Untargeted Metabolomics Analysis Revealed Lipometabolic Disorders in Perirenal Adipose Tissue of Rabbits Subject to a High-fat Diet

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Research

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Abstract

High-fat diet (HFD) has been widely recognized as a significant modifiable risk for insulin resistance, inflammation, type 2-diabetes (T2D), atherosclerosis and other metabolic diseases. The biological mechanisms responsible for disturbances in perirenal adipose tissue (PAT) and other tissues in rodents fed a HFD are well understood. However, the biological mechanism responsible for key metabolic disorders in PAT of rabbits subject to HFD remains unclear.

Methods: Here, untargeted metabolomics (LC-MS/MS) combined with liquid chromatography (LC) and high resolution mass spectrometry (MS) were used to evaluate PAT metabolic changes. Histological observations showed that the adipocytes cells and density of PAT was significantly increased in HFD rabbits. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) showed noticeable changes in PAT metabolites between the HFD and standard normal diet (SND) rabbit groups.

Results: Our study revealed 206 differential metabolites (21 up-regulated and 185 down-regulated), and then the 47 differential metabolites (13 up-regulated and 34 down-regulated), mainly phospholipids, fatty acids, steroid hormones and amino acids, chosen as potential biomarkers to help explain metabolic disorders caused by HFD. These metabolites were mainly associated with biosynthesis of unsaturated fatty acids, the arachidonic acid metabolic pathway, the ovarian steroidogenesis pathway, and the platelet activation pathway. Our study revealed that a HFD caused significant metabolic disorders in rabbit PAT.

Conclusion: High levels of phospholipids, fatty acids, steroid hormones and l-methionine may inhibit oxygen respiration by increasing the adipocytes cells and density cause mitochondrial and endoplasmic reticulum dysfunction, produce inflammation, and finally lead to insulin resistance, thus increasing the risk of T2D, atherosclerosis, and other metabolic syndromes.

1. Introduction (Background)

In recent years, the occurrence rate of obesity is fleetly increased which poses risk for many medical diseases, causing concern for many public and health-related professionals. Obesity is a serious medical, social, and economic problem that has caused millions of disabilities, concomitant diseases, and deaths [1, 2]. The prevalence of obesity in humans is widespread across all ages and both sexes and can be attributed to the interaction between environment and physiological factors [3, 4]. Nowadays, the universality of a HFD is one of the major contributors to the development of obesity. Moreover, many analyses across various species (rat, mice, pig) have indicated that HFD is associated with multiple metabolic syndromes such as insulin resistance (IR), T2D, cardiovascular disease, fatty liver, hypertension, Alzheimer’s disease, and cancer [5–12]. PAT is a kind of white adipose tissue that supporting triglyceride (TG) storage for energy demands and endocrine function. Broadly study revealed that PAT plays a significant role in controlling lipid mobilization and reproductive function as well as
modulating multiple metabolic pathways [13, 14]. Accumulated evidence shows that the main mechanisms leading to these metabolic diseases include endoplasmic reticulum stress and mitochondrial dysfunction [15], excessive accumulation of metabolites in adipose tissue, imbalance of energy supply and metabolic homeostasis [16], reduction of reverse cholesterol transport [17], aggravation inflammation and reduction of insulin sensitivity [18]. Above all, one of the most significant and destructive complications is abnormal lipid metabolism, which will definitely worsen in the future [19].

With the popularity of metabolite research, the detection methods of metabolites have also been innovated. Metabonomics is usually used as a tool to discover biomarkers, which can analyze metabolites in biological fluids, tissues, and cells [20]. Moreover, untargeted metabolomics analysis is a kind of metabonomics that can detect and analyze all small molecule metabolites simultaneously without bias. Untargeted metabolomics analysis can evaluate metabolites in detail, explain the categories of metabolites, and examine the relationship between related metabolites from multiple ways [21]. Metabolites maintain homeostasis in response to adverse biological responses. Previous studies have also shown that the use of untargeted metabolomics analysis provides a basis for metabolic syndrome in HFD fed rats [22, 23]. Therefore, untargeted metabolomics is used to identify the phenotype-related metabolites and metabolic pathways, to explain the specific functions of metabolites, and to understand the physiological effects.

Rabbits are economically important domestic animals raised primarily as a source of animal protein, and more recently used as a practical model for obesity-related studies [24]. A previous study have reported that the subcutaneous adipose tissue of HFD fed New Zealand white rabbits after 5 or 10 weeks plays a significant role in obesity-associated systemic low-grade inflammation [25]. Additionally, there was a study that evaluated changes in blood vessel function using a HFD rabbit model [26]. However, the overall metabolic change of PAT in rabbits fed a HFD has yet to be elucidated. Thus, to gain further understanding of the molecular consequences of obesity, we investigated the metabolic change in PAT from the obese rabbit induced by a HFD by using untargeted metabolomics.

2. Materials And Methods

2.1 Ethics statement

This study was approved by and conducted in strict accordance with the ethical standards of the Institutional Animal Care and Use Committee of the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China.

2.2 Animals and experimental design

A total of 16 female Tianfu black rabbits (35 days of age) at the teaching farm of the Sichuan Agricultural University were randomly divided into two groups, a control group fed a SND (n = 8) and an experimental group fed a HFD (SND plus 10% lard, n = 8). Detailed information on the feeding procedures
can be found in a previous study [27]. Briefly, rabbits from both groups were fed twice a day with free access to water and under a light/dark cycle of 12 h per day. The room temperature is about 22 to 26°C. According to the previous research method, the animals were classified as obese [27]. In short, body weight, body length, bust length and adipose tissue weight were used as the criteria for selecting obese rabbits. After eliminating the substandard rabbits, 6 rabbits were selected from the HFD (n = 6) and SND (n = 6) groups respectively for sampling. After being fed for 5 weeks, the selected rabbits were humanely slaughtered by electrical stunning with exsanguinations for sampling. Rabbit PAT samples were rapidly collected after rabbits were euthanized and stored in eppendorf tubes at -80°C for subsequent analysis.

