Toward the Development of Personalized Syndrome Discriminant Systems: A Discriminant System for Hypertension with Liver Yang Hyperactivity Syndrome

Guang-yao Shang,1 Lei Zhang,2 Lin Lin,3 Hai-qiang Jiang,3,4 Chao Li,1,4 Feng Jiang,1 Dong-mei Qi,5 Yun-lun Li,1,3,4 and Wen-qing Yang3,4

1Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250014, China
2Faculty of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250355, China
3Innovation Research Institute of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250355, China
4Shandong Province Engineering Laboratory of TCM Precise Diagnosis and Treatment of Cardiovascular Disease, Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250355, China
5Experimental Center, Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250355, China

Correspondence should be addressed to Yun-lun Li; yunlun.lee@hotmail.com and Wen-qing Yang; wenqing-yang@hotmail.com

Received 20 August 2021; Accepted 23 October 2021; Published 15 November 2021

1. Introduction

Hypertension is considered to be the most common threat to public health worldwide [1] and constitutes the primary cause of numerous irreversible cardiovascular events [2]. China, in particular, is facing an enormous population health challenge owing to the high prevalence of hypertension among adults [3]. Although modern medicine has made considerable progress regarding antihypertensive treatment, several limitations remain as indicated by the intricate pattern of the associated pathology. One potential approach is through traditional Chinese medicine (TCM). Notably, as TCM originally harbored no concept of blood pressure, it treated patients primarily by differentiating the syndromes according to the symptoms and signs caused by hypertension, paying more attention to relieving symptoms and improving patient quality of life [4]. Moreover, a growing body of evidence supports the utility of TCM for providing effective hypertension treatments [5].
In TCM, liver Yang hyperactivity syndrome constitutes a primary pathogenesis of hypertension [4], encompassing a characteristic combination of syndromes and pathogeneses. The early stage of liver Yang hyperactivity syndrome is mainly excess syndrome. The excessive function of the liver controlling conveyance and dispersion can lead to many pathological symptoms. Its symptoms include dizziness, headache, tinnitus, bitter taste in the mouth, dry mouth, distending pain in the hypochondrium, impatience and irritability, flushing, redness of the eyes, red tongue, yellow fur, and stringy pulse. Therefore, clarifying the altered pathophysiological state associated with hypertension in liver Yang hyperactivity syndrome (HLYH) is of high priority to better understand this disorder.

Rats with hypertension of the liver Yang hyperactivity type represent a commonly used model that exhibits the characteristic behaviors and physiology of HLYH [6] and is frequently employed for drug discovery and to investigate the mechanisms of drug action [7, 8]. Although consensus-based screening guidelines exist for the application of physical signs of this model, microscopic biomarkers and the combination of suitable macro- and microdiscriminant factors have not yet been fully elucidated. Currently, numerous challenges remain regarding the evaluation of this rat model. Previous studies have emphasized the roles of several specific pharmacological indices or partial functional changes; however, such studies lacked evaluation indicators adapted to liver Yang hyperactivity syndrome, preventing these systems from truly elucidating the therapeutic mechanism(s) of TCM treatments based on syndrome differentiation.

Specifically, Uncaria rhynchophylla, Rhizoma Gastrodiae, and Concha Haliotidis are the major components included in TCM for HLYH. Previous studies have suggested that Uncaria rhynchophylla contains vasodilation-mediating active compounds, especially indole alkaloids [9]. Gastrodin is the main bioactive constituent of Rhizoma Gastrodiae. The antihypertensive activity of gastrodin may be attributed to its effects on the balance of endothelin and nitric oxide levels in the plasma and the protection of vascular endothelial cells [10]. In turn, Ca²⁺ plays a central role in a number of important physiological processes that regulate hypertension [11]; notably, the use of Concha Haliotidis has been shown to increase serum calcium and decrease blood pressure [12]. Therefore, in the present study, we exploited the specific therapeutic effects of Uncaria rhynchophylla, Rhizoma Gastrodiae, and Concha Haliotidis to extract the core discriminant factors of HLYH. The aim of this study was not to evaluate the antihypertensive effects of the drugs isolated from Uncaria, Rhizoma Gastrodiae, and Rhizoma Gastrodiae but rather to disprove the rat model of liver Yang hyperactivity syndrome using drugs with antihypertensive effects to suppress liver hyperactivity, thereby subsiding Yang.

