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Engineering 5 (2019) 916–923

Contents lists available at ScienceDirect

Engineering

journal homepage: www.elsevier.com/locate/eng

Research
Traditional Chinese Medicine—Article

Traditional Chinese Medicine-Based Subtyping of Early-Stage Type 2 Diabetes Using Plasma Metabolomics Combined with Ultra-Weak Photon Emission

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ARTICLE INFO

Article history:
Received 5 July 2018
Revised 30 September 2018
Accepted 5 March 2019
Available online 19 June 2019

Keywords:
Type 2 diabetes mellitus
Plasma metabolites
Disease subtypes
Ultra-weak photon emission
Correlation networks

ABSTRACT

The prevalence of type 2 diabetes mellitus (T2DM) is increasing rapidly worldwide. Because of the limited success of generic interventions, the focus of the disease study has shifted toward personalized strategies, particularly in the early stages of the disease. Traditional Chinese medicine (TCM) is based on a systems view combined with personalized strategies and has improved our knowledge of personalized diagnostics. From a systems biology perspective, the understanding of personalized diagnostics can be improved to yield a biochemical basis for such strategies; for example, metabolomics can be used in combination with other system-based diagnostic methods such as ultra-weak photon emission (UPE). In this study, we investigated the feasibility of using plasma metabolomics obtained from 44 pre-T2DM subjects to stratify the following TCM-based subtypes: Qi-Yin deficiency, Qi-Yin deficiency with dampness, and Qi-Yin deficiency with stagnation. We studied the relationship between plasma metabolomics and UPE with respect to TCM-based subtyping in order to obtain biochemical information for further interpreting disease subtypes. Principal component analysis of plasma metabolites revealed differences among the TCM-based pre-T2DM subtypes. Relatively high levels of lipids (e.g., cholesterol esters and triglycerides) were important discriminators of two of the three subtypes and may be associated with a higher risk of cardiovascular disease. Plasma metabolomics data indicate that the lipid profile is an essential component captured by UPE with respect to stratifying subtypes of T2DM. The results suggest that metabolic differences exist among different TCM-based subtypes of pre-T2DM, and profiling plasma metabolites can be used to discriminate among these subtypes. Plasma metabolomics thus provides biochemical insights into system-based UPE measurements.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic, devastating, and complex disease. T2DM is characterized by increased fasting plasma glucose levels, impaired postprandial insulin secretion, decreased insulin sensitivity, and impaired pancreatic beta-cell function [1]. In addition, patients with T2DM have increased levels of inflammatory factors such as TNFα, IL-6, IL-8 and of reactive active species [2,3]; altered levels of hormones, peptides, proteins, and enzyme activity; and other metabolic perturbations [4]. Strikingly, nearly all of these metabolic changes are often present years before the patient presents with clinical symptoms leading to a diagnosis of T2DM [5,6].

Based on epidemiology studies, an estimated 2.85×10^8 individuals are affected by diabetes worldwide, and this number continues to increase [7]. Furthermore, this number is likely an

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https://doi.org/10.1016/j.eng.2019.03.011

underestimate, as many individuals are not diagnosed in an early stage due to insufficient knowledge regarding the multi-symptom relationships at a systems level [8,9]. Receiving a diagnosis only in a later stage of diabetes—together with the severe complications associated with disease progression—can lead to high costs and can reduce the efficacy of treatment [10]. For example, long-term dysglycemia increases the risk of severe complications such as hypertension, blindness, renal failure, and cardiovascular disease [11,12]. These complications reduce quality of life and are a major cause of morbidity, hospitalization, and mortality among patients with diabetes. Current diagnostic tests are based primarily on a single screening tool such as the oral glucose tolerance test or measuring fasting plasma glucose. Understanding the symptoms that develop in an early stage of the disease and developing indicators of disease progression would likely contribute to improving both prevention and treatment strategies, including strategies based on changes in lifestyle. Moreover, treatments based on generic observations—which have led to the notion of one drug–one target–one disease (or one-size-fits-all)—are extremely limited, particularly in early stages of the disease. Therefore, system-based approaches are needed in order to achieve personalized approaches.

