Metabolomics study of regulatory effects of exercise training on db/db type 2 diabetic mice

Li Xiang

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THESIS TITLE: Metabolomics Study of Regulatory Effects of Exercise Training on db/db Type 2 Diabetic Mice

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Metabolomics Study of Regulatory Effects of Exercise Training on \textit{db/db} Type 2 Diabetic Mice

XIANG Li

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Principal Supervisor: Prof. CAI Zongwei

Hong Kong Baptist University

March 2018
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

I have read the University’s current research ethics guidelines, and accept responsibility for the conduct of the procedures in accordance with the University’s Committee on the Use of Human & Animal Subjects in Teaching and Research (HASC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical approval, and acknowledged my obligations and the rights of the participants.

Signature: __________________

Date: March 2018
ABSTRACT

Type 2 diabetes mellitus (T2DM) is mainly caused by genetic modifications and inappropriate life styles. The complexity of T2DM has brought us challenges for a comprehensive understanding of altered metabolic pathways that contributing to the development of T2DM. Therefore, a comprehensive metabolic analysis is needed. To date, taking regular exercise is a common and effective therapeutic way known to antagonize the metabolic disorders of T2DM. However, the regulatory effects of exercise on T2DM or T2DM-induced complications have not been clearly characterized.

We investigated the effect of physical activity on biochemical changes in diabetic db/db mice in plasma, urine, skeletal muscle, kidney and liver samples. Based on the techniques of liquid chromatography-high resolution Orbitrap mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), untargeted and targeted metabolomics studies were developed to delineate metabolic signatures in various kinds of biofluid and tissue samples. Targeted quantification methods on acylcarnitines and acyl-CoA were developed. Untargeted metabolomics analysis by GC-MS and LC-MS were also developed to draw a more comprehensive view of the metabolic changes in response to T2DM and exercise on db/db diabetic mice. The transcript expressions of mRNA in pathways of interest were measured to confirm the hypothesis.

Firstly, untargeted metabolomics study was carried on plasma samples to give a general view of exercise effect on db/m+ control and db/db diabetic mice. A total of 24 differential metabolites were identified contributing to the metabolic differentiation of the 2 types of mice. The levels of three biomarkers, including creatine, uridine and lysine, were substantially reversed by exercise on db/db mice. Notably, the level of palmitoylcarnitine and pantothenic acid, which participate in FAO, were significantly
increased by exercise on db/db mice rather than on db/m+ mice. The findings indicated that db/db diabetic mice may be more susceptible to exercise for energy expenditure. The results demonstrated that physical activity might have the ability to mitigate insulin resistance in T2DM mice through improving FAO and eliminating overloaded intermediate which contribute to insulin resistance.

Based on the findings from plasma sample, we noticed that acylcarnitines that play an important role in fatty acid β-oxidation (FAO) have been substantially changed between db/m+ and db/db mice. In order to acquire more information of the disturbance of this pathway, a targeted quantification method of acylcarnitines was developed. Acylcarnitines exert a variety of biological functions depending on the differences in lengths, saturation levels and conjugation groups, which to a great extent contributes to the challenges of acylcarnitines quantifications due to the various kinds of isomers. Here, we describe a novel method by using high resolution parallel reaction monitoring (PRM) on LC-MS platform. Both reversed-phase and normal-phase columns were used in order to get accurate, reliable, widespread quantification of acylcarnitines, without tedious sample preparation procedure. The method provided the most comprehensive acylcarnitine profile with high resolution MS and MS/MS confirmation to date. A total of 117 acylcarnitines were detected from plasma and urine samples. The application of targeted profiling of acylcarnitines in db/m+ control and db/db diabetic mice showed incomplete amino acid and fatty acid oxidation in diabetic mice. Interestingly, the reduction of medium odd-numbered chain acylcarnitines in urine samples was firstly observed between db/m+ and db/db mice. The high resolution PRM method allowed monitoring the widespread metabolic changes of the acylcarnitines in response to stimuli. Besides, the accurate MS and MS/MS spectra data
of the 117 acylcarnitines could be used as mass spectrometric resources for the identification of acylcarnitines.

Skeletal muscle is the major tissue that responsible for insulin sensitivity. Any changes in skeletal muscle mass, responsiveness to hormones or metabolic rate could substantially influence the overall homeostasis of energy in the body. In Chapter 4, untargeted metabolomics analysis was performed to investigate the effect of regular exercise on db/db mice in skeletal muscle. Both LC-MS and GC-MS were carried out to monitor a wide range of regulated metabolites. Ninety-five metabolites were identified which contribute to the discrimination of db/m+ control and db/db diabetic mice. The regulatory effects of exercise on these metabolites were mainly associated with attenuating the levels of long-chain fatty acids (C14 to C18) and medium- to long-chain acylcarnitines (C12 to C18), indicating that exercise might play a positive role in inhibiting the accumulation of excessive lipids, which contributed to insulin resistance.

In addition, uric acid that is a risk factor for inflammation, cardiovascular complications, and fatty liver in diabetic patients, together with its intermediates (such as inosinic acid, hypoxanthine, etc.) were also substantially down regulated after exercise, indicating that exercise might also be protective against hyperuricemia related risks in T2DM. These findings revealed that regular exercise played a positive role in improving the efficiency of lipid metabolism and meanwhile enhancing uric acid clearance to prevent lipid accumulation in skeletal muscle, which might contribute to improved body fitness and body muscle composition.

In addition, the regulatory effect of exercise on diabetic nephropathy, a major complication of T2DM recognized to cause severe morbidity and mortality in diabetic patients, was also investigated in chapter 5. Untargeted and targeted metabolomics studies based on GC-MS and LC-MS were performed. The results demonstrated that
exercise exhibited beneficial effect in reducing hyperlipidemia, expression levels of inflammatory markers (TNFα, IL-6 and COX2) and fibrosis markers (Collagen 1), and alleviated DN induced mesangial expansion in kidneys of diabetic mice. The results of metabolic changes in kidney of DN mice revealed that the accumulation of acyl-CoA, phospholipids and hydroxylated acylcarnitines were substantially ameliorated by exercise, and the reduction of important enzymes CTP1α and Acadl in FAO were partially reversed. In addition, branched-chain amino acids (BCAA) metabolism that positively related to inflammation (TNFα) was down-regulated in diabetic mice. However, exercise up-regulated BCAA catabolism via reducing the level of leucine and increasing the expression of Acad8, Bcat2, Hibch and Hmgcl in this pathway. Furthermore, the accumulation of uric acid, which contributes to inflammation and tubulointerstitial fibrosis in kidney disease, together with its six precursors (guanosine, succinyladenosine, inosine, guanine and xanthine) were substantially reduced. The above results demonstrated that exercise might play beneficial effect in alleviating lipotoxicity through improving FAO efficiency in diabetic kidneys. In addition, exercise also ameliorated diabetic induced inflammation and fibrosis via promoting BCAA catabolism and accelerated the elimination of uric acid.

Together, mass spectrometry-based metabolomics study is a powerful tool to investigate the regulatory effect of exercise on complex metabolic diseases. The obtained results in this study may provide informative insights into the underlying the mechanism of exercise on T2DM and T2DM-induced complications.
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<tbody>
<tr>
<td>4P-Pan</td>
<td>4'-phosphopantothenate</td>
</tr>
<tr>
<td>Acadm</td>
<td>Medium-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>Acads</td>
<td>Short-chain acyl-CoA dehydrogenase</td>
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<tr>
<td>Acadsb</td>
<td>Short-branched chain acyl-CoA dehydrogenase</td>
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<td>ACN</td>
<td>Acetonitrile</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>AMP</td>
<td>Adenosine 5’-monophosphate</td>
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<tr>
<td>ANOVA</td>
<td>Analyses of variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BCAAs</td>
<td>Branched-chain amino acids</td>
</tr>
<tr>
<td>Bcat2</td>
<td>Branched chain amino acid transaminase 2</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CACT</td>
<td>Carnitine-acylcarnitine translocase</td>
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<tr>
<td>CE</td>
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<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Gluc-1P</td>
<td>Glucose-1-phosphate</td>
</tr>
<tr>
<td>Gluc-6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>HCD</td>
<td>Higher-energy collisional dissociation</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>Hibch</td>
<td>3-hydroxyisobutyril-CoA hydrolase</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HMDB</td>
<td>Human Metabolome Database</td>
</tr>
<tr>
<td>Hmgcl</td>
<td>3-hydroxymethyl-3-methylglutaryl-CoA lyase</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>ILDLD</td>
<td>Intermediate-density lipoprotein</td>
</tr>
<tr>
<td>IM</td>
<td>Inner side of the mitochondria</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner side membrane of mitochondria</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin injection test</td>
</tr>
<tr>
<td>Ivd</td>
<td>isovaleryl-CoA dehydrogenase</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
</tbody>
</table>
LC-MS  Liquid chromatography-mass spectrometry  
LDL  Low-density lipoprotein  
LE  $db/m^+$ mouse group treated with exercise  
LOD  Limit of detection  
LOQ  Limit of quantification  
LS  $db/m^+$ mouse group treated with sedentary  
m/z  Mass to charge ratio  
MeOH  Methanol  
MRM  Multiple reaction monitoring  
MS  Mass spectrometry  
MS/MS  Tandem mass spectrometry  
na  Not available  
NIST  National institute of standards and technology  
NMR  Nuclear magnetic resonance  
ns  Not significant  
OE  $db/db$ mouse treated with exercise  
OGTT  Oral glucose injection test  
OM  Outside of the mitochondria  
OMM  Outside membrane of mitochondria  
OPLS  Orthogonal projections to latent structures  
OS  $db/db$ mouse treated with sedentary  
PanK  Pantothenate Kinase  
PCA  Principal component analysis  
PCr  Phosphocreatine  
PLS-DA  Partial least squares Discriminant Analysis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>PPAR δ</td>
<td>Peroxisome proliferator-activated receptor δ</td>
</tr>
<tr>
<td>PRM</td>
<td>Parallel reaction monitoring</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>QQQ</td>
<td>Triple quadrupole</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard derivation</td>
</tr>
<tr>
<td>s/n</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected ion monitoring</td>
</tr>
<tr>
<td>SM</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen-free</td>
</tr>
<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>UDP/UTP</td>
<td>Diphosphate/uridine triphosphate</td>
</tr>
<tr>
<td>UD</td>
<td>Under detection limit</td>
</tr>
<tr>
<td>VIP</td>
<td>Variable importance in the projection</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
</tbody>
</table>
Chapter 1 General Introduction

1.1 Type 2 diabetes mellitus

1.1.1 Diabetes mellitus

Diabetes mellitus is a kind of metabolic disease that characterized by defects of insulin secretion or lack of cell response to insulin that has been produced [1-3]. High blood sugar levels often exhibited over a prolonged period. There are many symptoms accompanied with high blood sugar, such as increased thirst, hunger, and frequent urination.

Diabetes mellitus can be classified into three main broad etiopathogenetic types. One is type 1 diabetes mellitus (T1DM). The cause of T1DM is an absolute deficiency of insulin secretion. It is a form of diabetes mellitus that is induced by the autoimmune destruction of insulin production from β cells in the pancreas, which will lead to the increase of glucose in blood and urine. Responsiveness and sensitivity to insulin are usually normal in the early stages.

Another type of diabetes mellitus is type 2 diabetes mellitus (T2DM), which is much more prevalent than the former one. T2DM is characterized by hyperglycemia (high blood glucose) as a result of insulin resistance and relative inadequate of insulin supply. Obesity is generally thought to be the principal cause of T2DM in patients with diabetes [4, 5]. Rates of T2DM have increased significantly since 1960 accompanied with obesity. The third main type of diabetes mellitus is gestational diabetes mellitus (GDM). It is involving a combination of relatively insufficient insulin secretion and responsiveness. About 2-10% of pregnant women may develop GDM, and often disappear after delivery [6, 7]. Unlike other types of diabetes mellitus, GDM is fully treatable with careful medical supervision during pregnancy.
The basic dysfunction of diabetes ranges from autoimmune destruction of β cells of the pancreas resulted insulin deficiency to abnormalities in insulin resistance [8]. The consequences of the abnormalities will lead to dysfunction in fat, carbohydrate and protein metabolism. Deficient action of insulin often results from inadequate secretion of insulin and/or reduced tissue response to insulin. This phenomenon frequently coexists in the same patient. Untreated diabetes could cause many complications [9, 10], such as nonketotic or ketoacidosis hyperosmolar syndrome [11]; chronic kidney disease [12]; retinopathy; peripheral neuropathy; cardiovascular symptoms [13, 14]; hypertension; food ulcers and sexual dysfunction [15, 16].

1.1.2 Prevalence and social burden caused by T2DM

The number of patients with diabetes is increasing in parallel with population growth, urbanization, aging and increased prevalence of obesity and physical inactivity. T2DM makes up about 90% of cases of diabetes mellitus [17]. Appraisal of current and future prevalence of diabetes mellitus has been reported [18-20] that the global prevalence of diabetes in the year 2000 and prediction in 2030 are serious (Figure 1.1). The number of patients with diabetes was 175 million in 2000. However, this number may increase to 353 million by 2030 [17, 21], with the greatest increase in India (14 million increased to 25 million) and in other developing countries (87.78 million to 200.2 million). Therefore, the high prevalence of T2DM will cause us a severe high social burden.
Figure 1.1 Prevalence of diabetic patients in 2000 and prediction for 2030. Source: reference 17.

Furthermore, the cost of caring for patients with diabetes is vast. The global emergence of diabetes is as serious an economic problem as it is a health problem [22, 23]. Epidemic obesity and diabetes induce a considerable economic burden to the societies. Lots of evidence has demonstrated that the medical and treatment expenses for diabetes siphon considerable resources from the health system. We can see that from Figure 1.1 that more than 50% of people with diabetes will come from developing countries. Many of the high-priced and specialist-care treatments will definitely drive the growth of medical expenses which is poorly suited for health systems of developing countries. Besides, the burden of obesity and diabetes contributing to health systems just
only reflects a small proportion of the financial disruptions. Lots of evidence also shows that the economic burden of diabetes will also result in a lower returns on education [24, 25], reduced earnings, incomes and wages [26, 27], and increased risk of premature unemployment and retirement [28, 29]. Over time, these potential impacts can be influenced more severely than the conditions themselves on people with diabetes. It would be a huge economic burden and reduction of the quality of life for individuals with diabetes, especially for those patients in the developing countries.

1.1.3 T2DM and physical activity

For decades, physical activity along with diet and medication, have been regarded as a cornerstone of diabetes mellitus management. A large number of cohort studies have found that higher levels of physical activity (especially aerobic fitness) are related with markedly lower risk of cardiovascular and overall mortality [30-32]. Stewart and colleagues [33] have reported that the potential mechanism of the regulatory effect of exercise on diabetes patients may through decreasing the accumulation of abdominal visceral fat, improving the function of endothelial vasodilator, reducing diastolic dysfunction and decreasing systemic inflammation. Generally, the effects of exercise on blood glucose and lipids are relatively modest among non-diabetic population [34, 35]. As the intensity of exercise increase, a greater reliance will be aroused on carbohydrate, especially when sufficient amounts are available in muscle or blood [36, 37]. The reason is that during moderate-intensity of exercise, the increase in peripheral glucose intake is relevantly equivalent by an equal increase in hepatic glucose production.
However, exercise programs with higher intensity on diabetic subjects can cause a greater reduction of triglycerides and elevation of high-density lipoprotein (HDL) cholesterol in plasma [38]. Some studies have also reported that taking regular exercise could significantly reduce body weight [39, 40], which might result from stimulated fat oxidation and storage in muscle [36, 41, 42]. What’s more, more prolonged or intense exercise has been reported to have benefits in insulin action [43-46]. Acute improvements in insulin sensitivity have been found in women with T2DM [46]. In addition, Lots of studies reveal that enhanced whole-body insulin action after aerobic exercise training seems related to gains in peripheral [47, 48] function. However, although a large quantity of studies have been exerted to investigate the regulatory effect of exercise on T2DM and T2DM induced complications, the mechanism of physical activity on T2DM management and prevention are still not completely clarified.

1.2 Metabolomics technology

1.2.1 Metabolomics: building a bridge from biochemistry to guidance of human health

Nowadays, healthcare of humans is facing with many challenges, and the use of biochemistry knowledge could provide us a renewed purpose. For people who keep in good health, all of the metabolic pathways have to function in a relatively appropriate way and their metabolic pathways have to be balanced by appropriate nutritional inputs. When individuals are in bad health, such as metabolic disease, including obesity, atherosclerosis, hypertension, osteoporosis or diabetes, the balance of metabolic pathway will be broken [49]. The healthcare system at various levels has been recruited to solve these challenges,
and biochemistry technology played a critical role to solve these problems [50].
However, biochemistry must be coupled to a new tool in order to figure out the
multi-factorial nature and complex of these metabolic diseases. The tool should
have the ability to profile the hallmark metabolites that related to chronic
metabolic diseases. Fortunately, as the analytical technologies improving, most
of the known endogenous metabolites can be detected by one of the newly
existing and powerful analytical platforms [51, 52] which usually combined
with computational technologies, can be able to give an indiscriminate overview
of the changes between health and sickness people with chronic metabolic
diseases. These demands and technologies drive the rapidly emerging field,
metabolomics.
Metabolomics is the scientific study of chemical changes involving in
endogenous metabolites. Specifically speaking, metabolomics is the quantitative
and systematic study of all metabolites responding to the stimuli (drug, disease,
diet and physical activity) in an organism [53]. Nowadays, metabolomics
technology has been applied in many areas. Firstly, metabolomics can be
applied in toxicity assessment. Metabolomic profiling could detect the
physiological changes that caused by toxic intervention, such as chemicals.
Many studies have found that the observed metabolite changes have a
relationship with specific syndromes. For instance, the invasion of a toxic
chemical might cause a specific lesion to organs, such as liver or kidney.
Besides, this application might be of particular relevance to pharmaceutical
industries who are willing to measure the toxicity of potential drug candidates
[54]. Another important application is focusing on functional genomics.
Metabolomics can be used as an excellent tool for the determination of the
phenotype that caused by a genetic manipulation. For example, metabolomics has the ability to detect phenotypic changes in plants that have been genetically manipulated. Furthermore, metabolomics can also be used for predicting the function of unknown genes through comparing with the metabolic changes of known genes [55]. Besides, metabolomics technology can be also applied in human nutrition. In general, the metabolome in the body fluid is mainly influenced by endogenous factors, such as genetics, sex, age as well as underlying pathologies. Moreover, diet and physical activity can also cause shifts in metabolome. Metabolomics can be used to determine the metabolic fingerprint of a specific diet or drug administration, which reflects the metabolome shift or balance of specific forces on the metabolism of individuals.

1.2.2 Analysis platform of metabolomics technology

Figure 1.2 shows the general work flow of untargeted metabolomics study. After extraction from various kinds of biofluid and tissue, the samples will be put onto different instruments for data collection. Then the raw data will be extracted and applied for multivariate statistical analysis. After that, the differentiated metabolites will be picked out. In addition, verification from gene and protein levels are necessary to help to verify the potential mechanism based on identified biomarkers.
Figure 1.2 General workflow of metabolomics technology.

Among the process of metabolomics, the first and primary objective is to detect all metabolites within a biological sample which comprises as a highly complex mixture. The metabolites in biological samples involve in a much wider distribution of compound compositions and physical natures. Therefore, in the present, there is no single technological platform with the ability to detect and identify all metabolites in a single biological sample simultaneously. There are two basic approaches used in metabolomic analysis at present. The first approach is nuclear magnetic resonance (NMR) spectroscopy, which is a tactic method to analyzed samples [56]. The second approach for analyzing
metabolites is a bottom up method where metabolites are separated into a single molecular by chromatography and detected by mass spectrometry (MS).

NMR spectroscopy is a relatively quantitative and nondestructive analytical approach used to obtain spectra of a variety of biological samples, such as cells, tissues and biological fluid [57]. Comparing with other analyzing platforms of metabolomics, NMR technology has many advantages. For example, it is a high reproductive method with simple sample preparation procedure. Moreover, this method does not rely on separation of metabolites, so the samples can be recovered for further analysis. As a result, all kinds of metabolites can be analyzed simultaneously. So in this sense, it is a universal detector. However, the sensitivity is not as high as the other analyzing platforms of metabolomics.

Another widely applied platform for metabolomics analysis is mass spectrometry, which is usually combined with chromatography for separation of complex analytes. This kind of approach can analyze hundreds and thousands of small molecular weight of endogenous metabolites comprehensively in the samples. MS is continually improving as the sensitivity, dynamic mass range and accuracy improves. To date, the most frequently used MS based platforms for metabolomics technology, namely gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS).

GC-MS is one of the most widely used approaches for analyzing endogenous metabolites [58-60]. There are many advantages for metabolomics study in using GC-MS. Firstly, the sensitivity is high among all platforms, which could significantly decrease the amount of raw biological samples that needed for accurate measurements. Secondly, better separation performance of compounds in the gas phase than in the liquid phase. Thirdly, extensive compound databases
(such as national institute of standards and technology (NIST)) and experimental protocols are available, since GC-MS technique has been applied in fundamental mechanism investigation and clinical diagnosis for a long time. To date, lots of studies have applied GC-MS in metabolomics analysis. Biomarkers of adrenarche were analyzed in a large scale study of 400 subjects by using GC-MS [61], and identification of metabolites in ryegrass was carried out by 2D-TOF-MS [62]. However, although GC-MS has been widely used for metabolomics, the biological samples require chemical derivatization. Although the preparation of samples was relatively complex, GC-MS could not be replaced by other platforms due to its remarkable advantages in analyzing less polar small molecular, such as amino acids and short chain fatty acids. (Shown in Figure 1.3).

While high performance liquid chromatography - mass spectrometry (HPLC-MS) is another widely used approach for metabolic analysis. Electrospray ionization (ESI) is the most commonly used ionization technique applied in LC-MS analysis. This is a kind of soft ionization technique, and suitable for polar molecules with ionizable functional groups. When compared with GC-MS, although HPLC-MS have a lower chromatographic resolution, generally it does not require derivatization for polar molecules in biological samples. Samples can be directly injected for separation after sample deproteinization, which largely simplifies the procedure of sample preparation. However, derivatization usually applied to improve the retention and sensitivity of mass spectrometry. HPLC-MS has the advantage that a greater amount of analytes can be detected with a much higher sensitivity than GC-MS approach [63]. However, LC-MS also has disadvantages in separating isomers and extremely polar metabolites
(such as glucose). Therefore, in order to get a full span of metabolites in biological samples, the integration of GC-MS and HPLC-MS analysis also carried out to give a more detailed overview of metabolic changes between different samples.

Figure 1.3 Metabolites that can be detected by GC-MS and LC-MS.

1.2.3 Untargeted metabolomics

Generally speaking, metabolomics strategies have been split into two approaches, untargeted and targeted metabolomics. An untargeted metabolomics analysis is usually designed to profile all differential metabolites in a single experiment, and have the advantage in collecting novel information. Figure 1.4 shows the general work flow of untargeted metabolomics. Metabolite extraction is designed to maximize the coverage of metabolites in order to include broad detection of various kinds of compounds, such as amino acids, amines, organic acids, nucleotides, lipids, etc. A great number of studies reveal that untargeted metabolite extraction can be achieved by using organic/aqueous solvents (such as 80% methanol) [64]. Internal standards usually added during sample
extraction step to monitor the stability of the analysis, and the output data can further be normalized using computational procedures before statistical analysis. A wide variety of normalization methods have been developed [65]. Generally, the most frequently used normalization methods are normalization by the sum, the median, or by reference feature, etc. Different normalization method could be tried to achieve better results. Although normalization procedure is a pivotal step in untargeted metabolomics analysis, little consensus has been achieved which normalization method is the most robust.

During non-targeted metabolomics analysis, the goal of metabolic profiling experiments is to find out the relative differences of the metabolomes between two or more systems. Therefore, an essential step of unbiased metabolic fingerprinting is the use of multivariate statistic analysis approaches to identify those biologically relevant features for further analysis. Generally, the most frequently used multivariate statistic analysis methods are principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA). In essence, the two methods aim to differentiate in/between classes in highly complex data set. Briefly, PCA is to arrive at a linear transformation that preserves as much of the variance of the original data as possible into the lower dimensionality output data which finally could make the high-dimensional datasets visualizable. However, such unbiased application of dimensionality reduction only reveals group structure when the variation within -group is sufficiently less than between-group. Thus, a supervised multivariate statistic analysis method rely on the class membership of each observations are also usually applied in non-targeted metabolomics profiling experiments, which allows the algorithm to better separations between classes in score space.
After multivariate statistic analysis, the ions whose variable importance in the projection (VIP) values are more than 1.2 and meanwhile the $p$ value less than 0.05 are selected out for identification. For LC-MS based untargeted metabolomics, the accurate MS and MS/MS are used for identification of potential metabolites through online free data base (METLIN (https://metlin.scripps.edu), HMDB (http://www.hmdb.ca/), MassBank (http://www.massbank.jp), etc.) first, and further verified by authentic standards (retention time, $m/z$ and MS/MS fragments). For GC-MS based untargeted metabolomics, the MS and MS/MS data are firstly searched in the database NIST (National Institute of Standards and Technology, www.nist.gov). And then, verification by authentic standards is also performed.

Figure 1.4 MS-based untargeted and targeted metabolomics.
VIP, variable importance in the projection. NIST, National Institute of Standards and Technology. MRM, multiple reaction monitoring. SRM, selected reaction monitoring. SIM, selected ion monitoring. PRM, parallel reaction monitoring.

1.2.4 Targeted metabolomics

Although untargeted metabolomics analysis is a powerful tool in observing the comprehensive metabolic changes in stimuli and offers opportunities for novel biomarker discovery, the coverage of the metabolites is still restricted as a result of the methodologies of sample preparation, and the limitation of specificity and sensitivity of the existing analytical technique employed. In addition, the principal challenges of untargeted metabolomics analysis lie in characterizing and identifying unknown metabolites, the reliance on the wide breadth coverage of small molecules of the platform employed, which unavoidably bias towards the detection of molecules with high abundance, and blunder away those low abundant molecules but with important biological significance.

