1. Introduction

Kidney-yang deficiency syndrome (KYDS) is a kind of basic syndrome which often leads to extensive pathophysiological change in the body. Modern research has shown that KYDS can cause various degrees of disorders in the hypothalamus–pituitary-target gland. Once the intake of exogenous corticosteroid is stopped, the inhibition state of the hypothalamus–pituitary-target gland function will be exposed and cannot be restored in a short time.

Lipidomics, a branch of metabolomics, could provide a powerful technique for discovery of lipid molecules to reveal disease status and drug efficacy. The Shenqi pill (SQP) is a representative prescription for clinical application in the prevention and treatment of kidney-yang deficiency syndrome (KYDS). However, its effect mechanism is still not clear. This article aims to reveal the intervention effect of SQP on KYDS from the perspective of lipid metabolism. In this study, SQP was used to intervene in the rat model of KYDS, on the foundation of successfully replicating the rat model of KYDS induced by corticosterone. The MetaboAnalyst tool was used for analysis of the serum metabolic profile and pattern recognition of KYDS model, based on UPLC-SYNAPT-G2-Si-HDMS. Finally, twenty-two potential lipid biomarkers related to the KYDS model were characterized, and the effects of SQP on regulating potential lipid markers in serum of KYDS model were analyzed. There were 10 biomarkers and seven metabolic pathways closely related to SQP therapy for KYDS were found. The action mechanism and targets of SQP in treating KYDS were explored based on high-throughput lipidomics. This work could provide valuable data and scientific evidence in subsequent studies for the treatment of KYDS.
2. **Experiment**

2.1 **Chemical reagents**

Methanol and acetonitrile of UPLC were both obtained from Dikma Company (USA). Water for UPLC was made by Milli-Q system (USA). Methanoic acid of UPLC was supplied by Honeywell company (USA); sodium pentobarbital (no. 20080603, purchased from Shanghai Chemical Reagent Factory); corticosterone (analytical grade, purchased from Sigma); olive oil (Oliver grade) was produced from Beijing Huasheng Biological Co., Ltd. leucine-enkephalin (SIGMA Technology Co., Ltd., USA); SQP was provided by Beijing Tongren Tang Drug Store (Harbin, China).

2.2 **Animals**

Wistar rats (SPF), male, weighting 226 g ± 10 g, purchased from Beijing Witonglihua Laboratory. Feeding temperature was controlled at 21 ± 2 °C and the range of humidity was from 65% to 75%. The light and dark by turns per 12 h and with natural drink and food. All the Wistar rats were acclimatized to the environment for one week before model establishment. All the rats were raised and supervised by GAP of Heilongjiang University of Chinese Medicine.

2.3 **Reproduction of KYDS rat model**

According to the weight of rats, they were assigned randomly to three groups: control group, model group and SQP group with each group of 10 rats. The model group was subcutaneously injected with 10 mg mL⁻¹ corticosterone solutions, which were diluted with olive oil, continuous injection at 1 mL kg⁻¹ for 21 days. And then lavage with purified water for 21 consecutive days, injected daily with the same dosages of olive oil last 21 days for the control group. During the first 21 days, rats of SQP group were administrated in exactly the same way as the model group. On the 22nd day, the SQP group was lavage with precise concentrations of SQP (SQP were crushed and dissolved in distilled water to 0.36 g mL⁻¹), 10 mL kg⁻¹ as the weight of rats for 21 consecutive days. The experimental procedures were approved by the Animal Care and Ethics Committee at Heilongjiang University of Chinese Medicine and all experiments were performed in accordance to the declaration of Helsinki.

2.4 **Preparation and collection of sample**

On the last day of the experiment, all rats were taken blood by the hepatic portal vein. The blood samples were kept at 4 °C for 30 minutes, and centrifuged at 4000g for 10 minutes. The upper serum was taken and stored in the refrigerator at −80 °C. The serum sample was defrosted at room temperature before analysis. Then we add 800 μL of methanol into 200 μL of the thawed serum samples to precipitate protein. Centrifuge separates the samples at 13 000g for 10 minutes after vortexing for 30 seconds. The supernatant was dried by nitrogen on water bath at 40 °C, and the residue was dissolved with 200 μL of 80% methanol, following centrifuged at 13 000g for 10 minutes. The supernatant was filtered byPTFE membrane (0.22 μm) for UPLC-HDMS analysis.

2.5 **Instruments and experimental conditions**

Waters Acquity TM UPLC combined with Waters Synapt G2-Si High Definition Mass Spectrometer (HDMS) system and the MassLynX™ 4.1 workstation were utilised for following data acquisition and processing.

