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Chapter 2

Urine metabolomics combined with the personalized diagnosis guided by Chinese Medicine reveals subtypes of pre-diabetes

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Metabolomics with the personalized diagnosis reveals subtypes of pre-diabetes

Abstract

The prevalence of type 2 diabetes continuously increases globally. A personalized strategy applied in the pre-diabetic stage is vital for diabetic prevention and management. The personalized diagnosis of Chinese Medicine (CM) may help to stratify the diabetics. Metabolomics is regarded as a potential platform to provide biomarkers for disease-subtypes. We designed an explorative study of 50 pre-diabetic males, combining GC-MS urine metabolomics with CM diagnosis in order to identify diagnostic biomarkers for pre-diabetic subtypes. Three CM physicians reached 85% diagnosis consistency resulting in the classification of 3 pre-diabetic groups. The urine metabolic patterns of group 1 ‘Qi-Yin deficiency’ and 2 ‘Qi-Yin deficiency with dampness’ (subtype A) and group 3 ‘Qi-Yin deficiency with stagnation’ (subtype B) were clearly discriminated. The majority of metabolites (51%), mainly sugars and amino acids, showed higher urine levels in subtype B compared with subtype A. This indicated more disturbances of carbohydrate metabolism and renal function in subtype B compared subtype A. No differences were found for hematological and biochemical parameters except for levels of glucose and γ-glutamyltransferase that were significantly higher in subtype B compared with subtype A. This study proved that combining metabolomics with CM diagnosis can reveal metabolic signatures for pre-diabetic subtypes. The identified urinary metabolites may be of special clinical relevance for non-invasive screening for subtypes of pre-diabetes, which could lead to an improvement of personalized interventions for diabetics.
Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia with disturbances of carbohydrates, fat and protein metabolism and its prevalence continuously increases globally[1-3]. Evidence shows that both lifestyle regulation and early pharmacotherapy during pre-diabetes are effective in slowing down the onset and progression of T2DM [1, 3, 4]. However, due to its multi-factorial causes resulting from the interaction between a genetic predisposition and behavioral and environmental risk factors, the control of T2DM represents a considerable therapeutic challenge[1, 2] and to diagnose and treat T2DM only based on the glucose level seems insufficient. Glycaemic control as evidenced by the reduction of glycated hemoglobin (HbA1c) with existing agents was found to have a weak and non-significant effect on the incidence of cardiovascular complications [1]. A more personalized and system based strategy to control the factors beyond glycaemia management (e.g. hypertension, dyslipidaemia, insulin resistance, obesity) is vital in reducing the diabetic morbidity and complications. The challenge towards personalized treatment is how to stratify patients to provide a diagnosis considering the uniqueness and the interaction of person with his environment as a whole [5].

The concept of personalized health and system diagnostic principles is not new, as it has long been the basis of Chinese Medicine (CM), in which the focus is the ‘diseased person’ instead of the ‘person’s disease’ [6]. CM does not focus solely on the disease defined by pathological changes but the overall maladjustments of functional status called ‘syndrome type’ [6]. The syndrome type is a functional status which is caused by the reaction to or interaction with environmental changes and pathogenic factors [7]. It is a manifestation profile of a group of signs and symptoms and its essence is the imbalance of the human system resulting in the perturbation of the metabolic or biological network [7]. CM aims to restore the self-regulatory ability of the human system, instead of antagonizing specific pathogenetic targets [6]. Metabolomics, defined as the “comprehensive quantitative and qualitative analysis of all small molecules in a system”, has been increasingly applied to study complex disease mechanisms to discover health-disease associated mechanistic biomarkers and it is regarded as a unique bridge between different healthcare perspectives on personalized medicine [8]. Urine metabolomics is of special interest because the urine collection is non-invasive and it amplifies the circulating levels of metabolites by renal concentration, which consequently ensures urine a distinct representation of metabolic response [9]. Several studies [10-13] combining metabolomics and CM syndrome types have revealed different molecular and metabolic patterns of patients with the Western Medicine (WM) diagnosed diseases, which provided new opportunities to
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improve patient stratification and personalized intervention. In China, integration of CM and WM has accomplished better therapeutic effects for diabetics [14-17], manifesting as the improvement of insulin sensitivity, lipid profile and quality of life and the alleviation of diabetic foot ulcer. However, to implement CM diagnosis under WM system is not easy; as the diagnosis is not based on objective instruments. By using four diagnostic methods: observation, listening and smelling, question and pulse feeling (Figure 1), CM physicians attempt to establish the health status through qualitative symptoms collection based on one’s appearance, behaviors, mental status, bio-rhythms, life style as well as his interaction and adaptation to both natural and social environment. Specifically, ‘observation’ method focuses on the behavior, movement, figure, facial color, tongue, throat and finger veins of a patient; with ‘listening and smelling’ method, CM physicians will evaluate the speech of a patient (e.g. pitch, tone, tempo), the way a patient is coughing or breathing and smell his breath and secretion (e.g. sweat). ‘Question’ is one the most important diagnostic techniques and CM physician will carefully ask and analyze the complaints and symptoms of a patient. Normally the following information will be covered [18]: medical history, response to cold/heat weather or food, sweat, symptoms of head, chest and body, pain, diet and appetite, sense organs, sleep quality and life style; for females there will be questions related to menstrual cycle and pregnancy experiences. ‘Pulse feeling’ is an unique diagnostic method in CM, by feeling the rate, depth and tension of the pulse, CM physicians can speculate the health status of the whole system. CM physicians will then cluster the symptoms/signs together according to CM diagnostic principles to get the CM syndrome type and provide personalized treatment. Due to the fact that CM diagnosis is conceptual and relies entirely on clinical signs discerned by CM physicians, the inter-physician consistency on CM diagnosis is of importance to both scientific research and clinical practice [19].