2.3 Histological examination

In order to examine the histological changes of PAT, selective rabbits were humanely slaughtered and PAT was stained with hematoxylin-eosin (H&E). In brief, tissue samples were fixed with a 10% neutral formaldehyde fixator for about 24 h and then washed with clean water. After that, the tissue samples were dehydrated, embedded in paraffin, and stained with H&E. And then, the PAT sections (6 to 8 µm) were collected using a microtome (RM2235, Leica, Nussloch, Germany). Furthermore, an alternative microscope (DM1000, Leica, Nussloch, Germany) was used to capture images at a 200 × field of view. In the end, PAT slices of rabbits from the two groups were analyzed by ImageJ software (National Institutes of Health).

2.4 Sample preparation

Approximately 100 mg of PAT sample from each rabbit was ground into powder in liquid nitrogen. Homogenized samples were resuspended and vortexed by adding a prepared mixture composed of 80% methanol and 0.1% formic acid. Then, samples were placed on ice and incubated for 5 minutes followed by centrifugation at 15000 g and 4°C for 5 minutes. The methanol concentration of the supernatants was reduced to 53% by LC-MS/MS grade water. After samples were transferred to a new eppendorf tube, they were centrifuged at 15000 g and 4°C for 10 minutes. Lastly, the supernatants were incorporated into the LC-MS/MS system to conduct the LC-MS/MS analysis [28].

2.5 Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis

Chromatographic separation of differential metabolites was accomplished by injecting PAT samples into a Hypesil Gold HPLC column (2.1 × 100 mm, 1.9µm) (Thermo Fisher, USA) using a Vanquish UHPLC System (Thermo Fisher, Germany) coupled with a Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher, Germany) located at Novogene Co., Ltd. (Beijing, China). Detailly, the Hypesil Gold HPLC column was kept at 40°C with a flow rate of 0.2mL/min using a 17-minutes linear gradient. The eluent B (Methanol) was the same for both positive and negative polarity modes. However, the eluent A differed in positive (0.1% FA in Water) and negative (5 mM ammonium acetate, pH 9.0) polarity modes. The solvent gradient was set as follows: 2% B, 1.5 minutes; 2 to 100% B, 12.0 minutes; 100% B, 14.0 minutes; 100 to 2% B, 14.1 minutes; 2% B, 17 minutes. The Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer was operated in both positive and negative electron spray
ionization (ESI+)/(-ESI) mode with a spray voltage of 3.2 kV. Moreover, the settings were 320°C for capillary temperature, 40 arb for sheath gas flow rate, and 10 arb for aux flow rate.

2.6 Data processing and analysis

The raw data files generated by UHPLC-MS/MS were processed with Compound Discoverer 3.1 (CD 3.1, Thermo Fisher) to determine the peak value for each metabolite. The detailed parameters are set as follows: a) the retention time tolerance was 0.2 minutes; b) the actual mass tolerance was 5 ppm; c) the signal intensity tolerance was 30%; d) the signal ratio was 3; e) the minimum intensity was 100000. Then, peak values were matched with the mzCloud (https://www.mzcloud.org/), mzVault, and MassList databases to obtain exact and relative quantitative results. To better observe the inter-group distributions and otherness, the program metaX was used for PCA and PLS-DA. The quality of the PLS-DA models was assessed using R2X, R2Y, and Q2. R2X and R2Y are fractions of sum of squares explained by a given principal component. Q2 represents the predictive ability of the PLS-DA model. Permutation tests with 200 permutations were used to validate models.

2.7 Identification and analysis of metabolites

Differently expressed metabolites were identified based on the value of VIP, P-value, and fold change (FC). Detailely, the metabolites with variable importance in projection (VIP) > 1, P-value < 0.05, and FC ≥ 2 or FC ≤ 0.5 were considered to be differential metabolites. Moreover, volcano plots were used to filter metabolites of interest using log2 (FC) and -log10 (P-value). Besides, differential metabolites were annotated with Human Metabolome Database (HMDB, https://hmdb.ca/metabolites), and LIPID MAPS (http://www.lipidmaps.org/) databases respectively to obtain a systematized overview. The differential metabolites-related metabolic pathways and physiological functions were explored by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/pathway.html). A differential metabolic pathway was considered to be significantly enriched when the observed frequency of a metabolite (x/n) > expected frequency of a metabolite (y/n) and pvalue < 0.05.

2.8 Statistical analysis

In this study, statistical software available in R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS Linux (CentOS release 6.6) were used to analyze the metabolic data. Univariate t-test was applied to calculate the statistical significance, and only P-value < 0.05 was considered as statistically significant.

3. Results

3.1 PAT histological observations

The H&E stained PAT samples showed that the number of adipocytes cells and density were increased in rabbits fed a HFD (Fig. 1).

3.2 Quality control of metabolomics data
Pearson correlation coefficients between positive and negative quality control (QC) samples are shown in Additional file 1. The Pearson correlation coefficient between QC samples was higher than 0.991 for both ESI+ and ESI−, indicating that stability of detection and quality of data was excellent. The PCA score plots show that the distributions of PAT metabolites from the SND and HFD groups differed (Fig. 2A-B). All the PLS-DA score plots for the SND and HFD groups (Fig. 2C-D) were within the 95% confidence interval, except for the 17th HFD sample. The values of the PLS-DA statistics were R2Y = 0.92 and Q2 = 0.67 for ESI+ data, and R2Y = 0.91, Q2 = 0.66 for ESI− data. These R2Y and Q2 values indicate that the PAT metabolic differences between SND and HFD rabbits were quite significant. Permutation tests were used to prevent over-fitting of PLS-DA models. The validation included 200 random permutation tests, which generated intercepts of R2 = 0.76 and Q2 = -0.69 for ESI+ data and R2 = 0.68 and Q2 = -0.91 for ESI− data (Fig. 2E-F), indicating that the PLS-DA models were credible and without over-fitting. Thus, the PLS-DA models showed excellent predictive ability and reliability to determine significant PAT metabolic disturbances in HFD rabbits.