Based on this stratagem, in this study, we dynamically collected multilevel data of animal models using a variety of technological methods, from which we extracted the core discriminant factors of the animal models. We then explored the potential to build a discriminant system for HLYH by integrating macroscopic and microscopic parameters, with the goal of rendering the discriminant system as an ideal tool to elucidate the essence of the syndrome and the mechanisms of therapeutic efficacy.

2. Materials and Methods

2.1. Experimental Drugs and Reagents. Radix Aconiti Lateralis Preparata, Uncariae, Rhizoma Gastrodiae, and Concha Haliotidis were purchased from Huqiao Pharmaceutical Co., Ltd. (Anhui, China). These Chinese medicinal materials were authenticated by Professor Lingchuan Xu (School of Pharmacy, Shandong University of Traditional Chinese Medicine). High-performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA, USA) was used to determine the effective constituents of Radix Aconiti Lateralis Preparata, Uncariae, and Rhizoma Gastrodiae. Acid-base titration was used to determine those of Concha Haliotidis.

Enzyme-linked immunosorbent assay kits for angiotensin II (Ang II), adrenaline (E), norepinephrine (NE), dopamine (DA), and 5-hydroxytryptamine (5-HT) were purchased from R&D Systems (Minneapolis, MN, USA). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Formic acid (HPLC grade) was purchased from Fisher Scientific (Waltham, MA, USA). Distilled water was produced using a Milli-Q Reagent water system (Millipore, Billerica, MA, USA). Other reagents were of analytical grade.

2.2. Animals. A total of 56 male spontaneously hypertensive rats (SHRs), specific-pathogen-free (SPF) level, 8-week-old, 186.35 ± 8.15 g, and seven male Wistar Kyoto rats (WKY), 8-week-old, 190.15 ± 6.37 g, were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China), certificate number SCXK (Beijing) 2012-0001. The animals were maintained under SPF laboratory conditions with free water and food ad libitum, at a temperature of 22 ± 2°C on a 12 h light/dark cycle during the experimental period. The study was approved by the Animal Ethics Committee of our institution. The study was performed according to the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals.

2.3. Animal Preparation and Grouping. One week following adaptation, all SHRs were randomly divided into eight groups, with seven rats in each group: model (M), disease syndrome (DS), disease syndrome rats with Uncaria extract intervention (DSGT), SHR with Uncaria extract intervention (MGT), disease syndrome rats with Rhizoma Gastrodiae extract intervention (DSTM), SHR with Rhizoma Gastrodiae extract intervention (MTM), disease syndrome rats with Concha Haliotidis extract intervention (DSSJM), and SHR with Concha Haliotidis extract intervention (MSJM). WKY served as the normal control group (N).

2.4. Intervention Measures. First, "liver Yang hyperactivity syndrome" was induced in the DS, DSGT, DSTM, and DSSJM groups with Radix Aconiti Lateralis Preparata...
extract using a standard protocol, as described previously [6]. Briefly, the rats were intragastrically administered Radix
Aconiti Lateralis Preparata extract at a dose of 20 mL/kg once daily (9:00–10:00 a.m.) for 6 weeks to induce liver Yang
hyperactivity syndrome. Compared with SHRs, facial tem-
perature and water intake of the DS rats were increased,
while pain threshold and rotation time were reduced.

2.5. Tests for Experimental Indices. The blood pressure of rats
was determined using a tail-cuff sphygmomanometer with an
automated system photoelectric sensor (ALC-Non-In-
vasive Blood Pressure System, Shanghai Alcott Biotech Co.,
Ltd., China). The pain threshold of rats was detected using a
tenderness-measuring instrument (ZXC-A, Shandong
Academy of Medical Sciences, China). The rotation time of
rats was determined by placing the rat on a rotary platform
at a speed of 45 r/min, and the time to fall was recorded; if
rats remained stable for 2 min, the experiment was termi-
nated. An infrared thermometer (GM550, Shenzhen Jumao
Source Technology Co., Ltd., China) was used to measure rat
facial temperature. In addition, the water intake of the rats
was measured for three continuous days each week.