In this explorative study, we diagnosed 50 pre-diabetic males with CM syndrome types and applied urine metabolomics to search for biomarkers of pre-diabetic subtypes, with the following aims and hypotheses: (1) to assess the inter-physician concordance of CM diagnoses; (2) to find the relationships between classifications of pre-diabetics according to CM diagnosis and metabolomics. We hypothesize that combining CM diagnosis with metabolomics could help us identify pre-diabetes subtypes with related urinary metabolic patterns. The latter can provide quantitative biological evidence for CM diagnosis.
Figure 1. CM diagnosis and treatment paradigm.

Experimental section

Subjects and study design

The study was conducted at TNO (Zeist, the Netherlands) and 69 overweight pre-diabetic males were recruited from TNO’s candidate database. A pre-study screening comprised of a physical examination, clinical laboratory tests and the
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evaluation of anamnesis and life style was performed in order to confirm that the recruited subjects have no other clinical abnormalities than pre-diabetes. Subjects were declared eligible when meeting the inclusion and exclusion criteria. Inclusion criteria: 1) males aged 30–70 years; 2) Body Mass Index (BMI) 26 – 35 kg/m²; 3) pre-diabetes as determined by fasting glucose level of 6.1 – 6.9 mmol/L; 4) normal Dutch eating habits. Exclusion criteria: 1) smokers; 2) alcohol consumption ≥28 units/week; 3) unexplained weight fluctuation of > 2kg in the month before the pre-study screening; 4) currently on CM therapies. Finally 50 overweight pre-diabetic males, all of Dutch ancestry, were included in the study. All study subjects signed the informed consent and their baseline characteristics are summarized in Table 1. The study was approved by the Medical Ethics Committee of Tilburg (METOPP; Dutch acronym for medical ethical research in patients and volunteers).

| Table 1. Baseline characteristics of 50 pre-diabetic males |
|---------------------------------------------|----------|----------|
| Male (n = 50) | Mean ± SD | Range |
| Age /years | 57 ± 8 | 40 – 69 |
| Body weight /kg | 94.2 ± 10.1 | 75.4 – 117.6 |
| BMI /kg m⁻² | 28.8 ± 2.4 | 25.6 – 35.5 |
| BP systolic /mm Hg⁻¹ | 137.7 ± 17.8 | 93 – 177 |
| BP diastolic /mm Hg⁻¹ | 83.0 ± 10.1 | 56 – 104 |
| Waist circumference /cm | 105.0 ± 8.1 | 92 – 128 |
| Blood glucose /mmol L⁻¹ | 6.3 ± 0.3 | 5.9 – 6.9 |

The study was designed as an explorative study without intervention (Figure 2). Fifty pre-diabetic males were diagnosed by three different CM physicians separately and in a randomized order. The CM physicians had to have least ≥ 5 year CM training with a valid certificate and ≥ 10 year clinical experience in the Netherlands. Each CM examination took 15- 20 minutes and the physicians were not allowed to exchange ideas or know the diagnosis from each other. They were asked to apply CM diagnostic theories of organ and Qi-Blood-Fluid to give the personalized descriptions with percentage scaling to each subject. CM physicians first questioned subjects with symptoms and signs, then summarized and clustered these signs into CM diagnostic terms/syndromes (Figure 1). Finally CM physicians wrote down all the diagnosed syndromes, often 3–4 syndromes for each subject, and then based on severity and frequency of these syndromes ranked with percentage scaling for each diagnosed syndrome under 100% in total.
**Figure 2. Study design and objectives**