3.3 Differential metabolites analysis

The VIP of the first principal component of the PLS-DA model combined with p-value was used to ascertain metabolites with differential expression in the SND and HFD groups. The thresholds were VIP > 1.0, FC > 1.5 or FC < 0.665, and p-value < 0.05 [29–31]. A total of 206 metabolites were detected, of which 21 were significantly up-regulated, and 185 were significantly down-regulated (Additional file 2–3). The volcano maps in Fig. 3A-B visually show the overall distribution of differential metabolites. Based on KEGG, HMDB and LIPID MAPS databases, fatty acids, phospholipids, and sterol lipids [ST] were identified as main metabolites. Then, on this basis, the metabolites chosen as potential biomarkers to help explain metabolic disorders caused by HFD were those with VIP values above 1 and p-value below 0.05 [32]. A total of 47 metabolites were detected, of which 13 were significantly up-regulated, and 34 were significantly down-regulated. These results further illustrate that the metabolism of PAT was disturbed by HFD.

3.4 Metabolic pathway analysis

To study the changed pathways induced by HFD, an exhaustive KEGG pathway analysis was conducted. Significance was ascertained using a hypergeometric test with a threshold p-value ≤ 0.05 to filter out pathways with p-value higher than 0.05. Detailly, the major identified metabolic pathways were platelet activation, arachidonic acid metabolism, ovarian steroidogenesis, biosynthesis of unsaturated fatty acids, ferroptosis, and vitamin B6 metabolism (Additional file 4–5). The top 20 signaling pathways are shown in the positive (Fig. 4A) and negative (Fig. 4B) KEGG enrichment plots obtained with MetaboAnalyst. Among these pathways, vitamin B6 metabolic signaling pathway, arachidonic acid metabolic pathway, biosynthesis of unsaturated fatty acids, platelet activation, serotonergic synapse, ovarian steroidogenesis, and ferroptosis were highly enriched in the HFD rabbit group. Given that PAT is an organ for lipid storage and metabolism, we picked some pathways associated with the lipid cycle for a more detailed analysis of metabolites (Fig. 4C). These metabolites are main phospholipids, fatty acids,
steroid hormones, and L-methionine, implying that these molecules may be the key molecules during the development of obesity.

4. Discussion

Feeding a HFD is the most common method for animal models of obesity, thus HFD continues to be an indispensable method for discovering mechanisms of metabolic syndromes [33]. Similarly, untargeted metabolomics, an effective method to measure metabolites, plays an important role in understanding the physiological functions of metabolites and the potential causes of metabolic disorders [34].

Feeding rabbits with a HFD will result in damage to the normal function of PAT, destruction of the balance between lipid formation and degradation, and over accumulation of lipids in PAT. The LC-MS/MS metabolite analyses showed that the PAT lipid cycle in rabbits fed a HFD was disturbed, resulting in significant changes in the levels of phospholipids, fatty acids, steroid hormones, and L-methionine (Fig. 4C). Similarly, feeding mice with a HFD caused a metabolic imbalance that resulted in metabolic disorders such as IR and nonalcoholic steatohepatitis [35].

The symptoms of obesity closely resemble the spectrum of metabolic changes in PAT, including phospholipids, fatty acids, steroid hormones, and amino acids, among which phospholipids and lysophosphatides are the most abundant metabolites. Phospholipids are the main components of plasma membranes including phosphatidylethanolamines (PEs) and phosphatidylcholines (PCs), which are precursors of lysophosphatidylethanolamine (LPEs) and lysophosphatidylcholines (LysoPCs/LPCs), respectively [36]. In our study, PCs and PEs were the most frequently found phospholipids (most of them down-regulated in HFD rabbits), whereas levels of all LPCs were reduced. Concerning the 14 PCs and 8 PEs in PAT, some PCs, such as PC (18:4e/20:5) and PC (17:2/22:6), increased in HFD rabbits, which was similar to a previous study in humans where some PCs to be significantly higher in the obese group than that in the control group [37]. The level of PE (18:2/18:2) was lower in mice fed a HFD than mice fed a normal diet [38], consistent with our results. Further, the level of LPCs (LPC 15:0, LPC 19:0) decreased in rabbits fed a HFD for 4 weeks, which was partially in agreement with a decrease in plasma in human Obesity and T2D [39], and low-abundance of LPCs in serum of hyperlipidemic mice fed a HFD [40]. And PE is methylated to PC [41]. Changes in phospholipid levels can inhibit calcium ion transport and affect the transfer of phospholipids between the endoplasmic reticulum (ER) and mitochondria, inducing ER stress and mitochondrial dysfunction, which will decrease fatty acid oxidation and acetyl CoA levels [42].

In addition, an imbalance in the PC/PE ratio will affect the mitochondria-associated ER membranes, leading to an excessive accumulation of sphingomyelin (SM) in the ER, inducing the activation of PKC, inhibiting the activity of AKT, and disrupting energy supply and metabolic homeostasis [16]. SM is produced by a group transfer in phosphatidylcholine combined with the associated skeleton, which is closely related to sphingomyelin synthase (SMS). A significant increase in SM may reduce reverse cholesterol transport, increasing the risk of atherosclerosis lesions and other metabolic diseases [17]. Further, as an important signal molecule, reduced levels of LPC combined with some cell-specific G-coupled protein receptors can cause an increase in insulin secretion through glucose stimulation, which
will damage β cell function and lead to insulin resistance, stimulate the production of adipocytes, and aggravate the risk of obesity and other diseases [43, 44]. These are the factors that may cause insulin resistance. The decrease of LPCs in rabbits from this study may be related to an increase in insulin resistance, thus LPCs could be considered as potential biomarkers for metabolic diseases caused by obesity due to HFD. Our results here indicated that SM levels were significantly up-regulated in the HFD compared to the SND rabbit groups, in agreement with reports of obesity and insulin sensitivity in obese adult humans [45] and a study on the plasma metabolic fingerprints of atherosclerosis rabbits [46]. These results suggest that changes of phospholipids levels may reduce insulin sensitivity, lead to insulin resistance, and increase the risk of atherosclerosis.