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Fig. 1 Flow diagram for data processing and analysis.
Fig. 2  Metabolic profiling analysis. (A) Serum samples of the control group in positive ion mode; (B) serum samples of KYDS model group in positive ion mode; (C) serum samples of the control group in negative ion mode; (D) serum samples of KYDS model group in negative ion mode.
Chromatographic conditions: ACQUITY UPLC™ HSS T3 (100 mm × 2.1 mm i.d., 1.8 μm, Waters, USA) was chosen as the chromatographic column. The temperature of column was 40 °C and the autosampler was set at 4 °C. Mobile phase system comprise 0.1% methanoic acid–acetonitrile (A) and 0.1% methanoic acid–water (B), with flow velocity of 0.4 mL min⁻¹. Injection volume of sample was 3 μL for both ESI⁺ and ESI⁻ mode. Gradient elution as follows: 0–2 min, 5–50% A; 2–4 min, 50–60% A; 4–7 min, 60–80% A; 7–10 min, 80–100% A; 10–12 min, stay 100% A.

Mass spectrometry conditions: the ionization source of ESI was operated in ion mode of positive (ESI⁺) with 30 V of sample cone bore voltage, and 3.0 kV of capillary voltage. Temperature of desolvation was 400 °C, and ion source was 110 °C. Desolvent and cone gas flow velocity were 500 L h⁻¹ and 50 L h⁻¹, respectively. In the ion mode of negative (ESI⁻), the sample cone bore voltage was 40 V, desolvation temperature was 300 °C, velocity of desolvation gas flow was 550 L h⁻¹. Other parameters of the negative ion mode were the same with the positive ion mode. Data were captured under centroid mode and the range of mass scan was 50–1000 Da. For precise mass detection, leucine-enkephalin ([M + H]⁺ = 556.2771 and [M – H]⁻ = 554.2615) was selected as internal standard, and the lock-mass solution with the concentration of 0.2 ng mL⁻¹.

2.6 Data processing and screening

As displayed in Fig. 1, all the source data of serum metabolomics were imported into Progenesis QI 1.0 software for peak processing and normalization. By matching and screening, only the lipid ion peaks were analyzed. The pretreatment data were processed by the method of multivariate statistical analysis, include PCA (principal component analysis) and OPLS-DA (orthogonal partial least squares discriminant analysis) via the EZInfo 3.0.3 software. The value of VIP greater than 1 and P < 0.05 in T-test was selected as potential biomarkers for further identification.

Fig. 3 Score plots of serum samples of control group and KYDS model group. (A) PCA score plots for control and KYDS model group in positive mode; (B) 3D plots of OPLS-DA based on serum metabolites discriminating control and KYDS model group in positive mode; (C) PCA score plots for control and KYDS model group in negative mode; (D) 3D plots of OPLS-DA based on serum metabolites discriminating control and KYDS model group in negative mode.
3. Results

3.1 Analysis of serum metabolism profile in rats with KYDS model

The samples of blank group and model group were collected and processed according to the established analysis conditions of serum endogenous metabolites, and through full scanning analysis by UPLC-HDMS. Typical metabolic profiles of both positive and negative ion pattern were shown in Fig. 2.

![Fig. 4](image_url) The biomarkers in the VIP and S-plot between control and KYDS model group analyzed by OPLS-DA. (A) In positive ion mode; (B) in negative ion mode.

![Fig. 5](image_url) The heatmap showed the relationship between control group and model group, and the comparison of contents of lipid metabolites. Each color blocks correspond to the value of content of each biomarker.
3.2 Identification of potential lipid biomarkers of KYDS
When used the Progenesis QI to identify compounds, we selected lipidomics and basic lipids as search parameters, and then multiple database searches also be used for data matching and identification, such as LIPID MAPS® Lipidomics Gateway, Metaboanalyt, KEGG, etc. Combining the above databases with the potential biomarkers met the criteria (VIP > 1, \( P < 0.05 \)), we regarded the screened compound as the lipid biomarkers of KYDS model.

3.3 Multivariate statistical analysis of metabolite profiling
Software of MarkerLynx 4.2 was used for preprocess the raw data of UPLC-HDMS. The results of PCA analysis by EZinfo 3.0.3 software were shown as Fig. 3, included 2D and 3D PCA plots in both positive and negative ion model. As displayed, the control group and the KYDS model group were clustered into two groups with no obvious interaction, it means that the endogenous metabolic network in serum of rats changed significantly after corticosterone injection. VIP-plot (shown in Fig. 4) was obtained by further analysis of OPLS-DA based on serum metabolic profile data, and we chose the ions that satisfy both VIP > 1 and \( P < 0.05 \) (t-test) as the target ions. Eventually, according to relative signal intensity of the potential lipid biomarkers, there were 22 lipid biomarkers be discovered. In addition, the significant difference in the lipid metabolites of the control and model group was shown in clustering heatmap as Fig. 5.