Integrative holistic forms of medicine such as traditional Chinese medicine (TCM) provide descriptions of disease syndromes and subtypes at a systems level, including descriptions that can be used to diagnose early syndromes of chronic diseases. Based on the Chinese theory for syndrome differentiation of pre-T2DM, one important syndrome in TCM for pre-diabetes is "Qi-Yin deficiency" (QYD). Other pathological factors such as dampness or stagnation interact with the QYD, leading the development of the disease in two different directions or toward two different statuses. From a pharmacological perspective, QYD can be used to reflect hypermetabolism-induced mild inflammatory status and chronic fatigue syndrome. Both dampness and stagnation are closely related to an unhealthy lifestyle and poor diet: Dampness can lead to complications such as metabolic syndrome, hypertension, and anginosis, while stagnation may be associated with pressure and tension, thereby leading to emotional problems, impaired blood circulation, and diabetic peripheral neuropathy. Such descriptions can be used as a guide or reference in order to achieve personalized medicine. In this respect, TCM has provided descriptions of pre-T2DM syndromes, indicating its potential for assisting in the development of personalized medicine [13,14]. To build a bridge between TCM and western medicine, evidence-based scientific data is needed at the biochemical level. Modern systems biology research—including metabolomics—is a promising approach for exploring the biochemistry underlying TCM subtyping.

Metabolic disorders are often present for years before the appearance of clinical disease, and metabolomics is a widely used technique for predicting and diagnosing disease [15]. Metabolomics provides a comprehensive profile of small molecular metabolites in biological systems and can be used as a readout of an organism's physiological status [16]. In principle, this approach is well suited to studying complex TCM-based diagnostics. Metabolomics is generally performed on fluids such as blood, urine, and cerebrospinal fluid. Urine is commonly used for metabolomics, as it easily obtained, contains information regarding the excretion of products, and can reflect how metabolic processes change during the disease process. Several studies have used urine metabolomics to explore TCM-based diagnostics and T2DM syndrome subtypes [17,18]. Like urine, blood contains information regarding the body's regulatory status and dynamics. Thus, performing metabolomics on different fluids can provide complementary information, thereby improving our understanding of T2DM. An explorative study at the Netherlands Organization for Applied Scientific Research[†] (TNO; Zeist, the Netherlands) was designed in which 44 pre-T2DM subjects received a diagnosis by a panel of three TCM-trained physicians [17], and we explored these TCM-based subtypes using plasma metabolomics.

Recently, a sensitive, non-invasive technique has been proposed for supporting TCM-based diagnostics [19]. This technique, called ultra-weak photon emission (UPE), is used to measure spontaneous photon emissions from the skin's surface [20]. Because UPE reflects the body's physiological and pathological status, it represents a promising tool for use in clinical diagnostics at a systems level [21–23]. The underlying biochemistry of UPE is related to metabolism and is correlated with reactive oxygen species in oxidative metabolic processes [24–27]. Although the use of UPE properties for characterizing TCM-based diagnostics has been summarized previously [19,20,28], further understanding of the molecular basis of UPE is needed. Metabolomics can be combined with TCM-based diagnostics to investigate the biological meaning of UPE and to explore the added value of each technology. It is important to note that UPE was used previously to subtype the same cohort of 44 subjects with pre-T2DM [28], thereby enabling us to study the correlation between UPE and plasma metabolomics.