By contrast, targeted metabolomics is a methodology in detecting a group of chemically characterized or biochemically annotated metabolites. Quantitative or semi-quantitative measurement of interested metabolites can be undertaken by the use of internal standards. This method exhibits advantages of a comprehensive understanding of a wider breadth of metabolic enzymes, their end products and kinetics, and the contribution to the known biochemical pathways. When performing targeted metabolomics analysis, sample preparation procedure can be optimized. Enriching of low abundant and reducing of high abundant molecules in the analysis can be achieved to obtain a relatively comprehensive observation of interested species of metabolites.
Due to the advances in the development of mass spectrometry (gas chromatography, liquid chromatography, etc.), multiple reaction monitoring (MRM) or selected reaction monitoring (SRM), selected ion monitoring (SIM) and parallel reaction monitoring (PRM) detection modes (Figure 1.5) have been widely applied in targeted metabolomics analysis [66-68]. All of them exhibit excellent quantification ability. Generally, SIM is a mass spectrometry scanning that only a limited number of selected ions (precursors) are transmitted by the instrument [69]. Compared with the full spectrum range, SIM mode could significantly improve the sensitivity. MRM (SRM) is a quantification method used in tandem mass spectrometry. Firstly, precursor ion was selected in Q1 (first filter), then fragmented in Q2, the selected product ion of the precursor ion from Q2 is selected (second filter) in Q3, then the product ion is detected by the detector [69]. MRM is highly selective for its two-step filtration, which exhibits greater sensitivity, specificity, speed and quantification of interested analytes. PRM is a recently developed technology in targeted metabolomics analysis. In comparison with MRM, all the product ions generated in Q2 collision cell are transferred into orbitrap (high resolution) analyzer to allow the parallel detection of all fragments of the precursor ion. Due to massive information of high resolution fragments of the precursor, PRM yields quantitative data over a broader dynamic range than MRM (or SRM) in complex matrix since PRM’s high selectivity in the mass-to-charge (m/z) domain. A great quantity of studies have investigated the application of targeted quantification method in detection various kinds of molecules. Zongwei Cai and colleagues have developed a targeted method in detecting 52 ribonucleosides in urine by using MRM method [70]. Li Liang and colleagues developed a targeted MRM method in detecting
355 acylcarnitines and 65 acylglycines in urine through MRM modes [71, 72].

Besides, a great number of studies have reported the application of targeted quantification of low abundant molecules, such as detection of acyl-CoA [73], which barely detected by untargeted metabolomics study.

In summary, both untargeted and targeted metabolomics have advantages and disadvantages. Generally, non-targeted metabolomics studies carry out first to obtain an overview of metabolite changes in response to stimuli, then targeted metabolomics studies will be performed on interested pathways that based on the non-targeted metabolomics study. In order to get more comprehensive information of disturbed metabolites, the integration of both untargeted and targeted metabolomics approaches has been widely used to investigate the metabolic changes in response to stimuli [74-76].
Figure 1.5 Comparison of SIM, MRM (SRM) and PRM in targeted metabolomics analysis.

1.2.5 Application of metabolomics in studying T2DM

Metabolomics is a powerful tool in study complex disease, such as diabetes. T2DM is characterized by cells that do not respond to produced insulin from beta cell. A comprehensive understanding of the pathophysiology of T2DM is necessary for the development and protecting of this disease. Particularly, identification of early metabolic changes is promising in the study of pathogenic factors and may be able to help figure out the mechanism of T2DM and
interventions of T2DM. A number of biomarkers have been performed as indicators of T2DM risks, such as fasting glucose level in blood, triglycerides [77], glycated hemoglobin A1C [78], high density lipoprotein (HDL) cholesterol [79], adiponectin [80, 81], inflammatory markers [82], liver enzymes and fetuin-A [83]. However, a majority of existing biomarkers failed to grasp the complexity of the pathology of T2DM [78]. A high-throughput analytical technique, metabolomics, which has the ability to study a numerous of low-molecular weight compounds simultaneously, might be helpful to reveal the potential mechanisms. These differential compounds (also called metabolites) from metabolomics analysis, represent intermediates and end products of metabolic changes could reflect physiological dysfunctions rapidly [84].

A large number of studies have reported the metabolic shift of pathology and intervention of T2DM. As shown in Figure 1.4 [85], glucose and related carbohydrate metabolites have been confirmed as biomarkers of diabetes. As the serum concentration of complex sugars increase, glycolipid and glycoprotein will be metabolized into N-acetylglucosamine, sialic acid moieties or 1,5-anhydroglucitol (1,5-AG). We have found that the 1-deoxy form of glucose is a marker of short-term glycemia [86, 87]. The branched-chain amino acids (BCAAs, valine, leucine and isoleucine) are all increased in diabetes group, which indicates that it might be induced by an impaired short-term metabolic control [88]. This phenomenon is concordant with other studies such as measurement in diabetic mice, which also found that the concentration of BCAAs was increased as well [89]. Besides, db/db (leptin receptor deficiency) diabetic mice present significant evidence of increased gluconeogenesis, which might result in a strongly decreased concentration of gluconeogenic amino acids,
such as glycine, serine and alanine [89]. Other studies have also observed metabolic changes in diabetes, such as bile acids in guts [90], fatty acids and phospholipids in adipose tissue [91], and so on. All these findings showed that metabolomics is an efficient and powerful tool in investigating the pathological mechanism of diabetes and intervention for treating diabetes.

Figure 1.6 A systemic view of metabolic changes that associate with diabetes. BCAAs, branched-chain amino acids. Source: reference 84.

1.3 Objectives

Considering the regulatory effect of exercise on T2DM and T2DM induced complications have not been clearly clarified, and metabolomics analysis is a powerful tool in investigating the mechanism of interventions of disease with complex metabolic disorders, in this study, mass spectrometry based metabolomics techniques (both untargeted and targeted metabolomics) have been applied to reveal the regulatory effect of exercise on db/db diabetic mice in
various kinds of biofluid and tissues. Due to the large range of polarities of the endogenous metabolites, LC-MS and GC-MS analysis will be performed according to the need of the experiments. Targeted analysis of some endogenous metabolites has also been established to give a relatively more comprehensive view of the metabolic changes in response to T2DM and exercise. Besides, verification of several pathways in transcript RNA level has also been carried out. The detailed objectives are listed as follows:

1) Establish robust LC-MS and GC-MS analysis methods for untargeted metabolomics analysis of biological samples for a better understanding of regulatory effect of exercise on db/db diabetic mice.

2) To establish targeted metabolomics platform for acylcarnitines and acyl-CoA species by high resolution PRM or SRM methods.

3) Untargeted metabolomics study in plasma samples to give an overview of metabolic changes on db/db mice in response to exercise.

4) Untargeted metabolomics assay in skeletal muscle to investigate the regulatory effect of moderate intensity of exercise on db/db mice.

5) Untargeted and targeted analysis on db/db mice in kidney samples to reveal the regulatory effect of exercise in alleviating diabetic nephropathy induced inflammation.

6) Relevant expression of RNAs in lipid metabolism and BCAA catabolism pathways in the kidney of db/db mice was measured to verify the regulatory effect of exercise in improving inflammation that induced by diabetic nephropathy.
Chapter 2 Metabolomics study of regulatory effects of regular exercise on

$db/db$ mice in plasma

2.1 Introduction

Type 2 diabetes mellitus (T2DM) is one of the most common diseases worldwide [92, 93]. This disease causes an annual cost of about 132 billion US dollars for the United States Healthcare System [94]. T2DM is mainly characterized by dysfunction of insulin secretion and insulin resistance [95, 96], and closely associated with obesity [97-99]. Recently, the growing evidences on pathophysiologic mechanisms, such as pancreas islet axis dysfunction, increase in glucagon secretion, insulin resistance in brain, impaired ability of glucose reabsorption in kidney and lipotoxicity, advance the understanding of this disease [100-102]. Thus, T2DM is caused by multiple set of pathogenesis. Those complexities face challenges for a comprehensive understanding of metabolic disorders that contribute to the development of this disease, which also add difficulty for the prevention and treatment of T2DM.

For decades, exercise along with diet and medication, has been considered as one of the effective approach of diabetes management [103-105]. Regular exercise training is highly recommended for patients with T2DM since physical activity may have beneficial effects on regulating metabolic dysfunction during the development of diabetes complications [106]. Lots of studies have been carried out to investigate the mechanism of exercise on T2DM [107-110]. However, the impingement of physical activity on metabolism in T2DM has not been fully understood so far.

Process in investigating the exercise effect on multi-pathogenesis diseases (such as T2DM) can be promoted by advanced technologies for comprehensive
metabolic analysis, which termed as “metabolomics”. Metabolomics is a quantitative measurement of low molecular weight endogenous metabolites in response to pathophysiological or physiological stimuli [111, 112]. It enables simultaneous quantification of endogenous metabolites in a small sample volume which prepared from any type of samples (body fluid and tissues) [113]. Metabolomics studies had been applied to study the differences between insulin sensitive and resistant patients with obesity [91], and compare metabolic changes between diabetic and lean individuals [114]. Jane Sharer et al. [109] used metabolomics approach to study metabolic response to exercise on lean and diet-induced obese mice. Thus, this systematic and unbiased method is a powerful tool in investigating whether taking regulatory exercise alters the basal metabolite profile and regulates T2DM induced shifts in metabolic spectra.

In the current study, liquid chromatography mass spectrometry (LC-MS) based metabolomics approach was applied to study the exercise effect on db/db diabetic mice. Db/db mouse is the most frequently used T2DM mouse model. It derives from an autosomal recessive mutation which is traced to db gene (encoded for leptin receptor) on chromosome 4 on C57BL/KsJ mouse [115, 116]. Lacking of functioning leptin receptor can lead to failure to control food intake, cause obesity, insulin resistance, hyperglycemia and dyslipidemia [117, 118]. Relative to homozygote db/db diabetic mouse, the heterozygote mouse db/m+ mouse is selected as non-diabetic control mouse. In this study, metabolomics study found that when comparing the regulatory effects of physical activity on db/m+ control mice and db/db diabetic mice, it was found that exercise might exert good effect on the dysfunction of energy metabolism
induced by T2DM, especially on fatty acid metabolism, which may finally play a role on improving diabetic induced insulin resistance.

2.2 Experiment

2.2.1 Chemicals and materials

HPLC grade Acetonitrile was bought from Tedia Company (Fairfield, OH, USA). HPLC grade methanol was bought from RIC Labscan Ltd. Co., (Bangkok, Thailand). Pure water was prepared from a Milli-Q Ultrapure water system (Millipore, Billerica, MA, USA). Formic acid (FA) was bought from Sigma - Aldrich (St. Louis, MO, USA). ACQUITY UPLC HSS T3 column (100 × 2.1 mm i.d., 1.7 µm particle size) (Waters, Milford, MA) was used in this study. Authentic standards of L-proline, L-lysine, acetylcarnitine, L-arginine, palmitoylcarnitine, glutamine, L-carnitine, fumaric acid, L-tyrosine, uridine, pantothenic acid and creatine were bought from Sigma- Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

2.2.2 Animal experiments

In this study, C57BL/KsJ db/db mouse (male) was selected as type 2 diabetes mellitus (T2DM) model mouse, while C57BL/KsJ db/m+ mouse (male) was used as control mouse. Each type of mouse was randomly assigned to sedentary and exercise treated groups. The amount of mouse in each group was listed as follows: db/db mouse group treated with sedentary, n = 8; db/db mouse group treated with exercise n = 8; db/m+ mouse group treated with sedentary, n = 9; db/m+ mouse group treated with exercise, n = 9. The animal experimental procedures were conformed and approved by local ethics committee, the Universite’ catholique de Louvain and the Rangueil Hospital animal ethics
committee. All mouse were kept in a specific pathogen-free (SPF) colony (inverted 12 hours daylight cycle and lights-off at 10:00 a.m.), and fed standard laboratory chow (mouse No. 3 Breeding, Special Diets Services) and ad libitum [119]. The detailed information of exercise protocol was listed as follows: mouse was treated under moderate intensity of exercise on the treadmill (Columbus Instruments) in the morning, run at 8 m/min, lasted for 30 min, 6 days a week and continuously for 4 weeks. To stimulate the stress induced by physical activity, sedentary animals were also put in a stationary treadmill for acclimation. Animals were rested for 24 h to negate any direct effects of physical activity [120] at the end of exercise period.

After that, all animals were weighed. The animals were firstly anesthetized with pentobarbital, whole blood was collected to a 1.5 mL micro-centrifuge tube (moistened by heparin sodium). Blood was centrifuged at 13,200g for 10 min at 4 °C, the supernatant were collected and stored at – 80 °C until analysis. The blood samples were collected with overnight fasting between 8:00 a.m. to 10:00 a.m. The Blood glucose level was measured with a glucose meter (Roche Diagnostics, Meylan, France), and the level of insulin was also measured as described previously.

### 2.2.3 Sample preparation

After thawed, 200 μL of methanol were added to 50 μL aliquots of plasma samples (Figure 2.1). The mixture was vortexed (1 min), and centrifuged at 13,200 rpm for 10 min (4 °C). The supernatant was collected, dried under gentle nitrogen stream, then stored at -80 °C before analysis. The dried samples were reconstituted in 50% methanol (methanol/water (v/v) = 1:1) for analysis.
Quality control (QC) sample was prepared by pooling the same volume (10 μL) of each reconstituted plasma sample. QC sample was injected randomly throughout the experiment to observe the stability of analysis system in terms of retention time, masses and intensities. In order to minimize the carry-over throughout the analysis, blank sample (acetonitrile) was also injected every 3 sample injections. Stock solution was firstly prepared in methanol, and then diluted to an appropriate concentration for verification analysis.

![Diagram of plasma sample preparation](image)

Figure 2.1 Process of plasma sample preparation.

### 2.2.4 Instrument analysis

Analyses were operated on a Thermo Scientific quaternary Accela 1250 pump coupled to a LTQ Orbitrap TM XL mass spectrometer (Thermo Fisher Scientific, MA, USA). Column oven was kept at 40 °C, flow rate was 0.4 mL min⁻¹. The mobile phases were acetonitrile (B) with 0.1% formic acid and ultrapure water
(A) with 0.1% formic acid. In this study, experiments were operated in both negative and positive ionization modes. The injection volume was 8 μL in negative and 10 μL in positive ionization modes. The elution gradients are shown in Table 2.1. The detailed parameters of MS were set as follows: Sheath Gas Flow Rate: 50 arb; I Spray Voltage: 3 kV; Heater Temp.: 350 °C; Capillary Temp.: 300 °C; Capillary Voltage: 50 V; Tube Lens: 80.00 and Aux Gas Flow Rate: 5 arb. The mass scan range was m/z 100 to 1000. MS/MS data was analyzed in targeted MS/MS mode and with 3 collision energies, 10 eV, 20 eV and 40 eV, respectively.

Table 2.1 LC gradient in negative and positive ionization modes.

<table>
<thead>
<tr>
<th>No.</th>
<th>Negative ion mode</th>
<th>Positive ion mode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (min)</td>
<td>Time (min)</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>0.0%</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>0.0%</td>
</tr>
<tr>
<td>3</td>
<td>9.0</td>
<td>50.0%</td>
</tr>
<tr>
<td>4</td>
<td>16.0</td>
<td>100.0%</td>
</tr>
<tr>
<td>5</td>
<td>20.0</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

2.2.5 Quality control

Stability and repeatability were measured to evaluate the performance of LC-MS system. According to different masses (m/z) and polarities, five ions have been selected for method validation in terms of the variation of their retention times and peak intensities. The data from QC samples were applied for evaluation of repeatability and stability of the large-scale analysis. Stability was evaluated by 6 QC samples analyzed throughout the sequence. Repeatability was evaluated by continuous injection of QC samples for 6 times. Peak intensity and retention time and of the five extracted ions were used to analyze the variation.
2.2.6 Data processing and multivariate statistical analysis

Raw LC-MS data were initially converted into mzXML format. Then extracted by R with the program of XCMS [121] (https://xcmsonline.scripps.edu/). Retention time was aligned and peak intensity was extracted in each chromatogram. A table including the information of retention time, m/z and ion intensity was generated. The extracted data were imported into SIMCA-P software (Ver. 11, Umetrics, Umea, Sweden) for further multivariate statistic analysis. Both partial least squares Discriminant Analysis (PLS-DA) and Principal component analysis (PCA) was applied for multivariate statistic data analysis. Comparison of the metabolic changes was carried out between OS and LS groups. Notably, quality control analysis was important prior to sample analysis. One-way analyses of variance (ANOVAs) were chosen for significant analysis. Differences were considered when $P < 0.05$.

2.3 Results

2.3.1 Exercise performance of mice on Pharmacological parameters

In $db/db$ mice, leptin resistance results in rapid gains in body weight. As was shown in Figure 2.2A, the average weight in the $db/db$ sedentary (OS) group and $db/db$ exercise training (OE) group were significantly increased compared with the $db/m+$ sedentary (LS) group. However, there was no significant regulatory effect from exercise training in both the $db/db$ and $db/m+$ mice groups on weight loss. Besides, we all know that the increased blood glucose level is an important indicator of diabetes, while insulin is the principal hormone in regulating the uptake of glucose from blood into the cells of the body. In this study, blood glucose levels were examined on $db/m+$ and $db/db$ mice regardless
of the sedentary or physical activity treatment. We can see from Figure 2.2B that the level of blood glucose significantly increased in the obesity mice groups (OS and OE) compared with the lean mice groups (LS and LE). However, physical activity did not show any regulatory effect on the increased blood glucose levels in obese mice. An oral glucose tolerance test (OGTT) was also carried out, and we can see from Figure 2.2C that after oral glucose injection, the blood glucose levels (120 min) significantly increased in the OS and OE groups, but did not account for any statistical significance between the two groups after exercise training. However, the results of the insulin tolerance test (ITT) showed a significant decrease in blood glucose levels (120 min) for db/db mice after exercise training compared with the OS group (Figure 2.2D), indicating that physical activity might have the ability to help improve insulin resistance which is a pathological condition that fail to respond normally to the hormone insulin. All the physiological characteristic results indicated that the model of diabetes was successful and exercise training might exert positive regulatory effects on insulin resistance in diabetic mice.

Figure 2.2 Regulatory effect of exercise on pharmacological parameters.
Note: Body weight (A), blood glucose (B), OGTT (C) and ITT (D)).

### 2.3.2 Method validation

To assess system reliability throughout sequencing analysis on LC-MS, stability and repeatability was performed (Table 2.2). For the validation of system stability, the relative standard derivations (RSDs) of these 5 ions were less than 1.28% for retention times and 7.66% for peak intensities. For the validation of system repeatability, the RSDs of selected 5 ions were less than 1.36% for retention time and 7.78% for peak area intensities. The data demonstrated that the stability and repeatability of this proposed method was satisfactory for the large-scale sample sequence analysis.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Repeatability</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area (RSD %)</td>
<td>r.t. (RSD %)</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>1.35</td>
<td>0.3</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>6.14</td>
<td>1.36</td>
</tr>
<tr>
<td>12-HETE</td>
<td>3.64</td>
<td>0.04</td>
</tr>
<tr>
<td>Palmitoylcarnitine</td>
<td>4.05</td>
<td>0.11</td>
</tr>
<tr>
<td>Acetylleucine</td>
<td>7.78</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Note: RSD, relative standard deviation. r.t., retention time.

### 2.3.3 Multivariate statistical profiling analysis

In this study, multivariate statistical analysis PCA and PLS-DA approach was applied in metabolic profiling analysis for biomarker identification related to the development of T2DM. $R^2_X$, $R^2_Y$ and $Q^2$ (cum) are important parameters to evaluate the PLS-DA model. In order to acquire a high predictive ability of the model and prevent the model from over-fitting, the value of the three parameters should be close to 1. The established PLS-DA model in this study using cross
validation could describe 84.0% of the variation in X ($R^2_X = 0.84$), 99.0% of variation in Y ($R^2_Y = 0.99$), and with a predictive ability of 86.6% ($Q^2_{(cum)} = 0.866$) in negative ion mode. Meanwhile, the value of $R^2_X$, $R^2_Y$ and $Q^2_{(cum)}$ were 0.72, 1.00 and 0.992 respectively in positive ion mode. All the parameters indicated that the established models obtained satisfactory grouping and predicating ability.

2.3.4 Identification of metabolites in response to T2DM and exercise training

Based on PLS-DA analysis (Figure 2.3), detected ions (1315 ions in negative ionization mode and 1392 ions in positive ionization mode) which contributed to the discrimination and clustering were selected according to the threshold of variable importance in the projection (VIP) values (VIP > 1.5). At last, 24 features (15 ions in positive ionization mode and 9 ions in negative ionization mode) were identified by searching MS and MS/MS fragments in Biofluid Metabolites Database (http://metlin.scripps.edu) and Human Metabolome Database (HMDB) (http://www.hmdb.ca), and part of them were confirmed by authentic standards (Including L-lysine, glutamine, L-proline, L-valine, L-arginine, L-tyrosine, malic acid, L-palmitoylcarnitine, pantothenic acid, fumaric acid and uridine). The detailed information of identified biomarkers was listed in Table 1 and Table 2. These biomarkers were mainly classified into amino acids and their derivatives, organic acids and lipids. Three biomarkers, including creatine, uridine and lysine (Figure 2.6) were successfully reversed by exercise training that induced by T2DM. Interestingly, the concentration of pantothenic acid, palmitoylcarnitine and hydroxyphenyllactic acid (Figure 2.7) were specifically and significantly increased after physical activity in diabetic mice.
Figure 2.3 PLS-DA score plots of db/m+ and db/db mice in plasma samples.

Note: (a) Score plot from positive ion mode. Each red dot represents a sample from db/db sedentary group (OS); each black square represents a sample from db/m+ sedentary group (LS). (b) Score plot from negative ion mode. (c) Loading plot of (a). Each triangle represents a detected ion (observation). The selected red triangle represents VIP > 1.5. The further away of the triangles from the center, the larger of the VIP value.
Figure 2.4 Example of metabolite identification.
Figure 2.5 PLS-DA score plots of exercise effect on $db/m+$ control and $db/db$ diabetic mice.

Note: (a) PLS-DA score plot in negative ion mode; (b) PLS-DA score plot in positive ion mode.
Figure 2.6 PCA score plots of \(db/m+\) control and \(db/db\) diabetic mice in plasma samples.

Note: N1-5: PCA score plots in negative ion mode. P1-5: PCA score plots in positive ion mode. LS: \(db/m+\) mice treated with sedentary, black square; LE: \(db/m+\) mice treated with exercise training, red dot; OS: \(db/db\) mice treated with sedentary, blue diamond; OE: \(db/db\) mice treated with exercise training, green triangle.
<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>R.T.</th>
<th>m/z</th>
<th>Ion modes</th>
<th>Errors (ppm)</th>
<th>Fragment ions</th>
<th>Metabolites category</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lysine (^a)</td>
<td>0.60</td>
<td>147.1125</td>
<td>positive</td>
<td>0</td>
<td>130.0826; 84.0808</td>
<td>Amino acids and derivatives</td>
</tr>
<tr>
<td>2</td>
<td>Glutamine (^a)</td>
<td>0.68</td>
<td>145.0621</td>
<td>negative</td>
<td>1</td>
<td>127.0514; 109.0410</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Proline (^a)</td>
<td>0.75</td>
<td>116.0711</td>
<td>positive</td>
<td>4</td>
<td>70.0650</td>
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</tr>
<tr>
<td>4</td>
<td>Valine (^a)</td>
<td>0.79</td>
<td>118.0863</td>
<td>positive</td>
<td>0</td>
<td>72.0807; 55.0541</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Creatine (^b)</td>
<td>0.96</td>
<td>132.0766</td>
<td>positive</td>
<td>0</td>
<td>114.0658; 90.0549</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Arginine (^a)</td>
<td>1.11</td>
<td>175.1187</td>
<td>positive</td>
<td>0</td>
<td>158.0922; 157.1082; 70.0650</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4-Guanidinobutanoic acid (^b)</td>
<td>1.42</td>
<td>146.0924</td>
<td>positive</td>
<td>0</td>
<td>128.0817; 87.0440</td>
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<td>8</td>
<td>Tyrosine (^a)</td>
<td>2.92</td>
<td>182.0812</td>
<td>negative</td>
<td>0</td>
<td>165.0547; 136.0753; 123.0440</td>
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</tr>
<tr>
<td>9</td>
<td>Hippuric acid (^b)</td>
<td>6.33</td>
<td>178.0508</td>
<td>negative</td>
<td>0</td>
<td>134.0611</td>
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</tr>
<tr>
<td>10</td>
<td>N-Acetylleucine (^b)</td>
<td>6.78</td>
<td>172.0978</td>
<td>negative</td>
<td>0</td>
<td>130.0872</td>
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<tr>
<td>11</td>
<td>DL-2-Amino-octanoic acid (^b)</td>
<td>7.40</td>
<td>160.1333</td>
<td>negative</td>
<td>0</td>
<td>114.1278</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Acetyl-tryptophan (^b)</td>
<td>9.19</td>
<td>247.1080</td>
<td>positive</td>
<td>1</td>
<td>229.0973; 205.0965; 201.1023; 188.0708</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Malic acid (^a)</td>
<td>0.79</td>
<td>133.0141</td>
<td>negative</td>
<td>1</td>
<td>115.0036; 71.0137</td>
<td>Organic acids</td>
</tr>
<tr>
<td>14</td>
<td>Hydroxyphenyllactic acid (^b)</td>
<td>5.76</td>
<td>181.0505</td>
<td>negative</td>
<td>0</td>
<td>163.0400; 135.0449</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Suberic acid (^b)</td>
<td>7.04</td>
<td>173.0818</td>
<td>negative</td>
<td>0</td>
<td>111.0815</td>
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<tr>
<td>16</td>
<td>12-HETE (^b)</td>
<td>13.95</td>
<td>319.2274</td>
<td>negative</td>
<td>1</td>
<td>301.2170; 179.1071</td>
<td>Lipids</td>
</tr>
<tr>
<td>17</td>
<td>L-Palmitoyl carnitine (^a)</td>
<td>17.78</td>
<td>400.3430</td>
<td>positive</td>
<td>0</td>
<td>341.2688; 239.2371</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>LysoPC(16:0) (^b)</td>
<td>17.25</td>
<td>496.3410</td>
<td>positive</td>
<td>0</td>
<td>478.3309; 184.0738</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>LysoPC(20:0) (^b)</td>
<td>20.81</td>
<td>552.4030</td>
<td>positive</td>
<td>0</td>
<td>534.3923; 184.0735</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>choline (^a)</td>
<td>0.72</td>
<td>104.1056</td>
<td>positive</td>
<td>1</td>
<td>60.0819</td>
<td>Others</td>
</tr>
<tr>
<td>21</td>
<td>Fumaric acid (^a)</td>
<td>0.80</td>
<td>115.0036</td>
<td>negative</td>
<td>0</td>
<td>71.0138</td>
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<tr>
<td>22</td>
<td>Uridine (^a)</td>
<td>2.76</td>
<td>243.0617</td>
<td>negative</td>
<td>0</td>
<td>200.0560; 110.0243</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>p-Coumaric acid (^b)</td>
<td>2.92</td>
<td>165.0546</td>
<td>positive</td>
<td>0</td>
<td>147.0440; 119.0491; 91.0543</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Pantothenic acid (^a)</td>
<td>5.59</td>
<td>220.1182</td>
<td>positive</td>
<td>1</td>
<td>202.1075; 184.0969; 90.0549</td>
<td></td>
</tr>
</tbody>
</table>

Note: \(^a\) represents metabolites that identified by authentic standards; \(^b\) represents metabolites that identified by accurate MSMS spectrum.
2.4 Discussion

Multivariate analysis methods PLS-DA and Principal Component Analysis (PCA) have been applied to give an overview of the regulatory effect of exercise training on different types of mice. As we can see in Figure 2.5 and Figure 2.6, *db/db* diabetic mice groups were clustered far away from *db/m+* mice groups in both positive and negative ion modes regardless of the treatment of sedentary and exercise training, indicating that there were notable metabolic changes between the two types of mice. When assessing the regulatory effect of exercise training on the two types of mice, we found that LE group was overlapped with LS group in both positive and negative ion modes (Figure 2.5 and Figure 2.6), showing that the metabolic change was small between the two groups, and indicating that exercise has no significant regulatory effect on lean mice (*db/m+* mice). However, OE group can be clearly separated from OS group in both ionization modes, suggesting that exercise might affect body metabolism in diabetic mice. Furthermore, when evaluating the metabolomic alterations of diabetes based on identified biomarkers, we found that 20 identified biomarkers were significantly changed between LS and OS groups. Meanwhile, when evaluating the metabolomic alterations of exercise training on *db/m+* and *db/db* mice, we found that only one of the Branched-chain amino acids (BCAAs), valine, was significantly changed after exercise training on *db/m+* mice, which in accordance with the findings that the concentration of valine were lower after short-term acute exercise training in human skeletal muscle [123]. However, there was no significant changes of valine between OS and LS group, indicating that the changes of valine might not related to diabetes, but related to exercise regardless of control mice or diabetic mice. Interestingly, 9 biomarkers,
including hippuric acid, DL-2-aminoctanoic acid, hydroxyphenyllactic acid, lysoPC(16:0), L-palmitoylcarnitine, uridine, pantothenic acid, L-lysine, and creatine were significantly changed after exercise training on diabetic mice.