3.4 Intervention and profile analysis of SQP against KYDS
According to the analytical methods mentioned above, three-dimensional information (Rt_m/z_intensity) in serum samples of the control group, model group and SQP group were analyzed, to determine the relative content and the relationship of potential lipid biomarkers in blood of the three groups. The 2D and 3D plots of PLS-DA analysis by EZinfo 3.0.3 software were shown as Fig. 6. The results showed that after SQP

![Fig. 6](image_url) Score plots of serum samples of control group, KYDS group and SQP group. (A) PCA score plots for the three groups in positive mode; (B) 3D plots of OPLS-DA based on serum metabolites discriminating the three groups in positive mode; (C) PCA score plots for the three groups in negative mode; (D) 3D plots of OPLS-DA based on serum metabolites discriminating the three groups in negative mode.
treatment, the contents of the 22 potential biomarkers mentioned above all tended to be called back to the control group. Finally, 10 biomarkers with significant differences were identified, compared with the model group. For more detailed information and the intervention effect of SQP on lipid biomarkers based on KYDS were specified in Table S1.† The relative signal intensity of them was displayed in the bar graph as Fig. 7.

4. Discussion

Mass spectrometry (MS)-based omics has developed rapidly in recent years and has become a predictor and interpreter of the relationship between complex phenotypes and metabolic characteristics.24–32 MS is a sensitive, efficient and high throughput method of medicine development, which can provide especially useful structural information for medicine analysis.33–41 In recent years, exponential growth in the application of MS in lipidomics, and separation technology based on MS also got remarkable progress.42–53 It has enabled the discovery of potential biomarkers and explore disease pathogenesis,54–64 disease diagnosis,65–71 prognosis,72–77 and treatment.78–83

In our study, based on metabolomics and technology of UPLC-HDMS, the mechanism of SQP related to lipid metabolism was analyzed and characterized, in the model of KYDS induced by corticosterone. Results of PCA and OPLS-DA score plots showed that the metabolic profiling of rats with SQP administration was gradually moving away from the model group, and close to the control group. It means that SQP has a tendency to prevent the occurrence and development of metabolic trajectory in KYDS model. Through various of database and analysis methods, we eventually discovered 22 lipid markers can be regulated by SQP in the model of KYDS, among which, 10 markers were significantly regulated. Combined with the visualized KEGG analysis results, these biomarkers were input into the MetPA platform to analyze the topological characteristics of metabolic pathways, to search the highly correlated metabolic pathways with them. The underlying mechanisms of SQP intervention on KYDS was clarified from the perspective of lipid metabolism as Fig. 8.

Research suggests that there was significant changes of multiple lipid metabolites in the model of KYDS,84,85 and renal damage was also clearly associated with changes in fatty acid metabolism,86 phospholipid level can be used as an indicator of clinical diagnosis and it is a marker of chronic

Fig. 7 The relative signal intensities of the lipid biomarkers in control, model and SQP group. Compared with KYDS model group: *p < 0.05, **p < 0.01.
renal failure. All of the above indicated that lipid metabolism disorders were intimately linked to kidney diseases. Glycerophospholipid metabolism includes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycer (PG), lyso phospholipids and so on. Moreover, PC as precursor of alpha-linolenic acid can influence mitochondrial activity and participate in the tricarboxylic acid cycle through the metabolism of alpha-linolenic acid. In the experiment, we found that PC, PE, PG and lysophospholipid were disturbed intensely, however, compared with the model group, SQP has significant callback effect on PE (O-18:0/0:0), PG (18:1 9Z)/20:1 (11Z)), PC (P-16:0/20:3 (5Z, 8Z, 11Z)), PC (16:0/18:2 (9Z, 12Z)) and SM (d16:1/18:1). Linoleic acid (d4) and 2-oxo-4-methylthiobutanoic acid (P < 0.05) related to fatty acid metabolism also can be up-regulated tends to normal.

Steroid hormone synthesis pathway is an important pathway in the KYDS model, abnormal steroid levels are common in patients with KYDS. In our study, the steroid content in KYDS model of rats was obviously different from the control group, whereas SQP group can effectively reverse 5beta-pregnan-3,20-dione and pregnenolone in steroid hormone synthesis pathway (P < 0.05).

5. Conclusions

This work mainly emphasizes the establishment of a combination of lipidomics and UPLC-MS technology to clarify the characteristics of lipid metabolism and regulation effect of SQP on KYDS. Based on the current lipidomics platform, we have characterized 22 potential lipid biomarkers associated with the KYDS, and found 10 lipid biomarkers closely related to SQP treating for KYDS. The efficacy of SQP may have significant related to the regulation of 10 potential biomarkers. Consider these markers as the possible drug targets, perturbations in the concentration of them may affect the regulation of relevant lipid metabolic pathways, including ether lipid metabolism, glycerophospholipid metabolism, steroid hormone biosynthesis, cysteine and methionine metabolism. It reveals that SQP could play an intervention role in treatments for KYDS. The UPLC-SYNAPT-G2-Si-HDMS-based lipidomics is a powerful tool to study the essence of disease syndrome. This study also could provide the data basis and scientific method for the further study of SQP therapy for KYDS.

Conflicts of interest

There are no conflicts to declare.

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