Arachidonic acid (ARA) and adrenic acid are omega-6 polyunsaturated fatty acids. In the current study, levels of ARA were higher in the HFD than in the SND rabbit group, in agreement with a significant increase in serum ARA levels in rats fed a HFD [47]. According to our identification results, ARA was the main metabolite of the arachidonic acid metabolic, ovarian steroidogenesis, biosynthesis of unsaturated fatty acids, and ferroptosis metabolism pathways. However, a ‘one-to-many’ type of relationship was pointed out between metabolic pathways that have been annotated and identified compounds. ARA matched 18 associated metabolic pathways, which showed that at least ARA was comparatively important for PAT. The most important metabolic pathway for ARA in this study was platelet activation ($P < 0.01$). Under normal circumstances the release of fatty acids in PAT is strictly controlled to meet energy requirements. Conversely, metabolic disorders cause excessive release of fatty acids relative to tissue requirements. It is widely accepted that disturbances in fatty acid metabolism lead to increased inflammatory signaling, which are central factors in IR [18]. ARA promotes the production of several prostaglandins, which are associated with lipopolysaccharide (LPS) induced inflammation [48]. It is hypothesized that the increase of ARA is associated with PAT metabolic disorders and may induce inflammation to further produce insulin resistance. Previous studies have shown that the concentration of body fat and adipocytokines in the (HFD + ARA) group was significantly increased after six weeks of induction [49]. Further, excessive levels of ARA can cause oxidative stress and activate pro-inflammatory signals that induce endoplasmic reticulum (ER) stress leading to IR [50, 51]. Hence, ARA can be used as an indicator of PAT metabolic disorders in obese patients consuming HFD. Endogenous adrenal acid is produced by ARA, and it is mainly oxidized in the peroxisome. Consistent with results here, plasma levels of adrenal acid were found to be up-regulated in an adipose hepatitis model, and primarily caused by instability of peroxidase β-oxidation [52]. Docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) are long-chain omega-3 polyunsaturated fatty acids (PUFAs). The levels of DPA and DHA were significantly elevated in PAT from HFD-fed rabbits compared to SND-fed rabbits. The higher levels of DPA and DHA in the HFD rabbit group disagreed with significantly lower levels of DPA and DHA in 12-wk old rats fed a HFD relative to rats in the control group. Further, a strong positive association existed between the reduced levels of these two metabolites and the insulin sensitivity index [53]. A possible explanation is that a higher concentration of n-3 PUFAs inhibits the release of free fatty acids from PAT [54], which in turn inhibits the inflammatory signaling pathway and decreases the risk of IR, thus playing a protective role. In addition, DHA and DPA have strong anti-inflammatory effects and can activate peroxisome, thus
increasing insulin sensitivity [54, 55]. The levels of DPA and DHA in the HFD rabbit group were significantly increased, which may indicate a protective effect. Therefore, rabbits in the HFD group may have IR and other metabolic syndromes, but they may also produce metabolites such as DHA and DPA to protect them against adverse factors. However, no free fatty acids were detected in plasma/serum of HFD rabbits, thus additional research is needed.

Steroid hormones such as testosterone, 2-hydroxyestradiol, and epitestosterone were also found in this study. These hormones play vital roles in the production and metabolic function of adipose tissue via hormone receptors. Levels of testosterone, 2-hydroxyestradiol, and epitestosterone were significantly lower in PAT from the HFD than the SND rabbit group. The hormone 2-hydroxyestradiol has strong inhibitory effects on NADPH during lipid peroxidation in rat microsomes. In addition, lipid peroxides depend on specific ions at the initial stage, and are strongly inhibited by oxygen absorption [56], which may inhibit the PAT lipid metabolic pathway and cause lipid accumulation. Changes in testosterone were the result of rats fed HFD to induce IR [57], strongly suggesting that changes in testosterone levels are due to rabbits fed HFD induced insulin resistance. Furthermore, a study showed that testosterone gradually increased in visceral fat rather than in subcutaneous adipose tissue in human females [58]. This result agreed with the significant increase adipocytes cells and density of PAT in HFD rabbits relative to SND rabbits. Thus, changes in 2-hydroxyestradiol and testosterone levels in this study may have led to lipid accumulation by increasing adipocytes cells and density of PAT and inhibiting the absorption of oxygen by lipid metabolism. However, the specific mechanism of steroid hormones in PAT needs further study.

L-methionine produces methionine, and it functions not only as an essential amino acid but also as a physiological effector [59]. We found higher levels of L-methionine in HFD than in SND rabbits. The methionine cycle provides methyl units for various reactions including methylation in lipids. The S-adenosine methionine (SAM) is used as a major methyl donor molecule and it is synthesized from the essential amino acid methionine [60]. Choline, produced by phosphatidylcholine, and the subsequent substances produced by choline oxidation, such as betaine, can not only help to adjust cell volume, but also act as methyl donors in the homocysteine-methionine (HM) cycle, transporting excess fatty acids to corresponding organelles for metabolism [61]. Methionine supplementation increases homocysteine (Hcy) concentration and is associated with vitamin B6. Therefore, in our study, one possible explanation is the excessive accumulation of fatty acids in PAT of rabbits fed with a HFD, and the increase of L-methionine level, thus further increasing the level of Hcy, and finally disturbing the HM cycle. However, previous studies have been reported that high circulating Hcy concentrations are related to elevated risk of atherosclerosis, steatohepatitis and lipid metabolic disturbances [62, 63]. And changes in HM cycle after feeding lean Iberian sows with a HFD were associated with obesity-related diseases and T2D [64], indicating that the higher levels of L-methionine may be related to atherosclerotic diseases and T2D by affecting the HM cycle.