2. Materials and methods

2.1. Inclusion criteria for the selection of pre-T2DM subjects and the diagnosis of syndrome subtypes based on TCM

The recruitment of subjects and the diagnosis of pre-T2DM subtypes by TCM-trained physicians have been described previously [17]. In brief, clinical parameters were obtained from 44 male Dutch subjects who met the following inclusion criteria: 30–70 years of age, body mass index of 26–35 $kg \cdot m^{-2}$, and a fasting glucose level of 6.1–6.9 $mmol \cdot L^{-1}$. No other clinical abnormalities or evidence of diabetic complications were detected. The subjects were then diagnosed separately in a blinded study by three TCM-certified physicians with at least five years of training in TCM and at least ten years of clinical experience. Three categories were based on 26 TCM-based diagnostic terms, and 85% consensus was reached among the three TCM physicians with respect to diagnosing the subjects. These three categories are defined as follows: QYD (n = 15 subjects), Qi-Yin deficiency with dampness (QYD_Damp, n = 20 subjects), and Qi-Yin deficiency with stagnation (QYD_Stag, n = 9 subjects). Blood samples were collected after overnight fasting and used for the metabolomics study. In addition, UPE was measured from the palmar and dorsal surfaces of both hands.

2.2. Ethics statement

This explorative study was designed and conducted by TNO and was approved by the Medical Ethics Committee of Tilburg (METOPP).

2.3. Data acquisition

2.3.1. Plasma metabolomics profiling

Metabolic profiles were measured by TNO. Heparinized blood samples were collected, and plasma was obtained by centrifugation (4000 $r \cdot min^{-1}$ at 4 °C for 15 min). The plasma samples were aliquoted and stored at −20 °C prior to metabolite extraction and mass spectrometry.

Using a gas chromatography–mass spectrometry (GC–MS) platform, a large variety of metabolic classes were measured, including amine metabolites, organic acids, sugars, and lysophosphatidic

[†] https://clinicaltrials.gov/ct2/show/NCT00469287.

acid (LPA)-derived metabolites. The details of the extraction and the GC–MS analysis protocol have been published previously [29]. In brief, 100 μL aliquots of plasma were spiked with a mixture of internal standards (ISTDs) and deproteinized with methanol. After centrifugation, the supernatant was transferred to a new sample vial for evaporation and two-step derivatization. The derivatized extracts were then analyzed using an Agilent 6890 gas chromatograph (Agilent Technologies, Inc., USA) on a J&W BD-5 ms capillary column (30 m × 250 μm i.d., 0.25 μm film thickness; Agilent Technologies, Inc., USA) coupled to an Agilent 5973 mass selective detector (Agilent Technologies, Inc., USA); helium was used as the carrier gas at a flow rate of 1.7 $mL \cdot min^{-1}$ for temperature-programmed gradient chromatographic separation. The raw data were preprocessed and exported using ChemStation G1701CA software (version D.01.02; Agilent Technologies, Inc., USA), providing response ratios to the appropriate ISTD for each metabolite; these ratios were used for further statistical analysis.

For liquid chromatography–tandem mass spectrometry (LC–MS) lipid measurements, seven classes of lipids, including polar lipids (e.g., phosphatidylcholine, phosphoethanolamine, lyso-phosphatidylcholine, lyso-phosphoethanolamine, and sphingomyelin) and non-polar lipids (e.g., cholesterol esters (ChEs) and triglycerides (TGs)), were investigated using targeted analysis, as reported previously by Van Wietmarschen et al. [30] and Draisma et al. [31]. In brief, 10 μL aliquots of plasma were deproteinized by the addition of isopropanol containing a mixture of ISTDs. The lipids were separated and analyzed using a TSQ Quantum Discovery Triple Quad mass spectrometer coupled to a Surveyor MS HPLC System (Ultimate 3000, TSQ Endura; Thermo Fisher Scientific, Inc., USA) on an Alltech Prosphere C4 300 Å column (150 mm × 3.2 mm, particle size of 5 μm; Alltech Associates, Inc., USA) in combination with a Symmetry 300 C4 guard column (2.1 mm × 10 mm, particle size of 3.5 μm; Waters, USA) in positive ionization mode. The peak areas of the target lipids were integrated, and raw data were exported using LCQUAN software (version 2; Thermo Fisher Scientific, Inc., USA), yielding response ratios to the appropriate ISTD for each metabolite; these ratios were used for further statistical analysis.

