Effects of Gancao Nourish Yin Decoration on Liver Metabolic Profiles in hTNF- α Transgenic Arthritic Mouse Model

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Abstract

Background

Gancao Nurish Yin (GCNY) decoction has been applied to clinical rheumatoid arthritis (RA) patients and it had shown effectiveness not only in disease activity controlling but also on improving patients' physical status. However, its mechanism of function has not been investigated. Metabolic perturbations have been associated with RA and targeting the metabolic profile is one of the ways to manage the disease. The aim of this study was to observe the effect of GCNY on metabolic changes of human tumor necrosis factor alpha (hTNF- α) transgenic arthritic mouse model.

Methods

hTNF- α transgenic arthritic model mice were divided into control and GCNY groups with 6 mice in each group. After 8 weeks of treatment, liver tissues of mice in both groups were obtained for liquid chromatography-mass spectrometry (LC/MS) analysis. Significantly regulated metabolites by GCNY treatment were first identified, followed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and network analysis.

Results

Of the 126 metabolites detected in the liver, 17 metabolites were with significantly altered between control group mice and mice treated with GCNY. Specifically, thiamine, gamma-L-Glutamyl-L-valine, pantothenic acid, pyridoxal (Vitamin B6), succinic acid, uridine 5'-diphospho-glucuronic acid (UDP-D-Glucuronate), uridine, allantoinic acid, N-Acetyl-D-glucosamine, nicotinamide ribotide and N₂,N₂-Dimethylguanosine were down-regulated by GCNY treatment whereas isobutyrylglycine, N-Acetylcadaverine, N-Carbamoyl-L-aspartic acid, L-Anserine, creatinine and cis-4-Hydroxy-D-proline were up-regulated. Six metabolic pathways were significantly altered including the alanine, aspartate and glutamate metabolism, pyrimidine metabolism, thiamine metabolism, amino sugar and nucleotide sugar metabolism, pantothenate and CoA biosynthesis and citrate cycle (TCA cycle). Integrative metabolic network analysis suggested the possibility of GCNY having both positive and negative effects on RA through the suppression of angiogenesis and promotion of leukocyte extravasation into the synovium, respectively.

Conclusions

GCNY can induce a change in the metabolic profiles of hTNF- α transgenic arthritic mouse model. Further optimization of this decoction may lead to better therapeutic effects on RA patients.

1. Background

Rheumatoid arthritis (RA) being a chronic autoimmune disease affects approximately 0.5-1% of the population. RA is responsible for progressive articular damage, functional loss of joints and comorbidity[1]. Treatment for RA has changed profoundly over the past decades but modern medicine

still lacks in terms of symptom relief and mending joint structural damage[2]. RA in Traditional Chinese Medicine (TCM) is known as Bi - the physical pain or numbness caused by wind, cold and humidity which can cause the stagnation of Qi and blood stasis[3]. Multiple TCM treatment methods including acupuncture, moxibustion and herbal medicine have been applied as complementary management of RA and many effective benefits have been reported[3]. Among the plethora of herbal medicine available, *Gancao Nurish Yin* (GCNY) can be an effective complementary medicine for RA with our clinical work showing dramatic benefits for RA patients. GCNY is a herbal formula developed from The Golden Chamber which is a classical medical book authored by Zhang Zhongjin during the Han dynasty (AD. 150 - 154). The formula was associated to acute or chronic gastrointestinal inflammation and Huhuo disease (similar to Behcet's syndrome, an autoimmune disorder)[6]. Although its constituents such as gancao (glycyrrhizae)[7], ginseng[8], ginger[9] and others were reported to be anti-inflammatory by an abundance of research, little is known about the integrative mechanism of the formula which will be more of significance from a clinical perspective.