5. Conclusion
Histological examination and untargeted metabonomics analysis revealed that rabbits fed a HFD exhibited PAT metabolic disorders affecting unsaturated fatty acid synthesis, and the arachidonic acid metabolic, ovarian steroidogenesis, and platelet activation pathways. Phospholipids and excessive levels of ARA may cause mitochondrial dysfunction, inflammation, and induce ER stress leading to insulin resistance. Steroid hormones may inhibit oxygen absorption by increasing the adipocytes cells and density of PAT. L-methionine may increase the risk of T2D and atherosclerosis by affecting the HM cycle. Contrary to previous studies, we found significantly elevated levels of DHA and DPA which are inversely associated with obesity in both humans and animals. This aspect merits further research. The metabolic changes and biomarkers identified in this study may serve as a foundation for future therapeutic interventions against lipometabolic disorders.

**Abbreviations**

PAT: perirenal adipose tissue
HFD: high-fat diet
SND: standard normal diet
HPLC: high performance liquid chromatography
MS: mass spectrometry
PCA: principal component analysis
PLS-DA: partial least squares discriminant analysis
IR: insulin resistance
T2D: type 2 diabetes
FC: fold change
QC: quality control
ST: sterol lipids
ARA: arachidonic acid
PEs: phosphatidylethanolamines
PCs: phosphatidylcholines
SM: sphingomyelin
SMS: sphingomyelin synthase
ER: endoplasmic reticulum
SAM: s-adenosine methionine
SAH: methyltransferase inhibitor S-adenosylhomocysteine
LPS: lipopolysaccharide
DPA: docosapentaenoic acid
DHA: docosahexaenoic acid
PUFAs: polyunsaturated fatty acids
LPEs: lysophosphatidylethanolamine
LysoPCs/LPCs: lysophosphatidylcholines
Hcy: homocysteine
HM: homocysteine-methionine
LC: liquid chromatography
LC-MS/MS: liquid chromatography-tandem mass spectrometry
TG: triglyceride
H&E: hematoxylin-eosin
KEGG: kyotoencyclopedia of genes and genomes.

**Declarations**

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**Availability of data and materials**

All data generated or analyzed during this study are included within the article and its supplementary information files.
Ethics approval and consent to participate

The authors confirm that this study was performed in accordance with the guidelines of Good Experimental Practices adopted by the Institute of Animal Science (Sichuan Agricultural University, Chengdu, China). All experimental protocols involving animal were approved by the Biological Studies of Animal Care and Use Committee, Sichuan Province, China.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors’ contributions

Conceived and designed the study: JW XJ SJL. Collected data and conducted the research: TT YHL TFL MCG YM; Wrote the paper: SQX JHS. Mauricio A. Elzo revised the paper.

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References

1. Ng M, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013[J]. Lancet, 2014. 384(9945):766–781.

2. Wyatt SB, Winters KP, Dubbert PM. Overweight and obesity: prevalence, consequences, and causes of a growing public health problem[J]. Am J Med Sci. 2006;331(4):166–74.

3. Reilly J, et al. Determining the worldwide prevalence of obesity[J]. The Lancet, 2018. 391:1773–1774.

4. Elobeid MA, D.B.J.C.O.i.E D, Allison. and Obesity. Putative environmental-endocrine disruptors and obesity: a review[J]. 2008. 15(5):403–408.

5. McLaughlin T, et al. Role of innate and adaptive immunity in obesity-associated metabolic disease[J]. J Clin Invest. 2017;127(1):5–13.
6. Bhupathiraju SN, Hu FB. Epidemiology of Obesity and Diabetes and Their Cardiovascular Complications[J]. Circ Res, 2016. 118(11):1723–1735.

7. O'Sullivan J, et al. Obesity and gastrointestinal cancer: the interrelationship of adipose and tumour microenvironments[J]. Nat Rev Gastroenterol Hepatol, 2018. 15(11):699–714.

8. Liu Z, et al. High-fat diet induces hepatic insulin resistance and impairment of synaptic plasticity[J]. PLoS One, 2015. 10(5):e0128274.

9. Picone P, Carlo MD, Nuzzo D. Obesity and Alzheimer's disease: Molecular bases[J]. Eur J Neurosci. 2020;52(8):3944–50.

10. Lira FS, et al. Supplementing alpha-tocopherol (vitamin E) and vitamin D3 in high fat diet decrease IL-6 production in murine epididymal adipose tissue and 3T3-L1 adipocytes following LPS stimulation[J]. 2011. 10(1):1–5.

11. Wang Q, et al. FGF21 Attenuates High-Fat Diet-Induced Cognitive Impairment via Metabolic Regulation and Anti-inflammation of Obese Mice[J]. Mol Neurobiol, 2018. 55(6):4702–4717.

12. Rodríguez RR, et al. The Iberian pig fed with high-fat diet: a model of renal disease in obesity and metabolic syndrome[J]. Int J Obes (Lond). 2020;44(2):457–65.

13. Luo L, Liu M. Adipose tissue in control of metabolism[J]. J Endocrinol, 2016. 231(3):R77-R99.

14. Bocquier F, et al. Effects of photoperiod and feeding level on perirenal adipose tissue metabolic activity and leptin synthesis in the ovariectomized ewe[J]. Reprod Nutr Dev, 1998. 38(5):489–498.