**WM diagnosis**
Pre-diabetic male
FPG: 6.1-6.9 mmol/L
BMI >25 kg/m²

**CM Personalized diagnosis**
Subjects with high diagnosis consistency are categorized into CM syndrome type groups

**CM Syndrome type group**
Each with a personalized CM diagnosis

3 CM physicians diagnose subjects separately; Diagnosis consistency is assessed

Find relationships between classifications according to CM diagnosis and metabolomics or clinical parameters

Clinical parameters + Urine metabolites
For example, the diagnosis was written down per subject by each physician in the form of “55% liver yang ascending, 35% yin deficiency and 10% liver qi stagnation”. CM diagnoses were considered consistent when ≥ 80% interphysician agreement was reached based on generalized procrustes analysis (GPA) results [20]. GPA is a multivariate exploratory technique that involves transformations (i.e., translation, rotation, reflection, isotropic rescaling) of individual data matrices to provide optimal comparability [20]. Subjects who were diagnosed differently by three CM physicians were excluded from further data analysis. The blood and the first void urine samples of study subjects were collected after an overnight fast (≥ 12 h). The blood samples were used for clinical laboratory measurements and urine samples for GC-MS metabolomics. The relationships between classifications of pre-diabetics according to CM diagnosis and metabolomics or clinical parameters were studied.

**Clinical parameter analysis**

Blood samples were obtained from antecubital vein of forearms and collected in tubes containing clot activator for serum and in anticoagulant tubes (Vacutainer Systems, Becton Dickinson, UK) of either K₃EDTA or Li-heparin for plasma. The K₃EDTA tubes were used for Hb1Ac determination while the Li-heparin tubes were used for the determination of glucose and lipids. Blood samples were centrifuged for 15 min at 2000 × g at 4 °C within 30 min after collection. Samples were aliquoted and stored at -20°C till analysis. All biochemical determinations in blood were analyzed with enzymatic techniques on the Olympus analytical equipment (Olympus-Diagnostica, Germany), including the haematology profile and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, bilirubin, urea, creatinin, total- and high density lipoprotein-cholesterol (HDL-C) and the ratio of total cholesterol and HDL-C, triacylglycerols, glucose.

**GC-MS of urinary metabolomics**

**Urine sample preparation**

After collection, the first void spot urine samples were aliquoted and then stored in -20°C till gas chromatography–mass spectrometry (GC-MS) analysis. Urine samples were first thawed to room temperature, then homogenized by vortexing, and afterward centrifuged for 20min at 3500 RPM. One hundred µL urine sample of each study subject was added to 10 µL internal standard (IS) mixture (nr.1) consisting of leucine-D3, glutamic acid-D3, phenylalanine-D5, cholic acid-D4 with a
final IS concentration of 10ng/µL in a clean PTFE vial. Afterwards the urine samples with IS mixture (nr.1) were frozen at -80 °C and lyophilized or freeze dried. Subsequently, 10µL IS mixture (nr.2) consisting of alanine-D4 and glucose-D7 with final IS concentration of 10 ng/µL were firstly added to the dried extracts. The dried extracts were then oximized with 20 µL of 16 mg/mL pyridine hydrochloride solution and 20 µL of 56mg/mL ethoxamine hydrochloride solution in pyridine for 90 min at 40 °C, followed by adding 10 µL IS mixture (nr.3) consisting of DCHP, trifluoroacetyl- anthracene (TFAA) and DFBP with corresponding final concentrations of 18ng/ µL, 19 ng/µL and 20 ng/µL in IS nr.3. Finally, the extracts were silylated for 50 min at 40 °C with 200 µL of MSTFA and centrifuged for 20 min at 3500 RPM.

Instrument settings

The derivatized (oximation and silylation) urine extracts were analyzed with an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass selective detector. The GC-MS method applied in the study can analyze a broad range of metabolites and was reported by Koek el al. [21]. The 1-µL aliquots of the extracts were injected into a HPS-MS capillary column (30 m × 250 µm i.d., 0.25-µm film thickness; J&W Scientific, Folson, CA) using PTV injection (Gerstel CIS4 injector) in the splitless mode. The temperature of the PTV was 70 °C during injection, and 0.6 min after injection, the temperature was raised to 300 °C at a rate of 2 °C /s and held at 300 °C for 51 min. The initial GC oven temperature was 70 °C; 5 min after injection the GC oven temperature was increased with 6 °C /min to 325 °C and held for 3 min at 325 °C. Helium was used as a carrier gas and pressure programmed such that the helium flow was kept constant at a flow rate of 1.7 mL/min. Detection was achieved with MS using electron impact ionization and full scan data acquisition (m/z 15-800).