Many evidences suggest that cellular metabolic alterations fuel and dictate the inflammatory state of cells. Data from RA patients demonstrated a strong link between the degree of systemic inflammation and the development of insulin resistance[10]. The induction of arthritis in mice resulted in a global inflammatory state that is characterized by defective carbohydrate and lipid metabolism in different tissues[11]. Therapeutic strategies based on tighter control of inflammation provide promising approaches to normalize and prevent metabolic alterations associated with RA[12].

For animal models of RA, human tumor necrosis factor alpha (hTNF- α) mimics human RA clinical presentations including polyarticular swelling, impairment of movement, synovial hyperplasia and cartilage and bone erosion[13]. The systematical and joints manifestations are stable and has been applied in metabolic researches[14]. Thus, in this study, we investigate the effects of GCNY decoction on the metabolic profiles of hTNF- α transgenic arthritic mouse model.

2. Materials And Methods

2.1. Animals and grouping

Human TNF- α transgenic arthritic model mice were bought from Guangdong Experimental Animal Monitoring Institute, China. The transgenic mouse line was produced using a human TNF/ β -globin (TNF-globin) recombinant gene construct which contained a 2.8kb fragment of entire coding region and promoter of the hTNF α gene fused to a 0.77kb fragment of 3' untranslated region (UTR) and polyadenylation site of human β -globin replacing that of the hTNF α gene. The fragment was then microinjected into the pronuclei of FVB/J inbred strain fertilized eggs which were subsequently implanted into the fallopian tube of 8-week-old female pseudo-pregnant ICR mice. Transgenic lineages were established by back-crossing the transgenic founder individuals to the FVB/J inbred strain[13].

The hTNF- α transgenic arthritic model mice at 16 weeks of age (all male, 28-33g) were divided into control and GCNY groups with 6 mice in each group. The GCNY group was treated with GCNY decoction

(provided by Qianzheng Health Technology Development Co., Ltd. Shanghai, China) by free drinking (equivalent to 6.3 times the amount of a 60 kg adult) whereas the control group mice were fed with routine water. The treatment lasted for 8 weeks. Then, the mice were put into anesthesia by intramuscular injection of Zoletil 50 (Virbac, France) and liver tissues were collected into biopsy boxes. Mice were put to death by neck broken.

2.2. Liquid chromatography-mass spectrometry-based metabolomics

25 mg of live tissue sample was weighed into a centrifuge tube and 500 µl of extract solution (acetonitrile:methanol:water = 2:2:1) was added. After 30s of vortex, the samples were homogenized at 35 Hz for 4 min and sonicated for 5 min in ice-water bath. The homogenization and sonication cycle was repeated 3 times. Then, the samples were incubated at -40 °C. 400 µl of supernatant was transferred to a fresh tube and dried in a vacuum concentrator at 37 °C. Next, the dried samples were reconstituted in 100 µl of 50% acetonitrile by sonication on ice for 10 min. The constitution was then centrifuged at 12000 rpm for 15 min at 4 °C and 75 µl of the supernatant was transferred to a fresh glass vial for liquid chromatography-mass spectrometry (LC/MS) analysis. The quality control (QC) sample was prepared by mixing an equal volume of aliquots of the supernatants from all of the samples.

The ultra-high-performance liquid chromatography (UHPLC) separation was carried out using an Agilent 1290 Infinity series UHPLC System (Agilent Technologies) equipped with a UPLC BEH Amide column (2.1*100 mm, 1.7 µm, Waters). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 nmol/L ammonia hydroxide in water (pH = 9.75) (solution A) and acetonitrile (solution B). The elution gradient was set as follows: 0 - 1.0 min, 95% solution B; 1.0 - 14.0 min, 95% - 65% solution B; 14.0 - 16.0 min, 65% - 40% solution B; 16.0 - 18.0 min, 40% solution B; 18.0 - 18.1 min, 40% - 95% solution B; 18.1 - 23.0 min, 95% solution B. The flow rate was 0.5 mL/min whereas the column temperature was 25 °C. The auto-sampler temperature was 4 °C and the injection volume was 2 µL (positive) or 2 µL (negative). An Agilent 6495 triple quadrupole mass spectrometer (Agilent Technologies) was apply for the assay development and the parameters were as follows: capillary voltage = +3000/-2500 V, N₂ temperature = 170 °C, N₂ flow rate = 16 L/min, sheath gas temperature = 350 °C, sheath gas flow rate = 12 L/min, nebulizer pressure = 40 psi, fragmentor voltage = 380 V.