15. Cheng H, et al. The Molecular Mechanisms Underlying Mitochondria-Associated Endoplasmic Reticulum Membrane-Induced Insulin Resistance[J]. Front Endocrinol (Lausanne), 2020. 11:592129.

16. Blouin CM, et al. Plasma membrane subdomain compartmentalization contributes to distinct mechanisms of ceramide action on insulin signaling[J]. Diabetes, 2010. 59(3):600–610.

17. Meikle PJ, Summers SA. Sphingolipids and phospholipids in insulin resistance and related metabolic disorders[J]. Nat Rev Endocrinol, 2017. 13(2):79–91.

18. Solinas G, Becattini B. JNK at the crossroad of obesity, insulin resistance, and cell stress response[J]. Mol Metab, 2017. 6(2):174–184.

19. Fu S, et al. Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity[J]. Nature, 2011. 473(7348):528–531.

20. Johnson CH, Ivanisevic J, Siuzdak G. Metabolomics: beyond biomarkers and towards mechanisms[J]. Nature reviews. Molecular cell biology, 2016. 17(7):451–459.

21. Zamboni N, Saghatelian A, Patti GJ. Defining the metabolome: size, flux, and regulation[J]. Mol Cell, 2015. 58(4):699–706.

22. Zhu H, et al. Untargeted metabonomics reveals intervention effects of chicory polysaccharide in a rat model of non-alcoholic fatty liver disease[J]. Int J Biol Macromol. 2019;128:363–75.

23. Gowda SGB, et al. Untargeted Lipidomic Analysis of Plasma from High-fat Diet-induced Obese Rats Using UHPLC-Linear Trap Quadrupole-Orbitrap MS[J]. Anal Sci, 2020. 36(7):821–828.
24. Martínez-Álvaro M, et al. Correlated responses to selection for intramuscular fat in several muscles in rabbits[J]. Meat Sci, 2018. 139:187–191.
25. Lowry JE, et al. Effect of high-fat diet on peripheral blood mononuclear cells and adipose tissue in early stages of diet-induced weight gain[J]. Br J Nutr. 2019;122(12):1359–67.
26. Alarcon G, et al. High fat diet-induced metabolically obese and normal weight rabbit model shows early vascular dysfunction: mechanisms involved[J]. Int J Obes (Lond), 2018. 42(9):1535–1543.
27. Shao J, et al. Growth, behavioural, serum biochemical and morphological changes in female rabbits fed high-fat diet[J]. J Anim Physiol Anim Nutr (Berl), 2021. 105(2):345–353.
28. Want EJ, et al. Solvent-dependent metabolite distribution, clustering, and protein extraction for serum profiling with mass spectrometry[J]. Anal Chem, 2006. 78(3):743–752.
29. Heischmann S, et al. Exploratory Metabolomics Profiling in the Kainic Acid Rat Model Reveals Depletion of 25-Hydroxyvitamin D3 during Epileptogenesis[J]. Sci Rep. 2016;6:31424.
30. Haspel JA, et al. Circadian rhythm reprogramming during lung inflammation[J]. Nat Commun, 2014. 5:4753.
31. Sreekumar A, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression[J]. Nature, 2009. 457(7231):910–914.
32. Ma N, et al. Feces and liver tissue metabonomics studies on the regulatory effect of aspirin eugenol eater in hyperlipidemic rats[J]. Lipids Health Dis. 2017;16(1):240.
33. Kleinert M, et al. Animal models of obesity and diabetes mellitus[J]. Nature Reviews Endocrinology. 2018;14(3):140–62.
34. Johnson CH, Ivanisevic J, Siuzdak G. Metabolomics: beyond biomarkers and towards mechanisms[J]. Nat Rev Mol Cell Biol, 2016. 17(7):451–459.
35. Cai X, et al. Pu-erh tea extract ameliorates high-fat diet-induced nonalcoholic steatohepatitis and insulin resistance by modulating hepatic IL-6/STAT3 signaling in mice[J]. J Gastroenterol, 2016. 51(8):819–829.
36. Facchini L, et al. Structural characterization and profiling of lyso-phospholipids in fresh and in thermally stressed mussels by hydrophilic interaction liquid chromatography-electrospray ionization-Fourier transform mass spectrometry[J]. Electrophoresis, 2016. 37(13):1823–1838.
37. Whiley L, et al. Evidence of altered phosphatidylcholine metabolism in Alzheimer’s disease[J]. Neurobiol Aging, 2014. 35(2):271–278.
38. Kim HJ, et al. Metabolomic analysis of livers and serum from high-fat diet induced obese mice[J]. J Proteome Res. 2011;10(2):722–31.
39. Barber MN, et al. Plasma lysophosphatidylcholine levels are reduced in obesity and type 2 diabetes[J]. PLoS one, 2012. 7(7):e41456.
40. Jin S, et al. Preventive effects of turmeric on the high-fat diet-induced hyperlipidaemia in mice associated with a targeted metabolomic approach for the analysis of serum lysophosphatidylcholine using LC-MS/MS[J]. Journal of Functional Foods. 2014;11:130–41.
41. Ye C, et al. A Metabolic Function for Phospholipid and Histone Methylation[J]. Mol Cell, 2017. 66(2):180–193.

42. Cheng H, et al. The Molecular Mechanisms Underlying Mitochondria-Associated Endoplasmic Reticulum Membrane-Induced Insulin Resistance[J]. Frontiers in endocrinology, 2020. 11:592129.

43. Samuel VT, Shulman GIJC. Mechanisms for insulin resistance: common threads and missing links[J]. 2012. 148(5):852–871.

44. Pories WJ, Dohm GLJDC. Diabetes: Have We Got It All Wrong?: Hyperinsulinism as the culprit: surgery provides the evidence[J]. 2012. 35(12):2438–2442.