2.6. Collection and Preparation of Serum Samples. All rats
were sacrificed by anesthesia using 2% sodium pentobarbital
(3 mL/kg). Serum samples were collected and centrifuged at
3,500 r/min for 15 min at 4°C. The serum samples were
divided into two equal parts. One part of serum was used to
measure the levels of Ang II, E, NE, DA, and 5-HT by using the
appropriate enzyme-linked immunosorbent assay kit
according to the manufacturer’s instructions. Another part
of the serum was used for metabonomics. Prior to
metabonomics analysis, 300 μL serum was mixed with
600 μL acetonitrile and vortexed for 1 min. The samples were
then centrifuged at 15,294 × g for 15 min at 4°C, and the
complete supernatant was transferred into vials and filtered
through a 0.22 mm membrane to obtain the prepared sample
extracts for ultraperformance liquid chromatography-mass
spectrometry (UPLC-MS).

2.7. Chromatography and MS Conditions. Metabolic profil-
ing analysis was performed using a UPLC-QExactive-MS
system (Thermo Scientific, Waltham, MA, USA). Chromo-
tographic analysis was performed using an UltiMate 3000
UPLC system (Thermo Scientific). Chromatographic sepa-
rations were performed using a Halo-C18 column
(2.1 × 100 mm, 2.7 μm, America Advanced Material Tech-
nology Corp., Phoenix, AZ, USA) with a binary solvent
system (solvent A: water with 0.1% formic acid; solvent B:
acetonitrile with 0.1% formic acid). The flow rate was 0.3 mL/
min and the injection volume was 5 μL. The column tem-
perature was set at 45°C. The gradient elution of B was
performed as follows: 2% B at 0-1 min, 2–20% B at 1–3 min,
20% B at 3-4 min, 20–40% B at 4–7 min, 40–70% B at 7–9 min,
and 70–98% B at 9–15 min. The column was then returned to
the initial status and reequilibrated for 3 min. All the samples
were maintained at 4°C during the entire analysis.

MS detection was performed using a Q Exactive™ hybrid
quadrupole-Orbitrap mass spectrometer (Thermo Scientific)
in both positive (ESI+) and negative (ESI–) ionization
modes. Nitrogen was used as the cone and desolvation gas.
The range of data collection was 80–1,000 m/z and (S)-lens
RF level was 55. The ion source temperature was 300/320°C
(±), and the sheath gas was 45 arb/40 arb (±). The capillary
capillary voltage was set at 3.5 kV/2.8 kV (±). The capillary tem-
perature was 300°C, and the auxiliary gas was 10 arb.

To ensure system stability and repeatability, the method
was validated using a quality control (QC) sample that
contained all the biological information. As the sequence
was running, the QC sample was analyzed six times at the
beginning of the experiment and randomly arranged after
ten unknown serum samples.

2.8. Data Analysis. Unprocessed LC/MS raw files were
converted to mzXML format using Proteowizard software
(v3.0.8789) (http://proteowizard.sourceforge.net). Baseline
correction, peak discrimination and alignment, and reten-
tion time correction were performed using the R (v3.3.2)
(https://www.r-project.org) XCMS package with default
settings. A visual data matrix containing retention time, m/z
pairs, sample names, and normalized ion intensities was
generated and exported to MetaboAnalys 3.0 (http://www.
metaboanalyst.ca) for multivariate data analysis. Unsuper-
vised principal component analysis (PCA) was used to afford
different metabolic pattern recognition. Supervised or-
thogonal partial least-squares discriminate analysis (OPLS-
DA) was applied to find differential metabolites among
different groups [13]. Variable importance projection (VIP)
produced during OPLS-DA was applied to identify variables
that substantively contributed to the classification. VIP >1
and VIP-Plot exhibiting a reliable confidence interval [14]
were considered to be statistically significant and treated as
candidate difference variables [15]. In addition, the t-test and
fold change (FC) were also applied to discover the con-
tributing variables for classification. Finally, the variables
with VIP values > 1, P < 0.05, and FC value ≥ 2 or FC val-
ue ≤ 0.5 were treated as potential biomarkers. The exact mass
of potential biomarkers was searched in databases such as
the Human Metabolome Database (HMDB; http://www.
hmdb.ca), METLIN (https://metlin scripps.edu), and
Kyoto Encyclopedia of Genes and Genomes (KEGG; http://
www.genome.jp/kegg/) for biomarker identification.
The data related to physical signs and enzyme-linked immunosorbent assays were expressed as the mean ± standard error. Multiple-group comparisons were analyzed using a one-way analysis of variance followed by the Tukey post hoc multiple range test. Data were analyzed using SPSS software (version 22.0; IBM, Armonk, NY, USA). 