2.3. Preprocessing of raw data

Missing data in the raw data was first imputed using the half minimum method[15]. Next, individual peaks with > 50% null value in the peak area data and relative standard deviation (RSD) of > 20% were removed[16]. Lastly, the normalization of the data was performed with respect to the total ion current.

2.4. Statistical analysis

Statistical analysis was performed using SIMCA v15.0.2 (Sartorius Stedium Data Analytics AB, Umea, Sweden). First, multivariable analyses using principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) was applied to the preprocessed data.

The OPLS-DA model was verified using a 7-fold cross-validation technique and a permutation test (200 iterations) was performed to evaluate the fitting of the model[16]. The variable importance in the projection (VIP) score was calculated for each metabolite. Next, univariable *t*-test was used to compare the metabolite levels across the two groups. Hierarchical clustering was performed by calculating the Euclidean distance matrix and clustered using the complete linkage method. Lastly, Pearson's correlation coefficient *r* was calculated for the difference of the metabolite levels between the two groups. A *P* value < 0.05 was considered statistically significant.

2.5. KEGG pathway and network analysis

First, significantly regulated metabolites were annotated using various databases that include the Human Metabolome Database (HMDB), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Pubchem. After annotations, all the pathways that contain the significantly regulated metabolites were identified using the Mus musculus KEGG pathway database. In order to reveal pathways that were highly correlated to GCNY treatment, enrichment analysis and topology analysis were carried out to find the *P* value and impact value, respectively. A pathway's impact value was evaluated as the total importance measures of the matched metabolites divided by the total importance measures of all metabolites in each pathway[18].

3. Results

3.1. Identification of Significantly Regulated Metabolites

Preprocessing of the raw data revealed a total of 126 metabolites peaks as shown in Table S1. Unsupervised PCA showed the clustering and almost total separation of the treatment and control group with no outlier present (Figure 1A). Figure 1B shows the supervised fitted model of OPLS-DA and it can be seen that the separation of the two groups was much more distinct with an R^2X , R^2Y and Q^2 of 0.332, 0.977 and 0.590, respectively, indicating the model has good predictability[18]. After 200 permutations, the R^2Y and Q^2 values were generally lower than that of the original model and the Q^2 intercept value was < 0 at (0, -0.69) (Figure 1C). These showed that there was no overfitting for the OPLS-DA model[20, 21]. Based on the criteria of *t*-test *P* value < 0.05 and VIP > 1[20, 21], 6 significantly up-regulated and 11 significantly down-regulated metabolites were identified (Figure 1D and Table 1). Hierarchical clustering of the 17 significantly regulated metabolites further validated the specific up- or downregulatory effects of GCNY in the treatment group as compared to the control group (Figure 2A).

Figure 1: Multivariate analyses of metabolite signatures and identification of significantly regulated metabolites. (A) PCA score scatter plot of metabolite profiles in control mice and mice treated with GCNY. Each point represents a metabolite profile of a biological replicate. All points fall within the Hotelling's T² ellipse (95% confidence interval). PC[1] and PC[2]: principal component 1 and 2. (B) OPLS-DA score scatter plot of metabolite profiles in control mice and mice treated with GCNY. R^2X , R^2Y and Q^2 were 0.332, 0.977 and 0.590, respectively. Each point represents a metabolite profile of a biological replicate.

