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# Exploration of The Mechanism of Qianliexin Capsule Against Chronic Nonbacterial Prostatitis Using Metabolomicsv

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**[&]Author Contributions:**

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## 1. Abstract

**1.1. Objective:** Qianliexin Capsule (QLX), a traditional Chinese medicine, has been widely used to treat Chronic Nonbacterial Prostatitis (CNP) in China. Here, metabolic profiling was utilized to clarify the bio-targets of QLX in CNP treatment.

**1.2. Methods:** An estradiol-induced prostatitis rat model was used to mimic the hormonal imbalance-induced CNP. The urine and serum were collected to analyze metabolic profiles by ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS).

**1.3. Results:** An anti-inflammatory role for QLX was seen in the estradiol-induced prostatitis rats, and the Lower Urinary Tract Symptoms (LUTS) were relieved by QLX. The unique metabolic profiling in CNP was significantly changed by addition of QLX. Thirty-nine metabolites identified in serum and urine, that are involved in multiply pathways, were significantly reversed by QLX in the CNP rats. Among them, glycerophospholipid metabolism, sphingolipid metabolism and fructose and mannose metabolic pathways were the most significantly influenced by QLX and were closely associated with the anti-inflammatory role of QLX in CNP. Conclusion: The changed metabolic profile after QLX addition may be associated with the pharmacologic function of QLX in CNP.

## 2. Introduction

Prostatitis is defined as painful inflammation of the prostate gland. Around 8.4% Chinese men and 8.2% American men have reported prostatitis-like symptoms [1, 2]. Based on the National Institutes of health classification there are four main types of prostatitis: acute bacterial prostatitis, chronic bacterial prostatitis, chronic non-bacterial prostatitis/chronic pelvic pain syndrome (CNP/CPPS), and asymptomatic inflammatory prostatitis [3]. CNP is the most common type of prostatitis, accounting for 90-95% of the clinical incidence, and has become an important clinical problem [2]. CNP is associated with Lower Urinary Tract Symptoms (LUTS) including increased urinary frequency, urgency incontinence, nocturia and chronic pelvic pain syndrome that may become serious [4]. Currently, antimicrobial analgesics, anti-inflammatory drugs, muscle

relaxants, thermotherapy, surgery and biofeedback physiotherapy have been proposed and studied for CNP treatment [5]. Additionally, herbal therapies, especially traditional Chinese medicine (TCM), have become a new intriguing treatment for CNP.

Qianlixin Capsule (QLX), a TCM, was originally recorded in a traditional Chinese medicine work "Empirical Prescription" for treating chronic prostatitis during the Qing Dynasty. QLX contains 14 Chinese herbs including Commiphora myrrha (Nees) Engl., Paeonia lactiflora Pall., etc. (Table 1). Preclinical and clinical studies have indicated that QLX improved the quality of life, improved LUTS syndrome [6] and exerted strong anti-inflammatory and anti-inflammasome activity in hormonal imbalance and carrageenan-induced CNP models. QLX significantly relieved LUTS in both hormonal imbalance and carrageenan-induced CNP- model rats by reducing prostate enlargement, epithelial thickness, pain-response time, urine volume and bleeding time, and by improving prostatic blood flow. The proinflammatory cytokines interleukin (IL)-1β, the proinflammatory transcription factor nuclear factor kappa- light-chain-enhancer of activated B cells (NF-κB), and inflammasome components were reduced by QLX addition in CNP and BPH tissues (unpublished data, see reference paper). The pharmacological mechanism of QLX in CNP including the change in signaling pathways is still unclear, therefore the metabolomics pattern was investigated in an effort to understand the target of QLX action.

Metabolomics, defined as the comprehensive analysis of metabolites in a biological specimen, has been increasingly applied to the discovery of biomarkers and identification of signaling pathways [7]. Metabolome studies were able to qualitatively and quantitatively analyze changes in small molecules (< 1,000 Da) in organisms in response to a certain stimulus at large scale [8]. Using a "top-down" strategy, metabolomics were able to reflect the functional and metabolic changes of complete organisms caused by interventions in a holistic context [9]. The metabolome studies of TCM has been reported. The antidepressant function of Danzhi Xiaoyao Powder (DZXY), a TCM, was associated with 38 differential metabolites identified in the plasma by using UPLC-MS/MS metabonomics platform [10]. Rhizoma Drynariae (RD), a Chinese herb, was used to protect osteoporosis. RD-induced metabolites changes were enriched in linoleic acid metabolic, glycerophospholipid metabolism and arachidonic acid metabolism pathways which may reduce the reduce the risk of osteoporosis [11]. Therefore, metabolomics studies have been used to understand the mechanism of TCM, since TCM acts on multiple targets and multiple pathways.

Here, a metabolites study was performed in a QLX-treated CNP animal model to understand the pharmacological mechanism of QLX on CNP. Results from this study may be helpful in guiding rational clinical drug use.