2.4.1 Regulatory effect of exercise on creatine may reveal good effect on diabetes induced dysfunction of energy metabolism

Here we detected creatine (Figure 2.7). Indeed, creatine generally behaves as phosphocreatine (PCr) and has the ability to increase the stores of PCr, potentially through increasing the ability of muscle to regenerate ATP from ADP to match energy demands. In this study, we found that the concentration of creatine was decreased (p < 0.05) in OS group when compared with LS group. The observation was in accordance with the findings by Gavin E. Duggan et al. [109], who found that the level of creatine was also significantly decreased in obesity mice under high fat diet. The results indicated that decreased concentration of creatine might cause shorter storage of PCr in muscle and might further cause accumulation of fatty acids which contribute to body fat mass [124, 125]. Interestingly, it was found that exercise could significantly reverse the decreased concentration of creatine to normal level (LS group), indicating that moderate exercise might have good reverse effects on the dysfunction of energy metabolism that induced by diabetes.
Figure 2.7 Significantly regulated metabolites by exercise.

Note: UDP: Uridine diphosphate; CK: Creatine Kinase; Gluc-6P: glucose-6-phosphate; Gluc-1P: glucose-1-phosphate. #: $P$ value < 0.05, OS vs. LS; ##: $P$ value < 0.01, OS vs. LS; ###: $P$ value < 0.001, OS vs. LS; ☆: $P$ value < 0.05, OE vs. OS.

2.4.2 Regulatory effect of exercise on uridine may indicate positive effect on promoted insulin sensitivity

Uridine, which contributes to the intracellular uridine diphosphate/uridine triphosphate (UDP/UTP) pool, is involved in the process of glycogen synthesis. In glycogenesis (Figure 2.7), glucose is first phosphorylated to glucose-6-phosphate (Gluc-6P) under participation of adenosine triphosphate (ATP) and converted to glucose-1-phosphate (Glucose-1P). Then, uridine diphosphoglucose
UDP-glucose is formed from glucose-1-P and UDP. In the final step, glycogen is generated from UDP-glucose together with the release of UDP. Since oral administration of glucose will increase the plasma concentration of uridine [126], it is suggested that UDP which is released from UDP-glucose, increases sharply along with abrupt decrease of UDP-glucose [127, 128] through glycogenesis process. Luciano Rossetti and colleagues [129] found that UDP-glucose depletion was effective in inducing skeletal muscle insulin resistance by infusion of uridine alone. In other words, extracellular uridine enhances UDP-glucose accumulation and reduces insulin sensitivity. In our study, it was found that the concentration of uridine in plasma was increased in OS group, indicating that db/db mice might induce insulin resistance through dysfunction of uridine metabolism. It is likely that exercise training could significantly increase plasma uridine to normal level by comparing OE group with LS group, indicating that uridine in blood might be an important indicator to reflect insulin sensitivity promoted by exercise training in diabetic mice.

2.4.3 Exercise effect on fatty acid metabolism pathway

Additionally, the induction of insulin resistance is induced by dysfunction of glucose, lipid metabolism and its metabolic sequelae. Of particular importance is that fatty acids can be metabolized via mitochondrial fatty acid β-oxidation (FAO) to prevent insulin resistance [130] (Figure 2.9). During this process, coenzyme A (CoA) plays an important role in tricarboxylic acid (TCA) cycle at the beginning of this pathway. Pantothenic acid, which is required in the formation of CoA, plays a critical role in the metabolism and synthesis of proteins and nutrient supplies [131, 132]. However, FA needs to be activated before binding to carnitine to generate “acylcarnitine” to enter the inner
membrane of mitochondrial (IMM) for β-oxidation. Firstly, the free FAs in cytosol are bonded to CoA to form acyl CoA. Secondly, acyl CoA is transferred to carnitine molecule and combined with hydroxyl group of carnitine by carnitine acyltransferase I (CPTI) to form acylcarnitine on the outer side of mitochondrial. Thirdly, acylcarnitine will transport through carnitine-acylcarnitine translocase inside the mitochondrial matrix. Finally, with the help of carnitine acyltransferase II (CPTII) located on the inner side of mitochondrial membrane, acylcarnitine is converted to acyl CoA and free FAs which will be metabolized through β-oxidation to obtain usable energy (Adenosine triphosphate (ATP)) through the TCA cycle. Pantothenate Kinase (PanK) is the rate controlling step at the beginning of the biosynthesis of CoA through the formation of 4’-phosphopantothenate (4P-Pan) \[133]\] from pantothenic acid. In this study, we have detected that the concentration of pantothenic acid (Figure 2.9) that required for the formation of CoA was dramatically increased after exercise training between T2DM groups (OE vs. OS) but not between lean control mice groups (LE vs. LS), indicating that diabetic mice might be more sensitive to physical activity than lean control mice for FA β-oxidation through increasing the concentration of obligatory activator of CoA. Interestingly, the regulatory effect of pantothenic acid on obese patients in plasma were also significantly increased after exercise training \[134]\], which in accordance with our hypothesis that physical activity might have good regulatory effect on improving the efficiency of FAO. Furthermore, worth mentioning is palmitoylcarnitine (C16-CN), Roberta Leonarni and colleagues \[135]\] have reported that palmitoylcarnitine plays a positive role in the reversal of acetyl-CoA inhibition of PanK2 (Figure 2.9). In this study, the concentration
of palmitoylcarnitine was significantly increased only in diabetic mice group by exercise training but not in lean mice group. Combined with the phenomenon of regulatory effect of pantothenic acid, it is indicated that diabetic mice might be more susceptible than lean mice in improving FAO to meet the increased energy demands during exercise training. However, many studies have also reported that the increased concentration of palmitoylcarnitine could also play a role in cytotoxicity in Caco 2 and IEC-18 cells [136], indicating that in addition to good regulatory effect on diabetic mice through improving the efficiency of FAO, exercise training might also cause cytotoxicity specifically on diabetic mice.

Figure 2.8 Exercise effects on pantothenic acid, palmitoylcarnitine and hydroxyphenyllactic acid.

Note: ☆ P value < 0.05, OE vs OS. #: P value < 0.05, OS or OE vs LS; ##: P value < 0.01, OS or OE vs LS; ###: P value < 0.001, OS or OE vs LS; Data is displayed as mean ± SD.
Figure 2.9 Pathway of pantothenic acid related to fatty acid β-oxidation.

Note: IM: inner side of the mitochondrial; OM: outside of the mitochondrial; IMM: inner side membrane of mitochondrial; OMM: outside membrane of mitochondrial. Note: 4P-Pan: 4'-phosphopantothenate; Pan: pantothenic acid; FAs: fatty acids; PanK2: pantothenate kinase 2; AC: acylcarnitines; CACT: carnitine-acylcarnitine translocase; CPT1: Carnitine palmitoyltransferase I, CPT II: Carnitine palmitoyltransferase II; TCA: Citric acid cycle.

Besides, we all know that lysine and methionine provide the carbon backbone for carnitine. Randall S. et al. have found that an dietary supplementation of lysine could lower the concentration of free carnitine in rats’ plasma [137]. Klaus Eder and colleagues [138] also reported that high lysine diet had lower concentration of total and free carnitine in plasma, kidney, liver and skeletal muscle in pigs. In this study, the concentration of lysine was significantly lower in OS group when compared with db/m+ mice groups (Figure 2.7), indicating that the decreased concentration of lysine might associate with a reduction of free carnitine which plays a critical role in transporting FA into mitochondrial matrix to yield energy through β-oxidation. However, the concentration of lysine was significantly increased when compared with OS group after exercise.
training on diabetic mice, indicating that physical activity might also get positive effect on regulating FA β-oxidation via carnitine metabolism pathway. Furthermore, Hydroxyphenyllactic acid (Figure 2.8), metabolite of tyrosine, has been shown that may function as a natural anti-oxidant by decreasing reactive oxygen species (ROS) production in mitochondria [139]. In this study, the concentration of hydroxyphenyllactic acid has been dramatically increased (5.07 fold change, OE vs OS (See Supplementary Table 1)) specifically in diabetic mice group after exercise training, indicating that diabetic mice might be more susceptible than db/m+ mice in decreasing mitochondria ROS production during exercise training.

In summary, our results presented that moderate exercise training might got positive regulatory effect in improving energy metabolism and insulin sensitivity. However, the results also showed an altered fatty acid β-oxidation in mitochondrial defect [140, 141] and the dysfunction of mitochondrial ROS production. This could be that a greater length of time, along with a more intense exercise of training that might be needed in our future experiment to particularly investigate whether the defect of β-oxidation in T2DM mice can be improved.

2.5 Summary of this chapter

Untargeted metabolomics profiling of plasma from db/db diabetic mice with and without exercise revealed that physical activity might have positive regulatory effects on energy metabolism associated with creatine/phosphocreatine system. Of note, the level of plasma uridine was also reversed by exercise training in a good agreement with the change of plasma insulin level, which indicated that uridine in blood might be an important indicator to reflect insulin sensitivity that
promoted by exercise training. Meanwhile, FA catabolism through β-oxidation was more susceptible to physical activity on diabetic mice than lean mice. These metabolic markers demonstrated in plasma could support our notion that physical activity did have positive impacts on T2DM mice and advance our understanding of the underlying mechanism linked to insulin sensitivity.

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Chapter 3 Comprehensive analysis of acylcarnitine species in db/db mouse using a novel method of high resolution parallel reaction monitoring reveals widespread metabolic dysfunction induced by diabetes

3.1 Introduction

Carnitine [3-hydroxy-4-(trimethylazaniumyl)butanoate] is an amino acid derivative mainly obtained from diet which plays a crucial role in fatty acid oxidation. Within a biological system, carnitine can be conjugated with fatty acids (FAs, both endogenous and exogenous) to form acylcarnitines (ACs), which facilitates FAs transport into the inner membrane of mitochondria where fatty acids β-oxidation (FAO) takes place[142]. Various kinds of ACs can serve as biomarkers for the disturbed intermediary metabolism, including metabolic changes related to disease pathogenesis (inborn errors of metabolism, renal tubular disease, and type 2 diabetes mellitus [143-146]). For instance, the accumulation of even-numbered chain ACs is a positive reflection of incomplete FAO, whereas the accumulation of short chain ACs such as C3 and C5 ACs is related to elevations of branched-chain amino acids[144]. Long chain hydroxylated ACs are highly abundant in mouse heart. Dicarboxylic ACs, particularly short- to medium-chain dicarboxylic ACs have been identified as important biomarkers of acidemia and imminent cardiovascular events [147, 148].

Considerable effort has been carried out on comprehensive quantification of ACs. In comparison with many other methods such as ultraviolet and visible spectrum (UV) and nuclear magnetic resonance (NMR), liquid chromatography coupled with mass spectrometry (LC-MS) is the most frequently used approach for targeted analysis of small molecules due to its high sensitivity, and its ability
of simultaneous analysis in a broad range of complex biological mixtures in a relatively short time. Reversed phase and normal phase LC coupled with mass spectrometry has been widely used in targeted analysis of ACs owing to the fact that ACs generate common fragments on the basis of multiple reaction monitoring (MRM)[72, 149-153]. Although this method shows high precision and accuracy, the coverage of ACs may be limited, because the analysis of the exact structures usually remains elusive due to isomeric compounds.

Therefore, in order to get a comprehensive coverage of ACs, we developed a novel method for the robust, highly sensitive and rapid quantitative profiling of all ACs reported by previous studies [72] with the common fragments generated form ACs. Owing to the highly polar character of several short-chain acylcarnitines, both reversed phase and normal phase LC coupled to a high resolution Orbitrap mass spectrometer using parallel reaction monitoring (PRM) were performed (Figure 3.1). The PRM method could isolate targeted precursor ions, generate fragments from the precursor, and then detect the resulted product ions as well as precursors in the Orbitrap mass analyzer. Quantification was carried out based on the selected high resolution fragment ion coupled with high resolution precursor ion. This method could eliminate most interferences, providing accurate detection and quantification with confident targeted fragments confirmation.
Figure 3.1 Schematic representation of PRM method performed on Orbitrap-MS instrument.

The method applicability was demonstrated from the quantification of ACs in plasma and urine samples carried out on lean (db/m+) and diabetic obese mice (db/db). Ample evidence has revealed that type 2 diabetes and obesity could induce global metabolic changes of ACs. The excessive ACs supply and its accumulation interferes the insulin response[154], which might induce gluco-lipotoxicity[155] and diabetic ketosis[156]. Therefore, a more reliable method to quantify broader range of acylcarnitines is necessary to provide a comprehensive view of metabolic changes in response to the disease.

3.2 Material and methods

3.2.1 Reagents

HPLC grade methanol and acetonitrile were purchased from RIC Labscan Ltd. Co., (Bangkok, Thailand) and Tedia Company (Fairfield, OH, USA), respectively. Formic acid and ammonium acetate were acquired from Sigma - Aldrich (St. Louis, MO, USA). Water was prepared from a Milli-Q Ultrapure water system (Millipore, Billerica, MA, USA). The authentic standards, including $^2$H$_9$-carnitine ($d9$-C0-CN), $^2$H$_3$-acetyl-carnitine ($d3$-C2-CN), $^2$H$_3$-propionyl-carnitine ($d3$-C3-CN), $^2$H$_3$-butyryl-carnitine ($d3$-C4-CN), $^2$H$_9$-isovaleryl-carnitine ($d9$-C5-CN), $^2$H$_3$-octanoyl-carnitine ($d3$-C8-CN), $^2$H$_9$-myristoyl-carnitine ($d9$-C14-CN) and $^2$H$_3$-palmitoyl-carnitine ($d3$-C16-CN) were obtained from Cambridge Isotope Laboratories , Inc. (CIL, Andover, MA, USA). Free carnitine (C0-CN), acetyl-carnitine (C2-CN), propionyl-carnitine (C3-CN), isobutyryl-carnitine (isoC4-CN), 2-methylbutyryl-L-carnitine (C5-M-
CN), glutaryl-L-carnitine (C5-DC-CN), octanoyl-carnitine (C8-CN, decanoil-L-carnitine (C10-CN b), lauroyl-L-carnitine (C12-CN), myristoyl-carnitine (C14-CN), and palmitoyl-carnitine (C16-CN) were purchased from Sigma-Aldrich. 

$^2$H$_3$-Methylmalonyl-DL-carnitine ($d$3-C4-DC-CN), malonyl-L-carnitine (C3-DC-CN), succinyl-carnitine (C4-DC-S-CN), 3-hydroxybutryl-carnitine (C4-OH-CN) and 3-hydroxyisovaleroyl-carnitine (C5-OH-CN b) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). All other reagents were of analytical grades unless specified.

### 3.2.2 Animal experiments and sample collection

In this study, C57BL/KsJ $db/db$ male mice ($n = 8$) were selected as type 2 diabetes mellitus (T2DM) model animal, $db/m+$ male mice ($n = 9$) at similar age were selected as control mice. All animal experimental procedures were confirmed with and approved by the Hong Kong Government Department of Health, the Animal Research Ethical Committee of the Chinese University of Hong Kong and was consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Animals were weighed, and the blood glucose level, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were assessed with a glucose meter (Roche Diagnostics, Meylan, France) as described previously [157]. Urine was collected (12 h, fasted) before execution. At last, blood was collected into heparin sodium coated tubes, then centrifuged with a speed of 4000 rpm for 10 min at 4 °C to separate the plasma. All the samples were stored under -80 °C until analysis.
3.2.3 Sample preparation and metabolites extraction

For preparation of stock solution, the authentic standards were firstly dissolved in methanol, then diluted to appropriate concentration with 20% acetonitrile (acetonitrile/H$_2$O (v/v) = 20/80) and stored at -20 °C until analysis. For plasma samples, 4 times of ice cold methanol with appropriate concentration of internal standards (IS) was added to 50 µL of plasma for deproteinization. After vortex, the mixture was centrifuged with a speed of 14,200 rpm at 4 °C for 10 min. The supernatant was transferred into to a new Eppendorf tube, dried under vacuum desiccation at 4 °C. For preparation of urine, 100 µL of ice cold methanol (with appropriate concentration of IS) was added to 100 µL of urine, then treated the same way as the process of plasma. All the dried samples were stored under –80 °C until analysis.

3.2.4 Liquid chromatography coupled with Orbitrap mass spectrometry

The analysis was carried out on Ultimate 3000 UHPLC (Dionex), coupled with Orbitrap Fusion™ Tribrid™ MS (Orbitrap Fusion-MS, Thermo Scientific) system for compound separation and detection. A reversed phase liquid chromatography column, Luna C18 (2.1 mm i.d. ×100 mm, 1.6 μm particle size, Phenomenex) and Syncronis HILIC column (2.1 mm id × 100 mm, 1.7 μm) from Thermofisher (Runcorn, UK) were used in this study by two separate injections. For reversed phase column, the mobile phase A: water with 0.1% of formic acid, and mobile phase B: methanol with 0.1% of formic acid. The flow rate was set at 0.2 mL/min. The linear gradient was listed in Table 3.1. For HILIC column, the mobile phase A: 10 mM ammonium acetate aqueous solution with 0.1% formic acid, mobile phase B: 10 mM ammonium acetate in
95% acetonitrile (acetonitrile/water, v/v = 95/5) with 0.2% formic acid. The flow rate was also 0.2 mL/min, and the linear gradient was also listed in Table 3.1.

Table 3.1 Gradient of C18 column and HILIC column

<table>
<thead>
<tr>
<th>Reversed phase C18 column</th>
<th>HILIC column</th>
</tr>
</thead>
<tbody>
<tr>
<td>r.t.(min)</td>
<td>% A</td>
</tr>
<tr>
<td>0.0</td>
<td>98.0</td>
</tr>
<tr>
<td>1.5</td>
<td>98.0</td>
</tr>
<tr>
<td>14.5</td>
<td>0.0</td>
</tr>
<tr>
<td>21.0</td>
<td>0.0</td>
</tr>
<tr>
<td>21.1</td>
<td>98.0</td>
</tr>
<tr>
<td>25.0</td>
<td>98.0</td>
</tr>
</tbody>
</table>

r.t., retention time

Orbitrap Fusion-MS was equipped with HESI probe. The parameters are as listed: sheath gas, 40; auxiliary gas, 10; sweep gas, 0; heater temperature, 320 °C; capillary temperature, 300 °C; S-lens, 55; spray voltage, 3.6 kV in positive ion mode; Mass range, m/z 50-600. Resolution was set at 30000. The maximum injection time was 100 ms. Automated gain control (AGC) was targeted at $5 \times 10^4$. The cycle time was set at 3 seconds and with acceptable dwell times (between 8 and 20 ms, assessed based on internal standards). The isolation width of the precursor ion was set at 0.4 (m/z), high energy collision dissociation (HCD) was 25% with stepped collision energy (± 5%).

3.2.5 Liquid chromatography coupled with triple quadruple mass spectrometry

The detailed experiment parameters by LC-QQQ-MS were listed as follows: The product ion scan was carried out on an Ultimate 3000 rapid separation liquid chromatography (RSLC) coupled with TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific, USA). The chromatographic separation
column, mobile phase and any other LC conditions were the same as the PRM assay. For MS part, the optimized MS parameters of Quantiva were listed as follows: The source voltage was 3,000 V in positive ion mode. The resolution of Quartile 1 and Quartile 3 was 0.7 FWHM (Full width half maximum). Pressure of sheath gas and Aux gas was 40 arb and 10 arb, respectively. The collision gas was 1.5 mTorr. Temperature of ion transfer tube and vaporizer was set at 320 ℃ and 300 ℃, respectively. The collision energy was optimized and shown in Figure 3.2
3.2.6 Quantification and method validation

Calibration was performed by spiking plasma (fetal bovine serum (FBS) instead) and urine samples with appropriate concentration of authentic standards and with the same sample (plasma and urine) preparation procedure as before. The calibration curves were performed by addition of increasing concentration of standards and appropriate concentration of internal standards (IS). Limit of detection (LOD) and limit of quantification (LOQ) were assessed by stable isotope dilution in plasma or urine matrix. Recovery, stability, repeatability and accuracy studies were carried out in plasma and urine samples for method validation. In this study, recovery was assessed by comparing the difference of standard addition before samples extraction (addition of standards in extraction solvent with appropriate concentration of IS) and after samples extraction (addition of standards in constitution solvent with appropriate concentration of IS) in three different concentrations. Stability was evaluated by comparing the response of samples that directly extracted and injected with samples stored at -4 °C for 12 hours, or stored at -20 °C for 48 hours, or samples with 3 freeze-thaw cycles. Repeatability of this method was determined by multiple analyses of plasma and urine samples. Accuracy was evaluated by testing three different concentrations of standards (with appropriate concentration of IS) spiked into plasma and urine samples.

3.2.7 Peak extraction

Raw data collected from LC-MS were processed on Thermo Xcalibur Processing Setup-Quan-Identification software. The peak was extracted with
trace of accurate mass of parent and product ions (Base Peak, \( m/z \) 85.0284), and the mass tolerance was set at 10 ppm. Background signal to noise ratio was set at 3. The \( m/z \) value of parent ion and product ions of each compound was listed in Table 3.2. The chromatograms of several acylcarnitines detected from C18 column and HILIC column were shown in Figure 3.3 and Figure 3.4. Notably, two peaks of carnitine and succinylcarnitine were observed in Figure 3.4. The reasons might be inappropriate proportion of organic solvent in reconstitute solvent or inappropriate pH value of the mobile phase when using HILIC column for analysis.
Figure 3.3 Chromatograms of acylcarnitines detected by reversed phase column.

Figure 3.4 Chromatograms of acylcarnitines detected by normal phase HILIC column.

3.2.8 Statistical analysis

Unlike other body fluids, urine volume can vary widely due to water consumption and other physiological conditions, such as obesity and diabetes. Therefore, the concentrations of endogenous metabolites in urine sample vary
widely. As a result, normalization of the urine samples is necessary. In this study, acylcarnitines in urine sample was normalized by the concentration of total carnitine (sum of free carnitine and acylcarnitine esters) [158]. Principal component analysis (PCA) was operated on SIMCA-P software (Ver. 11, Umetrics, Umea, Sweden). The heatmap was performed by HemI software (Heatmap Illustrator, version 1.0)[159]. Pathway analysis was performed on KEGG pathway database (http://www.genome.jp/kegg/). Levels of significance between the two types of mice were evaluated by two-sided independent Student’s t-test. While P value < 0.05 was considered as significant.

3.3 Results

3.3.1 Simultaneous detection of acylcarnitines

A comprehensive method was developed for simultaneous detection of ACs in plasma and urine samples (Table 3.2). Mass spectrometric analysis of ACs was carried out on PRM acquisition in positive ion mode. Targeted MS/MS analysis was performed, and the major fragments of each ACs are listed in Table 3.2. As shown in Figure 3.5, acylcarnitine species generate a common product ion at m/z 85.028 (+CH₂-CH=CHCOOH) (Figure 2) owning to the neutral loss of trimethylamine moiety [m/z 59.073 (NH(CH₃)₃)] of the butyric acid chain on the carnitine backbone. It is regarded as a specific and the most abundant product ion of acylcarnitine fragmentation [160]. The fragment ion at m/z 60.080 (trimethylamine moiety) and fragments owing to neutral loss of trimethylamine moiety (loss of m/z 59.073 (NH(CH₃)₃)) were also detected. In addition, fragments owing to another neutral loss of m/z 143.094 ((CH₃)₃+CH₂-CH=CHCOO⁻) commonly observed give rise to positively charged fatty acid group, and the peak at m/z 144.101
[(CH₃)₃+NCH₂CH=CHCOOH, due to loss of ester group of the carnitine backbone)] were also observed commonly in ACs [161, 162]. Functional group such as hydroxyl and carboxyl groups attached to the acyl group can be observed in acylcarnitines. For those 3-hydroxy conjugated acylcarnitine species (Figure 3.5(a)), characteristic fragment \( m/z \) 145.049 (HOC=CH₂OCH₂CH₂OHC=O⁺) was observed due to the loss of trimethylamine and fatty acid chain with the cleavage between \( \alpha \)-C and \( \beta \)-C, which might be used to differentiate them from other hydroxyl conjugated isomeric species at different position along the chain [152]. In addition, carnitine conjugates of dicarboxylic acids, such as pimelylcarnitine (Figure 3.6), its fragments \( m/z \) 245.1017 (correspond to the neutral loss of trimethylamine) to \( m/z \) 199.0965 was due to the loss of carboxyl group (\( m/z \) 46.0052) from the fatty acid chain [72].