The performance of the applied GC-MS platform was assessed through the repeated analysis of the quality control (QC) samples. The QC samples, used to monitor the GC-MS response in time, were prepared by pooling aliquots of 50 urine samples to represent the full biochemical diversity of the study samples and allow the calculation of the analytical precision for all metabolites measured. The QC sample data is also used to correct systematic errors such as batch to batch response differences by a single point calibration model [22, 23]. Twenty QC samples were processed exactly in the same way as the study samples and analyzed after every 2-3 study samples with double injections. Furthermore, method performance was carefully monitored using multiple internal standards and duplicate analysis of samples.
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Metabolomics data preprocessing

The GC-MS raw data was processed by Agilent ChemStation software. Only metabolites with 80% of the samples having a value were kept in the dataset. Data for each sample was corrected for the recovery of the IS for injection. Batch to batch differences in data were removed by synchronizing medians of QC-samples per batch. Duplicate measurements were combined into a single measurement. When both analytical duplicates had a zero value or when both had a non-zero value, measurements were averaged. The single value was taken when only one of the duplicates was above zero. Metabolites with high imprecision (i.e. relative standard deviation ≥50%) were removed from the dataset. Finally, the GC-MS dataset contained 128 metabolites and 46 of which were identified.

Data analysis

In order to assess the inter-physician diagnostic consistency, generalized procrustes analysis (GPA) was performed [20]. GPA is a method for comparing shapes of objects, in which the shape of an object is defined in a mathematical context as all the remained geometrical information after location, scale and rotational effects being filtered out. Three objects (CM physicians) were defined in the study, namely a matrix with concept-scores for each of three CM physicians. Each matrix size was 50 (number of pre-diabetic males) × 26 (number of CM diagnostic terms with percentage scaling). To explore urinary metabolic patterns and its relation to CM diagnosed groups, an unsupervised Principal component analysis (PCA) [24] was carried out in MATLAB (version 7.7.0471, the Mathworks) with the PLS toolbox (version 5.0.3, Eigenvector Research, InC.). Partial least squares discriminant analysis (PLS-DA) [25] was further applied to identify the specific metabolites which contribute most to discriminate between the subtypes; and PLS-DA models were validated using double cross validation (DCV) [26]. To avoid the possibility that a few high-intensity variables dominate the final results [27], metabolomics dataset was standardized per variable, meaning that first the means of each variable were subtracted, and then all variables were divided by their standard deviation (SD). Statistical differences of blood clinical parameters and urine metabolite regulation between subtypes of pre-diabetics were assessed by univariate data analyses by SPSS 17.0 and the results are presented as means ± SD unless indicated otherwise, with p value < 0.05 as statistical significance. To correct for false positives, the multiple test correction (MTC) of Benjamini and Hochberg false discovery rate (FDR) analysis[28] was applied to adjust p values derived from the univariate results of the metabolomics data.
Results

The diagnostic consistency of CM physicians and the CM syndrome types

Twenty six CM diagnostic terms (data not shown) were used to provide the personalized descriptions for each subject. The GPA analysis revealed a diagnostic consensus proportion of 85% among three CM physicians. The residuals from GPA can provide information of the inter-physician diagnosis agreement for a subject. The higher the residual, the lower agreement the CM physicians have upon this subject. Six subjects with relatively high residuals (≥ 0.02) were excluded from further analysis. An example of subjects with high and low inter-physician diagnostic consistency is illustrated in Figure 3. The GPA residual value was low for subject 35 and high for subject 11. Obviously, the similar diagnoses of three CM physicians were given for subject 35 (Figure 3A) while different diagnoses were given for subject 11 (Figure 3B).

The chief CM physician categorized the remaining 44 study subjects into three CM syndrome type groups: group 1 “Qi-Yin deficiency” (n=15), group 2 “Qi-Yin deficiency with dampness” (n=20) and group 3 “Qi-Yin deficiency with stagnation” (n=9). The description and main symptoms under each syndrome type were summarized in Table 2. All subjects actually had symptoms of CM syndrome type “Qi-Yin deficiency”, mainly manifesting as fatigue, spontaneous or night sweat and afternoon heat. Only subjects in group 1 were diagnosed solely as “Qi-Yin deficiency” while subjects in group 2 and 3 had basic symptoms of “Qi-Yin deficiency” with additional CM pathological factors, either “dampness” or “stagnation”.