45. Hanamatsu H, et al. Altered levels of serum sphingomyelin and ceramide containing distinct acyl chains in young obese adults[J]. Nutr Diabetes, 2014. 4(10):e141.

46. Liu YT, et al. UPLC-Q/TOF MS standardized Chinese formula Xin-Ke-Shu for the treatment of atherosclerosis in a rabbit model[J]. 2014. 21(11):1364–1372.

47. Liu TW, et al. High-Fat Diet Alters Serum Fatty Acid Profiles in Obesity Prone Rats: Implications for In Vitro Studies[J]. Lipids, 2015. 50(10):997–1008.

48. Sonnweber T, et al. Arachidonic Acid Metabolites in Cardiovascular and Metabolic Diseases[J]. Int J Mol Sci, 2018. 19(11):3285.

49. Mak IL, et al. Arachidonic acid exacerbates diet-induced obesity and reduces bone mineral content without impacting bone strength in growing male rats[J]. J Nutr Biochem. 2019;73:108226.

50. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease[J]. Cell, 2010. 140(6):900–917.

51. Johnson AM, Olefsky JM. The origins and drivers of insulin resistance[J]. Cell, 2013. 152(4):673–684.

52. Horas H, Nababan S, et al. Adrenic acid as an inflammation enhancer in non-alcoholic fatty liver disease[J]. Archives of Biochemistry and Biophysics, 2017. 623–624:64–75.

53. Huang JP, et al. Docosapentaenoic acid and docosahexaenoic acid are positively associated with insulin sensitivity in rats fed high-fat and high-fructose diets[J]. J Diabetes, 2017. 9(10):936–946.

54. Guo XF, et al. n-3 Polyunsaturated Fatty Acids and Metabolic Syndrome Risk: A Meta-Analysis[J]. Nutrients, 2017. 9(7):703.

55. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology[J]. Nature, 2014. 510(7503):92–101.

56. Miura T, Muraoka S, Ogiso T. Inhibition of lipid peroxidation by estradiol and 2-hydroxyestradiol[J]. Steroids, 1996. 61(6):379–383.

57. Patel R, Shah G. High-fat diet exposure from pre-pubertal age induces polycystic ovary syndrome (PCOS) in rats[J]. Reproduction, 2018. 155(2):141–151.

58. Wiik A, et al. Metabolic and functional changes in transgender individuals following cross-sex hormone treatment: Design and methods of the GEnder Dysphoria Treatment in Sweden (GETS) study[J]. Contemp Clin Trials Commun. 2018;10:148–53.

59. Shim J, et al. L-Methionine Production[J]Adv Biochem Eng Biotechnol. 2017;159:153–77.
60. Bauerle MR, Schwalm EL, Booker SJ. Mechanistic diversity of radical S-adenosylmethionine (SAM)-dependent methylation[J]. The Journal of biological chemistry, 2015. 290(7):3995–4002.

61. Lever M, Slow S. The clinical significance of betaine, an osmolyte with a key role in methyl group metabolism[J]. Clin Biochem, 2010. 43(9):732–744.

62. Blachier F, Andriamihaja M, Blais A. Sulfur-Containing Amino Acids Lipid Metabolism[J]. J Nutr. 2020;150(Supplement_1):2524s–2531s.

63. Shen W, et al. Homocysteine-methionine cycle is a metabolic sensor system controlling methylation-regulated pathological signaling[J]. Redox Biology, 2020. 28:101322.

64. Sanz-Fernandez MV, et al. A Cross-Sectional Study of Obesity Effects on the Metabolomic Profile of a Leptin-Resistant Swine Model[J]. 2020. 10(3):89.

