDS

sodium bicarbonate supplementation, anaerobic performance, physiological profile, gastrointestinal reactions, metabolomics

Introduction

During high-intensity exercise, the muscles mainly supplies energy through anaerobic metabolism to meet the energy demand (Gastin, 2001). In this process, it is inevitable to produce some metabolic acidic substances, such as lactate. The muscle lactate will cross the muscle cell membrane and enter the blood through diffusion or transfer. If the lactate cannot be removed in time, it will be decomposed into lactate and H^+ , resulting in H^+ accumulation (Zinner et al., 2011). The increase of H^+ will lead to an imbalance of acid-base balance and hinder the production of ATP (Juel, 2001), which will damage the contractile ability of muscles and ultimately affect exercise ability and performance. Therefore, it is very necessary to take reasonable measures to promote muscle acid-base balance during exercise.

Regulating the acid-base balance through nutrition is one of the important methods to alleviate fatigue and promote physical recovery (Naderi et al., 2016) quickly. During the past decades, numerous studies have demonstrated that increases in the extracellular buffer concentration *via* the oral ingestion of an alkaline solution, such as sodium bicarbonate may enhance human exercise performance (Wilkes et al., 1983; McNaughton et al., 1999; Requena et al., 2005; Lindh et al., 2008; Zabala et al., 2008; Ferreira et al., 2019). Sodium bicarbonate ingestion leads to an increase in plasma bicarbonate (HCO_3^-) concentration. The increase in extracellular pH leads to a greater transmembrane H^+

concentration gradient, stimulating the co-transport of H^+ and lactate from the exercising muscle cell. It has also been established that ingestion of Na^+ can increase the plasma volume (Greenleaf and Brock, 1980), which could also benefit anaerobic activity by creating an enlarged buffering potential through dilution of the H^+ concentration. Other studies have identified that acute or chronic exogenous HCO_3^- may improve performance in 400 m races, high-intensity cycling, the Wingate test, and other anaerobic activities (Pouzash et al., 2012; Saunders et al., 2014; Dalle et al., 2021). Previous studies on the effect of sodium bicarbonate on anaerobic capacity were fewer. One study explored different levels of sodium bicarbonate intake that influenced acid-base balance and performance during high-intensity exercise (DouroudosFatouros et al., 2006), and another study examined the effect of sodium bicarbonate ingestion on performance and perceptual responses (Zabala et al., 2008).

Based on a comprehensive review of the international Society of Sports Nutrition position on sodium bicarbonate and exercise performance, the ergogenic effects of sodium bicarbonate are mostly established for exercise tasks of high-intensity that last between 30 s and 12 min (Driller et al., 2012; Mündel, 2018; Grgic et al., 2021; Grgic, 2022). The Wingate test is a common method to evaluate anaerobic exercise performance and has been used more often in studies related to sodium bicarbonate. However, the results of exploring the performance of sodium bicarbonate on the Wingate test were found to be ambiguous (Zabala et al., 2008; Zabala et al., 2011; Saunders et al., 2014). Based on previous

literature (Zinner et al., 2011), the present study adapted the exercise protocol and used four Wingate anaerobic 30s cycling tests as the exercise protocol. Sodium bicarbonate supplementation (doses from 0.2 to 0.5 g/kg) may improve the performance of muscular endurance activities. Although 0.2 g/kg of sodium bicarbonate appears to be the minimum dose needed to improve exercise performance, there are still findings of gastrointestinal discomfort at this dose (Gough et al., 2017). Based on the literature and pre-experimental results, an intake dose of 0.2 g/kg was used in this study.

Sodium bicarbonate supplementation has the possibility of gastrointestinal discomfort, resulting in symptoms such as nausea, stomach pain, diarrhea, and vomiting (Burke and Pyne, 2007). One study also discovered that abdominal distress was significantly more prominent in the sodium bicarbonate trial than the placebo, resulting in an increase in stomach cramping, stomach ache, and diarrhea in the participants immediately after consumption. A larger increase in symptoms may have impacted performance (Miller et al., 2016). By applying a novel ingestion strategy to an enteric coating (containing hydroxypropyl methylcellulose, shellac, etc.), it resists dissolution in the gastric acid environment, and acid-sensitive components such as sodium bicarbonate can bypass the gastric (Barbosa et al., 2017). Since GI distress during sodium bicarbonate application may be partly attributable to degradation in the stomach (Turnberg et al., 1970), enteric coating can reduce the neutralizing effect of gastric acid and largely reduce side effects. Some studies have reported that enteric-coated sodium bicarbonate can attenuate gastrointestinal symptoms following acute bicarbonate loading on aerobic exercise performance have been investigated (Hilton et al., 2020). Evidence suggests that athletes may be deterred from supplementing with sodium bicarbonate due to the risk of GI symptoms during training or competition (Heibel et al., 2018). However, fewer studies have been conducted on the effects of acute enteric-coated sodium bicarbonate supplementation on the physiological profile, anaerobic performance, and symptoms of gastrointestinal discomfort.

Metabolomics is a new approach after genomics, proteomics, and transcriptomics with the advantages of high throughput, high specificity, and high sensitivity. Metabolomics is routinely applied as a tool for biomarker discovery to understand the systems-level effects of metabolites (Johnson et al., 2016). It is the profiling of metabolites in biofluids, cells, and tissues. The use of urine provides an ideal non-invasive method that results in a large overview of the metabolite matrix (Daskalaki et al., 2015). With the recent emergence of metabolomics analyses, metabolomics is now being applied to choosing exercise and nutritional supplements (Nieman et al., 2015; Al-Khelaifi et al., 2018). Metabolomics analysis can be either targeted (that focused on quantitative measurements of usually small numbers of

metabolites) or untargeted (that focused on metabolic profiling of the total complement of metabolites for the studied samples) (Emwas et al., 2021). Targeted metabolomics focuses on analyzing several selected metabolites, such as studies related to specific metabolic pathways, drug toxicology, and specific effects of certain foods/nutrients. However, metabolomics analysis methods are less applied in the study of enteric-coated sodium bicarbonate.

Some studies have examined sodium bicarbonate supplementation during high-intensity exercise affects performance. However, the findings are equivocal, and some studies did not find significant differences between sodium bicarbonate and placebo conditions (Joyce et al., 2012; Driller et al., 2013). The researchers hypothesized that the finding may have been due to the unique subject characteristics (highly trained members of national teams) (Edge et al., 2004; Carr et al., 2011; Wang et al., 2019). Based on these findings, healthy college students were chosen to be the participants of this study instead of highly trained athletes, to better understand the possible impact of sodium bicarbonate ingestion when coupled with high-intensity exercise.

The results of past studies led us to develop a research project that would investigate the effects of acute enteric-coated sodium bicarbonate supplementation on anaerobic performance, heart rate, blood lactate, and symptoms of gastrointestinal discomfort in healthy young men. The target metabolomics based on the multi-reaction monitoring technology (MRM) method was used to analyze the specific metabolic pathway. It was hypothesized that both supplementation conditions would improve anaerobic performance. However, enteric-coated sodium bicarbonate was more effective in reducing the incidence of gastrointestinal side effects.

Materials and methods

Participants

Fifteen healthy, college-aged men participated in the study (but three participants failed to complete the entire trial, $n=12$). The basic information of the participants is presented in Table 1. Participants were excluded from the study if they were smokers, taking medication, drinking any beverages other than water, or having any chronic diseases. No participants reported taking any performance-enhancing supplements or supplements that would be classified as either intracellular or extracellular buffers. Participants were asked to refrain from maximal exercise 48 h before each trial, and each subject was advised to maintain a consistent diet and activities. All participants were informed of both the benefits and the potential side effects of the study (both

TABLE 1 Basic information of participants (mean $\pm$ SD).

Sex	Number	Age (Years)	Height (cm)	Weight (kg)	Bmi
Male	12	24.25 $\pm$ 0.75	179.08 $\pm$ 2.42	74.43 $\pm$ 6.12	23.19 $\pm$ 1.60

TABLE 2 HR responses during three experimental conditions.

Time	PL (beats/minute)	ES (beats/minute)	GS (beats/minute)
Before test	80.25 $\pm$ 8.18	79.25 $\pm$ 9.07	73.25 $\pm$ 8.94
First (Immediately)	165.42 $\pm$ 18.84	167.50 $\pm$ 13.33	166.08 $\pm$ 16.64
First (4min)	114.42 $\pm$ 16.26	111.08 $\pm$ 10.11	105.75 $\pm$ 13.53
Second (Immediately)	175.58 $\pm$ 13.75	172.33 $\pm$ 11.74	171.00 $\pm$ 13.76
Second (4min)	128.33 $\pm$ 13.55	127.00 $\pm$ 14.89	121.25 $\pm$ 15.33
Third (Immediately)	174.17 $\pm$ 13.66	173.92 $\pm$ 12.48	171.08 $\pm$ 13.87
Third (1min)	148.25 $\pm$ 18.76	150.42 $\pm$ 13.67	145.50 $\pm$ 15.53
Third (4min)	123.50 $\pm$ 14.49	125.50 $\pm$ 14.43	119.08 $\pm$ 14.34
Third (7min)	115.67 $\pm$ 9.99	115.92 $\pm$ 11.82	113.08 $\pm$ 15.02
Third (10min)	112.83 $\pm$ 14.57	109.75 $\pm$ 11.31	108.17 $\pm$ 11.01
Third (15min)	106.17 $\pm$ 9.86	107.50 $\pm$ 10.56	107.33 $\pm$ 15.28
Third (20min)	105.17 $\pm$ 9.65	105.58 $\pm$ 11.05	103.75 $\pm$ 13.82
Third (25min)	101.92 $\pm$ 7.46	102.17 $\pm$ 10.02	99.42 $\pm$ 12.59
Third (30min)	101.17 $\pm$ 8.99	100.58 $\pm$ 7.79	97.08 $\pm$ 12.35

Values are means $\pm$ SD. First (n min): n minutes after the first WACT; Second (n min): n minutes after the second WACT; Third (n min): n minutes after the third WACT.

TABLE 3 RPE in three conditions at each moment.

Time	PL	ES	GS
Before test	8.33 $\pm$ 1.67	8.00 $\pm$ 1.86	8.58 $\pm$ 1.31
First (Immediately)	14.33 $\pm$ 1.50	13.58 $\pm$ 1.31	13.92 $\pm$ 1.84
First (4min)	12.33 $\pm$ 1.67	11.42 $\pm$ 1.78	12.33 $\pm$ 1.50
Second (Immediately)	15.33 $\pm$ 1.56	15.00 $\pm$ 1.48	15.33 $\pm$ 1.15
Second (4min)	13.50 $\pm$ 1.62	13.17 $\pm$ 2.08	13.67 $\pm$ 1.50
Third (Immediately)	15.92 $\pm$ 1.44	16.08 $\pm$ 1.62	16.42 $\pm$ 1.56
Third (1min)	15.58 $\pm$ 1.00	15.75 $\pm$ 1.66	15.83 $\pm$ 1.59
Third (4min)	14.08 $\pm$ 1.51	14.00 $\pm$ 2.09	14.42 $\pm$ 1.78
Third (7min)	12.58 $\pm$ 1.62	12.92 $\pm$ 1.78	13.50 $\pm$ 1.51
Third (10min)	11.67 $\pm$ 1.67	11.92 $\pm$ 1.88	12.50 $\pm$ 1.93
Third (15min)	11.00 $\pm$ 1.71	11.50 $\pm$ 1.68	11.58 $\pm$ 2.15
Third (20min)	10.83 $\pm$ 1.27	11.08 $\pm$ 1.62	11.42 $\pm$ 2.27
Third (25min)	10.58 $\pm$ 1.24	11.08 $\pm$ 1.93	11.25 $\pm$ 2.26
Third (30min)	10.50 $\pm$ 1.31	10.58 $\pm$ 1.98	11.17 $\pm$ 2.44

Values are means $\pm$ SD. First (n min): n minutes after the first WACT; Second (n min): n minutes after the second WACT; Third (n min): n minutes after the third WACT.

verbally and in writing) before providing written informed consent. The institutional research ethics committee of Capital University of Physical Education and Sports granted ethical permission (2021A42).

Procedures

The study was divided into three trials including enteric-coated sodium bicarbonate (ES, containing enteric coating and sodium bicarbonate), general sodium bicarbonate (GS, containing sodium bicarbonate), and placebo (PL, containing cornstarch), and was conducted in a randomized crossover design. The supplementation dose was 0.2 g/kg body mass in the form of unmarked oval tablets for each group of experiments. The recommended timing of sodium bicarbonate intake was 90–180 min prior to exercise or competition (Jones et al., 1977; Bishop et al., 2004; Yuen, 2010; Grgic et al., 2021). Enteric-coated sodium bicarbonate is chosen to be ingested 180 min before exercise due to the additional time required for digestion and absorption in the intestine (Maderuelo et al., 2019). ES and PL were ingested 180 min prior to testing, and GS was ingested 90 min prior to testing. Each experiment was separated by at least 7 days (1 week was considered a sufficient washout period to eliminate any ergogenic effect of sodium bicarbonate (Bishop and Claudius, 2005)). All trials were performed at the same time of day to minimize diurnal variation. The Wingate anaerobic 30-s cycling test (WACT, resistance factor was set at 0.075 kg/kg BW) was used in this study. Prior to the test, participants adjusted handlebar and seat position (same position in trials), and then participants performed a standardized warm-up

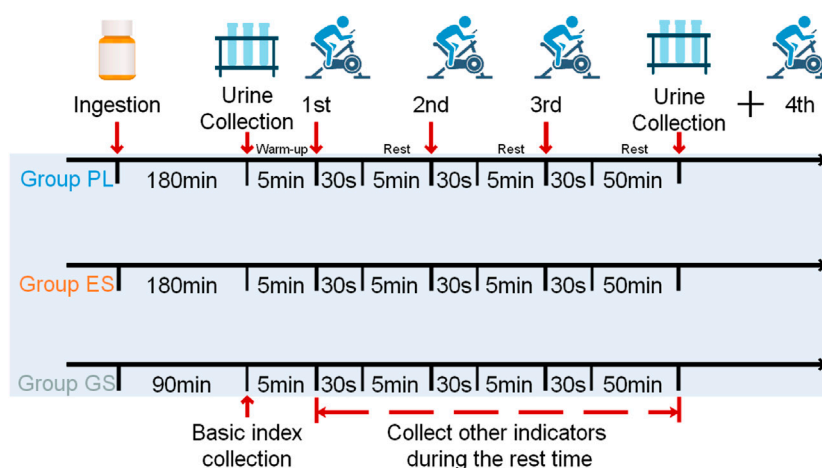


FIGURE 1

Main procedure of the trials. placebo (PL), enteric-coated sodium bicarbonate (ES), general sodium bicarbonate (GS). 1st: the first WACT; second: the second WACT; third: the third WACT; fourth: the fourth WACT.

(power: 60 W; time: 5 min). Participants performed the first three WACTs to test anaerobic capacity with a 5-min interval each time (passive recovery, trying to remain seated; small amounts of water allowed, totaling 200 ml). This was followed by a third interval of 50 min (passive recovery; no water or other food intake to avoid interfering with urine sampling), and a fourth WACT was performed to test recovery of anaerobic capacity. Throughout the test, participants were given verbal encouragement and asked to complete the WACTs as quickly as possible. The specific experimental procedure is shown in [Figure 1](#).

Experimental design

Upon arrival at the laboratory, participants were asked to ingest ES, GS, or PL for the next 30 min. At a specified time after the start of supplement intake (ES and PL: 180 min, GS: 90 min), participants started performing four Wingate anaerobic 30-s cycling tests with a Monark 894E (Ergonomic 894E, Sweden) after a baseline (pre-trial) capillary blood sample was taken. Samples were taken at the start, in the middle, and at the end of WACT. The participants had the index collection to establish basal measurements (blood lactate (BLA), heart rate (HR), ratings of perceived exertion (RPE), gastrointestinal-symptoms assessment questionnaire (GSAQ), and urine) before the first WACT. Capillary blood samples to determine BLA concentration were collected from the ear lobes *via* the h/p/cosmos Sirius® lactate test meter (manufacturer: SensLab GmbH, Germany) at the third minute of the five-minute interval after the first three WACT. In the third WACT, measurements of BLA were

conducted immediately at 1, 4, 7, 10, 15, 20, 25, and 30 min after the cessation of exercise. The HR was monitored by the Polar Rs 800cx telemetry heart rate monitor (manufacturer: Polar, Finland) from the resting state to the recovery period for analyzing the heart rate. RPE was measured with the Borg 15-point scale, ranging from 6 (very, very light) to 20 (very, very heavy).

HR and RPE were measured immediately after the first three WACTs and the end of the third WACT, as well as 1, 4, 7, 10, 15, 20, 25, and 30 min after the third WACT. Due to the gastrointestinal side effects associated with sodium bicarbonate ingestion ([Carr et al., 2012](#)), participants completed a GSAQ before the test. This was followed by collecting for GSAQ (immediately after the third WACT and before the fourth WACT). The numerical rating scale (NRS) (scale 0–10, with zero reflecting no gastrointestinal discomfort and 10 indicating the most severe gastrointestinal discomfort) was used to classify the intensity of these symptoms ([Dworkin et al., 2005](#)). After that, the fourth WACT was conducted. The mean power (MP) and peak power (PP) of the test were calculated *via* Monark Anaerobic Testing software after every WACT. Urine was collected before warm-up and 50 min after the third WACT.

Urine metabolomics

The metabolomics in this study is a targeted metabolomics analysis based on the MRM approach. This detection method covers important metabolites in the tricarboxylic acid cycle, the glycolytic pathway, and oxidative phosphorylation processes. The MRM principle uses selective response/multi-reaction monitoring technology (SRM/MRM) to detect and analyze

specific metabolites in standard samples. Absolute quantitative results of the target metabolites can be obtained, which have the characteristics of high specificity, high sensitivity, and high accuracy.

The samples were removed from -80°C , slowly dissolved at 4°C , and 1000 μl of pre-cooled methanol acetonitrile solution (1:1, v/v) was added to each group of samples, Vortex for 60 s, -20°C for 1 h to precipitate the protein, 14000rcf, centrifuge at 4°C for 20 min, freeze dry the supernatant, -80°C to store the samples.

The samples were separated using an Agilent 1290 Infinity LC Ultra-Performance Liquid Chromatography system. The mobile phase contained A = 10 mM aqueous ammonium acetate solution and B = acetonitrile. The samples were in the automatic sampler at 4°C , and the column temperatures were kept constant at 45°C . The gradients were at a flow rate of 300 $\mu\text{l}/\text{min}$, a 2 μl aliquot of each sample was injected. The relevant liquid phase gradients were as follows: the gradient was 90% B linearly reduced to 40% in 0–18 min, and then added to 90% in 0.1 min, and then maintained at 90% from 18.1 to 23 min. The sample cohort is set up with one QC sample for each interval of a certain number of experimental samples, which is used to detect and evaluate the stability and reproducibility of the system. The sample cohort is set up with a mixture of standards of energy metabolites for chromatographic retention time correction.

Mass spectrometry was performed using a 5500 QTRAP mass spectrometer (AB SCIEX) in negative ion mode. The 5500 QTRAP ESI source conditions are as follows: source temperature 450°C , ion Source Gas1 (Gas1): 45, Ion Source Gas2 (Gas2): 45, Curtain gas (CUR): 30, ionSapary Voltage Floating (ISVF)-4500 V; Adopt the MRM mode detection ion pair.

Statistical analysis

Data were entered into Microsoft Excel 2010 and analyzed with SPSS 26.0. Data were analyzed using parametric tests following confirmation of a normal distribution *via* the Shapiro-Wilks-W-test and are presented as mean $\pm$ standard deviation (SD). Normally distributed data sets were analyzed with appropriate parametric statistical tests. Repeated-measures ANOVA compared conditions for significant differences among the three trials (MP, PP, BLA, HR, and RPE). Simple effect analysis was performed for the horizontal comparison of between-group factors when there was an interaction, and longitudinal comparison of within-group factors was performed when there was no interaction. In order to evaluate differences following the ingestion of GS and ES, effect sizes were calculated for each variable. In all cases, the significance level was set at $P < 0.05$. In addition, urine sample data was extracted by Multiquant software for peak area and retention time. The retention time was corrected using standards of energy metabolites for metabolite identification.

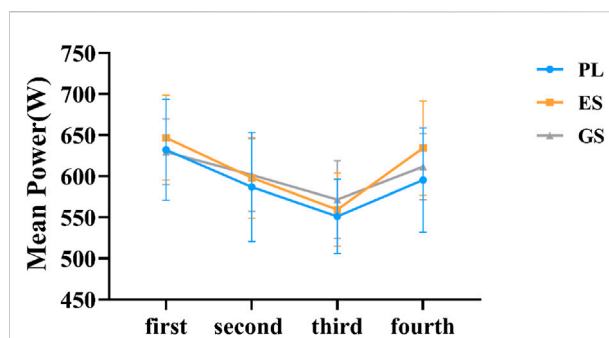


FIGURE 2

Trend of mean power. The horizontal coordinate represents the order of WACT in the experiment. The figure represents the mean $\pm$ SD of mean power data for three conditions.

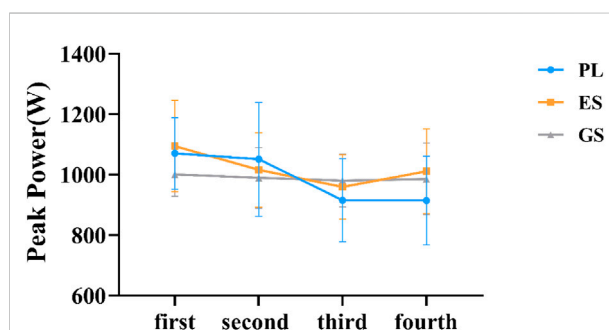


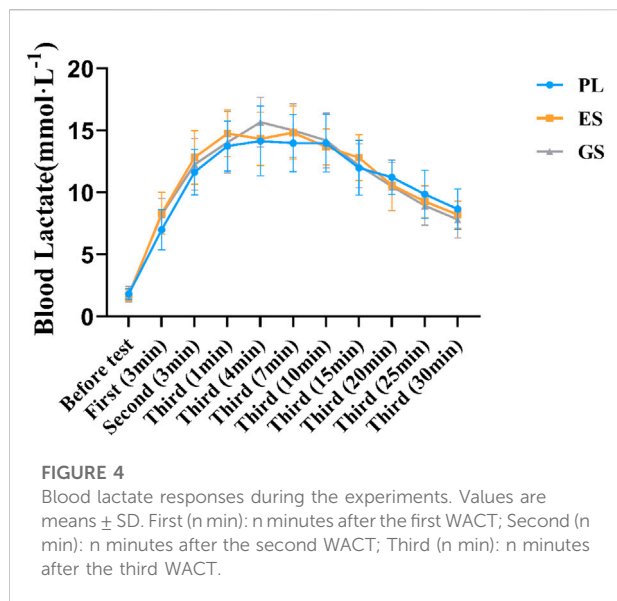
FIGURE 3

Trend of peak power. The horizontal coordinate represents the order of WACT in the experiment. The figure represents the mean $\pm$ SD of peak power data for three conditions.

Results

Wingate anaerobic 30s cycling test

An overall main effect for WACT test was not apparent ($p > 0.05$) (Figures 2, 3). A comparison of the trials indicated no differences in MP completed among the three conditions of trials (MP: $F_{2,0,33} = 0.541$, $p = 0.587$, $\eta^2 = 0.032$; PP: $F_{2,0,33} = 0.526$, $p = 0.596$, $\eta^2 = 0.031$). There was a significant main effect of time on MP ($F_{2,867,94,606} = 37.103$, $p = 0.000$, $\eta^2 = 0.529$) and PP ($F_{2,821,93,108} = 6.929$, $p = 0.000$, $\eta^2 = 0.174$), but no interaction effect between condition and time (MP: $F_{5,734,94,606} = 1.166$, $p = 0.332$, $\eta^2 = 0.066$; PP: $F_{5,643,93,108} = 1.973$, $p = 0.082$, $\eta^2 = 0.107$). However, ingestion of ES and GS slightly increased MP compared with PL in the three experimental conditions (The fourth WACT MP: ES = $634.36 \pm 57.21\text{W}$, GS = $611.74 \pm 40.32\text{W}$, PL = $595.40 \pm 63.37\text{W}$; $p > 0.05$), despite no significant differences being observed in all the performance variables



analyzed between successive tests (Figure 2). The PP of the ES and GS decreased slower than that of the PL from the second WACT (The fourth WACT PP: ES = 1011.31 ± 139.76 W, GS = 985.18 ± 118.40 W, PL = 914.53 ± 146.75 W; $p > 0.05$), based on the changing trend of four peak powers (Figure 3).

Blood lactate

In the three experimental conditions, there was no difference in the resting BLA, either in the first two WACT or other periods of the third WACT (see Figure 4).

ES and GS ingestion did not alter BLA ($F_{2,0, 33,0} = 0.191$, $p = 0.827$, $\eta^2 = 0.011$) despite significant changes post-exercise ($F_{5,971, 197,037} = 289.572$, $p = 0.000$, $\eta^2 = 0.898$), with no condition $\times$ time interaction ($F_{11,942, 197,037} = 1.703$, $p = 0.069$, $\eta^2 = 0.094$). However, at 7 min after the third WACT under experimental conditions, BLA values were higher in ES and GS than in PL (ES = 14.83 ± 2.13 mmol L⁻¹, GS = 15.00 ± 2.16 mmol L⁻¹, PL = 13.98 ± 2.30 mmol L⁻¹; $p > 0.05$). The results of all three experiments showed that the BLA of the ES and GS was lower than that of the PL on the descent after 10 min of the third WACT (After 20 min of the third WACT: ES = 10.59 ± 2.03 mmol L⁻¹, GS = 10.48 ± 1.96 mmol L⁻¹, PL = 11.22 ± 1.39 mmol L⁻¹; $p > 0.05$). From the downward trend in Figure 4, the BLA of the ES and GS decreased more than the PL.

Heart rate

The HR was increasing with time during the whole experiment ($F_{4,847, 159,967} = 581.113$, $p = 0.000$, $\eta^2 = 0.946$).

However, no significant differences were found between conditions ($F_{2, 33} = 0.418$, $p = 0.662$, $\eta^2 = 0.025$), and there was no significant condition $\times$ time interaction ($F_{9,695, 159,967} = 0.565$, $p = 0.836$, $\eta^2 = 0.033$).

Enteric-coated sodium bicarbonate consumption had no influence on HR (e.g., before the test: ES = 79.25 ± 9.07 , GS = 73.25 ± 8.94 , PL = 80.25 ± 8.18 ; at 30 min after the third exercise: ES = 100.58 ± 7.79 , GS = 97.08 ± 12.35 , PL = 101.17 ± 8.99 ; $p > 0.05$). As with GS, there was no difference between the three experimental conditions at these moments during the four WACTs (Table 2, all $p > 0.05$).

Gastrointestinal-symptoms assessment questionnaire

Among all three conditions, the greatest incidence of GI side effects was recorded among the GS group. Overall, two participants in the ES and two participants in the PL reported mild side effects. However, seven participants suffered from obvious side effects during the GS study. Side effects emerged after general sodium bicarbonate supplementation, most often immediately after the third WACT.

Figure 5 indicates the main symptoms of gastrointestinal reactions before exercise, immediately after the third WACT, and 50 min after the third WACT. Analysis of the circumstances of acute GI discomfort in the GS indicated more cases of diarrhea, flatulence, gastrointestinal discomfort, and nausea. Figure 5 shows a significant increase in all of the parameters measured at 90 min after general sodium bicarbonate ingestion. However, the ES had no obvious gastrointestinal tract symptoms after exercise.

Ratings of perceived exertion

There were no differences in either RPE ($F_{2,0, 33,0} = 0.362$, $p = 0.699$, $\eta^2 = 0.021$) between conditions (Table 3), although there were significant increases in RPE during the experiment ($F_{3,552, 117,209} = 143.397$, $p = 0.000$, $\eta^2 = 0.813$). No significant condition $\times$ time interactions were revealed for neither RPE ($F_{7,104, 117,209} = 0.628$, $p = 0.734$, $\eta^2 = 0.037$).

Urine metabolomic analyses

The targeted metabolomics tests cover important metabolites in the tricarboxylic acid cycle, glycolytic processes, and oxidative phosphorylation processes. Eight samples were screened from each group of urine samples for metabolomics analysis. QC samples were prepared by mixing all samples in equal quantities, and the QC samples were used to evaluate the stability and reproducibility of the data. The RSD (relative standard deviation) results of the measured substances in the

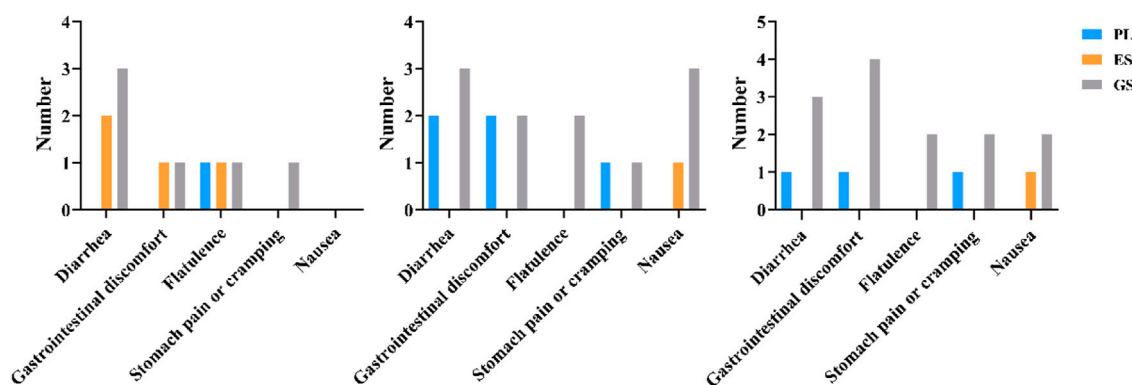


FIGURE 5

Gastrointestinal-symptoms assessment questionnaire results before exercise, immediately, and 50 min after the third WACT.

QC samples are presented in Figure 6, where the energy metabolism with RSD is less than 30%, indicating that the data in the samples were stable and reliable. The metabolite hierarchy clustering plots are presented in Figure 6. When the candidate metabolites are screened reasonably well and accurately, the same condition of samples can appear in the same cluster through clustering. At the same time, metabolites clustered in the same cluster have similar expression patterns and may be in closer reaction steps in the metabolic process.

After testing and analysis, eighteen metabolites were observed in this study. A total of five differential metabolites were screened 50 min after the experiment with the three conditions for differences in urinary metabolites, namely 3-phospho-D-glycerate, D-Glucose 6-phosphate, pyruvate, cis-aconitate, oxaloacetate, and citrate. Screening analysis of pre-exercise after supplementation found that the level of lactate was significantly higher in ES than in PL. However, there was no significant difference after exercise.

There were five metabolic differences between the three experimental conditions at 50 min after the third WACT (see Figure 7). ES exhibited greater increases in 3-phospho-D-glycerate ($p < 0.001$), D-Glucose 6-phosphate ($p < 0.001$), pyruvate ($p < 0.05$), cis-aconitate ($p < 0.01$) than GS. The levels of 3-phospho-D-glycerate ($p < 0.01$), D-Glucose 6-phosphate ($p < 0.01$), pyruvate ($p < 0.05$), and cis-aconitate ($p < 0.05$) in GS were significantly lower than that in PL, while the level of citrate in GS was significantly higher than that in ES ($p < 0.05$) and PL ($p < 0.001$). Compared to PL, the level of oxaloacetate ($p < 0.05$) was higher after exercise in ES.

Discussion

The main findings of this analysis were that, compared to PL, acute ingestion of ES and GS did not significantly improve either

peak power or mean power during the WACT, regardless of the number of tests performed. However, compared to placebo, the intake of ES and GS increased the values of peak and mean power during the third and fourth WACTs (Figure 2). In addition, some additional changes occurred in ES and GS, including a decrease in the average power drop range. Our data suggest that in the population studied, enteric-coated and general sodium bicarbonate may have some positive impact on the Wingate tests on anaerobic capacity. One possible explanation for this finding is that power is more related to glycolytic metabolism and is limited by H^+ accumulation (Lancha Junior et al., 2015; Lopes-Silva et al., 2019). Therefore, the buffering action of sodium bicarbonate could be more effective at minimizing fatigue and increasing mean power during the test, particularly in later stages (Lopes-Silva et al., 2019). Based on the literature reviewed, the failure of alkalosis to increase Wingate performance may be explained by an insufficient time during the exercise to allow a significant difference in H^+ ion efflux from the muscle fibers or an inability to generate a sufficient difference in H^+ ion gradient to produce a difference between trials (Mainwood and Worsley-Brown, 1975; Linderman and Fahey, 1991; Marx et al., 2002; Forbes et al., 2005; Street et al., 2005). Overall, these results suggest that sodium bicarbonate may have an ergogenic effect on repeated WACT test performance (Grgic, 2022). The likely explanation is also that recovery between bouts of exercise is enhanced by enhanced buffering capacity. However, one study has also suggested that sodium bicarbonate ingestion results in significant shifts in the acid-base balance of the blood and has a small but non-significant effect on anaerobic power and capacity (Parry-Billings and MacLaren, 1986). Our data are similar to the results of the study, but there is no significant change. This may be due to the higher intake dose in these studies than in the study we designed, although our dose is also effective in some studies (Durkalec-Michalski et al., 2020).

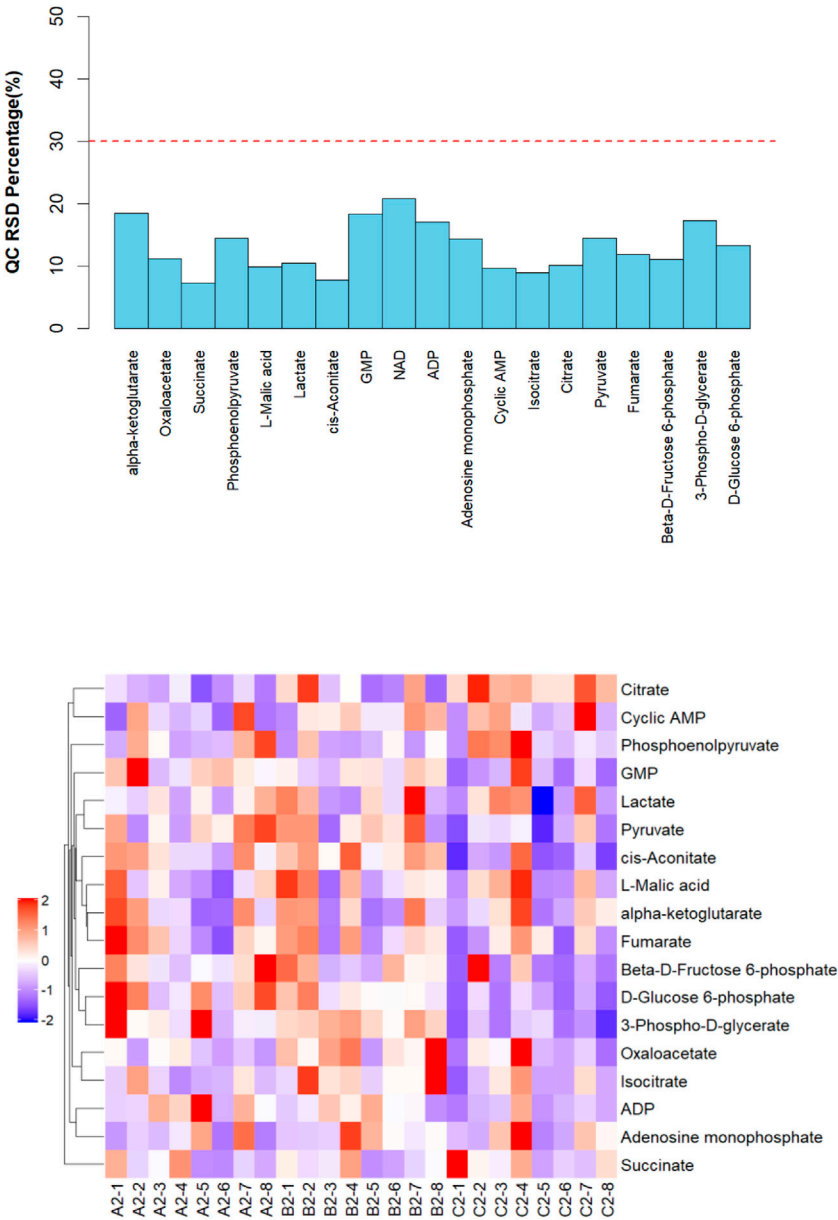
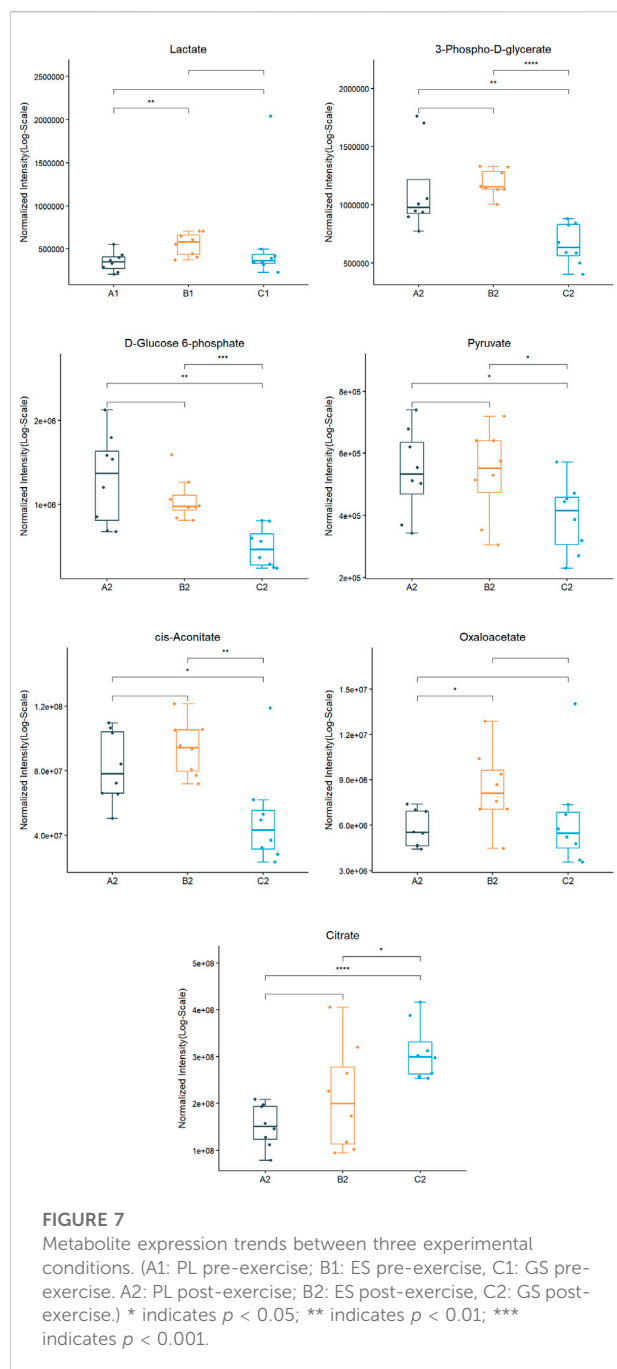


FIGURE 6
RSD distribution of QC samples, and urine metabolite hierarchy clustering plots post-test.

The findings demonstrated that BLA was not significantly different among experimental conditions in all tests. From the overall trend, the BLA of participants in enteric-coated and general sodium bicarbonate decreased more rapidly during the decline of BLA. We speculated that enteric-coated and general sodium bicarbonate might promote lactate clearance after exercise, reducing lactate accumulation. Some studies have indicated that sodium bicarbonate can change the content of blood lactate (Hartono and Sukadiono, 2017; Rezaei et al., 2019). Evidence suggests that sodium bicarbonate promotes non-

oxidative energy metabolism, as observed through greater muscle lactate production and muscle glycogen utilization during intermittent exercise performed in the severe-intensity domain (Percival et al., 2015). However, if lactate is not removed in time, it will be decomposed and converted into lactate and cause a large amount of H^+ accumulation, resulting in muscle acidification, causing acidosis (Zinner et al., 2011). The increase in buffering capacity achieved by ingesting sodium bicarbonate before exercise has previously been well documented (Siegler et al., 2010). Although our data suggest no significant difference



among three conditions in the BLA ($p > 0.05$), the supplementation of enteric-coated and general sodium bicarbonate positively impacted the lactate clearance trend after exercise.

Three participants experienced serious problematic GI effects during our three experiments after taking general sodium bicarbonate and failed to complete the entire experiment. Previous studies have suggested that general sodium bicarbonate can adversely affect GI comfort (Cameron et al.,

2010; Carr et al., 2011; Saunders et al., 2014). The accumulation of CO_2 in the stomach, resulting from supplementation with sodium bicarbonate, may cause bloating, nausea, vomiting, and abdominal pain (Kahle et al., 2013). The incidence and severity of these side-effects increase linearly with the dose of sodium bicarbonate ingested and should be considered in terms of their overall effect on performance (Cameron et al., 2010). In this study, gastrointestinal discomfort was shown to be significantly more prominent in the general sodium bicarbonate trial than the placebo and enteric-coated sodium bicarbonate, resulting in an increase in stomach cramping, stomach ache, and diarrhea in the participants in sports. There was a larger increase in symptoms that may have impacted on performance. Therefore, following general sodium bicarbonate supplementation, these side effects may negatively impact exercise performance. More recent evidence suggests that enteric-coated sodium bicarbonate is a possible strategy to minimize the likelihood and severity of these side effects (Hilton et al., 2020). Our results support this study.

At baseline, no differences were observed among the three trials for HR. As expected, exercise resulted in increases in HR; all measures returned toward baseline during recovery from exercise (Table 2). This is similar to the study suggesting no improvement in heart rate recovery after exercise following sodium bicarbonate supplementation (Wang et al., 2019).

Based on RPE data, ES or GS did not affect RPE during a series of WACTs. This result disagrees with those published previously (Swank and Robertson, 1989; Swank and Robertson, 2002) showing lower RPEs under alkalotic conditions relative to placebo. Similar results have been demonstrated in the other study, showing that RPE measured immediately after each WACT was not affected by sodium bicarbonate ingestion (Zabala et al., 2008). This may be due to the excessive exercise intensity and short intervals during each WACT, resulting in little subjective feeling.

The targeted metabolomics tests cover important metabolites in the tricarboxylic acid cycle, glycolytic, and oxidative phosphorylation processes (see Figure 8). The metabolites of the participants in the three conditions were found to be diverse at that time. Studies have demonstrated that due to the high intensity and short movement duration, it is characterized by an energy supply derived primarily from glycolytic metabolism. In the ES, excluding citrate, the levels of 3-phospho-D-glycerate, pyruvate, cis-aconitate, and oxaloacetate were increased 50 min after exercise compared with the levels of exercise in the PL and GS, while D-Glucose 6-phosphate decreased. D-Glucose 6-phosphate, 3-phospho-D-glycerate, and pyruvate are the intermediate products of glucose catabolism. Glucose is the main energy-supplying substance in humans. ES more potently induced circulating glucose metabolism and the TCA cycle than GS and PL. It appears that both greater muscle buffering capacity and enhanced removal of protons result in increased glycolytic ATP production (Chycki et al., 2018).

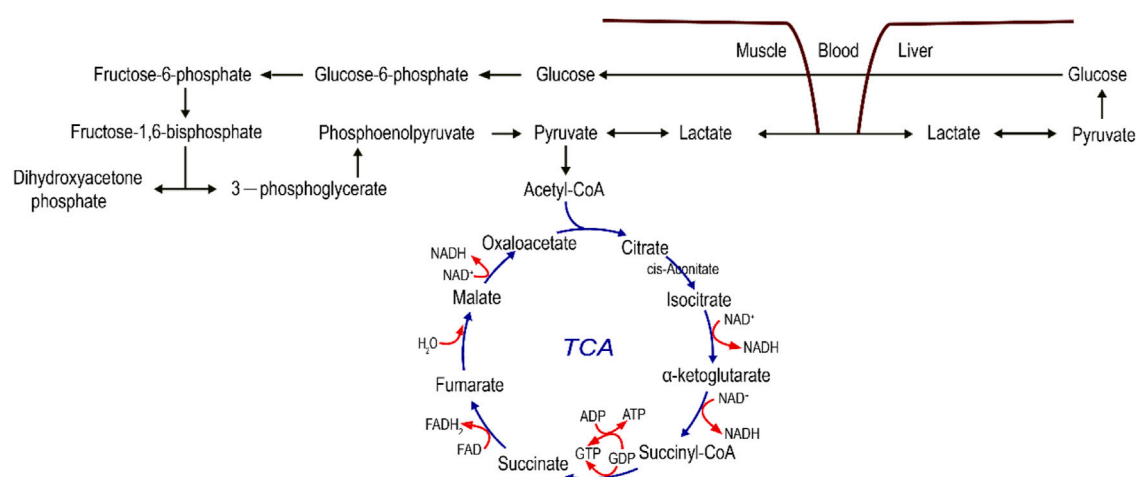


FIGURE 8
Pathways of related energy metabolites.

Citrate, oxaloacetate, and cis-aconitate are the intermediate products of the TCA cycle. The tricarboxylic acid cycle (TAC) is a necessary metabolic pathway in tissue energy supply and is distributed in the mitochondria. From the results, it is clear that the ES and GS affect the metabolic substances of the TAC metabolic pathway. Compared with the PL, an increase in intermediates of the TCA cycle was observed in the ES, including cis-aconitate, oxaloacetate, and citrate. It is hypothesized that the increased amount of TCA circulating intermediate in the blood may be due to the positive effect of ES intake on acidic substances clearance, which can promote the production of more energy in mitochondria for consumption by the system. The results showed that the differential metabolites related to the tricarboxylic acid cycle, aconitate and oxaloacetate decreased significantly, and citrate increased significantly, indicating the disorder of the tricarboxylic acid cycle. It may be due to side effects affecting the state of the exercise.

Lactate is a metabolite produced by the anaerobic enzymatic supply of glycogen and glucose outside the mitochondria in a state of oxygen deprivation. Various studies have confirmed that sodium bicarbonate increases extracellular pH and promotes H^+ and lactate efflux from active muscles (Driller et al., 2012; Rezaei et al., 2019). The level of lactate in the ES of the pre-test was higher than that in the PL. It is hypothesized that ES supplementation promotes lactate clearance.

There are some limitations to the current study. Our study employed a limited sample of solely healthy young males, limiting its generalizability to other demographics. Coaches and athletes may consider using these supplements to give a possible performance edge, taking into personal account response.

Conclusion

Based on these results, it was concluded that acute enteric-coated sodium bicarbonate and general sodium bicarbonate do not improve anaerobic exercise performance. Based on the analysis of target metabolomics, enteric-coated sodium bicarbonate and general sodium bicarbonate supplementation can affect the metabolism of substances, including 3-phospho-D-glycerate, D-Glucose 6-phosphate, pyruvate, cis-aconitate, oxaloacetate, citrate, and lactate. In healthy young men, the supplementation of acute enteric-coated sodium bicarbonate and general sodium bicarbonate can enhance some of the weak effects of blood lactate clearance during anaerobic exercise, which may be beneficial for glycolytic energy supply. An important point to note is that general sodium bicarbonate is a serious gastrointestinal problem, while enteric-coated sodium bicarbonate supplements can relieve gastrointestinal distress.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the institutional research ethics committee of Capital University of Physical Education and Sports granted ethical permission (2021A42). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, HW; methodology, XW and NZ; validation, YF; formal analysis, NZ; investigation, HW; resources, JW; writing—original draft preparation, NZ; writing—review and editing, YF and NZ; supervision, HW; project administration, HW; funding acquisition, HW. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

Author JW was employed by the company Qingdao Shengbang Health Food Co.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict.

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Attenuated inflammatory profile following single and repeated handgrip exercise and remote ischemic preconditioning in patients with cerebral small vessel disease

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Background: Similar to remote ischemic preconditioning bouts of exercise may possess immediate protective effects against ischemia-reperfusion injury. However, underlying mechanisms are largely unknown. This study compared the impact of single and repeated handgrip exercise versus remote ischemic preconditioning on inflammatory biomarkers in patients with cerebral small vessel disease (cSVD).

Methods: In this crossover study, 14 patients with cSVD were included. All participants performed 4-day of handgrip exercise (4x5-minutes at 30% of maximal handgrip strength) and remote ischemic preconditioning (rIPC; 4x5-minutes cuff occlusion around the upper arm) twice daily. Patients were randomized to start with either handgrip exercise or rIPC and the two interventions were separated by > 9 days. Venous blood was drawn before and after one intervention, and after 4-day of repeated exposure. We performed a targeted proteomics on inflammation markers in all blood samples.

Results: Targeted proteomics revealed significant changes in 9 out of 92 inflammatory proteins, with four proteins demonstrating comparable time-dependent effects between handgrip and rIPC. After adjustment for multiple testing we found significant decreases in FMS-related tyrosine kinase-3 ligand (Flt3L; 16.2% reduction; adjusted *p*-value: 0.029) and fibroblast growth factor-21 (FGF-21; 32.8% reduction adjusted *p*-value: 0.029) after single exposure. This effect did not differ between handgrip and rIPC. The decline in Flt3L after repeated handgrip and rIPC remained significant (adjusted *p*-value = 0.029), with no difference between rIPC and handgrip (adjusted *p*-value = 0.98).

Conclusion: Single handgrip exercise and rIPC immediately attenuated plasma Flt3L and FGF-21, with the reduction of Flt3L remaining present after 4-day of

repeated intervention, in people with cSVD. This suggests that single and repeated handgrip exercise and rIPC decrease comparable inflammatory biomarkers, which suggests activation of shared (anti-)inflammatory pathways following both stimuli. Additional studies will be needed to exclude the possibility that this activation is merely a time effect.

KEYWORDS

exercise, remote ischemic preconditioning, cerebral small vessel disease, inflammation, proteomics

Introduction

Ischemia-reperfusion (I/R)-injury represents a contributor to cerebral- and cardiovascular-related morbidity and mortality (Hausenloy et al., 2017; Heusch, 2020). While reperfusion is essential for restoring blood flow to tissue and preventing tissue necrosis from ischemia (e.g., during an infarction or surgery), the rapid reintroduction of blood flow paradoxically causes additional injury. In addition to timely reperfusion previous studies revealed that remote ischemic preconditioning (rIPC) may reduce this I/R-injury. rIPC refers to the exposure of remote tissue (e.g., a limb) to repeated periods of short-lasting ischemia (e.g., 4 times 5-min) (Hess et al., 2015) which improves resilience to a period of prolonged ischemia in a target organ (e.g., the brain and heart) (Przyklenk et al., 1993; Benstoem et al., 2017). Despite the success of pre-clinical studies, a series of clinical trials evaluating the efficacy of rIPC (Hausenloy et al., 2015; Meybohm et al., 2018; Hausenloy et al., 2019) failed to translate this into improved clinical outcome. It is not exactly known by which mechanisms preconditioning might work. Previous studies suggest that rIPC causes the release of humoral factors that can affect the target organ (e.g., heart or brain) *via* the circulation (Iadecola and Anrather, 2011). These humoral factors possibly relate to inflammatory biomarkers.

Interestingly, within the past few years, a series of studies in animals and humans have suggested that exercise also induces immediate preconditioning effects (Seeger et al., 2015; Thijssen et al., 2018). Whether exercise and rIPC share underlying pathways related to the possibility to protect cerebral and cardiac tissue against I/R-injury is largely unknown (Thijssen et al., 2018). Exercise has been shown to also influence inflammatory pathways, but the molecular effects (e.g., inflammatory, metabolic, cardiovascular and immune pathways) are very complex (Contrepois et al., 2020). This makes it likely that exercise also activates (inflammatory) pathways that are not influenced by rIPC. Insight into this hypothesis will help to understand the mechanisms underlying the effects of exercise, but also to understand the overlap, or lack thereof, between exercise and rIPC in activation of inflammatory pathways. Understanding these mechanisms could help guide the translation of remote ischemic preconditioning to clinical application.

Whilst the majority of studies have focused on the impact of single exposure to rIPC, little work explored whether repeated bouts of preconditioning may be more effective than single application. Repeated exposure to preconditioning may enhance the efficacy of the preconditioning stimulus (Thijssen et al., 2016), possibly through combining the effects of the early-phase (1–2 h post-preconditioning) and late-phase protection (starting 12–72 h after the stimulus and persists for days to weeks) (Dirnagl et al., 2009). Therefore, the purpose of this study was to compare the impact of single and repeated handgrip exercise versus remote ischemic preconditioning on inflammatory biomarkers (using proteomics analysis) in patients with cerebral small vessel disease (cSVD). For this purpose, we selected cSVD since this patient group has an increased risk for cerebro-cardiovascular events (Rost and Etherton, 2020; Suzuyama et al., 2020) and shows increased susceptibility to injury following cerebral I/R (Leech et al., 2019). We performed proteomics analysis to compare the release of blood-borne markers of inflammation between exercise and rIPC. We hypothesised that both exercise and rIPC alter inflammatory biomarkers, with strong overlap between both stimuli, which would highlight the potential of exercise as a preconditioning stimulus.

Materials and methods

Study population and design

In this cross-over intervention study, we included 14 patients with clinical symptoms of cSVD (i.e., lacunar stroke or transient ischemic attack, cognitive disturbances, motor disturbances and/or depressive symptoms) with accompanying MRI markers of cSVD from the Radboud University Nijmegen Diffusion tensor and Magnetic resonance imaging Cohort (RUNDMC) study, which is a prospective study that investigated risk factors and clinical consequences of cSVD. The full protocol of the RUNDMC study has been described previously (van Norden et al., 2011). Patients were selected from the participants that completed the most recent follow-up measurements of the RUNDMC study in 2021. Inclusion criteria were: 1) age above 60 years, 2) mentally able to give informed consent. Exclusion criteria were: 1) upper extremity injury or edema contra-indicating handgrip exercise or rIPC, 2) mastectomy on both

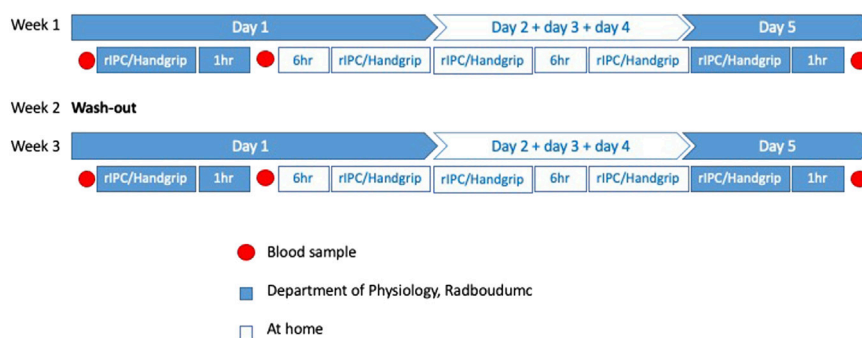


FIGURE 1

Overview study design. An overview of all the measurements in this cross-over study. rIPC = Remote ischemic preconditioning.

sides. All patients signed informed consent before participating. The study was approved by the ethical committee (CMO Arnhem-Nijmegen, registration number 2020-6781).

Intervention

All participants underwent the handgrip exercise and rIPC interventions with at least 9-day wash-out period in between and were randomly assigned to start with either handgrip or rIPC. Handgrip involved a protocol of 4 cycles of 5 min of handgrip exercise, divided by 5 min of rest. To determine the setting for the dynamometer maximal handgrip strength was assessed during screening. The dynamometer was thereafter set at 30% of maximal handgrip strength and grip and relaxation were alternated every second. rIPC was performed by inflating a blood pressure cuff around the upper arm at 200 mmHg (after making sure that no patient had a blood pressure that exceeded 180 mmHg). The rIPC intervention was performed in 4 cycles of 5 min with 5 min of reperfusion in between.

The first intervention cycle of both the handgrip and rIPC took place at the department of Physiology, Radboudumc, under supervision of a trained researcher (a research physician). This was always performed on Monday morning and timing was consistent for both interventions within each individual participant. Thereafter, participants independently continued the intervention at home twice daily for 4 days, with at least 6 hours of rest between the two interventions each day. On the morning of the fifth day (Friday), the participants again visited the Radboudumc to perform the last intervention, resulting in 9 cycles per participant over the course of 5 days.