Metabolomics patterns and the relevant clinical characteristics

Metabolomics patterns

The GC-MS urine metabolomics data matrix of 44 subjects × 128 metabolites was used for PCA. The first two principal components (PCs) were selected, which described 45.4% of the total variance of the urine metabolome for 3 CM groups (Figure 4). A clear separation was observed (gray line in Figure 4) of group 1 (Qi-Yin deficiency) with group 2 (dampness) versus group 3 (stagnation). Thirteen out of 15 subjects (87%) of group 1 were clustered with subjects of group 2; while only three subjects numbered 22, 29 and 31 of group 1 were closer with subjects of group 3. Eight of 9 subjects of group 3 appeared at the right side of PCA score plot, except for subject 4 who was clustered with subjects diagnosed with ‘dampness’ and ‘Qi-Yin deficiency’ (Figure 4). This separation in PCA indicated
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underlying concentration differences of urine metabolites between two potential pre-diabetic subtypes: ‘group 1 plus 2’ (subtype A) versus ‘group 3’ (subtype B). PLS-DA with DCV was used to further identify urine metabolites that differed in concentrations between subtypes A and B. The DCV error rate was 11%, indicating that 5 out of 44 subjects were misclassified. Among top 10 metabolites that contributed most to discriminate between subtype A and B, only three were identified. Gluconic acid and tryptophan showed significantly higher concentration in subtype B subjects (Figure 5B) while D-xylose appeared no significant differences between the two subtypes (data not shown).

Subsequently, quantitative differences in urine metabolites between subtypes A and B were tested by an independent t-test with MTC-FDR to correct for false positives. This resulted in 65 out of 128 urine metabolites (51%) with significant higher concentrations ($p = 0.00 - 0.02$) and 38% metabolites with higher concentration trend in subtype B compared with subtype A (Figure 5A). Twenty four out of the 65 significantly changed metabolites could be identified, mainly sugars, amino acids and organic acids, and were ranked based on the coefficients of PLS-DA regression vector (Figure 5B). The urine levels of these 24 metabolites were found to be 60 – 460% higher in subtype B compared to subtype A.

Table 2. Descriptions and main symptoms of three CM syndrome types

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qi-Yin deficiency (n= 15)</td>
<td>Qi-Yin deficiency with Dampness (n= 20)</td>
<td>Qi-Yin deficiency with Stagnation (n=9)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>The similar symptoms as “Qi-Yin deficiency” plus symptoms</td>
<td>The similar symptoms as “Qi-Yin deficiency” plus symptoms below:</td>
</tr>
<tr>
<td>Short of breath</td>
<td>Uncomfortable fullness of belly</td>
<td>Chest oppression;</td>
</tr>
<tr>
<td>Palpitation</td>
<td>Inactive, lack of appetite</td>
<td>Tingling in feet or limbs</td>
</tr>
<tr>
<td>Dizziness</td>
<td>Heaviness of the body</td>
<td>Dry skin</td>
</tr>
<tr>
<td>Spontaneous or night sweat</td>
<td>Obesity</td>
<td>Cold finger tips or toes</td>
</tr>
<tr>
<td>Dry mouth and throat</td>
<td>Edema</td>
<td>Unexplained pain</td>
</tr>
<tr>
<td>Thirsty</td>
<td>Feels sick and vomiting;</td>
<td>Light purple lips</td>
</tr>
<tr>
<td>Insomnia and irritation</td>
<td>Bad breath</td>
<td>Dilated veins under the tongue</td>
</tr>
<tr>
<td>Hot flush</td>
<td></td>
<td></td>
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<tr>
<td>Afternoon heat</td>
<td></td>
<td></td>
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<tr>
<td>Concentration problems</td>
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</tbody>
</table>
**Figure 3.** Illustration of inter-physician diagnostic consistency. (A) Subject 35 was with a low GPA residual meaning consistent inter-physician diagnoses; (B) subject 11 was with a high GPA residual meaning discrepant inter-physician diagnoses.
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**Figure 4.** PCA score plot of urine metabolomics data reflecting pre-diabetic subtypes based on CM diagnosis. Three CM syndrome groups (n=44): group 1 ‘Qi-Yin deficiency’, group 2 ‘Qi-Yin deficiency with dampness’, group 3 ‘Qi-Yin deficiency with stagnation’. Group 3 was clearly separated from group 1 and 2. The gray line highlighted the separation between “group 1 plus 2” and “group 3”.