Due to limited availability of authentic standards, the identification of acylcarnitine species is difficult. However, high resolution MS and MS/MS spectrum could make the identification more accurate and confident. We can see from Figure 3.5, succinyl-carnitine (C₄-DC-S-CN) and 3-hydroxy-isovaleryl-carnitine (C₅-OH-CN b) can be easily differentiated from accurate parent ion. Besides, the comparison of MS/MS fragments of C₇-DC-CN a and C₈-OH-CN a from triple quadrupole and Orbitrap detectors (Figure 3.6) has been performed, high resolution MS/MS fragments confirmation showed more confident and accurate identification of acylcarnitine species. Firstly, we can distinguish C₇-DC-CN a (\( m/z \) 304.1757, \( C_{14}H_{26}NO_{6}^+ \), 0.7 ppm) from C₈-OH a (\( m/z \) 304.2119, \( C_{15}H_{30}NO_{5}^+ \), -2.6 ppm) through the accurate MS. Besides, the accurate MS/MS fragments (< 5 ppm) confirmation of the structures could make the identification
more confident than low resolution detector. What’s more, the interference MS/MS peaks that may lead to false assignment can be easily excluded [163-165]. Therefore, in this study, putative identification of acylcarnitines was carried out based on accurate MS/MS spectra and retention time information. Based on common fragments shared by each acylcarnitine compound and previous studies [72, 150, 166, 167], the ions with equal or more than 3 characteristic (m/z 85.028 and m/z 60.080 must be contained) MS/MS spectra were selected as potential acylcarnitines when authentic standards were not available. In this study, acyl groups with unsaturated double bonds are named and abbreviated by the number of carbon in the fatty acid chain and number of double bonds. Hydroxyl conjugated acylcarnitines were named by the number of hydroxyl (OH) groups, for mono hydroxyl conjugated acylcarnitines with the observation of characteristic fragment m/z 145.049 (HOC=CH₂OCHCH₂CH₂OHC=O+) were named as 3-OH conjugated acylcarnitines. For carboxyl conjugated acylcarnitines, the MS/MS spectra of acylcarnitines with the observation of characteristic loss of m/z 46.005 (carboxyl group) were abbreviated as “DC” in Table 3.2. In addition, according to the elemental formula, saturation level, MS/MS fragments and information from other studies [150], two compounds, m/z 266.1385 and m/z 280.1536 (in positive ion mode) likely to be provided by the presence of an aromatic ring, was abbreviated as C1-Phe-CN and C2-Phe-CN, respectively.
Figure 3.5 Product fragments spectra and proposed fragmentation patterns of 3-hydroxyisovaleryl-carnitine (a) and succinyl-carnitine (b).
Figure 3.6 Proposed fragmentation pattern of C7-DC-CN a and C8-OH-CN a by LC-QQQ-MS product ion scan and LC-Orbitrap-MS PRM scan.
Note: (a) MS/MS spectrum of C7-DC-CN a by LC-QQQ-MS product scan. (b) MS/MS spectrum of C7-DC-CN a by LC-Orbitrap-MS PRM scan. (c) MS/MS spectrum of C8-OH-CN a by LC-QQQ-MS product scan. (d) MS/MS spectrum of C8-OH-CN a by LC-Orbitrap-MS PRM scan.
<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds (name or formula [M-H]+)</th>
<th>Abbreviation</th>
<th>r.t. (min)</th>
<th>Parent (m/z)</th>
<th>Product (m/z)</th>
<th>Analytes/IS allocations</th>
<th>MS/MS fragments</th>
</tr>
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<tbody>
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<td>1</td>
<td>Carnitine</td>
<td>C0-CN&lt;sup&gt;std&lt;/sup&gt;</td>
<td>7.18/7.62</td>
<td>162.1123&lt;sup&gt;U&lt;/sup&gt;</td>
<td>85.0284</td>
<td>C0-CN/d9-C0-CN</td>
<td>60.0808, 85.0284, 102.0913, 103.0389</td>
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<tr>
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<td>Acetyl-carnitine</td>
<td>C2-CN&lt;sup&gt;std&lt;/sup&gt;</td>
<td>6.17</td>
<td>204.1229&lt;sup&gt;U&lt;/sup&gt;</td>
<td>85.0284</td>
<td>C2-CN/d3-C2-CN</td>
<td>60.0808, 71.0492, 85.0284, 103.0387, 144.1018, 145.0494</td>
</tr>
<tr>
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<td>Propionyl-carnitine</td>
<td>C3-CN&lt;sup&gt;std&lt;/sup&gt;</td>
<td>4.66</td>
<td>218.1384&lt;sup&gt;U&lt;/sup&gt;</td>
<td>85.0284</td>
<td>C3-CN/d3-C3-CN</td>
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<td>Malonyl-carnitine</td>
<td>C3-DC-CN&lt;sup&gt;std&lt;/sup&gt;</td>
<td>8.59</td>
<td>248.1125&lt;sup&gt;U&lt;/sup&gt;</td>
<td>85.0284</td>
<td>C3-DC-CN/d3-C4-DC-M-CN</td>
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<td>C4-OH-CN&lt;sup&gt;std&lt;/sup&gt;</td>
<td>6.83</td>
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<td>C4-OH-CN/d3-C2-CN</td>
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<td>C4-DC-S-CN&lt;sup&gt;std&lt;/sup&gt;</td>
<td>7.17/7.94</td>
<td>262.1281&lt;sup&gt;U&lt;/sup&gt;</td>
<td>85.0284</td>
<td>C4-DC-S-CN/d9-C0-CN</td>
<td>60.0808, 73.0283, 85.0284, 103.0389, 144.1016, 185.0441, 203.0548</td>
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<td>276.1438&lt;sup&gt;U&lt;/sup&gt;</td>
<td>85.0284</td>
<td>C0-CN/d9-C0-CN</td>
<td>60.0808, 85.0284, 87.0440, 103.0389, 115.0389, 144.1018, 199.0599, 217.0704</td>
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<td>262.1650&lt;sup&gt;U&lt;/sup&gt;</td>
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Note: Detailed information of acylcarnitines detected from plasma and urine samples. \( ^p \), represents extracted from plasma sample. \( ^u \), represents extracted from urine sample. \( ^{std} \), represents verified by authentic standards. r.t., retention time. IS, internal standards. ND, not detected. \( ^{d9} \)-C0-CN, \( ^{d9} \)-carnitine; \( ^{d3} \)-C2-CN, \( ^{d3} \)-acetyl-carnitine; \( ^{d3} \)-C3-CN, \( ^{d3} \)-propionoyl-carnitine; \( ^{d3} \)-C4-CN, \( ^{d3} \)-butyryl-
carnitine; \( ^{d9} \)-C5-CN, \( ^{d9} \)-isovaleryl-carnitine; \( ^{d3} \)-C6-CN, \( ^{d3} \)-octanoyl-carnitine; \( ^{d9} \)-C14-CN, \( ^{d3} \)-tetradecanoyl-carnitine; \( ^{d3} \)-C16-CN, \( ^{d3} \)-palmitoyl-carnitine. For the abbreviation column (the third column), “C” represents carbon; “OH” represents hydroxyl group; “DC” represents dicarboxylic group; “Phe” represents phenyl group. The number of C indicates the number of carbons in esterified acyl groups; the number of “C:” indicates the number of double bounds; the number of “OH” indicates the number of hydroxyl groups; CN, carnitine.
Table 3.3 Calibration data.

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Note: Calibration curves were generated by plotting peak area ratio of analytes to IS against the spiked concentrations. LOD and LOQ of isoC4-CN and C5-M-CN were determined as the LOD and LOQ of d3-C4-CN and d9-C5-CN, respectively. IS, internal standards; LOD, limit of detection; LOQ, limit of quantitation. na, not available.
**Table 3.4 Stabilities**

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Stabilities in plasma and urine samples were evaluated in three conditions. Inter-day: stored at 4 °C for 12 hours; intra-day, stored at -20 °C for two days. RFT: repeated freeze-thaw cycle for three times. Stabilities were calculated in percentage in comparison with samples extracted and directly injected. Coefficient of variations (CV) of each acylcarnitines in different conditions was also listed in the table. “-” Represents under detection limit. Four individual samples were prepared in each matrix.
Table 3.5 Repeatability of acylcarnitines in plasma and urine samples.

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Repeatability was assessed by injecting five individual samples. The results were displayed by CVs of peak area ratio of acylcarnitines to internal standards.

Table 3.6 Recoveries

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<th>Recovery (%)</th>
<th>CV (%)</th>
<th>Spiked concentration (pmol/μL)</th>
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73
Note: Recoveries in plasma and urine samples were calculated by percentage of peak area ratio of pre-spiked sample (add standards before sample extraction, corrected by endogenous levels of plasma and urine samples) and post-spiked sample (add standards when reconstitution) in three different concentrations. Five individual samples were prepared. SE, standard error. Concentration was shown as mean ± SE. UD, under detection limit.

Table 3.7 Accuracies

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<td>C14-CN</td>
<td>C16-CN</td>
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</table>

Note: The accuracies were assessed by the concentration in percentage of the actual spiked concentration in three different levels. Five individual samples were prepared for each matrix. SE, standard error. The data was displayed as percentages of total carnitine (sum of free carnitine and its esters, mean ± SE). UD, under detection limit.
### Table 3.8 Concentration of acylcarnitines in plasma sample

| Acylcarnitines |  
|----------------|--------------------------
<table>
<thead>
<tr>
<th></th>
<th>$db/m+$ (pmol/μL, mean ± SE)</th>
<th>$db/db$ (pmol/μL, mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0-CN</td>
<td>47.86±7.01</td>
<td>32.08±8.77</td>
</tr>
<tr>
<td>C2-CN</td>
<td>31.19±1.78</td>
<td>58.38±5.46</td>
</tr>
<tr>
<td>C3-CN</td>
<td>1.48±0.11</td>
<td>2.74±0.36</td>
</tr>
<tr>
<td>C3-DC-CN</td>
<td>0.088±0.019</td>
<td>0.55±0.11</td>
</tr>
<tr>
<td>C4-OH-CN</td>
<td>0.85±0.16</td>
<td>3.90±1.07</td>
</tr>
<tr>
<td>C4-DC-S-CN</td>
<td>1.35±0.24</td>
<td>2.68±0.65</td>
</tr>
<tr>
<td>C4-DC-M-CN</td>
<td>UD</td>
<td>0.0051±0.0051</td>
</tr>
<tr>
<td>C5-OH-CN b</td>
<td>0.026±0.0074</td>
<td>0.14±0.045</td>
</tr>
<tr>
<td>C4:1-CN</td>
<td>UD</td>
<td>0.0060±0.0032</td>
</tr>
<tr>
<td>C6-DC-CN a</td>
<td>0.11±0.014</td>
<td>0.17±0.0032</td>
</tr>
<tr>
<td>isoC4-CN</td>
<td>0.29±0.045</td>
<td>0.74±0.11</td>
</tr>
<tr>
<td>C4-CN</td>
<td>1.51±0.20</td>
<td>2.24±0.18</td>
</tr>
<tr>
<td>C7-DC-CN a</td>
<td>0.022±0.011</td>
<td>0.046±0.015</td>
</tr>
<tr>
<td>C7-DC-CN b</td>
<td>0.034±0.0093</td>
<td>0.055±0.013</td>
</tr>
<tr>
<td>C5-DC –CN</td>
<td>0.018±0.0051</td>
<td>0.026±0.0051</td>
</tr>
<tr>
<td>C5:1-CN</td>
<td>0.0023±0.00095</td>
<td>0.019±0.0049</td>
</tr>
<tr>
<td>C5-M-CN</td>
<td>0.62±0.17</td>
<td>0.64±0.44</td>
</tr>
<tr>
<td>C5-1-CN</td>
<td>1.11±0.13</td>
<td>3.65±0.063</td>
</tr>
<tr>
<td>C5-V-CN</td>
<td>1.11±0.13</td>
<td>3.65±0.063</td>
</tr>
<tr>
<td>C1-Phe-CN</td>
<td>0.0020±0.0011</td>
<td>0.0097±0.0033</td>
</tr>
<tr>
<td>C6:1-CN</td>
<td>0.0014±0.0010</td>
<td>0.0047±0.0026</td>
</tr>
<tr>
<td>C2-Phe-CN</td>
<td>UD</td>
<td>0.0332±0.019</td>
</tr>
<tr>
<td>C6-CN a</td>
<td>0.022±0.0083</td>
<td>0.072±0.022</td>
</tr>
<tr>
<td>C6-CN b</td>
<td>0.64±0.090</td>
<td>1.01±0.18</td>
</tr>
<tr>
<td>isoC7-CN</td>
<td>UD</td>
<td>0.00010±0.00010</td>
</tr>
<tr>
<td>C7-CN</td>
<td>UD</td>
<td>0.00010±0.00010</td>
</tr>
<tr>
<td>C10:3-CN a</td>
<td>UD</td>
<td>0.0023±0.0014</td>
</tr>
<tr>
<td>C10:3-CN b</td>
<td>0.0037±0.0018</td>
<td>0.021±0.0048</td>
</tr>
<tr>
<td>C10:3-CN c</td>
<td>0.0037±0.0018</td>
<td>0.021±0.0048</td>
</tr>
<tr>
<td>C8-CN</td>
<td>0.0022±0.0020</td>
<td>0.016±0.0029</td>
</tr>
<tr>
<td>isoC9-CN</td>
<td>0.0071±0.0028</td>
<td>0.016±0.00096</td>
</tr>
<tr>
<td>C12:3-CN a</td>
<td>0.020±0.00053</td>
<td>0.029±0.00093</td>
</tr>
<tr>
<td>C10:1-CN a</td>
<td>0.041±0.0023</td>
<td>0.054±0.0014</td>
</tr>
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<td>C10:1-CN b</td>
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<td>0.023±0.00059</td>
</tr>
<tr>
<td>C10-CN b</td>
<td>0.045±0.0042</td>
<td>0.078±0.0078</td>
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<td>C14:2-OH-CN</td>
<td>0.018±0.00027</td>
<td>0.021±0.0011</td>
</tr>
<tr>
<td>isoC11-CN</td>
<td>0.0018±0.0018</td>
<td>0.0060±0.0029</td>
</tr>
<tr>
<td>C14:3-CN</td>
<td>0.017±0.00016</td>
<td>0.018±0.00032</td>
</tr>
<tr>
<td>C12:1-CN b</td>
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<td>0.0062±0.00030</td>
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<tr>
<td>C11-CN</td>
<td>0.0013±0.00064</td>
<td>0.0024±0.00071</td>
</tr>
<tr>
<td>C14:2-CN b</td>
<td>0.0074±0.00044</td>
<td>0.011±0.00089</td>
</tr>
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<td>C12-CN</td>
<td>0.0088±0.00074</td>
<td>0.013±0.0012</td>
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<td>C14:2-CN c</td>
<td>0.0034±0.00043</td>
<td>0.0040±0.00034</td>
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<td>0.012±0.0012</td>
<td>0.018±0.00097</td>
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<tr>
<td>C16:3-CN</td>
<td>0.0043±0.000055</td>
<td>0.0048±0.000090</td>
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<tr>
<td>C16:2-CN</td>
<td>0.011±0.00047</td>
<td>0.016±0.0011</td>
</tr>
<tr>
<td>C14-CN</td>
<td>0.023±0.0019</td>
<td>0.032±0.0024</td>
</tr>
</tbody>
</table>
Concentration of acylcarnitines in plasma samples on \textit{db/m+} and \textit{db/db} mice.
SE, standard error. Concentration was shown as mean ± SE. UD, under detection limit.

Table 3.9 Concentration of acylcarnitines in urine sample

<table>
<thead>
<tr>
<th>Acylcarnitines</th>
<th>(db/m^+) (pmol/μL, mean ± SE)</th>
<th>(db/db) (pmol/μL, mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0-CN</td>
<td>55.25±7.12</td>
<td>15.63±3.77</td>
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<tr>
<td>C2-CN</td>
<td>10.16±2.05</td>
<td>15.06±1.81</td>
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<tr>
<td>C3-CN</td>
<td>0.34±0.12</td>
<td>0.071±0.011</td>
</tr>
<tr>
<td>C3-DC-CN</td>
<td>0.088±0.013</td>
<td>UD</td>
</tr>
<tr>
<td>isoC4-CN</td>
<td>1.68±0.263</td>
<td>1.48±0.56</td>
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<tr>
<td>C4-CN</td>
<td>0.076±0.031</td>
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<td>2.75±1.09</td>
<td>0.51±0.047</td>
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<td>C4-OH-CN</td>
<td>3.69±0.55</td>
<td>3.97±0.56</td>
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<td>C5:1-CN</td>
<td>0.029±0.0060</td>
<td>0.054±0.011</td>
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<td>0.030±0.012</td>
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<td>0.0032±0.0016</td>
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<td>0.016±0.0062</td>
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<td>0.0034±0.0012</td>
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<td>0.11±0.017</td>
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<td>0.061±0.014</td>
<td>0.13±0.022</td>
</tr>
<tr>
<td>C10-DC-OH-CN b</td>
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<td>0.10±0.017</td>
</tr>
<tr>
<td>C11-CN</td>
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<td>UD</td>
</tr>
<tr>
<td>C11:1-CN</td>
<td>0.0045±0.0018</td>
<td>UD</td>
</tr>
<tr>
<td>Acylcarnitine</td>
<td>Concentration (db/m+)</td>
<td>Concentration (db/db)</td>
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<tr>
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<td>-----------------------</td>
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<tr>
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<td>0.0025±0.0012</td>
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<tr>
<td>C12:3-OH-CN</td>
<td>0.0037±0.0017</td>
<td>0.0035±0.0013</td>
</tr>
<tr>
<td>C12:4-CN</td>
<td>0.28±0.055</td>
<td>0.11±0.020</td>
</tr>
<tr>
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<td>0.0045±0.0017</td>
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<tr>
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</tr>
<tr>
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<td>UD</td>
</tr>
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<tr>
<td>C14:2-OH-CN</td>
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<td>UD</td>
</tr>
<tr>
<td>C16-CN</td>
<td>0.12±0.045</td>
<td>0.039±0.015</td>
</tr>
<tr>
<td>C16:1-CN</td>
<td>0.0092±0.0052</td>
<td>UD</td>
</tr>
<tr>
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<td>UD</td>
</tr>
<tr>
<td>C18-CN</td>
<td>0.032±0.013</td>
<td>0.021±0.0084</td>
</tr>
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<td>0.071±0.029</td>
<td>0.015±0.006</td>
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<tr>
<td>C18:2-CN</td>
<td>0.094±0.036</td>
<td>0.036±0.014</td>
</tr>
<tr>
<td>C18:3-CN</td>
<td>0.0034±0.0025</td>
<td>UD</td>
</tr>
</tbody>
</table>

Concentration of acylcarnitines in urine samples on db/m+ and db/db mice. SE, standard error. Concentration was shown as mean ± SE. UD, under detection limit.
3.3.2 Quantification of acylcarnitines in biological samples

In this study, calibrations were performed by addition of different concentration of ACs with the same appropriate concentration of IS mixture to a pool of plasma (FBS instead) and urine samples. As shown in Table 3.3, 13 ACs and 9 deuterated AC species were used for the generation of calibration curves, respectively. Free carnitine (C0-CN), acetyl-carnitine (C2-CN), propionyl-carnitine (C3-CN), malonyl-carnitine (C3-DC-CN), succinyl-carnitine (C4-DC-S-CN), and 3-hydroxyisobutyryl-carnitine (C4-OH-CN) were analyzed by using a HILIC column, the left 7 ACs listed in Table S2 were analyzed by using a reversed phase C18 column. The ratio of mass response of analytes to relevant IS was calculated for generation of calibration curves. The obtained curves were linear with correlation coefficients ($R^2$) of greater than 0.99 for all ACs. For those ACs without authentic standards, they were quantified by the calibration line of AC with the closest retention time and the same analytes/IS allocations (shown in Table 1). The detailed information of analytes-IS allocations was listed in Table 1. The representative LOD of deuterium-labeled acylcarnitines were determined as the concentration giving a signal to noise of equal or more than 3, while the LOQ were determined as that with a signal to noise of equal or more than 10. The results shown in Table 3.3 indicated that the method provided good sensitivity for the quantification of AC species in plasma and urine sample.

3.3.3 Assessment of sample stability

The stabilities of ACs in plasma and urine samples were evaluated by comparing different
storage conditions with the samples that were directly extracted and measured. The conditions include storage at 4 °C for 12 hours and storage under -20 °C for 2 days after sample extraction. As summarized in Table 3.4, most of the ACs showed a high stability when stored at 4 °C for 12 hours or stored under – 20 °C for 2 days, demonstrated that the ACs got satisfactory stability (80% - 120%) across intra- and inter-day analyses. However, several ACs, especially dicarboxy conjugated ACs, such as glutaryl-carnitine (C5-DC-CN), Octanediocarnitine (C8-DC-OH-CN), Adipoylcarnitine (C6-DC-CN a), showed poor stability on inter-day study in plasma or urine samples. Besides, crotonyl-carnitine (C4:1-CN) and isoC9-CN were not stable on inter-day study either. In this study, thawing of the plasma and urine samples in repeated freeze-thaw cycles (n = 3) was also assessed. Although most of the ACs showed a high stability, hydroxylated and dicarboxylated ACs showed dramatic deduction after three freeze-thaw cycles (C5-DC-CN, C5-OH-CN a, C5-OH-CN b, C4:1-CN, C6-DC-CN a, C6-OH-CN b, C7-DC-CN b, C8:1-DC-CN a, etc.). Thus, we suggest that the samples should be analyzed as soon as possible and stored properly in order to minimize the degradation of those frangible ACs (particularly dicarboxyl conjugated ACs).

3.3.4 Method validation

The recoveries were calculated as the percentage of area ratios of pre-spiked sample and post-spiked sample in three different concentrations. The results showed that the recoveries of most ACs were between 80% and 120% (Table 3.6), with a few expectations that in low (isoC4-CN in urine matrix and C5-M-CN in plasma matrix). Accuracy of this method was measured by spiking
different concentrations (including low, medium and high concentration in the calibration range) of standards in plasma and urine samples. The results (Table 3.7) indicated that the accuracies of most ACs were between 75% and 125%, with a few expectations that in low (free carnitine (C0-CN), acetyl-carnitine (C2-CN) and isobutyryl-carnitine (isoC4-CN) in urine samples) or high (propionyl-carnitine (C3-CN), 3-hydroxy-isobutyryl-carnitine (C4-OH-CN), dodecanoyl-carnitine (C12-CN) and tetradecanoyl-carnitine (C14-CN) in plasma samples) concentrations were overestimated. The repeatability of the current method was assessed by injecting 5 individual samples with the same preparation procedure as described before. The results showed that coefficient of variation (CV) value of area ratio of the 13 ACs were less than 13.4 % (Table 3.5).

3.3.5 Targeted profiling of acylcarnitines in response to type 2 diabetes in mice in plasma and urine samples

Targeted quantification of ACs in plasma and urine samples was carried out on db/db diabetic mice and db/m+ control mice. The db mutation was traced to the mutation in leptin receptor [168], appears to behave the highest plasma cholesterol and triglyceride levels[169], which is regarded as the most frequently used mice model for diabetic dyslipidemia. Besides, db/db mice showed substantial effect on insulin resistance, accompanied by a significant increase of body weight and dramatic increase in blood glucose level[157].

The changes of ACs in plasma and urine samples (Table 3.8 and Table 3.9) in response to T2DM were in accordance with the findings reported previously[161, 170]. As shown in Figure 3.7 and Figure 3.8, compared with db/m+ mice, the concentration of free carnitine decreased in db/db
mice group in both plasma and urine samples. Short-chain acylcarnitines (SCAC, C2-C5, also classified as lipid or lipid-like derivatives according to HMDB) were the most abundant ACs in plasma and urine samples. The total amount of SCAC increased in both type of samples from \( db/db \) mice. C2-CN, butyryl-carnitine (C4-CN), C4-OH-CN, isovaleryl-carnitine (C5-I-CN) and valeryl-carnitine (C5-V-CN), malonyl-carnitine (C3-DC-CN) and succinyl-carnitine (C4-DC-S-CN) that are produced through branched-chain amino acids (BCAA) catabolism increased substantially in plasma sample. Similarly, most SCAC increased in urine from \( db/db \) mice except for C4-DC-S-CN, which decreased. The level of medium-chain acylcarnitines (MCAC, C6 to C12) in plasma sample increased substantially (Figure 3.9). However, the regulatory effect of MCAC in urine sample was different according to the length of ACs (Figure 3.10). Generally, the concentration of C6 to C10 ACs with dicarboxyl- and hydroxyl-conjugated groups increased in response to T2DM. On the contrary, the level of C8 to C12 saturated and unsaturated MCAC decreased significantly in response to T2DM in urine sample. For the long-chain acylcarnitines (LCAC, C13 to C22), a vast majority of these metabolites increased substantially in plasma samples in \( db/db \) mice group, while decreased in urine samples.
Figure 3.7 Percentage of acylcarnitines in plasma (a) and urine (b) samples on the basis of different length of acylcarnitines.

Note: C0-CN, free carnitine; SCAC: short-chain acylcarnitine (C2 – C5); MCAC: medium-chain acylcarnitine (C6 – C12); LCAC: long-chain acylcarnitine (C13 – C22).

Figure 3.8 Short-chain acylcarnitines in response to diabetes in plasma (a) and urine (b) samples.

Note: *, represents $p$ value < 0.05; **, represents $p$ value < 0.01; ***, represents $p$ value < 0.001, $db/db$ vs. $db/m+$ group. Total carnitine was calculated by the sum of free carnitine and all esterified acylcarnitines.
Figure 3.9 Heatmap of acylcarnitines in plasma samples. (a) Heatmap of medium-chain acylcarnitines. (b) Heatmap of long-chain acylcarnitines.

Note: * $p$ value < 0.05.
Figure 3.10 Heatmap of acylcarnitines in urine samples. (a) Heatmap of medium-chain acylcarnitines. (b) Heatmap of long-chain acylcarnitines.