Blood sampling

Venous blood was drawn at three time points in both intervention weeks: 1) baseline (directly before the start of the

first intervention), 2) 1 hour after completion of the first intervention, and 3) 1 hour after the last intervention on day 5, resulting in 6 samples per patient: rIPC baseline, rIPC single, rIPC repeated, Handgrip baseline, Handgrip single and Handgrip repeated. Timing of blood sampling was consistent for both intervention weeks within each individual patient. **Figure 1** represents an overview of the study design. At each time point 40 ml of venous blood was drawn and was collected in lithium-heparin tubes. The samples were centrifuged at 1.400 RCF for 10 min at 4°C. The plasma (~20 ml per sample) was collected and stored at -80°C.

Proteomics analysis

As a primary outcome the effect of handgrip and rIPC on inflammatory biomarkers was explored. For the proteomics analysis all 84 samples were analyzed simultaneously for 92 unique protein biomarkers using a pre-designed Proseek Multiplex® immunoassay panel; Inflammation (OLINK Proteomics, Uppsala, Sweden). The entire list of proteins can be found in **Supplementary Table S1**. Plasma was analyzed using Proximity Extension Assay (PEA), which has a high specificity and sensitivity (Assarsson et al., 2014). Validation data and limits of detection (LOD) are available at the manufacturer's webpage (<http://www.olink.com>). The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD037267.

The Olink assay was performed with a fixed proportion of the plate designated for control samples. Samples were randomly allocated to wells. The outcome data was normalized using standard Olink workflows to produce relative protein abundance on a log2 scale ('NPX'). Quality assessment was performed by examination of Olink internal controls. Following this step, one sample with poor-quality was removed. 37 proteins had more than 75% of the samples

TABLE 1 Baseline characteristic.

Table 1. Baseline characteristics

Total (N = 14)

Age (years)	70.4 (± 3.6)
Sex, male n (%)	8 (57.1%)
BMI (m/kg ²)	24.7 (± 3.9)
Blood pressure	
Systolic (mmHg)	142 (± 17)
Diastolic (mmHg)	81 (± 9)
Medical history, n (%)	
Atrial fibrillation	1 (7.1)
Hypertension	8 (57.1)
TIA	2 (14.3)
Stroke	2 (14.3)
Diabetes Mellitus	1 (7.1)
Alcohol use (yes), n (%)	11 (78.6)
Smoking status, n (%)	
Never	2 (14.3)
Former	11 (78.6)
Active	1 (7.1)
Max handgrip strength (kg)	32.4 (± 11.5)
MRI markers of cSVD	
White matter hyperintensity volume (ml)	5.1 [1.2, 43.5]
Lacunes (n)	0 [0,2]
Microbleeds (n)	0 [0,1]

Values are mean ($\pm$ standard deviation), number (percentage) or median [min, max].
Abbreviations: TIA, Transient ischemic attack; BMI, body mass index.

below the limit of detection and were excluded from the final analysis. For the remaining 55 proteins, the change in NPX for each protein between baseline and after single application was determined and compared between rIPC and handgrip. Additionally, the difference in NPX levels for each inflammatory biomarker after repeated preconditioning was analyzed for both rIPC and handgrip.

Statistical analysis

Analyses were performed using RStudio (TEAM, 2019) (Team, 2019). All outcomes were checked for normality. The differences in NPX for each protein were investigated with two linear mixed models (baseline *versus* single and baseline *versus* repeated). Linear mixed models were performed using the lmer function in the lme4 package (BATES et al., 2015) with a random intercept. The fixed variables were time (baseline, single and repeated) and treatment (rIPC and handgrip), including an interaction term for time*treatment. We used the Benjamini-Hochberg method to correct for multiple testing of the 55 inflammatory biomarkers. Adjusted *p*-values <0.05 were considered statistically significant, but because of the exploratory

nature of this study, we also reported all proteins that had unadjusted *p*-values <0.05.

Results

Fourteen cSVD patients participated. Their mean age was 70.4 ± 3.6 years and 57% was male. All baseline characteristics are presented in Table 1.

Single exercise and rIPC. The mixed model analysis showed a significant main effect in 9 (out of 55) proteins (Table 2; Figure 2) after single intervention. We found five proteins with a significant reduction over time (before versus after single exposure: Flt3L, FGF-21, TRANCE, MMP-1, and MCP-1) and five proteins with a significant difference between exercise and rIPC (TRANCE, CCL19, CCL11, CXCL5, and CD6). There were no significant interaction effects. After adjustment for multiple testing, we found significant decreases in FMS-related tyrosine kinase 3 ligand (Flt3L) and fibroblast growth factor 21 (FGF-21). The NPX for Flt3L reduced from 8.12 to 7.87 (16.2% reduction on a linear scale; adjusted *p*-value: 0.029; Figure 3) and FGF-21 from 3.83 to 3.25 (32.8% reduction on a linear scale; adjusted *p*-value: 0.029; Figure 4). We found no difference in changes in Flt3L and FGF-21 between handgrip and rIPC (adjusted *p*-value: 0.86 and 0.91, respectively).

Repeated exercise and rIPC. Analysis comparing baseline with repeated handgrip exercise or repeated rIPC largely supported the findings of the initial analysis, in that we found a significant change in 7 out of the 55 proteins (table 3). After adjustment for multiple testing, the decline in Flt3L after repeated handgrip and rIPC remained significant (NPX for Flt3L reduced from 8.12 to 7.84 (17.2% reduction on a linear scale; adjusted *p*-value: 0.029; Figure 3), with no difference between rIPC and handgrip (adjusted *p*-value: 0.98). We found no difference between the effect size of single versus repeated handgrip exercise or rIPC on Flt3L (adjusted *p*-value: 0.95) or on any other inflammatory biomarker.

Discussion

The purpose of our study was to examine the impact of single and repeated (handgrip) exercise and rIPC on inflammatory biomarkers through targeted proteomics analysis. Targeted proteomics revealed a significant reduction in various inflammatory proteins upon exposure to single handgrip or rIPC, with no further changes thereafter. Changes in inflammatory proteins following handgrip exercise and rIPC were in part overlapping, with most prominent reductions in the pro-inflammatory biomarkers Flt3L and FGF-21 after both interventions. Despite this overlap, some proteins demonstrated a distinct pattern between handgrip and rIPC. This suggests that, at least partly, there is overlap in inflammatory biomarkers in

TABLE 2 Levels of inflammatory biomarkers before and after single preconditioning.

	Handgrip		rIPC		<i>p</i> -values (unadjusted)			<i>p</i> -values (adjusted)		
	Baseline	Single	Baseline	Single	Treatment	Time	Time* <i>Treatment</i>	Treatment	Time	Time* <i>Treatment</i>
Flt3L	8.09 ± 0.41	7.82 ± 0.49	8.15 ± 0.46	7.91 ± 0.46	0.24	<0.001	0.72	0.86	0.029	0.79
FGF-21	3.83 ± 1.64	3.10 ± 1.27	3.82 ± 1.18	3.41 ± 1.03	0.33	<0.001	0.29	0.91	0.029	0.86
TRANCE	2.95 ± 0.66	2.71 ± 0.65	3.15 ± 0.64	2.92 ± 0.55	0.004	0.001	0.98	0.18	0.06	0.99
CCL19	8.18 ± 0.97	8.16 ± 1.05	8.34 ± 0.87	8.37 ± 0.99	0.02	0.94	0.76	0.57	0.97	0.97
MMP-1	12.51 ± 0.75	12.41 ± 0.85	12.70 ± 0.89	12.42 ± 0.96	0.24	0.03	0.30	0.86	0.71	0.87
MCP-1	11.52 ± 0.82	11.28 ± 0.92	11.55 ± 0.89	11.43 ± 0.89	0.26	0.03	0.51	0.86	0.73	0.96
CCL11	8.63 ± 0.14	8.60 ± 0.16	8.66 ± 0.18	8.67 ± 0.11	0.044	0.74	0.44	0.73	0.97	0.96
CXCL5	9.88 ± 0.80	9.85 ± 0.69	10.19 ± 0.74	10.01 ± 0.84	0.047	0.38	0.50	0.73	0.94	0.96
CD6	4.14 ± 0.32	4.08 ± 0.47	4.23 ± 0.30	4.24 ± 0.32	0.049	0.70	0.57	0.73	0.97	0.96

The bold values represent statistically significant results.

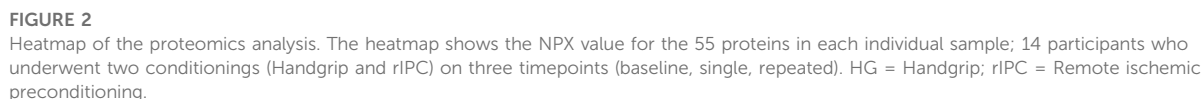
Values are presented as mean NPX (Normalised protein expression; relative protein abundance on a log2 scale) ± standard deviation for each protein on baseline and after single handgrip/rIPC., Linear mixed model factors: Treatment, Time and Time**Treatment* (unadjusted and adjusted).

response to (repeated) bouts of exercise and ischemia as preconditioning stimuli in patients with cSVD.

In line with our hypothesis, we found that a single bout of handgrip exercise has immediate effects on inflammatory biomarkers. This effect of a single bout of handgrip exercise was in line with rIPC, which supports the concept that exercise, similar to rIPC, has preconditioning effects (Thijssen et al., 2018). Interestingly, previous studies reported reduced efficacy of rIPC in older patients with endothelial dysfunction (Seeger et al., 2016; van den Munckhof et al., 2013). As patients with cSVD chronically suffer from damage to the smaller vessels of the brain due to endothelial dysfunction (Quick et al., 2021), we expected an attenuated or even absent efficacy of rIPC compared to exercise. Although we have not compared the effects in patients with cSVD with healthy controls and are therefore unable to provide insight into a potentially attenuated efficacy of a single bout of preconditioning, our results suggest that exercise and rIPC both have immediate effects on inflammatory protein expression in this population of older patients with cSVD. One possible reason for this observation is selection bias, consequently resulting in the inclusion of a relatively healthy, physically active, population of cSVD patients. In a small proof-of-concept study, physical activity indeed restored the efficacy of ischemic preconditioning in senescent rat hearts (Abete et al., 2000). Similarly, in previous work we have demonstrated that regular physical activity has beneficial effects, and may restore preconditioning effects in older individuals or patients with heart failure (Seeger et al.,

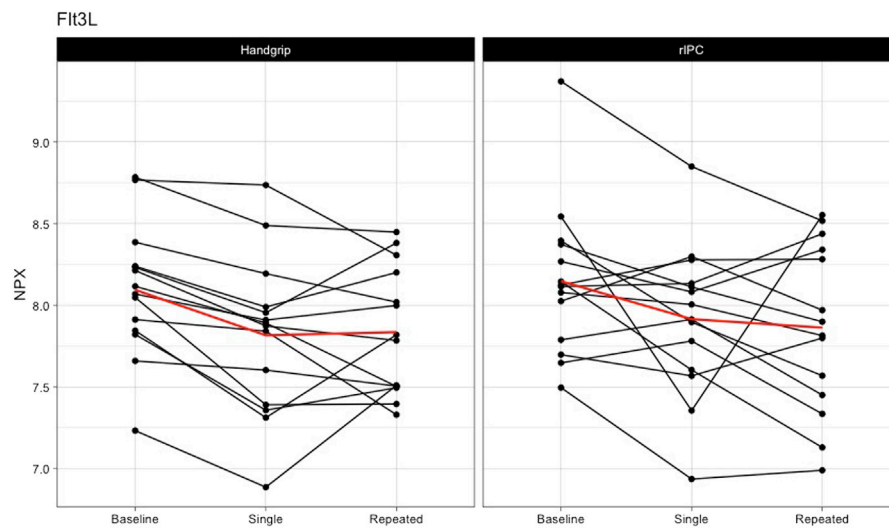
2016; Seeger et al., 2015; van den Munckhof et al., 2013). At least, our data indicate the ability of handgrip exercise and rIPC to influence inflammatory protein expression in a population of cSVD patients. Similarly, the inclusion of a relatively healthy group may also explain why repeated exposure does not further increase the magnitude of this response.

Findings from our explorative proteomics analysis identified reduction of Flt3L after handgrip exercise and rIPC. Flt3L may play a role in development of cardiovascular disease as upregulation of Flt3L leads to dendritic cell development, subsequently mediating blood pressure elevation and development of atherosclerosis (Zernecke, 2015; Lu et al., 2020). Similarly, a previous study found increased Flt3 expression following I/R-injury in a human neuroblastoma cell line (Dong et al., 2019), whilst silencing of Flt3 prevented I/R-injury in these cells, suggesting a mediating role for Flt3 during I/R-injury (Dong et al., 2019). Our study is the first to show a decrease in Flt3L after exercise and rIPC in cSVD patients. In contrast with our findings, previous studies reported an upregulation of Flt3L and the Flt3L-system after a single bout of whole-body exercise in healthy subjects and patients with multiple sclerosis (Bonsignore et al., 2002; Zaldivar et al., 2007; Deckx et al., 2015). These different outcomes may relate to the timing of the blood draws after exercise (immediate vs. 1 h post), study populations and type of exercise; whole-body cycling/running exercise vs. local handgrip exercise. Such differences in exercise volume and intensity, with

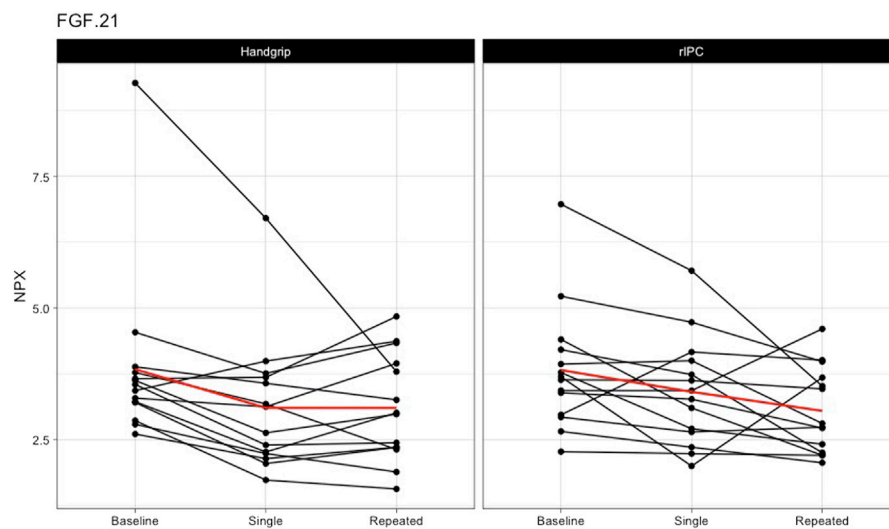


We also found a decrease in FGF-21 following handgrip exercise and rIPC. In line with Flt3L, FGF-21 may play a role in CVD. For example, FGF-21 infusion resulted in a lack of protection against I/R injury was observed in obese, but not healthy, rodent hearts (Patel et al., 2014). Similarly, other studies found that FGF-21 signaling is affected during cardiovascular

disease (Wu et al., 2020). This suggests an altered FGF-21 signaling during cardiovascular disease, which might also occur in sCVD patients. Studies examining the acute effects of exercise on FGF-21 have presented conflicting results, ranging from an increase of FGF-21 following whole-body exercise in healthy individuals (de Sousa et al., 2021; Domin et al., 2021; Jurimae et al., 2021), small FGF-21 changes in obese subjects and even lower FGF-21 in older men (Taniguchi et al.,

**FIGURE 3**

Changes in Flt3L after single and repeated preconditioning. The changes in the NPX of Flt3L (on the y-axis) between baseline and after single and repeated intervention (on the x-axis) for both the handgrip intervention (on the left) and the remote ischemic preconditioning (on the right). Each black line represents one participant and the red line is the mean for all the participants together. rIPC = Remote ischemic preconditioning. NPX = Normalised protein expression; Flt3L = FMS-related tyrosine kinase 3 ligand.

**FIGURE 4**

Changes in FGF-21 after single and repeated preconditioning. The changes in the NPX of FGF-21 (on the y-axis) between baseline and after single and repeated intervention (on the x-axis) for both the handgrip intervention (on the left) and the remote ischemic preconditioning (on the right). Each black line represents one participant and the red line is the mean for all the participants together. rIPC = Remote ischemic preconditioning. NPX = Normalised protein expression; FGF-21 = fibroblast growth factor 21.

2016; Khalafi et al., 2021). The local handgrip exercise bout performed in our study makes it difficult to compare our results with these previous studies that have typically adopted whole-body exercise. Taken together, our results suggest that changes

in Flt3L and FGF-21 may play a role in the effects of exercise and rIPC, but follow-up measurements would be required to better understand the link between these proteins and protection against I/R-injury.

TABLE 3 Levels of inflammatory biomarkers before and after repeated preconditioning.

	Handgrip		rIPC		<i>p</i> -values (unadjusted)			<i>p</i> -values (adjusted)		
	Baseline	Repeated	Baseline	Repeated	Treatment	Time	Time*Treatment	Treatment	Time	Time*Treatment
Flt3L	8.09 ± 0.41	7.84 ± 0.39	8.15 ± 0.46	7.86 ± 0.52	0.54	<0.001	0.85	0.98	0.029	0.99
FGF-21	3.83 ± 1.64	3.10 ± 1.01	3.82 ± 1.18	3.04 ± 0.82	0.87	0.002	0.93	0.99	0.13	0.99
CCL20	6.96 ± 0.70	6.66 ± 0.64	6.66 ± 0.63	6.99 ± 0.74	0.92	0.90	0.01	0.99	0.99	0.63
MCP-1	11.52 ± 0.82	11.31 ± 0.99	11.55 ± 0.89	11.40 ± 0.99	0.44	0.04	0.74	0.98	0.98	0.99
CXCL5	9.88 ± 0.80	9.80 ± 0.51	10.19 ± 0.74	10.10 ± 1.22	0.046	0.57	0.96	0.98	0.98	0.99
IPN-gamma	6.75 ± 1.03	6.76 ± 0.69	6.53 ± 0.89	6.39 ± 1.07	0.047	0.66	0.63	0.98	0.98	0.98
TRANCE	2.95 ± 0.66	2.85 ± 0.62	3.15 ± 0.64	2.96 ± 0.64	0.047	0.06	0.54	0.98	0.98	0.98

The bold values represent statistically significant results.

Values are presented as mean NPX (Normalised protein expression; relative protein abundance on a log2 scale) ± standard deviation for each protein on baseline and after repeated handgrip/rIPC. Linear mixed model factors: Treatment, Time and Time*Treatment (unadjusted and adjusted).

Despite the overlap in inflammatory proteins between handgrip exercise and rIPC, especially those with the strongest downregulation (i.e., Flt3L and FGF-21), we also found differences between interventions in five proteins (i.e., TRANCE, CCL19, CCL11, CXCL5, and CD6). This suggests that handgrip exercise has distinct effects on some proteins compared to rIPC. Although none of these differences remained significant after adjustment for multiple testing, these findings may suggest that the effects of handgrip exercise and rIPC, at least partly, work through distinct pathways.

Limitations. Our study also has some limitations. An obvious limitation is that we did not implement a control condition, which makes it difficult to directly relate the changes over time we found to either handgrip exercise or rIPC. The changes in inflammatory biomarkers may be attributed to the time of day, especially since inflammatory markers (e.g., FGF-21) fluctuate during the day (Bookout et al., 2013). However, no such evidence for a circadian rhythm of Flt3L has previously been reported. Future studies are warranted to explore whether handgrip exercise can also be translated to clinically meaningful effects in humans. Secondly, we cannot rule out that different exercise protocols could alter the magnitude of the effect compared to handgrip exercise. We adopted a handgrip exercise protocol, rather than whole-body exercise, because it allows for a more valid comparison with the local rIPC stimulus. Whole-body exercise has a wide variety of systemic effects (e.g., sympathetic nervous system, heart rate), which makes it difficult to compare exercise with rIPC. Moreover, handgrip exercise has potent clinical validity, since handgrip exercise will be easy to translate to clinical situation, including patient populations. A previous study showed that high-intensity cycling exercise protects against arterial dysfunction after prolonged ischemia, whereas moderate intensity exercise did not provide this same protective effect (Seeger et al., 2015). Similarly, the handgrip intervention performed in this study adopts an intermittent protocol of higher intensity exercise of the forearm muscles. One may question whether the magnitude of protection can be further enhanced by increasing the intensity and/or the volume of active muscle mass. In contrast, other studies showed that already moderate-intensity exercise can trigger preconditioning-related pathways (Thijssen et al., 2018). These studies suggest that presence of local (muscle) ischemia during exercise is not obligatory for the potential preconditioning effects. At least, our study is the first to demonstrate that even handgrip, activating a relatively small number of muscles, is sufficient to influence inflammatory protein expression that could potentially be signaling molecules associated with preconditioning.

In conclusion, single handgrip exercise performed by people with cSVD immediately attenuated plasma Flt3L and FGF-21, which seems comparable with the effects of rIPC. The reduction of Flt3L remains present across 4 days of repeated handgrip

exercise. Flt3L and FGF-21 may both serve as biomarkers for these preconditioning interventions. Our data suggest that there is partial overlap in the inflammatory pathways activated by a single and repeated (4-day) exposure to local handgrip and upper arm ischemic preconditioning stimuli in patients with cSVD.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The studies involving human participants were reviewed and approved by the CMO Arnhem-Nijmegen. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TL was the coordinating investigator and was involved in protocol development, gaining ethical approval and obtaining funding together with YS, F-EL, and DT. LU and IH performed

most of the key measurements described in this manuscript. Statistical approval and writing of the manuscript was done by TL. All authors critically revised the manuscript and approved the final version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2022.1026711/full#supplementary-material>

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Study on potential markers for diagnosis of renal cell carcinoma by serum untargeted metabolomics based on UPLC-MS/MS

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Objective: Renal cell carcinoma (RCC) is the most common malignancy of the kidney. However, there is no reliable biomarker with high sensitivity and specificity for diagnosis and differential diagnosis. This study aims to analyze serum metabolite profile of patients with RCC and screen for potential diagnostic biomarkers.

Methods: Forty-five healthy controls (HC), 40 patients with benign kidney tumor (BKT) and 46 patients with RCC were enrolled in this study. Serum metabolites were detected by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), and then subjected to multivariate statistical analysis, metabolic pathway analysis and diagnostic performance evaluation.

Results: The changes of glycerophospholipid metabolism, phosphatidylinositol signaling system, glycerolipid metabolism, D-glutamine and D-glutamate metabolism, galactose metabolism, and folate biosynthesis were observed in RCC group. Two hundred and forty differential metabolites were screened between RCC and HC groups, and 64 differential metabolites were screened between RCC and BKT groups. Among them, 4 differential metabolites, including 3-β-D-Galactosyl-sn-glycerol, 7,8-Dihydroneopterin, lysophosphatidylcholine (LPC) 19:2, and γ-Aminobutyryl-lysine (an amino acid metabolite), were of high clinical value not only in the diagnosis of RCC (RCC group vs. HC group; AUC = 0.990, 0.916, 0.909, and 0.962; Sensitivity = 97.73%, 97.73%, 93.18%, and 86.36%; Specificity = 100.00%, 73.33%, 80.00%, and 95.56%), but also in the differential diagnosis of benign and malignant kidney tumors (RCC group vs. BKT group; AUC = 0.989, 0.941, 0.845 and 0.981; Sensitivity = 93.33%, 93.33%, 77.27% and 93.33%; Specificity = 100.00%, 84.21%, 78.38% and 92.11%).

Conclusion: The occurrence of RCC may involve changes in multiple metabolic pathways. The 3-β-D-Galactosyl-sn-glycerol, 7,8-Dihydroneopterin, LPC 19:

2 and γ -Aminobutyryl-lysine may be potential biomarkers for the diagnosis or differential diagnosis of RCC.

KEYWORDS

renal cell carcinoma, benign kidney tumor, untargeted metabolomics, lipid metabolism, amino acid metabolism, potential biomarkers

1 Introduction

Kidney carcinoma is one of the most common malignancies of the urinary system, and renal cell carcinoma (RCC) accounted for more than 85% of kidney carcinoma (Nabi et al., 2018). According to the GLOBOCAN 2020 reports, there are more than 430,000 new cases of RCC and nearly 180,000 new deaths annually worldwide (Sung et al., 2021). In the early stages of RCC, patients are usually asymptomatic, but RCC patients' conditions usually progress to the intermediate or advanced stage, when they present with the typical classic clinical triad of gross hematuria, flank pain, and palpable abdominal mass (Gray and Harris, 2019). Although modern imaging techniques are widely available to detect most kidney masses, 20%–30% of patients with RCC have metastases at diagnosis (Petejova and Martinek, 2016). Moreover, most patients with RCC are almost always detected by brightness-mode ultrasound, computed tomography, or magnetic resonance imaging when screening for other diseases not related to the kidney (Capitanio et al., 2019). Therefore, early diagnosis of RCC is essential to improve treatment outcomes and reduce mortality in patients with RCC.

The commonly used kidney function indicators in clinic, such as urea, creatinine (Cr), uric acid (UA), cystatin C (CysC), complement C1q (C1q), neutrophil gelatinase-associated lipocalin (NGAL), and estimated glomerular filtration rate (eGFR), can evaluate the kidney function of patients with RCC, but cannot indicate kidney tumors. To date, few reliable tumor-specific markers are available for clinical use in kidney carcinoma. Although kidney biopsy is the "gold standard" for the diagnosis and identification of kidney tumors, its clinical application is often limited because of its invasive nature and the possible risk of needle tract implantation and metastasis.

In recent years, metabolomics technology has been widely used to screen for potential biomarkers of diseases, especially tumors, and to explore the occurrence and development of diseases through the metabolic pathways of substances *in vivo* (Wang et al., 2021a). Some early studies investigated the urinary metabolomics of patients with RCC using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and found a metabolite panel consisting of cortolone, testosterone and L-2-aminoadipate adenylate, and that combined detection of L-3-hydroxykynurenine, 1,7-dimethylguanosine and Tetrahydroaldosterone-3-glucuronide may be used for distinguishing RCC from benign kidney tumor (BKT) (Liu et al., 2019a; Zhang et al., 2020a). Another study confirmed

that analysis using blood samples could more accurately reflect the metabolic changes in tumor tissues than using urine samples (Ganti et al., 2012). Additionally, some scholars found by plasma metabolomics (Liu et al., 2020) that combined detection of diaminopimelic acid, 12,13-DHOME, 5-L-glutamyl-L-alanine, PC (38:4), 4,8-dimethylnonanoyl carnitine and cholesteryl 11-hydroperoxy-eicosatetraenoate could be used for the differential diagnosis of benign and malignant kidney tumors. However, these screened potential biomarkers had low diagnostic performance, sensitivity and specificity for identifying RCC. Furthermore, it has been shown that the metabolomic analysis of serum samples is more reliable than plasma samples (Yu et al., 2011; Sotelo-Orozco et al., 2021).

Lipids are important components in serum, and a study found that disturbed lipid metabolism was strongly associated with disease severity of RCC and may be a risk factor for RCC development (Li et al., 2020). Based on the above theories and research findings, we propose a hypothesis: compared with healthy subjects or BKT, RCC patients have abnormal metabolic pathways of some substances. In this study, we analyzed the serum metabolomic profile of patients with RCC and BKT by untargeted metabolomics based on UPLC-MS/MS. We also investigated the relationship of metabolites with common indicators for kidney function and lipid indicators. The results of this study may provide new insights into the pathogenesis of RCC and provide evidence for screening new potential biomarkers for its diagnosis and differential diagnosis.

2 Materials and methods

2.1 Subjects

Between March 2021 and March 2022, 86 patients with kidney tumors, including 46 cases with RCC and 40 cases with BKT, who were admitted to the Department of Urology, Mianyang Central Hospital were enrolled in the study.

Inclusion criteria: 1) age ≥ 18 years; 2) All patients were diagnosed as RCC or BKT by pathological biopsy after finding abnormalities in computed tomography (CT) or magnetic resonance imaging (MRI) examination. Exclusion criteria: 1) patients with failure of blood sample collection; 2) women in pregnancy or lactation; 3) patients complicated with other metabolic diseases and tumors; 4) patients with RCC who underwent treatment with radiotherapy or immunotherapy before enrollment; 5) patients who had undergone

nephrectomy. In addition, all diagnoses were confirmed by senior clinicians according to clinical diagnostic criteria.

Forty-five healthy subjects who underwent physical examination during the same period were enrolled as healthy controls (HC). Inclusion criteria: 1) age ≥ 18 years; 2) healthy volunteers with normal examination indexes during the same period; 3) subjects who did not take drugs that may affect kidney function within the last month. Exclusion criteria: 1) patients with failure of blood sample collection; 2) women in pregnancy or lactation.

Detailed clinical information of the subjects is shown in **Supplementary Table S1**. This study was approved by the Medical Ethics Committee of Mianyang Central Hospital (P2020030), and all patients signed the informed consent.

2.2 Sample collection

After fasting overnight, all study subjects were subjected to blood collection (approximately 5.0 ml of venous blood each) from 8:00–10:00 a.m. The SSTTM II Advance vacuum blood collection tube (BD Vacutainer®, United States) containing separation gel and coagulant was used. After centrifugation at 3,000 *g* for 10 min, the serum was collected and equally divided into two parts. One part was tested for kidney function and lipid indicators within 2 h. The other part was stored at -80°C for metabolomics analysis.

2.3 Detection of common kidney function and lipid indicators

The indicators for kidney function and lipid indicators were measured on LST008 automatic biochemical analyzer (Hitachi, Japan). The C1q kit was provided by Shanghai Beika Biochemical Reagent Co., Ltd. (Shanghai, China). The kits for other indicators were provided by Sichuan Maker Biotechnology Co., Ltd. (Sichuan, China).

2.4 Ultra-high performance liquid chromatography-tandem mass spectrometry analysis

2.4.1 Sample preparation and analysis

A mixture of 190 μl of serum sample, 10 μl of internal standard (10 $\mu\text{g}/\text{ml}$, clenbuterol and chloramphenicol mixture) and 800 μl of methanol-acetonitrile (*v/v* = 1:1) solution was sonicated at 4°C for 10 min, then the mixture was incubated at -20°C for 1 h, followed by centrifugation at 13,000 *g* for 15 min at 4°C to obtain the supernatant. The supernatant was filtered by 0.22 μm microporous membrane. Finally, 3 μl of the filtrate-solution was transferred by an autosampler and injected into the

UPLC-MS/MS system for metabolomic analysis. In addition, 10 μl of serum from each sample was mixed as a quality control (QC) sample. QC samples were processed in the same way as the study samples. Serum metabolomics analysis was performed with Agilent® 1290 Infinity II UPLC system (Agilent Technologies Inc., United States) and AB Sciex® Triple TOF 5600+ mass spectrometer system (AB Sciex, United States). UPLC-MS/MS analytical conditions used previous methods of our lab (Xu et al., 2021). In addition, QC samples were tested after every 10 samples in the analysis sequence to evaluate the reliability of the large-scale metabolomics analysis and the stability of the instrument (Dudka et al., 2020; Zhu et al., 2021).

2.4.2 Metabolite identification and analysis

Raw data were collected using Analyst TF 1.7 software (AB Sciex, United States of America). The metabolomics data were subjected to a series of processing workflow, including peak picking, quality assessment, missing value imputation, normalization, transformation and scaling. The details were as follows: 1) the XCMS algorithm was applied to peak extraction based on the One-MAP platform (<http://www.5omics.com>) provided by Dalian Dashuo Information Technology Co. (Dalian, China). 2) quality analysis of the data based on the stability of the QC samples. The percentage of relative standard deviation (RSD) of metabolic mass spectrometry features in QC samples that were less than 50% should exceed 80%. Calibration of QC was performed using the statTarget analysis (Luan et al., 2018). 3) the 80% rule was used to exclude metabolic features with more than 20% of non-zero values in any category of samples in the metabolic features. Missing values were filled with the smallest value in the variable. 4) normalization of data was performed using the MetNormalizer method of QC-based support vector regression analysis and the internal standards was used for checking the stability of the instrument performance. 5) the feature variables were processed by auto scaling in principal component analysis (PCA) and partial least squares analysis (PLS-DA). This was to eliminate differences in the order of magnitude of the concentration of different metabolites. The characteristic peaks of metabolites were carried out by molecular weight error (<20 ppm), signal-to-noise ratio and summation ions to predict their molecular formulae. Metabolite identification was annotated by scoring each peak based on matches to accurate masses, retention times, and MS/MS fragmentation to the standard compounds databases (containing information of 1,550 metabolic standards), and custom databases including METLIN (<http://metlin.scripps.edu/>), Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.kegg.jp/kegg/pathway.html>), LipidMaps (<https://www.lipidmaps.org/>), Human Metabolome Database (HMDB) (<https://hmdb.ca/>), MassBank (<https://massbank.eu/>), and PubChem Database (<https://pubchem.ncbi.nlm.nih.gov/>), parameters setting: $\Delta m/z$ (MS1) $\leq 0.01000\text{Da}$; $\Delta m/z$ (MS2) $\leq 0.05000\text{Da}$; MS2 Score Method = Forward; Reference Noise of

TABLE 1 Common renal function and lipid indicators of the study subjects.

Group	HC (<i>n</i> = 45)	BKT (<i>n</i> = 40)	RCC (<i>n</i> = 46)	χ^2/F , <i>P</i>
UA ($\mu\text{mol/L}$)	332.0 (269.3, 371.7)	282.4 (229.0, 350.8)	333.3 (236.4, 417.1)	4.354 ^a , 0.113
C1q (mg/L)	207.0 (175.5, 235.5)	193.5 (168.4, 229.0)	191.5 (166.6, 221.8)	1.366 ^a , 0.505
NGAL ($\mu\text{g/L}$)	95.0 (78.5, 113.0)	124.0 (85.3, 180.0) *	130.1 (93.0, 164.8) *	13.799 ^a , 0.001
Urea (mmol/L)	5.10 (4.13, 5.64)	5.37 (4.07, 6.60)	5.43 (4.46, 6.59)	4.215 ^a , 0.122
Cr ($\mu\text{mol/L}$)	66.0 (53.4, 78.0)	65.6 (54.2, 87.6)	80.1 (61.7, 105.7) *	11.775 ^a , 0.003
CysC (mg/L)	0.91 (0.79, 1.00)	0.92 (0.76, 1.18)	1.07 (0.90, 1.26) *	13.391 ^a , 0.001
eGFR (ml/min/1.73m ²)	88.59 $\pm$ 12.29	85.75 $\pm$ 24.14	73.09 $\pm$ 20.85 *	8.057 ^b , 0.001
TC (mmol/L)	4.58 $\pm$ 0.59	4.89 $\pm$ 1.11	5.17 $\pm$ 1.37 *	3.297 ^b , 0.040
TG (mmol/L)	0.98 (0.87, 1.19)	1.29 (0.99, 2.37) *	1.39 (0.98, 1.97) *	13.261 ^a , 0.001
HDL-C (mmol/L)	1.38 (1.14, 1.55)	1.31 (1.17, 1.64)	1.22 (1.05, 1.45)	2.717 ^a , 0.257
LDL-C (mmol/L)	2.60 (2.27, 2.96)	3.13 (2.26, 3.56) *	2.99 (2.32, 4.31) *	9.355 ^a , 0.009
Apo-A1 (g/L)	1.55 $\pm$ 0.21	1.53 $\pm$ 0.31	1.48 $\pm$ 0.27	0.834 ^b , 0.437
Apo-B (g/L)	0.81 $\pm$ 0.14	0.93 $\pm$ 0.24*	1.00 $\pm$ 0.30*	7.263 ^b , 0.001

Note: n, case; a is the χ^2 value; b is the value of F; HC, healthy control; BKT, benign kidney tumor; RCC, renal cell carcinoma; Cr, creatinine; UA, uric acid; CysC, cystatin C; C1q, complement C1q; NGAL, neutrophil gelatinase-associated lipocalin; eGFR, estimated glomerular filtration rate; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; Apo-A1, apolipoprotein A1; Apo-B, apolipoprotein B. Compared with HC, group, * $p < 0.05$. Compared with BKT, group, $p < 0.05$.

Unknown MS2 to remove = 1.000000; Reference Noie of Standard MS2 to remove = 200.000000; the number of near fragments at least to merge peaks cluster = 2; investigate the maximum number of fragments = 2. According to the formal definition of metabolite annotation and identification specified by the Chemical Analysis Working Group of the Metabolomics Standards Initiative (MSI), the metabolites determined in this study would be considered as putative identification (levels 2) (Viant et al., 2017). Untargeted metabolomics does not need a reference standard, but structural information was obtained using MS/MS data and combined with mass-to-charge ratio (m/z) and retention time. Metabolic pathways of the differential metabolites were analyzed by the Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/kegg>) and MetaboAnalyst database exploration (<http://www.metaboanalyst.ca/>).

2.5 Statistical analysis

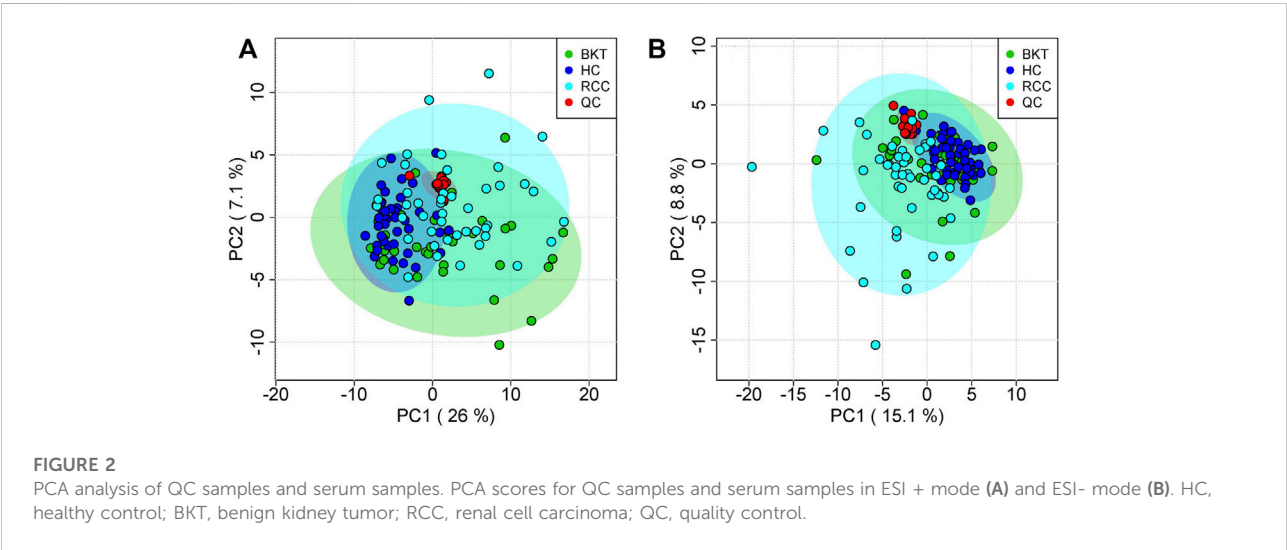
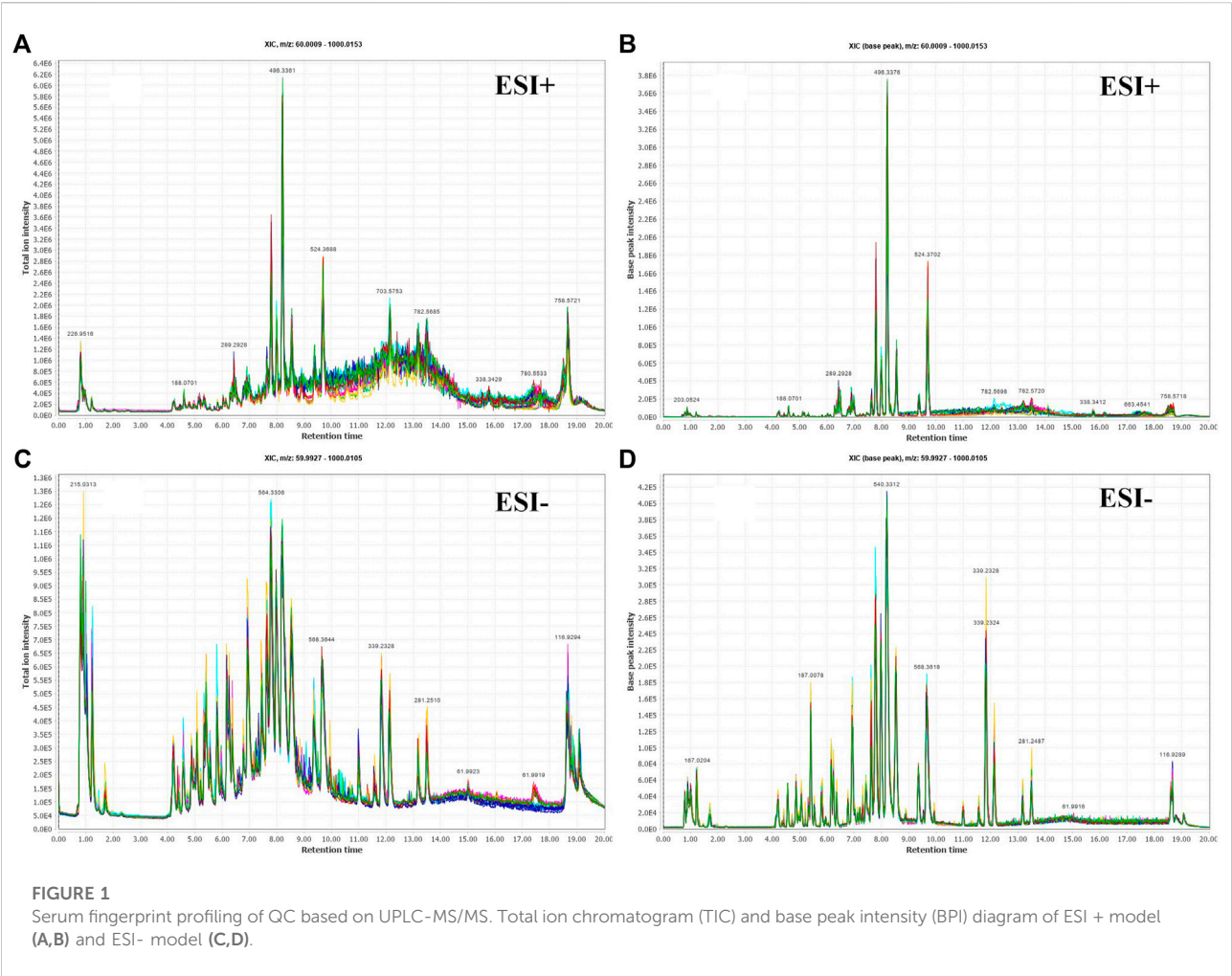
Statistical analysis was performed using SPSS 26.0 software (International Business Machines Corp., United States). Normally distributed data were expressed as mean $\pm$ standard deviation, and compared with one-way ANOVA followed by LSD test. Non-normally distributed data were expressed as median (interquartile range), and analyzed with Kruskal–Wallis H test. Count data were compared using chi-square test. Spearman correlation was used for correlation analysis. PCA and PLS-DA were performed using SIMCA

14.1 software (Umetrics AB, Umea, Sweden). The validity of the PLS-DA model was examined using a random permutation test (100 times). Receiver operating characteristic curve (ROC) analysis was used to evaluate the diagnostic performance of the differential metabolites. When AUC = 0.50–0.59, 0.60–0.69, 0.70–0.79, 0.80–0.89, or ≥ 0.90 , it means that the diagnostic performance is fail, poor, fair, good, or excellent, respectively (Nahm, 2022). $p < 0.05$ indicated that the difference was statistically significant.

3 Results

3.1 Kidney function and lipid indicators

As shown in Table 1, the kidney function and lipid indicators of NGAL, Cr, CysC, eGFR, triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and apolipoprotein B (Apo-B) were statistically significant among the HC, RCC and BKT groups (all $p < 0.05$). Pairwise comparison analysis showed that compared with the HC group, NGAL ($z = 3.542$, $p < 0.001$), Cr ($z = 3.352$, $p = 0.001$), CysC ($z = 3.511$, $p < 0.001$), TG ($z = 3.521$, $p < 0.001$), LDL-C ($z = 2.650$, $p = 0.008$) and Apo-B ($t = 0.368$, $p < 0.001$) were significantly increased in the RCC group, whereas eGFR ($t = 3.443$, $p = 0.001$) was significantly decreased in the RCC group. NGAL ($z = 2.705$, $p = 0.007$), TG ($z = 2.420$, $p = 0.016$), LDL-C ($z = 2.650$, $p = 0.008$) and Apo-B ($t = 0.240$, $p = 0.018$) were significantly increased in the BKT group than in the HC group. Compared with the BKT group, Cr



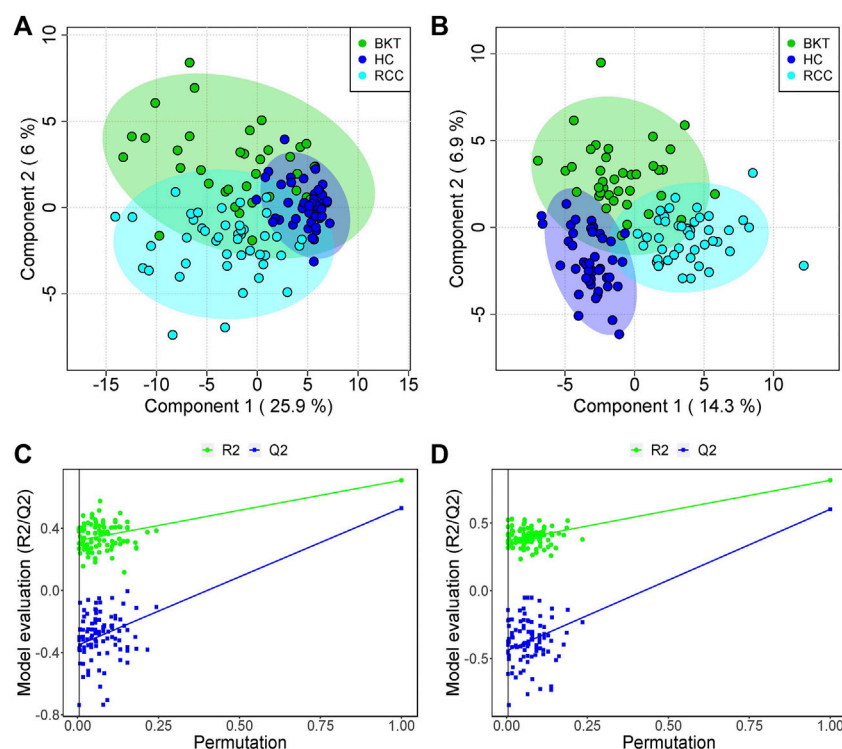


FIGURE 3

PLS-DA analysis and permutation test. PCA scores for the HC, BKT, and RCC groups in the ESI+ model (A) and ESI- model (B). Permutation test in the ESI+ model (C) and ESI- model (D) among HC group, BKT group, and RCC group. The criterion for PLS-DA not overfitting is that the R2 and Q2 values of all alignments on the left side are lower than the corresponding original points on the right side. The regression line of point Q2 intersects the horizontal coordinates or is less than 0. R2X and R2Y denote the degree of explanation of the PLS-DA model for the categorical variables X and Y, respectively. Q2 denotes the predictiveness of the PLS-DA model. HC, healthy control; BKT, benign kidney tumor; RCC, renal cell carcinoma.

($z = 2.250$, $p = 0.024$) and CysC ($z = 2.561$, $p = 0.010$) were significantly increased in the RCC group, while eGFR ($t = 2.631$, $p = 0.010$) was significantly decreased in the RCC group. These results showed that kidney function and lipid indicators were abnormal in patients with RCC to different degrees.

3.2 Serum metabolic profiling

The 131 serum samples and 14 QC samples were analyzed by UPLC-MS/MS in positive ion (ESI+) and negative ion (ESI-) modes. The results were analyzed by PCA analysis. The PCA score plots, total ion chromatogram (TIC) and base peak intensity (BPI) diagram of QC samples indicated that the metabolomics dataset of this study had good stability and reproducibility (Figure 1), while those of the serum samples showed that the serum components of the HC, BKT and RCC groups were not effectively separated (Figures 2A,B). Further analysis with PLS-DA resulted in effective separation (Figures 3A,B). To avoid overfitting of the PLS-DA model, 100 random permutation tests were performed. The results showed that for

the HC, BKT and RCC groups, the degree of explanation of X (R2X), the degree of explanation of Y (R2Y) and the predictability of the model (Q2) were 0.32, 0.76, and 0.54, respectively, in the ESI+ model, and were 0.37, 0.82 and 0.60, respectively, in the ESI- model (Figures 3C,D). These results reveals that the PLS-DA model has high goodness-of-fit and predictive power, and can effectively separate serum metabolite profiles of the HC, BKT, and RCC groups.

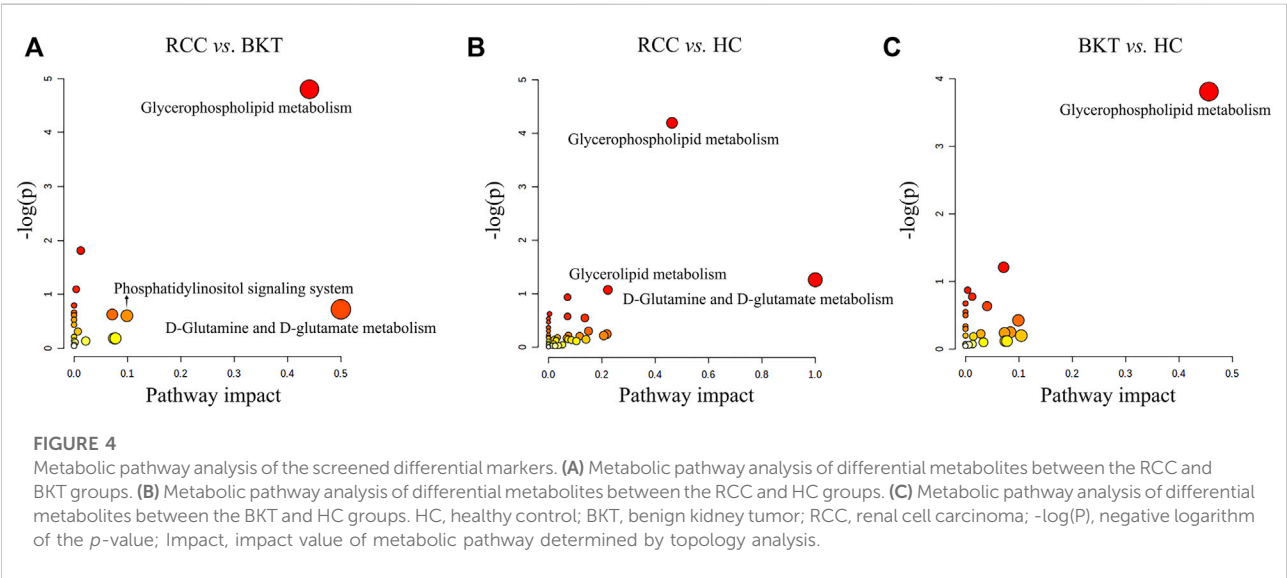
3.3 Screening of markers and analysis of their metabolic pathways

The results of MS/MS experiments were subjected to data filtering, which yielded 6938 and 6868 peaks in the ESI+ and ESI- modes, respectively. Qualitative analysis of these peaks using the standard compounds databases, customized databases, and integrated databases showed that a total of 910 metabolites were identified in ESI+ mode and 917 metabolites were identified in ESI- mode. According to the criteria of variable importance for the projection >1, fold change >1.2 or <5/6, and

TABLE 2 Metabolic pathways significantly altered between groups.

Group	Pathway	−log(<i>P</i>)	Impact	Hits	Compounds
RCC vs. BKT	Glycerophospholipid metabolism	11.10	0.44	8	PE 21:5, PC 34:2, LPC 19:2, PA 23:2, PS 18:3, PG 14:0, LPG 18:3 and PI 18:4
	Phosphatidylinositol signaling system	1.39	0.10	2	PI 18:4 and PA 23:2
	D-Glutamine and D-glutamate metabolism	1.67	0.50	1	Glutamic acid
RCC vs. HC	Glycerophospholipid metabolism	9.65	0.46	10	PE 21:5, PC (34:2), LPC 19:2, Choline, PA 23:2, PS 18:3, LPE 16:0, LPI 18:5, LPG 24:0 and PI 16:0
	Glycerolipid metabolism	8.48	0.22	3	PA 23:2, MG (18:1) and MGDG 22:3
	D-Glutamine and D-glutamate metabolism	2.90	1.00	1	Glutamic acid
BKT vs. HC	Glycerophospholipid metabolism	8.77	0.46	8	PE 22:1, PC 34:2, LPC 19:2, PA 23:3, PS 23:4, LPE 20:1, PG 14:0 and PI 16:0

Note: HC, healthy control; BKT, benign kidney tumor; RCC, renal cell carcinoma; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; MG, monoglyceride; MGDG, monogalactosyldiglyceride. −log(*P*), negative logarithm of the *p*-value of the statistic; Impact, impact value of metabolic pathway determined by topology analysis; Hits, the number of differential metabolites matching the pathway.



$p < 0.05$ (Zhang et al., 2020b; Lu et al., 2021), 240 differential metabolites between the RCC and HC groups, 175 differential metabolites between the BKT and HC groups, and 64 differential metabolites between the BKT and RCC groups were identified. Pathway enrichment was used to analyze the metabolic pathways in which these differential metabolites may be involved. The results showed that among the 64 differential metabolites between the BKT and RCC groups, 8 differential metabolites were enriched in the glycerophospholipid metabolism pathway, 2 differential metabolites were enriched in the phosphatidylinositol signaling system, and 1 differential metabolite was enriched in the D-glutamine and D-glutamate metabolism pathway. Among the 240 differential metabolites between the RCC and HC groups, 10 differential metabolites were enriched in the glycerophospholipid metabolism pathway,

3 differential metabolites were enriched in the glycerolipid metabolism pathway, and 1 differential metabolite were enriched in the D-glutamine and D-glutamate metabolism pathway. These results indicate that the main metabolic pathways in patients with RCC may be lipid metabolism and amino acid metabolic pathways (Table 2 and Figure 4). Details of metabolic pathways matched to specific metabolites are shown in Supplementary Tables S2–S4.

3.4 Diagnostic performance of the screened markers

With reference to the literature (Nahm, 2022), we selected differential metabolites with good or excellent diagnostic

TABLE 3 Diagnostic performance evaluation of candidate biomarkers.