**Table 1:** The detailed parameters of metabolomics data analysis

| Parameters | Urine samples in positive mode | Urine samples in negative mode | Serum samples in positive mode |
|---|---|---|---|
| Initial retention time (min) | 2 | 1 | 1 |
| Final retention time (min) | 14 | 14 | 22 |
| Low mass | 50 | 50 | 50 |
| High mass | 1500 | 1500 | 1500 |
| XIC window (Da) | 0.1 | 0.1 | 0.1 |
| Mass window | 0.5 | 0.5 | 0.5 |
| Retention time window | 0.2 | 0.2 | 0.2 |
| Noise elimination level | 10 | 10 | 10 |
| Peak width at 5% height (seconds) | 1 | 1 | 1 |
| Marker intensity threshold (counts) | 10000 | 10000 | 30000 |

## 3. Materials and Methods

### 3.1. Medicines and Chemical Reagents

The components of QLX capsules including the amounts of individual herbs are list in Table 1. QLX capsules (batch number: 1904009, 1903002, 1903004) were manufactured based on The Pharmacopedia of People's Republic of China 2020 (p1387-1388) and the Chinese patent (CN1742903B), and provided by the manufacturer, Shandong Hongjitang Pharmaceutical Group Co. Ltd.

### 3.2. Animals and Experiment Program

Twelve-week-old SPF healthy male SD rats (weighing180-220 g) were provided by Liaoning Changsheng Biotechnology Co., Ltd (Shenyang, China; Certificate of Conformity: SCXK (Liao) 2015-0001). Experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the Shenyang Pharmaceutical University (Permit No. 211002300042526).

Three groups including the control group, model group and QLX group were created. Rats in control groups received a sham operation and solvent injection. Rats in the model group and QLX group received 0.25 mg/kg of 17 β-estradiol subcutaneously for 17 days, beginning three days after the castration operation. Then, 4 g/kg QLX or water was given orally 3 times a day for 45 days.

### 3.3. Sample Pretreatment

The urine samples (100 μL) were further diluted by adding 100 μL of water, and the supernatant was collected after 10 min centrifuge

at 13000 rpm and the supernatant was further pass to 0.22 μM filter. Methanol (100 μL) was added to 50 μL plasma to precipitate protein for 10 min on ice, and the supernatant was collected for sample analysis after after 10 min centrifuge at 13000 rpm.

### 3.4. UPLC-MS Conditions

#### 3.4.1. Conditions for Urine Analysis

The UPLC-MS analysis was performed using a Waters Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm) with a flow rate of 0.2 mL min-1 maintained at 40 °C. Mobile phases were water with 0.1% formic acid (A) and acetonitrile (B). The gradient was 0-20 min, 2-95% B; 20-22 min, 95-45% B; 22-23 min, 45-2% B; 23-25 min, 2% B. The sample injection volume was 10 μL. The MS was performed in in Waters Quattro microTM API Mass Spectrometer in the conditions as follows: electrospray ion source; source temperature: 120 °C; desolvation gas temperature: 400 °C; desolvation gas flow: 450 L h-1; extract cone voltage: 25 V; capillary voltage: 3 kV; full scan range: 50-1500 Da. The equipment was controlled by Masslynx 4.1 (Waters® Corporation) software.

#### 3.4.2. Conditions for Blood Samples

Mobile phases were water with 0.1% formic acid (A) and acetonitrile (B). The gradient was 0-10 min, 2-30% B; 10-12 min, 30-95% B; 12-13 min, 95% B; 13-16min, 2% B. The sample injection volume was 10 μL. The MS was performed the same as above.

### 3.5. Metabolomics Data Analysis

Masslynx 4.1 was used to analyze the raw data obtained from the UPLC-MS. Peak matching, peak alignment and other operations was performed in Markerlynx XS (Waters Corporation). Multivariate statistical analysis, including principal component analysis (PCA) and partial least squares discriminant analysis (PLSDA) was performed using SIMCA (Umetrics version 14.0, Umea, Sweden). The variable importance in the projection (VIP) was used to find potential biomarkers. Displacement inspection was used to verify the model fitting. The variables with VIP values > 1.5 were selected as potential biomarkers for the pathway analysis. The metabolic pathways were analyzed using the HMDB database (https://hmdb.ca/) by Metaboanalyst (www.metaboanalyst.ca). The detailed parameters were listed in (Table 1).

### 3.6. Statistical Analysis

SPSS software was used for one-way ANOVA analysis and back testing. The LSD test was used for homogeneity of variance and the Dounnet T3 test was used for heterogeneity of variance. $P < 0.05$ was considered to be a statistically significant difference.

## 4. Results

### 4.1. QLX Relieves The LUTS Syndrome in Estradiol-Induced CNP

As hormonal imbalance is one of the factors causing CNP, estradiol was used to induce prostatitis in rats [12]. Hematoxylin and eosin stain (H&E) staining indicated that the estradiol- induced proinflammatory cell infiltration was increased, and QLX addition clearly reduced the proinflammatory cell number in the prostate (Figure 1A). Consistently, the proinflammatory cytokine IL-1β in prostate tissues was significantly reduced in estradiol-treated rats after QLX treatment (Figure 1C). The prostate index indicated that prostate enlargement was significantly reduced by estradiol addition, and QLX addition significantly reversed the reduced prostate index (Figure 1B). Consistently, H&E staining indicated that the thickening of the epithelium was increased by QLX addition compared to estradiol-induced CNP models. Estradiol-induced narrowed prostate gland chambers, irregularly arranged epithelial cells, and damaged epithelial cells were reduced by QLX addition. A large amount cell apoptosis were observed by tunel staining in estradiol-induced CNP model, and QLX addition reduced the cell apoptosis (Figure 1A). This suggested that QLX exerted anti-inflammatory and anti-apoptotic effects in CNP.