2.9. Metabolic Pathway Construction. To further explore the interactions between potential biomarkers and to visualize metabolic networks, the MetaboAnalyst 3.0 pathway analysis module (https://www.metaboanalyst.ca) was used to carry out enrichment analysis and topological analysis of metabolic pathways. In addition, the MetScipe plug-in in Cytoscape 3.2.1 (https://cytoscape.org) was used to build an interaction network between potential biomarkers, to integrally observe the association between potential biomarkers.

2.9.1. Structure Discrimination System. Through the integration of physical signs, biochemical indicators, and metabolic markers, we extracted the core discriminant factors of the rats. To these, we applied partial least-squares regression (PLS) to construct the macro-micro discriminant system.

3. Results and Discussion

3.1. Extraction of Macroscopic Integral Discriminant Factors.
To clarify the integral discriminant factors of HLYH at the macroscopic level, we first investigated the changes in blood pressure following treatment (Figures 1(a) and 1(b)). During exposure to Uncaria, Rhizoma Gastrodiae, and Concha Haliotidis, the systolic pressure (F = 29.57, P < 0.001) and diastolic pressure (F = 5.20, P < 0.001) of the drug intervention groups were significantly reduced, and a significant difference was also noted between the SHR and HLYH model groups. We next compared the clinical diagnostic criteria of HLYH with the physical signs of the rat models. We found that the physical signs gradually improved only in rat models of HLYH, which is consistent with the pathological basis of sympathetic-adrenal medullary hyperfunction in liver Yang hyperactivity syndrome [23]. Moreover, compared with those of the SHRs, we found that Ang II (F = 4.02, P = 0.001), E (F = 2.64, P = 0.016), NE (F = 4.22, P = 0.001), and DA (F = 3.65, P = 0.02) were elevated in the serum of Radix Aconiti Lateralis Preparata-induced rat models (Figure 2). This indicates that the sympathetic-adrenal medulla function was enhanced in rat models of HLYH, which is consistent with the pathological basis of sympathetic-adrenal medulla hyperfunction in liver Yang hyperactivity syndrome [23].

3.2. Extraction of Microscopic Specificity Discriminant Factors.
To further clarify the pathological nature of HLYH, we utilized metabonomics to explore the metabolic characteristics of the rat models of HLYH from systematic and overall viewpoints. We analyzed the physiological function and metabolic networks of the potential biomarkers and identified metabolic pathways with abnormal perturbations. Overall, we aimed to extract the microscopic specificity discriminant factors for distinguishing HLYH.

Representative total ion chromatograms (TICs) of typical samples in each group are shown in Figures S1(a) and S1(b). Differences were observed in peak intensity and retention time among the different groups, suggesting that the endogenous metabolism of rats, under different interventions, was changed. We further utilized QC to ascertain whether the system error of the whole experiment was within a controllable range. As shown in Figures S1(c) and S1(d), the QC samples were clustered relative to the experimental samples. The relative standard deviation (RSD) of the peak area of all metabolites was below 20%, which demonstrated good stability and reproducibility.