Biomarker	Scan mode	Rt (s)	m/z	Adducts	AUC (95%CI)	Se (%)	Sp (%)	YI
RCC vs. BKT								
3-β-D-Galactosyl-sn-glycerol	—	—	—	—	0.989 (0.936–1.000)	93.33	100.00	0.933
γ-Aminobutyryl-lysine	—	—	—	—	0.981 (0.924–0.999)	93.33	92.11	0.854
7,8-Dihydroneopterin	—	—	—	—	0.941 (0.867–0.981)	93.33	84.21	0.775
LPC 19:2	—	—	—	—	0.845 (0.748–0.916)	77.27	78.38	0.557
RCC vs. HC								
3-β-D-Galactosyl-sn-glycerol	ESI-	481.035	253.083	M-H	0.990 (0.942–1.000)	97.73	100.00	0.977
γ-Aminobutyryl-lysine	ESI-	545.151	230.155	M-H	0.962 (0.898–0.991)	86.36	95.56	0.819
7,8-Dihydroneopterin	ESI-	481.173	254.086	M-H	0.916 (0.838–0.964)	97.73	73.33	0.711
LPC 19:2	ESI+	440.903	556.346	M + Na	0.909 (0.829–0.959)	93.18	80.00	0.732
6-Keto-prostaglandin F1α	ESI+	668.909	371.242	M + H	0.897 (0.815–0.952)	88.64	82.22	0.709
17α,21-Dihydroxypregnenolone	ESI+	698.951	349.237	M + H	0.830 (0.735–0.901)	77.27	77.78	0.551
γ-Glutamylphenylalanine	ESI-	283.958	293.113	M-H	0.823 (0.728–0.896)	77.27	80.00	0.573
RCC vs (BKT + HC)								
3-β-D-Galactosyl-sn-glycerol	—	—	—	—	0.990 (0.953–0.999)	97.78	97.56	0.953
γ-Aminobutyryl-lysine	—	—	—	—	0.971 (0.924–0.992)	86.67	95.12	0.818
7,8-Dihydroneopterin	—	—	—	—	0.928 (0.868–0.966)	95.56	78.05	0.736
LPC 19:2	—	—	—	—	0.879 (0.808–0.930)	93.18	71.60	0.648
(RCC + BKT) vs HC								
3-β-D-Galactosyl-sn-glycerol	—	—	—	—	0.763 (0.679–0.834)	65.06	93.18	0.582
γ-Aminobutyryl-lysine	—	—	—	—	0.692 (0.604–0.771)	48.19	97.73	0.459
7,8-Dihydroneopterin	—	—	—	—	0.691 (0.602–0.770)	63.86	72.73	0.366
LPC 19:2	—	—	—	—	0.803 (0.722–0.869)	81.48	70.45	0.519

Note: HC, healthy control; BKT, benign kidney tumor; RCC, renal cell carcinoma; Rt, retention time; m/z, mass-to-charge ratio; LPC, lysophosphaticholine; AUC, area under ROC, curve; 95%CI, 95%confidence interval; Se, sensitivity; Sp, specificity; YI, youden index.

performance ($AUC \geq 0.80$) as potential markers for diagnosis. The diagnostic performance of the candidate markers was evaluated by ROC analysis, and the results showed that among the 64 differential metabolites between the BKT and RCC groups, there were 4 differential metabolites with area under the ROC curve ($AUC \geq 0.80$), namely 3-β-D-Galactosyl-sn-glycerol, γ-Aminobutyryl-lysine, 7,8-Dihydroneopterin, and lysophosphatidylcholine (LPC) 19:2 (Table 3). Among the 240 differential metabolites between the HC and RCC groups, there were 7 differential metabolites with $AUC \geq 0.80$, namely 3-β-D-Galactosyl-sn-glycerol, γ-Aminobutyryl-lysine, 7,8-Dihydroneopterin, LPC 19:2, 6-Keto-prostaglandin F1α, 17α,21-Dihydroxypregnenolone, and γ-Glutamylphenylalanine (Figure 5). The m/z of parent ion and product ion of the 7 differential metabolites is shown in Supplementary Table S5. Serum levels of 6-Keto-prostaglandin F1α, 17α,21-Dihydroxypregnenolone and γ-Glutamylphenylalanine were significantly higher in the RCC group compared with the HC group (Table 4). Compared with the HC and BKT groups, serum levels of 3-β-D-Galactosyl-sn-glycerol, γ-Aminobutyryl-lysine, 7,8-Dihydroneopterin and LPC 19:2 were significantly lower in the RCC group, and the normalized peak intensities of

these four metabolites were shown in Figure 6. These results suggest that 3-β-D-Galactosyl-sn-glycerol, γ-Aminobutyryl-lysine, 7,8-Dihydroneopterin and LPC 19:2 could distinguish patients with RCC from patients with BKT and healthy subjects.

3.5 Correlation analysis of markers with common kidney function and lipid indicators

Spearman correlation was used to analyze the correlation of the four metabolites with common kidney function and lipid indicators (Table 5). The results showed that 3-β-D-Galactosyl-sn-glycerol was negatively correlated with Cr ($r = -0.268$, $p = 0.002$), CysC ($r = -0.268$, $p = 0.002$) and NCAL ($r = -0.176$, $p = 0.047$), while positively correlated with eGFR ($r = 0.268$, $p = 0.002$). 7,8-Dihydroneopterin was negatively correlated with Cr ($r = -0.214$, $p = 0.016$), CysC ($r = -0.193$, $p = 0.030$) and NGAL ($r = -0.202$, $p = 0.022$), whereas positively correlated with eGFR ($r = 0.193$, $p = 0.030$). LPC 19:2 had negative correlation with urea ($r = -0.181$, $p = 0.042$), Cr ($r = -0.222$, $p = 0.012$), CysC ($r = -0.202$, $p = 0.023$) and TG ($r = -0.183$, $p = 0.040$), but positively

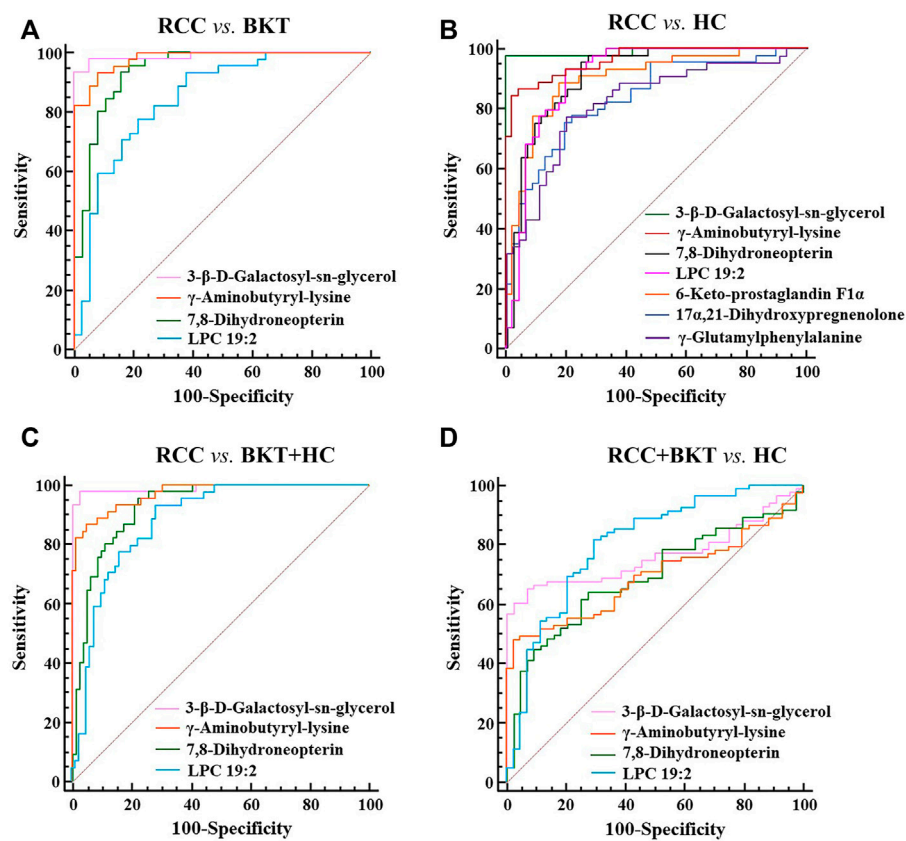
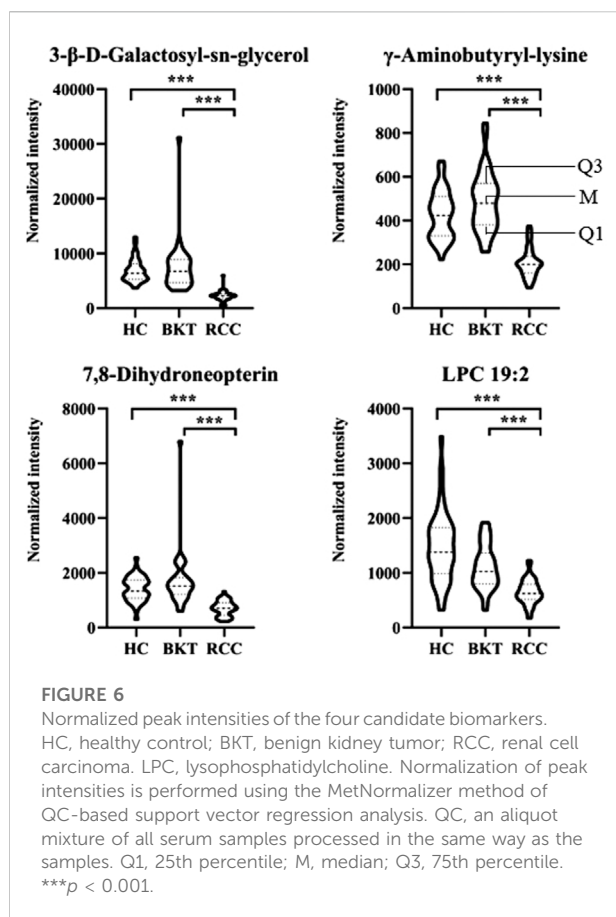


FIGURE 5 ROC analysis of candidate biomarkers. (A) ROC analysis of candidate biomarkers for discriminating RCC from BKT. (B) ROC analysis of candidate biomarkers for discriminating RCC from HC. (C) ROC analysis of candidate biomarkers for discriminating RCC from BKT and HC. (D) ROC analysis of candidate biomarkers for discriminating RCC and BKT from HC. HC, healthy control; BKT, benign kidney tumor; RCC, renal cell carcinoma. LPC, lysophosphatidylcholine.

TABLE 4 Significantly differential metabolites of RCC vs. BKT groups and RCC vs. HC groups.

Group	FC	VIP	P	Trend	Pathway
RCC vs. BKT					
3-β-D-Galactosyl-sn-glycerol	0.296	3.723	<0.001	↓	Galactose metabolism
γ-Aminobutyryl-lysine	0.425	4.769	<0.001	↓	Amino acid metabolism
7,8-Dihydroneopterin	0.419	3.349	<0.001	↓	Folate biosynthesis
LPC 19:2	0.589	4.611	<0.001	↓	Glycerophospholipid metabolism
RCC vs. HC					
3-β-D-Galactosyl-sn-glycerol	0.322	3.853	<0.001	↓	Galactose metabolism
γ-Aminobutyryl-lysine	0.485	3.687	<0.001	↓	Amino acid metabolism
7,8-Dihydroneopterin	0.500	3.167	<0.001	↓	Folate biosynthesis
LPC 19:2	0.441	3.239	<0.001	↓	Glycerophospholipid metabolism
6-Keto-prostaglandin F1α	2.800	3.042	<0.001	↑	Arachidonic Acid Metabolism
17α,21-Dihydroxypregnenolone	2.119	2.722	<0.001	↑	Steroid hormone biosynthesis
γ-Glutamylphenylalanine	1.932	1.659	0.006	↑	Amino acid metabolism

Note: HC, healthy control; BKT, benign kidney tumor; RCC, renal cell carcinoma; LPC, lysophosphatidylcholine; FC, fold change; VIP, variable importance for the projection.



correlation with eGFR ($r = 0.202$, $p = 0.023$). Although the correlation coefficient is weak (Akoglu, 2018; Schober et al., 2018), these results indicated that the reduced serum levels of 3-β-D-Galactosyl-sn-glycerol, 7,8-Dihydroneopterin and LPC 19:2 in patients with RCC may have a certain degree of correlation with kidney impairment and dyslipidemia.

4 Discussion

RCC is a metabolic disease and analyzing its metabolic profile is essential for identification of biomarkers. Metabolomics based on UPLC-MS/MS technology have been widely used to explore new biomarkers of diseases (Liu et al., 2019b; Crestani et al., 2020; Wilkinson et al., 2020; Ai et al., 2022). Studies have shown that comparing with plasma samples. Serum can avoid the influence of anticoagulants on the concentration of certain metabolites (such as amino acids) compared to plasma samples, and the higher concentration of metabolites in serum may provide more sensitive results in biomarker detection, so the metabolomic analysis of serum samples may be more reliable than plasma samples (Yu et al., 2011; Sotelo-Orozco et al., 2021). Take into account this factor, serum was selected as the sample for this

study. We found that there were significant differences in serum metabolic profiles among healthy subjects, patients with BKT, and patients with RCC. The glycerophospholipid metabolism pathway, D-glutamine and D-glutamate metabolism pathway, phosphatidylinositol signaling system, glycerolipid metabolism pathway, galactose metabolism pathway, and folate biosynthesis were significantly abnormal in patients with RCC. In addition, 3-β-D-Galactosyl-sn-glycerol, γ-Aminobutyryl-lysine, 7,8-Dihydroneopterin, and LPC 19:2 were identified as potential markers for the diagnosis of RCC.

Dysregulated lipid metabolism affects a variety of cellular physiological processes, such as cell proliferation, differentiation and motility, which are closely associated with cancer transformation, progression and metastasis (Perrotti et al., 2016; Butler et al., 2020). In the present study, most of the markers screened from RCC were lipid and lipid-like metabolites, among which serum levels of 3-β-D-Galactosyl-sn-glycerol and LPC 19:2 were significantly reduced, while serum levels of 6-Keto-prostaglandin F1α and 17α,21-Dihydroxypregnenolone were significantly increased. The reduced serum levels of lipid and lipid-like metabolites in patients with RCC may be attributed to increased demand for phospholipids by the rapidly proliferated cancer cells to generate new cell membranes; and, the increased serum levels of lipid and lipid-like metabolites may because the enhanced exogenous lipid uptake and activated endogenous lipid synthesis in tumor cells to provide energy (Butler et al., 2020). It is worth noticing that LPC 19:2 in serum has not been reported in the literature. In this study, the retention time, MS1 and MS2 data obtained through MS/MS analysis matched with the integrated database, and LPC 19:2 was identified. After metabolic pathway analysis, it was found that LPC 19:2 involved in the glycerol phospholipid metabolic pathway. Glycerophospholipid metabolism is one of the important pathways of lipid metabolism *in vivo*, and changes in glycerophospholipid levels may affect cellular function, cytotocytosis, cytospin, cytoskeletal regulation, and membrane fusion (Wang et al., 2021b). Previous studies have found that there is dysregulated glycerophospholipid metabolism pathway in some cancers, such as gastric cancer (Yu et al., 2021), hepatocellular carcinoma (Yu et al., 2022), prostate cancer (Xu et al., 2021), non-small cell lung cancer (Chen et al., 2018), ovarian cancer (Gan et al., 2020), colorectal cancer (Gumpenberger et al., 2021), and pancreatic ductal adenocarcinoma (Martin-Blazquez et al., 2020). In this study, we found altered glycerophospholipid metabolism pathway in patients with RCC, further confirming that the glycerophospholipid metabolism pathway may be associated with the occurrence of RCC. However, the mechanism underlying the altered the glycerophospholipid metabolic pathway in RCC needs further study. In addition, abnormal lipid metabolism in RCC may also be due to abnormal expression of key genes of lipogenesis, such as fatty acid synthase, ATP citrate lyase, sterol regulatory element-binding protein 1, and

TABLE 5 Correlation analysis of markers with common renal function and lipid indicators (*r*, *P*).

Compounds	3-β-D-Galactosyl-sn-glycerol	γ-Aminobutyryl-lysine	7,8-Dihydroneopterin	LPC 19:2
Urea (mmol/L)	-0.159, 0.093	-0.029, 0.748	0.037, 0.680	-0.181, 0.042*
Cr (μmol/L)	-0.268, 0.002*	-0.095, 0.290	-0.214, 0.016*	-0.222, 0.012*
UA (μmol/L)	-0.084, 0.350	-0.074, 0.407	-0.002, 0.986	-0.041, 0.650
CysC (mg/L)	-0.271, 0.002*	-0.138, 0.123	-0.190, 0.033*	-0.203, 0.023*
eGFR (ml/min/1.73m ²)	0.271, 0.002*	0.138, 0.123	0.190, 0.033*	0.203, 0.023*
C1q (mg/L)	0.081, 0.367	-0.015, 0.869	0.006, 0.949	0.029, 0.746
NGAL (μg/L)	-0.169, 0.063	-0.131, 0.152	-0.218, 0.016*	-0.125, 0.173
TC (mmol/L)	-0.057, 0.523	-0.054, 0.547	-0.015, 0.169	-0.101, 0.263
TG (mmol/L)	-0.113, 0.204	-0.132, 0.138	-0.169, 0.057	-0.176, 0.049*
HDL (mmol/L)	0.084, 0.345	0.102, 0.255	0.104, 0.243	0.115, 0.203
LDL (mmol/L)	-0.053, 0.555	-0.044, 0.626	0.044, 0.620	-0.150, 0.094
APO-A (g/L)	0.101, 0.262	0.102, 0.255	-0.063, 0.486	0.123, 0.173
APO-B (g/L)	-0.071, 0.427	-0.076, 0.395	-0.007, 0.939	-0.168, 0.062

Note: LPC, lysophosphatidylcholine; Cr, creatinine; UA, uric acid; CysC, cystatin C; C1q, complement C1q; NGAL, neutrophil gelatinase-associated lipocalin; eGFR, estimated glomerular filtration rate; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; Apo-A1, apolipoprotein A1; Apo-B, apolipoprotein B. **p* < 0.05.

hydroxy acyl-CoA dehydrogenase alpha subunit (Heravi et al., 2022). Further studies at gene level are needed to understand the mechanisms.

Glutamine metabolism and glutamic acid metabolism are one of the important pathways to obtain nutrients during the growth and proliferation of cancer cells. Glutamine is not only the nitrogen source for cancer cell biosynthesis (such as nucleotide synthesis and protein synthesis), either directly or indirectly by conversion to glutamic acid, but also a carbon source for amino acid and fatty acid synthesis in cancer cells (Altman et al., 2016). Additionally, it can be converted to α-ketoglutarate to enter the tricarboxylic acid cycle, thus providing energy for cancer cell growth and proliferation (Altman et al., 2016). One study found (Shroff et al., 2015) that the metabolites of the glutaminolytic pathway were elevated in RCC tissues compared to normal kidney tissues, suggesting that RCC survival may be related with glutamine metabolism. Consistently, the current study confirmed that serum levels of glutamic acid, which is involved in the D-glutamine and D-glutamate metabolism pathway, were elevated in patients with RCC. These findings indicate that elevated glutamic acid in RCC may provide sufficient energy and substances for growth and proliferation of RCC.

In this study, 3-β-D-Galactosyl-sn-glycerol, γ-Aminobutyryl-lysine and 7,8-Dihydroneopterin were identified to be possible potential biomarkers to distinguish patients with RCC from patients with BKT and healthy subjects. The chosen kidney function and lipid indicators for comparative analysis in this study are commonly used in clinical diagnosis of kidney function damage or to judge whether the blood lipid level is normal. They are intermediate or end products of substance metabolism in the body. Because of their high level in blood, they can be accurately detected

by conventional chemical analysis methods. In contrast, the new biomarkers screened in this study were obtained by untargeted metabolomics technology, which can only perform qualitative and semi-quantitative analysis on the analyzed substances (that is, the level of substance in the sample can only be estimated roughly according to the peak height or area of the mass spectrum). However, several potential biomarkers for diagnosis or differential diagnosis of RCC, which were not previously noticed, were found through the analysis of serum samples of subjects using untargeted metabolomics technology in this study. Many studies have also confirmed that untargeted metabolomics can comprehensively and systematically analyze small molecular substances in biological samples (Hou et al., 2020; Castelli et al., 2022; Khoramipour et al., 2022), and it is an excellent analytical tool for screening biomarkers at present. In this group of subjects, no indicators consistent with the observed kidney function and blood lipid indicators were found by UPLC-MS/MS method. This is because we use untargeted metabolomics technology to screen different markers in different settings. However, the observed kidney function and blood lipid indicators have high concentrations in each group we set, so they may be ignored as non-differential indicators during the analysis. Some new biomarkers related to them were also found. However, due to different detection methods, the correlation between these new biomarkers and any of the observed kidney function and blood lipid indicators is not good enough. However, this is enough to show that the reduced serum levels of 3-β-D-Galactosyl-sn-glycerol and 7,8-Dihydroneopterin in patients with RCC may have a certain of correlation with kidney impairment and dyslipidemia. However, the underlying mechanisms need further investigation. 3-β-D-Galactosyl-sn-glycerol is involved in the galactose metabolism pathway and it is reduced in the serum of patients with RCC.

Lactose is hydrolyzed into to glucose and galactose in the intestine by lactase (Forsgard, 2019), which can enter the circulation through the same transporter in the intestinal epithelium and participate in metabolism. The glucose requirements of cancer cells are greater than those of normal cells because they mainly depend on aerobic glycolysis for their energy source. Therefore, reduced serum levels of 3- β -D-Galactosyl-sn-glycerol metabolites involved in galactose metabolism in patients with RCC may be attributed to the competitive inhibition of galactose uptake in intestinal epithelial cells. Although the 7,8-dihydroneopterin in other diseases has been reported, the mechanism of its association with RCC occurrence needs to be further investigated. 7,8-Dihydroneopterin are organic compounds of biopterins and their derivatives, which have antioxidant effects (Janmale et al., 2020). The current study identified γ -Aminobutyryl-lysine, a dipeptide present in the human brain, whose relationship with RCC has not been reported.

In this study, serum levels of 6-Keto-prostaglandin F1 α , 17 α ,21-Dihydroxypregnenolone and γ -Glutamylphenylalanine in RCC patients were found to be elevated which may be potential biomarkers to distinguish RCC patients from healthy individuals. However, the diagnostic performances of these three biomarkers for distinguishing RCC and BKT were not good (all AUC<0.80, the data were not displayed). 6-Keto-prostaglandin F1 α belongs to the prostaglandin and related compounds. Prostaglandin is unsaturated fatty acids produced by the cyclooxygenase-catalyzed arachidonic acid metabolism and is widely present in various vital tissues and body fluids in humans. It is reported that cyclooxygenase-2 overexpression in RCC may be associated with metastasis of tumor cells, tumor invasion and angiogenesis (Kaminska et al., 2014; Ching et al., 2020). Therefore, elevated 6-Keto-prostaglandin F1 α in RCC patients may be attributed to the overexpression of cyclooxygenase. 17 α ,21-Dihydroxypregnenolone belongs to the organic compounds known as 21-hydroxysteroids, and is involved in steroid hormone biosynthesis pathway. Increased serum levels of 17 α ,21-Dihydroxypregnenolone in patients with RCC may be related to the expression and function of steroid hormone receptors. These receptors can act as ligand-dependent transcription factors or induce gene expression through ligand-independent pathways and play an important role in tumor growth and differentiation (Bennett et al., 2014). γ -Glutamylphenylalanine is a dipeptide composed of γ -glutamic acid and phenylalanine, which is a proteolytic breakdown product of larger proteins. It may be formed by γ -glutamyl transpeptidase catalyzing the transpeptidation between glutathione and the corresponding amino acid. γ -glutamyl transpeptidase is an enzyme primarily involved in cellular glutathione homeostasis, and although it has been studied in other cancers, such as gastric cancer (Wang et al., 2017), intrahepatic cholangiocarcinoma (Zhang et al., 2021), hepatocellular carcinoma (Ince et al., 2020) and oral squamous cell carcinoma (Mujawar et al., 2020), its mechanism of action in RCC has not been reported. In addition, some compounds have not been screened as potential diagnostic biomarkers of RCC in this study, such as 21-Deoxycortisol, Dehydrocholic acid, Leucyl leucine,

Ethylene brassylate, Leu Ala OH and Leukotriene F4, etc. However, they also show high FC, VIP scores and significant *p*-values. Therefore, these compounds may also be worthy of attention and further research.

In the screening process of biomarkers, it was found that some compounds involved two or more metabolic pathways, such as phosphatidic acid (PA) 23:2 and phosphatidylinositol (PI) 18:4, and were associated with glycerophospholipid, phosphatidylinositol and glycerolipid pathways. Since these biomarkers are provided by database comparison, are they the intersections of metabolic pathways or subset of other pathways? Unclear. Fortunately, these substances have little significance for this study, so they are only cared about very little. Although these metabolites are not as important as those screened in this study, they actually involve some metabolic pathways. Perhaps these metabolites could be related to a certain disease in a particular way, but they cannot be observed in this study. Similarly, in the process of pathway analysis, it is found that some metabolic pathways only involve the changes of 1-2 markers, for example, only PA 23:2 and PI 18:4 were hit to the phosphatidylinositol metabolism pathway and only glutamic acid was hit to the D-glutamine and D-glutamate metabolism pathway. Although these substances were discovered by screening, the final results only focused on 4 biomarkers, indicating that the changes of 1-2 compounds may not affect the changes of the whole metabolic pathway. In addition, the changes in their quantity (peak intensity and area of MS) of these substances are not large enough among the different study groups. Therefore, they were not finally screened as differential biomarkers.

Based on the above theories and research findings, we propose a hypothesis: there are some changes in metabolic pathways of substances in the body of RCC patients, which at least include lipid metabolism, amino acid metabolism, galactose metabolism and folate biosynthesis. Studying the signal pathways involved in these metabolic pathways may find a new way to explore the pathogenesis of RCC. This study only provides a new way for screening biomarkers for diagnosis and differential diagnosis of RCC. In the follow-up study, we will verify and evaluate the diagnostic performance of these potential biomarkers of RCC found in this study through targeted metabolomic analysis technology based on the research results, so as to explore the possibility of these biomarkers in clinical practice.

5 Conclusion

In summary, this study analyzed serum metabolic profiles of healthy subjects, patients with BKT and patients with RCC using UPLC-MS/MS. Four potential biomarkers for the diagnosis of RCC were screened by multivariate statistical analysis and ROC analysis, namely LPC 19:2 involved in the glycerophospholipid metabolism pathway, 3- β -D-Galactosyl-sn-glycerol involved in the galactose metabolism, 7,8-Dihydroneopterin involved in the folate biosynthesis and γ -Aminobutyryl-lysine, an amino acid

metabolite. In addition to the above metabolic pathways, there may also be changes in the phosphatidylinositol signaling system, the D-glutamine and D-glutamate metabolism pathway, and the glycerolipid metabolism pathway in patients with RCC. These results suggest that the occurrence of RCC may be associated with changes in lipid metabolism, amino acid metabolism, galactose metabolism, and folate biosynthesis. These four metabolites may become markers for the diagnosis or differential diagnosis of RCC. However, this study is limited in the small sample size and the lack of targeted metabolomics validation. Therefore, follow-up studies with larger sample size and validation with other omics such as genomics and proteomics are warranted to clarify the metabolic mechanism of RCC and to further identify tumor markers for clinical application.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Materials](#), further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Mianyang Central Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception and design of research: JW and J-FF; collected and assembled data: JW, W-YY, X-HL, and BZ; contributed to

methodology: BX; analyzed and interpreted data: JW, W-YY, Y-WY, and J-FF; prepared figures: X-HL and C-MD; wrote the original draft of manuscript: JW; reviewed and edited the manuscript: J-FF; approved final version of manuscript: JW, W-YY, X-HL, BX, Y-WY, BZ, C-MD, and J-FF.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2022.996248/full#supplementary-material>

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Preliminary screening of biomarkers in HAPE based on quasi-targeted metabolomics

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High altitude pulmonary edema (HAPE) is a serious threat to the physical and mental health of people who quickly enter high plateaus, deserves more attention and in-depth research. In our study, through the detection of various physiological indexes and other phenotypes in a HAPE rat model, the HAPE group showed a significant decrease in oxygen partial pressure and oxygen saturation, and a significant increase in pulmonary artery pressure and lung tissue water content. The lung histomorphology showed characteristics such as pulmonary interstitial thickening and inflammatory cell infiltration. We applied quasi-targeted metabolomics to compare and analyze the components of metabolites in arterial-venous blood in control rats and HAPE rats. Using Kyoto Encyclopedia of Genes Genomes (KEGG) enrichment analysis and two machine algorithms, we speculate that after hypoxic stress and comparing arterial blood and venous blood products in rats, the metabolites were richer, indicating that normal physiological activities, such as metabolism and pulmonary circulation had a greater impact after hypoxic stress; D-mannose^{DOWN}, oxidized glutathione^{DOWN}, glutathione disulfide^{DOWN}, and dehydrocholic acid^{DOWN} in arterial blood play key roles in predicting the occurrence of HAPE; in venous blood, L-leucine^{DOWN}, L-thyroxine^{DOWN}, and cis-4-hydroxy-D-proline^{DOWN} may have key roles, which can be considered biomarkers of HAPE. This result provides a new perspective for the further diagnosis and treatment of plateau disease and lays a strong foundation for further research.

KEYWORDS

high altitude pulmonary edema, quasi-targeted metabolomics, arterial-venal blood differences, machine algorithms, biomarkers

1 Introduction

Acute mountain sickness is a serious threat to the physical and mental health of people who quickly enter high plateaus. As a severe type of acute altitude sickness, HAPE can occur in first-time visitors or in those who return to high plateaus after living at lower altitudes (Jensen and Vincent, 2022). HAPE is caused by sudden exposure to the plateau environment, which causes pulmonary artery pressure, pulmonary blood volume, pulmonary circulation disorders, and microcirculation internal fluid leakage to the lung interstitia and alveoli (Gudbjartsson et al., 2019).

As the final product of gene expression, metabolites have become the main focus in related diseases in recent years. And with the continuous development of metabolomics, more and more evidence shows that metabolites play an important role in the development of diseases. For

example, untargeted metabolomics analysis of lung tumors showed that compared with normal lung tissue of mice, glutathione was increased in tumors and accumulated in NSCLC lesions (Luengo et al., 2019). The study of Schaarschmidt B et al. also suggested that targeted metabolomics are an emerging field and can be used to diagnose or assess stages and severity of different liver diseases such as cirrhosis and fibrosis (Schaarschmidt et al., 2018). Hocher B et al. reviewed the metabolomic characteristics of non-diabetes chronic kidney disease (CKD) such as IgA nephropathy, and revealing amino acids and their metabolites, tryptophan metabolites, uric acid and other purine metabolites, lipids and acylcarnitines as promising markers (Hocher and Adamski, 2017). While, there are few studies on the changes of metabolites in the process of HAPE. In 2012, Luo Y et al. (Luo et al., 2012) found the changes of some metabolites in the plasma of HAPE patients by using proton (^1H) NMR metabolomics. However, the related components of metabolites in arterial and venous blood during HAPE are not clear. We used quasi-targeted metabolomics of metabolites in normal rats and HAPE blood to further clarify the metabolism changes and preliminarily screen for biomarkers of HAPE. Our findings could have great clinical importance in studying the pathogenesis and treatment of HAPE.

2 Materials and methods

2.1 Animals

60 specific pathogen-free (SPF) Sprague–Dawley rats, weighing 180–220g, were purchased from the Laboratory Animal Center of Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. License No. SCXK (Beijing) 2021–0011. The feeding environment was $25^\circ\text{C} \pm 1^\circ\text{C}$, relative humidity 50%–60%, and light/darkness for 12 h circulation. Rats are allowed to eat and drink freely. Before treatment, the body quality of rats was monitored. Animals and experimental protocol were conducted according to the guidelines and ethical standards of the Animal Care and Use Ethics Committees and were approved by the Science and Technology Ethics Committee of Qinghai University.

2.2 Establishment and grouping of animal models

60 rats were randomly divided into two groups: the control group and the HAPE group. Control rats were kept in the animal room of the Medical College of Qinghai University. The HAPE model rats were treated in a 6,000 m, 0.6 m/s hypobaric chamber with hypoxic stress for 48 h to establish a lung injury rat model. Rat weights in each group were measured daily at a fixed point during rearing. After treatment, the rats were anesthetized *via* intraperitoneal injection of pentobarbital sodium at 45 mg/kg. The arterial blood and venous blood of the rats were collected in a heparin sodium collector, and the control group was randomly divided into a blank control venous blood group (CV group) and an arterial blood group (CA group). The HAPE group was randomly divided into a HAPE venous blood group (HV group) and a HAPE arterial blood group (HA group). Whole blood was extracted from the abdominal aorta using a blood collector, and 1 ml of blood was taken for analysis of oxygen saturation and partial pressure of

oxygen in the abdominal aorta using an automatic blood gas analyzer (Sysmex, Japan). The remaining whole blood was centrifuged at 3,000 rpm for 10 min at 4°C , and the serum was collected and stored at -20°C . In addition, changes in pulmonary arterial pressure waveforms were observed using PowerLab physiological loggers (ADI, Australia), and pulmonary arterial pressure was measured using a pressure sensor. Finally, the lung tissue was collected, and the water content of the lung was calculated; the lung tissue was used for subsequent experiments.

2.3 Hematoxylin and eosin (H&E) staining

Lung tissue sections were dewaxed and stained with H&E staining (Servicebio, China) for pathological studies. Each lung tissue section was evaluated using a trinocular microscope (BA200Digital, Mike Audi, China).

2.4 Transmission electron microscope (TEM)

Lung tissue was taken from the lower tip of the right lung, cut into a 1-mm three tissue block, and fixed in a frozen tube with precooled 2.5% glutaraldehyde solution for 10 h and washed with PBS buffer. Tissue sections were fixed in osmic acid, dehydrated using gradient alcohol, embedded in pure acetone and mixed embedded liquid for 4 h, and then embedded overnight. Lead staining solution was used, and ultrastructural changes in the lung tissue were visualized using a TEM.

2.5 Metabolomics and LC-MS analysis

The samples (100 μL) were placed in the EP tubes and resuspended with prechilled 80% methanol by a good vortex. Then the samples were incubated on ice for 5 min and centrifuged at 15,000 g, 4°C for 20 min. Some of the supernatant was diluted to a final concentration containing 53% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube and then were centrifuged at 15,000 g, 4°C for 20 min. Finally, LC-MS/MS (Want et al., 2010; Dunn et al., 2011) analyses were performed using an ExionLCTM AD system (SCIEX) coupled with a QTRAP[®] 6,500 + mass spectrometer (SCIEX) in Novogene Co., Ltd. (Beijing, China). The detection of the experimental samples using Multiple Reaction Monitoring (MRM) was based on a novocaine in-house database. The data files generated by HPLC-MS/MS were processed using the SCIEX OS Version 1.4 to integrate and correct the peak.

These metabolites were annotated using the KEGG database. Partial least squares discriminant analysis (PLS-DA) was performed at metaX. We applied univariate analysis (t-test) to calculate the statistical significance (*p*-value). The metabolites with VIP > 1 and *p* < 0.05 and fold change ≥ 2 or FC ≤ 0.5 (Sreekumar et al., 2009; Haspel et al., 2014; Heischmann et al., 2016) were considered to be differential metabolites. Volcano plots were used to filter metabolites of interest-based on Log₂ (FC) and -log₁₀ (*p*-value) of metabolites by ggplot2 in R language. The functions of these metabolites and metabolic pathways were studied using the KEGG database. The metabolic pathways enrichment of differential metabolites was

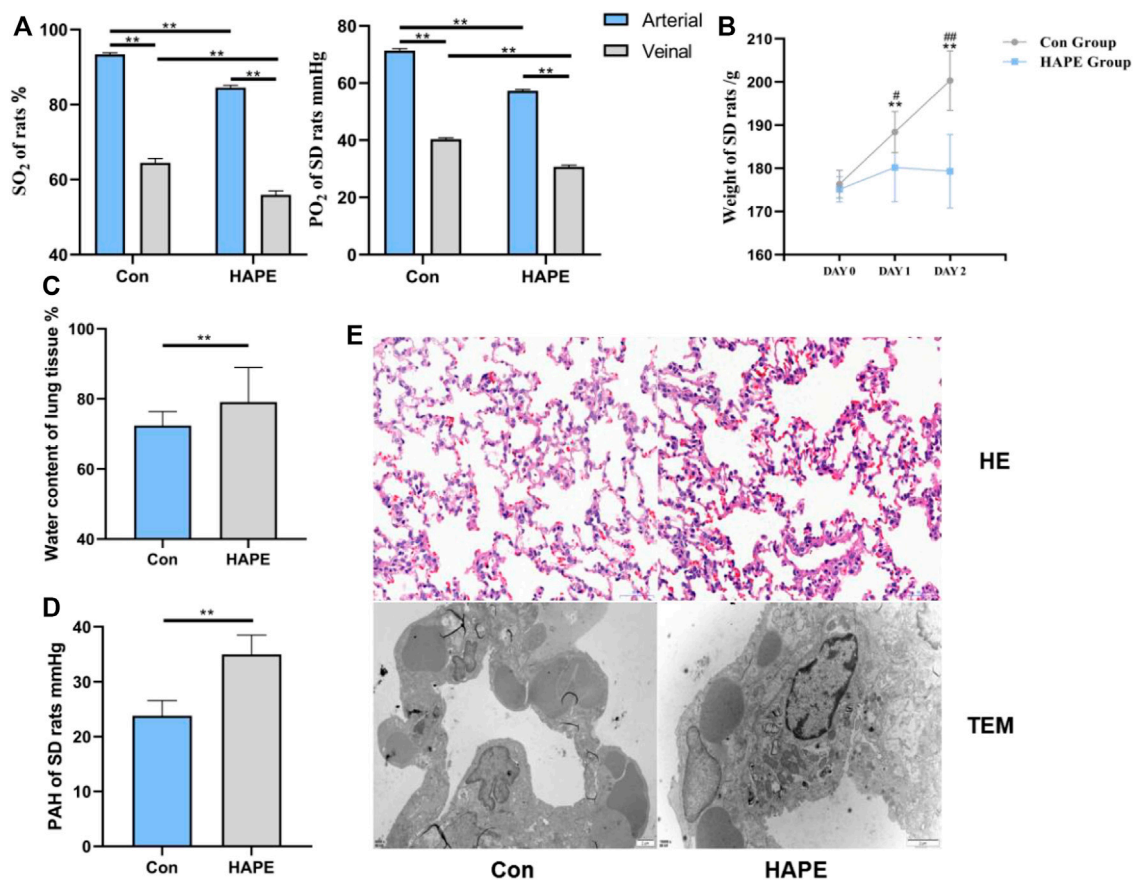


FIGURE 1
Physiological indexes and Lung tissue morphology of HAPE rats.

performed, when the ratio was satisfied by $x/n > y/n$, metabolic pathways were considered as enrichment, when $p < 0.05$, metabolic pathways were considered statistically significant enrichment.

2.6 Biomarker screening

Two machine learning methods were selected to construct the prediction model. Differential biomarkers in the top 15 positions were screened by two researchers: random forest (RF) and support vector machines (SVM), which had a key role in the grouping. Then, we used receiver operating characteristic curve to screen the biomarkers for HAPE.

2.7 Statistical analysis

All data were analyzed using SPSS 22.0 (IBM, USA) statistical analysis software, and shown as mean $\pm$ standard deviation (SD). The one-way analysis of variance (ANOVA) and two-tailed Student's t-test were applied to analyze the significant differences between the groups. $p < 0.05$ was significant difference.

3 Result

3.1 Physiological indexes and lung tissue morphology of HAPE rats

On analyzing blood gas in the rats, we found that the oxygen saturation and oxygen partial pressure in the venous blood (Figure 1A) of the HAPE and control groups were significantly lower than that in the arterial blood ($p < 0.01$). Through comparative analysis of the body weight of rats in each group (Figure 1B), we found that body weight in control rats increased gradually with an increasing feeding time ($p < 0.01$), whereas the body weight of rats in the HAPE group did not increase significantly with an increasing feeding time, and there was no significant difference in body weight within 3 days. However, compared with controls, the body weight of rats was significantly decreased ($p < 0.01$ or $p < 0.05$). Compared with controls, the oxygen partial pressure and oxygen saturation in the HAPE group were significantly decreased ($p < 0.01$). Compared with controls, water content of the lung tissue (Figure 1C) and the pulmonary artery pressure (Figure 1D) in HAPE rats were increased significantly ($p < 0.01$).

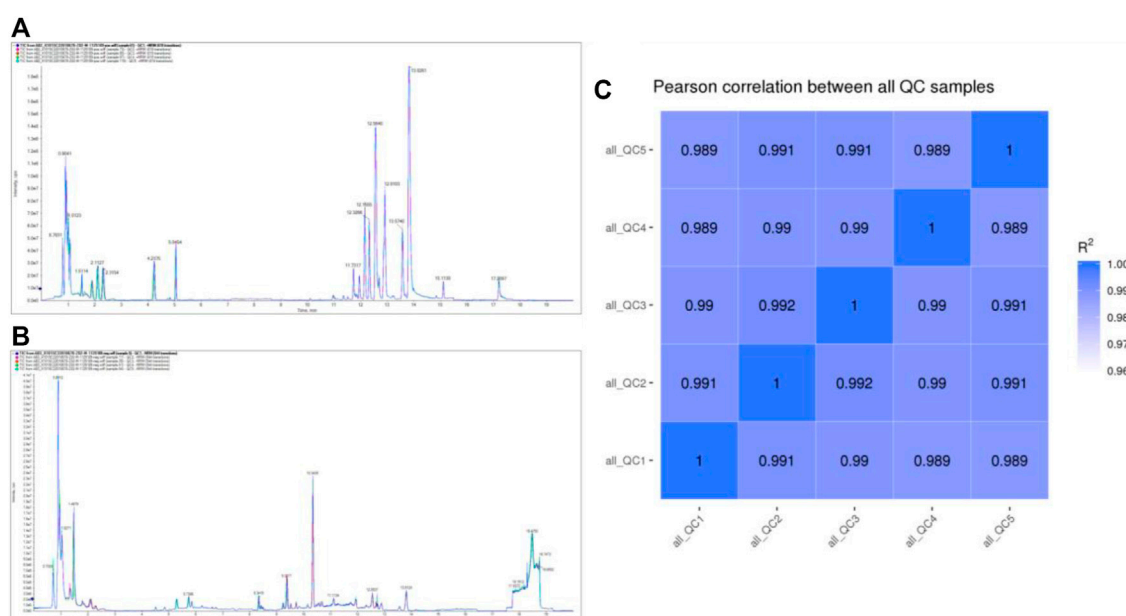


FIGURE 2
QC analysis of the metabolome data.

3.2 Morphological characteristics of lung tissue in HAPE rats

After H&E staining, the morphology of the rat lung tissue was observed under a light microscope, and the ultrastructural changes of the rat lung tissue were observed under a TEM. The lung tissue of the control group showed normal alveolar structure under a photoelectric microscope. Rats in the HAPE group showed injuries such as widening of the alveolar septum, infiltration of a large number of red blood cells, and inflammatory cells under light microscopy. Obvious swelling of mitochondria and shedding of lamellar bodies were observed *via* TEM (Figure 1E).

3.3 Analysis of the metabolome data

3.3.1 QC analysis

TIC overlapping display analysis shows that the technical repeatability of metabolites is good (Figure 2A; Figure 2B). The Pearson correlation coefficients of QC samples calculated by the relative quantitative values of metabolites are between 0.989–1.000 (Figure 2C), suggesting that the better the stability of the whole detection process, the higher the data quality. The above data indicate that the quality of this data is high, laying the foundation for follow-up relevant research.

3.3.2 Basic information of differential metabolites

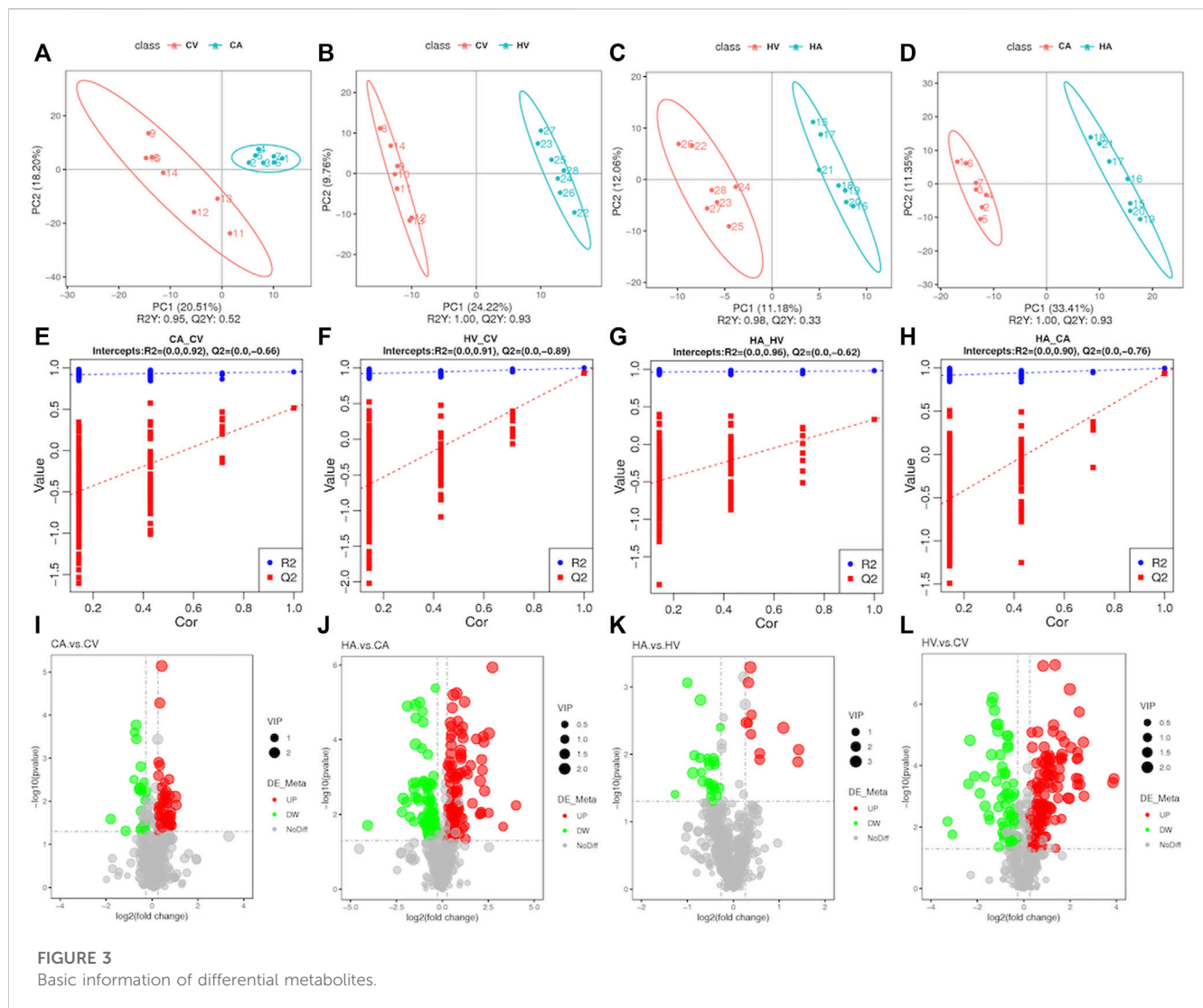
We applied PLS-DA to each of the four groups for statistical analysis (Figures 3A–D), and obtained model evaluation parameters R^2 and Q^2 by 7-fold cross-validation (Figures 3E–H), with the results suggesting that the models were all over-fitted and could

proceed to the next step of analysis. After perfecting the analysis of targeted metabolic data, we found that the difference in metabolites between groups was obvious, among which a total of 664 metabolites were tested (Figures 3I–3L; Supplementary Figure S1). The total difference in metabolites in CV versus CA was 74, of which 52 metabolites showed an upward trend and 22 metabolites showed a downward trend. HV versus HA totaled 36 differential metabolites, among which 11 metabolites showed an upward trend and 25 metabolites showed a downward trend. For CA versus HA, a total of 217 differential metabolites were observed, of which 155 metabolites showed an upward trend, and 62 metabolites showed a downward trend. For CV versus HV, the total difference in metabolites was 195, of which 113 metabolites showed an upward trend, and 82 metabolites showed a downward trend. In a comparison among groups, the differences of different products among groups were further clarified.

We found that differences in the composition of arterial blood and venous blood metabolites in rats after hypoxic stress tended to decline compared with that in rats in the normal group. However, after hypoxic stress and comparing arterial blood and venous blood products in rats with normal rats, the metabolites were richer, indicating that normal physiological activities, such as metabolism and pulmonary circulation after hypoxic stress, had a greater impact.

3.3.3 KEGG enrichment analysis in different groups

After KEGG enrichment analysis of differential metabolites (Figure 4), compared with the CA group, the CV group had differential metabolites that were mainly enriched in lysine (Lys) degradation and in a wide range of metabolic pathways ($p < 0.05$). The difference between the HV group and CV group was mainly enriched arginine (Arg) and proline (Pro) metabolism, tryptophan



(Trp) metabolism, and the protein digestion and absorption pathway ($p < 0.05$), metabolites were also enriched and differentially expressed in central carbon metabolism and thyroid hormone (TH) synthesis in cancer ($p > 0.05$). Comparing the HA group with the CA group, differential metabolites were mainly enriched in TH synthesis and fructose and mannose metabolic pathways ($p < 0.05$), metabolites were also enriched and differentially expressed in the central carbon metabolism and in starch and sucrose metabolic pathways in cancer ($p > 0.05$). Compared with the HA group, starch and sucrose metabolism, synthesis and degradation of ketone bodies, the FC ϵ RI signaling pathway, asthma, fructose and mannose metabolism, propanoate metabolism, and other metabolic pathways were mainly enriched in the HV group, but with $p > 0.05$, suggesting no statistical significance.

3.3.4 Analysis of the key metabolites

Key differential metabolic pathways were screened based on KEGG enrichment analysis, and the key metabolites in the pathway were screened for further analysis (Supplementary Figure S2), the percentage stacking map of metabolites is shown in Figure 5.

3.3.4.1 Three major nutrients

Glucose metabolism is the core of body metabolism. Sequencing data of quasi-targeted metabolomics in the arteriovenous blood of the normal body show that the levels of glucose-related metabolites in venous blood are significantly higher than those in arterial blood, which is mainly related to micro-circulation in the body. After hypoxia stimulation, glucose metabolism obviously strengthens, such as D-glucose 6-phosphate (G6P), D-mannose 6-phosphate (M6P), D-fructose 6-phosphate (F6P), with the levels of glucose-related metabolites in both arterial blood and venous blood significantly higher than those in normal controls.

Levels of acetoacetate (ACAC) and other ketones in arterial blood were significantly higher than those in venous blood in the present study. After hypoxia stress, the level of ACAC in the arterial and venous blood of the body was significantly higher than that in the control group, suggesting that ketone metabolism in the body is further activated after hypoxia stimulation.

The KEGG enrichment analysis of the metabolome data of amino acids showed that serum differential products in the hypoxia group were mainly enriched in the metabolic pathways of Arg, Lys, Trp and revealed disorders of Arg and histidine (His) metabolism. Specifically,

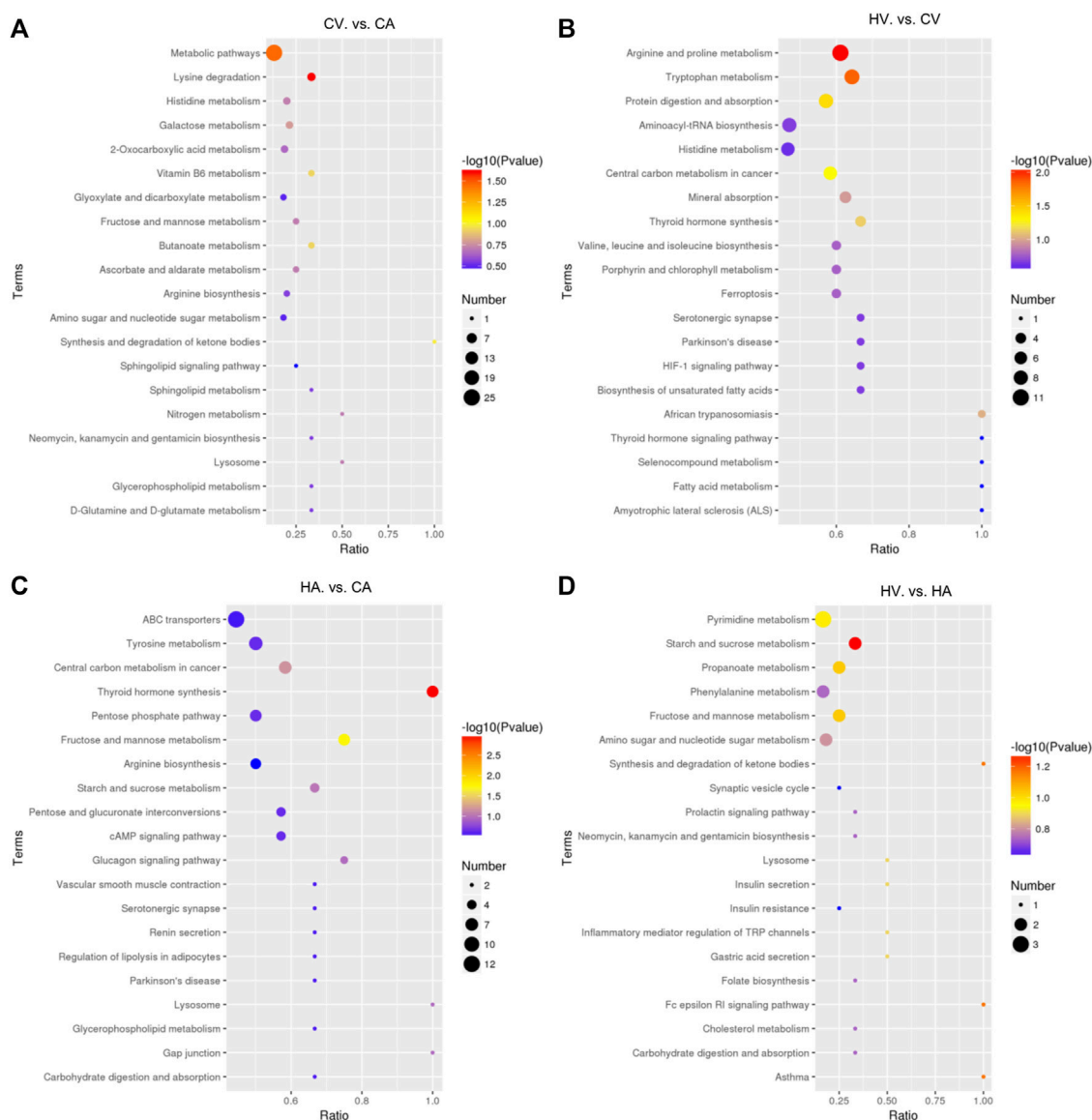


FIGURE 4
KEGG Enrichment analysis in different groups.

under hypoxia stress, the expression of key amino acids, such as citrulline (Ccp), Lys, Pro, alanine (Ala), glycine (Gly), cystine (Cys), Arg, aspartic acid (Asp), and n-acetyl-L-tyrosine (N-Ac-L-Tyr) in rat arterial serum was increased significantly, whereas the expression of lysine butyric acid, sodium glutamate (MSG), isoleucine (Ile), and acetylneuraminic acid (ANA) decreased significantly.

3.3.4.2 Antioxidant substances

Compared with the control group, the concentrations of glutathione (GSH) and oxidized glutathione (GSSG) in the serum of HAPE rats were decreased significantly in both arterial blood and venous blood. In addition to GSH, lipoic acid (ALA) and other antioxidants were significantly decreased in the serum of the HAPE rat model. Interestingly, however, the concentration of vitamin C (VC), with strong antioxidant capacity, increased in rats of HAPE.

3.3.4.3 Bile acid

Through analysis of quasi-targeted metabolomics data of arteriovenous blood in HAPE rats, we monitored 27 kinds of bile acids (BA). The specific results are as follows: no difference was found for BA in arterial and venous blood of normal rats; however, there were significant changes in BA metabolism components in the arteriovenous blood of HAPE rats. Among them, levels of taurocholic acid (TCA) and sodium taurodeoxycholate (TUDCA) in the arterial group were significantly higher than those in the venous group, with glycodeoxycholic acid (GDCA) concentrations decreased significantly. After hypoxia stimulation, compared with the control group, Chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), β -ursodeoxycholic acid (β -UDCA), and sodium taurine porcine deoxycholic acid (THDCA) showed a significant

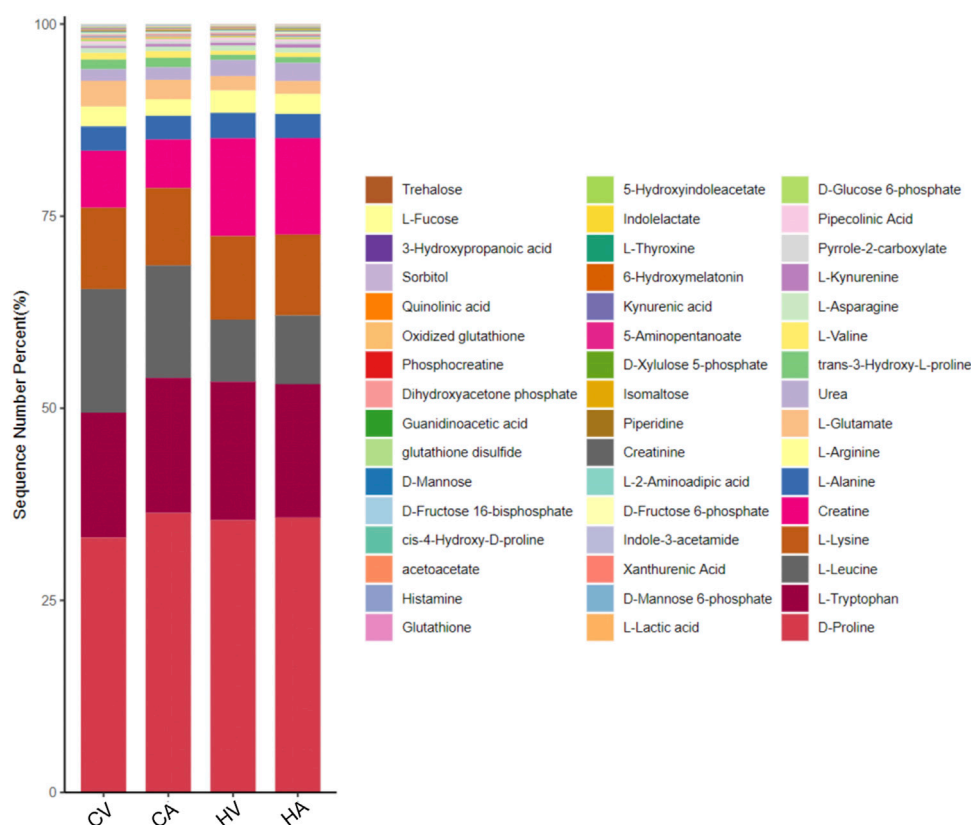


FIGURE 5

The key percentage stacking map of metabolites based on the KEGG pathways.

upward trend; 3 α ,6 α ,7 α -trihydroxy-5 β -cholic acid and beta mouse cholic acid (β -MCA) showed a significant downward trend, which suggested that significant changes take place in the BA pool during HAPE.