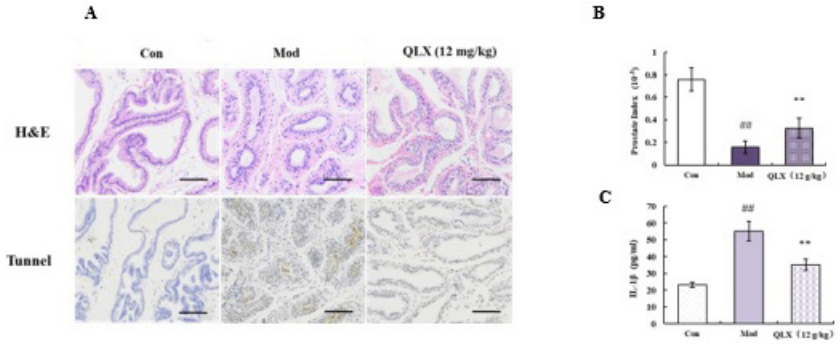


**Figure 1:** Pathological changes of the prostate and biochemical analysis before and after QLX treatment. (A) H&E staining and TUNEL staining were performed in prostate tissues. (B) The prostate index was calculated. (C) IL-1β level in prostate tissues was measured by ELISA. The rats were treated with sham operation (con), estradiol (mod) or QLX (QLX) as described in the Methods.

The chronic pelvic pain syndrome analyzed by pain response time was significantly increased about 2 fold after QLX addition in estradiol-treated mouse, and the urine volume recovered to the normal level after QLX addition in estradiol-treated mouse (Table 2). This suggested that QLX relieved the LUTS syndrome in estradiol-induced CNP. Moreover, urine metabolites including urea nitrogen, creatinine and uric acid were significantly increased in QLX-treated rats (Table 2). The reduction in key biomarkers of renal dysfunction in the urine indicated that QLX had prostate productive function.

**Table 2:** Improve effect of QLX on prostate LUTS and kidney function

| Group | Pain response time (s) | Urine volume (ml) | BUN (mg/dL) | Cr (μmol/L) | UA (μmol/L) |
|---|---|---|---|---|---|
| Con | 23.87 ± 2.52 | 16.88 ± 5.84 | 17.92 ± 1.41 | 40.31 ± 3.53 | 84.33 ± 21.40 |
| Mod | 14.08 ± 3.01## | 6.50 ± 2.52## | 23.04 ± 2.87## | 45.27 ± 3.77# | 175.67 ± 47.33## |
| QLX (12 mg/kg) | 29.47 ± 4.28** | 20.00 ± 4.43** | 19.74 ± 2.48* | 31.42 ± 5.60** | 109.25± 54.78** |

Values are mean ± S.D. of data from three separate experiments , n=8. $^{\#\#}P < 0.01$ when compared to Con; $^{**}P < 0.05$ when compared to Mod. Con, control group; Mod, TP-induced CNP group; QLX, QLX-treated CNP group.

### 4.2. Metabolic Profiles Were Analyzed by UPLC-MS

UPLC-MS was used to analyze the serum and urine samples from the control, estradiol- treated and QLX-treated groups to understand the changes of the metabolic profile after QLX addition.

Principal Components Analysis (PCA) was used to assess the stability of the instrument. Meanwhile, the Quality Control sample (QC) was prepared by mixing equal amounts of samples from the three groups. The obvious clustering of QC samples demonstrated good stability and reproducibility of the analytical method in the urine and serum samples in positive mode and urine samples in negative mode (Figure 2A-C). Partial least squares discriminant analysis (PLS- DA) was used to analyze modeling of three groups. In the score plot (Figure 2D-F), the control group (C) and the estradiol-treated group (M) are clearly distinguished, showing that the modeling was successful. The QLX-treated group (G) was concentrated and distinguished obviously from the estradiol-treated group (M), demonstrating that the drug could change the metabolic profiles in the estradiol-treated group and tended to be normal. The PLS-DA model conforms to the standard without over fitting in the permutation test (Figure 2G-I).

The volcano plot was used to show the metabolic trends of different groups (Figure 3A-D). In the diagram, green represents up-regulated metabolite content, red represents down- regulated metabolite content ($p < 0.05$ and fold change > 1.5). These ion peaks with fragmentation information were filtered and searched in the online database HMDB (www.hmdb.com) according to the high resolution m/z ions.