Note: * p value < 0.05.
3.4 Discussion

3.4.1 Identification of 117 acylcarnitines in plasma and urine samples

Acylcarnitines are important intermediates of fatty acids. Considerable studies have showed that acylcarnitines play an important role in fatty acid β-oxidation (FAO), amino acid metabolism and organic acid metabolism [171-173]. Profiling of acylcarnitines has been used in diagnosis, such as prenatal diagnosis[174], postmortem screening[175], diagnosis of diabetes[170]. Plenty of studies have established for quantification of acylcarnitines by using LC-MS. However, limited number of acylcarnitines has been reported due to complex matrix, isomers and low abundance. In this study, a high resolution PRM method was used with simple sample preparation (without solid phase extraction and derivatization). PRM is originally described as MRM with high resolution[176] (Figure 3.1), where precursor ions of compound is selected in Q1, then fragmented in Q2 with stepped collision energy. Subsequently all generated products (MS/MS) and precursor ions are detected in parallel by high-resolution and full scan orbitrap mass spectrometer. Finally, the peaks were extracted by Thermo Xcalibur Processing Setup-Quan-Identification software, with similar precursor-product ion pair as MRM but with high resolution (ppm < 10). PRM has been applied in targeted quantification of peptides and small molecules on high resolution LC-MS platforms [73, 163, 177] with reliable performance. In this study, PRM method was applied to targeted quantification of acylcarnitine species in different matrix.

Acylcarnitine species generate a common product ion at m/z 85.028 (Figure 3.3), which is regarded as a specific and the most abundant product ion of acylcarnitine fragmentation[160, 161]. In addition, m/z 60.080 and m/z 144.101 were also
characteristic fragment ions of acylcarnitines (Figure 3.3, Figure 3.4 and Table 3.2). Based on previous studies [72, 150, 166, 167], the ions with \( m/z \) 85.028, \( m/z \) 60.080 or \( m/z \) 144.101 were selected. Owing to the wide range of polarities of acylcarnitines, especially hydrophilic carnitine and short-chain acylcarnitines (SCAC) which have poor retention on reversed phase column, samples derivatization by using butanol or other reagents[161, 178] prior to LC-MS analysis is necessary. In this study, liquid chromatography with reversed phase C18 (RP-C18) column analysis allowed excellent separation of isomeric acylcarnitine species and normal phase HILIC column analysis that experted in the separation of high polar acylcarnitines species were both performed without tedious derivatization sample pretreatment. Finally, 117 acylcarnitines (8 acylcarnitines by HILIC column and 109 acylcarnitines by RP-C18 column) were detected from plasma and urine samples.

Outstanding separation of RP-C18 coupled with high resolution orbitrap detection allowed the separation and differentiation of isomeric acylcarnitine species. For instance, succinyl-carnitine and 3-hydroxy-isovaleryl-carnitine share the same MRM transition of 262/85[161] by using triple quadrupole detector. However, the two compounds can be easily differentiated through the accurate mass of parent ions (shown in Figure 3.3, \( m/z \) of succinyl-carnitine is 262.129, \( m/z \) of 3-hydroxy-isovaleryl-carnitine is 262.165). In addition, malonyl-carnitine (shown in Table 3.2, \( m/z \) 248.113) and hydroxylbutyryl-carnitine (\( m/z \) 248.149), glutaryl-carnitine (C5-DC-CN) (\( m/z \) 276.144) and hydroxylhexanoyl-carnitine (\( m/z \) 276.180), pimelyl-carnitine (\( m/z \) 304.175) and 3-hydroxy-octanoyl-carnitine (\( m/z \) 304.212), etc., could all be easily differentiated.
3.4.2 Satisfactory reliability and quantification ability of this method

The performance of this method has been evaluated. Repeatability studies (Table 3.5) showed that the coefficients of variation (CV) were less than 15%. For accuracy studies, accuracies of most of the ACs were between 75% and 125%, with a few expectations which were overestimated. In addition, vast majority of ACs got satisfactory stability within a day. However, although most of ACs showed satisfactory stabilities under the storage of -20 °C for 48 hours, several ACs, especially dicarboxy conjugated ACs showed poor stabilities. In addition, dicarboxyl- and hydroxyl- conjugated acylcarnitines, such as C5-DC-CN, C5-OH-CN, crotonyl-carnitine (C4:1-CN), etc., were significantly reduced after 3 freeze-thaw cycle, indicating that these kinds of acylcarnitines are frangible after repeated freezing and thawing. All these data indicated that this method shows high precision, stability and satisfactory recovery and accuracy for analysis of most of the ACs. However, we suggest that the samples should be analyzed as soon as possible and stored properly in order to minimize the degradation of those frangible ACs (particularly dicarboxy conjugated ACs).

3.4.3 Measurement of acylcarnitines in db/db mice in plasma and urine samples reveals substantial disturbed metabolic disorders

This method has been applied to analyze plasma and urine samples on db/m+ and db/db diabetic mice. The characteristic dysfunction of diabetes is excessive lipid deposition in the body, which is associated with insulin resistance (IR)[100, 179]. Besides, the disturbance of fatty acids and glucose metabolism is induced by accumulated lipids, leading to lipotoxicity in liver, skeletal muscle, kidney and other organs in the body[102]. Many studies have revealed positive correlation between lipid accumulation and increased glucose oxidation and inhibition of FAO. During FAO,
acylcarnitines, especially long-chain acylcarnitines are important intermediates to transport fatty acids into the inner membrane of mitochondria for fatty acid oxidation. In this study, targeted quantification of 117 acylcarnitines in plasma and urine samples were carried out. The PCA score plots (Figure 3.11) based on detected acylcarnitines showed that the \( db/m^+ \) and \( db/db \) mice can be clearly separated from each other. The level of free carnitine substantially decreased in urine sample when comparing \( db/db \) mice with \( db/m^+ \) mice (Figure 3.7). Although the level of free carnitine in plasma sample was not accounted for significant statistics, the reduction trend can still be observed in this study, which is due to the higher lipid load in \( db/db \) mice.
Figure 3.11 Multivariate statistic analysis of acylcarnitines in plasma and urine samples

Note: (a) PCA score plot of acylcarnitines in plasma sample. (b) Volcano plot of acylcarnitines of urine sample. (c) PCA score plot of acylcarnitines in plasma sample. (d) Volcano plot of acylcarnitines of urine sample. The postfix ‘-CN’ of abbreviation of each acylcarnitines in (c) and (d) has been omitted.

C2-CN was accumulated in plasma on db/db mice, while the elimination rate of C2-CN also increased in urine. C2-CN can be produced from both lipid and glucose metabolism, depending on the nutrient availability. C3-CN, C3-DC-CN, methylmalonyl-carnitine (C4-DC-M-CN), C4-DC-S-CN and C5-I-CN were all involved in organic academia [180, 181] (Figure 3.8). In this study, the levels of C3-
CN and C3-DC-CN were significantly increased in plasma on diabetic mice. However, no significant difference was observed between db/m+ and db/db mice in urine samples. In addition, C4-DC-S-CN was significantly reduced in urine on diabetic mice, which was in accordance with the accumulation of the precursor metabolites, succinic acid [182]. Besides, the increased level of C5-I-CN in plasma is associated with the accumulation of isovaleric acid and isovaleryl-CoA in the body, which lead to isovaleric acidemia [183] and can cause significant morbidity and mortality in human. All the results indicated that the accumulation of acidic SCAC in the body may provide a detailed analysis of ketoacidosis in diabetic mice. Besides, as shown in Figure 4, the metabolites of BCAAs, isoC4-CN, C4-OH-CN, C5-I-CN, 2-Methylbutyryl-carnitine (C5-M-CN), C5-OH-CN b and C5:1-CN were substantially increased in db/db mice in plasma or/and urine samples. The results were in accordance with the increased level of BCAAs in db/db mice by other studies[111]. Since the increased levels of BCAA on obese humans contribute to insulin resistance [144], and has been regarded as biomarkers of insulin resistance in diabetes [172, 184], we assume that acylcarnitines in BCAA catabolism pathway may also play an important role in insulin resistance.
Figure 3.12 Potential metabolic pathways of acylcarnitines.

Note: The pathway has been described in the discussion part. BCAA, branched-chain amino acid; FA, fatty acid; FAO, fatty acid β-oxidation; AA, amino acid; CN, carnitine; CPT I, carnitine palmityl transferase I; CoA, coenzyme A; TCA cycle, citric cycle. MCAC, medium-chain acylcarnitines; LCAC, long-chain acylcarnitines.

The levels of medium- to long-chain acylcarnitines were substantially increased in plasma sample, while MCAC were increased in urine sample except odd numbered-chain acylcarnitines, which were significantly decreased. Generally, acylcarnitines oxidized into acetyl-CoA except odd numbered-chain acylcarnitines degraded into one acetyl-CoA and one propionyl-CoA in the last round of FAO (Figure 3.12), then propionyl-CoA metabolized to succinyl-CoA, and finally all entered into TCA cycle. The positive correlation of C3-CN and propionyl-CoA [73] indicated that the increased level of C3-CN in plasma and decreased level of odd numbered-chain acylcarnitines,
especially isoC7-CN, C7-CN, C9-CN, C11:1-CN and C11-CN may contribute to the dysfunction of propionic acidemia (PA). In addition, combining with the findings that the level of propionyl-carnitine in urine sample was not changed on db/db mice as in the plasma, and the level of succinyl-carnitine was significantly decreased in urine, while several odd numbered-chain acylcarnitines (such as isoC9-CN and C1-Phe-CN) in plasma sample was increased, indicating that the intermediates involved in odd numbered-chain acylcarnitines were blocked in the body. Besides, the levels of long-chain acylcarnitines (LCAC) were substantially decreased in urine samples. The results indicate the accumulation of LCAC in the body, especially in skeletal muscle [171]. The metabolic changes of medium- to long-chain acylcarnitines might indicate insufficient FAO in the body on db/db diabetic mice which contributes to insulin resistance[171].

In summary, the targeted profiling of acylcarnitine species by using high resolution PRM method with simple sample preparation exhibited more features of acylcarnitines detected, which gave more information of the metabolic changes of acylcarnitine species in the body. However, there are several limitations in this study. Several isomeric acylcarntines, such as C5-M-CN, C5-I-CN and C5-V-CN were not well baseline separated. Therefore, a longer reversed phase column might be used in the future. Comparing with other studies [185], diastereomers can be poorly separated by the current method. Besides, carnitine and succinyl-carnitine got two peaks. Although both of them got quite satisfactory quantification results, the problem should be studied in the future. In addition, based on the high resolution fragments detected in this study, the proposed potential structures of acylcarntines, need to be further confirmed with synthesis of the authentic standards of acylcarnitines.
3.5 Chapter summary

A high resolution PRM targeted quantification method of acylcarnitine species that play a crucial role in glucose metabolism, fatty acid metabolism, ketone body metabolism, BCAAs and other amino acids metabolism in the body has been developed. The method avoided tedious sample preparation and provided reliable performance in the quantification of polar acylcarnitines in biological samples. Excellent separation and differentiation of isomers by high resolution PRM method with confident targeted fragments confirmation allowed much broader detection of acylcarnitine species compared to other methods, especially when authentic standards are not available. Metabolic profiling by this method on db/db diabetic mice showed insufficient supply of free carnitine and increased acylcarnitine production in the mouse body. The accumulation of SCAC in plasma and inefficient elimination of acylcarnitines through urine might lead to organic acidemia. Besides, the accumulation of medium- to long-chain acylcarnitines in plasma, and the reduction of odd-numbered medium-chain acylcarnitines and LCAC in urine, jointly indicated that incomplete fatty acid oxidation might have occurred on db/db mice, which might contribute to insulin resistance in db/db mice.
Chapter 4 Metabolomics study on db/db diabetic mice in skeletal muscle reveal effective clearance of overloaded intermediates by exercise

4.1 Introduction

Type 2 diabetes mellitus (T2DM) is a long term metabolic disorder. It is one of the most common diseases with over 150 million patients worldwide [186]. T2DM is characterized by high blood glucose and insulin resistance, later followed by impaired β-cell function. Long-term poor glycemic control leads to many complications, such as heart disease [187], diabetic nephropathy and physical disability [188], which cause high mortality among all the diseases [189]. T2DM primarily occurs as a result of over nutrition and a sedentary lifestyle, meaning lacking of sufficient exercise [190]. Therefore, regular exercise to control body weight is an effective way to prevent and even treat T2DM. However, the underlying mechanism of exercise on T2DM was not fully understood.

Metabolomics is a systematic study of metabolic changes of all small molecules in response to stimuli [191]. It is unique among omics technologies in exploring potential relationships of genotype-phenotype [192], which may reflect function more directly than protein or gene effects [193]. Recent advances in metabolomics technologies, especially mass spectrometry-based metabolomics studies, have greatly enhanced the observation of metabolic changes in response to stimuli. Metabolomics study has been applied to investigate the mechanism of T2DM for many years [157, 194, 195]. A large number of metabolomics studies on plasma and urine samples have revealed that the metabolic changes induced by T2DM were mainly involved in lipid metabolism, oxidative stress, mitochondrial dysfunction, etc. However, few studies have been carried out to study the mechanism of moderate intensity of exercise on T2DM in
skeletal muscle, which is the major tissue that responsible for insulin sensitivity in the body [196].

In this chapter, mass spectrometry-based metabolomics studies have been performed on type 2 diabetic db/db mice [197]. In order to get a comprehensive view of the regulatory effect of exercise on db/db mice in skeletal muscle, both liquid chromatography coupled with mass spectrometry (LC-MS) and gas chromatography coupled with mass spectrometry (GC-MS) were performed. In the current study, a number of biomarkers were found substantially increased in skeletal muscle from db/db diabetic mice, which might be due to the excessive accumulation of intracellular intermediates related to insulin resistance. Interestingly, exercise exerts a positive role in attenuating the accumulated intermediates on db/db mice, which might be a positive reflection of improved insulin resistance and improved body fitness.

4.2 Experiment

4.2.1 Chemicals and solutions

HPLC grade methanol was purchased from RIC Labscan Ltd. Co. (Bangkok, Thailand). HPLC grade acetonitrile was acquired from Tedia Company (Fairfield, OH, USA). Hexane was purchased from Rathburn Chemicals Ltd. (Rathburn, Scotland, UK). Formic acid, bis(trimethylsilyl)trifluoroacetamide (BSTFA + 1% TMCS), pyridine, methoxyamine and all other authentic standards were obtained from Sigma - Aldrich (St. Louis, MO, USA), Cambridge Isotope Laboratories (CIL, Andover, MA, USA) or Toronto Research Chemicals (Toronto, Ontario, Canada). Pure water was prepared from a Milli-Q Ultrapure water system (Millipore, Billerica, MA, USA). All other chemicals and solutions used in this experiment were of analytical grade otherwise specified.
4.2.2 Animal experiment

In this study, C57BL/KsJ \textit{db/db} male mice were used as type 2 diabetes mellitus (T2DM) model mice, while C57BL/KsJ male \textit{db/m+} mice were used as control mice. Other treatment on animals was the same as in the chapter 3. LE and OE groups were treated with moderate intensity of exercise at the age of 10 weeks. They were placed at the back of the treadmill lane (MK-680C, Muromachi Kikai Co., Ltd, Japan) with a mild electric shock (1 mA)\cite{120}. Considering animal acclimatization, exercise intensity and duration was gradually increased over the first week of exercise training. The speed was initially set at a targeted speed of 12 m/min for 30 min, which represented a daily forced exercise of 360 m. Then the speed was set at a targeted speed of 14 m/min for 40 min per day from the second week. The mice were exercised six days a week, and continuously for five weeks. The LS and OS groups were also put on the treadmill for 40 min without running.

After five weeks of exercise training, animals were weighed, and the blood glucose level, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were assessed with a glucose meter (Roche Diagnostics, Meylan, France). After the mice were sacrificed by CO$_2$ anesthesia, blood was collected from the inferior vena cava with a 1.5 mL micro-centrifuge tube moistened by heparin sodium. Skeletal muscles (quadriceps femoris) of each mouse were collected, washed with ice-cold phosphate buffered saline, blotted dry, weighed and immediately snap frozen with liquid nitrogen, and then stored under $-80$ °C before analysis.

4.2.3 Sample preparation

The skeletal muscles were weighed to a 2.0 mL micro-centrifuge tube, then homogenized with 800µL ice cold 80% of methanol/water (80/20, v/v). Samples were
mixed immediately with a vortexer for 2 min. After centrifuged at 14,200g for 10 min (4 °C), the supernatant was transferred to a new 1.5 mL micro-centrifuge tube. The residue was extracted with 400µL ice cold 80% of methanol/water again. The supernatant was combined, equally divided into three equal aliquots, then stored at -80 °C until analysis.

For LC-MS analysis, two aliquots were evaporated to dryness with appropriate concentration of internal standards (4-chlorophenylalanine), and reconstituted in 100 µL 30% acetonitrile/water (30/70, v/v) for positive and negative ion modes analysis, separately. For GC-MS analysis, the third aliquot was vacuum dried with an appropriate concentration of internal standard (ribitol). The dried residue was initially derivatized with 20 µL of methoxyamine (solution of methoxyamine hydrochloride in pyridine, 20 mg/mL) for 1 hour at 37 °C. Then trimethylsilylation with 60 µL of BSTFA plus 1% of TMCS, incubated for 1 hour at 55 °C. The derivatized samples were analyzed within one day.

Quality control (QC) sample is necessary to make sure the analysis by LC-MS and GC-MS is reliable. In this study, the QC sample was made by pooling of each extracted skeletal muscle samples, prepared the same as samples in LC-MS and GC-MS analysis described before, and injected randomly throughout the experiment to assess the instrument reliability in terms of mass to charge ratio (m/z), retention time and peak area. Blank sample (for LC-MS) was injected every four sample injections throughout the experiment to minimize the carryover. Stock solution of authentic standards was initially prepared in methanol, and then diluted to a proper concentration for verification.
4.2.4 Instrument analysis

For LC-MS analysis, separation was carried out on an UltiMate 3000, combined with Q Exactive™ Focus Quadrupole-Orbitrap MS (Thermo Fisher Scientific, Bremen, Germany). ACQUITY UPLC HSS T3 column (100 × 2.1 mm i.d., 1.7 µm particle size, Waters, Milford, MA) was chosen in this study. The column oven was held at 30 °C, and the flow rate was set at 0.3 mL min⁻¹. Mobile phase A was milli-Q water with 0.1% of formic acid, while mobile phase B was acetonitrile with 0.1% of formic acid. The sample injection volume was 7 µL in positive ion mode and 15 µL in negative ion mode. The gradient in both positive and negative ion mode was set as follows: 2% B at 0 min, hold for 1 min, 100% B at 19 min, hold for 2 min, 2% B at 21.1 min and hold for 3.9 min. Parameters of MS were set as below: Heater Temp., 350 °C; Sheath Gas Flow Rate, 45 arb; Aux Gas Flow Rate, 10 arb; Spray Voltage, 3 kV in positive ion mode and 2.5 kV in negative ion mode; Capillary Temp., 300 °C; Tube Lens, 55.00; and Capillary Voltage, 50 V. The mass scan range was m/z 100 to 1000. MS/MS analysis was carried out in targeted parallel reaction monitoring (PRM) mode under three stepped collision energies, 10 eV, 20 eV and 40 eV, respectively.

In the current study, GC-MS analysis was performed on an Agilent 7890B gas chromatography coupled with a 5977A mass spectrometric detector system in splitless mode. DB-5MS capillary column (coated with 5% diphenyl cross-linked 95% dimethylpolysiloxane, 0.25 µm film thickness, 30m × 250 µm i.d., Agilent J&W Scientific, Folsom, CA) was used. The experiment was performed under electron impact ionization source. Detailed parameters were set as follows: the scan range, m/z 50-650; the collision energy, 70 eV; solvent delay, 7 min; the helium carrier gas flow, 1.0 mL/min; the column inlet pressure, 70 kPa; and the injection temperature and interface temperature were both set at 250 °C. The temperature gradient was set as
follows: oven temperature was initially set at 70 °C, last for 2 min, then raised with a rate of 5 °C/min and up to 200 °C. Afterward, the temperature was increased to 300 °C at the rate of 10 °C/min.

4.2.5 Method validation

For untargeted LC-MS and GC-MS analysis, the assessment of method stability is very important. For LC-MS analysis, due to different polarities and m/z values, six ions (listed in Table 4.1, three from positive ion mode and three from negative ion mode) have been selected out for assessment of method reliability according to the coefficient of variation (CV) of their peak intensity from QC samples. For method evaluation of GC-MS, the reliability was assessed by observing the CV of peak intensity of the internal standard (Table 4.1) from QC samples. Besides, the clustering of QC samples in principal component analysis (PCA) score plots was also observed to monitor the reliability of the analysis methods.

Table 4.1 Stability assessment of LC-MS and GC-MS analysis

<table>
<thead>
<tr>
<th>Instrument used</th>
<th>Compounds</th>
<th>Variation of peak area (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-MS (pos)</td>
<td>Phenylalanine</td>
<td>6.2</td>
</tr>
<tr>
<td>LC-MS (pos)</td>
<td>Pantothenic acid</td>
<td>5.1</td>
</tr>
<tr>
<td>LC-MS (pos)</td>
<td>Tetradecanoylcarnitine</td>
<td>7.5</td>
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<tr>
<td>LC-MS (neg)</td>
<td>Glutathione</td>
<td>4.3</td>
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<tr>
<td>LC-MS (neg)</td>
<td>Fumaric acid</td>
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</tr>
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</tr>
<tr>
<td>GC-MS</td>
<td>Ribitol</td>
<td>17.0</td>
</tr>
</tbody>
</table>

CV, coefficient of variation. pos, acquired from positive ion mode. neg, acquired from negative ion mode.

4.2.6 Data extraction and metabolites identification

The data from GC-MS was extracted as descrip previously [198, 199]. The output files were performed by XCMS software, processed on open-source R statistical language (v 3.3.0). The raw data of LC-MS was directly extracted by SIEVE 2.2
(Thermo Fisher Scientific, MA, USA). The extracted LC-MS and GC-MS data were imported into simca-P software (Ver. 11, Umetrics, Umea, Sweden) for multivariate statistical analysis. Both principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were carried out. The ions whose thresholds of variable importance in the projection (VIP) values that > 1.2 were selected out for identification. For the identification of discriminating metabolites from GC-MS, their retention time and mass spectra were compared with standards available in our lab. After that, NIST library search was performed, in which the identification is based on a similarity of more than 70% (Figure S4.1). Discriminated metabolites from LC-MS were identified by comparison of MS and MS/MS fragments to Biofluid Metabolites Database (http://metlin.scripps.edu) and Human Metabolome Database (HMDB) (http://www.hmdb.ca). Furthermore, part of them was verified by standards available in our lab.

4.2.7 Statistic analysis

In this study, fold changes were calculated as mass response ratio between two arbitrary groups (LE vs. LS, OS vs. LS, OE vs. LE, and OE vs. OS). Analysis of variance (ANOVA) was carried out as significant analysis method. \( p \) value that less than 0.05 was considered as significant.

4.3 Results

4.3.1 Phenotypic characterization of \( db/m^+ \) and \( db/db \) mice in response to exercise

Over 5 weeks of exercise training, a substantial reduction of body weight was observed on \( db/db \) mice (Figure 4.1 (a)). The weight of skeletal muscles (quadriceps muscle) were significantly decreased with a fold change of 3.6 (Figure 4.1 (b)) when comparing \( db/m^+ \) sedentary group with \( db/db \) sedentary group. Although the weight of quadriceps
muscles was slightly but not significantly increased after exercise in *db/db* mice, the ratio of skeletal muscle mass to body weight (SM/BW) was significantly improved after exercise training in *db/db* mice (Figure 4.1 (c)). The result of OGTT (Figure 4.1 (d)) and ITT (Figure 4.1 (e)) revealed similarly impaired glucose metabolism in *db/db* mice after exercise.

Figure 4.1 Physiological characteristics of *db/db* and *db/m+* mice in response to exercise. (a) Body weight; (b) Weight of skeletal muscle (quadriceps muscle); (c) Ratio of quadriceps muscle mass to body weight; (d) Oral glucose tolerance test (OGTT); (e) Insulin tolerance test (ITT).

Note: LS: lean mice (*db/m+*) treated with sedentary; LE: lean mice (*db/m+*) treated with exercise; OS: diabetic mice (*db/db*) treated with sedentary; OE: diabetic mice (*db/db*) treated with exercise; SM, skeletal muscle; BW, body weight. *, P < 0.05, ***, P < 0.001, LE vs. LS, OS vs. LS or OE vs. LE; ★, P < 0.05, ★★★, P < 0.05, OE vs. OS.
4.3.2 Multivariate statistic analysis

As shown in Figure 4.2(a) and Figure 4.3, PCA score plots demonstrated that the lean and the obese mice separated clearly from each other. While the LE and LS groups were clustered together, OE and OS groups were separated, and OE group was closer to LS and LE groups. The results indicated substantial metabolic changes between lean and obese mice, and exercise exerts regulatory effect on the metabolic changes on db/db diabetic mice.

Figure 4.2 Multivariate statistic analysis.

Note: (a) PCA score plot in positive ion mode ($R^2X = 0.835$, $Q^2$ (cum) = 0.609). (橙) Orange open circle represents QC samples. (■) Black square represents db/m+ mice treated with sedentary (LS). (●) Red solid circle represents db/m+ mice treated with exercise (LE). (♦) Blue diamond represents db/db mice treated with sedentary (OS). (▲) Green triangle represents db/db mice treated with exercise (OE). (b) Volcano plot of ions in positive ion mode based on OS and LS groups. (c) Validation plot of PLS-DA model of OS and LS groups ($R^2 = (0.0, 0.674)$, $Q^2 = (0.0, -0.122)$, perturbation number = 100).
Figure 4.3 Multivariate statistic analysis of the results from LC-MS negative ion mode and GC-MS.

Note: (a) PCA score plot of the results from LC-MS negative ion mode ($R^2_X = 0.781$, $Q^2$ (cum) = 0.642). (b) PCA score plot of the results from GC-MS ($R^2_X = 0.764$, $Q^2$ (cum) = 0.590). (○) Orange open circle represents quality control (QC) samples. (■) Black square represents $db/m+$ mice treated with sedentary (LS). (●) Red solid circle represents $db/m+$ mice treated with exercise (LE). (♦) Blue diamond represents $db/db$ mice treated with sedentary (OS). (▲) Green triangle represents $db/db$ mice treated with exercise (OE).