3.3.4.4 Others

In the HAPE rat model, serum levothyroxine (L-T4) in both the HA group and HV group showed a significant downward trend compared with the control group, and the level of vitamin B2 (VB2) in rat venous blood decreased significantly in HAPE rats.

3.3.5 Machine algorithms for HAPE biomarkers prediction

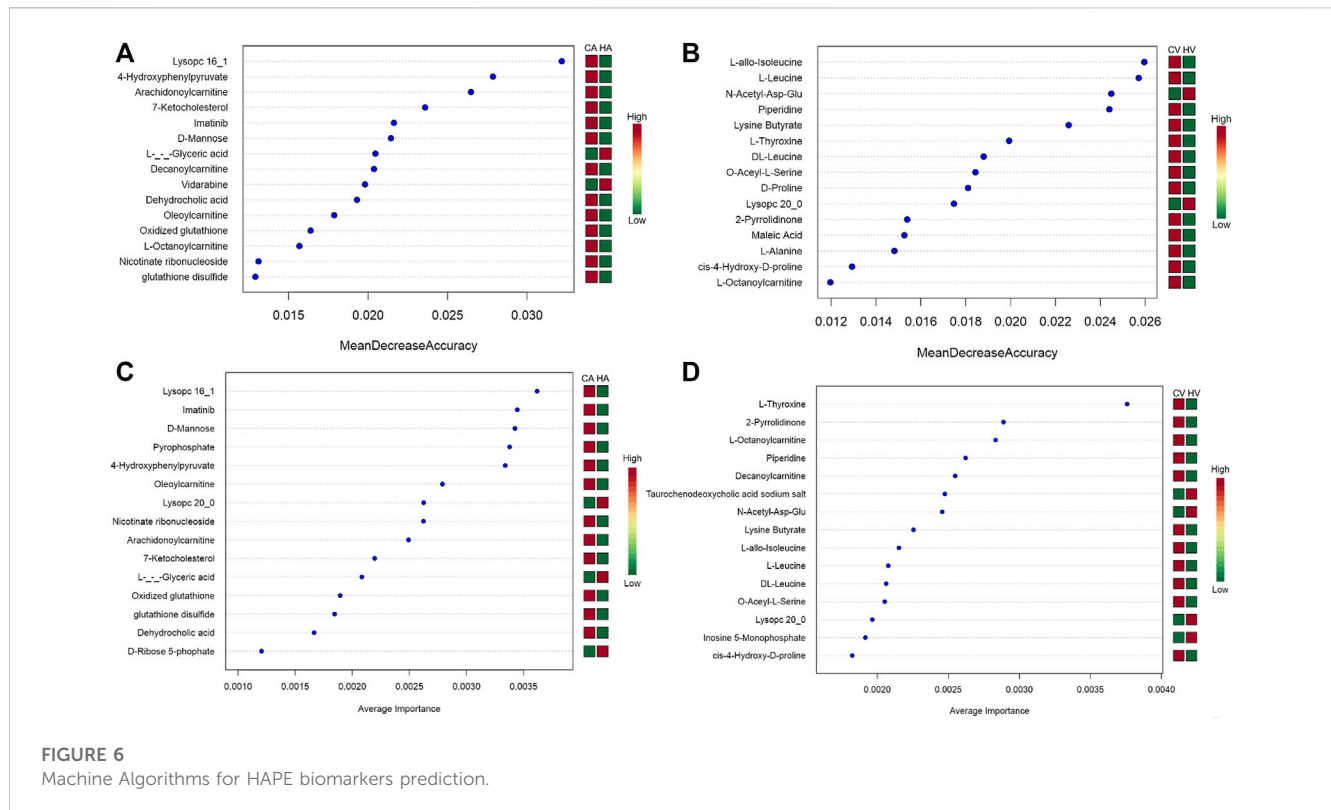
The metabolites with variable importance in projection (VIP) > 1.5 and $p < 0.01$ and fold change ≥ 2 or FC ≤ 0.5 were chosen and comparisons made among each group using RF (Figures 6A,B) and SVM (Figures 6C,D) to screen the top 15 metabolites. After combined analysis of the two machine algorithms, the representative difference products between the CA and HA groups were L-(α)-glyceric acid (L-GA), imatinib (IMA), lysoPC 16:1 (Lyp 16:1), oleoylcarnitine, 4-hydroxyphenylpyruvate (4-HPPA), D-mannose, arachidonoylcarnitine, glutathione disulfide, GSSG, dehydrocholic acid (DHCA), and nicotinate ribonucleoside (NAR). The representative difference products between the CV and HV groups were piperidine (PIP), O-acetyl-L-serine (OAS),

N-acetyl-asp-glu (NAAG), lysoPC 20:0 (Lyp 20:0), lysine butyrate, L-T4, L-octanoylcarnitine, L-leucine (L-Leu), L-allo-isoleucine(L-allo-Ile), DL-leucine(DL-Leu), cis-4-hydroxy-D-proline (4-D-Hyp), and 2-pyrrolidinone (2-P). We combined RF and SVM to screen out relevant metabolites and estimate AUC values (Table 1).

4 Discussion

With rapid ascent to high altitudes, HAPE seriously threatens the physical and mental health of people. We placed rats in a simulated low-pressure oxygen chamber at an altitude of 6,000 m, after hypoxic stress for 48 h. The blood oxygen saturation and oxygen partial pressure of rats decreased significantly, the pulmonary artery pressure and lung tissue water content increased significantly, and the morphology of lung tissues showed a state of injury and edema, suggesting that this method can successfully result in the construction of a HAPE rat model, thereby laying a foundation for further study of this disease.

During the occurrence and development of HAPE, changes in metabolites of the body are obvious. These changes are related to the body's adaptation to the hypoxic environment, the body's inflammatory damage, and so on. Herein, we discuss different types of metabolites as follows.



4.1 Three major nutrients

The normal metabolism of three major nutrients (sugar, fat, and amino acids) of the human body is an important basis for the survival of the organism. Glucose metabolism is the core of body metabolism. In the rat control group, the glucose-related metabolites in venous blood were significantly higher than those in arterial blood, which is mainly related to microcirculation in the body. Under the condition of hypoxia, the metabolic law of the normal state of body changes indicates that more energy will be consumed to meet its metabolic needs (Webster Keith, 2003). After hypoxia stimulation, the glucose-related metabolites in both arterial blood and venous blood were significantly higher than those in the normal control group, suggesting enhanced glycolytic activity in the body. Glycogen and gluconeogenesis are enhanced to maintain the stability of blood glucose, which helps to improve acute hypoxia tolerance and lung gas exchange and has a good effect on the advanced neural activities of hypoxic animals.

Among all ketones, those produced by fat metabolism play an important role in the energy homeostasis of the organism (Prins, 2008; Puchalska and Peter, 2017). AcAc, a key ketone, in the arterial blood was significantly higher than that in the venous blood in our study, which is related to normal microcirculation in the body. After hypoxic stress, the level of AcAc in the arterial and venous blood in the body was significantly higher than that in the control group, suggesting that ketone metabolism in the body is further activated after hypoxia stimulation. Previous studies have shown that ketone metabolism requires only a small amount of enzymatic reaction (Maalouf et al., 2007), which can efficiently complete productivity activities under hypoxia, improve metabolic efficiency, and reduce the production of reactive oxygen species (Gano Lindsey et al., 2014; Parra et al., 2017),

suggesting that the activation of ketone metabolism is an adaptive regulation mode of the body in a hypoxic environment.

A high-altitude hypoxic environment has a certain impact on the metabolism of normal amino acids, but there are few amino acid metabolisms and specific regulation mechanisms in a hypoxic environment (Dahl Rasmus et al., 2019). When the body first enters the plateau, the metabolism of proteins is characterized by weakened synthesis and enhanced decomposition (Gibson et al., 1981). The levels of endogenous glyconic amino acids (e.g., Gly, valine (Val), serine (Ser)) were significantly decreased after hypoxic stress, which may be related to the increase in gluconeogenesis of glyconic amino acids caused by hypoxia (Panjwani et al., 2007); With the sensitivity of Ala aminotransferase to hypoxia, Ala metabolism is inhibited after hypoxia stress (Akshay et al., 2021), resulting in the accumulation of Ala in the body and a significant increase of Ala in the body. Tyrosine (Tyr) can improve the working ability of the body in cold and high-altitude environments and reduce symptoms in a reaction to high altitude (Cooper et al., 2018). After hypoxic stress, the levels of Tyr and its related derivatives in the serum were significantly decreased, suggesting that the body's hypoxia tolerance decreased significantly or high-altitude injury had occurred in rats. Amino acids such as Asp are excitatory transmitters of the central nervous system. After hypoxia stimulation, metabolic disorders occur and amino acid levels increase significantly. Melatonin (MT) (Claustrat and Leston, 2015), which reduces the release of excitatory amino acids and which was significantly decreased in the body of the hypoxia group, further mediates the accumulation and neurotoxicity of excitatory amino acids and may even induce the occurrence of high-altitude cerebral edema (Ruan et al., 2000; Zhu and Xu, 2019).

TABLE 1 ROC of the key metabolites based on RF and SVM.

Group	Metabolites	ROC (AUC)	Trend
HV vs. CV	L-allo-Isoleucine	0.92	down
	L-Leucine	0.92	down
	N-Acetyl-Asp-Glu	1.00	up
	Lysine Butyrate	0.98	down
	L-Thyroxine	0.94	down
	O-Acetyl-L-Serine	0.92	down
	Lysopc 20–0	1.00	up
	cis-4-Hydroxy-D-proline	0.94	down
	L-Octanoylcarnitine	0.98	down
HA vs. CA	Lysopc 16–1	1.00	down
	Imatinib	1.00	down
	D-Mannose	1.00	down
	4-Hydroxyphenylpyruvate	1.00	down
	Oleoylcarnitine	1.00	down
	Nicotinate ribonucleoside	0.98	down
	Arachidonoylcarnitine	1.00	down
	L-(–)-Glyceric acid	1.00	up
	7-Ketocholesterol	1.00	down
	Oxidized glutathione	0.96	down
	glutathione disulfide	0.96	down
	Dehydrocholic acid	0.98	down

It is worth noting that a large number of B vitamins can reduce disorders of amino acid metabolism caused by hypoxia and that appropriate supplementation of vitamin B (VB) may play a certain role against hypoxic injury in the body (Liu et al., 2018). Our metabolome data results showed that VB2 in rat venous blood decreased significantly in HAPE, suggesting that VB2 levels may decrease exhaustively in a hypoxic environment. This may be related to the degree of injury and prognosis.

4.2 Thyroid hormone

To adapt to the hypoxic environment at high altitudes, a series of changes will take place in the body, including complex changes in the endocrine system and metabolic function. Produced by the largest endocrine gland in the human body, TH has many biological effects on the body. Research shows that it has excitatory effects on almost all tissues and has a certain correlation with energy metabolism (proteins, sugars, and fats), thermoregulation, tissue differentiation, and growth and development of bodies (Maria et al., 2019). Hypoxia at high altitudes leads to changes in thyroid function and structure (Naeije, 2010); however, the results of previous studies on the changes in TH caused by hypoxia are inconsistent (Rawal et al., 1993; Savourey et al., 2004; Barnholt et al., 2006). Thyroid function has been shown to be

enhanced, weakened, or even unchanged, which is related to many factors such as the exposure mode of hypoxia, altitude, duration, and availability of altitude adaptive training. Naoto Tani et al. (Tani et al., 2020a) found that an increase in thyroid-related hormones may indicate systemic hypoxia/ischemia, that is, thyroid-related hormones may be a marker of acute systemic hypoxia/ischemia. The metabolism in HAPE rats suggested that this may be an adaptive regulation method for cells to reduce the basic metabolic rate in a hypoxic environment and that the level of thyroid-stimulating hormone is closely related to the severity of hypoxia and prognosis of the disease. The lower the circulating levels of TH, the more serious the hypoxic injury (Tani et al., 2020b; Zeng et al., 2021). In conclusion, improving the examination of TH may further clarify the severity of hypoxia in patients with HAPE, suggesting that it may be used as a new biomarker for HAPE diagnosis.

4.3 Antioxidant substances

Under normal physiological conditions, the oxidation and antioxidant systems of the body are in dynamic balance. Previous studies have confirmed that hypoxic stress can induce oxidative stress in the body (Lin et al., 2020), in which GSH is a representative substance of antioxidants. It can directly remove reactive oxygen species (ROS) and protect cells from ROS damage under the action of GSH peroxidase. Under the stimulation of hypoxia, the levels of antioxidants such as GSH and GSSG in the body will decrease significantly, further leading to oxidative damage and inflammatory responses (Sinha et al., 2009; Chu et al., 2016). Compared with the control group, the levels of GSH and GSSG in the serum of HAPE rats were significantly decreased in both arterial blood and venous blood, suggesting that the antioxidant capacity of the body decreased. At present, GSH and GSSG are considered to be the main biomarkers after oxidative stress injury to tissues and cells (Coimbra-Costa et al., 2017). The above also further clarified the oxidative damage and inflammatory response in the process of HAPE occurrence and development.

In addition to GSH, ALA and other antioxidants were significantly decreased in the serum of HAPE model rats. Interestingly, however, VC levels, which have strong antioxidant capacity, increased in rats after hypoxia stimulation, which is inconsistent with some previous research results (Sureda et al., 2004; Wu et al., 2022). This may be related to the long half-life of VC and adaptive regulation of the body; however, further studies are needed to clarify this.

4.4 Bile acid

BA is a key component of the body's normal metabolism. In recent years, many studies have shown that metabolic disorders of BA are related to a variety of disease states in the body (Jia et al., 2017), and the hypoxic environment also leads to disorders of the BA pool in the body (Ramakrishnan et al., 2014). Zhang et al. (Staley et al., 2017) found that this may be related to the change in intestinal flora caused by hypoxia. When the body is in a hypoxic environment, the composition and quantity of intestinal microorganisms in the body change significantly. Additionally, as the key material of BA transformation, intestinal microorganisms will inevitably affect the normal steady state of the BA pool. Significant changes take place in the BA pool during HAPE that further affect the activation of various receptors and metabolism of

the abovementioned three major nutrients in the body (Xu, 2018). As an amphiphilic steroid molecule, metabolic disorders of BA lead to poor metabolism of fat and cholesterol (CHOL) (You et al., 2017; Jia et al., 2020). Previous studies have found that the steroid hormone synthesis pathway has a key role in acute hypoxic injury, and inflammatory factors in HAPE are significantly upregulated (Qian et al., 2018). The CHOL regulatory element protein 1c, a key molecule in the metabolism of CHOL and BA, can inhibit the over-activation of p38 MAPK/NF- κ B (Chen et al., 2004; Li et al., 2015), which has a role in inhibiting the inflammatory response in HAPE. As a target closely related to inflammatory factors, BA may provide a new direction for the prevention and treatment of altitude sickness. However, the specific reasons why all types of BA show different change characteristics in a hypoxic environment require further in-depth investigation, as do the differential changes in the BA pool; these may be related to many factors such as the hypoxic stress mode (time, altitude, speed of entry into high-altitude areas) and body physiological state.

There are obvious changes in the metabolites in the organism during the occurrence of HAPE, and the differences of metabolites in arteriovenous blood are also obvious. Combined with two machine algorithms and KEGG enrichment analysis, we speculate that D-mannose^{DOWN}, GSSG^{DOWN}, glutathione disulfide^{DOWN}, and DHCA^{DOWN} in arterial blood play key roles in predicting the occurrence of HAPE, whereas in venous blood, L-Leu^{DOWN}, L-T4^{DOWN}, and 4-D-Hyp^{DOWN} play key roles in predicting the occurrence of HAPE.

5 Conclusion

At present, the complex pathogenesis of HAPE remains unclear. This study systematically analyzed the metabolites of HAPE model rats using class-targeted metabolomics data, further clarifying the changes in metabolism of the body during HAPE and preliminarily confirming that D-mannose^{DOWN}, GSSG^{DOWN}, glutathione disulfide^{DOWN}, and DHCA^{DOWN} in arterial blood and L-Leu^{DOWN}, L-T4^{DOWN}, and 4-D-Hyp^{DOWN} in venous blood play key roles in predicting the occurrence of HAPE. Our results lay a strong foundation for further research. Interestingly, the biomarkers in arteriovenous blood after HAPE were inconsistent, which provides new ideas for clinical diagnosis and treatment and also points to the need for further research on this mechanism. Our findings may help in identifying useful molecular targets in the diagnosis and treatment of HAPE, providing a new perspective for further diagnosis and treatment of plateau disease. However, owing to the complexity of metabolomics data, not all metabolites were systematically analyzed in this study, only the key metabolites presented in this data were analyzed. In the follow-up study, we will further analyze various metabolites in the arteriovenous blood of HAPE rat model and people who rush to plateau, and further reveal their changes and mechanisms.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Laboratory Animal Ethics Committee, Medical College of Qinghai University.

Author contributions

XL and CD mainly reviewed relevant literature, collated literature and drafted the article; ZC is mainly responsible for the management and coordination responsibility for the research activity planning and execution; XP and TZ revised it critically for important intellectual content and approved the version to be published.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2023.1122026/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

The heatmap of metabolites in each group. (A, C) Heat maps of the CA and HV groups compared with the CV group metabolites; (B, D) Heat maps of the HV and CA groups compared with the HA group metabolites

SUPPLEMENTARY FIGURE S2

The key metabolites expression of each group. Compared with CV group, CA group ** $p < 0.05$; Compared with HA group, CA group ## $p < 0.05$, CV group ** $p < 0.05$; Compared with HV group, CA group ^{ss} $p < 0.05$, CV group ^{ss} $p < 0.05$, HA group ^{ss} $p < 0.05$

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Glossary

AMS Acute mountain sickness

HAPE High altitude pulmonary edema

KEGG Kyoto Encyclopedia of Genes and Genomes

CKD chronic kidney disease

SPF Specific pathogen-free

H&E Hematoxylin and eosin

TEM Transmission electron microscope

MRM Multiple Reaction Monitoring

PLS-DA Partial least squares discriminant analysis

RF Random forest

SVM Support vector machines

ANOVA The one-way analysis of variance

Lys Lysine

Arg Arginine

Pro Proline

Trp Tryptophan

TH Thyroid hormone

G6P D-glucose 6-phosphate

M6P D-mannose 6-phosphate

F6P D-fructose 6-phosphate

ACAC Acetoacetate

His Histidine

Ccp Citrulline

Ala Alanine

Gly Glycine

Cys Cystine

Asp Aspartic acid

N-Ac-L-Tyr N-acetyl-L-tyrosine

MSG Sodium glutamate

Ile Isoleucine

ANA Acetylneuraminic acid

GSH Glutathione

GSSG Oxidized glutathione

ALA Lipoic acid

VC Vitamin C

BA Bile acid

TCA Taurocholic acid

TUDCA Sodium taurodeoxycholate

GDCA Glycodeoxycholic acid

CDCA Chenodeoxycholic acid

DCA Deoxycholic acid

3 β -UDCA 3 β -Ursodeoxycholic acid

THDCA Sodium taurine porcine deoxycholic acid

β -MCA Beta mouse cholic acid

L-T4 Levothyroxine

VB2 Vitamin B2

L-GA L-(-)-Glyceric acid

IMA Imatinib

Lyp 16:1 LysoPC 16:1

4-HPPA 4- Hydroxyphenylpyruvate

DHCA Dehydrocholic acid

NAR Nicotinate ribonucleoside

PIP Piperidine

OAS O- acetyl-L-serine

NAAG N- acetyl-aspartate

Lyp 20:0 LysoPC 20:0

L-Leu L- Leucine

L-allo-Ile L-allo-isoleucine

DL-Leu DL-Leucine

4-D-Hyp Cis-4-hydroxy-D-proline

2-P 2- Pyrrolidinone

Val Valine

Ser Serine

Tyr Tyrosine

MT Melatonin

VB Vitamin B

ROS Oxygen species

CHOL Cholesterol



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Parental sex-dependent effects of either maternal or paternal eNOS deficiency on the offspring's phenotype without transmission of the parental eNOS deficiency to the offspring

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Background: Preclinical animal studies and clinical studies indicate that both maternal as well as paternal genetic alterations/gene defects might affect the phenotype of the next-generation without transmissions of the affected gene. Currently, the question of whether the same genetic defect present in the mother or father leads to a similar phenotype in the offspring remains insufficiently elucidated.

Methods: In this head-to-head study, we crossbred female and male mice with heterozygous endothelial eNOS knockout (eNOS+/-) with male and female wild-type (wt) mice, respectively. Subsequently, we compared the phenotype of the resulting wt offspring with that of wt offspring born to parents with no eNOS deficiency.

Results: Wt female offspring of mothers with heterozygous eNOS showed elevated liver fat accumulation, while wt male offspring of fathers with heterozygous eNOS exhibited increased fasting insulin, heightened insulin levels after a glucose load, and elevated liver glycogen content. By quantitative mass-spectrometry it was shown that concentrations of six serum metabolites (lysoPhosphatidylcholine acyl C20:3, phosphatidylcholine diacyl C36:2, phosphatidylcholine diacyl C38:1, phosphatidylcholine acyl-alkyl C34:1, phosphatidylcholine acyl-alkyl C36:3, and phosphatidylcholine acyl-alkyl C42:5 (PC ae C42:5) as well as four liver carbon metabolites (fructose 6-phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate and fumarate) were different between wt offspring with eNOS+/- mothers and wt offspring with eNOS+/- fathers. Importantly, fumarate was inversely correlated with the liver fat accumulation in female offspring with eNOS+/- mothers and increased liver glycogen in offspring of both sexes with eNOS+/- fathers. The qRT-PCR results revealed that the gene expression patterns were different between wt

offspring with eNOS+/- mothers and those offspring with eNOS+/- fathers. Different gene expression patterns were correlated with different observed phenotypic changes in male/female offspring born to mothers or fathers with a heterozygous eNOS genotype.

Conclusion: The identical parental genetic alteration (heterozygous eNOS deficiency), without being passed on to the offspring, results in distinct metabolic, liver phenotype, and gene expression pattern variations depending on whether the genetic alteration originated from the father or the mother.

KEYWORDS

maternal and paternal programming, eNOS, metabolomics, sex-dependent effects, offspring

Introduction

The “fetal programming” hypothesis suggests that during a crucial early stage of life, specific environmental and nutritional factors can lead to permanent changes in organ structure and function in response to environmental influences. These alterations may, in turn, increase the risk of developing cardiovascular and metabolic diseases later in life (Reichetzeder et al., 2016). The fundamental factors contributing to fetal programming encompass maternal undernourishment during pregnancy, overnutrition, obesity, diabetes, and exposure to harmful toxins (Reichetzeder et al., 2016). A novel mechanism, initially proposed by Hocher et al. (2000) and subsequently validated by other researchers (Masuda et al., 2002; van Beynum et al., 2006; Tsai et al., 2008; Miodovnik et al., 2012; Warrington et al., 2019), suggests that maternal genes may influence the fetal phenotype independently of the fetal genome. Similarly, existing studies have already suggested that paternal genes, even without being transmitted to the offspring, may also influence the phenotype of the offspring (Nelson et al., 2010; Chen et al., 2012; Li et al., 2016; Zhang et al., 2019; Liu et al., 2021; Zhang et al., 2022).

In our prior research, we conducted crossbreeding experiments involving female and male heterozygous eNOS knockout mice and male and female wt mice. We then compared the phenotype of the resulting wt offspring from these crosses with that of wt offspring born to parents who were both wt mice (Hocher et al., 2016; Hocher et al., 2022). We specifically selected eNOS knockout mice for these experiments due to the critical role that eNOS plays in regulating placental and vascular functions (Kulandavelu et al., 2012; Kusinski et al., 2012). Heterozygous eNOS deficiency has been conclusively linked to the development of an adverse intrauterine environment. Remarkably, this environmental factor can exert a significant influence on the vascular phenotype of offspring (Costantine et al., 2008). In addition, the presence of heterozygous eNOS deficiency in male mice can potentially lead to an unfavorable testicular microenvironment, primarily attributed to impaired testicular vascular function (Hocher et al., 2022). The findings from our prior research indicated that female offspring with wt genetics, born to mothers with heterozygous eNOS deficiency, developed fatty liver disease. Wt male offspring born to fathers with heterozygous eNOS deficiency exhibited elevated fasting insulin levels, as well as increased insulin levels following glucose ingestion (Hocher et al., 2016; Hocher et al., 2022). However, until now, we have not conducted a comprehensive study comparing the

effects of maternal and paternal eNOS deficiency on genetically healthy offspring head-to-head. Our overarching goal is to gain a deeper understanding of phenotypic variations and uncover potential molecular mechanisms, particularly in relation to differences in glycemic control.

Metabolomics is a rapid, robust, and efficient research tool that analyses many small molecules of biochemical pathways in tissue, blood, urine, and other biological fluids (Hocher and Adamski, 2017; Lu et al., 2018; Yong-Ping et al., 2020). Metabolites are influenced by both endogenous regulatory mechanisms and the environment (Bachlechner et al., 2016; Feldman et al., 2017; Boone et al., 2019). In this study, we thus used metabolomics to gain a more profound understanding of the distinctions between genetically healthy offspring originating from either mother or father with heterozygous eNOS deficiency.

Materials and methods

Breeding and study protocol

The whole study protocol received approval from the animal welfare committee in Berlin, Germany, and was conducted in accordance with the relevant local institutional guidelines. ENOS knockout mice (strain B6.129P2-Nos3tm1Unc/J) and C57BL/6J control mice were sourced from Jackson Laboratories (Bar Harbour, ME). Animals were housed at a controlled environment (21°C ± 2°C, 50% ± 10% relative humidity and a 12:12h light-dark cycle) and had access to food and water *ad libitum*. A comprehensive description of the breeding procedure was shown in **Supplementary Figure S1**. Female wild-type (wt) mice were bred with male eNOS knockout mice (eNOS-/-) to produce F1 offspring with heterozygous eNOS (eNOS+/-) genotypes. Subsequently, female F1 mice with heterozygous eNOS knockout and male F1 mice with heterozygous eNOS knockout were once again bred with male and female wt mice, respectively. Heterozygous animals used for breeding of the F2 generation were all derived from different dams, i.e., siblings were not used. In addition, the F1 mice chosen for breeding the subsequent generation were matched in terms of age. Following parturition by the female, we promptly standardized the litter size to ten pups (comprising five males and five females). These newborns were nurtured until weaning at 21 days of age. Only the F2 wt offspring resulting from this breeding procedure were included in the study, and

they were compared to wt offspring born to parents who were both wild type.

The male and female F2 offspring were raised for 24 weeks and subsequently underwent separate analyses, including measurements of birth weight, final body weight, and liver weight. Blood pressure was measured in the 24th week by using the tail-cuff method, as previously described (Quaschnig et al., 2007). In the 21st week, an intraperitoneal glucose tolerance test (IPGTT) was conducted. In brief, the animals underwent an overnight fasting period, followed by intraperitoneal injection of 2 mg glucose per gram of body weight. Blood samples were then collected from the tail vein at 0, 15, and 60-min intervals to measure plasma glucose and insulin levels, following previously established protocols (Kim et al., 2010; Wang et al., 2010; Du et al., 2012). The trapezoid rule was used to determine the area under curve (AUC) for glucose and insulin concentrations in each animal.

Liver morphology

Liver morphology was analyzed under the microscope using two different stainings: Hematoxylin and Eosin (H&E), and Red Oil Staining.

- a) H&E Staining: for preparation, the livers were washed with phosphate-buffered saline (PBS) buffer, then fixed with 4% (w/v) paraformaldehyde and embedded in paraffin. 3 μ m thick slices were obtained using a Microm HM230 Microtomy and then stained with H&E. We identified hepatic venules and their adjacent portal fields by the sinusoidal connection between them. Ten lobules of each slide were identified using a Zeiss Axiovert 100 microscope (200 x), photographed with a Leica EC3 digital camera, and saved using LAS EZ software (Leica Microsystems). We measured the linear lobular dimensions by determining the distance from the center of the hepatic vein to the center of three associated portal vein branches using ImageJ (version 1.410, NIH shareware). Subsequently, we calculated the mean radius of lobules for each animal.
- b) Oil Red O Staining was done as described before (Koopman et al., 2001). 30 pictures were taken per sample using an Olympus (Shinjuku, JP) BH-2 microscope (200x) and a digital camera CFW-1310C (Scion Corporation, Frederick, MD). The lipid content and the density of lipid droplets were quantified with ImageJ (version 1.410, NIH shareware).

Liver glycogen content

Liver tissue was incubated with 1N KOH (95°C, 30 min). Glycogen was precipitated using saturated sodium sulfate solution (Na₂SO₄) and 95% (v/v) ethanol and washed twice in 60% (v/v) ethanol. Resuspended glycogen was degraded with 0.1% (w/v) amyloglucosidase (Sigma-Aldrich, St. Louis, MO) in acetate buffer (0.2 M sodium acetate, 0.46% (v/v) acetic acid, pH 4.8) for 2 h at 40°C. Glucose concentration was measured photometrically using the Glucose (HK) Assay Kit (Sigma-Aldrich). Glycogen content was expressed in relation to liver weight.

Pancreas morphology

- a) H&E Staining: pictures of whole tissue slides and of every islet of Langerhans were taken using the Zeiss Axiovert 100 microscope (25x/200x) and the Leica EC3 digital camera. The islets were counted, and the islet area was measured using ImageJ software to calculate the islet density and the mean islet area per slide.
- b) Pancreas immunohistology: The beta cell content of islets of Langerhans was measured using immunohistological staining of insulin. We used an antibody against insulin (1:200, ab181547, abcam, Cambridge, United Kingdom) and a secondary antibody (1:500, ab97051, abcam, Cambridge, United Kingdom) diluted in antibody diluent (Dako, Glostrup, DK), and for visualization the ABC staining system (sc 2023, Santa Cruz Biotechnology, Santa Cruz, CA) following the instructions provided by the manufacturer. All islets per slide were photographed using an Olympus BH-2 microscope (200x) and a CFW-1310C digital camera. Thirty images for each sample were taken. The islet area and beta cell content were measured using ImageJ. The average islet size was obtained by total islet area/islet amount in each sample. The beta cell content was determined by the insulin positive staining area.

Metabolomic profiles in serum

At the end of the experiment, blood was obtained via retro-orbital collection. The blood samples were then centrifuged at 3,000 $\times$ g for 10 min at 4°C to obtain serum. Afterwards, we analysed serum metabolomic profiles using the Absolute IDQTM p150 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) and flow injection analysis-tandem mass spectrometry (FIA-MS/MS), following the kit's instructions. Detailed information on the procedure of metabolite quantification has been previously provided (Lu et al., 2018; Yong-Ping et al., 2020). We quantified a total of 163 targeted metabolites simultaneously from 10 μ L of serum. These included 92 glycerophospholipids [comprising 15 lysophosphatidylcholines (LPC) and 77 phosphatidylcholines (PC)], 40 acylcarnitines (acylC), free carnitine, 14 amino acids (13 of which were proteinogenic, plus ornithine), hexoses, and 15 sphingolipids (SM) (Yong-Ping et al., 2020). The quantification of metabolite concentrations [μ M] was based on internal standards.

Determination of central carbon metabolites in liver tissue

Liver tissues (15 to 30 mg each) were homogenized using a Fast Prep FP 120 homogenizer (Thermo Savant, Holbrook, NY) with 1 mL of phosphate buffer (pH 7.4) at a speed setting of 6.0, employing lysing matrix D. After homogenization, aliquots of the resulting homogenate were promptly frozen at -80°C until further analysis.

The concentrations of metabolites in the liver tissue homogenate were assessed using both gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS), following previously established protocols (Hofmann et al., 2008; Maier et al., 2010). We spiked defined volumes of liver homogenate (as outlined below) with an internal standard solution.

These samples were then subjected to evaporation until dryness using a stream of nitrogen, followed by derivatization. Subsequently, we conducted the analysis using gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was carried out using a 5975C inert XL MSD, coupled with a 7890A GC from Agilent Technologies in the Electron Impact (EI) mode.

Concentrations of 3-hydroxybutyrate, malate, citrate, and fumarate were determined in 5 μ L liver tissue homogenate using the respectively labeled analogs 3-hydroxy- 13 C₄ butyrate, 13 C₄ fumarate, 13 C₄ malate, and 13 C₄ citrate as internal standards. Following the evaporation step, the samples underwent derivatization to form methyloxime tert-butyldimethylsilyl derivatives (Hofmann et al., 2008).

To determine glucose 6-phosphate (G-6-P) and fructose 6-phosphate (F-6-P) concentrations, 25 μ L liver homogenate was spiked with the internal standards 13 C₆-G-6-P and 13 C₆-F-6-P, evaporated to dryness, and derivatized to the trimethylsilyl derivatives.

Concentrations of ribose 5-phosphate (Rib-5-P), ribulose 5-phosphate/xylulose 5-phosphate (Rib-5-P), sedoheptulose 7-phosphate (Sed-7-P), phosphoenolpyruvate (PEP), 6-phosphogluconate (6-PG), 2-/3-phosphoglycerate (3-PG), and fructose 1,6-bisphosphate (FBP) were determined in 10 μ L of liver homogenate and analysed by LC-MS-MS as described (Maier et al., 2010).

Quantitative real time PCR

We analysed a list of candidate genes involved in NO-synthase, metabolic process, energy homeostasis and fat storage, insulin-like growth factors and their binding proteins. RNA extraction from liver tissue and reverse transcription PCR were done like previously described (Hochoer et al., 2022). Primers were obtained from Sigma-Aldrich, Eurofins (Ebersberg, GER) and shown in the [Supplementary Table S1](#). The PCR was carried out on a Mx3000P thermal cycler (Stratagene, La Jolla, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), Sensi Mix or SensiFast low ROX kit (Bioline, London, United Kingdom) in accordance with the instructions. All samples were analysed in triplicate. The relative quantity of analysed genes was determined with the $\Delta\Delta$ Ct method as described before (Hochoer et al., 2022).

Statistics

Statistical analysis was performed using SPSS version 22.0. All values are presented as mean $\pm$ SEM. For all datasets, we applied a two-way analysis of variance (two-way ANOVA), followed by *post hoc* Tukey test. Metabolomics data of serum were analysed using MetaboAnalyst 3.0. To mitigate the false discovery rate (FDR), we employed the Benjamini-Hochberg (BH) procedure to adjust *p*-values. The BH procedure is defined as follows: $P_m \leq (m/M) \times q$, where “*m*” represents the rank of a given *p*-value, “*M*” is the total number of tests (*M* = 163), and “*q*” is the desired FDR threshold (set up at 0.05 in this study) (Madar and FastLSU, 2016). For normally distributed data, we used the Pearson correlation analysis. Statistically significant differences were defined as *p*-value $\leq$ 0.05.

Results

Parental eNOS deficiency had no impact on birth weight, body weight, organ weight, and blood pressure of wt offspring

Male and female wt offspring with either eNOS+/- fathers or eNOS+/- mothers showed no significant differences in birth weight, final body weight, relative liver weight, or blood pressure when compared to the control group ([Supplementary Table S2](#)).

Paternal eNOS deficiency led to an increase in fasting plasma insulin in male wt offspring

No differences in fasting plasma glucose were observed in male or female offspring born to eNOS+/- mothers/fathers. A significant increase in fasting plasma insulin was observed only in male wt offspring with eNOS+/- fathers ([Supplementary Table S2](#)).

Paternal eNOS deficiency led to increased plasma insulin after glucose intake in male wt offspring

IPGTT result showed that no differences in glucose concentrations at different time points after glucose intake and area under curve (AUC) of glucose in male or female offspring with eNOS+/- fathers/mothers ([Figures 1A–C](#)). Regarding insulin concentration, elevated insulin levels were observed at the 0 and 60 min after glucose intake in male offspring with eNOS+/- fathers ([Figure 1D](#)). Higher insulin levels at the 15 min after glucose intake were observed in female offspring with eNOS+/- fathers ([Figure 1E](#)). In addition, male offspring with eNOS+/- fathers had a significantly higher AUC of insulin ([Figure 1F](#)). No differences in insulin concentrations at different time points after glucose intake and AUC of insulin could be observed in male or female offspring born to eNOS+/- mothers.

Parental eNOS deficiency affected liver morphology of wt offspring

Liver lobule dimensions were consistent and similar among all groups. A significantly higher lipid droplet density and liver fat content were observed in female offspring born to eNOS+/- mothers ([Supplementary Table S2](#); [Figures 2A, B](#)). However, the liver glycogen content was significantly higher in animals of both sexes born to eNOS+/- fathers ([Figure 2C](#)).

Parental eNOS deficiency had no impact on pancreas morphology of wt offspring

The size and density of pancreatic islets of Langerhans, as well as the beta-cell content within the islets, exhibited no significant differences among all groups ([Supplementary Figure S2](#); [Supplementary Table S2](#)).

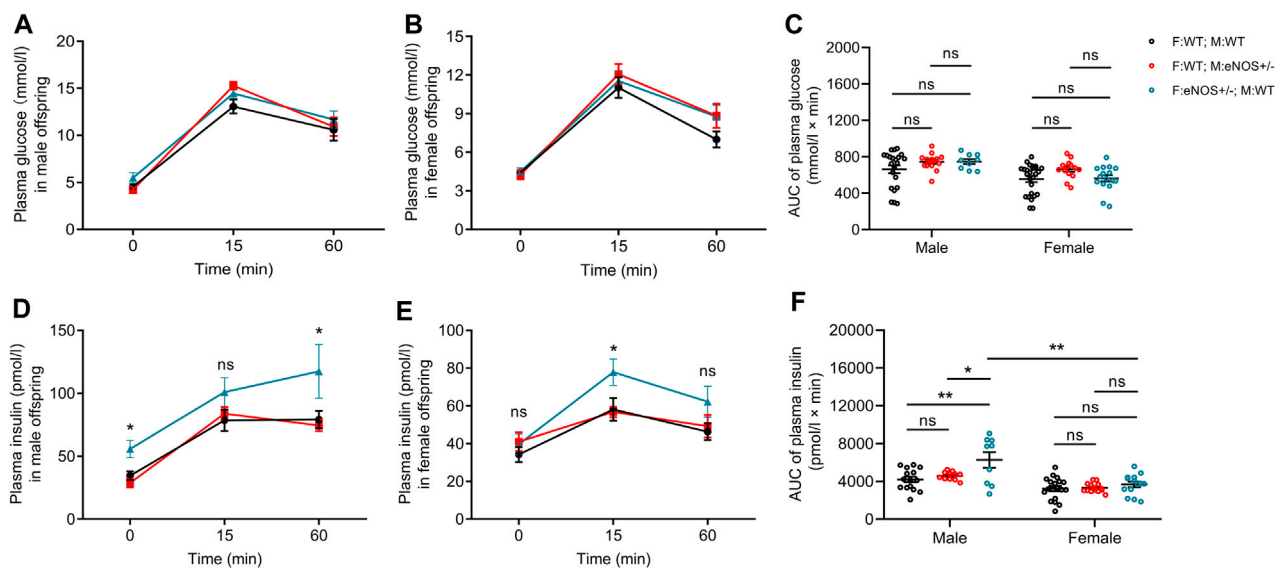


FIGURE 1

Comparison of plasma glucose (A,B) and insulin concentrations (D,E) during IPGTT in male (A,D) and female (B,E) offspring; AUC for IPGTT plasma glucose (C) and IPGTT plasma insulin (F) in male and female offspring. Black: F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers (male: $n = 22$ and female: $n = 28$); Red: F:WT; M: eNOS+/-: wildtype offspring of wildtype fathers and eNOS heterozygous mothers (male: $n = 15$ and female: $n = 14$); Blue: F: eNOS+/-; M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers (male: $n = 9$ and female: $n = 14$). The data were presented as mean $\pm$ SEM and analysed by two-way ANOVA followed by *post hoc* Tukey test. * $p < 0.05$; ** $p < 0.01$; ns: $p > 0.05$.

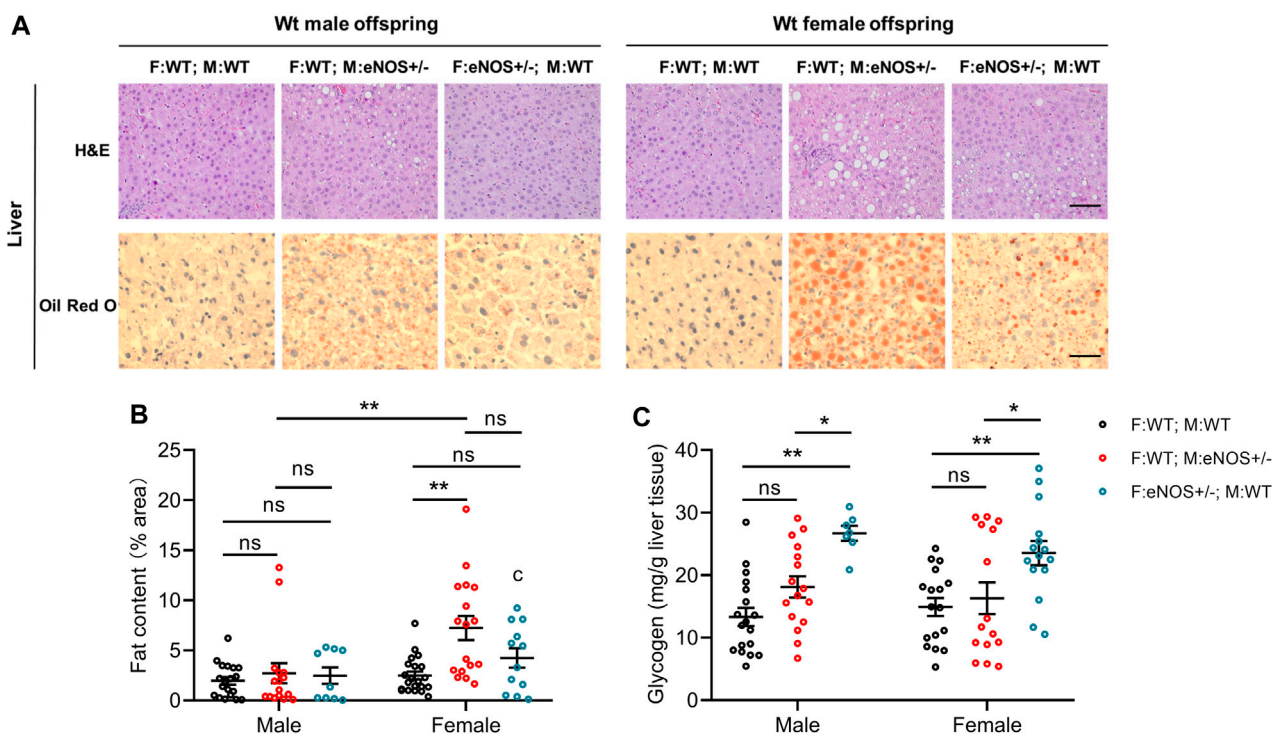
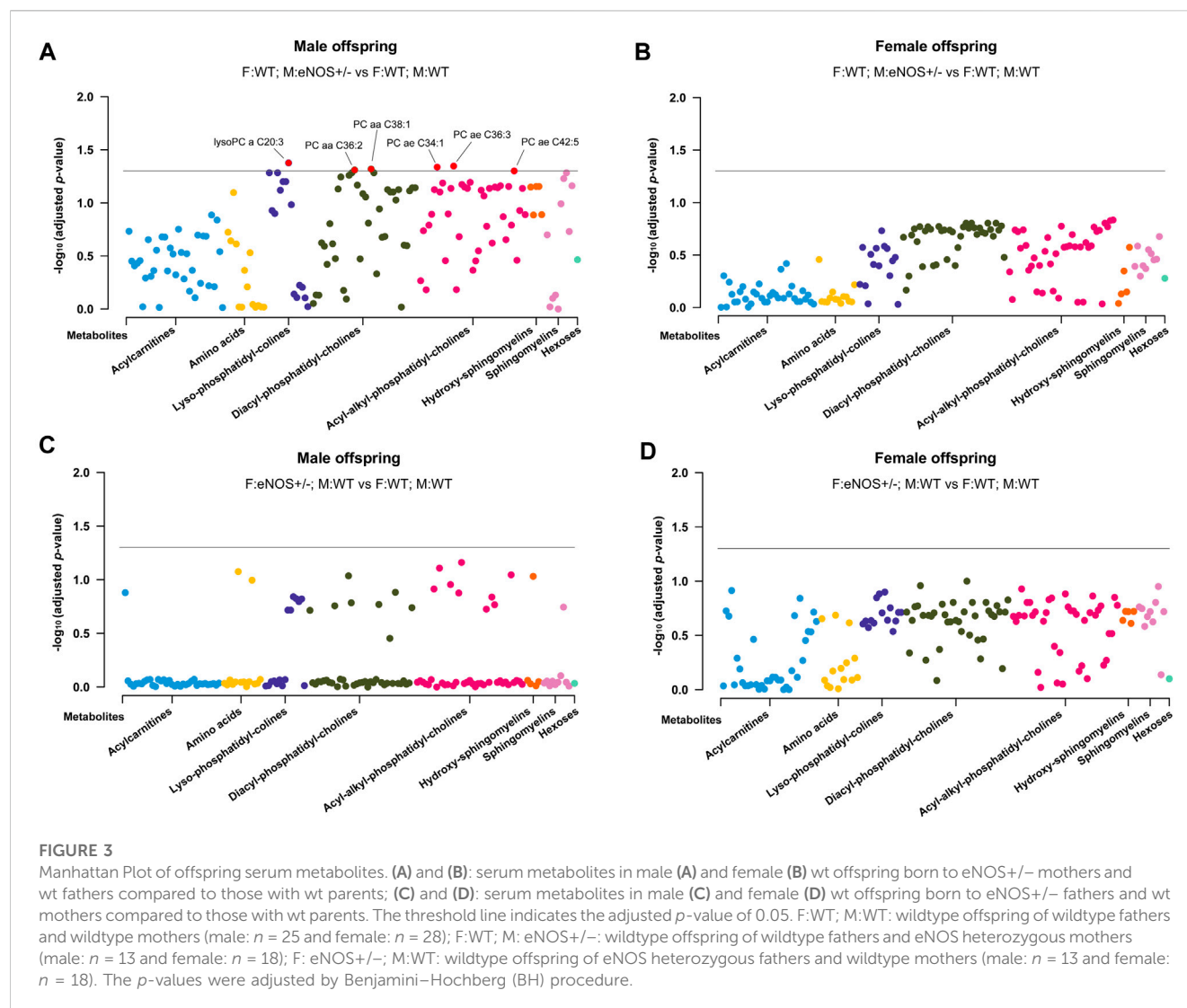


FIGURE 2

Morphological analysis of liver tissues. (A): Representative images of haematoxylin and eosin (H&E) and Oil red O staining of liver sections (magnification: $\times 200$ and scale bar: $50 \mu\text{m}$); (B): fat content and (C): glycogen in the liver of wt offspring. Black: F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers (male: $n = 19$ and female: $n = 21$); Red: F:WT; M: eNOS+/-: wildtype offspring of wildtype fathers and eNOS heterozygous mothers (male: $n = 16$ and female: $n = 17$); Blue: F: eNOS+/-; M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers (male: $n = 9$ and female: $n = 12$). The data in (B) and (C) were presented as mean $\pm$ SEM and analysed by two-way ANOVA followed by *post hoc* Tukey test. * $p < 0.05$; ** $p < 0.01$; ns: $p > 0.05$.



Parental eNOS deficiency led to differing metabolomic profiles in the serum of wt offspring

After adjusting the p -values using the Benjamini–Hochberg procedure, six metabolites (lysoPC a C20:3, PC aa C36:2, PC aa C38:1, PC ae C34:1, PC ae C36:3, and PC ae C42:5) of the 163 targeted serum metabolites were significantly reduced in male offspring born to eNOS+/- mothers and wt fathers ($p < 0.05$ and FDR < 0.05) (Figure 3). No metabolite was significantly different in female offspring with eNOS+/- mothers or eNOS+/- fathers and male offspring with eNOS+/- fathers (for more details see Supplementary Table S3).

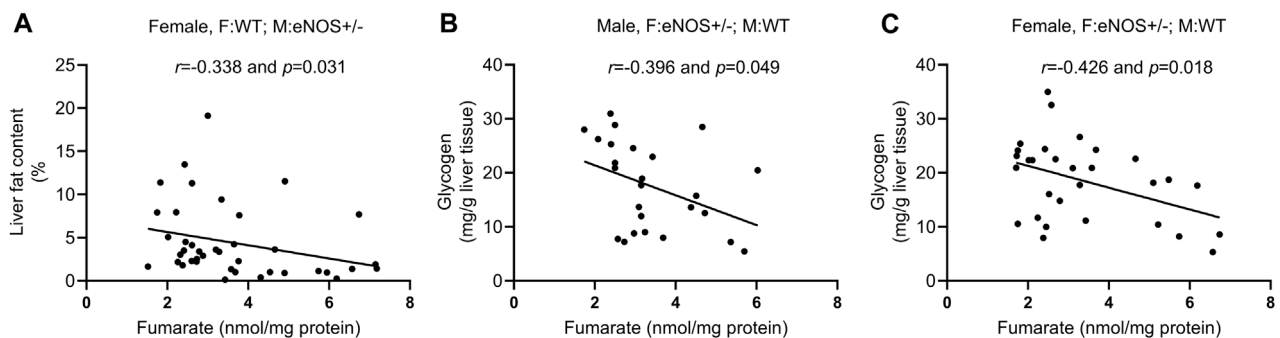
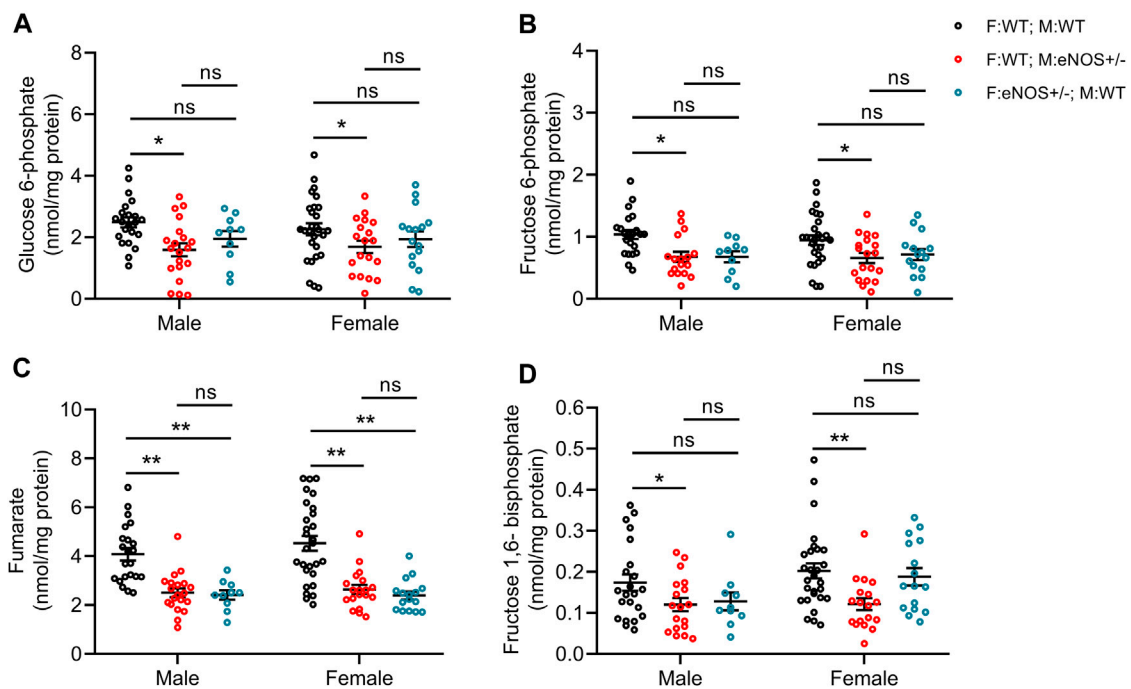
Parental eNOS deficiency led to differing liver carbon metabolites in liver tissue of wt offspring

We quantified selected substrates of glucose metabolism in liver tissue using both GC-MS and LC-MS-MS technology. In

both male and female offspring with eNOS+/- mothers, we observed significantly lower concentrations of fructose 6-phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate and fumarate. Regarding the offspring with eNOS+/- fathers, both male and female offspring displayed significant reduction in fumarate concentration (Figure 4; Supplementary Table S4).

Correlation analysis showed the relationship between altered metabolites in liver/serum and phenotype in wt offspring

In wt offspring with eNOS+/- mothers, glucose 6-phosphate was negatively correlated with fat content in male offspring ($r = -0.401$, $p < 0.05$). Glucose 6-phosphate ($r = 0.393$, $p < 0.05$), fructose 6-phosphate ($r = 0.388$, $p < 0.05$), and fructose 1,6-bisphosphate ($r = 0.404$, $p < 0.05$) were positively correlated with glycogen content in female offspring. Fumarate was negatively correlated with the liver fat content in female offspring ($r = -0.338$, $p < 0.05$) (Figure 5A). LysoPC a C20:3 was



positively correlated with the AUC of plasma insulin in female offspring ($r = 0.448$, $p < 0.05$). PC ae C42:5 was positively correlated with fat content in male offspring ($r = 0.368$, $p < 0.05$) (for more details see [Supplementary Table S5](#)).

In wt offspring with eNOS+/- father, fumarate was negatively correlated with glycogen content in both sexes ($r = -0.396$, $p < 0.05$; $r = -0.426$, $p < 0.05$) ([Figures 5B, C](#)). LysoPC a C20:3 had a positive

correlation with fat content in male offspring ($r = 0.394$, $p < 0.05$) and glycogen content in female offspring ($r = 0.375$, $p < 0.05$). PC aa C38:1 was negatively correlated with glycogen content in male offspring ($r = -0.459$, $p < 0.05$). LysoPC a C20:3 ($r = 0.460$, $p < 0.01$), PC aa C38:1 ($r = 0.404$, $p < 0.05$), and PC ae C34:1 ($r = 0.409$, $p < 0.05$) were positively correlated with AUC of plasma insulin in female offspring (for more details see [Supplementary Table S6](#)).

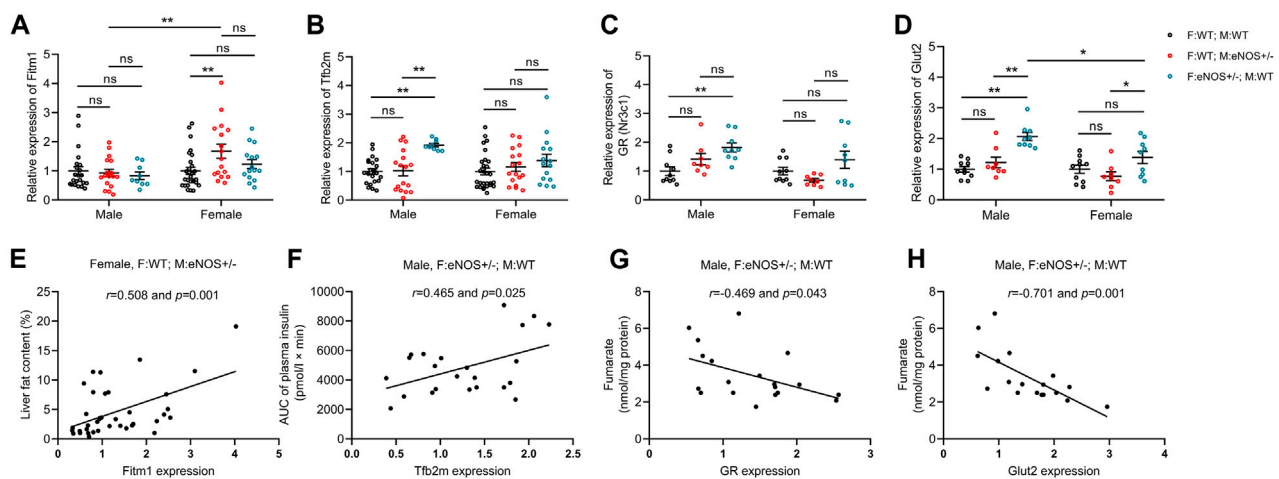


FIGURE 6

Gene expression (fold expression compared to control group) was analysed by real time PCR (A–D) and correlation analysis between altered genes and phenotypic changes (E, F) in wt offspring. (G, H): correlation between altered genes and fumarate in male wt offspring with eNOS^{+/-} fathers. Black: F: WT; M: WT: wildtype offspring of wildtype fathers and wildtype mothers (male: $n = 22$ and female: $n = 28$); Red: F: WT; M: eNOS^{+/-}: wildtype offspring of wildtype fathers and eNOS heterozygous mothers (male: $n = 17$ and female: $n = 17$); Blue: F: eNOS^{+/-}; M: WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers (male: $n = 9$ and female: $n = 15$). The data in (A–D) were presented as mean $\pm$ SEM and analysed by two-way ANOVA followed by *post hoc* Tukey test. * $p < 0.05$; ** $p < 0.01$; ns: $p > 0.05$.