Tables

Table1. Metabolites with significant difference were analyzed by LC-MS/MS to identify potential biomarkers of interest.
| Metabolites’ name | Formula        | \(^1m\)     | \(^2RT\) | \(^3VIP\)     | \(^4\) Trend and pvalue (HFD vs SND) |
|------------------|----------------|------------|---------|---------------|--------------------------------------|
| PC(2:0/16:1)     | C26H50NO8P     | 595.3491   | 13.322  | 1.563966765   | ↓**                                  |
| PC(2:0/16:0)     | C26H52NO8P     | 597.36482  | 13.637  | 1.678708      | ↓*                                   |
| PC (2:0/16:2)    | C26H48NO8P     | 533.312    | 13.083  | 1.257585      | ↓**                                  |
| PC (7:0/8:0)     | C23H46NO8P     | 495.296    | 13.64   | 1.709003      | ↓**                                  |
| PC (8:0/8:0)     | C24H48NO8P     | 509.3122   | 12.994  | 1.482654      | ↓*                                   |
| PC(14:0e/3:0)    | C25H52NO7P     | 509.3481   | 15.092  | 1.797233      | ↓**                                  |
| PC (14:0e/5:0)   | C27H56NO7P     | 537.3799   | 15.604  | 1.563913      | ↓**                                  |
| PC(14:0e/15:0)   | C37H76NO7P     | 660.4966   | 15.72   | 1.602117      | ↓**                                  |
| PC (15:1/18:2)   | C41H76NO8P     | 741.532    | 16.04   | 1.638059      | ↓*                                   |
| PC(16:0e/13:0)   | C37H76NO7P     | 767.5806   | 16.279  | 1.665696      | ↓*                                   |
| PC(16:0e/20:4)   | C44H82NO7P     | 709.5431   | 15.675  | 1.384283      | ↓**                                  |
| PC (16:2/18:5)   | C42H70NO8P     | 815.5423   | 15.998  | 1.250684      | ↑*                                   |
| PC (17:2/22:6)   | C47H78NO8P     | 783.5737   | 16.245  | 1.218645      | ↓*                                   |
| PC (18:1/19:2)   | C45H84NO8P     | 791.5449   | 15.76   | 2.130609      | ↓**                                  |
| PC (18:2/19:2)   | C45H82NO8P     | 795.5781   | 15.78   | 1.790827      | ↓**                                  |
| PC (18:4e/20:5)  | C46H76NO7P     | 791.5449   | 15.76   | 2.130609      | ↓**                                  |
| PC (18:4e/22:6)  | C48H78NO7P     | 791.5449   | 15.76   | 2.130609      | ↓**                                  |
| PC (19:2/18:4)   | C45H84NO8P     | 811.5374   | 15.781  | 1.790827      | ↓**                                  |
| PC (20:2/20:3)   | C48H86NO8P     | 835.5957   | 15.534  | 1.313735      | ↓*                                   |
| PC (22:3e/18:4)  | C48H84NO7P     | 817.594    | 15.407  | 1.361675      | ↓**                                  |
| PC (20:3/20:3)   | C48H84NO8P     | 833.5871   | 15.567  | 1.34977       | ↓*                                   |
| LPC 15:0         | C23H48NO7P     | 481.3167   | 14.571  | 1.951213      | ↓*                                   |
| LPC 19:0         | C27H56NO7P     | 597.40091  | 15.577  | 2.003144      | ↓*                                   |
| PE (2:0/16:2)    | C23H42NO8P     | 491.2647   | 13.081  | 1.33104       | ↓**                                  |
| PE (5:0/13:1)    | C23H44NO8P     | 493.2803   | 13.564  | 1.864231      | ↓**                                  |
| PE (2:0/18:1)    | C25H48NO8P     | 521.31072  | 14.223  | 1.958401      | ↓**                                  |
| Compound                  | Formula            | mW         | RT    | VIP       | Sign |
|--------------------------|--------------------|------------|-------|-----------|------|
| PE (17:1/18:1)           | C40H76NO8P         | 729.53193  | 16.438| 2.004323  | ↓**  |
| PE (18:2/18:2)           | C41H74NO8P         | 739.5142   | 16.165| 1.086091  | ↓*   |
| PE (18:2/20:4)           | C43H74NO8P         | 763.50059  | 15.487| 1.856928  | ↓**  |
| PE (20:3/20:4)           | C45H76NO8P         | 789.5234   | 15.926| 1.857655  | ↑*   |
| LPE 16:1                 | C21H42NO7P         | 451.2696   | 14.594| 1.583397  | ↓**  |
| LPE 17:0                 | C22H46NO7P         | 467.3011   | 14.966| 1.396921  | ↓**  |
| LPE 17:1                 | C22H44NO7P         | 465.2855   | 14.621| 2.160675  | ↓**  |
| LPE 20:4                 | C25H44NO7P         | 501.2857   | 14.407| 1.723687  | ↑*   |
| LPS 18:2                 | C24H44N O9 P       | 521.27548  | 13.894| 1.839273  | ↑**  |
| LPS 18:0                 | C24 H48NO9 P       | 525.30639  | 14.412| 1.545101  | ↓*   |
| PG (18:2/20:4)           | C44H75O10P         | 794.51067  | 15.605| 1.039475  | ↑*   |
| SM(d19:1/19:0)           | C43H87N2O6P        | 758.6277   | 15.612| 1.201017  | ↑*   |
| Arachidonic acid         | C20H32O2           | 304.23985  | 14.263| 1.235326  | ↑*   |
| Adrenic acid             | C22H36O2           | 332.27124  | 14.649| 1.732588  | ↑*   |
| Docosapentaenoic acid    | C22H34O2           | 330.2556   | 15.208| 2.095414  | ↑**  |
| Docosahexaenoic acid     | C22H32O2           | 328.23993  | 14.209| 1.90978   | ↑**  |
| Methyltestosterone       | C20H30O2           | 302.22456  | 14.414| 2.348698  | ↑**  |
| 2-Hydroxyestradiol       | C18H24O3           | 288.17234  | 10.513| 1.100492  | ↓**  |
| Epitestosterone          | C19H28O2           | 288.2084   | 13.648| 1.387     | ↓*   |
| Cholecalciferol          | C27H44O            | 384.3388   | 15.714| 1.61838   | ↓**  |
| 4-Pyridoxic acid         | C8H9NO4            | 183.0533   | 7.482 | 1.766454  | ↓**  |
| L-Methionine             | C5H11NO2S          | 149.0511   | 1.964 | 1.568533  | ↑*   |

NOTE:

1. m: molecular weight.
2. RT: retention time.
3. VIP: the importance projection of variables is used to reflect the contribution of quantitative value of each sample to the difference, generally, VIP > 1.
Trend and pvalue (HFD vs SND): *$P < 0.05$; **$P < 0.01$.

**Figures**

**Figure 1**

Hematoxylin-Eosin (H&E) staining of PAT in female rabbits fed SND (n=6) and HFD (n=6).
Figure 2

Quality control of metabolomics data. PCA score plots for SND (n=6) and HFD (n=6) groups in the positive (A) and negative (B) ion modes. PLS-DA score plots for SND (n=6) and HFD (n=6) groups in the positive (C) and negative (D) ion modes. Permutation tests from PLS-DA models for SND (n=6) and HFD (n=6) groups in the positive (E) and negative (F) ion modes.
Figure 3

Differential metabolites analysis. Volcano maps showing the overall distribution of differential metabolites in rabbit groups fed SND (n=6) and HFD(n=6) in the positive (A) and negative (B) ion modes. Red circles represent significantly higher numbers of metabolites and green circles indicate significantly lower numbers of metabolites in HFD (n=6) than in SND (n=6) rabbit groups.
Figure 4

Metabolic pathway analysis. KEGG positive (A) and negative (B) pathway enrichment plots obtained with MetaboAnalyst. Metabolic pathways are represented by circles, circle size and color shade are based on pathway impact and p-value (red being the most significant). (C) The network of metabolic pathways associated with lipid cycle. Metabolite levels are shown in color: red represents increased levels, and black represents decreased or undetected levels.
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