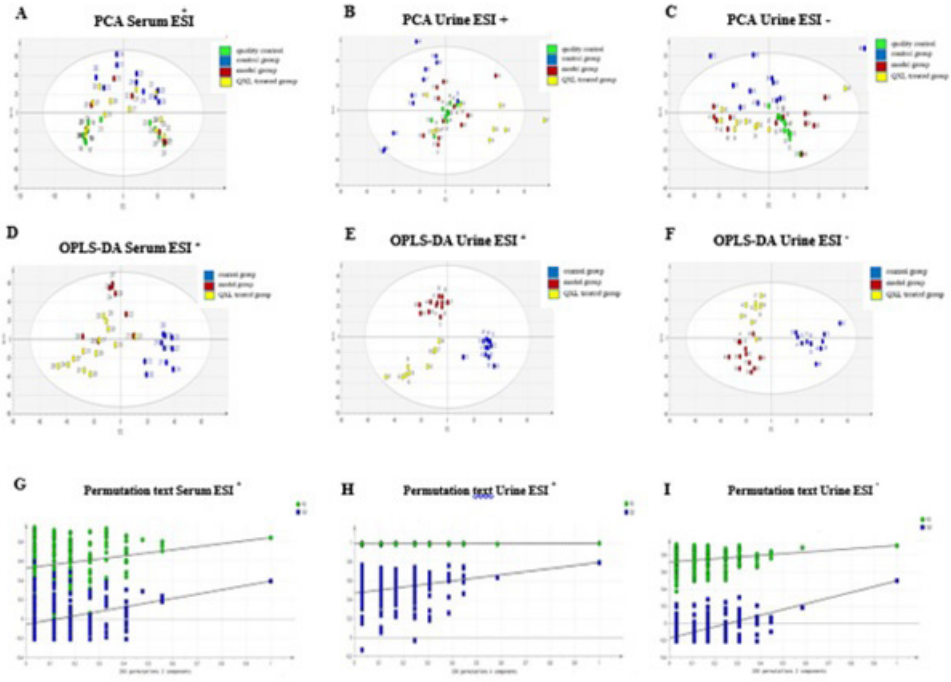


**Figure 2:** PCA score plot of indicated serum (A) and urine (B) metabolite changes in the positive model, and urine metabolite changes in the negative model (C). OPLS- DA analysis and permutation test were used to analyze the serum (D and G) and urine (E and H) metabolite changes in the positive model, and urine metabolite changes in the negative model (F and I). Serum and urine were collected from the rats that had been treated with sham operation (control group), estradiol (model group) or QLX (QLX treated group) as described in the Methods. Quality control was performed by mixing samples from the 3 groups at equal amounts.

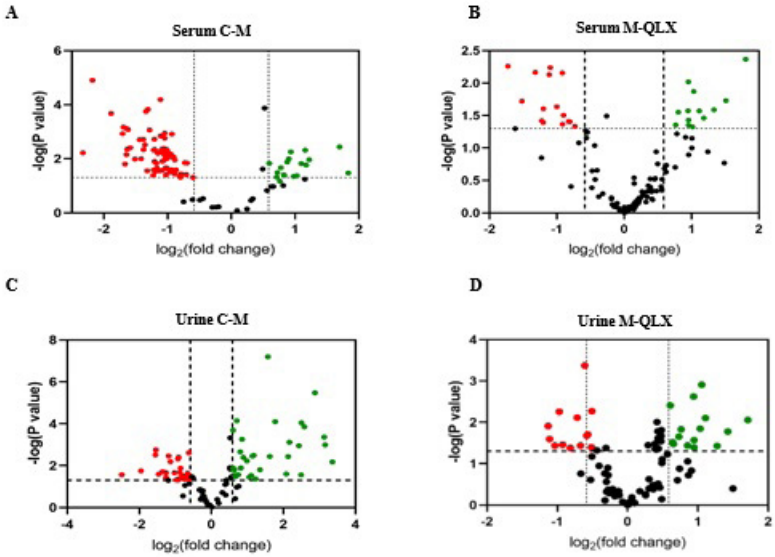


**Figure 3:** The corresponding S-plot of OPLS-DA. Serum samples from the control group vs. model group in the positive model (A), urine samples from the control group vs. model group (C). Serum samples from the model group vs. QLX group (B), urine samples from the model group vs. QLX group (D).

### 4.3. Potential Metabolic Biomarkers of QLX

As shown in the heat map (Figure 4A), a total of 112 different metabolites from serum were identified. QLX significantly reversed the estradiol-induced up-regulation and down-regulation of metabolites in serum (Figure 4B and Table 3). Compared with the control group, estradiol significantly increased the levels of 13 metabolites. Triglycerides and ceramide 1-phosphate was increased more than 3 fold; phosphatidylcholine, dimethyl phosphatidylethanolamine, perfluorooctane sulfonamide and phosphatidylinositol phosphate were increased about 2 fold; and phosphatidylserine, N-acetyldopamine, N-lactoylphenylalanine, 13Z,16Z-docosadienoyl CoA, 1,11-undecanedicarboxylic acid and 5'-deoxyadenosine were increased about 1.5 fold after estradiol addition. QLX treatment reduced these increased metabolites levels. QLX also increased eight metabolites, which were reduced after estradiol-stimulation in rats. This indicated that the anti-inflammatory effect of QLX could be associated with the indicated metabolites changes in CNP rats.

In urine, a total of 50 differential metabolites were identified after the QLX addition in estradiol-induced CNP rats (Fig. 4C and Table 4). QLX addition reduced the six up-regulated metabolites in estradiol -induced CNP rats. The increased lipids, which included 1, 11- undecanedicarboxylic acid, sphingomyelin, and monoerucic acid triglyceride, were blocked by QLX addition in the CNP rats. On the other hand, estradiol-induced down-regulation of ten metabolites including tetracosatetraenoyl carnitine, N-trifluoroacetyladriamycinol, phosphatidylglycerol (i-14:0/a-15:0), 2, 5-diamino-6-(5'-triphosphoryl-3',4'-trihydroxy-2'-oxopentyl)-amino-4-oxopyrimidine, cardiolipin (8:0/8:0/8:0/18:2(9Z,11Z)), UDP-4-dehydro- 6-deoxy-D-glucose, guanosine diphosphate mannose, spermine and 2-hydroxyundecanoate was reversed by QLX addition (Figure 4D).