We further applied PCA as a starting point for analysis to visualize possible intrinsic clusters and trends among the observations. PCA was additionally used to investigate whether each group was separated and to determine metabolic distinction [24]. The PCA score plots (Figure 3) indicated that the metabolic profile of the rat models of HLYH was disturbed. However, PCA displayed poor separation between different drug intervention groups. Therefore, to distinguish the endogenous metabolites and screen the differential metabolites to a larger extent, OPLS-DA was
Figure 1: Changes in blood pressure and physical signs following different drug interventions. Data represent means ± SEM. ▲ $P < 0.05$ vs. M groups. * $P < 0.05$ vs. DS groups. (a) Systolic pressure. (b) Diastolic pressure. (c) Pain threshold. (d) Rotation time. (e) Face temperature. (f) Water intake.
used to analyze the metabolomic data and for screening of differential metabolites as this method can maximize the difference between different groups, thereby significantly improving the effectiveness of the model and the ability to analyze data [25]. As shown in the OPLS-DA score plot in Figure 4, the different metabolic profiles of different rats are clearly reflected (a1, R2X = 61.4%, R2Y = 97.8%, Q2 = 96.6%; b1 R2X = 54.8%, R2Y = 98.3%, Q2 = 97.5%; c1, R2X = 73.3%, R2Y = 98.5%, Q2 = 98.2%; d1 R2X = 53.3%, R2Y = 97.6%, Q2 = 96.3%; e1, R2X = 58.3%, R2Y = 98.5%, Q2 = 98.2%; f1 R2X = 36.6%, R2Y = 92.4%, Q2 = 87.4%). To test the quality of the models, we extracted the parameters of the OPLS-DA models and performed a permutation test. The results indicated the excellent predictive capability and low risk of the models and were mined to extract differential variables. S-plot analysis was employed to determine the specific variation between the three groups. Metabolites for which VIP >1 were retained. In addition, FC and the t-test were

Figure 2: Changes in biochemical indicators after different drug interventions in each group. Data represent means ± SEM. ▲ P < 0.05 vs. M groups. * P < 0.05 vs. DS groups. (a) Content change of Ang II. (b) Content change of E. (c) Content change of NE. (d) Content change of DA. (e) Content change of 5-HT.
Figure 3: PCA score plot at positive (a) and negative (b) ion modes.

Figure 4: Continued.
used to assess significant differences of the different metabolites obtained.

The number of metabolites remaining in the three groups following analysis is shown in Figure 4(g). Notably, the overlap was observed between the different metabolites in each data matrix. The 46 metabolites with common variables between the three data matrices were classified as metabolites of HLYH. Moreover, the remaining variables of the N-DS and M-DS data matrix were eliminated and merged, identifying 53 variables as metabolites of liver Yang hyperactivity syndrome. In addition, following intervention with *Uncaria*, *Rhizoma Gastrodiae*, and *Concha Haliotidis*, the metabolic profile of the rats varied to different degrees. Therefore, from among the metabolite datasets, we selected differential metabolites exhibiting similar trends and near-normal conditions following the intervention of the three drugs as potential biomarkers. Finally, by comparing the retention time, MS, and MS/MS of ions with those of the standard or with information in databases, we identified 37 biomarkers considered to reflect the antihypertensive effect and mechanism of “calming the liver and suppressing Yang”.

ROC curves were used to screen markers by examining the area under the curve (AUC) of 37 potential biomarkers to identify the potential biomarkers with the discriminant ability for HLYH. Figure 5(a) shows the ROC curve assessing the predictive ability of the potential biomarkers. We found that 27 potential biomarkers exhibited an AUC > 0.8 [26] and demonstrated good sensitivity and specificity at the critical point. Therefore, these metabolites were selected as important microscopic indicators for HLYH. Information related to the potential biomarkers is shown in Table 1, and the changing trend following the intervention is shown in Figure 5(b).

To explore the metabolic pathway disturbance of HLYH, we utilized the MetPA database (https://www.metaboanalyst.ca) for pathway enrichment and topology analysis. Fourteen metabolic pathways were identified, including D-glutamine and D-glutamate metabolism, glycerophospholipid metabolism, and arachidonic acid (AA) metabolism. The results are shown in Figure 6(a). Moreover, we integrated the network diagram between potential biomarkers using MetScape (Figure 6(b)).

### 3.3. Structuring a Discrimination System.

We constructed a PLS model using 62 macro- and microcore discriminant factors (Table SI). The M group was found to be distinctly separated from the DS group (Figure 7). To test the predictive ability of the discrimination system for unknown samples, seven independent samples were introduced as the test set. We observed that the single sample selected from the DS group in the test set was accurately located in the area of the DS group, whereas the six samples selected from the drug intervention groups were distinct from the DS group and distributed separately. This indicated that the PLS model could accurately predict unknown samples. We used regression coefficients corresponding to the variables of VIP > 1 and VIP 95% confidence interval value to establish a regression model of HLYH (precise to three decimals) as follows.
Figure 5: Continued.
Table 1: Potential biomarkers for hypertension with liver Yang hyperactivity syndrome.