All points fall within the Hotelling's T2 ellipse (95% confidence interval). t[1]P: predicted principal component score of the first principal component; t[2]O: orthogonal principal component score. **(C)** Validation of the OPLS-DA model using permutation test of 200 random permutations. Intercepts of R²Y and Q² were (0, 0.9) and (0, -0.69), respectively. **(D)** Volcano plot identifying significantly regulated metabolites associated to GCNY treatment. A threshold of $P = 0.05$ ($-\log_{10}P\text{-value} = 1.3$) was used.

Table 1: Significantly regulated metabolites associated to GCNY treatment.

Metabolite	Retention time (s) ^a	HMDB	Direction of regulation
Thiamine	625.69	HMDB00235	Down
gamma-L-Glutamyl-L-valine	719.42	HMDB11172	Down
Pantothenic acid	503.88	HMDB00210	Down
Pyridoxal (Vitamin B6)	175.65	HMDB01545	Down
Succinic acid	742.40	HMDB00254	Down
Uridine 5'-diphospho-glucuronic acid (UDP-D-Glucuronate)	913.63	HMDB00935	Down
Uridine	282.02	HMDB00296	Down
Isobutyrylglycine	394.00	HMDB00730	Up
Allantoic acid	672.44	HMDB01209	Down
N-Acetylcadaverine	541.66	HMDB02284	Up
N-Acetyl-D-glucosamine	465.88	HMDB00215	Down
N-Carbamoyl-L-aspartic acid	805.11	HMDB00828	Up
Nicotinamide ribotide	884.74	HMDB00229	Down
N2,N2-Dimethylguanosine	338.43	HMDB04824	Down
L-Anserine	782.04	HMDB00194	Up
Creatinine	294.17	HMDB00562	Up
cis-4-Hydroxy-D-proline	665.11	HMDB60460	Up

^a: median retention time; HMDB: Human Metabolome Database

3.2. Correlation Analysis of the Significantly Regulated Metabolites

Figure 2B shows the correlation analysis results of the 17 significant metabolites. A positive correlation was observed between metabolites regulated in the same direction whereas an inverse correlation was

always observed between metabolites regulated in opposite directions. Among the up-regulated metabolites, creatinine was positively correlated to both N-carbamoyl-L-aspartic acid and L-anserine ($r = 0.631$, $P = 0.028$ and $r = 0.810$, $P = 0.001$, respectively) whereas L-anserine was positively correlated to N-carbamoyl-L-aspartic acid ($r = 0.720$, $P = 0.008$). On the other hand, notable very strong positive correlation ($r > 0.8$) among the down-regulated metabolites include gamma-L-Glutamyl-L-valine and UDP-D-Glucuronate ($r = 0.859$, $P < 0.001$), pantothenic acid and uridine ($r = 0.835$, $P = 0.001$), pantothenic acid and UDP-D-Glucuronate ($r = 0.837$, $P = 0.001$), pantothenic acid and gamma-L-glutamyl-L-valine ($r = 0.969$, $P < 0.001$), succinic acid and nicotinamide ribotide ($r = 0.835$, $P = 0.001$) and L-anserine and creatinine ($r = 0.810$, $P = 0.040$). There was only one very strong negative correlation ($r < -0.8$) observed for L-anserine and thiamine ($r = -0.837$, $P = 0.001$) (Figure 2B).

Figure 2: Analysis of significantly regulated metabolites. (A) Hierarchical clustering analysis for control mice and GCNY-treated mice. **(B)** Correlation analysis for control mice and GCNY-treated mice. The upper triangle denotes the Pearson's r whereas the lower triangle denotes the respective P values. Significant Pearson's r and P values are bolded.