**Table 3:** Significant different metabolite analysis in serum

| Compound ID | Metabolite | Molecular Formula | Mode | Ret. Time | Delta (ppm) | Measurd m/z | Fold Change | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | C-M FC | M-QLX FC |
| 3258 | Triglycerides | C69H122O6 | ESI+ | 18.2725 | 8 | 1047.94 | 3.57 | 0.33 |
| 3482 | Ceramide 1-phosphate | C34H68NO6P | ESI+ | 9.4885 | 37 | 1257.9 | 3.24 | 0.3 |
| 321 | Phosphatidylserine (DiMe(9,5)/ DiMe(13,5)) | C50H86NO12P | ESI+ | 8.4551 | 85 | 946.4975 | 1.71 | 0.53 |
| 458 | Phosphatidylcholine (22:2(13Z,16Z)/24:1(15Z)) | C54H102NO8P | ESI+ | 8.2218 | 23 | 946.7453 | 2.33 | 0.57 |
| 3003 | Guanosine triphosphate | C10H16N5O14P3 | ESI+ | 7.1821 | 85 | 1047.077 | 0.39 | 2.02 |
| 3041 | Dimethylphosphatidylethanolamine | C41H72NO8P | ESI+ | 20.4984 | 98 | 760.4145 | 2.26 | 0.63 |
| 4345 | N-acetyldopamine | C10H13NO3 | ESI+ | 7.7292 | 50 | 218.0896 | 1.76 | 0.43 |
| 5219 | N,N'-Diacetylchitobiosyldiphosphodolichol | C28H50N2O19P2 | ESI+ | 20.606 | 65 | 781.306 | 0.38 | 2.27 |
| 8571 | N-Lactoylphenylalanine | C12H15NO4 | ESI+ | 15.5911 | 5 | 497.1869 | 1.97 | 0.53 |
| 9588 | 1,11-Undecanedicarboxylic acid | C13H24O4 | ESI+ | 16.1506 | 63 | 511.3563 | 1.64 | 0.47 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 10467 | Phosphatidylethanolamine | C45H88NO7P | ESI+ | 20.0896 | 66 | 808.6722 | 0.31 | 3.49 |
| 10621 | Perfluorooctane sulfonamide | C8H2F17NO2S | ESI+ | 16.4722 | 93 | 521.8941 | 2.22 | 0.43 |
| 10940 | 13Z,16Z-docosadienoyl-CoA | C43H74N7O17P3S | ESI+ | 13.4225 | 2 | 1086.412 | 1.84 | 0.67 |
| 11387 | 5'-Deoxyadenosine | C10H13N5O3 | ESI+ | 18.7786 | 42 | 525.2147 | 1.91 | 0.74 |
| 11534 | Diglyceride | C47H80O5 | ESI+ | 7.2118 | 36 | 1472.244 | 0.32 | 2.01 |
| 11839 | Lysophosphatidylethanolamine (22:2(13Z,16Z)/0:0) | C27H52NO7P | ESI+ | 8.2053 | 38 | 1089.727 | 0.55 | 1.7 |
| 12367 | Phosphatidylinositol phosphate | C48H86O18P2 | ESI+ | 8.2039 | 77 | 1013.459 | 2.23 | 0.68 |
| 13855 | Cardiolipin (i-13:0/i-22:0/i-12:0/i-14:0) | C70H136O17P2 | ESI+ | 7.3167 | 10 | 1333.928 | 0.32 | 2.52 |
| 14155 | Phosphatidylserine (22:0/24:1(15Z)) | C52H100NO10P | ESI+ | 8.202 | 30 | 930.744 | 1.69 | 0.54 |
| 15023 | Lysophosphatidylethanolamin (20:3(8Z,11Z,14Z)/0:0) | C25H46NO7P | ESI+ | 7.2045 | 50 | 1029.54 | 0.47 | 1.93 |
| 15312 | Cholesterol fatty acid ester | C47H78O2 | ESI+ | 7.1953 | 27 | 1350.244 | 0.43 | 1.88 |

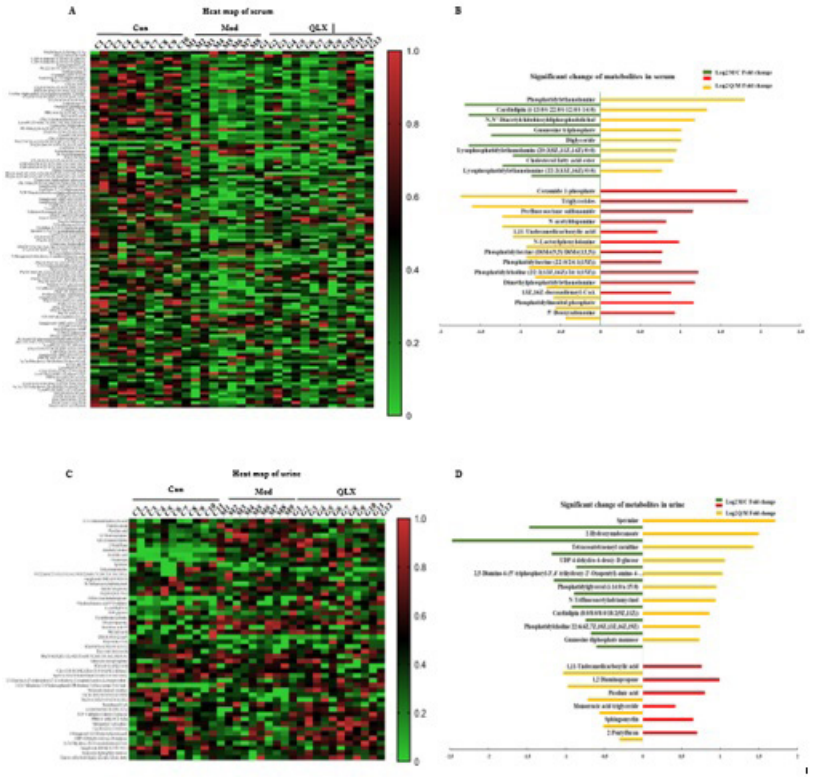

**Figure 4:** Heat map analysis of different metabolites in serum (A) and urine (C) from indicated groups (A). The significant metabolite changes in serum (B) and urine (D).