Parental eNOS deficiency resulted in different gene expression patterns in liver tissue of wt offspring

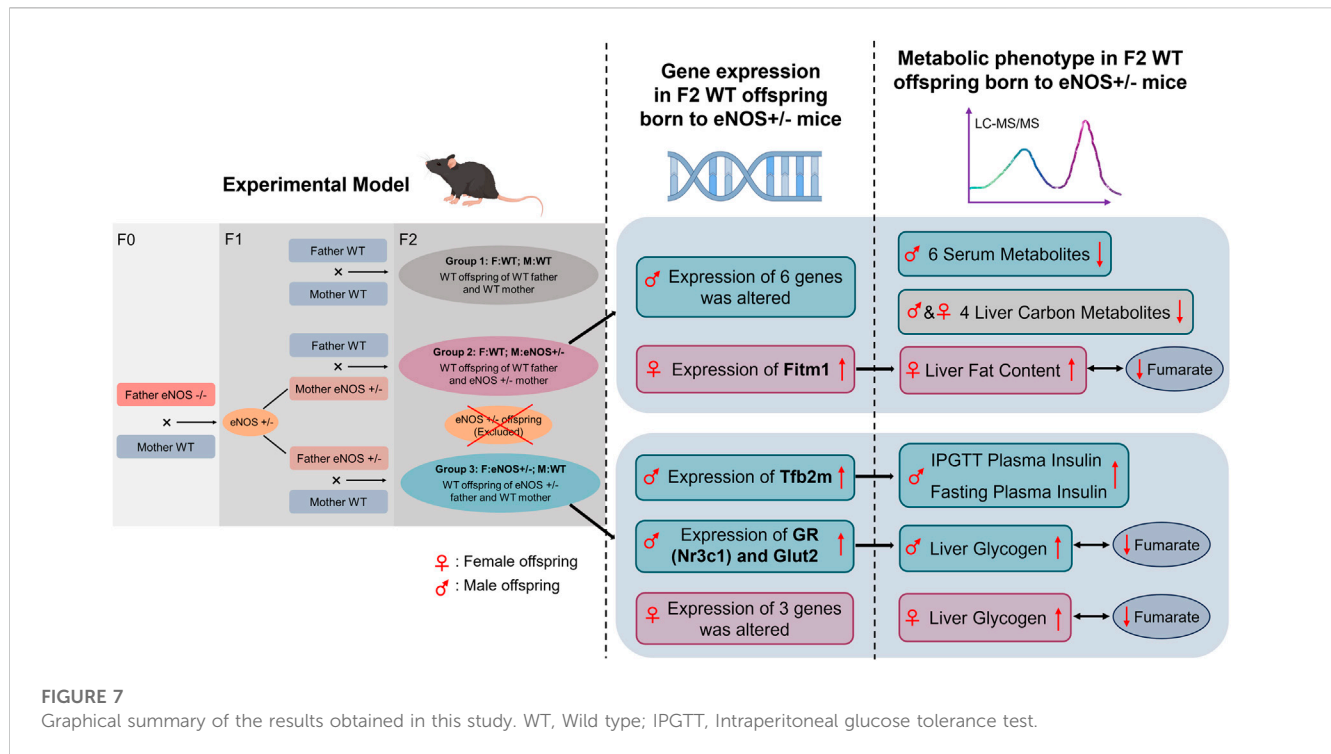
The qRT-PCR results revealed that the gene expression patterns were different between wt offspring with eNOS^{+/-} mothers and those offspring with eNOS^{+/-} fathers. In wt offspring with eNOS^{+/-} mothers, PPAR γ was significantly decreased while Pepck, Igf1, Igf2, Igfbp1 and Igfbp2 were significantly increased in male offspring. The expression of Srebp1c and Gck were significantly reduced while Fitm1 was significantly increased in female offspring. In wt offspring with eNOS^{+/-} fathers, 12 genes were differentially expressed in male offspring and 3 genes were differentially expressed in female offspring. See more details in the [Supplementary Table S7](#).

Correlation analysis showed the relationship between altered genes and phenotypic changes in wt offspring

As shown in [Figure 6](#), the liver fat ratio showed a significant correlation with the expression of Fitm1 ($r = 0.508$, $p < 0.01$) in female offspring with eNOS^{+/-} mothers. AUC of plasma insulin positively correlated with Tfb2m expression ($r = 0.465$, $p < 0.05$) in male offspring with eNOS^{+/-} fathers. Seven genes (Chrebp, GR, Tfam, Tfb2m, G6Pase, Glut2 and Igf2) showed a significant correlation with increased liver glycogen in male offspring with eNOS^{+/-} fathers ([Supplementary Table S9](#)). Fumarate negatively correlated with the expression of GR ($r = -0.469$, $p < 0.05$) and Glut2 ($r = -0.701$, $p < 0.01$) in male offspring with eNOS^{+/-} fathers. See more details in the [Supplementary Tables S8, S9](#).

Discussion

To investigate the advanced fetal programming hypothesis, which suggests that both maternal and paternal genes can influence the offspring's phenotype without the direct transmission of parental genes to the offspring, we employed a similar approach to our previous study. We crossed female and male heterozygous eNOS knockout mice with male and female wild type mice. Then, we conducted a head-to-head study to simultaneously analyse the paternal and maternal effects on the offspring's phenotype. Our study findings indicate that female wt offspring born to eNOS^{+/-} mothers showed elevated liver fat accumulation. In contrast, male wt offspring born to eNOS^{+/-} fathers displayed increased levels of fasting insulin, higher insulin levels following glucose intake, and elevated liver glycogen content ([Figure 7](#)). In addition, female wt offspring born to eNOS^{+/-} fathers also showed an increased liver glycogen content. Pancreas morphology, including the endocrine pancreas, was not affected by parental eNOS deficiency. Our study identified six serum metabolites [lysoPhosphatidylcholine acyl C20:3 (lysoPC a C20:3), phosphatidylcholine diacyl C36:2 (PC aa C36:2), phosphatidylcholine diacyl C38:1 (PC aa C38:1), phosphatidylcholine acyl-alkyl C34:1 (PC ae C34:1), phosphatidylcholine acyl-alkyl C36:3 (PC ae C36:3), and phosphatidylcholine acyl-alkyl C42:5 (PC ae C42:5)] in male wt offspring and four liver carbon metabolites (fructose 6-phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate and fumarate) in both sexes of wt offspring born to eNOS^{+/-} mothers were significantly changed compared with those in wt offspring born to eNOS^{+/-} fathers. These observations might be attributed to the adverse intrauterine environment in eNOS^{+/-} mothers. Notably, further correlation analyses showed fumarate was inversely correlated with the fat accumulation in the liver in female offspring with eNOS^{+/-} mothers and increased liver glycogen in offspring of both sexes with eNOS^{+/-} fathers ([Figure 7](#)). Gene expression analysis



revealed an elevated expression of liver *Fitm1* (fat storage inducing transmembrane protein 1) in female offspring with maternal eNOS deficiency, which was associated with increased liver fat accumulation in these offspring. Importantly, our study revealed that the expression of the *Tfb2m* (mitochondrial transcription factor B2) gene was increased in male offspring born to fathers with eNOS deficiency, which was significantly correlated with elevated insulin levels after glucose load in these male offspring. Seven genes were significantly correlated with increased liver glycogen in male offspring born to fathers with eNOS deficiency. Furthermore, in these genes, the increased expression of *GR* (glucocorticoid receptor) and *Glut2* (glucose transporter 2) genes was significantly correlated with the decreased levels of fumarate in male offspring with paternal eNOS deficiency (Figure 7).

Our data align with recent research (Nelson et al., 2010; Liu et al., 2021; Chen et al., 2022; Zhao et al., 2022), which suggests that maternal or paternal genes, even without direct transmission to the offspring, can influence the offspring's phenotype. This influence appears to depend on whether the gene defect was present in the mother or the father. One study investigated the transgenerational phenotypic effects of the fathers' Y chromosome on daughters' phenotypes and revealed that certain traits on the paternal Y chromosome (not inherited to daughters) significantly reduced anxiety levels among daughters (Nelson et al., 2010). Another study demonstrated that the mutation of paternal *Usp26* (ubiquitin-specific peptidase 26) increased the risk of having children with Klinefelter syndrome (Liu et al., 2021). A recent study showed that the maternal environment affects offspring by influencing the level of oocyte TET3 (tet methylcytosine dioxygenase 3). This, in turn, has an impact on the reprogramming of the paternal genome within the zygote. The impairment of DNA demethylation and epigenetic inheritance

specifically influences the expression of certain paternally hypermethylated genes involved in insulin secretion, including *Gck* (glucokinase), which plays a critical role in glucose metabolism. Consequently, this sensitizes the offspring to glucose intolerance (Chen et al., 2022). In addition, another study showed that deficiency of maternal *Ezh1/2* (enhancer of zeste homolog 1/2) caused compromised H3K27me3 (tri-methylation of lysine 27 on histone H3 protein) and affected pluripotent epiblast cells within late blastocysts, which subsequently results in impaired embryonic development (Zhao et al., 2022). To sum up, genetic changes in the parental germline can affect the offspring's phenotype, even if those changes are not encoded in the DNA sequence of the offspring. These changes can be influenced by many factors such as diet, stress, and exposure to toxins. The molecular mechanisms underlying these epigenetic effects induced by parental genes may involve DNA methylation, histone modification, or mediation through small RNAs.

The key molecule in this study is eNOS, an enzyme that produces nitric oxide (NO) in endothelial cells. NO plays a crucial role in locally regulating vascular resistance, promoting angiogenesis, and is also considered a potential regulator of placental steroid biosynthesis and nutrient uptake (Vatish et al., 2006). eNOS deficiency is linked to the occurrence of intrauterine growth retardation (IUGR) due to impaired placental blood flow and nutrient delivery (George et al., 2022). Parental stimuli, characterized by heterozygous eNOS deficiency, yield diverse phenotypic outcomes in their offspring, contingent upon the sex of the parent carrying the deficiency, while not being passed on to the subsequent generation. Female offspring of mothers with the eNOS^{+/-} genotype exhibited heightened liver fat accumulation, while male offspring of fathers with the same

genotype showed increased fasting insulin levels and enhanced liver glycogen storage. These distinct outcomes arising from the identical parental genetic defect (eNOS deficiency) may be attributed to varying effects of eNOS deficiency on the reproductive organs of males and females. Maternal eNOS deficiency can impact various aspects of reproductive and maternal physiology, including egg maturation, intrauterine development, nursing behavior, and lactation (Pallares et al., 2008; Teichert et al., 2008). It is important to note that maternal eNOS deficiency has negative effects on liver fat ratio most likely due to the influence on oocyte/intrauterine development induced by maternal eNOS deficiency. Paternal eNOS deficiency can have far-reaching effects, potentially influencing both the maturation and development of sperm (Mostafa et al., 2015). Furthermore, it may trigger transmissible epigenetic alterations within these sperm, giving rise to enduring epigenetic changes. These modifications can ultimately result in an adult phenotype characterized by elevated fasting insulin levels, heightened insulin response following glucose intake, and increased liver glycogen content. This explanation aligns with previous research that demonstrates how a paternal high-fat diet before conception can lead to impaired glucose tolerance in offspring, primarily due to epigenetic modifications in sperm and consequential alterations in target organs (Ng et al., 2010; Terashima et al., 2015; Chen et al., 2016; Nembhard et al., 2018), which correspond to our findings. In our prior research endeavours, we were able to establish disparities in both overall DNA methylation levels and gene-specific DNA methylation patterns, along with variations in the expression of specific candidate genes among wild-type offspring with either maternal or paternal eNOS deficiency. Notably, we observed a conspicuous correlation between DNA methylation patterns and the observed liver phenotype in these offspring (Hochoer et al., 2016; Hochoer et al., 2022). In the current study, we showed that hepatic fat accumulation in female wt offspring of eNOS+/- mothers was associated with increased expression of liver *Fitm1*. This gene was reported in our previous study (Hochoer et al., 2016). However, we added the corresponding expression data of this gene in wt offspring born to eNOS+/- fathers in this study. In addition, we found that increased expression of the *Tfb2m* gene in male offspring born to fathers with eNOS deficiency was significantly correlated with elevated insulin levels after glucose load in these male offspring. *Tfb2m* is a mitochondrial transcription factor involved in mitochondrial DNA transcription. Existing evidence have showed that *Tfb2m* played a critical role in insulin secretion (Adan et al., 2008; Fex et al., 2018). Our observations were consistent with these findings. What's more, we revealed that seven genes in male offspring born to fathers with eNOS deficiency were significantly correlated with increased liver glycogen in these male offspring. Particularly, we revealed the correlation among altered genes (*GR* and *Glut2*), metabolite (fumarate) and phenotypic changes (increased liver glycogen) in male offspring born to fathers with eNOS deficiency. These findings supplemented and added new evidence for a comprehensive understanding of how parental eNOS gene defects influence the phenotype of offspring, even when the offspring have not inherited the specific gene defect.

Sex differences on phenotypes were observed both in offspring of eNOS+/- mothers and of eNOS+/- fathers.

Sexual dimorphism in fetal programming in response to identical stimuli has been extensively documented in previous studies (Reichetzedler et al., 2016). This divergence can be attributed to several factors. Firstly, both male and female sex steroid hormones, which are produced by both the fetus and the placenta, play a role in modulating the impact of mild NO deficiency on epigenetic and phenotypic changes in the offspring in a sex-specific manner. Additionally, sex-dependent transcriptional variations in the offspring may contribute to these observed differences (Bermejo-Alvarez et al., 2011; Li et al., 2016; Reichetzedler et al., 2016).

In the present study, we employed metabolomics to investigate further links between the observed offspring phenotypes and maternal/paternal eNOS deficiency. Characteristic metabolites associated with fatty liver disease and increased insulin and liver glycogen storage were identified. The precise pathophysiological significance of the serum metabolites we observed remains not fully elucidated. Phosphatidylcholines, including lyso-phosphatidylcholines, diacyl-phosphatidylcholines, and acyl-alkyl-phosphatidylcholines, are vital constituents of cell membranes and lipoproteins (Cole et al., 2012). Beyond their fundamental structural role, these molecules seem to play a role in various physiological processes. Notably, they are implicated in the liver's secretion of very low-density lipoproteins and are also associated with glucose regulation (Cole et al., 2012; Furse and de Kroon, 2015; Boone et al., 2019). Phosphatidylcholines have also been demonstrated to enhance the cell proliferation effects of insulin and insulin-like growth factor-1 (Kiss, 1999). Furthermore, alterations in the concentrations of phosphatidylcholines were linked to cardiometabolic changes triggered by an excess of liver and visceral fat (Floegel et al., 2013) and atherosclerosis (Matsumoto et al., 2007). In line with these studies, we identified that lysoPC a C20:3 was specifically associated with the AUC of plasma insulin in female offspring born to eNOS+/- mothers/fathers. PC aa C38:1 and PC ae C34:1 had a positive relation with the AUC of plasma insulin in female offspring with eNOS+/- fathers. lysoPC a C20:3 was positively correlated with glycogen content in female offspring, while PC aa C38:1 was negatively correlated with glycogen content in male offspring born to eNOS+/- fathers. In summary, while the specific functions of the majority of metabolites observed in our study remain uncertain, there appears to be an association between these metabolites and alterations in plasma insulin levels, liver fat, and glycogen, induced by maternal or paternal eNOS deficiency.

Particularly, we found that fumarate had a significant correlation with increased fat accumulation in liver in female offspring born to eNOS+/- mothers and increased liver glycogen in offspring of both sexes born to eNOS+/- fathers. Fumarate plays a pivotal role as a key intermediate in the tricarboxylic acid cycle (TCA), facilitating the interconnection of carbon and nitrogen metabolism (Araujo et al., 2011; Hengist et al., 2019). One way in which fumarate can indirectly affect blood glucose levels is by influencing insulin signaling. Studies have shown that fumarate can enhance insulin sensitivity and improve glucose uptake by cells, which may help to lower blood glucose levels (Franko et al., 2022). Additionally, fumarate has been shown to activate AMP-activated protein kinase (AMPK) is a critical enzyme that plays a central role

in the regulation of glucose metabolism (Li et al., 2011). With regards to fat accumulation, fumarate may indirectly impact this process by affecting mitochondrial function. Fumarate has been demonstrated to stimulate mitochondrial biogenesis and improve mitochondrial function, which may help to reduce fat accumulation (Wang et al., 2021). When it comes to glycogen, fumarate can impact glycogen storage in the liver indirectly through its effects on ATP production, AMPK activation, and insulin sensitivity (Noster et al., 2019). Consistent with these previous studies, our research also illustrated that fumarate was significantly related to the changes in liver fat accumulation and liver glycogen, induced by maternal/paternal eNOS+/- deficiency in offspring.

Our study has also several limitations. The primary goal of our study was to analyze potential sex-dependent effects of parental eNOS deficiency on the offspring's phenotype and we indeed could demonstrate that it matters whether the parental heterozygous eNOS deficiency was present in the father or mother in our head-to-head study. However, sex dependent effects of the origin of homozygous eNOS deficiency in the grandfather/grandmother's generation could also affect the phenotype in the F2 generation. There is some evidence that it would have affected the phenotype of the heterozygous eNOS mice coming from earlier studies by Longo M et al., showing that eNOS heterozygous offspring born to eNOS knockout mothers had higher blood pressure, effects on glucose tolerance and insulin levels compared to those offspring born to eNOS knockout fathers most likely because eNOS knockout mothers had an abnormal uterine environment (Longo et al., 2016). This uterine effect is absent when starting with homozygous eNOS deficient fathers. Therefore, we used male F0 eNOS-/- mice as origin of eNOS deficiency in our study. Given the study by Longo M et al., it is very likely that if the origin of eNOS deficiency would be the grandmother in the breeding protocol it would have affected the phenotype in the F2 generation, but this represents another research question that merits to be addressed in an independent study designed to address this topic. Epigenetic alterations of the adult phenotype are mainly caused by intrauterine epigenetic alterations of gene expression and subsequent alterations of the function and morphology of organs (Reichetzeder et al., 2016). This is clearly an important task for further studies to better understand the early life epigenetic mechanisms of parental eNOS deficiency. In addition, we measured insulin and glucose levels at 0, 15, and 60 min during the IPGTT, with no further measurements to prioritize animal welfare. However, the duration of 60 min for IPGTT demonstrated differences among the groups and was also reported in previous studies (Kim et al., 2010; Wang et al., 2010; Hochoer et al., 2022). What's more, the specific metabolomics platform used in this study restricted our choice of metabolites to investigate. On the other hand, an advantage of this platform is that it includes a collection of metabolites that are both biologically and analytically well-defined. Another limitation of our study is the absence of an analysis on the potential impact of eNOS heterozygosity on the metabolism of the parents (F1 generation) and an analysis on the heterozygous eNOS fetuses of F2 generation. Some other studies already provided evidence on phenotype of eNOS heterozygous mice. Cook et al. have shown that eNOS^{-/+} heterozygous mice

were normotensive and had normal insulin sensitivity on a normal diet (Cook et al., 2004). Consequently, we did not investigate further on the metabolic phenotype of F1 eNOS heterozygous mice and mainly focused on the metabolic changes observed in the F2 generation with a healthy genotype. Additionally, we did not analyse the underlying epigenetic alterations. However, this was not done, because it was described recently by us using comparable experimental designs of the animal studies—increased liver glucocorticoid receptor and Ppargc1a gene expression attributed to altered methylation patterns of these genes when the father had eNOS deficiency and increased liver Fat Storage Inducing Transmembrane Protein 1 (*Fitm1*) and Cyclin-dependent kinase inhibitor 1A (*Cdkn1a*) gene expression resulted from altered methylation of these genes when the mother had eNOS deficiency (Kim et al., 2010; Wang et al., 2010; Hochoer et al., 2022).

In conclusion, this head-to-head study demonstrated that the identical parental genetic modification (heterozygous eNOS deficiency) without transmission to the offspring causes an offspring metabolic and liver phenotype and liver gene expression pattern depending on whether the alteration was present in the father or the mother. Female offspring with wildtype genes from mothers with a heterozygous eNOS deficiency showed increased liver fat accumulation. In contrast, male offspring with wildtype genes from fathers with a heterozygous eNOS deficiency had higher fasting insulin levels, increased insulin response after a glucose load, and elevated liver glycogen content. We identified six serum metabolites and four liver carbon metabolites that differed between wt offspring with eNOS+/- mothers and wt offspring with eNOS+/- fathers. The most prominent effects were observed regarding fumarate (strong correlations between fumarate and changes on liver histology induced by maternal/paternal eNOS deficiency). Moreover, the gene expression patterns were different between wt offspring with eNOS+/- mothers and those offspring with eNOS+/- fathers. Importantly, the changes in specific gene expression were found to be correlated with the observed phenotypic alterations in wt offspring with eNOS+/- mothers/fathers. Our findings enhance the understanding of how parental genetic defects may impact the phenotype of genetically healthy offspring. This provides a foundation for more precise genetic counselling and screening. The clinical implications of our study should be further investigated in monogenic inherited diseases such as thalassemia.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Animal welfare committee of the state of Berlin, Germany. The study was

conducted in accordance with the local legislation and institutional requirements.

Author contributions

XZ: Data curation, Formal Analysis, Writing—original draft. CR: Data curation, Writing—review and editing. YL: Writing—review and editing. J-GH: Writing—review and editing. AH: Data curation, Writing—review and editing. GL: Writing—review and editing. BK: Supervision, Writing—review and editing. LH: Writing—review and editing. BH: Conceptualization, Project administration, Supervision, Writing—review and editing.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2023.1306178/full#supplementary-material>

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Identification of the serum metabolomic profile for acute ischemic preconditioning in athletes

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Purpose: In recent years, ischemic preconditioning (IPC) has emerged as an effective strategy to increase tissue resistance against long-term ischemic damage and has been increasingly integrated into exercise regimens. However, further research is needed to explore the impact of IPC-mediated metabolic alterations from an exercise standpoint to conduct a comprehensive exploration of metabolic alterations and their exercise-related mechanisms during acute IPC.

Methods: Nontarget metabolomics was performed on blood samples obtained from 8 male athletes both before and after IPC. The studies included the identification of differentially abundant metabolites, analysis of receiver operating characteristic (ROC) curves, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for differentially abundant metabolites, and metabolite set enrichment analysis (MSEA).

Results: Nineteen differentially abundant metabolites were identified, with increasing levels of five metabolites, such as O-desmethyltramadol and D-gluconate, whereas 14 metabolites, including 9-hydroxy-10e, 12z-octadecadienoic acid (9-HODE), tetradione, 2-hexenal, (2,4-dichlorophenoxy)acetic acid (2,4-D), and phosphatidylserine (PS), decreased. ROC curve analysis revealed an AUC of 0.9375 for D-gluconate. Both KEGG enrichment analysis and MSEA revealed enrichment in the pentose phosphate pathway (PPP).

Conclusion: This study revealed that PPP, D-gluconate, O-desmethyltramadol, and D-2-aminobutyric acid could be upregulated within 5 min after acute IPC, whereas 2,4-D, PS, 9-HODE, 2-hexenal, and tetradione could be downregulated. These identified metabolites show promise for improving physical functional status and could be harnessed to enhance athletic performance.

KEYWORDS

ischemic preconditioning, metabolomic profile, taekwondo athletes, D-gluconate, pentose phosphate pathway

1 Introduction

Ischemic preconditioning is a well-established strategy that enhances tissue resilience against prolonged ischemic damage. Murry conducted repeated short-term ischemia-reperfusion on the hearts of dogs to stimulate the body's intrinsic protective mechanisms and reduce the degree of cardiac infarction. IPC was initially employed to safeguard the myocardium and mitigate myocardial damage resulting from prolonged ischemia (Murry et al., 1986). Additionally, it has a protective effect on the kidneys, liver, and cerebral nerves during extended periods of ischemia-reperfusion (Choi et al., 2020; Donato et al., 2021; Yang et al., 2020; Livingston et al., 2019). Recent studies have demonstrated that IPC can enhance the performance of elite athletes by briefly and repeatedly applying sufficient pressure to the limbs to occlude blood flow, thereby inducing brief ischemia-reperfusion (Caru et al., 2019). This process has been shown to stimulate athletic potential, leading to improvements in sports performance, including increased maximum oxygen uptake in cyclists (de Groot et al., 2010), enhanced performance in swimmers during the 100-meter race (Emilie Jean-St-Michel et al., 2011) and improved results in judo and taekwondo athletes during specialized tests (Ribeiro et al., 2019; Ou et al., 2024). These findings underscore the importance of future applications of IPC in sports training and competitions. However, the precise mechanism by which IPC enhances athletic performance remains unclear, highlighting the necessity of understanding this mechanism for the informed use of IPC in sports training to enhance performance and achieve competitive success.

Research has shown that IPC can help mitigate ischemia-reperfusion injury through the use of adenosine, bradykinin, opioids, and other substances, along with the selective activation of mitochondrial ATP-sensitive potassium (mK_{ATP}) channels (Nakano et al., 2000; Downey et al., 2007). Increased levels of mK_{ATP} and adenosine can lead to vasodilation, improve muscle blood supply (de Groot et al., 2010), and prevent vascular function decline following intense exercise (Kraemer et al., 2011). Additionally, some research has indicated that IPC can increase the maximum oxygen uptake in cyclists. Mitochondria are recognized as the primary intracellular effectors of IPC, with mK_{ATP} activation playing a critical role in cardioprotection (Heusch, 2020). IPC has the potential to stimulate muscle growth and metabolic changes, resulting in significant improvements in muscle endurance and strength in a short period, thereby enhancing athletic performance (Bailey et al., 2012; Lawson and Downey, 1993; Homer-Vanniasinkam, 2005). Recent studies have revealed that tourniquet-induced IPC can upregulate Mitofusin2 and help maintain muscle strength (Leurcharusmee et al., 2022). Furthermore, IPC can regulate microvascular dilation and exert anti-inflammatory effects through nitric oxide (NO). Improved vascular function during exercise enhances oxygen delivery efficiency, facilitates lactic acid removal, and maintains acid-base balance (Tapuria et al., 2008). Thus, IPC can influence the body's metabolism, with certain metabolic changes being beneficial for enhancing exercise performance. However, current research on the metabolite changes induced by IPC remains limited, necessitating further exploration of the metabolic profiles affected by IPC.

Although studies have demonstrated that ischemic preconditioning (IPC) induces relevant metabolites that enhance exercise performance, particularly aerobic activities, IPC may not universally improve performance across all forms of exercise (Caru et al., 2019). This underscores the necessity of comprehensively assessing the metabolic changes induced by IPC. Consequently, more thorough and in-depth methodologies are needed to investigate the metabolic characteristics of IPC and to elucidate its role in enhancing exercise capacity. In recent years, metabolomics research methods have gained increasing prominence in investigating mechanistic pathways by analyzing multiple metabolites in samples to uncover metabolic profiles and alterations in the body under different conditions (Jonsson et al., 2005). Nontargeted metabolomics has emerged as a valuable tool for studying the impact of IPC on the plasma metabolome. This approach can also be utilized to explore both potential and previously unidentified important metabolites. It was discovered that alpha-hydroxybutyrate could serve as a cardioprotective factor and biomarker for tissue ischemia (Laursen et al., 2017). A recent study also employed an untargeted metabolomic approach to explore the metabolic signature associated with long-term remote ischemic preconditioning, suggesting potential benefits for the diagnosis and treatment of cerebral ischemia-reperfusion injury (Du et al., 2023). Therefore, nontargeted metabolomics technology not only facilitates a deeper understanding of the characteristics of IPC-induced metabolic profiles but also explores the potential advantages of changes in these profiles for enhancing exercise performance and identifying biomarkers related to the mechanisms of IPC.

This study hypothesized that acute IPC influences the metabolic profile of taekwondo athletes, potentially enhancing their athletic performance. The primary objective of this research was to employ nontargeted metabolomics technology to assess metabolomic changes in athletes before and after acute IPC, with an emphasis on a detailed examination of exercise-related metabolic mechanisms and pathways.

2 Materials and methods

2.1 Subjects

In light of existing research demonstrating that ischemic preconditioning can significantly enhance the performance of certain sports and considering related studies on ischemic preconditioning and metabolomics technology, we ultimately selected eight taekwondo athletes for this investigation (Baranovicova et al., 2018; Baranovicova et al., 2021; Rong et al., 2022; Wu et al., 2022; Laursen et al., 2017). The participants had to meet specific criteria to ensure their good health. The inclusion criteria were as follows: (1) adult males over 18 years of age; (2) professional athletes at the national second level; (3) a minimum of 5 years of professional training in Taekwondo; and (4) maintenance of regular and standardized training for the past 3 months. The exclusion criteria were as follows: (1) acute or chronic diseases such as anxiety, depression, cardiovascular disease, respiratory disease, or metabolic disease; (2) use of creatine supplementation; (3) recent medication use within 3 months; (4) consumption of

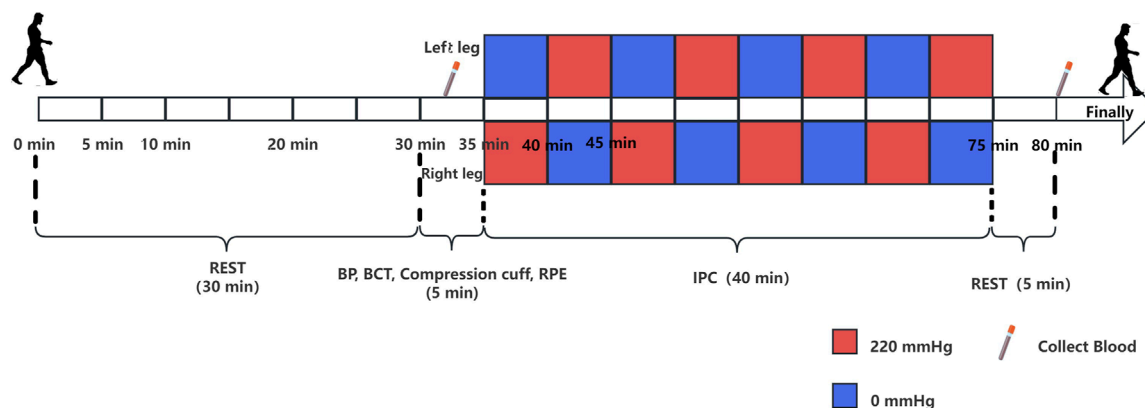


FIGURE 1

Experimental protocol for the study. The rectangle above the timeline represents the intervention for the left leg, and the rectangle below represents the intervention for the right leg. The red rectangle represents a pressure of 220 mmHg, whereas the blue rectangle represents a pressure of 0 mmHg. Each rectangle corresponds to a duration of 5 min, and the experiment proceeds from left to right.

alcohol and caffeine within the last 24 h before testing; and (5) engaging in aerobic or anaerobic exercise within the past 24 h (6) Having received ischemic preconditioning. All participants were informed about the benefits, discomfort, and potential risks associated with the study. Additionally, all participants received a thorough explanation of the study procedures, provided informed consent, and fasted for a minimum of 12 h prior to undergoing ischemic preconditioning.

This research was approved by the Ethics Review Committee of Guangzhou Sport University (ID Number: 2023LCCL-81) and conducted following the guidelines of the Declaration of Helsinki.

2.2 Experimental protocol of the study

The experimental plan consisted of several steps. The participants were instructed to abstain from alcohol, caffeine, and intense physical activity the day prior to the experiment. Upon arrival at the laboratory, the experimenter provided a detailed explanation of the procedures, obtained signed informed consent forms from the subjects, and allowed them to rest for 30 min. Subsequently, blood pressure (BP) was measured, and 5 mL blood samples were drawn from the middle cubital vein using an EDTA-K2 anticoagulated tube, which were then labeled Group A. A body composition test (BCT) was then conducted, with the participant wearing a compression cuff and lying down. Prior to the ischemic preconditioning intervention, fingertip blood was collected for analysis of lactic acid levels, along with an inquiry into the Rating of Perceived Exertion (RPE). During the second part of the experiment, the participants laid down for a total of 40 min of IPC. Following the IPC intervention, the participant remained still for an additional 5 min. At the end of this period, another blood sample (5 mL) was collected from the median cubital vein via an EDTA-K2 anticoagulant tube and labeled group B (Laursen et al., 2017). The experimental flow chart is depicted in Figure 1.

2.3 Basic indicator measurement methods

Blood pressure was assessed with an OMRON blood pressure meter (Model HEM-1020) via the authoritative oscillometric method (Alexandra et al., 2002). The tester held a colored rating of the Perceived Exertion scale and asked the experimental subjects, and the experimental subjects were subjectively scored according to their own feelings (Ritchie, 2012). Body composition, such as weight, fat mass and muscle mass, was measured via a body composition analyzer (Inbody 370).

2.4 IPC

Under normal oxygen conditions, the subject was placed in a supine position for IPC intervention. A blood pressure cuff was positioned on the upper third of both thighs (groin) of the subject. The cuff on the right leg was inflated to 220 mmHg, whereas the cuff on the left leg remained at 0 mmHg (Cheng et al., 2021; Paradis-Deschênes et al., 2020; Da Mota et al., 2019). After 5 min, the cuff on the right leg was deflated to 0 mmHg, and the cuff on the left leg was increased to 220 mmHg for another 5 min, completing one cycle. This protocol was repeated for 4 cycles, totaling 40 min.

2.5 Experimental methods for assessing metabolism

2.5.1 Chemicals

Sigma Aldrich provided ammonium acetate (NH_4AC), while Merck supplied acetonitrile. Fisher supplied ammonium hydroxide (NH_4OH) and methanol.

2.5.2 Sample collection and preparation

Blood was collected from the median cubital vein via EDTA-K2 anticoagulant tubes. After collection, the blood was centrifuged within 30 min at 4°C with a centrifugal force of $1,100 \times g$ for 15 min. The serum was separated on ice boxes and frozen at -80°C .

2.5.3 LC-MS/MS analysis

Metabolites were examined via nontargeted metabolomics. Following a gradual thawing process at 4°C, a portion of the sample was extracted and combined with a chilled mixture of methanol, acetonitrile, and water at a 2:2:1 ratio. The mixture underwent vigorous mixing and ultrasonication at low temperatures for 30 min, followed by a 10-minute incubation at −20°C. The mixture was subsequently centrifuged at 14,000 × g for 20 min at 4°C, after which the liquid portion was evaporated under vacuum. For mass spectrometry analysis, the dehydrated sample was reconstituted with 100 µL of an aqueous solution of acetonitrile (acetonitrile:water = 1:1), followed by mixing and centrifugation at 14,000 × g for 15 min at 4°C. The liquid portion was then used for sample analysis. Quality control (QC) samples were prepared by extracting 10 µL from each sample, with a QC sample analysis performed after every five samples were analyzed. The primary and secondary spectra were collected via an AB Triple TOF 6600 mass spectrometer. After HILIC separation, the ESI source conditions included gas settings such as Ion Source Gas1 (Gas1): 60, Ion Source Gas2 (Gas2): 60, and Curtain gas (CUR): 30. The source temperature was set at 600°C, and the IonSapary Voltage Floating (ISVF) was ±5500 V for both positive and negative modes. For mass spectrometry analysis, the TOF MS scan ranged from 60 to 1,000 Da, whereas the product ion scan ranged from 25 to 1,000 Da. The accumulation time for the TOF MS scan was 0.20 s/spectra, and that for the product ion scan was 0.05 s/spectra. The secondary mass spectrum was obtained via information-dependent acquisition (IDA) in high-sensitivity mode, with settings including a declustering potential (DP) of ±60 V for both positive and negative modes, a collision energy of 35 ± 15 eV, and the exclusion of isotopes within 4 Da. Moreover, the number of candidate ions to monitor per cycle was set at 10.

2.6 Data analysis

The raw mass spectrometry data were first converted to MzXML files via ProteoWizard MSConvert and then imported into the freely available XCMS software. Peak picking was performed with centWave using a mass-charge ratio tolerance of 10 ppm, a peak width ranging from 10 to 60, and prefilter values between 10 and 100. For peak grouping, the bandwidth was set to 5, the mzwid value was 0.025, and the minfrac value was 0.5. The Collection of Algorithms of MEtabolite pRofile Annotation was used for annotating isotopes and adducts. Only variables with more than 50% nonzero measurement values in at least one group were retained in the extracted ion features. Metabolite compound identification involved comparing accurate mass-charge ratio values (within 10 ppm) and MS/MS spectra with an in-house database of authentic standards. Missing data points were imputed via the K-nearest neighbor (KNN) method, and extreme values were removed. Finally, the total peak area data were normalized to ensure consistency across samples and metabolites.

Positive ion mode (POS) and negative ion mode (NEG) were utilized for metabolite detection. The data were subjected to systematic clustering analysis (Yuan et al., 2012), and the resulting dendrogram was generated via average linkage. Hierarchical clustering was conducted with the R package pheatmap (Kolde, 2015).

The screening criteria for identifying differentially abundant metabolites included a variable importance for the projection (VIP) of orthogonal partial least squares discriminant analysis (OPLS-DA) ≥ 1 and a significance level of $P < 0.05$ in the single-factor t-test (Han et al., 2022; Zhu et al., 2022; Deng et al., 2021). Additionally, cross-validation of the OPLS-DA model was performed. Fold changes (FCs) between the two groups were calculated, and a volcano plot was generated. A chart displaying the top 15 metabolites with the highest VIP scores from OPLS-DA was created (Yoon et al., 2020). To quantify metabolite composition and abundance variability among samples, correlation analysis of sample data was performed (Rao et al., 2016). Correlations were calculated via the R corrrp package (Wei T, 2017), and a heatmap was generated via the pheatmap package. A correlation coefficient approaching 1 indicates greater similarity in metabolic composition and abundance among samples.

Abundance normalization of differentially abundant metabolites was performed via the Z score, and hierarchical clustering with the R package pheatmap was employed (Kolde, 2015). The differentially abundant metabolites were then hierarchically clustered, samples were grouped, and a cluster heatmap was generated to visualize the cumulative difference between the two groups.

Receiver operating characteristic curve analysis was conducted via the R pROC package to assess the predictive ability of each discriminant metabolite. The AUC was determined by numerically integrating the ROC curves. The area under the ROC curve (AUC-ROC) was determined via the bootstrapping method to estimate the median and 95% confidence interval (95% CI).

The calculation formula for differentially abundant metabolite KEGG enrichment analysis is as follows:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

N represents the total count of metabolites labeled under the KEGG database, whereas n denotes the count of metabolites showing differential expression within N. Similarly, M represents the total count of metabolites labeled under a specific pathway, and m represents the count of metabolites showing differential expression within M. The p-value calculated underwent false discovery rate (FDR) correction, and a threshold of $FDR \leq 0.05$ was applied. Pathways satisfying these criteria were considered significantly enriched in differentially abundant metabolites.

Metabolic set enrichment analysis (Xia and Wishart, 2010) was applied to assess pathway overrepresentation with the MetaboAnalyst module. To conduct the analysis, the Small Molecule Pathway Database library was utilized. The overrepresentation analysis was performed via Fisher's exact test with the R package MSEAp (<https://rdrr.io/github/afukushima/MSEAp/>).

TABLE 1 Basic information of the study subjects.

	Age (Y)	Height (cm)	Weight (kg)	Years of training (Y)	BMI (kg/m ²)	Body fat percentage (%)	RPE
$\bar{X} \pm S$	20.25 $\pm$ 1.83	178.38 $\pm$ 5.32	71.64 $\pm$ 9.92	6.88 $\pm$ 1.36	22.45 $\pm$ 2.40	13.86 $\pm$ 6.17	6.25 $\pm$ 0.70

3 Results

3.1 Subjects

Basic characteristics of the 8 athletes before the intervention (Table 1).

3.2 Data quality control and metabolite statistical results

A total of 11,137 metabolites were identified in positive ion mode, comprising 1,468 known metabolites and 9,669 unknown metabolites. In negative ion mode, 9,001 metabolites were detected, with 789 known and 8,212 unknown metabolites. We labeled the blood samples before and after IPC as Group A and Group B, respectively. Principal component analysis (PCA) was performed on metabolites from Group A, Group B, and QC samples, resulting in a three-dimensional PCA diagram (Figure 2A). The analysis of the samples in the QC group demonstrated high stability, good data quality, and reliable data analysis. Additionally, a heatmap illustrating positive and negative ion correlations among samples was generated via Pearson correlation coefficients (Figure 2B).

3.3 Differential expression of serum metabolites

The serum metabolomic characteristics of the experimental subjects were analyzed via LC-MS/MS before and after ischemic preconditioning. Group A represents the serum before IPC, whereas group B represents the serum after IPC. Differentially abundant metabolites were screened via both positive and negative ion modes simultaneously, and 3D PCA plots were generated for both modes (Figure 2A).

The analysis revealed that there were no significant differences in the first, second, or third principal components among the samples from Group A and Group B; however, Group B presented a greater level of clustering than did Group A (Figure 2A). Differentially abundant metabolites between the two groups were identified via an OPLS-DA model, and positive and negative ion OPLS-DA score plots were generated (Figure 3A). The cross-validation results of the OPLS-DA model indicate that in positive ion mode, $R^2Y = 0.975$, whereas in negative ion mode, $R^2Y = 0.984$, demonstrating the model's high interpretability. A total of 19 differentially abundant metabolites were identified on the basis of the criteria of $VIP \geq 1$ in the OPLS-DA and $P < 0.05$ in the t-test of univariate statistical analysis. Among these metabolites, 5 presented increased levels in group B, including O-desmethyltramadol, D-2-aminobutyric acid, and D-gluconate,

whereas 14 metabolites presented decreased levels, such as N-butylamine, (2,4-dichlorophenoxy) acetic acid, 9-hydroxy-10e, 12z-octadecadienoic acid, tetradifon, PS, glycocholic acid, calcimycin, glycodeoxycholic acid (GDCA), and 2-hexenal. A statistical chart illustrating the differentially abundant metabolites associated with positive and negative ions was also generated (Figure 3B).

To clearly display the differentially abundant metabolites, we calculated the VIP values of the metabolites and generated an OPLS-DA VIP statistical chart (Figure 3C). Among these metabolites, glycochenodeoxycholate (GCDC) presented the highest VIP value, indicating its significant role in sample differentiation. We subsequently calculated the fold change in the abundance of the metabolites and created a volcano plot on the basis of the VIP and P values (Figure 3D). Pearson correlation coefficient analysis was conducted to examine the relationships among the metabolites, leading to the generation of a heatmap illustrating their correlations (Figure 3E). Furthermore, the differentially abundant metabolite clustering heatmap results (Figure 3F) highlighted distinct accumulation patterns among the comparison groups, demonstrating a clear aggregation trend.

Receiver operating characteristic curve analysis was conducted on the differentially abundant metabolites. The statistical chart (Figure 4) displaying the ROC curve and AUC values revealed that the AUC for the differentially abundant metabolite D-gluconate was 0.9375, which was the highest among all the differentially abundant metabolites.

3.4 Pathway analysis

3.4.1 KEGG annotations and enrichment analysis of differentially expressed metabolites

The Kyoto Encyclopedia of Genes and Genomes (Ogata et al., 1999) serves as the primary public database for pathways, identifying key biochemical metabolism and signal transduction pathways involving metabolites. This resource is valuable for conducting metabolic analysis and researching metabolic networks in organisms, allowing researchers to examine metabolites and their expression information within a comprehensive network.

The KEGG statistical plot results of all the metabolites (Figure 5A) indicated that a total of 1,067 metabolites were functionally associated with metabolism. Subsequently, KEGG enrichment analysis identified six candidate differentially abundant metabolites with pathway annotations: glycocholate, glycocholic acid, GCDC, PS, D-gluconate, and 2,4-D. Following ischemic preconditioning, a total of 14 enriched pathways were identified. These pathways include cholesterol metabolism (hits: glycocholate, glycocholic acid, and GCDC), primary bile acid biosynthesis (hits: glycocholate, glycocholic acid, and GCDC), secondary bile acid biosynthesis (hits: glycocholate, glycocholic acid, and GCDC),

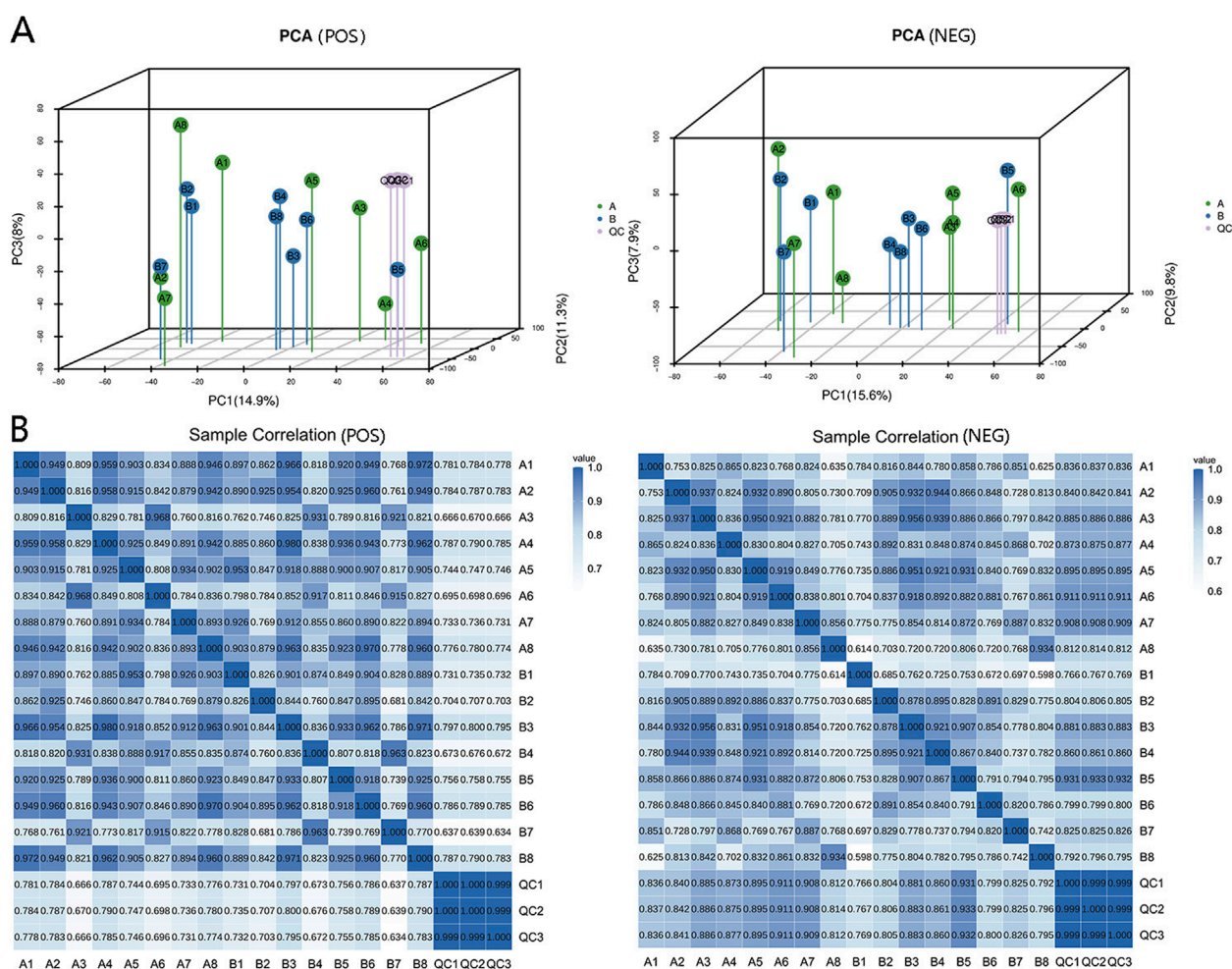


FIGURE 2

Data quality control and sample correlation. **(A)** Three-dimensional PCA plots demonstrating the metabolic variations within and between sample groups. Enhanced method stability and data quality are indicated by minimal differences among QC samples. The dense distribution of QC samples on the three-dimensional PCA chart ensures data reliability. **(B)** Sample correlation analysis was used to quantify and assess alterations in metabolite composition and abundance across samples via correlation data. A correlation value approaching 1 signifies a strong resemblance in metabolite composition and abundance among samples.

bile secretion (hits: glycocholate, glycocholic acid, and GCDC), systemic lupus erythematosus (hits: PS), leishmaniasis (hits: PS), the pentose phosphate pathway (hits: D-gluconate and PS), glycine, serine and threonine metabolism (hits: PS), glycerophospholipid metabolism (hits: PS), carbon metabolism (hits: D-gluconate), microbial metabolism in diverse environments (hits: D-gluconate and 2,4-D), biosynthesis of secondary metabolites (hits: D-gluconate and PS), and metabolic pathways (hits: D-gluconate, glycocholate, glycocholic acid, and PS), among which the first five pathways are significantly enriched. Consequently, a KEGG enrichment bar chart of differentially abundant metabolites was generated (Figure 5B). Pathway enrichment analysis was further conducted, resulting in the creation of a differentially abundant metabolite enrichment circle diagram (Figure 5C). The KEGG enriched pathway bubble plot (Figure 5D) highlights cholesterol metabolism as the pathway with the most significant differentially abundant metabolite enrichment among all the obtained pathways. Additionally, the differentially abundant metabolite D-gluconate,

enriched in the pentose phosphate pathway, was upregulated. Furthermore, KEGG enrichment difference analysis (Figure 5E) was conducted to illustrate the enrichment differences in each pathway.

Although six differentially abundant metabolites were annotated by enriched pathways, there are still unannotated metabolites, such as 2-hexenal, D-2-aminobutyric, O-desmethyltramadol, calcimycin, 2,4-D, and 9-HODE. These substances may indicate important changes that have occurred or are about to occur after IPC. Furthermore, the metabolites annotated in this study may play other significant roles in chronically trained taekwondo athletes. Therefore, we further explored the metabolomic characteristics of these differentially abundant metabolites related to exercise in chronically trained taekwondo athletes.

3.4.2 Metabolic set enrichment analysis

Our results revealed statistical significance ($P < 0.05$) for bile acid biosynthesis and the pentose phosphate pathway in

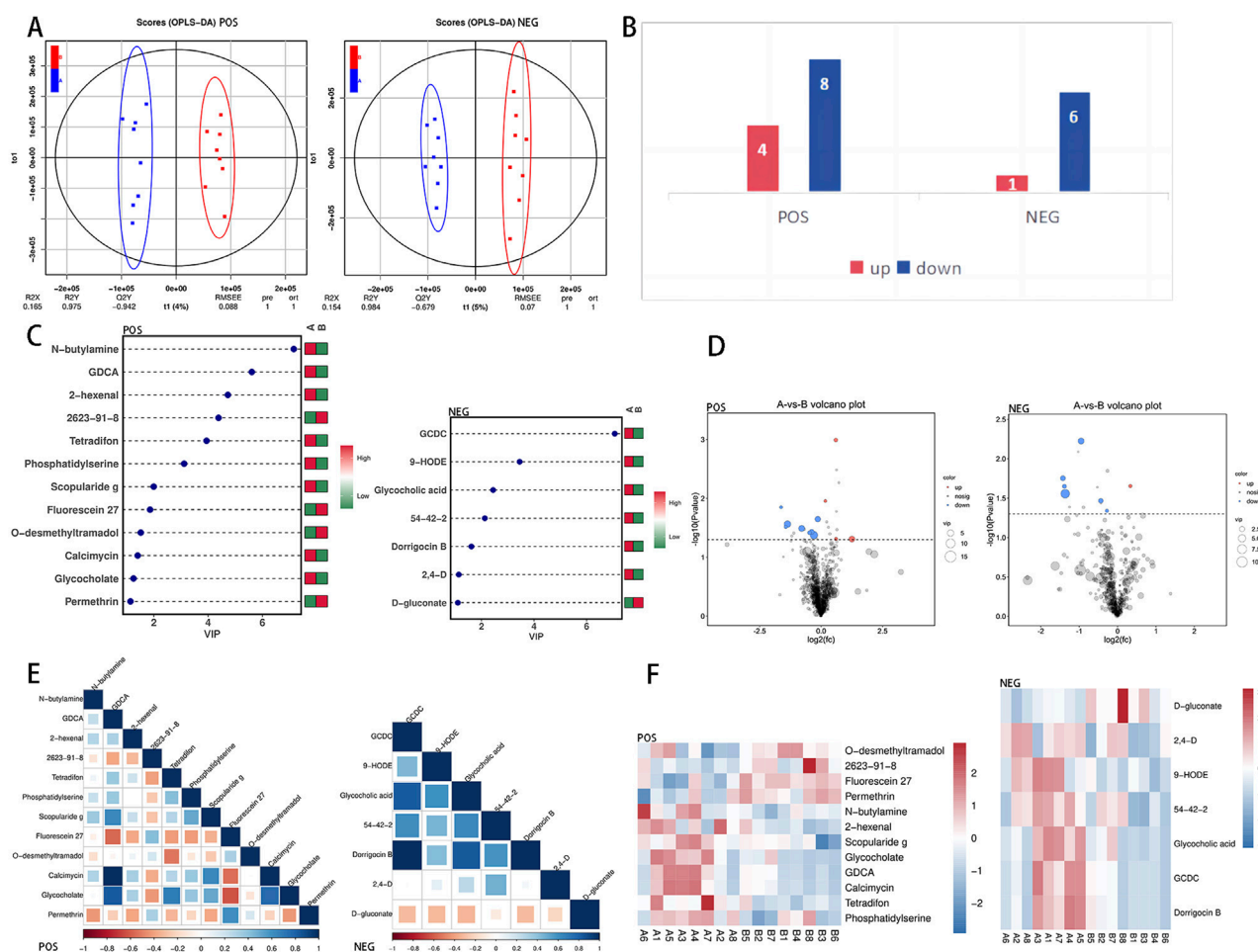


FIGURE 3

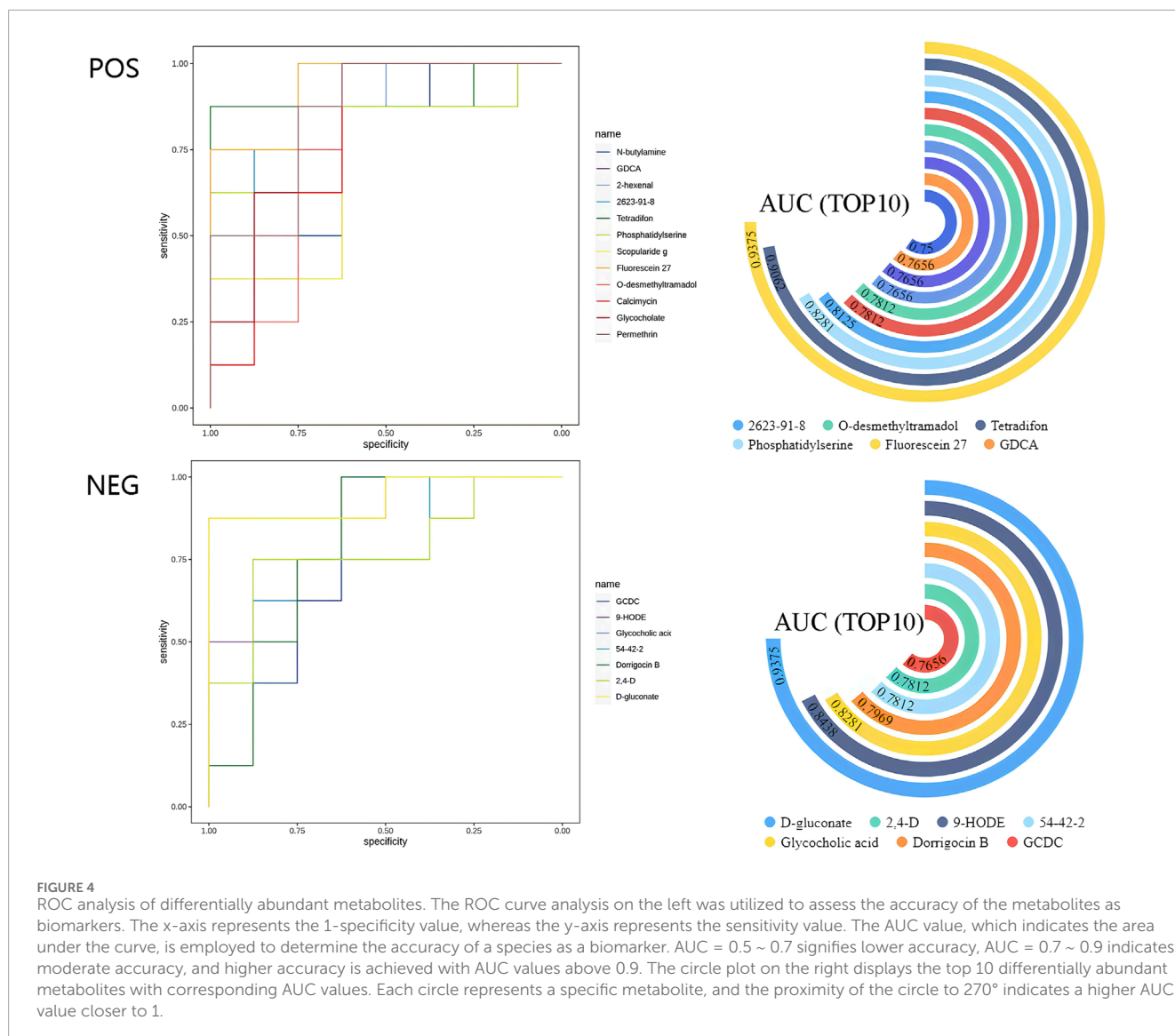
Differentially abundant metabolite identification. (A) Combining orthogonal signal correction (OSC) and PLS-DA simplifies the model, enhances explanatory power, and maintains predictive ability. This method optimizes the ability to distinguish between Group (A) and Group (B). The VIP value in OPLS-DA is used to identify important metabolites, with a VIP value greater than 1 indicating significance. (B) Statistical chart of differential metabolism upregulation and downregulation in positive and negative ion modes. (C) The x-axis shows the VIP value, the y-axis lists the differentially abundant metabolites, and the color legend indicates the abundance in different groups; red indicates upregulation, and green indicates downregulation. A higher VIP value indicates a stronger contribution to group distinction, with metabolites having a VIP value above 1 showing significant differences. (D) The x-axis depicts the $\log_2$ -fold difference in metabolite abundance for each control group, whereas the y-axis illustrates the $-\log_{10}$ of the P-value after the T-test. The dashed line perpendicular to the y-axis indicates the P-value threshold for screening differentially abundant metabolites. Red dots indicate upregulated differentially abundant metabolites ($FC > 1$) with $VIP \geq 1$ and $P < 0.05$, whereas blue dots represent downregulated differentially abundant metabolites ($FC < -1$) with $VIP \geq 1$ and $P < 0.05$. The size of the dots corresponds to the VIP value of the metabolite. (E) Differentially abundant metabolite correlation heatmap. Positive correlations are depicted by dark blue shading, which approaches 1, whereas negative correlations are shown by dark red shading, which approaches -1. The color gradient below the heatmap illustrates the Pearson correlation coefficient between the two differentially abundant metabolites. (F) In the heatmap, each row represents a metabolite, and each column represents a sample. The color intensity reflects the abundance of the metabolite, with red indicating higher abundance and blue indicating lower abundance. The differentially abundant metabolites presented diverse accumulation patterns between group (A) and group (B).

the metabolite set enrichment analysis, and the top 25 enriched pathways are visually represented in Figure 6.