**Clinical characteristics**

Clinical characteristics of two pre-diabetic subtypes were analyzed by independent t-test. No significant differences were observed for parameters of haematology (data not shown) or physical examination including age, BMI, waist circumference and blood pressures (BP) (Table 3). Subtype B showed significant higher levels of fasting plasma glucose ($p =0.04$) and γ-glutamyltransferase (γ-GT) ($p =0.03$) as compared to subtype A (Table 3). No other clinical parameters showed significant differences between the two subtypes.
Figure 5. Urinary metabolite level differences between subtypes A and B. (A) The distribution of urinary metabolite changes in subtype B (vs. subtype A); (B) Twenty four identified metabolites showed significantly higher urine levels in subtype B. The urinary metabolite level of subtype A was set to be 100% and relative changes of subtype B were illustrated in % compared with it (*p < 0.05; ** p < 0.01; *** p <0.001 after MTC-FDR). The number before each metabolite refers to the ranking of metabolite based on coefficients of PLD-DA regression vector.
Table 3. Clinical characteristics of two PCA separated CM syndrome type groups

<table>
<thead>
<tr>
<th></th>
<th>Subtype A</th>
<th>Subtype B</th>
<th>Subtype B</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Qi-Yin deficiency &amp;</td>
<td>Qi-Yin deficiency</td>
<td>Qi-Yin deficiency with</td>
<td>Stagnation</td>
</tr>
<tr>
<td></td>
<td>Qi-Yin deficiency with</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Dampness</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age /years</td>
<td>56±8</td>
<td>58±7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI ( kg/m²)</td>
<td>28.8±2.1</td>
<td>29.1 ±3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waist circumference / cm</td>
<td>105±7</td>
<td>105±12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mm/Hg)</td>
<td>137±17</td>
<td>135 ± 22</td>
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<tr>
<td>Diastolic BP(mm/Hg)</td>
<td>83±9</td>
<td>81 ± 13</td>
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<tr>
<td>Glucose (mmol/L)</td>
<td>6.1 ±0.4</td>
<td>6.4±0.6</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Hb1Ac (%)</td>
<td>5.8±0.3</td>
<td>5.9 ±0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.7±0.9</td>
<td>5.8 ± 1.0</td>
<td></td>
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</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.3±0.3</td>
<td>1.2 ±0.2</td>
<td></td>
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<tr>
<td>LDL-C (mmol/L)</td>
<td>3.7±0.8</td>
<td>3.7 ±1.0</td>
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<tr>
<td>Ratio TC/HDL-C</td>
<td>4.7±1.1</td>
<td>4.8 ± 1.0</td>
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<tr>
<td>TG (mmol/L)</td>
<td>1.7 ±0.8</td>
<td>1.9 ± 0.9</td>
<td></td>
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<tr>
<td>ALP (U/L)</td>
<td>79±20</td>
<td>72 ± 13</td>
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<td></td>
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<tr>
<td>ALT (U/L)</td>
<td>29±11</td>
<td>29 ± 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST( U/L)</td>
<td>23±5</td>
<td>22 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ -GT( U/L)</td>
<td>36±15</td>
<td>50±20</td>
<td>0.03</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>6.1±1.5</td>
<td>6.1 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>93±13</td>
<td>87 ± 14</td>
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NS: No significance, p > 0.05

Discussion

This is an explorative study combining urine metabolomics with CM syndrome type diagnosis to search for different metabolic patterns in pre-diabetic subtypes. First of all, this study demonstrated that three CM physicians have reached a high (85%) diagnostic consistency. Taken into account the many different CM diagnostic principles, the three CM physicians involved in the current study were
asked to apply CM theories of Organ and Qi-Blood-Fluid and to quantify the diagnoses in percentage scaling. In this way, the CM diagnostic theory was clarified and standardized in advance, which may increase the inter-physician diagnostic consistency. Zhang et al. [19] also reported that consensus on CM diagnostic criteria results in the improved agreement of diagnosis. Thus the standardization of CM diagnostic procedures including diagnostic principle selection and symptoms scaling can lead to a more quantitative and reproducible CM diagnosis.