**Table 4:** Significant different metabolite analysis in urine

| Compound ID | Metabolite | Molecular Formula | Mode | Ret. Time | Delta (ppm) | Measurd m/z | Fold Change | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | C-M FC | M-QLX FC |
| 3103 | Tetracosatetraenoyl carnitine Phosphatidylcholine | C31H53NO4 | ESI+ | 12.2698 | 21 | 1029.7624 | 0.44 | 2.69 |
| 318 | 22:6(4Z,7Z,10Z,13Z,16Z,19Z) | C52H80NO8P | ESI+ | 12.2052 | 34 | 878.5992 | 1.67 | 0.63 |
| 1020 | N-Trifluoroacetyladriamycinol | C29H30F3NO12 | ESI+ | 12.2161 | 44 | 1305.2762 | 0.53 | 1.92 |
| 1707 | Sphingomyelin | C23H51N2O5P | ESI+ | 12.2094 | 22 | 955.6757 | 1.57 | 0.7 |
| 1926 | Phosphatidylglycerol (i-14:0/a-15:0) | C35H69O10P | ESI+ | 12.2003 | 2 | 1383.9178 | 0.54 | 1.93 |
| 2955 | Monoerucic acid triglyceride | C66H110O6 | ESI+ | 12.2034 | 20 | 1021.7992 | 1.34 | 0.68 |
| 2992 | 2,5-Diamino-6-(5'-triphosphoryl-3',4'-trihydroxy-2'- Oxopentyl)-amino-4-oxopyrimidine | C9H18N5O14P3 | ESI+ | 12.4798 | 2 | 1027.0182 | 0.45 | 2.04 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3497 | Cardiolipin (8:0/8:0/8:0/18:2(9Z,11Z)) | C51H94O17P2 | ESI+ | 12.4761 | 64 | 1063.5182 | 0.6 | 1.81 |
| 3928 | UDP-4-dehydro-6-deoxy-D-glucose | C15H22N2O16P2 | ESI+ | 12.4917 | 89 | 1096.998 | 0.55 | 2.08 |
| 5003 | Guanosine diphosphate mannose | C16H25N5O16P2 | ESI+ | 12.2182 | 47 | 1211.2186 | 0.66 | 1.66 |
| 3558 | Spermine | C10H26N4 | ESI- | 12.4162 | 63 | 201.2211 | 0.36 | 3.27 |
| 3571 | 2-Hydroxyundecanoate | C11H22O3 | ESI- | 13.0895 | 31 | 201.1558 | 0.18 | 2.83 |
| 1872 | Picolinic acid | C6H5NO2 | ESI- | 2.3213 | 57 | 122.0178 | 1.74 | 0.61 |
| 2173 | 2-Pentylfuran | C9H14O | ESI- | 11.7888 | 4 | 137.0978 | 1.63 | 0.81 |
| 2073 | 1,2 Diaminopropane | C3H10N2 | ESI- | 1.9496 | 62 | 133.1065 | 1.98 | 0.51 |
| 1129 | 1,11-Undecanedicarboxylic acid | C13H24O4 | ESI- | 8.0451 | 40 | 243.17 | 1.69 | 0.49 |

In total, five types metabolites were found in serum and urine samples, namely glycerophospholipid, fatty acid ester, glucide and amino acid, among others (Figure 5). The increased phosphatidylcholine and 1, 11-undecanedicarboxylic acid and lipid metabolites were observed in both serum and urine in CNP rats, and QLX could reduce these levels by 50% (Figure 5B and D). This indicated that lipid metabolites may play a major role in response to QLX treatment in CNP.

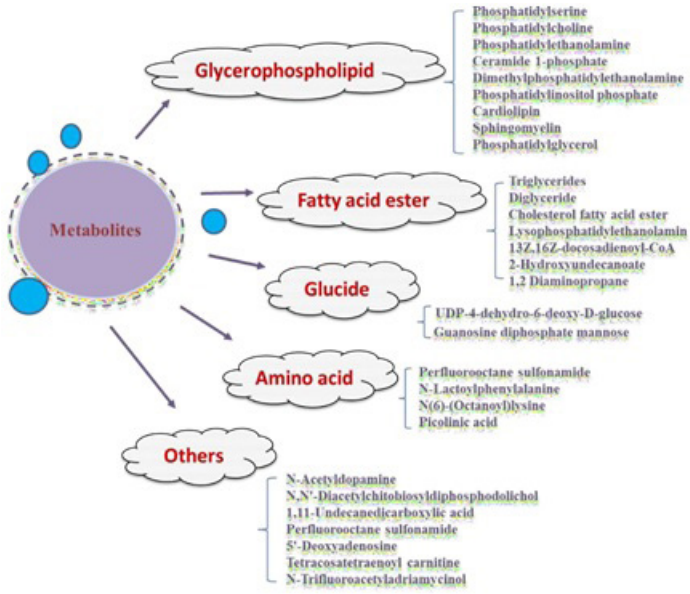


**Figure 5:** General classification of metabolites including glycerophospholipid, fatty acid ester, glucide and amino acid