During the GC–MS and LC–MS experiments, quality control (QC) samples were prepared by pooling equal amounts of plasma from each sample, and then dividing the pooled samples into aliquots; these QC samples were used to check the performance of the LC–MS platform and to identify temporal trends in the acquired data. The relative standard deviation (RSD) of each target peak in the QC samples was used to confirm the quality of the data acquired from each analytical platform.

2.3.2. UPE measurements

UPE signals were measured from the same cohort of 44 subjects. A photomultiplier system (provided by Meluna Research BV, the Netherlands) with two detecting heads located at the top of a dark chamber was used to measure UPE. Each detecting head contains a 9235QA photomultiplier tube within a spectral sensitivity range of 160–630 nm (Electron Tubes Enterprises Ltd., UK) and an electronically controlled shutter. The dark chamber was maintained at (20 ± 1.0) °C. The settings used to measure UPE have been described previously [32,33]. All measurements were controlled automatically via computer-driven software. UPE signals were measured at the following four hand surfaces: left dorsal (LD), right dorsal (RD), left palm (LP), and right palm (RP).

2.4. Data preprocessing and statistical analysis

2.4.1. Metabolomics data processing and analysis

Before performing statistical analysis on the metabolomics data, the log-transformed dataset was processed using various scaling options (i.e., autoscaling, range scaling, and Pareto scaling) using the online software package MetaboAnalyst 3.0[†] [34]. The Pareto-scaling approach (mean-centered and scaling by the square root of the standard deviation of each variable) was chosen because it provided the best grouping performance, consistently explaining the largest variabilities when considering the same number of principal components (both two-dimensional (2D) and three-dimensional (3D)) [35–37]. Preliminary selection of variables prior to multivariate analysis has been applied in order to: ① limit the dataset of variables in order to reliably separate the sample groups, ② remove irrelevant and/or confounding variables, and ③ decide which variables to retain for the further multivariate analysis. However, this selection is not with the aim to identify potential biomarkers, which have been applied in metabolic profiling studies [38] using *p*-values obtained from a one-way analysis of variance (ANOVA) ($p < 0.1$) in GC–MS and LC–MS. Multiple comparisons, including principal component analysis (PCA) and OPLS-DA, were conducted using MetaboAnalyst 3.0, which provides standard validation information including cross-validation and a permutation test to prevent overfit of the models to the data [34].

2.4.2. Acquisition of UPE data and derived parameters

From a 50 ms bin, the following ten UPE properties were calculated from all four hand surfaces: strength, FF0, FF1, FF2, alpha, gamma, theta, phi, SSI, and SSR [32,33,39]. Thus, a total of 40 UPE parameters were obtained from each subject.

2.4.3. Correlation analysis

The statistics software package R (version 3.0.3) was used to calculate Spearman's rank correlation coefficient in order to examine the relationship between the metabolites and UPE parameters. A graphical overview of the correlation networks was created using CytoScape version 3.3.0[‡] with the MetScape plugin [40,41]. Positive and negative correlations are indicated by positive and negative values of *r*, respectively.

3. Results and discussion

3.1. Subtyping based on plasma metabolomics

TCM-based diagnostics use important information on personalized phenotypes (so called "syndrome subtypes") at a holistic level. The identification of these syndrome subtypes is the most important concept guiding personalized interventions in TCM. Therefore, exploring TCM-based diagnostics may provide fresh insight into personalized healthcare and management strategies. However, TCM-based diagnostics are descriptive, phenomenological concepts that are based on several standard diagnostic steps, including inspection, listening and smelling, inquiry and questioning, and palpation. The outcomes from these steps are combined to create an individual profile, which is used to establish a diagnosis. Therefore, objective, evidence-based data are needed in order to support TCM-based diagnostics. In this study, 26 TCM-based diagnostic terms were determined in order to classify different syndrome subtypes in 44 pre-diabetic subjects [17]. From this exploratory study, plasma samples were used to obtain evidence-based information that was used to help subtype the pre-T2DM subjects.