4 Discussion

Our study revealed that PPPs can be enriched within 5 minutes following IPC, providing a reference for the potential use of IPC as an intervention to increase sports performance and its onset time. Additionally, our research clarified the effectiveness of IPC-induced PPP enrichment in human experiments. Furthermore, D-gluconate,

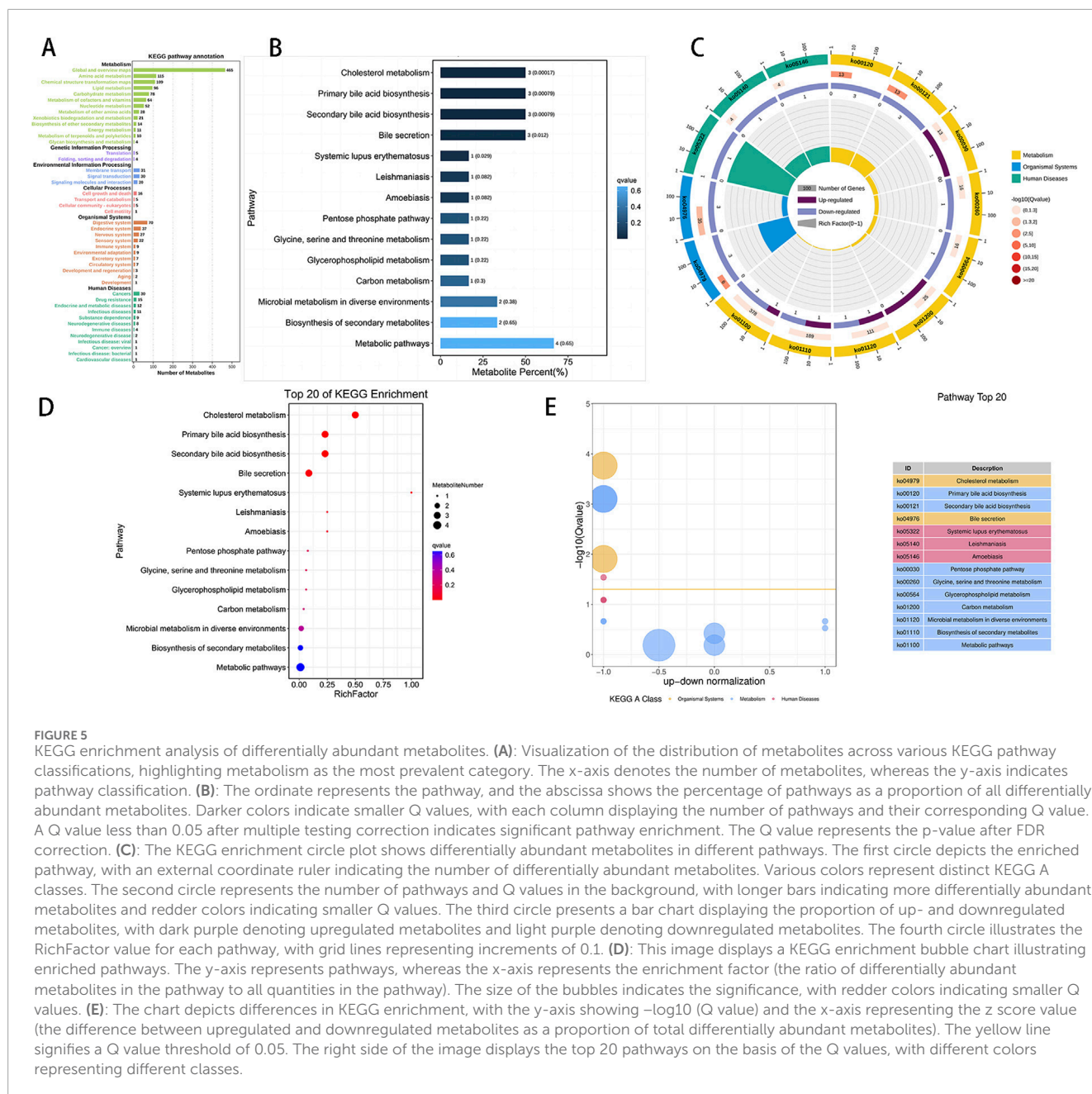
identified as a potential biomarker of IPC, can be utilized to ascertain whether the PPP-related protective mechanism of IPC is activated by measuring the concentration of D-gluconate. IPC may reduce the body's reliance on synthesizing ribose-5-phosphate 1-pyrophosphate (PRPP) via the oxidative pentose phosphate pathway (OPPP) through the upregulation of d-gluconate, which aids in ATP replenishment and the scavenging of accumulated free radicals. Metabolite analysis, which revealed changes in O-desmethylntramadol, D-2-aminobutyric, 2,4-D, PS, 9-HODE, 2-hexenal, and tetradenal, provides evidence supporting the potential of IPC to increase fatigue resistance and neutralize free radicals.



In our investigation, we observed a significant enrichment of the PPP in athletes undergoing IPC, which aligns with previous findings. However, earlier studies reported significant PPP activation 24 h after IPC and ischemia-reperfusion interventions in rats (Geng et al., 2019). In contrast, our results demonstrated that the PPP was already enriched in athletes just 5 min after IPC. This discrepancy may stem from limitations in the experimental sampling time, which could have restricted the detection of PPP activation in earlier studies. Furthermore, another study indicated that PPP was enriched during specific tests in Taekwondo athletes following IPC. This further suggests that PPP may be a potential mechanism by which IPC enhances athletic performance (Ou et al., 2024). Consequently, the enrichment of PPP after IPC implies that IPC may be utilized as an intervention method to improve exercise performance while also clarifying the effectiveness of PPP enrichment in human experiments post-IPC. Simultaneously, our study offers a reference for the optimal onset time of IPC. Additionally, ROC analysis revealed that the AUC value of the differentially abundant metabolite D-gluconate was 0.9375, identifying it as a promising

biomarker for IPC (Wei et al., 2011). The detection of D-gluconate may provide an effective method for rapidly assessing the success of IPC by reflecting the activation of the PPP. This aspect has rarely been mentioned in previous studies, suggesting that it may hold great significance in the practical application of IPC in sports training and warrants further research.

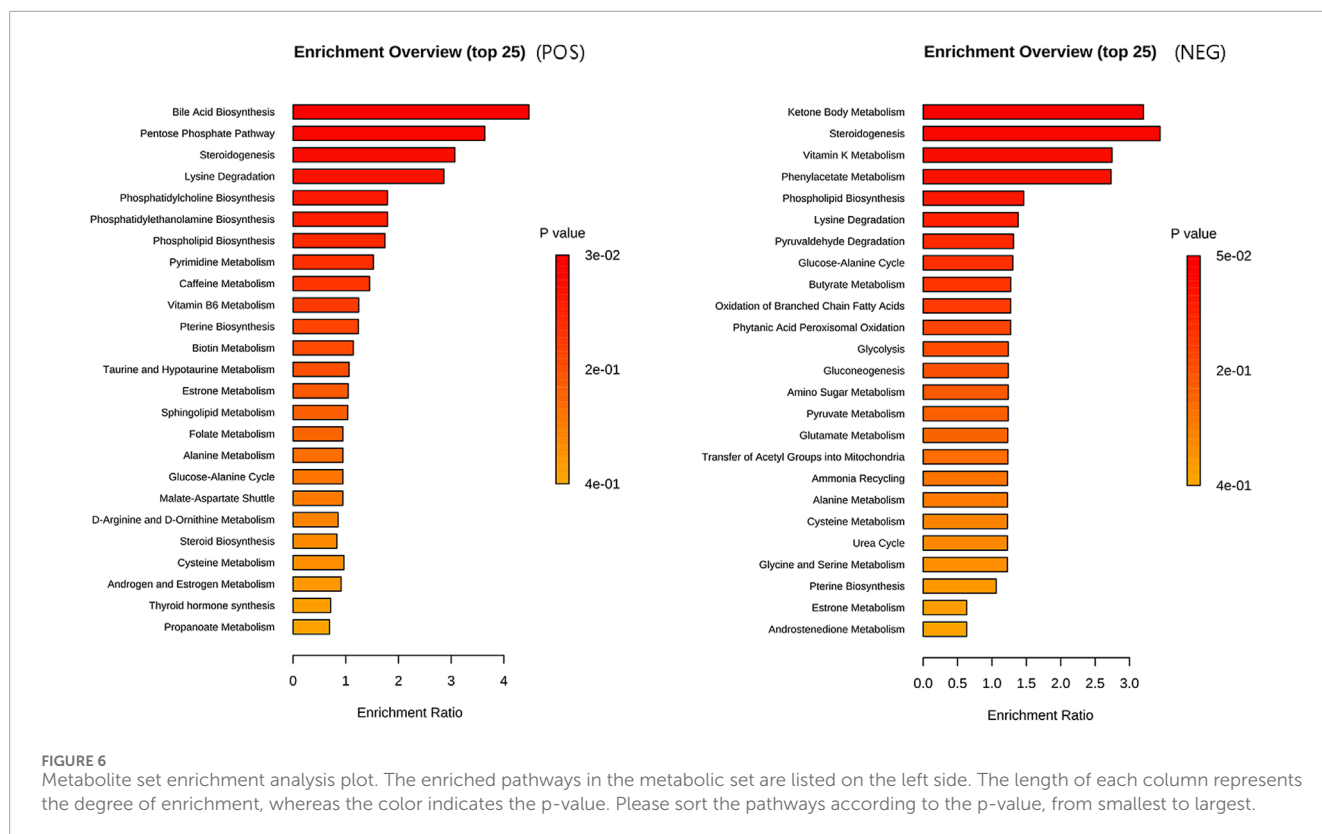
Notably, the IPC-induced upregulation of D-gluconate, along with the increased activation of the PPP, may contribute to increased antioxidant capacity in the body. This study underscores the importance of both the PPP and D-gluconate in the context of IPC, as demonstrated through metabolite analysis, KEGG enrichment analysis, and MSEA. The upregulation of the PPP and D-gluconate contributes to dihydronicotinamide-adenine dinucleotide phosphate (NADPH) and reactive nitrogen species (RNS) generation (Teslaa et al., 2023), such as NO, enhancing the body's resistance to oxidative stress and preventing free radical generation (Kloska et al., 2022). This has a positive impact on endurance sports performance (Reid, 2016a; Reid, 2016b; Henríquez-Olguín et al., 2019). Furthermore, NO can enhance



exercise performance by improving oxygen delivery, energy efficiency, contractility, and endurance levels (Reid, 2016b). NADPH oxidase plays a crucial role in IPC, offering protection against exercise-induced tachycardia (Sánchez et al., 2008; Bell et al., 2005). A study examining the effects of IPC intervention on the upper limbs in relation to swimming performance suggested that IPC could trigger the release of a protective factor and alter skeletal muscle tolerance to intense exercise (Emilie Jean-St-Michel et al., 2011), ultimately increasing maximum exercise capacity and endurance. This aligns with our own research findings.

Interestingly, an increase in plasma D-gluconate levels was observed, which may play a role in maintaining energy balance and preparing for a sustained energy supply. Research has indicated that the limited capacity of the oxidative pentose phosphate pathway

(OPPP) in bodily tissues constrains the production of ribose 5-phosphate (R5P), leading to an inadequate supply of ribose phosphate pyrophosphate pools. Consequently, this hinders the synthesis of adenine nucleotides, hampering ATP generation and restoration in tissues and causing the accumulation of free radicals, which is particularly noticeable in muscle tissue (Zimmer et al., 1990; Zimmer et al., 1973; Zimmer, 1992; Perl et al., 2011). Notably, our study revealed significant upregulation of the differentially abundant metabolite D-gluconate in athletes following ischemic preconditioning. D-gluconate can be converted to 6-phospho-D-gluconate by gluconokinase, and through subsequent catalysis by 6-phosphogluconate dehydrogenase and ribose 5-phosphate isomerase A, it bypasses the OPPP pathway to generate R5P, enhancing PRPP synthesis and ultimately replenishing bodily tissue



ATP. This finding aligns with prior research (Murry et al., 1986). Moreover, studies suggest that supplementing ribose *in vitro* can alleviate or prevent a decrease in ATP by bypassing the OPPP pathway (Zimmer, 1992). Hence, the increase in D-gluconate levels can effectively restore ATP in bodily tissues without relying on the OPPP pathway, thereby sustaining energy equilibrium. This phenomenon holds particular significance for skeletal muscle, where the OPPP pathway is most constrained (Zimmer et al., 1990). Although our study did not include exercise performance testing, the observed upregulation of D-gluconate and enrichment of the pentose phosphate pathway suggest that the mechanism by which ischemic preconditioning enhances exercise performance may be related to these factors. Consequently, further targeted research is warranted.

The upregulation of the PPP and D-gluconate is crucial for the body's redox system and energy balance. However, the functional properties of other plasma metabolites that are advantageous for exercise should not be overlooked. This study aligns with previous research demonstrating that IPC triggers the production of endogenous opioids in experimental subjects. O-desmethyltramadol, an opioid, is notably increased after ischemic preconditioning, leading to cardioprotection through the stimulation of μ -opioid (Schultz et al., 1998a) receptors and the activation of protein kinase C (PKC) (Fryer et al., 1999; Schultz et al., 1998b; Ytrehus et al., 1994). Research indicates that PKC and NO (produced from NADPH) can increase mK_{ATP} levels (Sato et al., 2000). Elevated mK_{ATP} levels promote vasodilation, preventing vascular function decline after high-intensity exercise and increasing blood flow to muscles (Kraemer et al., 2011; Enko et al., 2011). Additionally, NO has vasodilatory effects, improving

blood oxygen delivery capacity (Moncada et al., 1991). Enhanced vascular function during exercise facilitates oxygen transport, lactic acid removal, maintenance of acid-base balance, and overall body function (Cooper and Brown, 2008). O-desmethyltramadol also provides analgesic effects by inhibiting norepinephrine reuptake and stimulating serotonin release (Poulsen et al., 1996; Patel et al., 2009). The heightened levels of O-desmethyltramadol seem to alleviate acute pain associated with training, enabling athletes to fully execute their skills and tactics. Moreover, the metabolite D-2-aminobutyric acid significantly increased, potentially increasing intracellular glutathione levels through AMPK activation and offering protection against oxidative stress. Research has indicated that D-2-aminobutyric acid supplementation can increase myocardial and circulating glutathione levels, preventing doxorubicin-induced cardiomyopathy in mice (Irina et al., 2016). Consequently, ischemic preconditioning leads to the upregulation of O-desmethyltramadol and D-2-aminobutyric acid in plasma, which aids in vasodilation, enhances antioxidant capacity, improves oxygen transport, increases lactate clearance, and alleviates the negative effects of high-intensity exercise, particularly endurance training. This process also helps maintain vascular function, offering protective effects on the cardiovascular system and analgesic benefits. The metabolite changes induced by ischemic preconditioning seem to enhance the performance of athletes.

In addition, the plasma levels of the differentially abundant metabolites 2,4-D, PS, 9-HODE, 2-hexenal, and tetradenal were notably reduced following IPC. 2,4-D is a superoxide-producing oxidant that increases the expression of PPP metabolites and G6PD in dopaminergic cell lines, inducing oxidative stress and

neurotoxicity (Zhangxue et al., 2012; Higuchi et al., 2001). In this study, the downregulation of 2,4-D content resulting from IPC helped prevent the development of movement disorders (Tu et al., 2019). Exogenous PS supplementation has been shown to mitigate the emergency response of serum cortisol and creatine kinase to acute exercise (Monteleone et al., 1990; Monteleone et al., 1992; Fernholz, 2000), maintaining skeletal muscle movement under high-intensity exercise or hypoxic conditions, prolonging the time to fatigue, and ultimately improving exercise performance (Schumacher et al., 2021; Kingsley et al., 2005; Kingsley Mi et al., 2006). However, in our experiment, PS levels decreased, indicating its utilization in coping with emergency responses during IPC and activating relevant mechanisms through preadaptation, which helps to protect the exercise capacity of skeletal muscles. 9-HODE, a bioactive oxidized linoleic acid metabolite, is associated with oxidative stress and the inflammatory response in the body (Spiteller and Spiteller, 1997; Nieman et al., 2014). Research indicates that 9-HODE serves as an oxidative stress biomarker following acute exercise (Nieman et al., 2014). The reduction in 9-HODE after IPC suggests that IPC enhances the body's capacity to combat oxidative stress, thereby potentially improving exercise performance. Additionally, a reduction in 2-hexenal in the blood can lower the production of ROS, whereas decreased tetradinone levels can help prevent mutagenesis (Moriya et al., 1983) and damage the antioxidant system (Badraoui et al., 2007). The notable decrease in these plasma metabolites mediated by IPC suggests an increase in the body's antioxidant capacity and a reduction in the body's inflammatory response. All these factors are conducive to enhanced exercise performance.

The results revealed significant enrichment in cholesterol metabolism, primary bile acid biosynthesis, secondary bile acid biosynthesis, and bile secretion, with a decrease in the number of annotated differentially abundant metabolites. This contrasts with findings from a study on the metabolomic characteristics of long-term IPC training (Du et al., 2023), suggesting potential adaptive changes in the body following prolonged training. While reduced GCDC and Calcimycin levels were shown to aid in preventing hepatocyte ATP depletion and subsequent Ca^{2+} increase, thereby reducing toxicity to hepatocytes (Spivey et al., 1993; Pressman, 1976), changes in these substances have not been reported in studies on long-term IPC training. Notably, the PPP enrichment observed in this study has not been documented in long-term IPC training research. This discrepancy may stem from the differing mechanisms underlying acute IPC and long-term IPC, warranting further investigation.

5 Conclusion

Nontarget metabolomics technology is effective for identifying the metabolic profiles associated with acute IPC. Within 5 min following acute IPC, the levels of PPP, D-gluconate, O-desmethyltramadol, and D-2-aminobutyric acid were upregulated, whereas 2,4-D, PS, 9-HODE, 2-hexenal, and tetradenal were downregulated. Additionally, D-gluconate has the potential to serve as a biomarker for IPC, facilitating the rapid detection of IPC activation status. These changes in metabolic profiles and metabolic pathways may be the potential mechanism by which IPC improves

exercise performance. However, this study primarily examined metabolic changes in male athletes, highlighting the need for further research to explore sex differences. Additionally, the investigation was limited to metabolic changes occurring within 5 min following IPC, without assessing exercise capacity. Consequently, further research is warranted to explore the long-term effects of IPC and to investigate the potential of combined exercise regimens alongside metabolite profiling.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Ethics Review Committee of Guangzhou Sport University. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

ZO: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Validation, Writing—original draft, Writing—review and editing, Visualization. LY: Investigation, Methodology, Supervision, Writing—review and editing. MX: Conceptualization, Investigation, Writing—review and editing. XW: Supervision, Project administration, Writing—review and editing. GX: Conceptualization, Investigation, Methodology, Supervision, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Molecular mechanisms of lipid droplets-mitochondria coupling in obesity and metabolic syndrome: insights and pharmacological implications

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Abnormal lipid accumulation is a fundamental contributor to obesity and metabolic disorders. Lipid droplets (LDs) and mitochondria (MT) serve as organelle chaperones in lipid metabolism and energy balance. LDs play a crucial role in lipid storage and mobilization, working in conjunction with MT to regulate lipid metabolism within the liver, brown adipose tissue, and skeletal muscle, thereby maintaining metabolic homeostasis. The novelty of our review is the comprehensive description of LD and MT interaction mechanisms. We also focus on the current drugs that target this metabolism, which provide novel approaches for obesity and related metabolism disorder treatment.

KEYWORDS

lipid droplets, mitochondria, peridroplet mitochondria, obesity, metabolic syndrome, pharmacotherapy

1 Introduction

Obesity has escalated to epidemic proportions worldwide and constitutes a grave threat to public health (Seidell and Halberstadt, 2015). There is also a marked increasing in these conditions among children. The primary cause of obesity is the accumulation of abnormal body fat, which significantly increases the risk of metabolic syndromes that include non-alcoholic fatty liver disease (NAFLD) (Younossi et al., 2016), high blood cholesterol, and diabetes (Kaze et al., 2021). These conditions severely affect health and substantially increase the risk of mortality (Chen et al., 2019). Research into the mechanisms underlying abnormal fat metabolism may be the new perspective for treating obesity and metabolic syndromes.

LDs and MT work cooperatively in lipid metabolism and energy maintenance. LDs-associated MT reveals the existence of various MT groups in cell structures carrying unique protein sets and exhibiting diverse capabilities in fatty acid oxidation (FAO). As mitochondrial motility is a key parameter in mitochondrial fusion, peridroplet mitochondria (PDM) segregate from cytoplasmic mitochondria (CM) due to the distinct mechanisms of mitochondrial adhesion to LDs. Aerobic exercise diminishes the interactions between hepatic LDs and MT, alongside reducing LD size, which correlates with a less severe manifestation of NAFLD (Bórquez et al., 2024). Perilipin 5 (PLIN5) is a lipid

droplet-associated protein can regulate lipid metabolism through protein kinase A (PKA) phosphorylation (Gao et al., 2017; Keenan et al., 2021). In this paper, we have summarized the mechanism of LD-MT interaction in abnormal lipid deposition, which might be an influential factor in the etiology of obesity and metabolic syndrome. We also mention the current drugs that target LD-MT interaction which provide novel approaches for obesity treatment.

2 Lipid droplets

2.1 Structure of lipid droplets

LDs originate in the endoplasmic reticulum (ER) and are associated with general organelles through membrane contact sites. Generally distributed in both prokaryotic and eukaryotic cells (Ibayashi et al., 2024), LDs are lipid and phospholipid storage organelles that serve as centers of lipid metabolism (Zadoorian et al., 2023). Almost all types of cells have the ability to store excess energy in the form of triacylglycerol (TAG) in LDs. LDs store neutral lipids (NLs) and proteins involved in lipid metabolism and cell membrane synthesis, serving as hubs for metabolic processes (Gross and Silver, 2014). LDs consist of a core of NL surrounded by a phospholipid monolayer, which is modified by specific proteins called LDs-associated proteins (LDAP) (Jarc and Petan, 2020), Figure 1B.

2.2 Synthesis and catabolism of lipid droplets

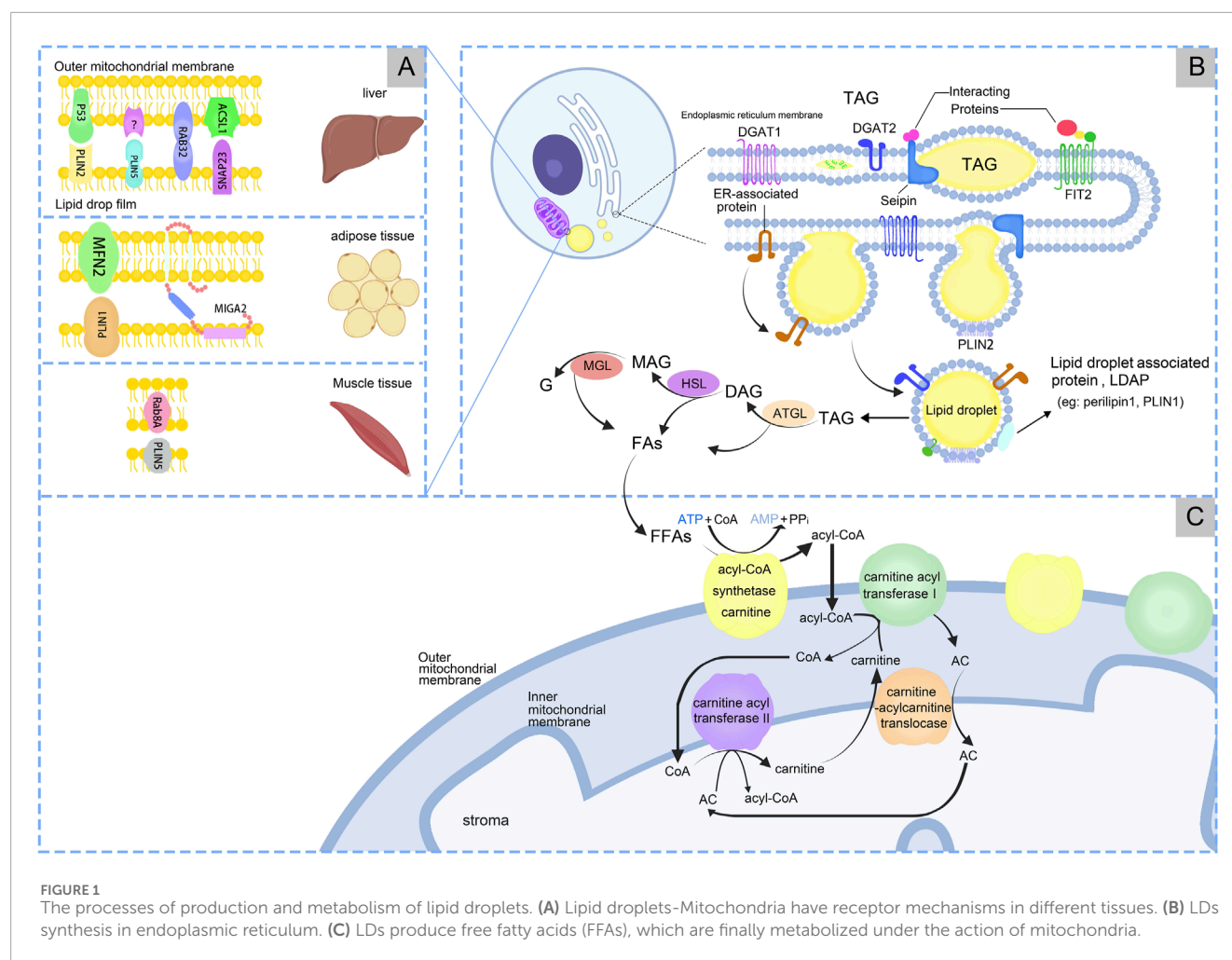
LDs formation is the process of synthesizing and assembling NL and phospholipid monolayers in the ER. This process involves the synthesis of NL within the ER, followed by nucleation, cytoplasmic outgrowth, and growth (Thiam and Ikonen, 2021). The NL stored in LDs are TAG and sterol esters (SE), with TAG being the predominant form. During LDs formation, diacylglycerol acyltransferases (DGAT), including DGAT1 and DGAT2, catalyze the covalent addition of a fatty acyl chain to diacylglycerol, resulting in the esterification and synthesis of TAGs (Wang et al., 2024). TAG is synthesized in the bilayers of the ER, and once a level of 5%–10% is reached, the proteins Fat Storage Inducing Transmembrane Protein 2 (FIT2) and Perilipin3 promote the development of TAG accumulates, LDs precursors (LDP) form “lens-like structures” (Walther et al., 2017). LDP is recruited to the site of LD biogenesis through interactions with LD markers, and at this site LDP bud from the ER into the cytoplasm (Cottier and Schneider, 2022). LDP originate from the ER and subsequently mature within the cytoplasm (Wang et al., 2016; Demirel-Yalciner et al., 2024), Figure 1B.

The process of lipolysis entails the sequential hydrolysis of TAGs to form diacylglycerols (DAGs) and monoacylglycerols (MAGs), with the liberation of a fatty acids (FAs) at each stage. The final FAs is released upon hydrolysis of the MAG, accompanied by the generation of glycerol (G). Enzymes associated with lipolysis

process include adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase/ α/β hydrolase domain-6 (MGL/ABHD6) (Ding et al., 2024). Lipophagy mediates the transfer and degradation of triglyceride-containing LDs through the lysosomal pathway (Kaushik and Cuervo, 2015; Wei et al., 2024). The resulting fatty acids undergo β -oxidation in MT. While the regulatory mechanisms of lipophagy remain unclear, this process significantly impacts hepatic metabolic disorders. Further research is needed to elucidate its precise role in liver metabolism (Kaushik and Cuervo, 2015). During the lipolysis process, ATGL breaks down TAG into DAG and FAs. Subsequently, HSL hydrolyzes DAG into MAG and FAs. Finally, MAG is converted into G and FAs by monoacylglycerol lipase (MGL). For a long time, HSL was believed as the rate-limiting enzyme in TAG breakdown. But currently ATGL is the rate-limiting enzyme that catalyzes the first step of TG breakdown to G and FAs (Xie et al., 2024). Mutations in the ATGL gene cause neutral lipid storage disease and myopathy, and reduced ATGL expression has been found in NAFLD (Ghosh et al., 2016). Disruptions in LD metabolism is associated with various metabolic disorders (Carotti et al., 2020), Figure 1B. LDs also response to inflammation, combined with MT, ER, and peroxisomes. These complexes undergo changes in inflammatory stimuli, can supply FAs for LD growth, and support FAs efflux from LDs. Macrophages utilize LDs for inflammatory lipid transport and influence inflammatory lipid mediators, indicating the importance of organelles in the regulation of inflammatory lipid metabolism (Zimmermann et al., 2024). Multi-spectral organelle imaging can comprehensively display the mapping of metabolic organelles including catalase, MT, Golgi apparatus and lysosomes, LD and ER, and their interactions with macrophages. This method can be applied in the study of metabolic changes in macrophages especially lipids which are rapidly responsive, opening up new avenues for potentially targeting the treatment of pathological conditions characterized by dysregulated lipid metabolism.

2.3 Lipid droplets associated proteins

LDs proteomics examined more than 200 proteins and functions that collaboratively regulate the droplets' formation, stability, metabolism, and growth. The aforementioned proteins can be broadly classified into these categories: membrane-associated structures of LDs that protect LDs and regulate their function, e.g., PLIN (Perilipin) and LSD2 (Lysine-Specific Histone Demethylase 2); lipid-metabolising enzymes on the surface of the ER and LDs that synthesise and degrade neutral lipids, e.g., DGAT1 and HSL. Membrane transporter proteins on the surface of the ER and LDs that regulate the interaction of LDs with other cellular structures, e.g., RAB18 (Ras-Related Protein Rab-18, Member RAS Oncogene Family); signalling proteins involved in inflammation and signal transduction, e.g., MAPK (Mitogen-Activated Protein Kinase); and degradation-associated proteins that degrade LD-related proteins, e.g., UBXD8 (UBX Domain-Containing Protein 8) also known as FAF2 (Fas Associated Factor Family Member 2). Furthermore, additional proteins, including ribosomal proteins, histones and actin, are implicated in protein translation, chromosome assembly under stress and LD motility, respectively. Among which perilipins



are ubiquitously found in the cytoplasmic LDs of mammalian cells, all play roles in LD function under differing conditions (Cinato et al., 2024). Five genes encode the five main perilipin (PLIN) family proteins (Sztalryd and Brasaemle, 2017), namely, PAT family including Perilipin A (PLIN A), Adipose Differentiation-Related Protein (ADRP), Tail-Interacting Protein 47 (TIP47), Adipocyte Protein S3-12(S3-12), and Oxidative Tissue-Enriched PAT Protein (OXPAT). Their function are various. PAT proteins regulate the lipases into LDs which promote LDs generation. Besides, PAT proteins can also control the lipolysis of stored NL via cytoplasmic lipases, maintain the morphology of LDs, and regulate the movement of LDs (Bickel et al., 2009). PLIN A is a surface protein on LDs that is identical to PLIN 1. PLIN A is predominantly expressed in WAT, and to a lesser extent is present in BAT, cardiac muscle liposarcoma, where its action functions in hormone-induced lipolysis large LD stabilization. PLIN one is a surface protein on LDs that facilitates the stabilization of LDs and lipolysis, which is mediated by lipases and cofactors. The PKA signaling is activated by PLIN one phosphorylation, then inducing the catabolism of TAG. This process has been linked to the development of metabolic diseases such as obesity, diabetes, endocrine disorders, and hypertension (Desgrouas et al., 2024). Furthermore, PLIN one was positively related to disease-free

survival (DFS) in lung squamous cell carcinoma (Kim et al., 2023). The depletion of B-cell receptor-associated protein 31 (BAP31) inhibited adipogenesis and lipolysis, but promoted the aberrant enlargement of LDs by reducing the proteasomal degradation of PLIN1 (Wei et al., 2023). ADRP is known as PLIN two that mainly expressed in liver, followed by premature adipocytes, macrophages, sebocytes, many gland epithelia, choriocarcinoma cells. The functions of PLIN2 include the differentiation of adipocytes, the generation of small LDs, and the stabilization of LDs. ADRP can target adenosine monophosphate-activated protein kinase alpha (AMPK α)-dependent lipophagy, mediated by the Perilipin 2-lysosomal acid lipase (PLIN2-LIPA) axis which ameliorates NAFLD (Fang et al., 2024). ADRP knockout inhibits the induction of platelet-derived growth factor (PDGF) and suppresses vascular smooth muscle cell (VSMC) proliferation in atherosclerotic lesions, as evidenced by a decline in extracellular signal-regulated kinase (ERK) activity and protein kinase B signaling pathways (Zhao et al., 2017). TIP47 is called PLIN three that mainly expressed in ubiquitous but also in skeletal muscle neutrophils, mast cells retinal pigment epithelium sebocytes and its action function is LD stabilization (compensation of PLIN2) PGE2 production intracellular trafficking. PLIN-3 induced apoptosis in CD8⁺ T lymphocytes and promoted Programmed Cell Death

1 Ligand 1 (PD-L1) and B7 Homologue 2 (B7-H2) expression in oral squamous cell carcinoma (OSCC). PLIN-3 knockdown in tumors reduces LDs and tumor migration (He et al., 2024). S3-12 is known as PLIN 4, predominantly expressed in WAT and secondarily occurs in hMSC (Musculin), which is induced during differentiation of skeletal muscle. PLIN four plays a major role in human adipocyte differentiation. Moreover, S3-12 binds to the surface of nascent LDs promoting TAG synthesis in a time-substrate- insulin-dependent manner in LDs generation (Wolins et al., 2003). OXPAT is named of PLIN 5, which is expressed predominantly in cardiac muscle BAT skeletal muscle and to a lesser extent in β -cells hepatic stellate cells, where it primarily plays a role in LD stabilisation FA supply to MT. Through augmentation of FAs uptake and elevation of the expression levels of enzymes associated with oxidative catabolism, OXPAT facilitates mitochondrial FAO (Wolins et al., 2006). The heterogeneity of LDAP in different cells and tissues is the result of a combination of factors, including differences in cell type and function, differences in the regulation of protein expression, differences in the intracellular environment, and differences in the interaction of LDs with other organelles. These differences enable lipid droplet proteins to fulfil specific roles in different physiological and pathological states. The proper functioning of LDAP is essential for maintaining cellular energy balance and metabolic health. However, disruptions in LDAP function are increasingly being recognized as significant contributors to various diseases.

3 Mitochondria

3.1 The structure of mitochondria

The outer membrane (OM) separates the MT from the cytoplasm. While the inner membrane (IM) forms the mitochondrial matrix. The IM is divided into an inner boundary membrane, parallel to the OM, and cristae. Mic60, a component of the MT's contact site and cristae organizing system, is systematically distributed at cristae junctions to support the mitochondrial structure (Stoldt et al., 2019). Carnitine palmitoyltransferase, a crucial enzyme in lipid β -oxidation, is located on the OM and catalyzes the conversion of palmitoyl CoA to palmitoyl carnitine, a process that is essential for the complete oxidative catabolism of FAs.

3.2 Distribution and classification of mitochondria

MT supply energy for the body, while liver, skeletal muscle, cardiovascular and brown adipocytes are the most energetic organs. In LDs metabolism, MT is involved not only in lipogenesis but also in FAO, processes that are intricately linked to mitochondrial dynamics, including movement, fusion, and fission. Microtubule stabilizers induce mitochondrial fusion, activate the mechanistic target of rapamycin (mTOR) signaling pathway and enhance ATP production (Cho et al., 2021; de Mello et al., 2018). Based on the specific proteins of MT and their roles in FAs and pyruvate oxidation, these organelles are categorized into PDM and CM, both of which coexist within the same cell to facilitate lipid oxidation

and synthesis (Benador et al., 2018). The functions of PDM and CM differ mainly because the different membrane surface proteins of each play different roles and are closely linked to the interaction with the microenvironment and other organelles. The growth and degradation processes of LDs, the concentration of surrounding FAs and the local redox state are bound to affect PDM (Mahdavi et al., 2017). In BAT, PDM has stronger pyruvate oxidation, electron transfer and ATP synthesis capabilities. It can also support LD amplification by esterifying FAs into triglycerides with the provision of ATP. CM shows stronger FAO ability (Benador et al., 2018). In WAT, PDM has stronger attachment to LDs through specific protein-protein or protein-lipid interactions, but has lower respiratory and ATP synthesis capabilities than CM. Moreover, the heterogeneity of PDM function can be determined by the size of LDs. The respiratory capacity of PDM is negatively correlated with the size of LDs (Brownstein et al., 2024). The reason for the gap between PDM attached to smaller LDs in WAT having higher respiratory capacity under pyruvate and PDM attached to larger LDs in BAT having higher ATP synthesis ability is that BAT has greater *de novo* lipogenesis and TAG turnover capacity, which is closely related to ATP synthesis and respiration, Figure 2. Naturally, PDM is not exclusive to adipose tissue, and it is likewise found in cardiomyocytes.

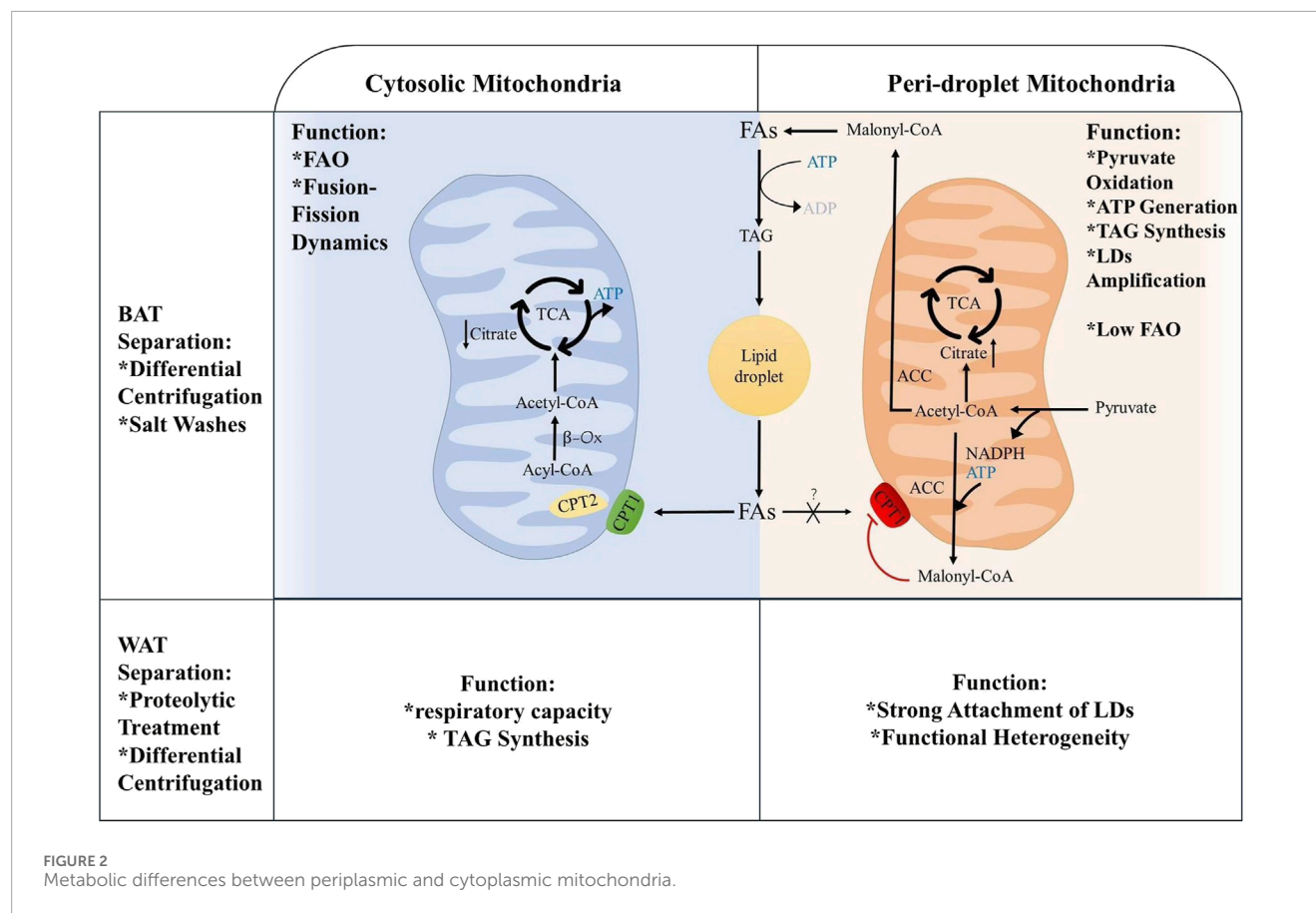
3.3 Identification and extraction of peridroplet mitochondria

Transmission electron microscopy (TEM) can directly identify PDM, allowing observation of its morphological features (Benador et al., 2018; Freyre et al., 2019; Tarnopolsky et al., 2007), including the arrangement of cristae in PDM towards LDs (Benador et al., 2018; Herms et al., 2015). Confocal imaging microscopy allows real-time observation of the dynamics of LD-MT (Stone et al., 2009; Wang et al., 2011) by double staining LD and MT markers in tissues marker (Benador et al., 2018). Besides, protein blot analysis and silver staining assay are applied to LD-MT mutual studies (Freyre et al., 2019; Cui et al., 2019; Yu et al., 2015), such as detecting PLIN5, mitochondrial proteins, and PLIN2. In addition, proximity ligation assay (PLA) and subcellular fractionation can also assess the status of mitochondria-associated endoplasmic ER membranes (Yang et al., 2019). Differential centrifugation and proteases could successfully separate PDM from LDs (Benador et al., 2018). However, centrifugation alone may not fully eliminate MT from LDs completely, proteases are required for efficient separation (Cui et al., 2019). In WAT, treating PDM with Proteinase K (Prot-K) before differential centrifugation could improve separation efficacy (Brownstein et al., 2024).

4 Lipid droplets-mitochondria interaction mechanism

4.1 Co-regulation of lipid metabolism by lipid droplets-mitochondria

MT produces ATP and participates in lipid metabolism within LDs. When the cell requires energy, TAG is hydrolyzed to



FAs, serving as energy carriers, **Figure 1B**. FAs are transported intracellularly to various cellular compartments by binding to the cytoplasmic lipid-binding protein, which maintains the solubility of FAs. MT convert FAs to lipoyl-coenzyme A (acyl-CoA) through the carnitine palmitoyltransferase (CPT1/2) system. Firstly, FFAs are catalyzed by acyl-CoA synthase and carnitine acyl transferase I (CPT1) on the OM of MT to form acyl-CoA and acyl-carnitine (AC). Carnitine-acylcarnitine translocase facilitates the transfer AC across the IM into the mitochondrial matrix. AC and CoA are enzymatically reconverted to acyl-CoA by carnitine acyl transferase II (CPT2) on the IM for β -oxidation (Talari et al., 2023), **Figure 1C**. FFAs are β -oxidized by highly FAO-capable CM to generate acetyl-CoA. Acetyl-CoA serves as a substrate in the tricarboxylic acid cycle (TCA) and oxidative phosphorylation to produce ATP for cells. The translocation of FAs from the LDs to the MT, which necessitates close contact between the two for lipid transfer, reduces FAs exposure and cellular lipotoxicity. In contrast, the enhanced ATP synthesis capacity of PDM facilitates the esterification of FAs to TAG and encourages LDs amplification. Additionally, PDM plays a pivotal role in the conversion of pyruvate to acetyl-CoA, which subsequently undergoes carboxylation by acetyl-CoA carboxylase (ACC) to form malonyl-CoA, an essential precursor for FA biosynthesis (Benador et al., 2018). When pyruvate oxidation through PDM is enhanced, acetyl-CoA levels significantly increase in the mitochondrial matrix. This elevation also impacted malonyl-CoA levels (Benador et al., 2018). At this juncture, malonyl-CoA acts as an inhibitory factor for

CPT1, thus effectively blocking the entry of FAs into the MT (McGarry et al., 1978), **Figure 2**.

4.2 The connection of lipid droplets-mitochondria interplay

The interactions between LDs and MT are facilitated by specific contact sites, **Table 1**. The expression levels and activities of proteins involved in the interaction between LDs and MT exhibit significant heterogeneity across different tissues. This heterogeneity can be attributed to the fact that the gene expression patterns of different tissue cell types are tailored (Elementaite et al., 2022) to meet the unique energy metabolism and cellular function requirements of their respective tissues (Ouyang et al., 2023; Ferreira et al., 2018). In liver, PLIN5 enhanced LDs formation and increased contacts between LDs and MT (Tan et al., 2019). When high fat diet (HFD), Rab32 localizes to LDs and MT, targeting LDAP-associated proteins Atgl and Plin5 to promote LDs accumulation and inhibition MT biosynthesis, fusion and oxidation. Inhibition of the Creb-Pgc1 α pathway blocked Rab32 localization to LDs and MT, suggesting that HFD may target LD-MT and then regulate hepatic LDs lipolysis (Song et al., 2020). Synaptosome-associated protein 23 (SNAP23) and long-chain acyl-CoA synthetase 1 (ACSL1) are situated on the OM of MT, which promote FAs β -oxidation (Che et al., 2023). SNAP23 may be crucial for the LD-MT complex (Jägerström et al., 2009), when ablation would inhibit the complex

TABLE 1 LD-MT interaction targets between different tissues.

Tissue	Contact site	Model	Result	References
Liver	Acs1(M), Snap23(L)	Mouse	Promote β -oxidation	Young et al. (2018)
	Acs1(M)	Mouse	Have a strong linear relationship with weight	Khamoui et al. (2020)
	RAB32 (M, L)	Human hepatocyte cell	Accumulate lipids	Li et al. (2016)
	RAB32 (M, L)	<i>P. fulvidraco</i> hepatocytes, human liver cancer cells	Regulating mitochondrial physiological processes	Song et al. (2020)
	P53(M), Plin2(L)	Mouse	Help drive mitochondrial fission	Zhou et al. (2018a)
	Unclear(M) Plin5(L)	Mouse	Promoted LDs Formation	Tan et al. (2019)
Adipose tissue	Miga (M, L)	3T3-L1, COS7 cells	Promotes lipid storage in LDs	Freyre et al. (2019)
	Mfn2(M) Plin1(L)	Mouse	Affected lipolysis	Boutant et al. (2017)
	Caveolin-1(L)	Caveolin-1 null mice	Regulates lipids metabolism	Cohen et al. (2004)
Skeletal muscle	Rab8a(M) Plin5(L)	Rat	Promote FAs oxidation	Ouyang et al. (2023)
	Plin5(L)	LE rats (Male)	Participate in lipolysis	Ramos et al. (2014)

Mshort for located on the Mitochondrial membrane; L: short for located on the LDs, film.
LE, rats: Long-Evans rats.

and β -oxidation. In adipose tissue, mitoguardin 2 (MIGA2) located on OM of MT, facilitate the synthesis of TAG from non-lipid precursors (Freyre et al., 2019). The interaction between PLIN1 and mitofusin 2 (MFN2) also facilitated the association of MT with LDs (Boutant et al., 2017). MT typically accumulate around LDs in adipocytes. Caveolin-1 is involved in LDs and MT interaction, when knockout in adipocytes display the disappearance of MT around LDs and alters the spatial structure between the LDs surface and the cytoplasm, resulting in reduced lipolysis (Cohen et al., 2004). In skeletal muscle, LD-associated PLIN5 established a binding complex with the mitochondrial receptor Rab8a at the surface of LDs, facilitating LDs hydrolysis and delivery of FAs to MT for β -oxidation (Ouyang et al., 2023), Figure 1A.

The contacts between LDs and MT are displayed dynamic and stable, which could not be completely separated during ultracentrifugation. Morphological studies reveal that the interaction between LDs and MT are highly dynamic, consistent with the 'kiss and run' model. Electron microscope observed that the interaction between LDs and MT in skeletal muscle increases with exercise (Tarnopolsky et al., 2007). Furthermore, there is experimental evidence from electron microscopy that contact between the two varies with experimental conditions (Valm et al., 2017). Despite the publication of a limited number of precise LD-MT interaction protein sites, the molecular biology of how target proteins regulate the binding or separation of the two remains unclear. This represents a significant avenue for future research.

4.3 Factors affecting the connection of lipid droplets-mitochondria interplay

The attachment of regulatory proteins associated with LDs and MT interactions represents a complex biological process. In addition to being influenced by the localization and expression of proteins, the spatial distance and nourishment are also significant factors. This section is dedicated to the non-protein factors, and how specific proteins regulate LDs and MT binding and segregation is detailed in subsequent sections.

4.3.1 Spatial distance

MT surrounding LDs were observed in adipocytes, with similar findings in liver cells (Kalashnikova and Fadeeva, 2006). TEM revealed direct contact between LDs and MT. Studies show an association between the size of LDs and their proximity to MT, indicating that larger LDs were more likely to be closely associated with MT, and more efficient FAs transportation. The spatial distance between LDs and MT may affect FAs transportation and lipid metabolism.

4.3.2 Nourishment

Insufficient nutrients activate autophagy to release FAs, which convert to acylcarnitines on the OM of MT by binding to the coenzyme A (COA) moiety. FAs are then transported into the MT matrix via the CPT1/2 system and participate in FAO and oxidative phosphorylation to generate ATP (Nguyen et al., 2017). When

TABLE 2 A meta-collection of drugs acting on LD-MT.

Name	Organ/tissue	Site of action	Result	References
Targeting LDs synthesis and catabolism				
Octyl gallate	Liver	Ppar- γ	Increase in LDs	Lima et al. (2020)
Ochratoxin A	Liver	Ppar- γ and Siah2	Increase in LDs	Zheng et al. (2021)
Syzygium simile leaves	Liver	CD36	Decrease in LDs	Yen et al. (2018)
Theaflavin-3,3'-digallate	Liver	TF3-PK-AMPK axis	Decrease in LDs	Zhang et al. (2020a)
Dihydroartemisinin	Liver	lncRNA-H19 and AMPK	Increase in LDs	Xia et al. (2021)
Oroxylin A	Liver	Hif-1 α	Decrease in LDs	Jin et al. (2018a)
Quercetin	Liver	PI3K/AKT (PKB)/mTOR	Decrease in LDs	Li et al. (2023a)
Vitamin D	Skeletal muscle	Plin2	Decrease in LDs	Li et al. (2018)
Glucagon like peptide-1 receptor agonists	Skeletal muscle	Sirt1 and Atrogin-1	Improve insulin resistance	Xiang et al. (2023)
Semaglutide	Skeletal muscle	Sirt1 and Atrogin-1	Improve insulin resistance	Xiang et al. (2023)
Polysaccharide CM1	Adipose tissue	Ppar- γ	Decrease in LDs	Yu et al. (2021)
Cycloastragenol	Adipose tissue	Ppar- γ	Decrease in LDs	Kim et al. (2024)
Lemon extract	Adipose tissue	Ppar- γ and Dgat-1mRNA	Decrease in LDs	Carota et al. (2021)
Polychlorinated biphenyls	Adipose tissue	Ppar- γ	Increase in LDs	Kim et al. (2017)
MHC	White adipose tissue	Ppar- γ and Fgfr1	Decrease in LDs	Zhou et al. (2024)
Curcumin and Synthetic Derivatives	unclear	PPAR- γ 、COX2、FAS	Decrease in LDs	Moetlediwa et al. (2024)
Clozapine	Adipose tissue	Leptin	Increase in LDs	Tsubai et al. (2017)
Suramin	Adipose tissue	Srebp1, C/Ebpa, C/Ebp β and Ppar- γ	Increase in LDs	Li et al. (2023b)
Rutin	Adipose tissue	Sirt1/Pgg-1 α /Tfam	Decrease in LDs	Yuan et al. (2017)
Olanzapine	Adipose tissue	Plin1, Plin2 and Plin4	Increase in LDs	Nimura et al. (2015)
Deoxyschizandrin	Adipose tissue	Liposome	Decrease in LDs	Liu (Liu et al., 2018)
Targeting the LD-MT physical linkage site				
Statins	Liver	Plin5	Reduce triglycerides in liver cells	Yuan et al. (2017)
Atorvastatin	Liver	Plin5	Promote PLIN5 phosphorylation to Promote fat breakdown	Bórquez et al. (2024)
Mammalian target of rapamycin 1	Liver	Rab32	Regulates lysosomal-mTOR transport	Liu et al. (2018)
Glycoumarin	Liver	Plin5-Sirt1 axis	Relieve lipotoxicity	Langhi et al. (2014)
Resveratrol	Liver	Tnf-a/Nf-kb/i Nos/Hif-1a axis	Protect the liver; Reduce accumulation of LDs	Ebrahim et al. (2022)
Paracetamol	Liver	p53	High doses cause acute liver failure	Zhang et al. (2020a)

(Continued on the following page)

TABLE 2 (Continued) A meta-collection of drugs acting on LD-MT.