### 4.4. Potential Metabolic Pathway of QLX

Pathway topology analysis (Figure 6) and pathway enrichment analysis (Figure 7) were performed using Metaboanalyst in samples from QLX-treated CNP rats. In the serum samples, 14 pathways were significantly changed after QLX addition (Figure 6A). Glycerophospholipid metabolism and sphingolipid metabolism pathways were the most affected pathways (influence values > 0.2) after QLX addition in estradiol-treated rats. Consistently, the glycerol phosphate shuttle and beta oxidation of very long chain fatty acids were highly and significantly enriched after QLX addition in CNP rats (Figure 7A).

In urine samples, the influence values of fructose and mannose metabolism, glycerophospholipid metabolism and amino sugar and nucleotide sugar metabolism pathways were more than 0.1 (Figure 6B). Pathway enrichment analysis indicated fructose and mannose degradation and saturated fatty acids oxidation pathways were significantly enriched in the QLX-treated CNP rats (Figure 7B). This indicated that the lipid and glucose metabolic pathways could be important for understanding the pharmacologic mechanism of QLX in CNP.

The pathway map was generated based on pathway enrichment analysis with log (P) values > 2 and pathway impact threshold > 0.15. Glycerophospholipid metabolism, sphingolipid metabolism and fructose and mannose metabolism pathways were identified as the major pathways that were affected by QLX in CNP rats (Figure 8). It suggested that the anti- inflammatory therapeutic effects of QLX in vivo may be associated with the metabolic pathway changes.

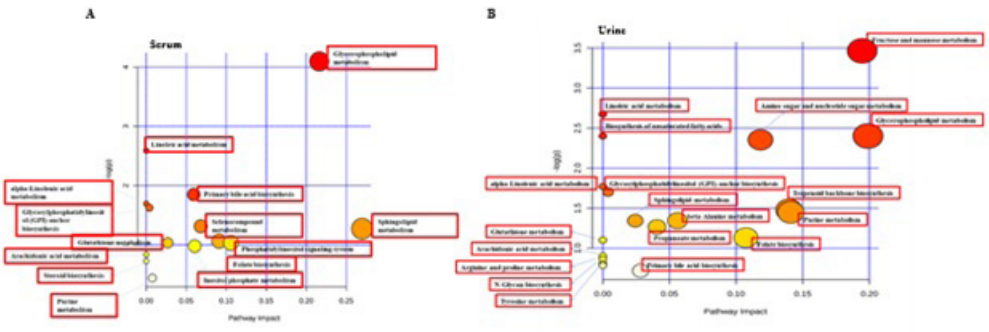


**Figure 6:** Pathway topology analysis was used to clarify the potential biomarkers in serum (A) and urine (B) responsible for the effect of QLX on prostatitis

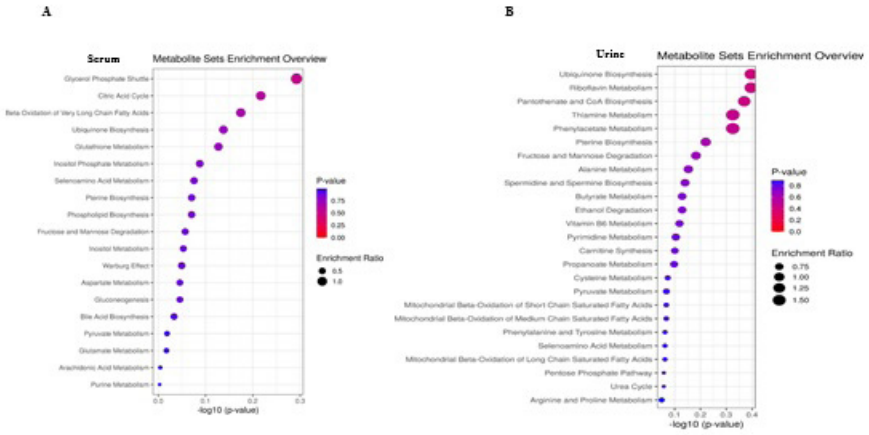


**Figure 7:** Pathway enrichment analysis was used to clarify the potential biomarkers in serum (A) and urine (B) responsible for the effect of QLX on prostatitis

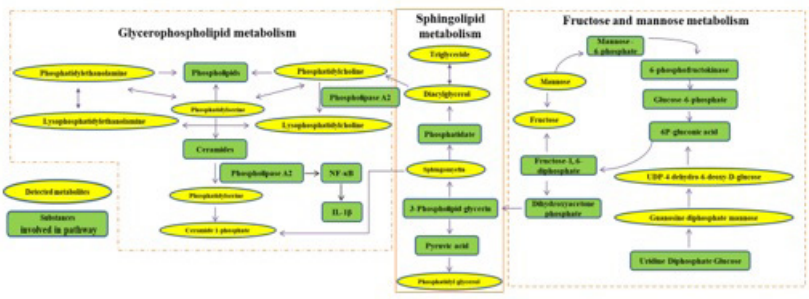


**Figure 8:** Primary related metabolic pathways

## 5. Discussion

This work confirmed the anti-inflammatory function and prostate productive effect of QLX. LUTS syndrome and key biomarkers of renal dysfunction in urine were also reduced by QLX in CNP models. As a TCM, QLX can control the progress and relieve symptoms of CNP by propelling the circulation of blood, eliminating blood stasis, reducing the release of inflammatory cytokines, relieving pain and balancing metabolic disorder based on clinical practice and the theory of herbal properties. Consistently, phenolic acid, a major active ingredient from QLX, exerts antioxidant, anti-inflammatory and anti-cancer functions [13]. QLX contains numerous polyphenols and polyphenolic metabolites, which have been reported to benefit management of CNP [14]. Therefore, QLX was useful to control the inflammation and LUTS of CNP patients.