3.3. Metabolic Pathway and Integrated Network Analysis

Utilizing HMDB, Pubchem and KEGG, all the pathways involving the significantly regulated metabolites were identified and are shown in Figure S1. Among these pathways, 11 metabolic pathways were associated with treatment using GCNY (Figure 3 and Table 2) with a high correlation. A combined consideration of the P and impact value revealed that 6 pathways were significantly altered: 1) alanine, aspartate and glutamate metabolism, 2) pyrimidine metabolism, 3) thiamine metabolism, 4) amino sugar and nucleotide sugar metabolism, 5) pantothenate and CoA biosynthesis and 6) citrate cycle (TCA cycle). Figure 4 shows an integrated metabolic network that connects 4 of the 6 significantly altered pathways (pantothenate and CoA biosynthesis, pyrimidine metabolism, alanine, aspartate and glutamate metabolism and TCA cycle). Three metabolites: pantothenate, succinate and uridine were found to be down-regulated (fold change of 0.40, 0.53 and 0.64, respectively) following GCNY treatment whereas only N-carbamoyl-L-aspartate, which was involved in both the alanine, aspartate and glutamate metabolism and pyrimidine metabolism pathways, was found to be significantly up-regulated with a fold change of 2.72.

Table 2: Metabolic pathways associated to GCNY treatment. Significantly altered pathways are bolded.

Pathway	Total ^a	Hits ^b	<i>P</i> value	Holm <i>P</i> ^c	Impact value
Alanine, aspartate and glutamate metabolism	24	2	0.011	0.934	0.000
Pyrimidine metabolism	41	2	0.032	1.000	0.035
Thiamine metabolism	7	1	0.048	1.000	0.400
Nicotinate and nicotinamide metabolism	13	1	0.088	1.000	0.000
Pantothenate and CoA biosynthesis	15	1	0.101	1.000	0.020
beta-Alanine metabolism	17	1	0.114	1.000	0.000
Propanoate metabolism	20	1	0.133	1.000	0.000
Citrate cycle (TCA cycle)	20	1	0.133	1.000	0.026
Butanoate metabolism	22	1	0.145	1.000	0.000
Amino sugar and nucleotide sugar metabolism	37	1	0.233	1.000	0.069
Purine metabolism	68	1	0.389	1.000	0.000

^a: Total metabolites in the pathway; ^b: Number of significantly regulated metabolites in the pathway; ^c: Holm-Bonferroni corrected *P* values for multiple comparisons

Figure 3: The metabolome view map of significantly altered pathways related to treatment with GCNY.

The x-axis represents the impact value in topology analysis whereas the y-axis represents the *P* value in enrichment analysis. Significantly altered pathways are labeled.

4. Discussion

The relationship between metabolism and inflammation has been profoundly investigated in recent decades. Although it is not yet known whether metabolic changes are a consequence of disease or whether primary changes to cellular metabolism might underlie or contribute to the pathogenesis of early-stage disease, changes to the metabolic profiles were observed in various diseases relating to inflammation and this includes RA as well[23]. Metabolic perturbations have been associated with RA whereby the hallmark swelling and heat observed in the joints of RA patients is considered to be a consequence of metabolic alterations. Daily whole-body resting energy expenditure is 8% higher in RA patients as compared to healthy individuals and this suggests that these metabolic changes in RA are significant and systemic in nature[23]. Furthermore, the catabolic condition “cachexia” occurs in RA patients with muscle atrophy and increase in body fat is associated with systemically elevated levels of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 β (IL-1 β), leukocyte inhibitory factor, interferon- γ (IFN- γ), and IL-6[23]. Regulation on metabolism, for example, by inhibiting

glucose metabolism exerted anti-inflammatory effects on immune cells[25] and therapeutic targets for RA from the metabolic aspect has been proposed[23].

In this study, the possible therapeutic mechanism of GCNY on RA was investigated. One of the

Figure 4:Metabolic alterations induced by GCNY treatment represented in an integrated metabolic network. Significantly regulated metabolites are bolded and the direction and magnitude of the fold change are denoted as arrows and value. Red and upwards arrows represent upregulated metabolites in the GCNY treatment group whereas blue and downwards arrows represent downregulated metabolites in the GCNY treatment group.

metabolic pathways GCNY altered was the TCA cycle via the down-regulation of succinate. Succinic acid is metabolized by body cells and has a role in the tricarboxylic acid cycle as a cycle media component. It also functions as an inflammatory signaling molecule which is elevated in animals subjected to metabolic and inflammatory diseases by inducing IL-1 β and HIF-1 α [26]. In the context of RA, the induction of HIF-1 α by succinic acid in turns lead to an induction of VEGF that will promote synovium angiogenesis[27]. It was also observed that the intra-articular microvascular blood flow has a high correlation with clinical synovitis in patients with RA[28], further suggesting that angiogenesis plays a significant role in the disease progression of RA. Thus, the reduction of succinic acid and angiogenesis by GNCY in RA patients might be one possible mechanism of action.