We used two validated metabolomics methods based on GC–MS and LC–MS. GC–MS yielded 147 untargeted metabolites, and LC–MS yielded 110 targeted metabolites; all of these metabolites were included in the total metabolomics profile. The metabolites detected by GC–MS included various metabolic classes, but

[†] http://www.metaboanalyst.ca/.
[‡] http://www.cytoscape.org.

primarily included amine metabolites, organic acids, sugars, and fatty acids such as LPA and LPA-derived metabolites. The metabolites detected by LC–MS could be classified into seven classes of lipids including polar lipids (e.g., phosphatidylcholine, phosphoethanolamine, lyso-phosphatidylcholine, lyso-phosphoethanolamine, and sphingomyelin) and non-polar lipids (e.g., ChEs and TGs). Given the relatively small number of subjects (44) compared to the large number of total variables (257), a first step of selecting variables was required before proceeding with a multivariate analysis. This step allowed us to optimize the variable/object ratio for discriminant type approaches and allowed us to remove potential irrelevant and/or confounding variables [38]. A total of 32 preliminary variables were selected based on an ANOVA ($p < 0.1$); these variables included 14 plasma metabolites identified by GC–MS and 17 plasma lipids identified by LC-MS. These variables were then used for subsequent multivariate analyses, including PCA, partial least squares discriminant analysis (PLSDA), and OPLS-DA (see Table S1 and Fig. S1).

The first step in our analysis focused on investigating whether plasma metabolomics could be used to discriminate between the three TCM-based syndrome subtypes of pre-T2DM (i.e., QYD vs. QYD_Damp, QYD vs. QYD_Stag, and QYD_Damp vs. QYD_Stag). A 3D PCA plot was used to visualize the natural distribution of the three groups in 3D space [38,42]. The first three principal components analyzed described 66.5% of the total variance in the plasma metabolome (Fig. 1). We found no large distance between the three subtypes reflected by PCA, which is not surprising given that their TCM-based diagnostic patterns are all linked (interrelated) and that TCM-based syndromes subtypes are not independent but show dynamic changes toward different directions [13,14]. However, we did observe a tendency toward clusters within the subtypes, with minor overlap in the PCA analysis.

Next, we used supervised models, including linear discriminant analysis (LDA), PLSDA, and OPLS-DA, in order to identify relevant plasma metabolites (Fig. S2). The OPLS-DA model provided the highest R^2 and Q^2 values and was therefore used to identify the most relevant variables based on score plots [43,44]. Furthermore, permutation tests with 1000 iterations ($p < 0.05$) showed a good performance of the model. Fig. 2 shows the OPLS-DA score plots for the first two principal components between each pair of subtypes (see also Fig. S3).

Table 1 summarizes the relevant metabolites (defined as the combination of covariance $|p[1]| > 0.7$ and correlation coefficient $|p(\text{corr})| > 0.3$ [44]) for each pair of groups, together with their contribution toward each pair of subtypes (QYD vs. QYD_Damp, QYD vs. QYD_Stag, and QYD_Stag vs. QYD_Damp). As shown in Table 1, 14 of the 18 metabolites that contributed to the differentiation between QYD and QYD_Damp are long-chain non-polar lipids (ten TGs and four ChEs); these metabolites were present in higher levels in the QYD_Damp group than in the QYD group. The TGs and ChEs (ten TGs and four ChEs) were also present in higher levels in the QYD_Stag group than in the QYD group, although two of those (C58_10_TG and C22_5_ChE) were not appeared in the comparison between QYD and QYD_Damp. Thus, we conclude that an increase in long-chain non-polar lipids is associated with the QYD_Damp and QYD_Stag groups.