Name	Organ/tissue	Site of action	Result	References
Targeting the LD-MT-related metabolic axis				
Pioglitazone	Skeletal muscle	Ampk	Decrease in LDs	Tan et al. (2020)
Simvastatin	Skeletal muscle	Akt/mTOR	Reduce glucose transport	Sanjeev et al. (2019), Bonifacio et al. (2015)
Simvastatin(PGC-1α is overexpressed)	Skeletal muscle	Fatp4	Increase fatty acid transport	Panajaticovic et al. (2021), Panajaticovic et al. (2020)
Dapagliflozin	Skeletal muscle	SGLT2	Increase LDs, increase FAO	Op den Kamp et al. (2022)

overnutrition, overproduced pyruvate, the high pyruvate oxidation capacity in PDM generates substantial malonyl-CoA, which inhibits CPT1 and negatively regulates the transfer of FAs between LDs and MT, thus reducing the rate of lipid metabolism and energy production (McGarry et al., 1978). Conversely, diminished pyruvate oxidation capacity in the CM results in decreased levels of citrate and malonyl-CoA, thereby promoting the translocation of FAs to MT by CPT1 for β -oxidation, Figure 2.

5 An overview of lipid droplets-mitochondria interactions in metabolic diseases

5.1 Liver tissues

The LD-MT interaction sites known from current studies in the liver are Rab32, ACSL1-SNAP23, p53-PLIN2, and PLIN5. The liver is indispensable in the regulation of lipid metabolism. Rab proteins (Nguyen et al., 2016) are associated with intracellular LDs, and Rab32 is the Rab GTPase associated with MT (Bui et al., 2010). The knockdown of Rab32 promotes lipolysis by indirectly increasing the expression of adipose ATGL (Li et al., 2016). Hepatic FAs significantly upregulate Pgc1 α and induce Rab32 localization to LD and MT (Song et al., 2020). NAFLD is one of the most common liver diseases worldwide, characterized by elevated levels of reactive oxygen species (ROS), and Bailey (Bailey et al., 2015) demonstrated that LDs can act as antioxidant organelles. Overexpression of PLIN5 promotes the formation of LDs and LD-MT contacts, reduces cellular levels of ROS and upregulates genes related to mitochondrial function. This represents a survival strategy adopted by cells in response to stress and is a promising new target (Tan et al., 2019). Moreover, oleic acid (OA) increases PLIN5 via the PI3K/PPAR(Peroxisome Proliferator-Activated Receptor) α pathway (Zhong et al., 2019), and IL-6 increases PLIN5 through the JAK/STAT3 axis (Krizanac et al., 2023). The increase in PLIN5 promotes the contact between LD and MT, reduces the cellular levels of ROS. In contrast, Chaperone-mediated autophagy (CMA) induces Plin5 degradation (Ma et al., 2020). In addition, lipid metabolism defects not only cause NAFLD but also insulin resistance (IR). Abnormal lipid metabolism in hepatocytes leads to increased ROS. These responses in turn affect insulin signalling

in the liver, resulting in decreased liver sensitivity to insulin. This leads to a reduction in hepatic uptake and utilisation of glucose and exacerbates IR (Tilg et al., 2021). The livers of Plin5-deficient mice exhibited activation of c-Jun N-terminal kinase, impaired insulin signal transduction, and IR, which impaired systemic insulin action and glycemic control. The re-expression of Plin5 reversed these effects (Keenan et al., 2019). Similarly, 17 β -HSD13 functions as a hepatocyte-specific LDAP, and its expression is upregulated in patients with NAFLD (s). Besides, comparative proteomic study reveals 17 β -HSD13 as a pathogenic protein in NAFLD (Su et al., 2014). Inadequate dietary choline intake is associated with choline deficiency (CD), which induces NAFLD (Michel et al., 2011). Knockdown of serine/threonine kinase (STK) on the surface of hepatic LDs increases lipolysis and protects hepatocytes (Kim et al., 2023). Another new entry point for the treatment of NAFLD is the NR4A1 (nuclear receptor subfamily four group A member 1)/DNA-PKcs (DNA-dependent protein kinase catalytic subunit)/p53 pathway. The regulation of p53 is bidirectional, driving MT fission on the one hand, and stalling MT autophagy on the other to maintain MT homeostasis. Furthermore, melatonin blocks the NR4A1/DNA-PKcs/p53 pathway, promotes mitochondrial autophagy, and enhances NAFLD (Zhou et al., 2018a). ACSL1 is situated in the mitochondrial OM. The direct interaction with CPT - one leads FAs from LDs to the MT for oxidation, while the tethering of SNAP23 is not required (Young et al., 2018). The liver injury results in increased energy expenditure and weight loss, with ACSL1 content being linearly correlated to body weight (Khamoui et al., 2020). Elevated ACSL1 expression decreases β -oxidation (Li et al., 2020). Furthermore, miR-34c promotes liver fibrosis by inhibiting ACSL1 expression (Li et al., 2021). PLIN2 deficiency attenuates hepatic steatosis. The lack of Cannabinoid receptor 1 (CB1) signaling results in a decrease in PLIN2 levels through the CB1-perilipin2 axis, which opens up ideas for the treatment of steatosis (Irungbam et al., 2020). In alcoholic fatty liver disease (AFLD), p53 interacts directly with ALDH2, inhibiting the formation of reactive tetramers and indirectly limiting pyruvate production (Yao et al., 2023). PLIN2 inhibits the adenosine 5'-AMPK/ULK1 (human) recombinant protein-lysosome pathway and promotes cell proliferation in hepatocellular carcinoma (HCC) (Liu et al., 2022). Additionally, RAB32 is a crucial target of miR - 30c - 5p in HCC. miR - 30c - 5p inhibits the growth and invasion of HCC cells via the miR - 30c - 5p - RAB32 axis (He et al., 2021).

5.2 Brown adipocytes

The LD-MT sites are mainly MIGA2, PLIN1-MFN2. High MIGA2 expression not only promotes LD-MT contact but also effectively activates adipocytes to synthesize TAG from non-lipid precursors (Freyre et al., 2019). The direct interaction between Mfn2 and PLIN1 is conducive to the entry of FAs into MT and FAO. Mfn2 knockout would impair the fat utilization in mice even under a low-fat diet (LFD), increasing the incidence of obesity (Boutant et al., 2017).

5.3 Skeletal muscle

Skeletal muscle activity requires a large supply of mitochondrial energy, and the known LD-MT sites are PLIN5-Rab8A and PLIN2. PLIN5 mediates LD-MT coupling (LDMC) to enhance mitochondrial respiratory capacity, and its abundance correlates with mitochondrial respiration rates (Bosma et al., 2012; Kien et al., 2022). Vitamin D upregulates PLIN2 levels. In myotubes, calcitriol (the active form of vitamin D) increases the mRNA of triglyceride synthesis genes DGAT1 and DGAT2, partially mediated by PLIN2 for mitochondrial oxidative function (Schnell et al., 2019). Interestingly, overfeeding upregulates PLIN2 expression, but has no effect on ROS release *in vivo* (Toledo et al., 2018). Vascular endothelial growth factor B (VEGFB) upregulates fatty acid transporter protein 4 (FATP4) and FATP1, inhibiting FASN production (Li et al., 2019). Caffeine promotes FAs utilization and FAO (Enyart et al., 2020).

5.4 Cardiovascular

Myocardial disease is the most common cause of mortality and disability globally (Christiansen et al., 2017; Wende et al., 2017). A certain degree of LDs can alleviate oxidative stress in cells (Jarc and Petan, 2019). Oxidative stress is an imbalance between ROS and antioxidants in cells (Burgoyne et al., 2012). Under physiological conditions, ROS regulates numerous cellular processes at low concentrations (Lennicke and Cochemé, 2021); however, overproduction of ROS results in impaired cellular components and function (Ong et al., 2015; Humeres and Frangogiannis, 2019), triggering adverse cardiac remodeling and progression to heart failure (De Geest and Mishra, 2022; D'Oria et al., 2020). ROS induces LDs accumulation by increasing PLIN2 expression, and PLIN2 modulates LDs formation via PPAR and CREBBP (CREB Binding Protein) signaling pathways (Jin et al., 2018a). In mouse hearts, Plin2 is upregulated during fasting-induced steatosis (Suzuki et al., 2009; Ueno et al., 2017). Moreover, Sato demonstrated that Plin2-induced cardiac steatosis leads to an increased incidence of atrial fibrillation in aged mice (Sato et al., 2019). PLIN5 expression inhibits ROS (Zheng et al., 2017). LDs prevent excess ROS production by decreasing FAO (Kuramoto et al., 2012). Plin5 knockout mice exhibit significantly reduced TAG accumulation in cardiomyocytes (Drevinge et al., 2016), increased cardiac hypertrophy, and elevated myocardial oxidative stress

following transaortic constriction (Wang et al., 2019). Thus, Plin5-deficient myocardium elevates levels of ROS (Zheng et al., 2017), suggesting that Plin5 deficiency reduces cardiac function.

6 Pharmacology research of lipid droplets-mitochondria - Related targets

There are few drug studies showing intervention at the LD-MT interaction site, and to better provide new insights into the treatment and prevention of obesity, we have collected a wide range of drug studies related to LD synthetic catabolism and LD-MT interactions.

6.1 Lipid droplets synthesis and catabolism

PPAR- γ , SREBP-1 and C/EBP α/β targets such as FASN, HSL, ATGL, fatty acid binding protein (FABP) and LDAP regulating LDs synthesis and catabolism. PPARs promote LDs accumulation in the liver. Octyl gallate (OG) (Lima et al., 2020) and p-coumaric acid (p-CA) (Yuan et al., 2023) upregulate PPAR- γ expression. Ochratoxin A (OTA) upregulates PPAR γ levels, and induces hepatic steatosis through the PPAR γ -CD36 axis (Zheng et al., 2021). Similarly, the extract from *Syzygium simile* leaves (SSE) decreases CD36 expression, hindering cellular LDs accumulation (Yen et al., 2018). Additionally, p-AMPK reduces LD accumulation. The TF3-PK-AMPK regulatory axis is a novel mechanism to alleviate lipid deposition. The theaflavin monomer theaflavin-3,3'-digallate (TF3) acts on the TF3-PK-AMPK regulatory axis and activates AMPK (Zhang et al., 2020a). Conversely, DHA inhibits AMPK phosphorylation (Xia et al., 2021). Additionally, oroxylin A inhibits HIF-1 α expression *in vivo*, suppressing LDs accumulation (Jin et al., 2018b). Furthermore, Quercetin induces NAFLD by inhibiting AKT via the PI3K/AKT pathway and promoting FAs synthesis (Li et al., 2023a).

In skeletal muscle, vitamin D reduced PPAR- γ levels *in vivo*, subsequently lowering PLIN2 expression and decreasing LDs in skeletal muscle (Li et al., 2018). Glucagon-like peptide-1 receptor agonists (GLP-1RA) and semaglutide enhance the Sirtuin1 (SIRT1) signaling pathway, leading to a downregulation of the atrophy-associated factor Atrogin-1 and an increase in myogenic factor expression, which alleviating muscle atrophy and enhancing IR (Xiang et al., 2023).

Also in adipocytes, Polysaccharide CM1 diminishes PPAR- γ , DGAT1, and DGAT2 (Yu et al., 2021). Lemon extract (LE) (Carota et al., 2021) and triterpenoid cycloastragenol (CAG) (Kim et al., 2024) downregulate PPAR- γ in 3T3-L1 cells. Curcumin and Synthetic Curcumin Derivatives also downregulate PPAR- γ , suppress COX2, inhibit FASN (Moetlediwa et al., 2024). Mulberry and Hippophae-based solid beverages inhibit TGF- β and PPAR- γ signaling pathways, restoring WAT dysfunction (Zhou et al., 2024). Polychlorinated biphenyls (PCBs) upregulate PPAR- γ and induce fat-specific protein 27 (Fsp27) expression, resulting in IR (Kim et al., 2017). Other pathways that modulated the increase in adipocyte LDs included clozapine, which significantly and directly reduced leptin secretion in 3T3-L1 adipocytes (Tsubai et al., 2017). In fascia-derived stromal cells (FSC), suramin significantly

increase PPAR- γ , consequently elevating the expression of PLIN2, causing LDs accumulation (Li et al., 2023b). Rutin directly activates the SIRT1/PGC-1 α /mitochondrial transcription factor (Tfam) signaling pathway increasing MT and UCP1 in BAT, ultimately enhancing energy expenditure (Yuan et al., 2017). Olanzapine can increase PLIN1, PLIN2 and PLIN4 expression (Nimura et al., 2015). Deoxyschizandrin (DS) and DS-liposomes (DS-liposomes) in 3T3-L1 adipocytes reduce LDL in the cytoplasm, alleviating NAFLD (Liu et al., 2018).

6.2 Drugs associated with lipid droplets-mitochondria

6.2.1 Drugs targeted on the lipid droplets-mitochondria connection

Drugs have the potential to directly target LD-MT interaction proteins to modify the oxidative catabolic process of LDs, aiming to intervene in obesity. Statins reduce hepatocyte TAG content by inhibiting PLIN5 expression (Langhi et al., 2014). Atorvastatin ameliorates NAFLD by enhancing PLIN5 phosphorylation, and reducing TAG accumulation in the liver (Gao et al., 2017). Additionally, glycoumarin (GCM) regulates the PLIN5-Sirt1 axis, alleviating hepatic lipotoxicity (Zhang et al., 2020b). Resveratrol (RES) inhibits the thioacetamide (TAA)-induced TNF- α (inflammatory)/NF- κ B (nuclear factor- κ B)/iNOS (nitrosative stress)/HIF-1 α axis (Ebrahim et al., 2022), and ameliorates hepatic steatosis (Zhou et al., 2018b). Tumor suppressor protein p53 reduces LDs (Borude et al., 2018).

6.2.2 Drugs targeted on lipid droplets-mitochondria-related axes

Drugs affect relevant LD-MT metabolites, which modulate lipid metabolism and influence obesity. Among them, CPT1/2, PGC-1 α (PPAR coactivator), and AMPK on the MT membrane play a central role. PGC-1 α serves as a transcriptional co-activator whose upregulation increases FAO. Pioglitazone upregulates PGC-1 α and ameliorates IR (Tan et al., 2020). Conversely, Simvastatin disrupts the Akt/mTOR pathway, hampered insulin receptor and mTORC2 function, inducing IR (Sanvee et al., 2019; Bonifacio et al., 2015). Interestingly, vincristine impairs glycogen muscle reserve in normal mice but not in PGC-1 α overexpressing mice, suggesting a role for PGC-1 α in preventing simvastatin-associated myotoxicity (Panajatovic et al., 2021; Panajatovic et al., 2020). In addition, dagliflozin treatment for 5 weeks increased CPT1 (Op den Kamp et al., 2022), Table 2.

7 Conclusions and perspectives

In lipid metabolism, LDs are involved in membrane synthesis and store neutral lipids and proteins, through interactions with membrane-contact sites in various organelles (Zadoorian et al., 2023; Gross and Silver, 2014). It also regulated functions such as lipase entry into the LDs and maintenance of LDs morphology and motility with the assistance of the LDAP protein family (Bickel et al., 2009). The pyruvate oxidation capacity of PDM in WAT was significantly greater than that of CM (Benador et al., 2018).

However, the mechanism behind this enhanced pyruvate oxidation capacity remains unknown. Moreover, PDM and CM have been individually researched in BAT and WAT, but the interconnections need to go deep.

There is substantial scientific evidence to suggest that LD-MT interplay is of great significance to human health (Fan and Tan, 2024). Despite there is a large number of data on LD-MT interplay, the molecular composition and regulation of LD-MT tethering is still unknown. Consequently, future research endeavors should prioritize the identification of novel interacting proteins. LD-MT interacts on the surfaces of LDs and MT with surface proteins, e.g., PLIN5 playing a significant role. Future research can continue exploring contact proteins that interact with PLIN5 in the liver, aiming to enhance understanding of the underlying mechanisms in studies centered on the interplay between LDs and MT.

Medications that target the LDs sites to regulate cellular lipid synthesis and lipolysis. Regarding LD-MT interaction sites, medications can directly impact the oxidative catabolic process of LDs. For instance, statins reduce hepatocyte TAG content by inhibiting PLIN5 expression (Langhi et al., 2014). There is limited research on drugs targeting the LD-MT target, and additional studies are necessary to investigate whether other drugs that promote fat reduction and metabolic enhancement inhibit or reduce body weight through this mechanism. We anticipate the development of potent and side-effect-free medications targeting alternative pathways for treating obesity-related conditions. These advancements will offer novel approaches for managing metabolic disorders associated with obesity.

Author contributions

CZ: Conceptualization, Software, Supervision, Writing—original draft, Writing—review and editing. MZ: Conceptualization, Methodology, Resources, Software, Writing—review and editing. RB: Conceptualization, Resources, Software, Validation, Writing—review and editing. JC: Software, Visualization, Writing—review and editing. HY: Project administration, Resources, Validation, Writing—review and editing. GL: Resources, Validation, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Urinary amino acid metabolomic profiling and its association with childhood obesity in prepubescent individuals

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Introduction: Amino acids are fundamental in several metabolic processes, and their levels can reflect metabolism impairments that contribute to obesity and related diseases. Our objective was to identify a urinary amino acid fingerprint in obese and overweight children in prepuberty and to correlate this profile with cardiometabolic alterations.

Methods: The study included 110 children, boys and girls aged 9–10 years, they were classified according to their BMI-for-age (Body Mass Index for age) into three groups: normal weight (NW) (n = 45), overweight (OW) (n = 21), and obese (OB) (n = 44). The 12-h urine samples were analyzed by LC-MS/MS to quantify 47 amino acids using the *Amino Acids Analysis Kit* (Zivak[®], Turkey), values were corrected by creatinine concentration. Anthropometric measurements, cardiovascular parameters, and biochemical profiles were assessed following standard protocols.

Results: When compared to NW, anthropometric measures, systolic and diastolic blood pressure, and serum uric acid levels were progressively elevated in the OW and OB groups. The OB group was characterized by elevated alpha-amino adipic acid, asparagine, cystathionine, 1-methyl-histidine, serine, tryptophan, phenylalanine, and tyrosine. In contrast, the OW group presented the most expressive levels of glutamine, alpha-diaminopimelic, and sarcosine.

Discussion: Our findings indicate that obese and overweight children exhibit a particular urinary amino acid fingerprint which is similar to that reported in studies with plasma. The altered amino acids, particularly tyrosine, are frequently associated with impairments in glucose

homeostasis, insulin resistance, and diabetes mellitus type 2. Potential mechanisms for increasing the levels of these amino acids in excess of weight may include enhanced protein degradation and impaired oxidative metabolism.

KEYWORDS

childhood obesity, prepuberty, urinary amino acids, metabolomics, biomarkers

Introduction

Childhood obesity prevalence has progressively increased worldwide. Over 340 million children and adolescents aged 5–19 years were overweight or obese in 2016 (World Health Organization, 2020; Singh et al., 2023). According to the World Obesity Federation's 2019 estimates, 206 million children and adolescents aged 5 to 19 will be obese by 2025, which is expected to increase to 254 million by 2030. Excessive fat accumulation in childhood is associated with an increased risk of the early development of cardiovascular diseases (Stefan, 2020), insulin resistance, diabetes mellitus (Hameed et al., 2020), and cancers (Di Angelantonio et al., 2016). Discovering biomarkers of predisposition to obesity and related comorbidities enables early diagnosis, identifying individuals who better benefit from non-medical interventions such as physical activity and healthy diet, and who need early treatment, thus contributing to disease prevention or better prognostic (Dessi et al., 2014).

Metabolomics is an emerging field recognized as a powerful tool for discovering the metabolic fingerprint of a given phenotypic change (Su et al., 2014). The metabolome represents the total metabolites in a given organism, such as sugars, organic acids, amino acids, lipids, vitamins, minerals, and other compounds. Genetic and epigenetic factors, age, environment, nutrition, drugs, and lifestyle reflect individual metabolome alterations. Moreover, the metabolites are not inert molecules, they can directly or indirectly regulate gene and protein expression and participate in the maintenance of homeostasis (Gerszten and Wang, 2008; Scalabre et al., 2017). Several metabolites have been correlated to disease phenotypes, as their presence or concentrations in biological fluids are directly related to pathogenic mechanisms (Su et al., 2014; Scalabre et al., 2017). Therefore, metabolomics enables the identification of biomarkers of exposure or susceptibility to several diseases, providing remarkable opportunities for better understanding exposure and predicting potential adverse health outcomes (Dessi et al., 2014; Handakas et al., 2022).

Obesity is a complex phenotype depending on several genetic and lifestyle factors. Despite being extensively studied, there is still no complete elucidation of the mechanisms underlying its development, particularly in childhood. Recent metabolomic studies have identified that the concentration of several metabolites including amino acids, lipids, monosaccharides, organic acids, and serotonin are altered in patients with obesity, thus being potential candidates as biomarkers (Hameed et al., 2020; Zhang et al., 2013; Rangel-Huerta et al., 2019; Szczerbinski et al., 2022). Nonetheless, there are fewer metabolomic studies targeting childhood obesity, partly due to methodological and ethical challenges in obtaining blood samples. These studies also need to be expanded, considering the bases of obesity, growth, and pubertal hormones.

Human urine is a rich biofluid for metabolomic studies, since it concentrates a wide range of metabolites that change with age, diet, nutritional status, and environmental exposure, thus being able to characterize a given phenotype (Bouatra et al., 2013; Slupsky et al., 2007; Wahl et al., 2012). Urine production is a constant process and its collection is easy and non-invasive (Slupsky et al., 2007). Urine metabolome has been well explored in studies with adults (Slupsky et al., 2007; Yu et al., 2012; Holmes et al., 2008), but only a few studies have been conducted with children (Zhang et al., 2013; Chiu et al., 2016; McCormack et al., 2013). Considering this, analyzing urine from prepubertal children is very attractive for metabolomic studies, as it targets the molecular mechanisms behind childhood obesity and cardiometabolic alterations.

This study aimed to investigate if there is an amino acid fingerprint in the urine of obese prepubertal children and explore possible mechanisms through which the altered metabolites can contribute to obesity and cardiometabolic diseases.

Materials and methods

Participants and ethical aspects

This is a retrospective cross-sectional study conducted with a sample of 110 pre-pubescent children, both sexes, aged 9–10 years who participated in previous studies to evaluate cardiovascular health and nutritional status in children from public and private elementary schools in Vitória-ES (Brazil) (Batista et al., 2015). All procedures were according to ethical standards and approved by the Ethics and Research Committee on Human Experimentation from the Universidade Federal de São Paulo (register number: 16613619.1.0000.5505). Informed consent was obtained from parents or legal guardians before enrollment.

Anthropometric measurements

Anthropometric parameters (weight, height, waist, and hip circumferences) were assessed following WHO recommendations described by Batista et al. (Batista et al., 2015). Briefly, body weight was determined using an electronic scale (Toledo®, model 2096, Brazil) with the participant standing barefoot wearing underwear only. The height was measured with a stadiometer (Seca®, model 206) attached to a flat wall. Using an anthropometric tape, the waist circumference was measured in the horizontal plane at the midpoint between the lowest rib and iliac crest (Sanny®).

Subjects were divided as normal weight (NW), overweight (OW), and obese (OB) according to their Body Mass Index classification for age and sex (BMI-for-age) using free

AnthroPlus® software based on WHO growth reference (de Onis et al., 2007). Waist-to-hip (WHiR) (Freedman et al., 1999) and waist-to-height (WHtR) (Vieira et al., 2017) ratios were calculated using primary anthropometric data.

Assessment of cardiovascular and biochemical parameters

Trained researchers conducted examinations in a controlled environment. Blood samples were collected under fasting conditions to measure urea, creatinine, uric acid, glucose, total cholesterol, LDL-cholesterol, HDL-cholesterol, VLDL-cholesterol, and triglycerides. All analyses were performed using commercial kits in a single laboratory (Central Laboratory of the Social Service of Industry, SESI-ES, Brazil). Blood pressure (BP) was measured in the left arm with an oscillometric device (OMROM® model HEM-705CP) using cuff size according to the manufacturer's recommendations. Children were comfortably seated with their feet flat on the floor. Three BP readings were taken with 2-min intervals between each, and the average of the last two measurements was calculated for systolic BP (SBP), diastolic BP (DBP), and heart rate (HR) (Batista et al., 2015).

Evaluation of urinary amino acids profile

A 12-h urine sample was collected from each participant and used for amino acid qualification and quantification. The concentration of thirty-nine amino acids was determined in urine samples by using the *Amino Acids Analysis Kit* (Zivak®, Turkey) by high-performance liquid chromatography (HPLC) coupled to an electrospray ionization (ESI) mass spectrometry (MS/MS) system. Regarding the chromatographic and mass spectrometry conditions, the analyses were strictly performed following the manufacturer's protocol for the Amino Acid Biological Fluids LC-MS/MS Analysis Kit (Plasma and Serum and Urine and Cerebrospinal Fluid) (Ref: ZV-3002-0200-10), produced by ZIVAK. A Thermo Scientific (TSQ Quantum Access Max) was employed with a quaternary pump (Accela 600 pump model) with an automatic sampler and a triple quadrupole mass spectrometer analyzer. The concentration of all amino acids was corrected by the urinary creatinine concentration. The amino acids measured were: 3-methyl-histidine (3-MeHIS); 5-Hydroxy-L-tryptophan (5-HTRP); Alanine (ALA); Alpha-aminoadipic acid (AAA); Alpha-aminobutyric acid (ABA); Alpha-aminopimelic acid (APA); Anserine (ANS); Arginine (ARG); Asparagine (ASN); Aspartic Acid (ASP); Beta alanine (BALA); Beta-aminoisobutyric acid (BAIB); Carnosine (CAR); Citrulline (CIT); Cystathionine (CTH); Cysteine (CYS); Cystine (C-C); Gamma-aminobutyric acid (GABA); Glutamic Acid (GLU); Glutamine (GLN); Glycine (GLY); Histidine (HIS); Homocystine (HC-HC); Hydroxylysine (HYL); Hydroxyproline (HYP); Isoleucine (ILEU); Leucine (LEU); Lysine (LYS); 1-methyl-histidine (1-MeHIS); Ornithine (ORN); Phenylalanine (PHE); Proline (PRO); Sarcosine (SAR); Serine (SER); Thiaproline (THPR); Threonine (THR); Tryptophan (TRP); Tyrosine (TYR) and Valine (VAL). The handling of calibrants, controls, and samples, as well as the settings of the analytical methods, were carried out following the manufacturer's instructions, and data were analyzed using

Xcalibur version 2.0 software (Thermo Fisher Scientific). Amino acids whose concentrations were below the detection capability of equipment (such as Homocysteine, Taurine, N-acetyl-L-tyrosine, O-Phospho-L-serine, Histamine, Methionine, and Serotonin) were not included in the analysis. The CAS numbers for each amino acid are listed in [Supplementary Table S1](#).

Statistical analyses

All data are expressed as medians $\pm$ SEM (standard error of the median). Differences between groups were assessed using ANOVA followed by Tukey's multiple comparison *post hoc* test, Kruskal-Wallis and Dunn's Test or Mood's median test, and Pairwise median test. The tests were chosen considering the variable's distribution and variability according to indications in the table legends. Statistical analyses were performed with R, version 3.6.2 (R Core Team, 2019). A value of $p \leq 0.05$ was considered to be statistically significant.

Raw metabolomic data were imported into the R software environment for preprocessing. Data normalization was performed using quotient normalization, and resulting data were log-transformed to stabilize variance and improve normality. Missing values were imputed using the k-nearest neighbor (k-NN) algorithm. Processed data were then analyzed using MetaboAnalystR (version 4.0) for comprehensive metabolomic data analysis. A volcano plot was generated to identify significantly different metabolites between the normal and excess weight (OW and OB) groups. Metabolites with a fold change (FC) greater than 1.25 and a p-value less than 0.05 were considered significant.

Results

Population characteristics

In this cohort, 54.5% of the participants were male and 45.5% were female. Regarding the nutritional status of the prepubescents, 40.9% showed normal weight, 19.1% were overweight and 40.0% were obese. All anthropometric data such as BMI-for-age (percentile and z-score), waist circumference, hip circumference, WHiR, and WHtR showed significant differences between the groups. As expected, there was a progressive increase in these parameters in the OW and OB groups compared to the NW group. Also, the OB group had higher height and height for age z-scores and percentiles than the NW group. These results are described in [Table 1](#).

Biochemical test values are described in [Table 2](#), and only the uric acid levels significantly increased in the OB group compared to the NW group (4.10 vs. 3.30, $p = 0.0013$). As expected, the OW and OB groups have shown higher systolic (109.00 and 110.00 vs. 102.00, $p = 0.0001$) and diastolic (67.00 and 66.75 vs. 60.00, $p < 0.0001$) blood pressures; however, no differences were observed in heart rate.

Urinary amino acids profile

In the amino acid analysis method, the limit of quantification (LOQ) is defined as the signal-to-noise ratio of 10. The results

TABLE 1 Anthropometric parameters and growth indicators according to the nutritional status of prepubescent children. Numbers within parentheses indicate the sample size. kg = kilograms, m = meters, cm = centimeters, BMI = Body Mass Index; WHiR = waist-to-hip ratio; WHtR = waist-to-height ratio; NS = non-significant. [#]Mood's median test and Pairwise median test. ^{*}Kruskal-Wallis and Dunn's Test. [~]ANOVA and Tukey HSD. ^B Normal Weight ≠ Obesity; ^C Overweight ≠ Obesity; ^D All groups differ from each other.

Parameters/Indicators	Normal Weight (NW)	Overweight (OW)	Obesity (OB)	p-value
Sex				
Male	20.9%	6.4%	27.3%	*0.0282 ^C
Female	20.0%	12.7%	12.7%	*NS
Age (years)	10.00 ± 0.09	9.00 ± 0.14	9.5 ± 0.10	*NS
Body Mass (kg)	31.14 ± 0.85	40.40 ± 1.44	50.13 ± 2.04	[#] < 0.0001 ^D
Height (m)	1.41 ± 0.01	1.44 ± 0.02	1.43 ± 0.01	[~] 0.0116 ^B
Percentile Height/a	64.70 ± 5.29	70.60 ± 7.96	84.85 ± 3.93	*0.0002 ^B
Z-score Height/a	0.38 ± 0.18	0.54 ± 0.28	1.03 ± 0.16	[~] 0.0002 ^B
BMI (kg/m ²)	16.27 ± 0.25	19.91 ± 0.23	23.64 ± 0.61	[#] < 0.0001 ^D
Percentile BMI-for-age	45.40 ± 4.63	92.40 ± 1.13	99.40 ± 0.20	[#] < 0.0001 ^D
Z-score BMI-for-age	-0.06 ± 0.14	1.44 ± 0.07	2.51 ± 0.10	[#] < 0.0001 ^D
Waist circumference (cm)	58.00 ± 0.85	69.50 ± 1.41	78.03 ± 1.57	[#] < 0.0001 ^D
Hip circumference (cm)	71.50 ± 0.90	81.00 ± 1.38	88.05 ± 1.51	[#] < 0.0001 ^D
WHiR	0.80 ± 0.01	0.84 ± 0.01	0.89 ± 0.01	[#] < 0.0001 ^D
WHtR	0.41 ± 0.01	0.48 ± 0.01	0.55 ± 0.01	[#] < 0.0001 ^D

obtained for the LOQs showed a linear range of 0.01–0.32 ng mL⁻¹ for AA with correlation coefficients (r) greater than 0.994 which can be seen in [Supplementary Table S2](#).

Several amino acids showed relevant statistical differences according to nutritional status, as shown in [Figure 1](#). Briefly, the concentration of the aromatic amino acids PHE, TRP, and TYR was significantly increased in the urine of OB group compared to the NW group (72.08 vs. 43.04, $p < 0.05$; 109.36 vs. 85.65, $p < 0.01$ and 140.54 vs. 85.07, $p < 0.001$, respectively), TYR levels were also higher in the OW group than NW group (109.74 vs. 85.07, $p < 0.05$) and TRP concentration was higher in OW group compared to NW group (113.55 vs. 85.65, $p < 0.05$). The OB group also presented augmented levels of AAA (61.18 vs. 44.89, $p < 0.01$), CTH (25.86 vs. 19.55, $p < 0.05$) and SER (332.40 vs. 210.97, $p < 0.05$) compared to NW group and increased concentration of urinary 1-MeHIS compared to OW group (190.62 vs. 149.59, $p < 0.05$). The most expressive concentrations of the amino acids APA, GLN, and SAR were found in the OW group. APA levels were higher in OW compared to NW and OB groups (12.64 vs. 10.45, $p < 0.05$ and 12.64 vs. 9.17, $p < 0.05$). Urinary concentration of GLN was higher in OW than OB group (1309.16 vs. 1024.30, $p < 0.05$) while SAR levels were increased in OW compared to OB group (78.75 vs. 62.36, $p < 0.05$). A table evaluating all 39 amino acids according to nutritional status is provided as supplementary material ([Supplementary Table S3](#)).

[Figure 2](#) presents a metabolomic approach to demonstrate the relationship between the statistical significance of an amino acid expression and the magnitude of the change in its expression (considering the mean). For this analysis, we considered two groups: excess weight (OW and OB) and normal weight (NW). Five metabolites (PHE, CTH, AAA, TYR, and LYS) presented significant fold change between the groups, being more expressed in the excess of weight group. The most expressive magnitude of fold change was observed in the following order: LYS, TYR, AAA, CTH, and PHE. TYR exhibited the most significant statistical difference between groups. [Supplementary Table S4](#) shows the fold change of the mean for the normal weight group compared to the excess weight group for all amino acids evaluated in the study ([Supplementary Table S4](#)).

Regarding Principal Component Analysis (PCA), PC1 accounts for 78.3% of the variance, while PC2 explains 15.7%. Despite PC1's high variance, its loadings cluster near the origin, indicating no strong driver of separation. Moreover, metabolites furthest from the origin in PC1 do not align with those statistically significant in the Volcano Plot, suggesting that separation arises from minor cumulative changes rather than distinct metabolic shifts. PC2 captures additional variation but remains limited. Thus, while statistical differences exist, PCA alone does not fully capture metabolic shifts, highlighting the need for complementary analyses ([Supplementary Figure S1](#)).

TABLE 2 Distribution of cardiovascular parameters, and biochemical tests according to nutritional status. “u” = urine sample measurement SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate; NS = non-significant. #Mood’s median test and Pairwise median test. *Kruskal-Wallis and Dunn’s Test. ANOVA and Tukey HSD. (A) Normal Weight ≠ Overweight; (B) Normal Weight ≠ Obesity; (D) All groups differ from each other.

Parameter	Normal Weight (NW)	Overweight (OW)	Obesity (OB)	p-value
Blood tests				
Total cholesterol (mg/dL)	156.00 ± 6.90	173.00 ± 9.14	170.00 ± 6.52	“NS
HDL cholesterol (mg/dL)	49.00 ± 2.31	50.00 ± 1.89	43.00 ± 2.05	#NS
LDL cholesterol (mg/dL)	94.00 ± 5.64	102.00 ± 7.09	99.40 ± 4.77	“NS
VLDL cholesterol (mg/dL)	12.40 ± 1.32	15.60 ± 3.20	15.90 ± 2.21	#NS
Triglycerides (mg/dL)	59.00 ± 6.24	78.00 ± 16.00	80.00 ± 11.30	#NS
Uric acid (mg/dL)	3.30 ± 0.17	3.60 ± 0.16	4.10 ± 0.20	*0.0013 ^B
Urea (mg/dL)	24.00 ± 1.41	24.00 ± 1.50	23.00 ± 1.37	“NS
Creatinine (mg/dL)	0.70 ± 0.01	0.70 ± 0.02	0.70 ± 0.02	*NS
Glucose (mg/dL)	88.00 ± 1.58	90.00 ± 2.42	90.00 ± 1.79	*NS
Urine tests				
Sodium u (mEq/L)	110.50 ± 12.84	132.50 ± 22.78	159.00 ± 15.31	*NS
Potassium u (mEq/L)	20.15 ± 2.82	21.10 ± 5.10	26.40 ± 4.18	#NS
Creatinine u (mg/dL)	28.55 ± 3.05	20.61 ± 3.70	33.24 ± 3.83	*NS
Cardiovascular parameters				
SBP (mmHg)	102.00 ± 1.67	109.00 ± 2.52	110.00 ± 1.73	“0.0001 ^{A,B}
Percentile SBP	46.00 ± 4.97	65.00 ± 6.50	73.00 ± 4.56	*0.0005 ^{A,B}
DBP (mmHg)	60.00 ± 1.60	67.00 ± 2.24	66.75 ± 1.40	*< 0.0001 ^{A,B}
Percentile DBP	49.00 ± 3.74	69.00 ± 4.87	66.50 ± 3.82	*< 0.0001 ^{A,B}
HR (bpm)	81.50 ± 2.20	80.50 ± 3.11	79.25 ± 1.91	*NS

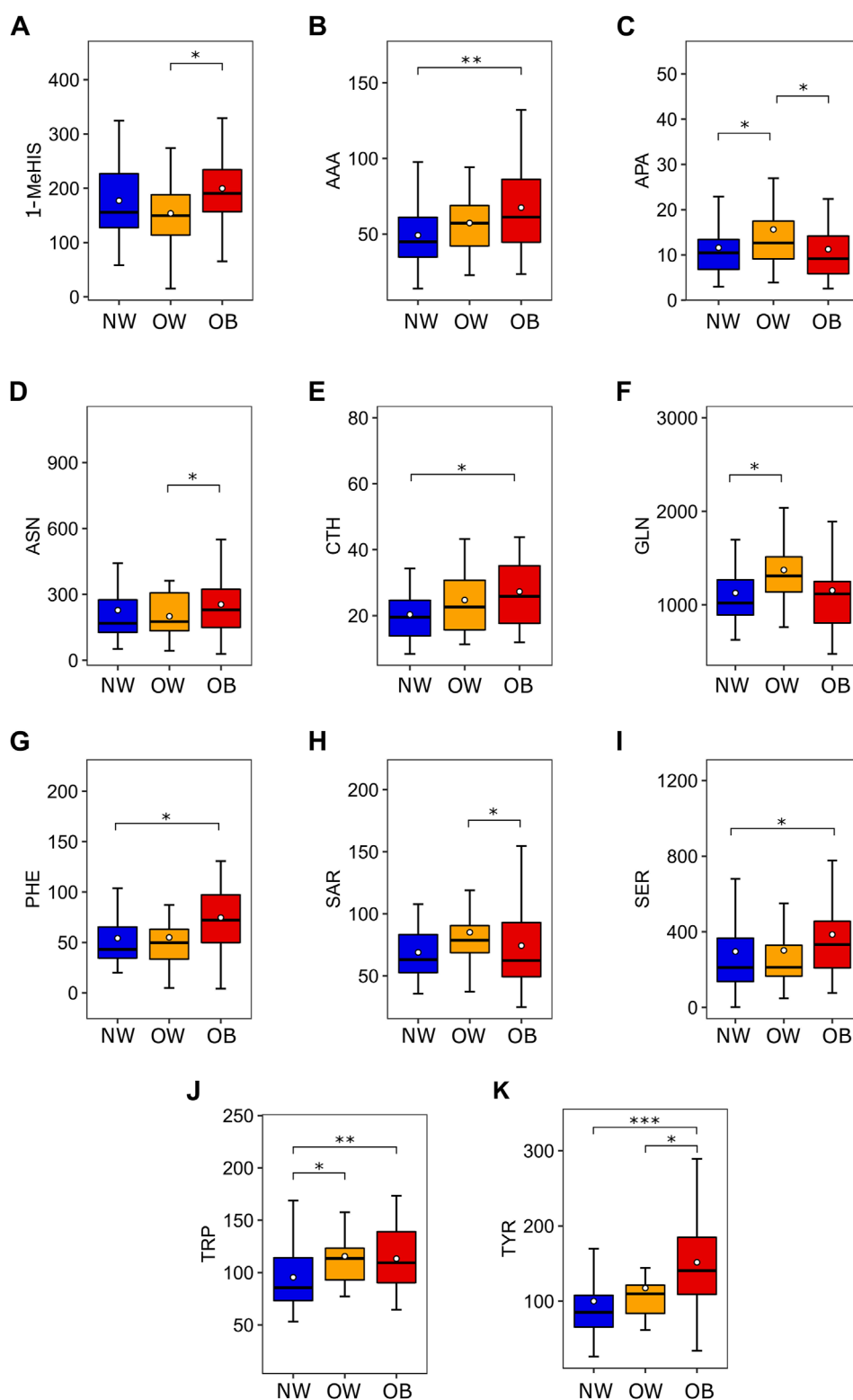
Discussion

Research groups and health organizations worldwide are unanimous in warning about the rapid increase in overweight and obesity prevalence and its consequences for individuals’ and communities’ health (Vieira et al., 2017; GBD, 2015 Obesity Collaborators et al., 2017; NCD Risk Factor Collaboration NCD-RisC, 2017). These consequences are directly related to higher health service costs, a high risk of disease development, and related mortality (Di Angelantonio et al., 2016; GBD, 2015 Obesity Collaborators et al., 2017; NCD Risk Factor Collaboration NCD-RisC, 2017).

Weight and BMI are anthropometric measures that reflect the quality of nutrition and healthiness of the living environment during childhood and adolescence. These parameters are good predictors of health and developmental outcomes throughout life (Park et al., 2012; Tanner, 1987). As expected, all anthropometric

measurements were progressively increased in the groups OW and OB, as shown in Table 1.

More important than total body fat, WHtR and WHiR predict fat distribution in the upper part of the body around the abdomen, which is associated with metabolic changes. WHiR and WHtR values of OW (0.84 ± 0.01 ; 0.48 ± 0.01 , $p < 0.0001$) and OB (0.89 ± 0.01 ; 0.55 ± 0.01 , $p < 0.0001$) groups, respectively, compared to NW (0.80 ± 0.01 ; 0.41 ± 0.01) suggest a progressive increase in abdominal fat in greater degrees of excess weight. Zeng et al. (Zeng et al., 2010) looking for biomarkers suggested that the waist-hip ratio, together with total triglycerides, total cholesterol, high-density lipoprotein, and low-density lipoprotein are the most critical parameters that correlate with the metabolic disturbances of childhood obesity (Lo et al., 2016). In a systematic review of thirty-nine studies, Park et al. (Park et al., 2012) observed evidence for associations between childhood BMI and type 2 diabetes, hypertension, and coronary heart disease, as in other studies (Lo et al., 2016; Libert et al., 2018;

**FIGURE 1**

Amino acids which are differentially expressed according to nutritional status. **(A)** 1-MeHIS concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. ANOVA ($p = 0.047$) followed by Tukey HSD ($OW \neq OB$). **(B)** AAA concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Kruskal-Wallis ($p < 0.01$) followed by Benjamini-Hochberg ($NW \neq OB$). **(C)** APA concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Mood's Median Test ($p = 0.046$) followed by Pairwise median test ($NW \neq OW$ and $OW \neq OB$). **(D)** ASN concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Mood's Median Test ($p = 0.049$) followed by Pairwise median test ($OW \neq OB$). **(E)** CTH concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Kruskal-Wallis ($p = 0.015$) followed by Benjamini-Hochberg ($NW \neq OB$). **(F)** GLN concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Mood's Median Test ($p = 0.029$) followed by Pairwise median test ($NW \neq OW$ and $OW \neq OB$). **(G)** PHE concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Kruskal-Wallis ($p < 0.01$) followed by Benjamini-Hochberg ($NW \neq OB$). **(H)** SAR concentration in urine (nmol/mg of urinary creatinine) according to

(Continued)

FIGURE 1 (Continued)

nutritional status. Mood's Median Test ($p = 0.024$) followed by Pairwise median test ($OW \neq OB$). **(I)** SER concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Kruskal-Wallis ($p = 0.049$) followed by Benjamini-Hochberg ($NW \neq OB$). **(J)** TRP concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Kruskal-Wallis ($p < 0.01$) followed by Benjamini-Hochberg ($NW \neq OW$ and $NW \neq OB$). **(K)** TYR concentration in urine (nmol/mg of urinary creatinine) according to nutritional status. Kruskal-Wallis ($p < 0.001$) followed by Benjamini-Hochberg ($NW \neq OW$ and $NW \neq OB$). Post-hoc tests: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Abbreviations: 1-MeHIS, 1-methyl-histidine; AAA, alpha-aminoadipic acid; APA, alpha-aminopimelic acid; ASN, asparagine, CTH, cystathionine; GLN, glutathione; PHE, phenylalanine; SAR, sarcosine; SER, serine; TRP, tryptophan; TYR, tyrosine; NW, normal weight; OW, overweight; OB, obesity.

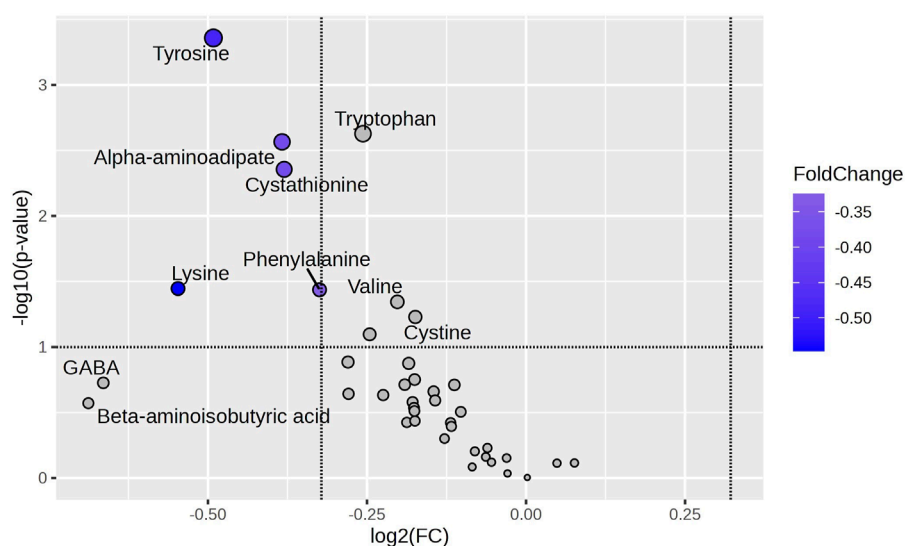


FIGURE 2

Amino Acid Variability Between NW and OW + OB Groups. The Volcano plot highlights amino acids differentially abundant between Normal Weight (NW) and Excess Weight (Overweight + Obesity; OW + OB) groups, using the NW group as the reference. Amino acids with a log2 fold change (FC) of less than -2 and a $-\log_{10}(p)$ value greater than 1.3 ($p\text{-value} < 0.05$) were considered significantly downregulated in the NW group compared to the OW + OB group. The amino acids phenylalanine, tyrosine, α -aminoadipic acid, cystathionine, and lysine fall within this category, indicating higher abundance in the OW + OB group. Blue highlights indicate amino acids with a $p\text{-value} < 0.05$. FC: Fold Change.

Juonala et al., 2011; Liu et al., 2021). Moreover, uric acid levels are substantially increased in obese subjects and proportionally associated with BMI (Weihrauch-Blüher et al., 2023), as observed in our results.

Metabolomics has been used to study the metabolic signature of obesity. It describes differential responses to dietary interventions, predicts health outcomes, and allows the study of the effects of specific nutritional patterns on obesity-related metabolites (Zhang et al., 2013; Rangel-Huerta et al., 2019; Szczerbinski et al., 2022). These biomarkers can represent disease diagnostic tools for developing new therapeutic protocols (Hameed et al., 2020; Zhang et al., 2013).

Investigation of serum metabolite concentrations in obese children may lead to new insights into biological mechanisms associated with childhood obesity, for example, branch-chained amino acids and various lipid metabolites, including phosphatidylcholines, cholesteryl esters, triglycerides (Zhang et al., 2013; Szczerbinski et al., 2022). Oberbach et al. (Butte et al., 2015) identified 163 serum metabolites, 12 of which were significantly related to obesity. Among those, GLY, GLN, and glycerophosphatidylcholine 42:0 (PCaa 42:0) serum concentrations were higher, whereas PCaa 32:0, PCaa 32:1, and PCaa 40:5

were decreased in the obesity group compared to the normal weight group. Wahl et al. (Wahl et al., 2012) analyzed serum samples from obese and normal-weight children aged six to 15. Fourteen metabolites and 69 metabolite ratios were significantly different in obese children compared to normal-weight children. Butte et al. (Butte et al., 2015) observed that obese Hispanic children had increased plasma concentrations of LEU, ILEU, and VAL but lower ASN, ASP, GLY, and SER concentrations. Plasma amino acid profile has shown a strong correlation with nutritional status (McCormack et al., 2013; Butte et al., 2015; Wu et al., 2024; Morris et al., 2012).

Despite urine being a promising biological fluid for metabolomics research, few studies have been conducted with this fluid compared to the numerous studies conducted with blood samples. However, urinary metabolomics research focuses on characterizing the metabolic profile present in urine, providing invaluable insights into both physiological and pathological processes. This comprehensive analysis facilitates the discovery of biomarkers for disease diagnosis, treatment monitoring, and elucidation of metabolic pathways (Scalbre et al., 2017; Szczerbinski et al., 2022; Bouatra et al., 2013; Cho et al., 2017; Chavira-Suárez et al., 2020). Detecting metabolites

in prepubertal urine represents a gap in the existing literature, and your investigation provides additional insights.

An amino acid signature characteristic of OB group was found in our study, with elevated levels of AAA, ASN, CTH, 1-MeHIS, SER, and aromatic amino acids, and reduced concentrations of APA, and SAR. The OW group presented most expressive urinary levels of APA, GLN, and SAR.

Urinary excretion of 3-MeHIS indicates protein catabolism, as it comes from skeletal muscle actin and myosin, and is also directly related to meat consumption (Cross et al., 2011). The higher levels of 1-MeHIS observed in the OB group may reflect dietary differences in meat intake. A controlled feeding study investigated 1-MeHIS and 3-MeHIS as potential biomarkers of meat intake and found a dose-dependent association between meat intake and urinary excretion of 1- and 3-MeHIS (Cross et al., 2011; Cross et al., 2014).

Our results show increased values of CTH and SER for the OB group. One-carbon metabolism is a metabolic network driven by three interrelated metabolic pathways: the folate cycle, the homocysteine-methionine cycle, and the transsulfuration pathway. If there is abundant methionine, the transsulfuration pathway will become active, by which homocysteine reacts with serine to form cystathionine by cystathionine β -synthase (Zhu et al., 2024; Dwight, 2020). Due to the inability to quantify homocysteine and methionine, their contribution to the increased levels of these other amino acids cannot be directly determined. Nonetheless, elevated levels of CTH and SER may indicate enhanced methionine metabolism and, consequently, increased homocysteine production through cystathionine β -synthase action (Dwight, 2020; Zhang et al., 2020). On the other hand, Butte et al. (Wu et al., 2024) found reduced serum levels of SER, which were associated with risk factors for insulin resistance, hypertriglyceridemia, and hyperuricemia.

Increased levels of AAA were observed in the OB group, as reported by Libert, Nowacki, and Natowicz (Libert et al., 2018), who also found elevated levels in adult subjects with obesity and diabetes. AAA can be generated by LYS metabolism. L-lysine is first converted to saccharopine by condensation with α -ketoglutarate, which is then reduced to 2-amino adipic semialdehyde, releasing GLU. Subsequently, 2-amino adipic semialdehyde is interconverted to AAA (Dwight, 2020). Catabolism of AAA forms 2-keto adipic acid, and in TRP catabolism, it also occurs through L-kynurenine degradation (Dwight, 2020). High values of AAA and TRP, but not LYS, may suggest changes in these metabolic pathways in the OB group. Wang et al. (Wang et al., 2013) observed that AAA predicted the development of diabetes in normoglycemic individuals and hypothesized that AAA levels were increased in response to hyperglycemia, increasing insulin secretion and contributing to a compensatory mechanism to maintain glucose homeostasis in early insulin resistance. It also suggests that AAA is a marker of diabetes risk and a potential modulator of glucose homeostasis in humans. Additional investigations should be done to link AAA to weight gain, insulin resistance, and T2DM.

In addition to AAA, GLN has also been linked to insulin resistance (Cheng et al., 2012; Newgard et al., 2009). Hanzu et al. (Hanzu et al., 2014) found high levels of GLN and ALA in the visceral adipose tissue of individuals with obesity. Due to the high gluconeogenic effect, the increased amount of these amino acids released by visceral adipose tissue contributes to hyperinsulinemia and the development of insulin resistance (Hanzu et al., 2014).

Higher GLN levels in the OW group suggest that being overweight may modify glucose metabolism due to increased body fat, especially visceral fat (Payab et al., 2021; Yan et al., 2023), evidenced by the augmented waist and hips circumferences and relative ratios. Despite the evidence, other groups have observed reduced levels of GLN in serum samples from children and urine samples from obese adolescents (Wahl et al., 2012; Cho et al., 2017).

Furthermore, it is well established that branched-chain and aromatic amino acids are indicators of the development of insulin resistance in normoglycemic young adults. This fact indicates a strong association between amino acids (Singh et al., 2023), particularly aromatics (TRP, PHE, and TYR), and the risk of future development of diabetes mediated partially by insulin resistance (Rangel-Huerta et al., 2019; Newgard et al., 2009; Martos-Moreno et al., 2005).

Under conditions of obesity, most studies demonstrate significant changes in blood and urinary values of aromatic (Butte et al., 2015; Cho et al., 2017; Haufe et al., 2016) and branched-chain amino acids (Szczerbinski et al., 2022; Butte et al., 2015; Bagheri et al., 2018). TRP, PHE, and TYR levels were elevated in the OB group, as other studies have also shown an association between increased concentrations of aromatic amino acids and obesity in both younger and older children, with different metabolic disturbances involved in the progression from overweight to obesity between the two age groups (Butte et al., 2015; Wu et al., 2024; Newgard et al., 2009; Payab et al., 2021; Bagheri et al., 2018; Kim et al., 2010; Yu et al., 2018).

Exploring amino acids through Volcano Plot analysis comparing children with normal weight (NW) and those with excess weight (OW + OB), using the NW group as a reference, the amino acids that exhibited a log₂ fold change of less than -2 and -log₁₀(p) of less than 1.3 — phenylalanine, tyrosine, α -amino adipic acid, cystathionine, and lysine — showed greater significance and were negatively regulated in the NW group. Notably, tyrosine emerged as the amino acid with the most significant changes and the most considerable magnitude of change.

Tyrosine levels were related to increased hepatic fat content, suggesting hepatic dysfunction associated with a metabolic disorder (Libert et al., 2018; Haufe et al., 2016). In addition, tyrosine contributes to the profile described in obese children (Payab et al., 2021). It may be a possible predictor of insulin resistance in these children and the most sensitive metabolite for the classification of obesity (Handakas et al., 2022; Butte et al., 2015; Martos-Moreno et al., 2005). Increased plasma levels of phenylalanine and tyrosine have been observed in most analyses of amino acid biomarkers in obesity and T2DM (Payab et al., 2021; Haufe et al., 2016; Zhao et al., 2016; Park et al., 2015). In this regard, elevated levels of PHE and its hydroxylation product, TYR, provided strong relevance as biomarker metabolites predictive of the development of cardiovascular disease and diabetes type 2. Suzuki et al. (2019) described that a state of unbalanced or increased amino acids associated with obesity may exacerbate obesity and insulin sensitivity (Suzuki et al., 2019).

Several limitations of the present study need consideration. The relatively small size of groups may have interfered with statistical significance. Moreover, our study used BMI-for-age to classify obesity, rather than body fat content and distribution, which would be a more accurate parameter of adiposity and metabolic changes.

However, determining criteria for cutting off body fat in children remains under discussion in the pediatric area. Additionally, other children's data should be compared with these results to trace the relationship between nutritional status and the metabolic profile of amino acids. Our working group is already outlining new data crossings.