| No. | RT (min) | Exact mass | Ion mode | KEGG | Identified metabolites | Formula | Pathway |
|-----|----------|------------|----------|------|------------------------|---------|---------|
| 1   | 3.62     | 121.0891   | +        | C05332 | Phenylethylamine        | C₈H₁₁N  | Phenylalanine metabolism |
| 2   | 3.85     | 179.0582   | +        | C01586 | Hippuric acid           | C₉H₉NO₃ | Phenylalanine metabolism |
| 3   | 9.81     | 837.7058   | +        | C01190 | Glucosylceramide (d18:1/26:1(17Z)) | C₅₀H₉₅NO₈S | Sphingolipid metabolism |
| 4   | 10.26    | 519.3325   | +        | C04230 | LysoPC(18:2(9Z,12Z))    | C₃₀H₅₀NO₇P | Glycerophospholipid metabolism |
| 5   | 0.92     | 147.0532   | _        | C00025 | L-Glutamic acid         | C₈H₇NO₄ | Arginine and proline metabolism |
| 6   | 0.92     | 192.0270   | _        | C00158 | Citric acid             | C₈H₈O₄  | Citrate cycle (TCA cycle)   |
| 7   | 1.06     | 118.0266   | _        | C00042 | Succinic acid           | C₈H₇O₄  | Citrate cycle (TCA cycle)   |
| 8   | 8.76     | 352.2250   | _        | C00584 | Prostaglandin E2        | C₂₀H₃₂O₃ | Arachidonic acid metabolism |
| 9   | 12.38    | 825.5309   | +        | C00157 | PC (18:4 (6Z, 9Z, 12Z, 15Z)/22:6 (4Z, 7Z, 10Z, 13Z, 16Z, 19Z)) | C₄₈H₇₆NO₈P | Glycerophospholipid metabolism |
| 10  | 12.56    | 677.4996   | +        | C00350 | PE (16:0/15:0)          | C₃₀H₇₂O₆P | Glycerophospholipid metabolism |
| 11  | 13.51    | 290.2245   | +        | C03917 | Dihydrotestosterone     | C₁₉H₃₀O₂ | Steroid hormone biosynthesis |
| 12  | 13.67    | 288.2089   | +        | C00535 | Testosterone             | C₁₉H₃₂O₂ | Steroid hormone biosynthesis |
| 13  | 13.79    | 917.6626   | +        | C06125 | 3-O-Sulfogalactosylceramide (d18:1/26:1(17Z)) | C₃₀H₇₆NO₁₅S | Sphingolipid metabolism |
| 14  | 13.84    | 889.6490   | +        | C01290 | Lactosylceramide (d18:1/18:0) | C₃₀H₇₂O₁₃ | Sphingolipid metabolism |
| 15  | 13.99    | 839.7214   | +        | C01190 | Glucosylceramide (d18:1/26:0) | C₃₀H₇₂O₈ | Sphingolipid metabolism |
| 16  | 14.15    | 392.2927   | +        | C02528 | Chenodeoxycholic acid    | C₂₄H₄₂O₄ | Primary bile acid biosynthesis |
| 17  | 14.20    | 702.5675   | +        | C00550 | SM (d18:0/16:1(9Z))     | C₃₀H₇₂O₄P | Sphingolipid metabolism |
| 18  | 14.21    | 663.6529   | +        | C00195 | Ceramide (d18:1/25:0)   | C₂₄H₄₂O₃N₃ | Sphingolipid metabolism |
Table 1: Continued.