In real CM practice, there are many CM syndrome/pattern diagnostic principles and CM physicians are free to choose one or more of them to provide a syndrome diagnosis, in order to provide an optimal personalized treatment. The diagnostic terms under these principles can be translated into one another in certain extent. As shown in figure 6, most frequently used CM diagnostic terms of T2DM were summarized in different diagnostic principle levels. In the organ level, CM terms under ‘Organ’ diagnostic principle were listed and they can be translated to the relevant higher ‘functional level’. For example, the diagnostic terms in ‘Organ level’ such as ‘Lung stomach heat’ can be categorized under diagnostic term ‘Yin deficiency with excessive heat’ under ‘functional level’. According to the ‘Standard for diagnosis and therapeutic effect evaluation of diabetes mellitus’ established by Chinese integrative medicine academy diabetes committee in the year 2005 [29], the T2DM and related disorders including metabolic syndrome and obesity were categorized into CM syndromes under CM ‘functional level’, which is based on the combination of ‘CM theories of Organ and Qi-Blood-Fluid’. As for research reasons, the first step to understand the subtypes in ‘functional level’ is more practical. Followed this concept, three CM physicians in current study were asked to freely write down the CM diagnostic terms ranked with percentage scaling based on the severity and frequency of the syndrome, which is a purely personalized diagnosis for each patient (Figure 2). Then 44 subjects with high CM diagnosis agreement were set to the CM syndrome types in CM ‘functional level’ based on the relations of CM syndromes shown in figure 6. Therefore, the CM diagnostic strategy applied in the current study is semi-unsupervised. The fully unsupervised CM diagnosis need much more study subjects and will be investigated in the future.

Furthermore, this study demonstrated that three different pre-diabetic syndrome types could be diagnosed based on CM principles: “Qi-Yin deficiency” (group 1), “Qi-Yin deficiency with dampness” (group 2) and “Qi-Yin deficiency with stagnation”. “Qi-Yin deficiency” can be described as abnormalities induced by hypermetabolism. Its symptoms showed certain similarities with “chronic fatigue syndrome” and/or “people with mild inflammatory status”. Its main complaints (Table 2) included fatigue, short of breath, dizziness, afternoon heat,
Metabolomics with the personalized diagnosis reveals subtypes of pre-diabetes concentration problems and sleep disorders. “Qi-Yin deficiency” is regarded as the main CM syndrome type of T2DM in many Chinese national protocols and guidelines for diabetes management [29, 30]; while “stagnation” and “dampness” are two CM pathological factors closely associated with T2DM and diabetic complications [29, 30]. In CM theory, “dampness” refers to the imbalanced metabolism due to dysfunction of liquid and humor, usually by an over intake of raw, cold, greasy, or sweetened food [31]; while “stagnation” covers a wide range of abnormalities including blood circulation disturbance (especially microcirculation), metabolic and immune disorders, connective tissue pathological changes and some mental problems [32].

Figure 6. Chinese Medicine syndrome type summary for T2DM and its related disorders including pre-diabetes, obesity and metabolic syndrome.
related to these CM syndrome types might provide us with an idea of the underlying biological mechanisms. The animal model of “dampness” was built by feeding animal with high carbohydrate and fat diets in the warm and humid environment to induce the symptoms analogous to humans such as fatigue, appetite loss and reduction of Na+-K+-ATPase activity [31]. Animal models of “stagnation” were made by using repetitive external stimuli (e.g. electronic needle, noise, light) to induce pain, stress, increased blood noradrenalin levels, a sympathetic hyperfunction and microcirculatory disturbances [33]. In Chinese population, “dampness” was more prevalent in patients with fatty liver [34], hypertension [35] and diabetic angiopathic complications [36]. “Stagnation” or its related CM syndromes (e.g. Yang deficiency) refers to T2DM patients with higher occurrence of proliferative diabetic retinopathy [11] or cerebral infarction [37]. Although CM syndrome type based animal models have many more varieties [31-33] and shall differ from real human situations, we still could infer that “dampness” is more associated with unhealthy life-style (i.e. intake of high energy diets) induced abnormalities; while “stagnation” may represent either catecholamines induced autonomic nervous system derangement or blood circulation problems.

The most importantly, this study demonstrated that we were able to distinguish two out of three defined pre-diabetic subtypes based on their urinary metabolic patterns: subtype A: “Qi-Yin deficiency” (group 1) and “Qi-Yin deficiency with dampness” (group 2), subtype B: “Qi-Yin deficiency with stagnation”. Although the “Qi-Yin deficiency” group could not be clearly distinguished from the other two groups based on the urinary metabolome, its biological background could be reflected by understanding the other two groups as they shared the same basic complaints. Subtype A was highlighted by “dampness” symptoms such as heaviness of the body, obesity and oedema while subtype B by “stagnation” symptoms such as cold and tingling limbs and pain. The two pre-diabetic subtypes showed a clear separation by PCA and PLS-DA and 51% of the measured urinary metabolites were significantly different between these pre-diabetic subtypes. Subtype B showed higher urinary levels of metabolites compared to subtype A.