Table 4.2 Detailed information of metabolites from GC-MS analysis

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<tr>
<th>No.</th>
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<th>R.Match</th>
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<td>891</td>
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<td>861</td>
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$^*$ represents verified by authentic standards. All the compounds were compared with NIST. The normal search mode “Match” and “R-Match” are similarity indices of mass spectrum calculated by NIST MS Search 2.0. TMS, trimethylsilyl. The number of TMS represents the number of trimethylsilyl-conjugated group.
Table 4.3 Detailed information of identified metabolites from LC-MS analysis.

<table>
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<tr>
<th>No.</th>
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Hexadecenoylcarnitine (C16:1-CN) 60.0811, 85.0283, 144.1018, 339.2527
Linoelaidylcarnitine (C18:2-CN) 60.0809, 69.0699, 81.0698, 85.0283, 97.1011, 144.1018
Hydroxy-octadecanoylcarnitine (C18-OH-CN) 60.0806, 85.0278, 144.1012
LysoPC(16:0) 86.0964, 104.1068, 184.0732, 478.3267
Vaccenylcarnitine (C18:1-CN) 60.0810, 85.0283, 95.0854, 144.1018, 367.2841
Stearoylcarnitine (C18-CN) S 60.0810, 85.0283, 144.1014, 369.2984
Arachidyl carnitine (C20:4-CN) 60.0807, 85.0278

Note: r.t., retention time. S, verified by authentic standards. For abbreviation of acylcarnitines, “C” represents carbon; “OH” represents hydroxyl group; “DC” represents dicarboxylic group; CN, carnitine; The number of C indicates the number of carbons in esterified acyl groups; the number of “C:” indicates the number of double bounds.

Table 4.4 Changes of identified metabolites in response to diabetes and exercise

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<tr>
<th>Class and derivatives</th>
<th>Metabolites</th>
<th>LE vs. LS</th>
<th>OS vs. LS</th>
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*, p < 0.05. **, p < 0.01. ***, p < 0.001. fc, fold change. ns, not account for significance.
4.3.3 Discriminated metabolites in response to diabetes and exercise

Finally, 94 metabolites (32 metabolites from GC-MS analysis, 62 metabolites from LC-MS analysis) were identified contributed to metabolic changes in response to T2DM (Table 4.2 and Table 4.3). Those biomarkers were mainly classified as amino acids, carboxylic acids, carbohydrates, fatty acids, nucleotides, nucleosides, phospholipids and acylcarnitines. They were mainly involved in branched-chain amino acid (BCAA, including valine, leucine and isoleucine) biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, glycine, serine and threonine metabolism, pantothenate and CoA biosynthesis pathway (Figure 4.4), etc.

Figure 4.4 Overview of pathway analysis based on discriminated metabolites between 
$db/m+$ and $db/db$ mice.
In this study, 30 acylcarnitines were detected to be substantially increased in db/db sedentary group when compared with db/m+ sedentary group. Interestingly, acylcarnitines characterized by their lengths, were differentially regulated by exercise in db/db mice (Figure 4.5). More specifically, the levels of short-chain acylcarnitines (SCAC, C2 to C5) together with free carnitine were significantly increased in db/m+ lean mice after exercise, but most of them were decreased in db/db mice after exercise. The levels of medium- to long-chain acylcarnitines (C8 to C20) were all markedly increased in db/db mice compared to db/m+ mice. Decanoylcarnitine (C10-CN), dodecanoylcarnitine (C12-CN), tetradecanoylcarnitine (C14-CN), etc. were significantly reduced after exercise in db/db mice after exercise, while, no change was observed in db/m+ mice after exercise. In addition, the levels of some other lipids, such as phospholipids (lysoPC(16:0) and lysoPC(16:1(9Z))) and long-chain fatty acids myristic acid (C14), palmitelaidic acid (C16:1), palmitic acid (C16), 9,12-octadecadienoic acid (C18:2) and 9-octadecenoic acid (C18:1)) were all specifically increased in db/db mice, which were substantially reduced after exercise.
Figure 4.5 Various kinds of lipids in response to diabetes and exercise. (a) Acylcarnitines. (b) Fatty acids. (c) Phospholipids.
Note: LS, db/m+ mice treated with sedentary. LE, db/m+ mice treated with exercise. OS, db/db mice treated with sedentary. OE, db/db mice treated with exercise. # represents p value < 0.05, LE vs. LS, OS vs. LS or OE vs. LE; ★ represents p value < 0.05, OE vs. OS. C14:0, myristic acid. C16:1, palmitelaidic acid. C16:0, palmitic acid. C18:2, 9, 12-octadecadienoic acid. C18:1, 9-octadecenoic acid. C18:0, stearic acid.

Other identified metabolites, such as BCAAs which were positively correlated with insulin resistance [144], were significantly increased on db/db mice when comparing with db/m+ mice. However, exercise did not exert regulatory effect on both types of mice. The levels of inosinic acid, adenosine, hypoxanthine, etc. that involved in purine metabolism pathway (Figure 4.6) were substantially increased on db/db diabetic mice. Interestingly, they were significantly reduced on db/db diabetic mice by exercise. Besides, several metabolites (Figure 4.7 and Figure 4.8) involved in histidine metabolism pathway (3-methyl-L-histidine and carnosine), citric acid (TCA) cycle (lactic acid, oxalic acid, etc.), galactose metabolism pathway (mannose, glucose, etc.), phenylalanine metabolism pathway (phenylalanine), etc., were reduced by exercise.

Figure 4.6 Metabolites relate to purine metabolism pathway in response to T2DM and exercise in skeletal muscle.
Figure 4.7 Metabolites related to carnitine metabolism in response to T2DM and exercise.

Note: * represents $p$ value < 0.05; ** represents $p$ value < 0.01; *** represents $p$ value < 0.001, LE vs. LS, OS vs. LS and OE vs. LE. ★ represents $p$ value < 0.05; ★★ represents $p$ value < 0.01, ★★★ represents $p$ value < 0.001, OE vs. OS.

Nicotinamide adenine dinucleotide.
Figure 4.8 Metabolites in other metabolism pathways in response to T2DM and exercise.

Note: * represents $p$ value < 0.05; ** represents $p$ value < 0.01; *** represents $p$ value < 0.001, LE vs. LS, OS vs. LS and OE vs. LE. ★ represents $p$ value < 0.05; ★★ represents $p$ value < 0.01, ★★★ represents $p$ value < 0.001, OE vs. OS.
4.3.4 Correlation analysis of acylcarnitines with SM/BW in response to T2DM and exercise

Skeletal muscle metabolism has been regarded as a determinant of overall metabolic rate [200]. It is responsible for insulin-mediated glucose disposal and ectopic lipid accumulation, which is important for maintaining and utilizing energy storage [201]. The accumulation of acylcarnitines in skeletal muscle from obese and diabetic mice has been found to be biomarkers owning to incomplete fatty acid β-oxidation (FAO), contributing to insulin resistance [171, 202]. Considering T2DM is associated with excessive loss of skeletal muscle [203], and most of the detected acylcarnitines were up-regulated in $db/db$ mice and some were down-regulated after exercise (Figure 4.5), correlation analysis of the SM/BW with acylcarnitines was carried out. We can see that there is a strong negative correlation between SM/BW and medium to long-chain acylcarnitines when comparing $db/db$ diabetic mice with $db/m+$ control mice (Figure 4.9 (a)), especially those hydroxyl-conjugated acylcarnitines (C18:1-OH-CN, C16:1-OH-CN, C14:1-OH-CN, etc.). Coupling the results of receiver operating characteristic curve (ROC) analysis (Figure 10), the area under the curve (AUC) value was up to 0.941, indicating that the accumulation of acylcarnitines in skeletal muscle might be a strong positive reflection of the loss of skeletal muscle mass.

Interestingly, the correlation analysis on $db/db$ mice between sedentary and exercise groups (Figure 4.9 (b)) showed that there was a strong positive correlation between SM/BW and hydroxy-conjugated medium to long-chain acylcarnitines (C18-OH-CN, C16:2-OH-CN, C14:2-OH-CN, etc.), and also part of the short-chain acylcarnitines (C0, tiglylcarnitine (C5:1-CN and butyrylcarnitine (C4)). However, most of the non-hydroxy conjugated medium to long-chain acylcarnitines, especially saturated medium to long-chain acylcarnitines, such as C10-CN, C12-CN and C14-CN showed strong
negative correlation with the SM/BW after exercise. The results indicated that exercise might exert positive regulatory effects on the elimination of the accumulation of hydroxy-conjugated acylcarnitines rather than non-hydroxy conjugated acylcarnitines, especially futile on those saturated ones to improve body muscle composition.
Figure 4.9 Correlation analysis of acylcarnitines with skeletal muscle mass to body weight ratio.

Note: SM/BW, skeletal muscle mass to body weight ratio.
Note: (a) Correlation analysis of acylcarnitines with SM/BW between \textit{db/db} sedentary and \textit{db/m+} sedentary groups. (b) Correlation analysis of acylcarnitines with SM/BW between \textit{db/db} sedentary and \textit{db/db} exercise groups.

Figure 4.10 Receiver operating characteristic curve analysis (ROC) of acylcarnitine changes in discriminating \textit{db/db} and \textit{db/m+} mice.

The correlation analysis and ROC analysis were performed on MetaboAnalyst (www. metaboanalyst. ca). AUC, area under the curve.

4.4 Discussion

It is well documented that exercise exerts favorable metabolic effects on weight loss [204], glycemic control [205], lipid profile[206], etc. in the context of obesity and insulin resistance. Skeletal muscle is the major tissue responsible for insulin sensitivity in the body [196]. Any changes in skeletal muscle mass, metabolic rate, or responsiveness to hormones would substantially influence the overall energy homeostasis. We showed that the SM/BW value was increased after exercise in \textit{db/db} mice, mainly due to body weight reduction.
Multivariate statistical analysis (Figure 4.2 and Figure 4.3) of skeletal muscle metabolites showed clear separation between db/m+ control and db/db diabetic mice, indicating that there are substantial metabolic changes between the two genotypes. Among the most changed metabolites, valine, leucine and isoleucine biosynthesis pathway, phenylalanine, tyrosine and tryptophan biosynthesis pathway, purine metabolism pathway, were significantly increased (Figure 4.6 and Table 4.4), which is possibly as result of the redundancy of intracellular intermediates induced by T2DM in skeletal muscle, as indicated by previous study [144]. More importantly, there is clear separation between sedentary and exercise db/db mice. Specifically, the level of several metabolites involved in glucose metabolism, such as lactic acid, glucose, mannose, and amino acids involved in TCA cycle, like phenylalanine, alanine, and serine, were reduced after exercise in db/db mice in skeletal muscle. The results might be a positive reflection of promoted efficiency of energy utilization thus better performance after exercise training in db/db mice.

![Valine, Leucine, Isoleucine](image)

Figure 4.11 Changes of branched-chain amino acids in response to diabetes and exercise.

Note: ** represents \( p \) value < 0.01; *** represents \( p \) value < 0.001, OS vs. LS, or OE vs. LE.
4.4.1 Regulatory effect of exercise on purine metabolism pathway reveals more efficient energy utilization on db/db diabetic mice

In this study, one of the most significantly changed pathways (Figure 4.4) between db/m+ and db/db mice is purine metabolism pathway. Uric acid, which is positively associated with serum glucose level [207] and insulin resistance, has been proved and regarded as a strong and independent risk factor among patients with T2DM [205]. Uric acid, which can be produced in the liver, muscle, and fat, is able to directly induce lipid accumulation in the liver, causing fatty liver; and also inhibit insulin signaling and AMPK activity to promotes insulin resistance [208, 209]. We also found that high uric acid accumulation in skeletal muscle of db/db mice was reduced after exercise. Moreover, the increases of several intermediates produced in uric acid biosynthesis, including adenylsuccinic acid, inosinic acid (IMP), hypoxanthine, adenosine, nicotinamide adenine dinucleotide (NAD), niacinamide and adenosine monophosphate (AMP) in db/db mice were also restored after exercise. These results indicated that T2DM might induce the accumulation of uric acid and its precursors in skeletal muscle, compromising ATP synthesis, and such effect can be alleviated by exercise. In addition, fructose can be also further metabolized to uric acid in hyperglycemic condition [210]. High fructose level causes rapid depletion of intracellular phosphate, which stimulates the activation of AMP deaminase, the enzyme that converts IMP to uric acid [211]. Several studies have reported that fructose-induced uric acid generation in the body causes mitochondrial oxidative stress, and plays a crucial role in stimulating fat accumulation [212, 213]. In our study, the decrease of uric acid and its precursors after exercise indicates a more efficient removal of intermediates, which is associated with more efficient energy utilization and muscle function in diabetic mice. However,
whether uric acid biosynthesis pathway is a causative factor of the beneficial effect of exercise on metabolism in diabetic mice requires further study.

4.4.2 Exercise exerts good role in maintaining the level of carnitine in skeletal muscle of db/db mice

Carnitine is a ubiquitously existing substance which plays crucial roles in intracellular metabolism, particularly in transportation of long-chain fatty acids into mitochondrial matrix through the inner mitochondrial membrane for fatty acid β-oxidation (FAO) [214, 215]. Carnitine in eukaryotic organisms is continuously biosynthesized from proteinogenic amino acids methionine and lysine [216]. As shown in Figure 4.12, carnitine can be biosynthesized from trimethyl-L-lysine (TML), a component of histone protein, generated via methylation of protein lysine residues by the action of S-adenosyl-L-methionine (SAMe)[217]. Besides, carnitine could be converted into 3-dehydroxycarnitine catalyzed by carnitine dehydrogenase [218]. In this study, the level of free carnitine and metabolites involved in carnitine biosynthesis, including TML, 5'-methylthioadenosine (MAT), SAMe, acetyl-L-lysine, betaine and 3-dehydroxycarnitine were all substantially reduced after exercise (Figure 4.7). The results showed that exercise might have a positive effect on attenuating the level of free carnitine [219] and its intermediates in skeletal muscle of diabetic mice.

4.4.3 Exercise exerts good effect in attenuating the accumulation of acylcarnitines to improve insulin resistance

Figure 4.5 indicated that acylcarnitines at different lengths were regulated differentially in response to T2DM and exercise. The increase of short-chain acylcarnitines (SCAC, C2 to C5), including C2-CN, C3-DC-CN, C4-CN, C5:1-CN, C5-M-CN and C5-I-CN, by exercise in lean mice might be related with the increased energy transport and
consumption by utilizing short-chain fatty acids in response to exercise [220]. However, the accumulation of SCAC in \(db/db\) sedentary mice might be due to the overload of incomplete fatty acid and amino acid (such as BCAA) oxidation. The results are in accordance with the metabolic changes of medium- to long-chain acylcarnitines (C8 to C22), both were increased in muscles from \(db/db\) mice. Besides, many studies have found that the activity of carnitine acyltransferase I (CPT I), which mediates the transfer of acyl-CoA to the hydroxyl group of free carnitine to generate acylcarnitines, was up-regulated in skeletal muscle of \(db/db\) diabetic mice [221]. This might lead to the intracellular accumulation of medium- to long-chain acylcarnitines, owing to incomplete fatty acid β-oxidation (FAO) [171, 222], and glucolipotoxicity [155].

Because we also observed improved glucose metabolism, the reduction of medium- to long-chain acylcarnitine could be a result of better FAO and/or improved glucose metabolism, which also supported by improved metabolic derangements of FAO, including the reduction of TML, SAMe, acetyl-L-lysine, betaine, 3-dehydroxycarnitine, pantothenic acid and free fatty acid (C14:0, C16:0 and C18:1) after exercise in \(db/db\) mice. Besides, the overview in Figure 4.9 showing strong negative correlation between SM/BW and long-chain acylcarnitines (LCAC) indicated that the accumulation of LCAC correlates with decreased body muscle composition. Interestingly, a positive correlation between SM/BW and some LCACs after exercise in diabetic mice was observed, together with the fact that the increased level of LCAC was positively related to insulin resistance [171, 222], indicating that the positive correlation of LCAC with improved body muscle composition might be a positive reflection of improved insulin resistance by exercise.
Figure 4.12 Overview of pathways based on differentiated metabolites between \( db/m+ \) control and \( db/db \) diabetic mice in skeletal muscle.

Note: BB, butyrobetaine; TML, N6, N6, N6-trimethyl-lysine; SAMe, S-adenosylmethionine; SAMa, S-adenosylmethioninamine; MTA, 5′-methylthioadenosine; 4GB, 4-guanidinobutanoic acid; DMG, dimethylglycine. PCA, Pyrrolidonecarboxylic acid; NAD, Nicotinamide adenine dinucleotide; FMN, Flavine mononucleotide; GTP, guanosine triphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; NADH, Nicotinamide adenine dinucleotide (reduced); FA, fatty acid; C16, palmitic acid; C18, stearic acid; C10-OH, 3-Hydroxycapric acid; VB5, pantothenic acid.

4.5 Summary of this chapter

In conclusion, metabolomics analysis on skeletal muscle in sedentary and exercised \( db/db \) diabetic mice provides us a comprehensive view of metabolic changes in response to exercise. Comparison between lean (non-diabetic) and obese (diabetic mice) showed the accumulation of various amino acids, fatty acids, and acylcarnitines species,
in diabetic mice, as a result of nutrient overload, which compromises energy metabolism, causing incomplete fatty acid oxidation, dysfunction of purine metabolism, BCAA metabolism and glucotoxicity. Exercise exerts positive regulatory roles in improving amino acid catabolism which provides substrates that can enter TCA cycle; removal of intermediates from metabolism of carnitine, medium- to long-chain acylcarnitines (C12 to C18), long-chain fatty acids (C14 to C18), phospholipids; and better clearance of uric acid and its precursors. Our findings suggested a positive regulatory role of exercise on energy metabolism, nutrient utilization, and waste disposal, which might help to improve fitness. Although we did not observe improvement of insulin sensitivity probably due to short treatment duration and age of mice, our study showed beneficial effects of exercise on overall metabolism in diabetic mice.
Chapter 5 Physical exercise ameliorates renal metabolic disorder during diabetic nephropathy in mice

5.1 Introduction

Emerging evidence suggests that diabetic kidney disease (DN), a common complication in diabetes mellitus, has long been acknowledged to cause severe mortality and morbidity [223]. Many studies have revealed that the pathogenesis of diabetic renal disease is complicated, and it involves multiple factors which contribute to the progression of this disease [224]. Lifestyle modification, such as regular physical activity is an effective way to manage diabetic nephropathy. Obesity and lack of physical activity are highly associated with risk of metabolic diseases [225, 226]. On the other hand, leanness and regular physical activity are positively associated with a lower risk of metabolic diseases. However, the underlying mechanism of physical activity on lowering the risk of metabolic diseases is not well understood. Therefore, it is important to investigate the mechanism of physical activity on regulating the metabolic dysfunction of diabetic renal disease.

Metabolomics analysis is a systematic study of all metabolites in an organism in relation to a biological perturbation [227, 228]. This technology has been widely applied in the diagnosis and treatment of T2DM [57, 229]. Previous studies using metabonomics technology have identified characteristic metabolic differences in kidney and urine samples on db/db diabetic mice by using nuclear magnetic resonance (NMR) [230]. However, little metabolomics study has been carried out to investigate the regulatory effect of physical activity on T2DM induced renal disease.

In this study, a frequently used T2DM animal model, db/db diabetic mice was selected. First of all, this model has a mutation in the leptin receptor gene, which will lead to obesity with progressive hyperinsulinemia, hyperglycemia and insulin resistance [231,
Secondly, this animal model appears to exhibit the most robust and consistent increase in urine albuminuria and mesangial matrix expansion. Last but not least, the renal dysfunction on \textit{db/db} diabetic mice, such as proteinuria, glomerular hypertrophy and glomerulosclerosis, are similar to human diabetic nephropathy. All these suggest that \textit{db/db} mice is a suitable model for studying the pathogenesis and therapeutic intervention in diabetic nephropathy [233-235]. In the current study, untargeted and targeted metabolomics studies were carried out by using liquid chromatography coupled with high resolution mass spectrometry (LC-MS) and gas chromatography coupled with mass spectrometry (GC-MS) to give a relatively comprehensive view of the metabolic changes related to DN and the effect of exercise on it.

5.2 Experiment

5.2.1 Chemicals and solutions

HPLC grade methanol and acetonitrile were phased from RIC Labscan Ltd. Co., (Bangkok, Thailand) and Tedia Company (Fairfield, OH, USA), separately. Formic acid (FA), oximation reagent methoxyamine hydrochloride, ammonium acetate, bistrimethylsilyl-trifluoroacetamide (BSTFA) plus 1% TMCS, and most of the authentic standards were acquired from Sigma - Aldrich (St. Louis, MO, USA). Urinary albumin mouse ELISA kit (ab108792) and urinary creatinine assay kit (ab204537) were purchased from (Abcam, Cambridge, MA). Pure water was prepared from a Milli-Q Ultrapure water system (Millipore, Billerica, MA, USA). ACQUITY UPLC HSS T3 column (100 × 2.1 mm, i.d., 1.7 μm particle size) (Waters, Milford, MA) was used in this study. All other chemicals and solutions used in this experiment were of HPLC grade.
5.2.2 Animal experiment

In this study, C57BL/KsJ \( db/db \) male mouse was selected as diabetic nephropathy (DN) model mouse, while C57BL/KsJ male \( db/m+ \) mouse was selected as control mouse. The two types of mouse were obtained from the Jackson Laboratory (Bar Harbor, ME) and then randomly distributed to either sedentary and exercise training groups. The number of each group was listed as follows: \( db/db \) sedentary (OS) group, \( n = 8 \), \( db/db \) exercise (OE) group, \( n = 9 \), \( db/m+ \) sedentary (LS) group, \( n = 8 \), and \( db/m+ \) exercise (LE) group, \( n = 9 \). The mouse was housed in a humidity- and temperature-controlled environment with 12 hours of light/dark cycle. All the mice were fed with standard laboratory chow (mouse No. 3 Breeding, Special Diets Services) and free to water. The whole experimental procedures were confirmed and approved by the Universite´ catholique de Louvain and the Rangueil Hospital animal ethics committee. \( Db/db \) and \( db/m+ \) exercise treated groups were taken moderate-intensity of exercise on the treadmill (Columbus Instruments), running at 8 m/min for 30 minutes in the morning, 6-days per week, and exercise for a total of 4 weeks. The sedentary treated groups (LS and OS) were also put in a stationary treadmill for adaption to induce the stress that caused by exercise training.

5.2.3 Plasma and urine parameters

After 5 weeks of exercise, urine was collected with an overnight fasting. The mouse was weighed, and then anesthetized with pentobarbital. Blood was collected with a 1.5 mL micro-centrifuge tube which was moistened with heparin sodium. The entire kidney of each mouse was collected and washed with phosphate buffered saline. It was then dried with filter paper, and snap froze with liquid nitrogen immediately. The kidney was stored under \( -80^\circ \)C before analysis. 3.5 \( \mu L \) of blood was used to measure
the blood glucose level with a glucose meter (Roche Diagnostics, Meylan, France). Meanwhile, the concentration of insulin was also assessed as described previously [236]. The detection of urinary creatinine and albumin in urine, and cytokines (TNFα and IL-6) in plasma were carried out by ELISA under the protocol of the assay kit. The measurements of total cholesterol, total triacylglycerol, high density lipoprotein (HDL) cholesterol, non-HDL cholesterol were also carried out by commercial kits.

5.2.4 Tissue processing and histological evaluation

The entire kidney of each mouse was collected and washed with phosphate buffered saline after execution. It was then dried with filter paper. One kidney of each mice were snap frozen with liquid nitrogen immediately. Part of kidneys were fixed in 10% buffered formaldehyde, and then embedded in paraffin. Five-µm-thick kidney sections were stained with hematoxylin-eosin. Representative renal corpuscles were photographed with a light microscope (Leica Microsystems Ltd.) fitted with a digital camera (Leica Application Suit, version 4.5.0). Forty glomeruli per kidney per mice were randomly chosen and examined for semi-quantitatively evaluation of the degree of mesangial matrix. Relative glomerular area was measured by ImageJ software [237].

5.2.5 Sample preparation for LC-MS and GC-MS analysis

Samples were thawed on ice on the day of extraction. Around 50 mg of kidney samples were weighed to a 2.0 mL micro-centrifuge tube, then homogenized with 800μL of ice cold methanol/water (v/v = 80:20) with an appropriate concentration of internal standards (IS). Samples were mixed using a vortex mixer for 1 min. After centrifuged at 14,200g for 10 min (4 °C), the supernatant was transferred to a new 1.5 mL micro-centrifuge tube. The residue was extracted again with 400 μL ice cold methanol/water (v/v = 80:20, with IS). The supernatant was combined and then separated into
appropriate amount of aliquot for further analysis in four independent platforms as described below (Figure 5.1). For LC-MS analysis in positive and negative ionization modes, each portion of aliquot was dried under vacuum desiccation and reconstituted in 100µL 50% methanol/water (v/v = 50:50) for injection. Targeted analysis of acyl coenzyme A (acyl-CoA) was reconstituted in 20% acetonitrile (with 50 mM ammonium acetate) and analyzed immediately within one day. For GC-MS analysis, the portion of aliquot was dried under vacuum desiccation with an appropriate concentration of ribitol as internal standard, which was used to monitor the performance of GC-MS and reproducibility of the method during a long time analysis. Afterward, the dried extracts were firstly derivatized using 30 µL of methoxyamine (solution of methoxyamine hydrochloride in pyridine, 20 mg/mL) for 1 hour at 37 °C. Trimethylsilylation with 70 µL of BSTFA plus 1% of TMCS was then followed for 1 hour at 55 °C. The derivatized samples were analyzed on GC-MS immediately within one day.

Figure 5.1 Work flow of this experiment.

A quality control (QC) sample is necessary to make sure the analysis is reliable on LC-MS and GC-MS. In this study, the QC sample was made by pooling 40 µL of each
extracts of kidney samples and prepared as described above in each experiment. The QC samples were analyzed regularly throughout sequence analysis to assess the instrument stability.

5.2.6 Instrument analysis

GC-MS analysis was operated in splitless mode on an Agilent 7890N gas chromatography coupled with a 5977A mass spectrometric detector system. The column used in the current analysis was DB-5MS capillary column, which coated with 5% diphenyl cross-linked 95% dimethylpolysiloxane (0.25 µm film thickness, 30m × 250 µm i.d., Agilent J&W Scientific, Folsom, CA). The experiments were operated under electron impact ionization, and the collision energy was set at 70 eV. The scan range was m/z 50-650. The gradient of oven temperature was listed in Table 5.1. The helium carrier gas flow was 1.0 mL/min, and the column inlet pressure was set at 70 kPa. The interface temperature and injection temperature were both set at 250 °C.