| No. | RT (min) | Exact mass | Ion mode | KEGG | Identified metabolites | Formula | Pathway |
|-----|----------|------------|----------|------|------------------------|---------|---------|
| 19  | 14.26    | 334.2508   | +        | C13713 | Tetrahydrodeoxycorticosterone | C_{21}H_{34}O_{3} | Steroid hormone biosynthesis |
| 20  | 14.32    | 312.2301   | +        | C14831 | 8 (R)-Hydroperoxylinoleic acid | C_{18}H_{32}O_{4} | Linoleic acid metabolism |
| 21  | 14.37    | 290.2245   | +        | C00523 | Androsterone | C_{19}H_{30}O_{2} | Steroid hormone biosynthesis |
| 22  | 14.46    | 481.4495   | +        | C00195 | Ceramide (d18:1/12:0) | C_{20}H_{30}NO_{3} | Sphingolipid metabolism |
| 23  | 14.49    | 912.8146   | +        | C00422 | TG (18:1 (9Z)/18:2 (9Z, 12Z)/20:0) [iso6] | C_{59}H_{108}O_{6} | Glycerophospholipid metabolism |
| 24  | 8.36     | 416.3290   | -        | C01673 | Calcitriol | C_{27}H_{44}O_{3} | Steroid biosynthesis |
| 25  | 8.93     | 430.3083   | -        | C17337 | 7α-Hydroxy-3-oxo-4-cholestenoate | C_{27}H_{44}O_{2} | Primary bile acid biosynthesis |
| 26  | 9.78     | 314.2457   | -        | C14828 | 9, 10-DHOME | C_{18}H_{34}O_{4} | Linoleic acid metabolism |
| 27  | 9.95     | 430.3083   | -        | C05455 | 7α-Hydroxy-cholestene-3-one | C_{27}H_{44}O_{2} | Primary bile acid biosynthesis |

KEGG, Kyoto Encyclopedia of Genes and Genomes; RT, retention time.

(a) Figure 6: Continued.
Although serum biomarkers of hypertension have been researched to varying extents, the ideal biomarkers for the detection or diagnosis of this condition remain elusive. Moreover, few biomarkers have been described that can explain the characteristics of HLYH. Further investigations are warranted to determine the relationship between the

\[
Y = -0.030X28 - 0.037X23 - 0.032X2 + 0.027X12 - 0.025X27 - 0.040X24 \\
\quad + 0.038X11 + 0.020X56 + 0.020X46 + 0.020X48 + 0.020X57 + 0.018X58 + 0.020X55 \\
\quad + 0.018X52 + 0.018X50 + 0.032X15 + 0.017X49 + 0.021X14 - 0.024X26 + 0.024X59 \\
\quad + 0.029X13 + 0.014X47 + 0.016X54 - 0.025X29 - 0.043X6 + 0.014X51 - 0.030X1 \\
\quad 0.024X36 - 0.031X7 + 0.041X17 + 0.017X53 + 0.009X45 + 0.008X44.
\]
internal disturbance and HLYH physical symptoms. The discriminant system constructed in the present study may reflect the important influence of biomarkers of cardiovascular injury on HLYH symptoms. This may then serve to provide a framework on which to determine future screening strategies and interventions.

A network diagram to intuitively reflect the relationship between internal changes and the external performance of the human body is shown in Figure 8.

Two themes emerge in this discriminant system. First, steroid hormones, which are associated with hypertension risk, may constitute intrinsic targets for the headache, impatience, and irritability observed with HLYH. In particular, a study in Ukraine has shown that hypertension is accompanied by a decrease in androgen among aging men and that restoring androgen balance represents an important measure to reduce the risk of cardiovascular disease [27]. In the present study, the levels of testosterone, dihydrotestosterone, and androsterone in the DS group were significantly lower than those in the N group. This result is consistent with the findings of Perusquia et al. [28] that showed the administration of SHR androgen therapy can dilate blood vessels and lower blood pressure. In turn, tetrahydrodeoxycorticosterone (THDOC) functions as an effective regulator of the GABA<sub>A</sub> receptor and can produce sedation, antianxiety, and anticonvulsant effects similar to benzodiazepines and barbiturates [29, 30]. Herein, we hypothesized that the aggressive behavior associated with HLYH rat models may be associated with decreased THDOC, resulting in weakened sedation. In addition, Medratta et al. [31] found that THDOC had an analgesic effect upon intraperitoneal injection in mice, which may be mediated by modulating GABA-ergic and opioidergic mechanisms and voltage-gated calcium channels. Therefore, we speculate a correlation may exist between the decrease in pain threshold and the decrease of THDOC in rat models of HLYH.