Firstly, some metabolites (i.e. D-glucose, pyruvic acid, D-ribose, citric acid, D-ribulose, L-cysteine, Meso-erythritol, nicotinamide) are related to carbohydrate and energy metabolism. The higher urinary concentrations of glucose and other sugars in subtype B may corroborate with a higher hyperglycemic status of these study subjects. The fasting plasma glucose level of subtype B also showed a significantly higher concentration \((p=0.04)\) than that of subtype A (Table 3). There might be enhanced endogenous glucose production from gluconeogenesis and the urinary loss of tricarboxylic acid (TCA) cycle intermediates such as citric acid and pyruvic acid in subjects of subtype B may indicate increased hepatic gluconeogenesis to provide extra pyruvate as a substrate for glucose [38]. Meso-
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eritritol and nicotinamide can provide beneficial effects for diabetics as food supplements [39-42].
Secondly, many amino acids (e.g. L-cysteine, L-serine, phenylalanine, tryptophan) were increased in the urine of subtype B. Pre-/diabetes is characterized by insulin resistance and catabolic/anabolic hormonal imbalance, which will affect the metabolism of the whole body. Insulin resistance can cause protein degradation and amino acid release [43, 44]. In addition to being used as substrate for gluconeogenesis, these amino acids are cleared from the circulation by the kidneys and thus appear in higher concentrations in the urine [43, 44]. We speculate that subtype B might have more insulin resistance or impaired insulin action compared with subtype A. Moreover, L-serine, a non-essential amino acid, was reported to be excreted significantly more in the diabetics than in healthy controls [45]. Phenylalanine and tryptophan are essential amino acids which are either incorporated into proteins or broken down for energy and metabolic intermediates [46]. Tryptophan is the precursor of brain neurotransmitter serotonin and phenylalanine is the precursor of catecholamines which are adrenalin-like substances such as tyramine, dopamine, epinephrine and norepinephrine in the body [42]. Increased phenylalanine and tryptophan in urine might reflect the catecholamines related autonomic nervous system derangement [47-50]. Interestingly, “stagnation” animal modeling was also developed with the increased blood noradrenalin level.
Finally, the higher urinary excretion of the majority of measured metabolites in subtype B subjects could indicate that they might have more potential disturbance of renal function; therefore they lost metabolites that are necessary for carbohydrate and energy metabolism. Specifically, hypoxanthine’s relation with renal problems has been well demonstrated. On one hand, when xanthine oxidase converts hypoxanthine to xanthine in the presence of molecular oxygen, superoxide radical is generated. Reactive oxygen species, which leads to a lipid peroxidation, protein denaturation and DNA oxidation, contributes to the microvascular dysfunction and exert direct renal tissue damage [51]. On the other hand, uric acid is the end product of hypoxanthine catabolism and could deposit in renal tissue and form kidney stones [51]. Increased urinary citric acids were observed in diabetics and created more risks of kidney stones [52-54].
Clinical parameters did not show significant differences among the two pre-diabetic subtypes, except for higher blood glucose and γ -GT in subtype B. Elevated γ -GT level is associated with diseases of the liver and pancreas as well as cardiovascular mortality [55, 56]. Though both are still within the clinical reference range, the potential diabetic related problems might be indicated, which need further investigation. This study is an early phase investigation on subtype of the pre-diabetics based on a small number of study subjects and CM
physicians. As such whilst this data is very promising, there needs to be larger investigations using more subjects and CM physicians to demonstrate the further reliability and external validity/ generalizability of this novel approach to diagnosing pre-diabetic subtypes.

**Conclusion**

This is the first study combining non-invasive GC-MS urine metabolomics with CM personalized diagnosis to find metabolic subtypes in pre-diabetics. This study demonstrated that 85% inter-physician consistency of CM diagnosis was reached when the diagnostic principles were standardized and symptom descriptions were clarified in advance. Based on 3 different CM syndrome groups, two pre-diabetic subtypes can be identified by urine metabolomics and had different urinary metabolic patterns. The subtype B excreted higher levels of sugars and amino acids compared to the subtype A, indicating more disturbances of carbohydrate metabolism and renal function. The identified urinary metabolites may be of special clinical relevance for easy and non-invasive screening for subtypes of pre/diabetes and uncovering the diabetic development and prognosis in some extent, which could lead to a better understanding and improvement of personalized interventions for diabetics. This study proved that the understanding and improvement of diagnosis plays a key role in bridging between CM and WM on personalized healthcare. Future studies are needed to validate the subtypes yielded in the current study and to assess the intervention response of these subtypes to metabolic or hyperglycemic drugs.

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