Surprisingly, uridine in the pyridine metabolism pathway was found to be downregulated in the GCNY treatment group. One of the hallmarks of RA - hyperplasia of the synovial lining layer, is caused by the excessive recruitment and accumulation of leukocytes in the synovium[29, 30]. The recruited leukocytes in turn will release pro-inflammatory cytokines which activate and stimulate the proliferation of resident synoviocytes[31]. Uridine was reported to have anti-inflammatory effects in an animal model of lung inflammation[32] as well as RA by suppressing extravasation of neutrophil, macrophage and T cells into the synovium and inhibited synovial expression of intercellular adhesion molecule 1 (ICAM-1), CD-18 and cytokine production[31]. The results from this study suggest that GCNY has a component that is detrimental to RA but this negative effect is less in magnitude than the positive effect due to the reduction of angiogenesis. Further research can be done to identify the component that leads to the downregulation of uridine and the efficacy of GCNY in treating RA with and without that particular component can then be compared. Besides uridine, N-carbamoyl-L-aspartate was found to be upregulated in this pathway as well. Since there was no significant correlation between N-carbamoyl-L-aspartate and uridine (Figure 2B), it suggests that the downregulation of uridine was independent of the change in N-carbamoyl-L-aspartate level.

Another metabolic pathway that was found to be significantly altered was the pantothenate and CoA biosynthesis pathway in which pantothenate was downregulated after treating with GNCY. Early research has shown that the level of pantothenate in RA patients was lower as compared to healthy people and the decrement of pantothenate level correlated with the severity of RA[33]. There exists weak evidence for the efficacy of pantothenate in treating RA whereby a 2 g daily intake of calcium pantothenate reduced

morning stiffness, pain and disability of RA patients[34]. However, the small sample size used in this study nor any further large scale, prospective study that has been performed still leave the therapeutic link between pantothenate and RA a debatable manner. The results of this study do not seem to support this claim.

Some of the other significant regulated metabolites are reported to be related to anti-inflammation. For example, creatinine and anserine, both upregulated in the GCNY treatment group, are attributed to anti-inflammatory actions[35] with the latter being able to alleviate thioacetamide-induced fibrosis[36]. On the other hand, some anti-inflammatory agents were found to be reduced in the liver after GCNY treatment. One of these downregulated metabolites was thiamine. Thiamine deficiency can result in the impairment of oxidative metabolism, excitotoxicity and inflammation in which the consequences include a series of events that set the stage for cerebral vulnerability[37]. Besides that, low circulation of pyridoxal was reported to be associated with the elevation of the inflammation marker C-reactive protein[38] and being a risk factor for inflammatory-related diseases including thrombosis and inflammatory bowel disease[39]. Gamma-L-Glutamyl-L-valine exhibits antiseptic activity by reducing the expression of pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β in the plasma and small intestine as well as inhibiting the phosphorylation of the signaling proteins c-Jun N-terminal kinases (JNK) and nuclear factor- κ B inhibitory factor γ (IkB γ) in a mouse model of LPS-induced sepsis[40]. These metabolites are not currently not associated with the pathogenesis or progression of RA but this study prompts further research to be done on investigating the relationship between these metabolites and RA to elucidate the underlying complex network mechanism.