As a high throughput method, metabolic profiling analyzed by UP-LC-MS found 50 different metabolites in urine and 112 metabolites in serum were affected by QLX addition. This indicated that QLX addition could affect the CNP-induced metabolic changes. The large amount of data generated here may be useful for further deep analysis to clarify the pharmacological targets of QLX. Pathway analysis indicated that QLX addition was able to significantly change the sugar and lipid metabolism, which was directly related to CNP progress.

Glycerophospholipid metabolism was significantly changed by QLX addition in CNP models. Phosphatidylethanolamine (PE), one product of glycerophospholipid metabolism, is produced through the decarboxylation of Phosphatidylserine (PS) by PS Decarboxylase (PSD) [15]. PS was reported to regulate inflammation and tumor necrosis factor-α (TNF-α) production in Kdo2-lipid A-stimulated RAW 264.7 cells in vitro [16]. Lysophosphatidylethanolamine (LysoPE), the hydrolysate of PE, contributes to cell proliferation and inflammation. LysoPE was reported to control the immune response by interacting with LysoPC [17]. In the present study, the decreases in PS, PC, LysoPS and LysoPC were significantly recovered after QLX addition in the CNP models. This indicated that QLX may play an anti-inflammatory role via glycerophospholipid metabolism. Because PSD is an important enzyme to control PS metabolism, QLX could change the PSD activity to control the glycerophospholipid metabolism-regulated inflammation in CNP.

Moreover, an elevation of PCs induced by QLX was observed. PCs and LysoPEs have been reported to compete for acyl receptors in the catalytic domain of cPLA2γ [18]. The elevation of PCs was associated with activation of phospholipase A2 (PLA2) [19]. Therefore, QLX may induce PLA2 activation. Meantime, PLA2 can strongly enhance nuclear p65 expression and phosphorylation, which is a component of the proinflammatory transcription factor NF-κB [20]. In our early research, a reduction in IL-1β by QLX addition may also be associated with regulation of NF-κB activation by glycerophospholipid metabolism.

Sphingolipids, an important component of the cell membrane, regulate cellular life activities. Ceramides, which are generated by sphingomyelinase (SMase) from sphingolipids, are able to induce NF-κB-activation [21, 22]. Ceramide 1-Phosphate (C1P), the phosphorylated version of ceramide, can translocate and be activated by cytosolic PLA2, and induce arachidonic acid and prostaglandin synthesis [23]. Increased C1P in CNP rats may induce arachidonic acid, which could induce an inflammatory response via oxidative stress [24]. The QLX-induced decrease in C1P may control the inflammation in CNP through sphingolipid metabolism. In addition, ceramide production is also negatively regulated by PS [25]. Therefore, the restored balance between sphingolipid metabolism and glycerophospholipid metabolism after QLX addition may contribute to controlling the inflammation in CNP.

The fructose and mannose metabolism pathway is closely related to sugar metabolism, which provide energy for various physiological processes. The activation of human T- lymphocytes and proinflammatory cytokine expression could be induced by hyperglycemia [26]. Mannose lectin, one mannose metabolism product, provides protection against pathogens and various immune diseases [27]. UDP-4-dehydro-6-deoxy-D-glucose is synthesized from UDP-glucose through the enzyme UDP-glucose 4,6-dehydratsase, and UDP-glucose is one of the precursors of glycogen, glycosides of glucose and polysaccharide. Glycogen metabolism controls the macrophage-mediated inflammatory responses. Glycogen metabolism also increases UDPG levels and the receptor P2Y14 in macrophages, through induction of UDPG levels and the P2Y14 receptor, which further induce the expression of STAT1 and proinflammatory cytokines [28]. The increased UDP-4-dehydro-6-deoxy-D-glucose in urine after QLX addition may be associated with the anti-inflammatory function of QLX in CNP.

Moreover, the crosstalk of glycerophospholipid metabolism, sphingolipid metabolism and fructose and mannose metabolism were involved in the anti-inflammatory function of QLX in CNP. The crosstalk of sphingolipids and glycerophospholipids are believed to be opposed/complementary forces in regulating lipotoxicity in metabolic diseases [29]. In obesity- related diseases, the sphingolipid and fructose and mannose metabolism pathways were also reported to work together to regulate inflammation [30]. Thus, QLX-induced changes in the metabolism network may contribute to regulate the inflammation status of CNP, rather than one or two specific molecules.

In summary, this work found that the unique metabolic profiling in CNP was significantly changed by QLX addition, which may be associated with the anti-inflammatory role of QLX in

CNP. Glycerophospholipid metabolism, sphingolipid metabolism and fructose and mannose metabolism pathways may be candidate bio-targets of QLX in CNP.

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