Conclusion

The search for a biomarker that indicates the development of obesity is essential in a population that has not yet reached the reproductive stage. Since it allows blocking the progression of obesity and related comorbidities from measures that encourage healthier habits, before the need for drug therapies. Analysis of amino acids in urine through metabolomics showed a strong association between childhood obesity and increased levels of AAA, CTH, SER, and aromatic amino acids, particularly TYR, which appears to be a good candidate for obesity biomarkers. Potential mechanisms for increased levels of these amino acids include increasing protein degradation and impairment of oxidative metabolism in some tissues. Additional investigations must be done to determine whether the metabolism of TYR and other aromatic amino acids could identify the metabolic profile of children with obesity and other disorders.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Ethics and Research Committee on Human Experimentation from the Universidade Federal de São Paulo (register number: 16613619.1.0000.5505). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MP: Data curation, Formal Analysis, Writing – original draft, Investigation. NA: Data curation, Formal Analysis, Writing – original draft, Investigation. MB: Methodology, Writing – review and editing, Validation. LF: Data curation, Writing – review and editing. MI: Formal Analysis, Writing – review and editing. MM: Resources, Visualization, Writing – review and editing. JM: Investigation, Resources, Visualization, Writing – review and editing. FB: Methodology, Writing – review and editing. DC: Conceptualization, Investigation, Writing – review and editing. LO: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Correction note

A correction has been made to this article. Details can be found at: [10.3389/fphys.2025.1703729](https://doi.org/10.3389/fphys.2025.1703729).

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2025.1524939/full#supplementary-material>

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Metabolomic profiling in a rat model of visual fatigue associated with liver-kidney yin deficiency syndrome

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Background: Visual fatigue, commonly attributed to excessive eye use or dry conditions, is traditionally associated with deficiencies in liver and kidney yin in Chinese medicine. However, its metabolic aspects remain largely unexplored.

Methods: Levothyroxine sodium combined with tail-clip stimulation induced a rat model of visual fatigue with liver and kidney yin deficiency. At 3 (M1), 7 (M2) and 14 (M3) days after induction, histopathological changes were observed, and metabolic profiling was completed using untargeted UHPLC-Q-Exactive Orbitrap/MS.

Results: The rats exhibited signs of liver and kidney yin deficiency and visual fatigue on days 3 and 7, respectively. Compared to the control group, we identified 127 and 96 differential metabolites in the serum on days 7 and 14, respectively, primarily lipids and organic nitrogen compounds. Moreover, we observed disruptions in sphingolipid metabolism and signaling pathways.

Conclusion: This study enriches our understanding of the metabolic profile associated with liver-kidney-yin deficiency type visual fatigue.

KEYWORDS

visual fatigue, liver and kidney yin deficiency, UHPLC-Q-Exactive Orbitrap/MS, metabolomics, signaling pathways

1 Introduction

Visual fatigue is caused by a variety of factors, including excessive eye use and environmental or pathological factors, which are mainly manifested as frequent eye discomfort, such as ocular acid, dryness, and blurred vision (1, 2). It is common in both children and adults. Statistically, the prevalence of visual fatigue in children (< 18 years old)

ranges from 12.4–32.2% (3, 4), and that in college students worldwide is 46–71% (5). In addition, the number of people suffering from visual fatigue is increasing annually. These people come from all age groups, especially those who frequently use digital devices such as computers (6). The dangers of visual fatigue are obvious, which not only affects the state of study and work but may also cause the progression of diseases such as dry eye and video terminal syndrome (7). Therefore, considering the impact of visual fatigue on physical and mental health, the identification of methods to alleviate visual fatigue is necessitated.

To improve clinical prevention and treatment strategies for visual fatigue, we first need to understand the exact mechanism underlying visual fatigue. According to traditional Chinese medicine (TCM) theory, visual fatigue is related to the liver, kidneys, and lungs (8). Its etiology involves multiple aspects such as liver-kidney yin deficiency, qi blood deficiency, and stomach weakness (9). Zeng et al. classified visual fatigue into three types according to the dialectics of TCM—heart-blood deficiency, liver-kidney deficiency, and qi-blood deficiency, of which liver-kidney yin deficiency is the most common (10). Liver and kidney yin deficiency may cause eye discomfort such as dry eyes, tired eyes, and blurry vision (11). However, the etiology and biochemical basis of visual fatigue with liver-kidney yin deficiency remain unknown. Understanding the effects of liver and kidney yin deficiency on visual fatigue is expected to provide valuable information for clinical applications.

In traditional and modern medicine, metabolomics is an important technology for studying the networks of biological systems. Metabolomics describes the metabolic profiles of endogenous substances and their dynamic changes to characterize different physiological and pathological states of an organism (12). Recent studies have emphasized the application of metabolomics in understanding the pathophysiological mechanisms of liver diseases (13, 14) and kidney diseases (15, 16), advancing precision medicine and patient care. However, traditional research methods often fail to effectively clarify the potential of TCM therapeutic interventions, hindering the modern exploration of TCM-derived functional compounds. Metabolomics can accurately capture the molecular interactions among disease-responsive metabolites, thereby characterizing the systemic fluctuations and molecular complexity of TCM-derived functional compounds (17, 18). Clinical examinations have revealed that individuals with symptoms of liver and kidney yin deficiency exhibit enhanced energy metabolism and disturbed free radical metabolism (19). Moreover, micronutrients affect the morphology and function of eye tissues by participating in multiple cellular metabolic pathways to maintain the body's homeostatic balance (20). Therefore, the identification of metabolite changes during the progression of visual fatigue can provide useful information for improving eye health. However, the metabolites and metabolic pathways involved in visual fatigue have not yet been identified.

High-resolution mass spectrometry (MS), especially UHPLC coupled with a Q-Exactive orbitrap/MS, is widely used to analyze the chemical composition of biological samples and herbal plants (21). The advantages of this technique include fast polarity

switching and high sensitivity. Therefore, in this study, we utilized levothyroxine sodium combined with a tail clamp and light/dark environment manipulation to create a rat model of visual fatigue with liver and kidney yin deficiency. Then, through untargeted UHPLC-Q-Exactive orbitrap/MS metabolomics analysis identified differential metabolites and perturbed pathways closely related to visual fatigue progression, especially in sphingolipid metabolism and sphingolipid signaling pathway. The obtained data can help to better understand the metabolic characteristics associated with visual fatigue and, reveal the link between sphingolipid metabolic disorders and visual fatigue in the context of liver and kidney yin deficiency. This offers a new perspective for modern neuro-ophthalmology, metabolically corroborating the TCM theory that “liver and kidney yin deficiency leads to inadequate eye nutrition” (11, 22), shows the complementarity of TCM and Western medicine, thereby providing a new scientific basis for the targeted treatment of this disease.

2 Methods

2.1 Animal preparation

Sprague-Dawley (SD) male rats (specific pathogen-free (SPF) grade, license number SCXK (Jing) 2021–0011), weighting 200 ± 20 g, 6–7 weeks old, were acquired from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in an SPF laboratory (license number SYXK (Shan) 2018–0009). Before performing the experiments, all animals were acclimatized under controlled conditions (20–25°C, 45–65% relative humidity, 12-h dark/light cycle) for five days, with free access to water and food. The research protocol was in accordance with the ethical requirements and authorized by the Animal Ethics Committee (ethics approval number 20220913). All methods were carried out in accordance with relevant guidelines and regulations and are reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>) for the reporting of animal experiments.

2.2 Induction of visual fatigue in rats and grouping

The rats were randomly assigned to four groups, namely normal control (K1) and treatment for 3 days (M1), 7 days (M2), and 14 days (M3), with 10 rats per group. Visual fatigue was induced using levothyroxine sodium gavage combined with tail-clamping stimulation. Briefly, rats in the model groups (M1, M2, and M3) were administered levothyroxine sodium (90 g/kg) by gavage in the morning at 1 mL/100 g. Afterward, the tails of the animals were clamped with a clip (approximately 3 cm from the tip of the tail) for 5 min once a day for effective stimulation to induce the symptoms of liver and kidney yin deficiency. The cages of the rats in the model group were covered with black cloth to simulate a dark environment, and an LED light was placed on the cages as the only light source. The light source was 10–15 cm away from the rat,

with an intensity of 3000–500 lx. During the modeling period, the cage was knocked every 15 min to stimulate the rats to keep their eyes open for 2 h per day, and water and food were forbidden.

2.3 Sample collection and preparation

At the end of light exposure on day 15, the rats were anesthetized intraperitoneally with 20% urethane and blood was harvested from the abdominal aorta. After standing for 2–4 min, the blood samples were centrifuged at 3000 r/min for 10 min, and the supernatants were isolated and stored at -80°C . Tissues, including the spleen, liver, lens, ciliary body, and eyelids, were rapidly excised. After washing with normal saline, the tissues were ground into a homogenate with a grinding rod in a cold environment and centrifuged at 4°C and 12,000 rpm for 10 min. Finally, supernatants were harvested and stored at -20°C until assayed.

2.4 Detection of biochemical parameters

According to the instructions of each kit, the levels of the following indicators were quantified: cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), and $\text{Ca}(2+)/\text{Mg}(2+)\text{-ATPase}$ in the lens and ciliary body; aquaporin 4 (AQP4), aquaporin 5 (AQP5), and lactate dehydrogenase (LDH) in the tarsal gland and palpebral muscle; $\text{Na}(+)/\text{K}(+)\text{-ATPase}$ and $\text{Ca}(2+)/\text{Mg}(2+)\text{-ATPase}$ in the liver and cAMP, cGMP, estradiol (E_2), testosterone (T), corticosterone (CORT), triiodothyronine (T_3), thyroxine (T_4), interleukin 2 (IL-2), interleukin 6 (IL-6), superoxide dismutase (SOD), and malondialdehyde (MDA).

2.5 Untargeted LC-MS/MS metabolomics analysis

2.5.1 Serum sample preprocessing

Serum samples were thawed at 4°C and cold methanol/acetonitrile/water solution was added in the ratio of 2:2:1 (v/v). The mixed samples were vortexed and sonicated for 30 min, followed by incubation at -20°C for 10 min. After centrifugation at $14,000 \times g$ and 4°C for 20 min, the supernatant was harvested and dried under vacuum. For MS analysis, 100 μL of acetonitrile/water solution (1:1, v/v) was added to the sample for re-dissolution, vortexed for 30 min, and centrifuged at $14,000 \times g$ for 15 min. Finally, the supernatant was collected for further analysis.

2.5.2 UHPLC-Q-Exactive orbitrap MS assay

MS separation of the samples was accomplished using a Vanquish LC UHPLC system coupled with a HILIC column. The column temperature was set at 25°C . Detection was carried out using a binary solvent system with mobile phases A (water containing 25 mM ammonium acetate and 25 mM ammonia) and B (acetonitrile). The specific gradient elution parameters were

as follows: 0 to 1.5 min, 98% B; 1.5 to 12 min, 98% B linearly declined to 2%; 12 to 14 min, 2% B; 14 to 14.1 min, 2% B straightly elevated to 98%; 14.1 to 17 min, 98% B. During the analysis period, the temperature of the autoinjector was maintained at 4°C , and the injection volume was 2 μL with a flow rate of 0.3 mL/min.

The MS1 and MS2 spectra were captured using a Q Exactive mass spectrometer, and the chromatographic data were analyzed and detected in the positive (pos) and negative (neg) ion modes of electrospray ionization (ESI). The specific parameters of the ESI source and mass spectrum were as follows: gas 1 and gas 2 were 60; CUR was 30 psi; ion source temperature was 600°C ; spray voltage of positive or negative was 5,500 V; the range of primary mass-to-charge ratio detection was 80–1200 Da, the resolution/secondary resolution was 60,000/30,000, and the cumulative scanning time was 50 ms.

2.6 Data preprocessing and analysis

The collected raw data were converted to mzXML format using ProteoWizard software (23), and XCMS software (24) was utilized for data processing operations, such as peak alignment, retention time correction, and peak area extraction. After matching the raw peak area information with the total ion flow profile, data analysis was conducted. First, the number and classification of the metabolites were identified using the in-house database (Shanghai Applied Protein Technology). Next, multivariate statistical analyses were performed to characterize metabolic perturbations and differences among the different groups, including principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). The variable importance for projection (VIP) was calculated based on the OPLS-DA model, which was applied to mine the differential metabolites with biological significance. The selection criterion was $\text{VIP} > 1$ ($P < 0.05$). Finally, to reveal the biological functions of the metabolites, KEGG pathway enrichment analysis was conducted for differential metabolites. Differences between groups were measured using Fisher's Exact Test, and entries with $P < 0.05$ were selected.

2.7 Statistical analysis

Results are expressed as mean $\pm$ SEM and were analyzed as well as plotted by GraphPad Prism (version 7.0). Outcomes between the groups were compared using a one-way analysis of variance (ANOVA) and Dunnett's test. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Rats with visual fatigue show symptoms of liver and kidney yin deficiency

Throughout the animal experiments (0–14 d), we monitored the body weight changes in all rats and found that the weight of the rats in

each group increased slowly, with no significant differences among the four groups (Figure 1A). There were no significant differences in the thymus and spleen indices between the K1 and the three model groups (Supplementary Figures S1A, S1B). Regarding the liver energy metabolism enzyme activities of rats, compared to the K1 group, the model groups displayed increased Ca(2+)/Mg(2+) ATPase and a decline in Na(+)/K(+) ATPase levels, especially in the M2 group (Figures 1B, C). We measured key parameters related to the crystalline lenses and tarsal glands of the rats. The results showed that in the model groups, the levels of GSH-Px, AQP5, cAMP, and cGMP in the lens were elevated, whereas those of T-AOC and Ca(2+)/Mg(2+) ATP were decreased; significant changes in most indicators were observed in the M2 and M3 groups (Figures 1D–I). However, the levels of AQP4 and AQP5 in the tarsal gland and LDH in the palpebral muscle did not significantly differ between the normal and model groups (Figures 1J–L).

Moreover, the levels of inflammatory factors, AQP, antioxidants, and hormones in the serum of rats are important indicators of visual fatigue. As shown in Figures 2A–I, the serum of the model groups presented high levels of IL-6, IL-2, AQP4, AQP5, cAMP, and cGMP, while exhibiting low levels of SOD and cAMP/cGMP. However, no significant differences in MDA levels were observed among the four groups. Compared to the normal (K1) group, the levels of serum hormones (E₂, T, CORT, T3, and T4) increased significantly in rats from the three model groups (Figures 3A–E). Overall, these findings indicate that rats in the M2 and M3 groups exhibited both liver and kidney yin deficiency and visual fatigue symptoms, confirming the success of our model construction. The rats in these two groups were selected for further analysis.

3.2 Serum metabolic profiles

In the positive and negative ion modes, the spectra of the total ion chromatogram showed that the intensities and retention times of the chromatographic peaks overlapped, indicating that instrument error was negligible (Supplementary Figure S2A). PCA revealed that the QC samples in the two modes were closely clustered together, indicating good replicability (Supplementary Figure S2B). Overall, the stability, precision, and reproducibility of the UHPLC-Q Exactive Orbitrap MS method were excellent.

3.3 Metabolite determination and multivariate statistical analysis

Statistically, 551 (neg) and 695 (pos) metabolites were detected. For M2 vs. K1, an examination of the PCA data revealed that the K1 and M2 samples could be clearly separated in both the pos and neg ion modes (Figure 4A). An OPLS-DA model was established to further verify the differences between the two groups. The validity of the model was examined using a permutation test. The R²_Y/Q² values for the Pos ions were 0.978/0.522 and 0.989/0.555 for the Neg

ion, suggesting that the model was stable and reliable (Figure 4B). Similarly, in the comparison between the M3 and K1 groups, we observed a significant separation between the two groups (Figure 5A). The R²_Y and Q² parameters of the OPLS-DA model under the pos and neg ion modes were satisfactory (pos: R²_Y, 0.996; Q², 0.462; neg: R²_Y, 0.943; Q², 0.106), implying that the model was not overfitted and that the results were reliable (Figure 5B).

3.4 Differential metabolite identification

At VIP > 1 and *P* < 0.05, 127 differential metabolites were identified between the K1 and M2 groups, including 62 in Pos and 65 in Neg (Supplementary Tables S1, S2). In the pos mode, these metabolites were clearly assigned to eight categories, most of which belonged to lipids and lipid-like molecules (21/62, 33.8%), followed by organic nitrogen compounds (9/62, 14.5%) and organic acids and derivatives (9/62, 14.5%). The relative abundances of fingolimod and phytosphingosine were significantly upregulated, whereas that of acebutolol, creatine, and 4-hydroxy-3-methoxybenzyl alcohol were markedly downregulated in the M2 group (Figure 6A). In the neg mode, these metabolites belonged to eight classes, primarily lipid-like molecules (23/65, 35.4%) and organic acids and derivatives (13/65, 20%). Notably, in the M2 group, the abundances of 6-phosphogluconic acid and isopentenyl pyrophosphate increased, whereas those of Psoralidin and Urocanate declined (Figure 6B).

Using the same threshold, 96 differential metabolites were identified between the K1 and M3 groups, with 56 in the Pos and 40 in the Neg modes (Supplementary Tables S3, S4). In Pos mode, these metabolites were explicitly divided into nine categories—lipids and lipid-like molecules (15/56, 26.8%), organic nitrogen compounds (10/56, 17.9%), and organoheterocyclic compounds (7/56, 12.5%). Compared to the K1 group, 43 metabolites (N-acetylhistamine and fingolimod) were elevated, and 13 metabolites (4-phenylbutan-2-ol and phenylalanine) were reduced in the M3 group (Figure 7A). Moreover, in the Neg mode, the 40 metabolites were classified into seven classes—lipids and lipid-like molecules (13/40, 32.5%), organoheterocyclic compounds (6/40, 15%), and organic oxygen compounds (6/40, 15%). We observed that the abundances of 31 metabolites including 6-phosphogluconic acid and 3-ketofusidic acid, increased, whereas those of 9 metabolites, such as shikimate and eicosapentaenoic acid, decreased in the M3 group compared to the K1 group (Figure 7B).

3.5 Functional pathway enrichment analyses

To explore the potential metabolic pathways involved related to the differential metabolites, pathway enrichment analysis was conducted. As shown in Figure 8A, B, the differential metabolites in the K1/M2 and K1/M3 compartments mainly influenced sphingolipid metabolism and the sphingolipid signaling pathway.

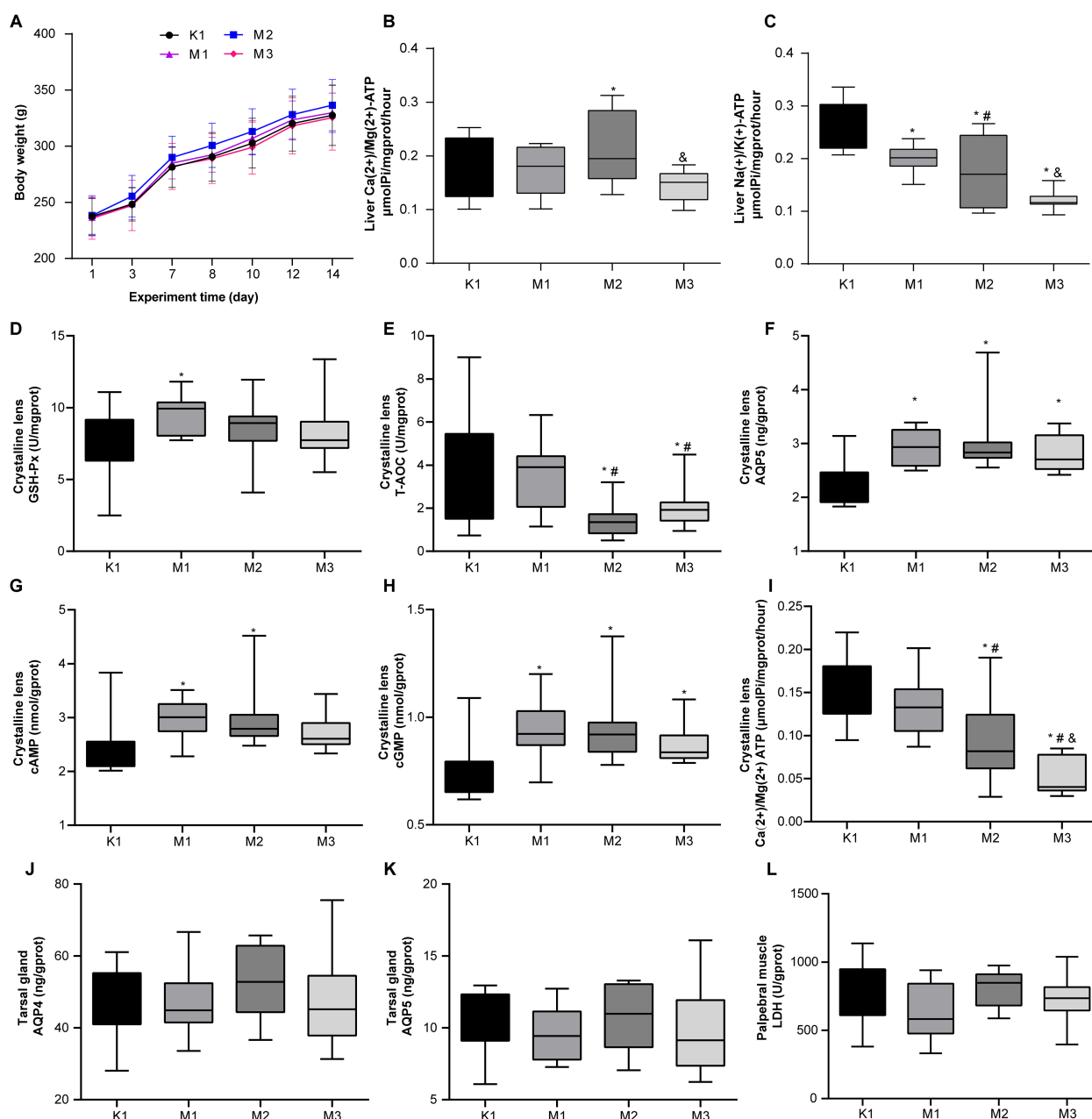


FIGURE 1

Biochemical and physiological alterations in rats with visual fatigue ($n = 10$). (A) Changes in body weight from day 1 to 14 after visual fatigue induction. (B) Liver $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATP levels in rats of each group. (C) Level of liver $\text{Na}^{+}/\text{K}^{+}$ ATP of each group. (D–I) Disordered lens-related indexes of GSH - Px (D), T-AOC (E), AQP5 (F), cAMP (G), cGMP (H), and $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATP (I) in rats with visual fatigue. (J–L) Tarsal gland AQP4 (J) and AQP5 (K) as well as LDH (L) levels in palpebral muscles in rats of each group. Data are shown as the mean $\pm$ SEM. K1, normal control group; M1, 3-day model group; M2, 7-day model group; M3, 14-day model group. * $P < 0.05$ vs. K1; # $P < 0.05$ vs. M1; & $P < 0.05$ vs. M2.

4 Discussion

Unlike books and printed materials, great information is transmitted through video display terminals, causing increased complaints of eye discomfort and fatigue (25). Common symptoms of visual fatigue include visual disturbances, ocular discomfort, and flickering of words within close range. TCM suggests that liver blood depletion and excessive use of the brain can lead to a lack of nourishment for the eyes, thus causing visual

fatigue, making liver-kidney yin deficiency evidence of a common visual fatigue pattern. However, little information is available on the development of visual fatigue in the liver-kidney yin deficiency syndrome. The identification of metabolic markers of visual fatigue provides a reference for elucidating the pathological processes and contributes to the development of precise interventions.

In this study, we established animal models of visual fatigue at different time points (3, 7, and 14 d). The results of biochemical indices showed that rats had disorders in the levels of sex hormones

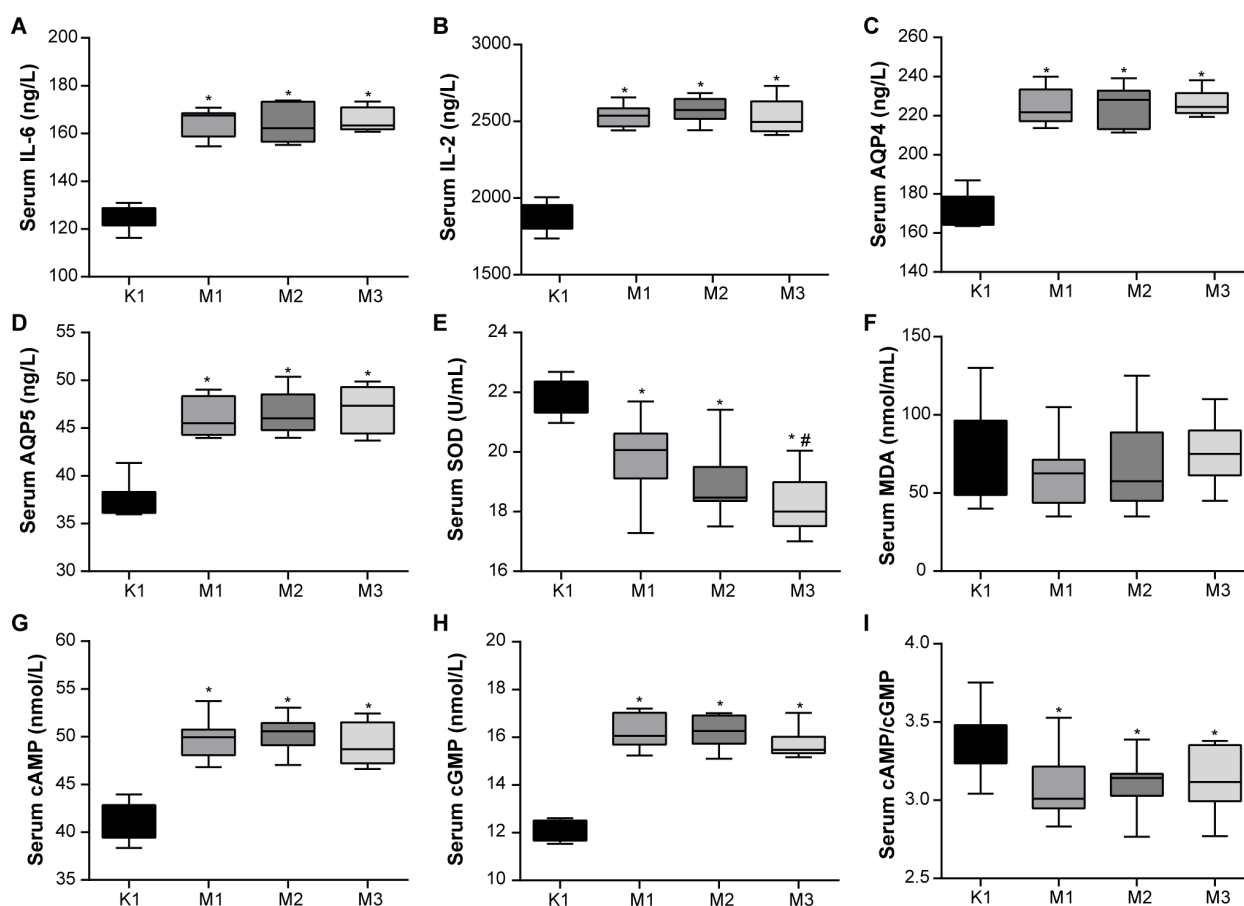


FIGURE 2

Abnormal blood inflammatory factors and antioxidant activity are observed in rats with visual fatigue ($n = 10$). (A) IL-6. (B) IL-2. (C) AQP4. (D) AQP5. (E) SOD. (F) MDA. (G) cAMP. (H) cGMP. (I) cAMP/cGMP. Data are shown as the mean $\pm$ SEM. K1, normal control group; M1, 3-day model group; M2, 7-day model group; M3, 14-day model group. * $P < 0.05$ vs. K1; # $P < 0.05$ vs. M1.

and thyroid hormones 3 days after modeling, which confirmed the appearance of liver-kidney yin deficiency. Seven days after modeling, the rats displayed an abnormal metabolism of free radicals, indicating that the visual fatigue model was successfully constructed. These results were confirmed in previous research (22). During the progression of visual fatigue, 127 and 96 significantly abnormal metabolites were identified at 7 and 14 days (compared to controls) after model induction, respectively. Pathway enrichment analysis revealed that these metabolites were involved in sphingolipid metabolism and sphingolipid signaling pathways.

Important causes of visual fatigue include damage to the structure or function of the ocular surface and fundus retina (26). Vision originates from the retina. Damage to the structure of the cells distributed in the retina, especially the retinal pigment epithelium (RPE), is affected (27). RPE cells are extremely active and require large amounts of oxygen to consume energy and nutrients; therefore, they are prone to produce reactive oxygen species (ROS) (28). This process can lead to oxidative stress if ROS are not released in a timely manner (29). On the other hand, continued overuse of the eyes or exposure to abnormal environments (such as bright light) increases the load on the retina and aggravates ocular oxidative stress, leading to dry eyes and eye fatigue (30). Decreased SOD activity has been detected in multiple eye-

related diseases, such as cataracts and glaucoma (31, 32), and is regarded as a potential diagnostic marker for oxidative stress-related diseases (33). Reduced SOD activity leads to intense lipid peroxidation (34). Abnormalities in this pathway have been implicated in the pathogenesis of cataracts. Specifically, lipid peroxidation alters the internal composition and conformation of cells by affecting the permeability of the cell membrane, causing loss of protein function, and ultimately leading to cataract formation (35). Moreover, Mg is a cofactor for several enzymes that maintain ionic homeostasis in the lens, such as $\text{Ca}(2+)\text{-ATPase}$ and $\text{Na}(+)/\text{K}(+)\text{ATPase}$ (36); Mg deficiency impairs ATPase function by enhancing oxidative stress (37). Thus, inhibition of $\text{Ca}(2+)\text{-ATPase}$ function leads to the loss of calcium homeostasis and calcium accumulation within the lens, which may eventually contribute to lens opacity (38). Consistent with the findings of the above studies, we observed decreased concentrations of serum SOD and lens $\text{Ca}(2+)\text{-ATPase}$ in the model rats. Accordingly, we speculated that visual fatigue can be alleviated by suppressing oxidative stress and restoring the Ca/Mg balance.

Furthermore, metabolite annotation results showed that the majority of the identified differential metabolites belonged to lipids and lipid-like molecules, organic nitrogen compounds, as well as organic acids and derivatives, such as fingolimod, phytosphingosine,

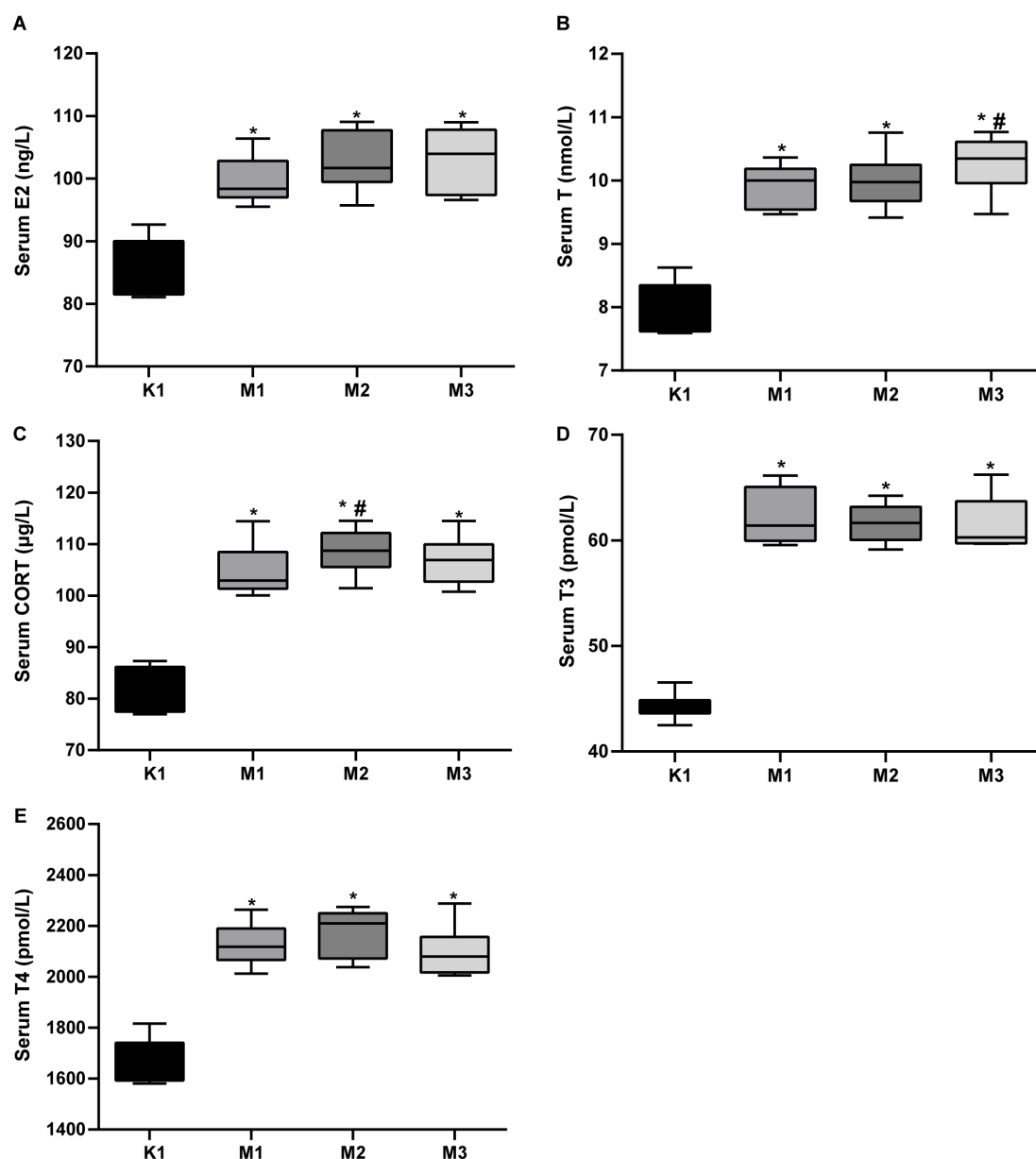


FIGURE 3

Elevated serum hormones in rats with visual fatigue ($n = 10$). (A) E₂. (B) T. (C) CORT. (D) T₃. (E) T₄. Data are shown as the mean $\pm$ SEM. K1, normal control group; M1, 3-day model group; M2, 7-day model group; M3, 14-day model group. * $P < 0.05$ vs. K1; # $P < 0.05$ vs. M1.

sphingosine, quillaic acid, phenylalanine and creatine. These metabolites also affect sphingolipid metabolism and the sphingolipid signaling pathway during liver and kidney yin deficiency - type visual fatigue progression. With the deepening of our understanding of ophthalmic diseases, numerous studies have revealed that lipid and sphingolipid signaling pathways, as well as organic nitrogen compounds and organic acids, are involved in the pathological mechanisms of ocular inflammatory diseases (39–41). Among the identified differential metabolites, organic acids are a significant proportion. For example, phenylalanine is an essential amino acid and neurotransmitter precursor, and creatine plays a key role in cellular energy metabolism and antioxidant defense. This study observed reduced levels of phenylalanine and creatine in the model group.

Combined with previous research reports, phenylalanine modification may reduce empty capsids to lower virus - vector - induced intraocular inflammatory responses (42). Creatine supplementation may delay cone secondary degeneration in retinitis pigmentosa and protect the retina by reducing oxidative stress and inflammation (43). Thus, maintaining or restoring the homeostasis of these organic acids may help delay visual fatigue progression.

Lipids, especially sphingolipids, are enriched in the nervous system and retina (44). Sphingolipid metabolism disorders are associated with visual dysfunction such as visual fatigue. For example, Green et al. found that patients with hereditary neuroretinas exhibit altered levels of complex sphingolipids (45). This study detected abnormal expression of the organic nitrogen

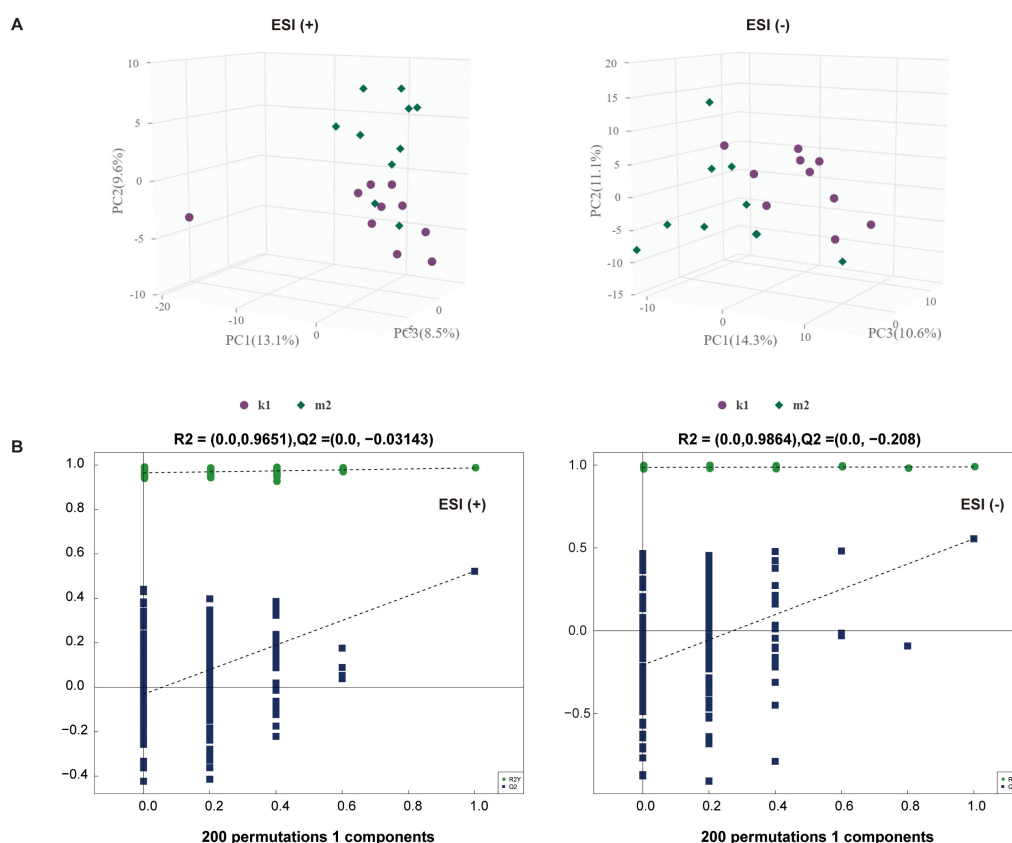


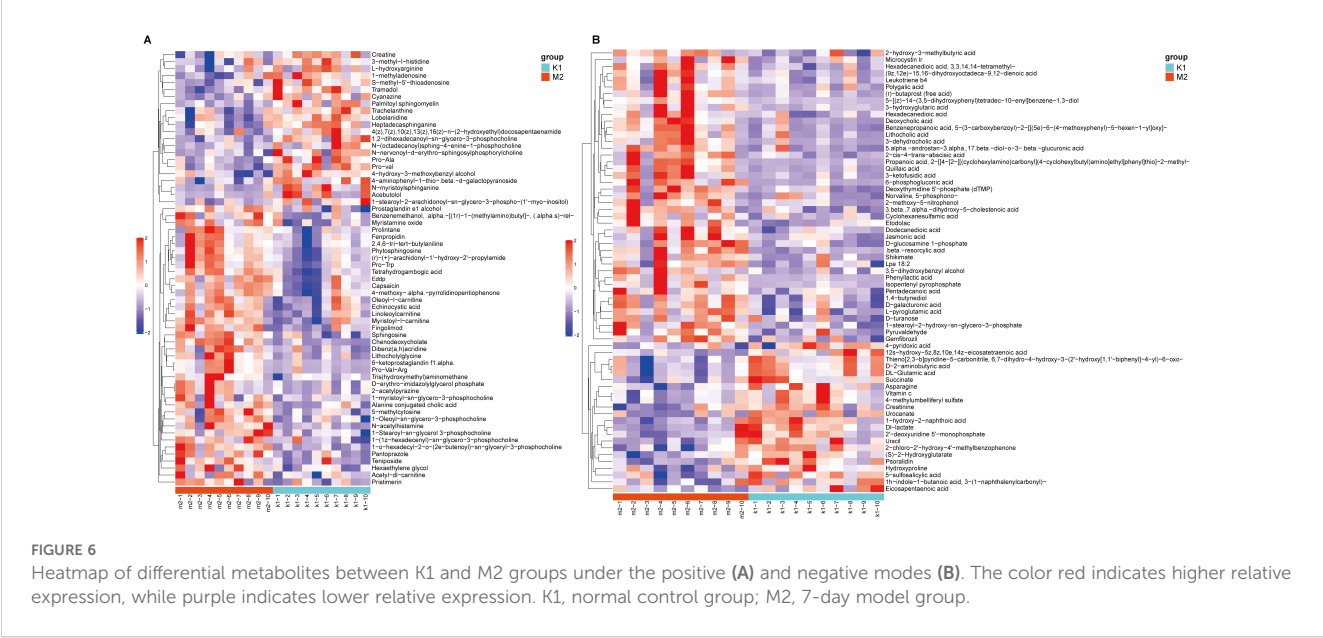
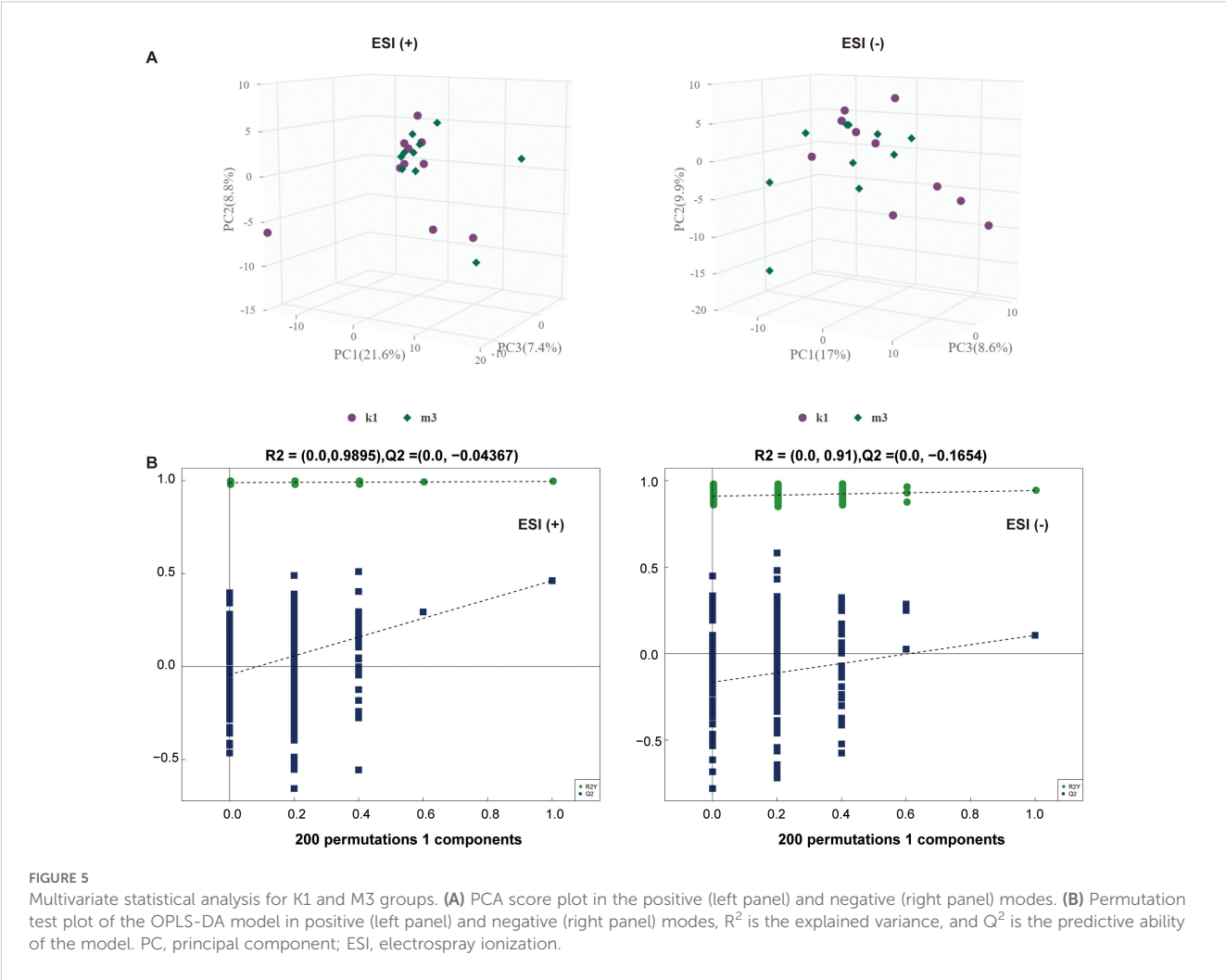
FIGURE 4

Multivariate statistical analysis for K1 and M2 groups. (A) PCA score plot in the positive (left panel) and negative (right panel) modes. (B) Permutation test plot of the OPLS-DA model in positive (left panel) and negative (right panel) modes, R^2 is the explained variance, and Q^2 is the predictive ability of the model. PC, principal component; ESI, electrospray ionization.

compound metabolite fingolimod. Fingolimod (also known as FTY720, a sphingosine analog) is used clinically as an S1PR agonist and immunomodulatory drug for the treatment of recurrent multiple sclerosis (46, 47). Interestingly, multiple sclerosis presents symptoms such as visual impairment and fatigue (48). Fingolimod/FTY720 is found to inhibit NF κ B and pro-inflammatory cytokines in the retina of diabetic rats, preventing disruption of the blood-retinal barrier; in addition, FTY720 reduces neuronal loss in rats with glaucoma (49, 50). In TCM theory, liver and kidney yin deficiency may relate to endocrine and immune disorders (51). As an immunomodulatory agent, we speculate that fingolimod may participate in the pathological process of liver and kidney yin deficiency type visual fatigue by regulating ocular immune response or neuroprotective mechanisms. Despite its promising applications, fingolimod has been documented to cause possible adverse ocular effects (such as macular edema) (52). Therefore, further studies are needed to confirm and evaluate the role of fingolimod in improving visual function disorders like visual fatigue. Porter et al. (53) showed that the sphingosine 1-phosphate signaling pathway is implicated in many key processes in the eye, ranging from light stress/apoptotic responses to retinal/vascular development, suggesting that the sphingolipid signaling pathway serves a role in both vision and apoptosis. Another differential metabolite, quillaic acid, exhibits various biological

activities, including anti-inflammatory, antiviral, and antineoplastic effects (54, 55). It also exhibits the ability to induce apoptosis in various cell types. Although there is currently no direct evidence of quillaic acid's role in visual fatigue, based on its known anti-inflammatory properties, we speculate that it may be beneficial in alleviating liver and kidney yin deficiency - induced visual fatigue symptoms. Taken together, the key differential metabolites and disrupted sphingolipid metabolic pathways found in this study's liver and kidney yin deficiency visual fatigue model suggest that they may contribute to visual fatigue development by affecting biological processes such as retinal inflammation and apoptosis. This provides a new perspective on understanding metabolic characteristics of visual fatigue, innovatively establishing a link between TCM syndromes and sphingolipid metabolic disorders, revealing the complementarity between the traditional TCM syndrome classification system and modern neuro - ophthalmic cognition. However, further research is needed to explore the specific molecular mechanisms.

Information on the signature metabolites associated with liver and kidney yin deficiency patterns in visual fatigue is limited, and our work fills this gap. The screened metabolites contribute to understanding the underlying causes of visual fatigue. However, this study has several limitations. First, the inherent physiological



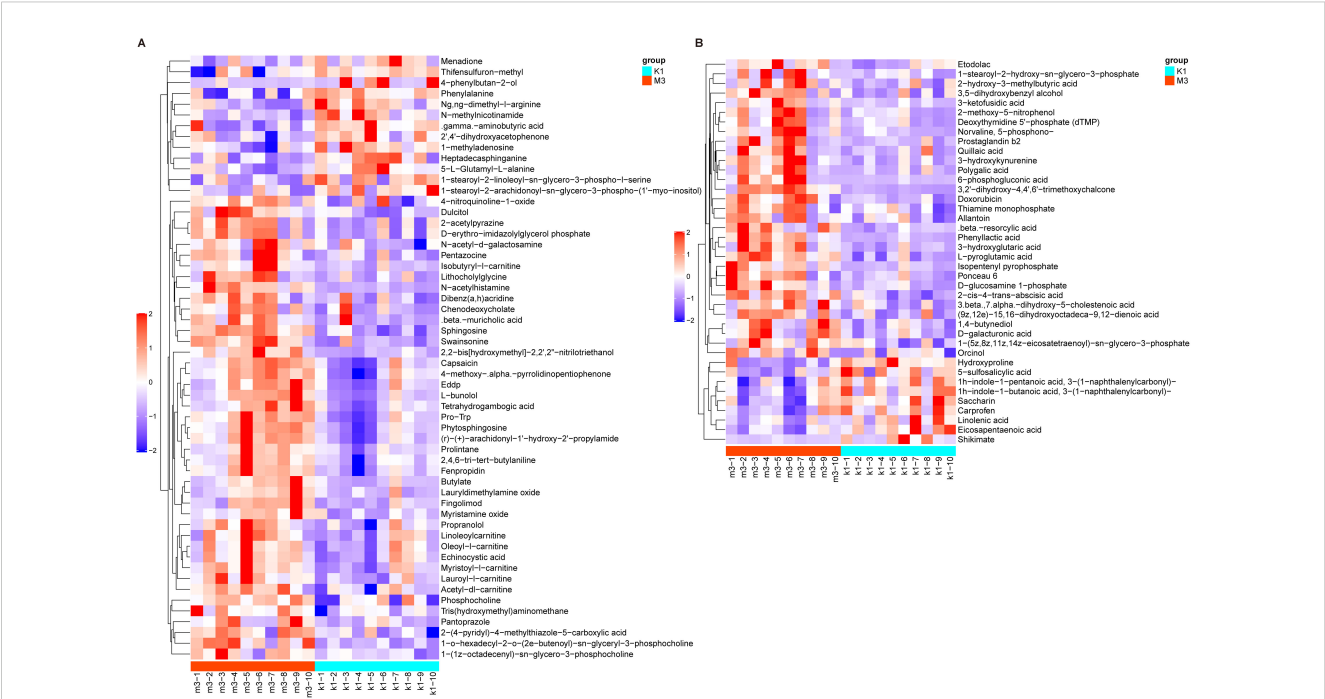


FIGURE 7 Heatmap of differential metabolites between K1 and M3 groups under the positive (A) and negative modes (B). The color red indicates higher relative expression, while purple indicates lower relative expression. K1, normal control group; M3, 14-day model group.

differences between rats and humans restrict the direct extrapolation of results. Second, the assessment of visual fatigue and liver-kidney yin deficiency in the animal model mainly depends on biochemical indicators. Future research should integrate more objective behavioral evaluations, such as optokinetic responses and pupil reaction, for a comprehensive visual function assessment. Moreover, the untargeted metabolomics analysis used here is

exploratory. The identified differential metabolites future need quantitative validation and mechanistic exploration through targeted metabolomics (e.g., LC-MS/MS) or functional experiments. Therefore, the results presented here should be interpreted with caution. Finally, the mechanisms of sphingolipid metabolism and signaling pathways implicated in visual fatigue need to be further explored in large populations.

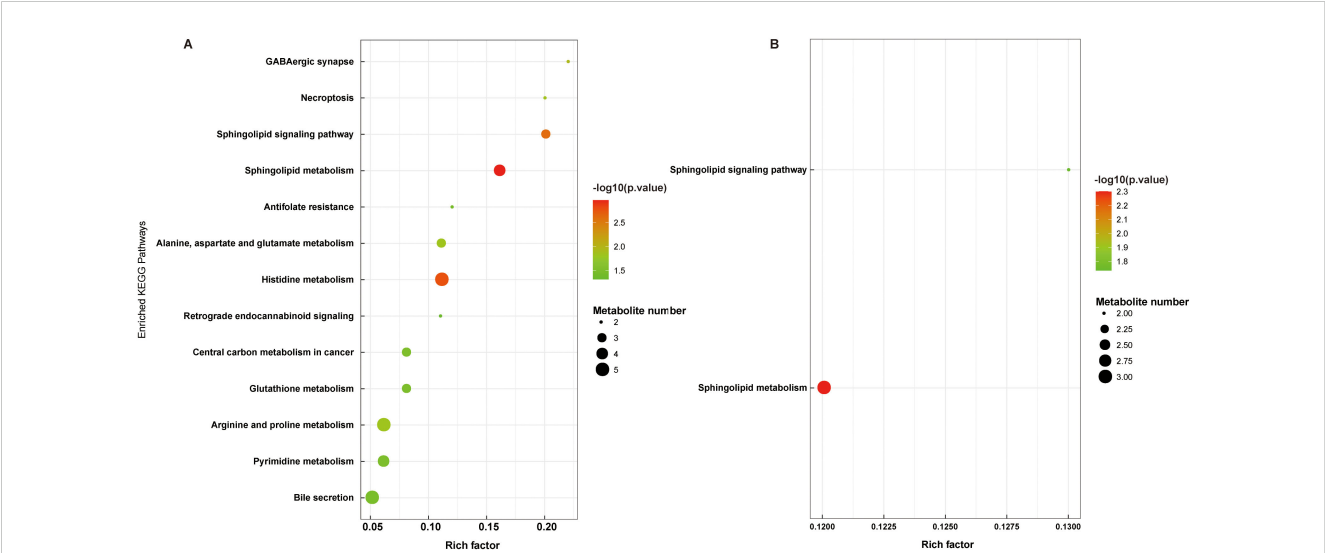


FIGURE 8 Functional pathway enrichment analyses of differential metabolites between K1 vs. M2 (A) and K1 vs. M3 (B). K1, normal control group; M2, 7-day model group; M3, 14-day model group.

5 Conclusions

Overall, we revealed the metabolic profiles of an animal model of visual fatigue with liver and kidney yin deficiency syndrome and identified metabolites and signaling pathways prominently associated with this disease. Sphingolipid metabolites are expected to be potential targets for alleviating visual fatigue. Further targeted metabolomics or functional studies are needed to quantitative validation our findings, confirm metabolite targets, and address animal model limitations. This study offers fresh insights into visual fatigue-related metabolic signatures, bridges TCM theory with modern neuro-ophthalmological metabolic mechanisms, and lays an initial scientific foundation for precise disease intervention.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was approved by The Food and Cosmetics Inspection and Authentication Center, Shaanxi Academy of Traditional Chinese Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XZ: Conceptualization, Data curation, Formal analysis, Writing – original draft. XL: Data curation, Methodology, Resources, Writing – original draft. ZH: Data curation, Investigation, Writing – review & editing. YC: Methodology, Writing – review & editing. YZ: Conceptualization, Writing – review & editing. JZ: Resources, Writing – review & editing. JD: Formal analysis, Project administration, Resources, Writing – review & editing. LC: Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2025.1586581/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Changes in thymus index (A) and spleen index (B) in rats in each group (n = 10). K1, normal control group; M1, 3-day model group; M2, 7-day model group; M3, 14-day model group.

SUPPLEMENTARY FIGURE 2

Peak intensity chromatograms of serum samples in positive and negative modes. (A): Total ion chromatogram. (B): Metabolic profiles of the serum samples in the PCA plot. PCA, principal component analysis; ESI, electrospray ionization.

SUPPLEMENTARY TABLE 1

List of differential metabolites between K1 and M2 groups under the positive mode.

SUPPLEMENTARY TABLE 2

List of differential metabolites between K1 and M2 groups under the negative mode.

SUPPLEMENTARY TABLE 3

List of differential metabolites between K1 and M3 groups under the positive mode.

SUPPLEMENTARY TABLE 4

List of differential metabolites between K1 and M3 groups under the negative mode.

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Glossary

MS	mass spectrometry	T4	thyroxine
TCM	traditional Chinese medicine	IL-2	interleukin 2
SD	Sprague-Dawley	IL-6	interleukin 6
SPF	specific pathogen-free	SOD	superoxide dismutase
cAMP	cyclic adenosine monophosphate	MDA	malondialdehyde
cGMP	cyclic guanosine monophosphate	pos	positive
GSH-Px	glutathione peroxidase	neg	negative
T-AOC	total antioxidant capacity	ESI	electrospray ionization
AQP4	aquaporin 4	PCA	principal component analysis
AQP5	aquaporin 5	OPLS-DA	orthogonal partial least squares-discriminant analysis
LDH	lactate dehydrogenase	VIP	variable importance for projection
E2	estradiol	ANOVA	analysis of variance
T	testosterone	RPE	retinal pigment epithelium
CORT	corticosterone	ROS	reactive oxygen species.
T3	triiodothyronine		

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