5. Conclusions

In conclusion, this study validated that GCNY treatment could change the metabolic profiles in the liver of hTNF- α transgenic arthritic mouse model. Alteration to the TCA cycle and pyrimidine metabolism pathway via the regulation of succinic acid and uridine, respectively, suggested GCNY can affect the angiogenesis and leukocyte extravasation processes in RA. Further studies should be carried out to illustrate the clear action mechanisms and explore other possible metabolic targets of RA.

Abbreviations

GCNY : *Gancao Nurish Yin*

HMDB : Human Metabolome Database

hTNF- α ; human tumor necrosis factor alpha

ICAM-1 : intercellular adhesion molecule 1

IFN- γ : interferon- γ

IL-1 β : interleukin-1 β

I κ B α : nuclear factor- κ B inhibitory factor α

JNK : c-Jun N-terminal kinases

KEGG : Kyoto Encyclopedia of Genes and Genomes

LC/MS : liquid chromatography-mass spectrometry

OPLS-DA : orthogonal projections to latent structures - discriminant analysis

PCA : principal component analysis

QC : quality control

RA : rheumatoid arthritis

RSD : relative standard deviation

TCM : Traditional Chinese Medicine

TNF : tumor necrosis factor

UDP-D-Glucuronate : uridine 5'-diphospho-glucuronic acid

UHPLC : ultra-high-performance liquid chromatography

UTR : untranslated region

VIP : variable importance in the projection

Declarations

• Ethics approval and consent to participate

The study protocol of the animal experiments were approved by the Ethics Committee of Integrated Traditional Chinese and Western Medicine hospital, Southern Medical University, China (approval No. NFZXYEC-2017-002).

• Consent to publish

Not applicable.

• Availability of data and materials

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

- **Competing interests**

The authors declare no competing interests.

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- **Authors' Contributions**

RBP and CHX conceptualized the study, KSC and YC reviewed the research proposal and drafted the manuscript. YJC, XWZ, and WTZ conducted the experiment. All authors have read and approved the final manuscript.

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Not applicable.

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Figures

Figure 1

Multivariate analyses of metabolite signatures and identification of significantly regulated metabolites. (A) PCA score scatter plot of metabolite profiles in control mice and mice treated with GCNY. Each point represents a metabolite profile of a biological replicate. All points fall within the Hotelling's T2 ellipse (95% confidence interval). PC[1] and PC[2]: principal component 1 and 2. (B) OPLS-DA score scatter plot of metabolite profiles in control mice and mice treated with GCNY. R2X, R2Y and Q2 were 0.332, 0.977 and 0.590, respectively. Each point represents a metabolite profile of a biological replicate. All points fall within the Hotelling's T2 ellipse (95% confidence interval). t[1]P: predicted principal component score of the first principal component; t[2]O: orthogonal principal component score. (C) Validation of the OPLS-DA model using permutation test of 200 random permutations. Intercepts of R2Y and Q2 were (0, 0.9) and (0,

-0.69), respectively. (D) Volcano plot identifying significantly regulated metabolites associated to GCNY treatment. A threshold of $P = 0.05$ ($-\log_{10} P\text{-value} = 1.3$) was used.

Figure 2

Analysis of significantly regulated metabolites. (A) Hierarchical clustering analysis for control mice and GCNY-treated mice. (B) Correlation analysis for control mice and GCNY-treated mice. The upper triangle

denotes the Pearson's *r* whereas the lower triangle denotes the respective *P* values. Significant Pearson's *r* and *P* values are bolded.

Figure 3

The metabolome view map of significantly altered pathways related to treatment with GCNY. The x-axis represents the impact value in topology analysis whereas the y-axis represents the *P* value in enrichment analysis. Significantly altered pathways are labeled.

Figure 4

Metabolic alterations induced by GCNY treatment represented in an integrated metabolic network. Significantly regulated metabolites are bolded and the direction and magnitude of the fold change are denoted as arrows and value. Red and upwards arrows represent upregulated metabolites in the GCNY treatment group whereas blue and downwards arrows represent downregulated metabolites in the GCNY treatment group.