For untargeted LC-MS analysis, separation was performed on a Thermo Scientific quaternary Accela 1250 pump with a PAL Sample Manager combined with Q Exactive™ Focus Quadrupole-Orbitrap MS (Thermo Fisher Scientific, MA, USA) (LC-QE-MS). ACQUITY UPLC HSS T3 column (100 × 2.1 mm i.d., 1.7 µm particle size) (Waters, Milford, MA) was selected in this study. The flow rate was 0.3 mL min⁻¹. The column oven was hold at 30 °C. The mobile phases were milli Q water (A) with 0.1% formic acid (FA) and acetonitrile (B) with 0.1% FA. The sample injection volume was 10µL in positive ion mode and 8µL in negative ion mode. The elution gradient in both ionization modes was as listed in Table 5.1. The parameters of mass detection method were set as follow: Sheath Gas Flow Rate was 45 arb; Heater Temp. was 350 °C; Aux Gas Flow Rate was 10 arb; Capillary Temperature was 300 °C; ISpray Voltage was 3.6
kV in positive ion mode and 2.5 kV in negative ion mode; Capillary Voltage was 50 V and Tube Lens was 55.00; The mass scan range was set from m/z 100 to 1000. The MS/MS analysis was acquired in targeted MS/MS mode under 3 stepped collision energies, which were 10 eV, 20 eV and 40 eV, respectively.

Table 5.1 Mobile phase gradients by LC-MS analysis

<table>
<thead>
<tr>
<th>No.</th>
<th>Non-targeted GC-MS analysis</th>
<th>Non-targeted LC-MS analysis in positive and negative ion modes</th>
<th>Targeted LC-MS analysis of acyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (min)</td>
<td>Temperature (°C)</td>
<td>Time (min)</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>70</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>70</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>22.0</td>
<td>190</td>
<td>19.0</td>
</tr>
<tr>
<td>4</td>
<td>34.0</td>
<td>250</td>
<td>21.0</td>
</tr>
<tr>
<td>5</td>
<td>36.0</td>
<td>300</td>
<td>21.1</td>
</tr>
<tr>
<td>6</td>
<td>45.0</td>
<td>300</td>
<td>25.0</td>
</tr>
</tbody>
</table>

For targeted acyl-CoA measurement, 21 acyl-CoA were analyzed on LC-MS based on previous studies [73]. Detailed information (including names, abbreviation, et al) was listed in Table 5.2. An example of fragmentation pattern was shown in Figure 5.2 (propionyl-dephospho-CoA). Experiment was performed on LC-QE-MS with the same separation column as untargeted analysis. In brief, for LC part, the sampler was kept under 4 °C. The mobile phase A: water with 5 mM ammonium acetate and mobile phase B: acetonitrile. Linear gradient is also listed in Table 5.1. The flow rate was 0.2 mL/min. For MS method of acyl-CoA analysis, Sheath gas, 30; Sweep gas, 2; Auxiliary gas, 10; Spray voltage, 3.6 kV in positive ion mode and 2.5 kV in negative ion mode; Scan range, m/z 100-1200; Isolation width of the precursor ion, 1.0 (m/z); Higher energy collisional dissociation (HCD), 18% ± 5%; the maximum injection time, 100 ms; automated gain control (AGC), 4 × 10⁵.
Figure 5.2 Schematic diagrams of fragmentation patterns of propionyl-dephospho-CoA.
Figure 5.3 Chromatograms from GC-MS analysis.
Figure 5.4 Example of identification of metabolites from GC-MS analysis (comparing with NIST).
Table 5.2 Detailed information of acyl-CoA in kidney samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Abbreviation</th>
<th>r.t. (min)</th>
<th>MS/MS fragment ions [M+H]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coenzyme A (CoA)</td>
<td>CoA</td>
<td>7.13</td>
<td>688.1530; 428.0344; 348.0685; 261.1255; 136.0612</td>
</tr>
<tr>
<td>2</td>
<td>Acetyl-dephospho-CoA</td>
<td>C2-CoA</td>
<td>7.87</td>
<td>730.1634; 428.0347; 348.0683; 303.1356; 136.0610</td>
</tr>
<tr>
<td>3</td>
<td>Succinyl-dephospho-CoA or methylmalonyl-dephospho-CoA</td>
<td>C4-DC-CoA</td>
<td>8.06</td>
<td>788.1675; 441.1054; 348.0690; 136.06134</td>
</tr>
<tr>
<td>4</td>
<td>Propiony-dephospho-CoA</td>
<td>C3-CoA</td>
<td>8.79</td>
<td>744.1788; 428.0349; 348.0685; 317.1512; 136.0610</td>
</tr>
<tr>
<td>5</td>
<td>Butyryl-dephospho-CoA or Isobutyryl-dephospho-CoA</td>
<td>C4-CoA</td>
<td>9.46</td>
<td>758.2040; 348.0687; 331.1668; 136.0604</td>
</tr>
<tr>
<td>6</td>
<td>Hexenoyl-CoA</td>
<td>C6:1-CoA</td>
<td>9.66</td>
<td>864.1771; 428.0342; 357.1823; 136.0608</td>
</tr>
<tr>
<td>7</td>
<td>Valeryl-dephospho-CoA or isovaleryl-dephospho-CoA or 2-methylbutyryl-dephospho-CoA</td>
<td>C5-CoA</td>
<td>9.93</td>
<td>772.1999; 348.0687; 345.1823; 136.0613</td>
</tr>
<tr>
<td>8</td>
<td>Hexanoyl-CoA</td>
<td>C6-CoA</td>
<td>10.02</td>
<td>866.1939; 428.0357; 359.1990; 136.0609</td>
</tr>
<tr>
<td>9</td>
<td>Decenoyl-CoA</td>
<td>C10:1-CoA</td>
<td>11.33</td>
<td>920.2382; 454.2272; 428.0348; 413.2453; 311.1778</td>
</tr>
<tr>
<td>10</td>
<td>Dodecenoyl-CoA</td>
<td>C12:1-CoA</td>
<td>11.8</td>
<td>948.2699; 441.2759; 428.0344; 136.0609</td>
</tr>
<tr>
<td>11</td>
<td>3, 5-Tetradecadienoyl-CoA</td>
<td>C14:2-CoA</td>
<td>12.04</td>
<td>974.2817; 467.2907; 428.0339; 261.1250; 136.0608</td>
</tr>
<tr>
<td>12</td>
<td>5-Tetradecenoyl-CoA</td>
<td>C14:1-CoA</td>
<td>12.37</td>
<td>976.3030; 469.3088; 428.0359; 136.0609</td>
</tr>
<tr>
<td>13</td>
<td>Lauroyl-dephospho-CoA</td>
<td>C12-CoA</td>
<td>12.6</td>
<td>870.3185; 443.2912; 348.0685; 136.0608</td>
</tr>
<tr>
<td>14</td>
<td>Myristoyl-dephospho-CoA</td>
<td>C14-CoA</td>
<td>13.26</td>
<td>898.3513; 471.3229; 348.0691; 136.0611</td>
</tr>
<tr>
<td>15</td>
<td>Linolenyl-dephospho-CoA</td>
<td>C18:3-CoA</td>
<td>13.47</td>
<td>948.3690; 521.3389; 348.0680; 136.0611</td>
</tr>
<tr>
<td>16</td>
<td>Hexadecenoyl-dephospho-CoA</td>
<td>C16:1-CoA</td>
<td>13.48</td>
<td>924.3665; 497.3384; 348.0689; 136.0613</td>
</tr>
<tr>
<td>17</td>
<td>Arachidonoyl-dephospho-CoA</td>
<td>C20:4-CoA</td>
<td>13.69</td>
<td>974.3800; 547.3529; 348.0687; 136.0606</td>
</tr>
<tr>
<td>18</td>
<td>Linoleoyl-dephospho-CoA</td>
<td>C18:2-CoA</td>
<td>13.74</td>
<td>950.3821; 523.3533; 348.0686; 261.1249; 136.0609</td>
</tr>
<tr>
<td>19</td>
<td>Palmitoyl-dephospho-CoA</td>
<td>C16-CoA</td>
<td>14.04</td>
<td>926.3819; 499.3542; 348.0685; 136.0610</td>
</tr>
<tr>
<td>20</td>
<td>Oleyl-dephospho-CoA</td>
<td>C18:1-CoA</td>
<td>14.21</td>
<td>952.3967; 525.3694; 348.0690; 261.1248; 136.0614</td>
</tr>
<tr>
<td>21</td>
<td>Stearyl-dephospho-CoA</td>
<td>C18-CoA</td>
<td>14.85</td>
<td>954.4100; 527.3851; 348.0688; 136.0610</td>
</tr>
</tbody>
</table>

Note: \(^5\) Verified by authentic standards
5.2.7 Method verification

The stability was monitored for the assessment of LC-MS and GC-MS approach with QC samples. As a result of different m/z and polarities, 6 ions (carnitine, hippuric acid and 3-Hydroxy-9-hexadecenoylcarnitine (C16:1-OH-CN) from positive ion mode and uric acid, tryptophan and fumaric acid from negative ion mode) from LC-MS analysis have been selected for method assessment according to the variation of their peak area intensity. The peak area intensity variation of ribitol from GC-MS analysis was also summarized in Table 5.3

Table 5.3 Stability assessment of LC-MS and GC-MS

<table>
<thead>
<tr>
<th>Instruments</th>
<th>Compounds name</th>
<th>RSD (%) of peak intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-MS (pos)</td>
<td>Carnitine</td>
<td>8.7%</td>
</tr>
<tr>
<td>LC-MS (pos)</td>
<td>Hippuric acid</td>
<td>7.2%</td>
</tr>
<tr>
<td>LC-MS (pos)</td>
<td>C16:1-OH-CN</td>
<td>8.0%</td>
</tr>
<tr>
<td>LC-MS (neg)</td>
<td>Uric acid</td>
<td>3.5%</td>
</tr>
<tr>
<td>LC-MS (neg)</td>
<td>Tryptophan</td>
<td>2.8%</td>
</tr>
<tr>
<td>LC-MS (neg)</td>
<td>Fumaric acid</td>
<td>2.5%</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Ribitol</td>
<td>14.9%</td>
</tr>
</tbody>
</table>

5.2.8 Preprocess of GC-MS data and LC-MS data and metabolites identification

The data extraction of GC-MS was analyzed following previously published method [198, 199]. Raw GC-MS data were firstly converted into AIA (CDF) files on the Agilent GC-MS 5975 Data Analysis software. The output files were operated by using XCMS software processed on the freely available R statistical language (v 3.3.0). For data extraction of LC-MS, the raw data was directly extracted by using SIEVE 2.2 software (Thermo Fisher Scientific, MA, USA). Finally, a list of ion features was generated including a three-dimensional matrix which consists of ion names (observations), arbitrary peak index (pair of m/z value and retention time), and peak area.
After normalization, the extracted GC-MS and LC-MS data were imported into Simca-P software (Ver. 11, Umetrics, Umea, Sweden). Both Principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) were operated for multivariate statistical analysis. The identification of metabolites was based on statistically significant threshold of variable importance in the projection (VIP) values > 1.2 and \( p \)-value < 0.05. For identification of metabolites form GC-MS analysis, the discriminating metabolites were firstly carried out by comparing the mass spectra and retention time of each compound with the standards available in our lab. Then, the remaining metabolites were identified by searching library NIST with a similarity of more than 70%. For identification of metabolites from LC-MS, the discriminating metabolites were identified by searching accurate MS and MS/MS fragments in Human Metabolome Database (HMDB) (http://www.hmdb.ca) and Biofluid Metabolites Database (http://metlin.scripps.edu). Moreover, part of them was also confirmed by authentic standards.

5.2.9 RT-PCR on kidney mRNA levels

Total RNA was isolated from kidney using Trizol reagent (Invitrogen). The mRNA was reverse transcribed to cDNA using iScriptTM cDNA synthesis kit (BioRad). Expression of targeted mRNA was measured with Taq DNA (Invitrogen) with reverse and forward primers. Sequences are listed in Table 5.4. Targeted gene expression was normalized to the internal control of \( \beta \)-action or GAPDH and presented as fold changes relative to control (LS group) values.
Table 5.4 Sequences of mRNA.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward</td>
</tr>
<tr>
<td><strong>Bcat2</strong></td>
<td>ACACTTTGAACATGAGCCTGC</td>
</tr>
<tr>
<td><strong>Hmgcl</strong></td>
<td>GGAGTGAAGATGCCGTCAGT</td>
</tr>
<tr>
<td><strong>Acad8</strong></td>
<td>ACCTGCTTCAGCGACTAGC</td>
</tr>
<tr>
<td><strong>Acads</strong></td>
<td>TGGCGACGGTTACACACTG</td>
</tr>
<tr>
<td><strong>Acadsb</strong></td>
<td>CCCAACCTGCTTGTCTCTTTG</td>
</tr>
<tr>
<td><strong>Acadm</strong></td>
<td>AGGGTTTAGTTTGAGTTGACGG</td>
</tr>
<tr>
<td><strong>Acadl</strong></td>
<td>TCTTTCTCAGGACGTGACA</td>
</tr>
<tr>
<td><strong>Hibch</strong></td>
<td>GTGGAAGCGTATAACGCTC</td>
</tr>
<tr>
<td><strong>Ivd</strong></td>
<td>ATCAACGGGCTAAACGAGGAG</td>
</tr>
<tr>
<td><strong>Cpt1a</strong></td>
<td>CTCCGCCTTAGCCATGAAG</td>
</tr>
</tbody>
</table>


5.2.10 Data analysis

Fold changes were calculated based on the average ratio of arbitrary groups (Table 5.5). Heatmap was performed by HemI software (Heatmap Illustrator, version 1.0) [159]. Pathway analysis was performed on KEGG pathway database (http://www.genome.jp/kegg/) and MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/). Receiver operating characteristic (ROC) curve analysis was carried out on SigmaPlot (version 10.0). Analysis of variance (ANOVA) was performed as significant analysis method. Significance was considered when \( p \) value < 0.05.
Table 5.5 Changes of identified metabolites in response to diabetic kidney disease and exercise

<table>
<thead>
<tr>
<th>Classification</th>
<th>Compounds</th>
<th>LS vs. LE</th>
<th>LS vs. OS</th>
<th>LE vs. OE</th>
<th>OS vs. OE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>fc</td>
<td>p-value</td>
<td>fc</td>
<td>p-value</td>
</tr>
<tr>
<td><strong>Amino acids metabolism pathway</strong></td>
<td>Asparagine</td>
<td>1.37</td>
<td>ns</td>
<td>1.75</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>1.02</td>
<td>ns</td>
<td>0.67</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>1.26</td>
<td>ns</td>
<td>1.41</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>0.97</td>
<td>ns</td>
<td>1.24</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>1.12</td>
<td>ns</td>
<td>1.38</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Saccharopine</td>
<td>1.26</td>
<td>ns</td>
<td>0.18</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>0.99</td>
<td>ns</td>
<td>1.32</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>0.97</td>
<td>ns</td>
<td>1.41</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>1.18</td>
<td>*</td>
<td>1.15</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>1.46</td>
<td>*</td>
<td>1.51</td>
<td>*</td>
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<td>ns</td>
<td>1.34</td>
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LysoPE(20:4)  1.06  ns  1.53  ***  1.03  ns  0.71  **
LysoPC(20:4)  1.04  ns  1.72  ***  1.19  ns  0.72  ***
LysoPC(22:5)  0.94  ns  1.45  ***  1.34  **  0.87  ns
LysoPE(16:0)  0.98  ns  1.32  **  1.12  ns  0.84  
LysoPC(18:1)a  1.00  ns  1.59  ***  1.19  ns  0.75  **
LysoPE(18:1)  1.03  ns  1.57  ***  1.06  ns  0.69  ***
LysoPC(18:1)b  1.00  ns  1.42  **  1.03  ns  0.73  **
PE(P-16:0)  0.91  *  1.58  ***  1.26  ns  0.73  **
LysoPC(P-16:0)  1.03  ns  1.52  ***  0.91  ns  0.61  ***
LysoPE(18:0)  0.99  ns  1.37  **  1.05  ns  0.76  *
LysoPC(18:0)  0.81  *  1.39  ***  1.34  **  0.78  **
C6:1-CoA  0.75  ns  1.06  ns  0.73  ns  0.57  ns
C5-CoA  0.94  ns  1.44  ns  1.19  ns  0.78  ns
C6-CoA  1.14  ns  1.57  ns  0.79  ns  0.57  ns
C10:1-CoA  0.63  ns  1.52  ns  0.79  ns  0.32  ns
C12:1-CoA  0.92  ns  2.01  ns  0.83  ns  0.38  *
C14:2-CoA  1.19  ns  1.21  ns  0.41  ns  0.41  *
C14:1-CoA  1.43  ns  2.56  *  0.78  ns  0.44  *
C12-CoA  1.02  ns  2.56  *  1.26  ns  0.50  ns
C14-CoA  1.22  ns  1.96  *  1.25  ns  0.78  ns
C18:3-CoA  1.15  ns  3.18  ***  2.23  *  0.81  ns
C16:1-CoA  1.17  ns  3.18  ***  1.84  ns  0.68  ns
C20:4-CoA  0.76  ns  0.93  ns  1.25  ns  1.03  ns
C18:2-CoA  0.99  ns  1.81  ***  1.61  *  0.88  ns
C16-CoA  1.18  ns  1.37  ns  1.23  ns  1.05  ns
C18:1-CoA  0.95  ns  1.90  **  2.04  **  1.02  ns
C18-CoA  0.79  ns  0.98  ns  1.43  ns  1.16  ns
Flavin  0.95  ns  1.36  **  1.22  *  0.85  *
Mononucleotide  1.31  ns  0.14  ***  0.27  ***  2.53  ns
Thyroxine glucuronide  1.36  ns  1.71  **  0.60  *  0.48  ***
Acetyl-L-methionine  0.96  ns  0.82  *  0.97  ns  1.15  ns
Carnitine

Note: fc, fold change. *, p-value < 0.05. **, p-value < 0.01. ***, p-value < 0.001.

5.3 Results

5.3.1 Exercise reduces DN induced inflammation, fibrosis and mesangial expansion

The db/db mouse model is the most widely used mouse model of T2DM as a result of leptin receptor (LepR<sub>db/db</sub>) defection [168, 238]. Following the development of diabetes, db/db mice induce progressive kidney disease which is similar to diabetic nephropathy on human. Therefore, db/db mice are regularly used to investigate the mechanisms of renal disease that induced by T2DM. As shown in Table 5.6, substantial increase of body weight was observed on db/db sedentary mice, while exercise exhibited significant body weight

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loss after five weeks of exercise. The kidney weight to body weight ratio is significantly increased might also owing to body weight loss after exercise on \(db/db\) mice. In addition, the dramatically reduced urinary creatinine (with fold change of 8.5) and elevated urinary albumin to creatinine ratio (with fold change of 9.2) in \(db/db\) diabetic mice, indicating diabetic induced renal dysfunction. However, there was no significant difference on DN mice after exercise.

What’s more, accumulation several risk factors, such as TNF\(\alpha\), Col1a1, COX-2, IL6, Fibronectin and \(\alpha\)-SMA in \(db/db\) sedentary mice in kidney samples indicating serious inflammation and fibrosis have been induced (Figure 5.5). However, exercise exerted significant effect on reversing risk factors related to proinflammation (TNF\(\alpha\), COX-2) and fibrosis (Col1a1).

As glomerular mesangial expansion is an important characteristic of DN, we examined the mesangial expansion in mice after five weeks of exercise. We found that severe mesangial expansion was induced in diabetic kidneys of 15 week old \(db/db\) mice, when compared with non-diabetic sedentary group (Figure 5.6). Exercise was able to alleviate mesangial expansion. Considering mesangial expansion plays a pathologic role for kidney injury in patients with diabetes \[239\], these results indicated that exercise was able to alleviate the renal injury in \(db/db\) mice prior to the improvement on renal function.

Table 5.6 Pharmacological parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>(db/m^+) mice</th>
<th>(db/db) mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sedentary</td>
<td>Exercise</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>29.44±0.084</td>
<td>30.17±0.11</td>
</tr>
<tr>
<td>Blood glucose level (mM)</td>
<td>5.1±0.3</td>
<td>4.3±0.4</td>
</tr>
<tr>
<td>Kidney weight/Body ratio</td>
<td>0.0077±0.000072</td>
<td>0.0073±0.000085</td>
</tr>
<tr>
<td>Urinary creatinine (mg/dL)</td>
<td>1.61±0.26</td>
<td>1.11±0.15</td>
</tr>
<tr>
<td>Urinary albumin/creatinine ratio</td>
<td>108.38±15.82</td>
<td>142.85±24.54</td>
</tr>
</tbody>
</table>
a, \( p \) value < 0.01; b, \( p \) value < 0.001, \( db/db \) sedentary mice vs. \( db/m+ \) sedentary mice, or \( db/db \) exercise mice vs. \( db/m+ \) exercise mice. ★, \( p \) value < 0.001, \( db/db \) exercise mice vs. \( db/db \) sedentary mice.

Figure 5.5 Regulatory effect of exercise on inflammatory and fibrotic markers in kidneys.

Note: #, \( p < 0.05 \), ##, \( p < 0.01 \), ###, \( p < 0.001 \), OS vs. LS or OE vs. LE. *, \( p < 0.05 \), **, \( p < 0.01 \), ***, \( p < 0.001 \). OE vs. OS.
Figure 5.6 Exercise effect on morphology changes of glomeruli. A, Light microscopic appearance of glomeruli from db/m+ sedentary (a), db/m+ exercise (b), db/db sedentary (c) and db/db exercise (d) mice in kidneys at 15 weeks of age. B, relative area of glomeruli. Results are showed as relative area (compared to LS group) of glomeruli (40 glomeruli per mice, 5 mice per group).

Note: #, p < 0.001, OS vs. LS or OE vs. LE. *, p < 0.001, OE vs. OS.

5.3.2 Regular physical activity reduces lipidemia

Furthermore, diabetic dyslipidemia is one of the major risk factors for the development of diabetic nephrology [240, 241]. We can see from Figure 5.7, the total triacylglycerol level, total cholesterol level, non-HDL cholesterol level etc. were all significantly increased on db/db sedentary group. However, the total cholesterol level was significantly reduced after exercise. In addition, the concentration of non-HDL which positively related to cardiovascular disease and atherosclerosis [242], was markedly reduced on db/db mice after exercise. Besides, other parameters, such as total/HDL, non-HDL/HDL were also significantly decreased after exercise on db/db diabetic mice, indicating that physical
activity is desirable in reducing cholesterol level in *db/db* diabetic mice, and may plays a positive role in improving the dysfunction of hyperlipidemia, which is induced by T2DM.

![Figure 5.7 Regulatory effect of exercise on lipidemia markers in plasma.](image)

Note: #, *p < 0.05, OS vs. LS or OE vs. LE. *, *p < 0.05, OE vs. OS.

### 5.3.3 Method assessment of LC-MS and GC-MS analysis

To evaluate the reliability of the established untargeted LC-MS analysis method in the current study, 6 ions from both positive and negative ionization modes were selected. The relative standard derivations (RSD) of each peak area of the six ions from QC samples have been used to evaluate the stability of this method. We can see from Table 5.3, the RSD% value of peak area drift were less than 8.7%. For the evaluation of stability of GC-MS analysis, the RSD% value of peak area drift of IS (ribitol) was less than 14.9%. Together with the tightly clustering of QC samples in PCA score plots (Figure 1 and Figure S2), these results indicated that the LC-MS and GC-MS systems were reliable for analysis.
5.3.4 Multivariate statistic analysis

In this study, multivariate statistical analysis methods, PCA and PLS-DA were used in metabolic profiling analysis of biomarkers (Figure 5.8), which contribute to the discrimination of different mouse model and treatment. $R^2_X$, $R^2_Y$ and $Q^2$ (cum) are important parameters to assess the model. $R^2_X$ represents cumulative interpretation ability of the current model. $R^2_Y$ represents the cumulative model variation in $Y$, while $Q^2_Y$ represents the cumulative predicted variation in $Y$. To acquire a credible predictive ability and prevent the model away from over-fitting, the value of the 3 parameters should close to 1. Besides, the validation plots (LS vs. OS) were also generated to assess if the current PLS-DA models were spurious. As shown in Figure 5.9, all $Q^2$ values to the left were lower than the original point to the right. Meanwhile, the regression lines ($Q^2$) intersected the vertical axis (on the left) were below zero. The results of the parameters in Figure 5.8 and Figure 5.9 showed that the PCA or PLS-DA models are valid and with satisfactory grouping and predicting ability.
Figure 5.8 Multivariate statistic analysis of ions from LC-MS and GC-MS analysis.

Note: (a) Principal component analysis ($R^2_X = 0.673$, $Q^2$ (cum) = 0.404). (■) Black square represents db/m+ mice treated with sedentary (LS). (●) Red circle represents db/m+ mice treated with exercise (LE). (♦) Blue diamond represents db/db mice treated with sedentary (OS). (▲) Green star represents db/db mice treated with exercise (OE). (○) Open orange circle represents quality control samples (QC). (b) Volcano plot of detected ions between OS and LS group. (c) PCA score plot by LC-MS analysis in negative ion mode ($R^2_X = 0.681$, $Q^2$ (cum) = 0.383). (d) PCA score plot by GC-MS analysis ($R^2_X = 0.516$, $Q^2$ (cum) = 0.29). (▼) Orange inverted triangle represents quality control samples (QC).
5.3.5 Changes of renal metabolites related to diabetic nephropathy

We can see from PCA score plots (Figure 5.8), \(db/db\) mice were clustered clearly away from the \(db/m+\) mice, indicating notable metabolic changes between the two types of mice (Figure 5.8 and Figure 5.9). Finally, together with targeted analysis of acyl-CoA, 73 metabolites were identified contributing to the discrimination of control and DN mice in kidney samples. These metabolites were mainly classed into amino acids, organic acids,
nucleosides, fatty acids, phospholipids, carbohydrates, acyl-CoA, acylcarnitines and acylglycines, and they were mainly involved in fatty acid metabolism, fatty acid anabolism, valine, leucine and isoleucine (BCAAs) degradation, purine metabolism, et al. (Figure 5.10, generated by MetaboAnalyst 3.0 [http://www.metaboanalyst.ca/] [243]. In this study, 16 phospholipids were found substantially increased on db/db mice in kidney samples (Figure 11(a)), and evidently contributing to the discrimination of control and DN mice (Figure 12(a)). In addition, five acylglycines were significantly decreased (Figure 5.11(b)) and seven acyl-CoA were found substantially increased (Figure 5.12(d)), they all got notable contribution to the discrimination of the two types of mice (Figure 5.12, AUC > 0.7). What’s more, acylcarnitines were also found significantly changed between control and DN mice (Figure 5.11(c)). However, saturated ones were significantly decreased while hydroxyl-conjugated ones were substantially increased, and they also got notable contribution to the discrimination of db/m+ and db/db mice in kidney (Figure 5.12(c) and Figure 5.12(e)). In addition to metabolites involved in lipid metabolism, 10 metabolites in purine metabolism (Figure 14(b)) were also significantly changed and highly contributing to the discrimination of control and DN mice (Figure 14(c)). What’s more, several amino acids and related metabolites, such as BCAAs (valine and isoleucine), tyrosine, phenylalanine, lactic acid, et al. were all significantly increased in DN mice (Figure 5.14). The results indicated that there are huge metabolic changes between control and DN mice in kidney.
Figure 5.10 Pathway analysis among differentiated metabolites between \textit{db/m}+ sedentary and \textit{db/db} sedentary groups.