Second, disordered lipid metabolism, reflected by the traditional serum biomarkers of hypertension, appears to constitute a key feature of HLYH. For example, phosphatidylcholine (PC) is a precursor of AA. As the

![Network diagram of the discriminant system. Red: potential markers; blue: biochemical indicators; orange: physical signs. Different colored lines indicate different metabolic pathways. The dotted line indicates indirect reaction generation.](image-url)
activity of the protein kinase C (PKC) pathway increases under hypertension, which then activates phospholipase A2, the rate of hydrolysis of PC to AA becomes accelerated and the content of AA increases significantly, whereas the content of PC decreases. Concurrently, large amounts of phosphatidylethanolamine (PE), which is the transient source of AA, are consumed in the inflammatory state. In the present study, the contents of PC and PE in the rat models of HLYH decreased significantly compared with those in the WKY controls. This suggested that the PC and PE conversion to AA was accelerated and promoted inflammation.

In addition, we found that the sphingomyelin (SM) was significantly reduced in the rat models of HLYH. It is presumed that phospholipase is activated under hypertension and that degradation of SM is accelerated. Hydrolysis of SM on a biofilm surface produces ceramide as a second messenger. Lactosylceramide (d18:1/18:0), glucosylceramide (d18:1/26:0), and 3-O-sulfoglucosylceramide (d18:1/26:1(17Z)), identified in the present study, constitute the intermediate metabolites of the ceramide signaling pathway. Considerable evidence supports that ceramide serves as the medium of the stress reaction, which participates in the destruction of the endangium barrier, alteration of vascular permeability, apoptosis induction, and promotion of inflammation [32–34]. In the present study, we found that ceramide and its intermediate metabolites were significantly lower in rat models of HLYH than those in WKY. It was presumed that disorders of the phospholipid metabolic pathway existed in rat models of HLYH. However, a previous study has reported that ceramide was elevated during hypertension, which differs from the results obtained in our study [35]. The causes of the large consumption of ceramide, therefore, require further investigation.

We also found that prostaglandin E (PGE) in AA metabolism and 8 (R)-hydroperoxylinoleic acid and 9,10-DHOME in linoleic acid metabolism were reduced in the DS group, suggesting that the fatty acids were altered in the HLYH rat models. Notably, PGE has a dual effect on blood pressure. PGE receptors 1 and 3 are involved in the accentuation of blood pressure by inhibiting adenylate cyclase and increasing intracellular calcium concentrations. Conversely, PGE receptors 2 and 4 exert antihypertensive effects by activating adenylate cyclase [36]. As fatty acids comprise the primary sources of energy in the body and catecholamine promotes metabolism and enhances energy mobilization, the decrease of these fatty acids may represent sympathetic-adrenal medulla hyperfunction, indicative of an imbalance between energy supply and demand in the rat models of HLYH. However, this study has certain limitations. Specifically, the sample size was small and the results were not validated in vivo. To verify our results, follow-up studies with larger sample sizes and including assessment of the mechanisms of potential biomarkers are warranted.

4. Conclusion

In summary, based on the theory of formula-syndrome relationships, we have extracted the core discriminant factors by integrating multilevel and multidirectional data information of HLYH. From this, we constructed a combined macro-micro personalized syndrome discriminant prediction system. Our results lay the groundwork for research related to the risk loci of HLYH. Our findings also broaden our understanding of the biological pathways involved in HLYH. In turn, these data will provide a basis for identifying the main effect components of the “calming the liver and suppressing Yang” drugs and constructing the interaction system of the “components-target-syndrome”.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

Animal care and experimental procedures used in the current study were approved by the Animal Ethics Committee of Shandong University of Traditional Chinese Medicine (No: SDUTCM20150409001).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

S-GY and ZL analyzed the data and wrote this report; JF conducted the animal handling; LL and LC conducted metabolomics experiments; Q-DM performed data analysis; L-YL revised the manuscript; Y-WQ designed experiments and contributed to the discussion. All the authors read and approved the final manuscript. Guang-yao Shang and Lei Zhang are co-first authors.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant nos. 81473653, 81774173, and 81974555), the Natural Science Foundation of Shandong Province (grant no. ZR2016HB50), the Shandong Province Key Research and Development Plan (No. ZR2018JC1157), and the Taishan Scholar Post Construction Fund (ts201712042).

Supplementary Materials

Figure S1: total ion chromatograms (TICs) of typical samples and Principal component analysis (PCA) score plot of quality control (QC) samples. (a) TICs in positive ion mode; (b) TICs in negative ion mode; (c) PCA score plot of QC samples at positive ion mode; (d) PCA score plot of QC samples at negative ion mode. Table S1: indicator variable identification table. (Supplementary Materials)
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