Note: BCAA, Branched-chain amino acid (valine, leucine and isoleucine). FDR, false discovery rate.
Figure 5.11 Heatmap of lipids in response to DN and exercise.

Note: (a) Heat map of phospholipids in response to DN and exercise. (b) Heat map of acylglycines in response to DN and exercise. (c) Heat map of acylcarnitines in response to DN and exercise. (d) Heat map of acyl-CoA in response to DN and exercise. *, P < 0.05, LE vs. LS, OS vs. LS, or OE vs. LE. ☆, P < 0.05, OE vs. OS.
Figure 5.12 Discrimination of various kinds of lipids in response to DN or exercise based on ROC analysis in kidneys.

Note: (a) Discrimination analysis of phospholipids in response to DN (between $db/m+$ sedentary and $db/db$ sedentary mice). (b) Discrimination analysis of phospholipids in response to exercise on DN mice (between $db/db$ sedentary and $db/db$ exercise mice). (c) Discrimination analysis of 3-hydroxy-acylcarnitines in response to DN. (d) Discrimination analysis of 3-hydroxy-acylcarnitines in response to exercise on DN mice. (e) Discrimination analysis of 3-hydroxy-acylcarnitines in response to exercise on DN mice. (f) Discrimination analysis of acyl-CoA in response to DN. (g) Discrimination analysis of acylglycines in response to DN. A, area under curve.
Figure 5.1 Diagrammatic sketch of fatty acid metabolism.

Note: FFA, free fatty acid. CoA, Coenzyme A. PL, phospholipids. TG, triglyceride. CPTI, Carnitine palmitoyltransferase I. CPTII, Carnitine palmitoyltransferase II. Acadl, long-chain specific acyl-CoA dehydrogenase. 3-OH-Acyl-CoA, 3-hydroxy conjugated acyl-CoA. 3-OH-Acylcarnitine, 3-hydroxy conjugated acylcarnitines. LS, db/m+ sedentary group. LE, db/m+ exercise group. OS, db/db sedentary group. OE, db/db exercise group. #, p < 0.05; ##, p < 0.01, OS vs. LS, OS vs. LS. **, p < 0.01, OE vs. OS.
Figure 5.14 Regulatory effect of exercise on metabolites in purine metabolism.

Note: (a) Schematic diagram of purine metabolism pathway related to pathogenic process of diabetic nephropathy. (b) Regulatory effect of exercise on metabolites in purine metabolism pathway. (c) Discrimination of metabolites in purine metabolism pathway in response to diabetic nephropathy based on ROC curve analysis. (d) Discrimination of metabolites in purine metabolism pathway in response to exercise on diabetic nephropathy.
mice based on ROC curve analysis. LE, db/m+ exercise group. OS, db/db sedentary group. OE, db/db exercise group. #, p < 0.05; ##, p < 0.01; ###, p < 0.001, OS vs. L or OE vs. LE. *, p < 0.05; **, p < 0.01; ***, p < 0.001, OE vs. OS.

Figure 5.15 Exercise effect on part of disturbed metabolites in BCAA catabolism and other amino acid metabolism pathways.

Note: 3-M-C5-CoA, 3-methyl-butyryl CoA. 2-M-C5-CoA, 2-methyl-butyryl CoA. IsoC4-CoA, isobutyryl-CoA. CN. LS, db/m+ sedentary group. LE, db/m+ exercise group. OS, db/db sedentary group. OE, db/db exercise group. *, p < 0.05; **, p < 0.01; ***, p < 0.001, OS vs. L or OE vs. LE. ★, p < 0.05; ★★, p < 0.01; ★★★, p < 0.001, OE vs. OS.

Figure 5.16 Regulatory effect of exercise on the activity of mRNA in BCAA catabolism pathway.

Note: Bcat2, branched chain amino acid transaminase 2. Acads, short-chain acyl-CoA dehydrogenase. Acadsb, Short-branched chain acyl-CoA dehydrogenase. Acadm, medium-chain acyl-CoA dehydrogenase. Hmgcl, 3-hydroxymethyl-3-methylglutaryl-CoA lyase. Hibch, 3-hydroxyisobutyryl-CoA hydrolase. Ivd, isovaleryl-CoA dehydrogenase. LS, db/m+ sedentary group. LE, db/m+ exercise group. OS, db/db sedentary group. OE, db/db
exercise group. *, p < 0.05; **, p < 0.01; ***, p < 0.001, OS vs. L or OE vs. LE. ★, p < 0.05; ★★, p < 0.01; ★★★, p < 0.001, OE vs. OS.

Figure 5.17 Diagrammatic sketch of disturbed metabolic pathways related to pathogenic process of diabetic renal disease.

5.3.6 Renal metabolic changes related to exercise

We can see from Figure 5.8, the LE group was clustered together with LS group. Although there was a slight overlap between OS and OE groups, a clear separation trend can be still observed. Based on the identified metabolites, only 4 metabolites were contributing to the
discrimination on control mice (LE vs. LS) after exercise, while 46 metabolites were significantly changed after exercise on DN mice (OE vs. OS). The results indicated that the metabolic changes after exercise training might be more susceptible on DN mice. Among significantly changed metabolites on DN mice after exercise, most of differentiated phospholipids were significantly reversed by exercise (Figure 5.11(a) and Figure 5.12(b), with high AUC value). In addition, part of acyl-CoA, acylcarnitines and acylglycines that involved in fatty acid metabolism was also substantially regulated after exercise. Besides, eight metabolites involved in purine metabolism were also significantly reversed by exercise (Figure 5.14, high AUC value). What’s more, several amino acids and their metabolites (Figure 5.15), such as isoleucine, tyrosine, phenylalanine, et al. were all substantially reversed by exercise. The transcript mRNA in BCAAs metabolism pathway, such as Bcat2, Hmgcl, Acad8 and Hibch were also substantially improved after exercise (Figure 5.16). All these results indicated that exercise may play a positive role in metabolic dysfunction of diabetic nephropathy in the kidney.

5.4 Discussion

5.4.1 Diabetic nephropathy and physical activity

Type 2 diabetes mellitus is an increasingly health problem worldwide with many complications. One of the complications is diabetic renal dysfunction. It is well known that kidney is particularly vulnerable to disease insults as a result of the high renal blood flow and also because it metabolizes, transports and concentrates chemicals that present in the tubular fluid. Diabetic kidney disease is a progressive renal dysfunction caused by poor control of blood glucose level, high blood pressure or advanced glycation end products formation that induced by diabetes. Recent research have found that regardless of aerobic physical activity or resistance exercise, might all play an effective therapeutic role for
T2DM [107, 205]. In this study, \( db/db \) diabetic mouse model with progressive renal injury [244-246] was selected to investigate the regulatory effect of moderate-intensity of physical activity on DN in kidney.

5.4.2 Exercise has good effect in alleviating lipidemia on DN mice

High level of lipids is a crucial risk factor contributing to diabetic nephrology. There is increasing evidence that the disorder of lipid metabolism and accumulation of lipids play an important role in the pathogenesis of renal disease induced by diabetes [247, 248]. In this model of DN, we have found that in addition to total triacylglycerol, various kinds of cholesterol parameters (total cholesterol, Non HDL cholesterol, Total/HDL cholesterol, Non HDL/HDL cholesterol and HDL cholesterol) were all significantly increased in \( db/db \) sedentary group (Table 1). However, the level of total cholesterol, non HDL cholesterol and total/HDL cholesterol were significantly reduced after exercise, indicating that exercise may have good regulatory effect in improving cholesterol degradation in the body. For metabolites in lipids metabolism (Figure 5.13), particularly fatty acid (FA) metabolism, it is well known that lipids turnover is a regular process in cells [249], it plays an important role in attaining the homeostasis of lipid composition of the body. We all know that the free fatty acid (FFA), especially long chain fatty acids can only be degraded through \( \beta \)-oxidation. During this process, mitochondrial enzyme carnitine acyltransferase I (CPTI) is the rate-limiting enzyme for the formation of acylcarnitines through catalyzing the transfer of acyl groups of long chain acyl-CoA from CoA to free carnitine. In this study, the expression of CPTI\( \alpha \) was significantly decreased in DN mice, which leads to the accumulation of long-chain acyl-CoA in the kidney. As a result, the levels of saturated acylcarnitines were decreased (Figure 5.11(c)). In the meantime, acylglycines (Figure 5.11(b)), which formed by conjugating glycine to the toxic acyl-CoA esters then be
excreted in the urine as a detoxification process in the kidney [250], was also reduced in DN mice in the kidney. In addition, the activity of Acadl, which is primarily responsible for fatty acid β-oxidation (FAO) within the mitochondria, was substantially decreased in DN mice, which also contributed to the accumulation of long-chain acyl-CoA in the kidney. Interestingly, the levels of 3-hydroxy conjugated acylcarnitines (3-OH-acylcarnitines) were accumulated in the kidney, which might be as a result of incomplete FAO in mitochondria during the last steps from 3-OH-acyl-CoA toward acetyl-CoA into TCA cycle. Combined with the results of accumulation of phospholipids in DN mice in the kidney, an evidently disturbed metabolism of various kinds of lipids which might contribute to lipotoxicity has been observed in DN mice. In this study, notable contribution of these lipids to the progression of DN (high AUC values, Figure 5.12) have also been observed, which verified the fact that dyslipidemia tightly correlated with the progression of renal injury [251, 252]. However, as shown in Figure 2, the levels of most of phospholipids (Figure 5.12(b), with high AUC values) and acylcarnitines, part of acylglycine and acyl-CoA were significantly reversed by exercise. Although the activity of CPTIα was not notably improved, the expression of Acadl in FAO pathway was substantially increased by exercise on DN mice. All the results indicated that exercise exerts good effect in alleviating DN induced hyperlipidemia in the kidney, especially plays a good role in improving the efficiency of FAO.

5.4.3 *Exercise exerts positive effect in improving DN induced metabolic acidosis*

Besides, we all know that hyperglycemia is an important risk factor for the development of diabetic nephropathy [253, 254]. The accumulation of glucose is a typical metabolic disorder, in which the body is not able to control the blood glucose level as a result of lacking insulin or insulin resistance. High concentration of glucose level is known to be
able to increase the synthesis of other matrix components (such as lactic acid, alanine, threonine, et al.) that contributes well to the loss of kidney functions [255, 256]. Although the level of glucose was not significantly reduced by exercise (Table 5.5), the accumulation of lactic acid which may owning to inefficient elimination from kidney into urine as a result of impaired renal function, has been significantly decreased. The regulatory effect of exercise on glycolysis indicated that exercise may play a positive role in improving hyperglycemia that contributing to renal injury. In addition, metabolic acidosis occurs when the body is lack of insulin and corresponding, which also leads to accumulation of glucose in the body [257]. In this study, four TCA cycle intermediates (maleate, fumarate, malate and citrate) were all significantly increased in db/db sedentary group, together with the accumulation of lactate in the kidney, and several intermediates (Figure 5.15, asparagines, tyrosine, phenylalanine and tryptophan) that involved in the formation of acids were also increased, indicating that the accumulation of acids might cause severe metabolic acidosis [258] in kidney (Figure 5.17). The phenomenon may due to inefficient elimination of acids and redundant intermediates from kidney into urine owing to impaired tissue sensitivity to insulin. However, in addition to lactate, the levels of citrate, as well as several intermediates (asparagines, tyrosine, phenylalanine and tryptophan) were also significantly reduced after exercise in DN mice. The results indicated that exercise might have positive effect in improving metabolic acidosis, which might also be a positive feedback of improved renal injury.

5.4.4 Exercise may alleviate DN induced inflammation through accelerating BCAA metabolism

Recently, epidemiological and experiment data revealed that branched-chain amino acids (BCAAs, including valine, isoleucine and leucine) contribute to the development of insulin resistance [144, 259]. In this study, we have found that the level of BCAAs (isoleucine and
valine) were significantly increased in db/db mice, which is in accordance with the findings of the other studies [111, 144]. In addition, the activity of genes in BCAA catabolism pathway (Figure 5.17), such as \textit{Bcat2}, \textit{Acadm}, \textit{Acads}, \textit{Acadsb}, \textit{Acad8}, \textit{Ivd} and \textit{Hmgcl} were all substantially decreased (Figure 5.16), which in accordance with the results of BCAAs. Interestingly, the concentration of isoleucine was significantly reduced in db/db mice in kidney samples by exercise, and the activities of genes in the downstream of BCAA catabolism pathway, such as \textit{Bcat2}, \textit{Acad8}, \textit{Hmgcl} and \textit{Hibch} were substantially increased after exercise in kidney. The results indicated that moderate-intensity of exercise training might play a positive role in improving insulin resistance through accelerating BCAAs catabolism in kidney on DN mice. However, the level of BCAAs in the kidney, which is related to renal functions, remains controversial. Several studies reported that decreased level of BCAAs might contribute to the development of renal fibrosis [260] and acute or chronic uremia [261]. Thus, more studies should be carried out to investigate the regulatory effect of exercise on DN.

5.4.5 Exercise may ameliorate DN induced inflammation through alleviating the accumulation of uric acid

Uric acid is an end product of purine metabolism, it is mainly generated in the liver, intestines, muscles and kidneys. In the last two decades, a great quantity of observational studies has reported the potential link between the accumulation of serum uric acid and chronic kidney disease [262, 263]. Some research has found that uric acid could adhere to the surface of renal epithelial cells and then induce inflammatory response to these cells [264]. Consequently, systemic cytokine production such as tumor necrosis factor $\alpha$ (TNF$\alpha$), and chemokines production such as cyclooxygenase 2 (COX-2) are increased. In addition, experimental studies have found that hyperuricemia could also cause tubulointerstitial fibrosis in the kidney [265]. Consistent with the previous studies, animal studies found that
the decrease of uric acid levels may slow the progression of chronic kidney disease. Notably, the reduction of uric acid levels could decrease tubulointerstitial fibrosis in diabetic nephropathy [266]. In this study, the accumulation of uric acid has been observed in DN mice in the kidney (Figure 5.14), which in accordance with the dramatic increase of risk factors related to inflammation (TNFα, COX-2 and IL-6) and fibrosis (Fibronectin, Collα1 and α-SMA) in the kidney (Figure 5.5). However, the level of uric acid and inflammation risk factors TNFα and COX-2 have substantially reduced by exercise. In addition, eight precursors of uric acid (succinyladenosine, IMP, AMP, guanosine, guanine, inosine, deoxyinosine and xanthine) in purine pathway have also been detected in accordance with the changes of uric acid and with great contribution to the progression of DN (Figure 5.14(c)). We can see from Figure 5.14(b), the levels of IMP, AMP and deoxyinosine were significantly decreased, which consistent with the accumulation of their downstream metabolites. Interestingly, although the level of IMP and AMP was not significantly increased by exercise, the level of deoxyinosine was significantly increased after exercise, and the several downstream metabolites (Figure 5.16(b), succinyladenosine, guanosine, guanine, inosine, hypoxanthine and xanthine) have also been substantially reversed. The regulatory effect of exercise on uric acid (AUC = 0.90) and its precursors (the AUC of succinyladenosine, guanosine, xanthine and hypoxanthine were 0.93, 0.90, 0.86 and 0.83, respectively) exerts a notable contribution to the classification of metabolic changes between DN sedentary and DN exercise mice. Consequently, all the results indicated that exercise may exert a good role in alleviating DN induced inflammation through reducing the levels of uric acid.

5.5 Summary of this chapter

In this study, we have evaluated the regulatory effect of moderate-intensity of exercise on DN through an integrated metabolomics technology on db/db diabetic mice in kidney
samples. Dysfunction of lipids (including phospholipids, acyl-CoA, acylcarnitines and acylglycines), metabolites involved in glycolysis, metabolic acidosis, BCAA catabolism and purine metabolism were observed in DN mice. Exercise may exert good regulatory role in improving the dysfunction of hyperlipidemia via improving the efficiency of fatty acid oxidation in the kidney. Besides, DN induced metabolic acidosis which may lead to renal injury were also improved by exercise. In addition, the dysfunction of BCAA catabolism which positively related to insulin resistance was partially reversed after exercise. What’s more, exercise may exert good effect in alleviating DN induced inflammation and fibrosis through attenuating the level of uric acid in the kidney. This study provides important baseline metabolic information in studying the mechanism of physical activity on diabetic kidney disease, which may underlie beneficial effects of exercise on diabetic kidney disease by lifestyle modification.
Chapter 6 Conclusions and future work

In this study, efforts were spent to investigate mass spectrometry-based metabolomics technologies to elucidate the regulatory effect of exercise on T2DM mice. Owning to widespread complications induced by T2DM, such as cardiovascular disease, diabetic nephropathy, and increasing mortality rate world widely, treatment of T2DM is urgent. Exercise has long been regarded as an effective way to treat T2DM. In order to investigate the regulatory mechanism of exercise on T2DM and T2DM-induced complications, both untargeted and targeted metabolomics studies have been carried out on different samples from db/m+ and db/db mice, regardless of sedentary or exercise treatment.

Ample evidence has indicated that T2DM and obesity can induce global metabolic changes of acylcarnitines. Excessive ACs supply and its accumulation could interfere insulin response, which may induce glucolipotoxicity and diabetic ketosis. Although a large number of studies have reported the quantification method of ACs by LC-QqQ-MS, a limited number of ACs have been quantified. In this study, in order to get a relatively comprehensive coverage of ACs, a high-resolution PRM method has been applied to quantify acylcarnitine species. Finally, a total of 117 acylcarnitines has been identified out from plasma and urine samples. The result of method assessment indicated that the current PRM assay exhibited excellent separation and differentiation of isomers. Confident and accurate fragments confirmation of ACs allowed much broader quantification of acylcarnitine species (especially those without authentic standards) compared to other methods. Application in detecting ACs on db/m+ and db/db mice in plasma and urine samples demonstrate insufficient supply of free carnitine and accumulation of ACs in the mouse body, which might contribute to incomplete fatty acid \( \beta \)-oxidation. Interestingly, the decrease of odd-numbered medium- to long-chain acylcarnitines in urine samples was firstly observed between db/m+ and db/db mice. Besides, the accurate MS and MS/MS
data can be deposit to HMDB (http://www.hmdb.ca/) data base for identification of this species. Finally, the described PRM method is applicable in targeted analysis of ACs, and provides a new perspective in targeted quantification of large-scale analogue metabolites.

Untargeted metabolomics analysis of exercise effect on plasma samples from *db/db* diabetic mice has been performed. The results found that exercise might have good regulatory effect on energy metabolism via regulating the dysfunction of cell signaling network that related to creatine/phosphocreatine system. The reduction of uridine level in plasma might also an indication of improved insulin sensitivity. Besides, the disorder of fatty acid β-oxidation can be observed in plasma samples, which is more sensitive to exercise on *db/db* mice.

In addition, untargeted analysis of the regulatory effect of exercise in skeletal muscle of *db/db* mice has also been carried out. Both LC-MS and GC-MS profiling of skeletal muscle samples have been analyzed. Comparison between *db/m+* control and *db/db* diabetic mice revealed that the accumulation of fatty acids, acylcarnitine species and various kinds of amino acids, might as a result of nutrient overload. The metabolic disorder may contribute to compromise energy metabolism of the body, then cause incomplete fatty acid and amino acid oxidation, and glucotoxicity. However, exercise exhibits a good regulatory role in accelerating amino acid catabolism which provides substrates that can enter TCA cycle. In addition, exercise exerts a positive role in the removal of intermediates from carnitine metabolism, lipid metabolism (medium- to long-chain acylcarnitines, long-chain fatty acid and phospholipids), and better clearance of uric acid and its precursors. The findings indicated that exercise plays a positive role in nutrient utilization and metabolic waste disposal, which may help to improve fitness. Although no significant improvement of insulin resistance has been observed after exercise (probably
due to short treatment period of exercise or age of mice), our studies still exhibited beneficial effects of physical activity on overall metabolism on diabetic mice. Moreover, we have evaluated the regulatory effect of exercise on DN mice in kidney samples. Both untargeted (by LC-MS and GC-MS) and targeted metabolomics studies have been carried out to give a more comprehensive view of the metabolic changes in response to exercise on DN mice. The results revealed that phospholipids, acyl-CoA and hydroxyl conjugated acylcarnitines instead of saturated acylcarnitines were more inclined to accumulate in diabetic kidneys. The results were in accordance with the expression of Acacm, Acadl and Cpt1a due to incomplete fatty acid β-oxidation. However, moderate intensity of exercise attenuated the accumulation of phospholipids, the levels of part of acyl-CoA and hydroxyl conjugated acylcarnitines, as well as the activity of Acadl was significantly reversed by exercise. In addition, exercise could increase the level of several acylglycines that play an important role in detoxification activity in the kidneys on DN mice. Moreover, exercise also significantly regulated the level of uric acid and precursors in the kidney of DN mice. Considering the fact that the accumulation of various kinds of lipids and uric acid has been reported positively related to inflammation (the activity of COX2 and TNFa have been substantially reduced by exercise) induced by DN, we hypothesized that exercise might play a positive role in improving DN induced inflammation. Besides, exercise also exerts a good effect in accelerating BCAA catabolism (reduce the level of isoleucine and increase the expression of Hmgcl, Bcat2, Acad8 and Hibch in BCAA catabolism pathway) that are positively related to insulin resistance and TNFα (inflammation factor), indicating that it may also a potential regulatory mechanism of exercise on DN mice in the kidney.

Application of mass spectrometry based untargeted and targeted metabolomics has enabled to comprehensively reveal the regulatory effect of exercise on db/db diabetic mice.
When taking an overview of the results from plasma, urine, skeletal muscle and kidney samples, we found that the metabolic changes induced by T2DM are mainly focused on fatty acid metabolism, BCAA catabolism, purine metabolism and other amino acid metabolism involved in TCA cycle. Interestingly, exercise showed positive effects in regulating most of them, which may exert good effects in improving insulin resistance and inflammation (in the kidney) on db/db mice. Our future work will be carried out on the basis of the previously discovered metabolomics results. On one hand, efforts will be carried out on studying the mechanism that why hydroxyl conjugated acylcarnitines instead of saturated acylcarnitines inclined to accumulate in the DN kidney, and which type of acylcarnitines contribute to the dysfunction of incomplete fatty acid β-oxidation. On the other hand, BCAAs metabolism has been substantially down-regulated on DN mice, however, exercise exerts a good role in regulating part of them (both metabolites and activities of transcript mRNA). Considering BCAA metabolism has been reported negatively regulated by TNFα in epididymal, based on the previous findings, the relationship between inflammation and BCAA will be investigated in tubule cells, and verified in the kidney of db/db DN mice to further unveil the mechanism of exercise in improving DN induced inflammation.

It is anticipated that the proposed future work may provide valuable insight in underlying the regulatory mechanism of exercise on T2DM-induced complications (such as diabetic nephropathy). Since exercise is an easy, economical, and scatheless way in treating T2DM, the results from this study will encourage patients with T2DM to take regular exercise to treat disease and improve their quality of life.
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Outcome of the thesis work

I. Publications

1. **Li Xiang**, Juntong Wei, Xiao Yu Tian, Bei Wang, Wan Chan, Shangfu Li, Zhi Tang, Hongsong Zhang, Wai San Cheang, Qian Zhao, Hongzhi Zhao, Yanjun Hong, Yu Huang*, Zongwei Cai*. Comprehensive analysis of acylcarnitine species in \(db/db\) mouse using a novel method of high resolution parallel reaction monitoring reveals widespread metabolic dysfunction induced by diabetes. *Analytical Chemistry*. http://dx.doi.org/10.1021/acs.analchem.7b02283

2. **Li Xiang**, Juntong Wei, Xiao Yu Tian, Hongsong Zhang, Hemi Luan, Shangfu Li, Hongzhi Zhao, Guodong Cao, Arthur C. K. Chung, Chunxue Yang, Yu Huang*, Zongwei Cai*. Metabolomics studies on \(db/db\) diabetic mice in skeletal muscle reveal effective clearance of overloaded intermediates by exercise. *Analytica Chimica Acta*. (Major revision)


6. Hongzhi Zhao, **Li Xiang**, Jiufeng Li, Zhiyi Yang, Jing Fang, Chao Zhao, Shunqing Xu, Zongwei Cai*. Investigation on fragmentation pathways of bisphenols by using


II. Conference and symposium presentations

1. 46th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Jeju, Korea
   
   Date: 5-9, November, 2017

   **Title of oral presentation:** Metabolomics studies on *db/db* diabetic mice in skeletal muscle reveal effective clearance of overloaded intermediates by exercise

2. Hong Kong Society of Mass Spectrometry (HKSMS) 2017. Hong Kong
   
   Date: 17, May, 2017

   **Title of oral presentation:** Untargeted and targeted metabolomics study of exercise effect on diabetic nephropathy mice.

   **Award:** Conference oral award

   
   Date: 8-10, May, 2017

   **Poster Title:** Untargeted and targeted metabolomics study of exercise effect on diabetic nephropathy mice.

   **Award:** Conference poster award

4. Hong Kong Society of Mass Spectrometry (HKSMS), Hong Kong.
   
   Date: 25, June, 2016.
Title of oral presentation: Metabolic signatures reveal exercise training on fatty acid β-oxidation in db/db type 2 diabetes mellitus mice.

Award: Conference oral award.

5. The 25th Australian and New Zealand Society for Mass Spectrometry (ANZSMS25) & 6th Asia Oceania Mass Spectrometry AOMSC6 conference held at the Brisbane Convention
Date: 19 to 22, July, 2015

Poster title: Metabolomics study of regulatory effects of exercise training on db/db diabetic mice in plasma.
CURRICULUM VITAE

Academic qualifications of the thesis author, Miss XIANG Li:

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