The physiological mechanisms that underlie the early phases of T2DM have been linked to lifestyle issues such as the consumption of a diet high in fat and calories [45–48]; in this aspect, the early phases of T2DM are similar to chronic fatigue syndrome and/or mild inflammatory status [17]. TGs are the precursors of phospholipids, which are the building blocks of cell membranes and play an important role in energy homeostasis. ChEs are a stored form of cholesterol that is normally exported as a high-density lipoprotein (HDL) and returned to the liver. High levels of cholesterol and TGs

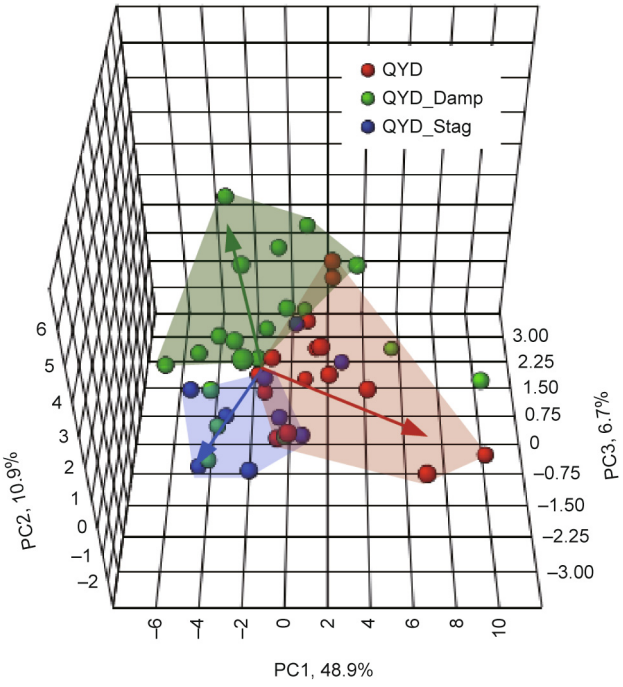

Fig. 1. 3D PCA score plot of the log-transformed and Pareto-scaled data for the three pre-T2DM subtypes. PC: principal component.

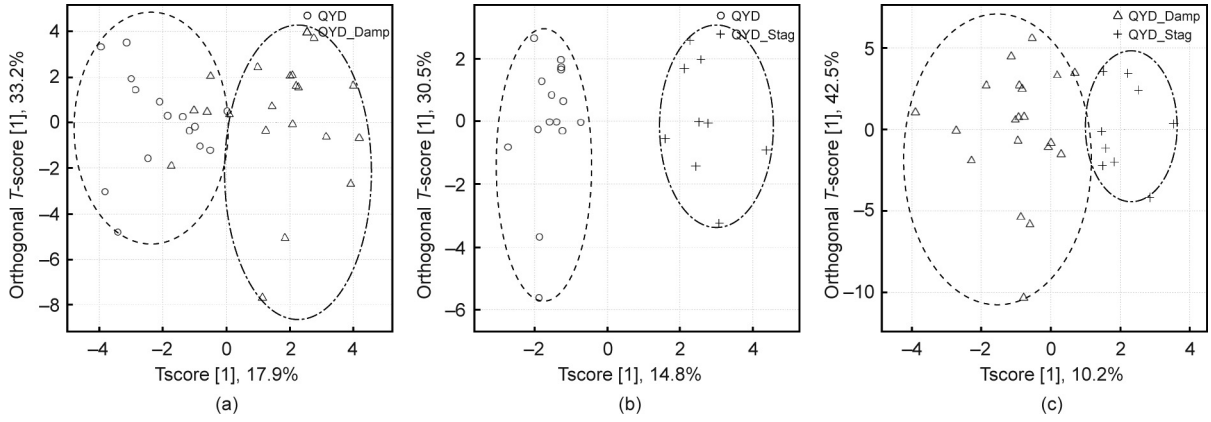

Fig. 2. OPLS-DA score plots of the first two principal components for each pair of subtypes. (a) QYD vs. QYD_Damp: $R^2Y = 0.61$, $Q^2 = 0.36$, permutation $p = 0.001$; (b) QYD vs. QYD_Stag: $R^2Y = 0.93$, $Q^2 = 0.62$, permutation $p < 0.001$; (c) QYD_Damp vs. QYD_Stag: $R^2Y = 0.69$, $Q^2 = 0.48$, permutation $p < 0.001$.

Table 1
List of relevant metabolites identified by OPLS-DA.

Pair of subtypes	Metabolite	Change
QYD_Damp vs. QYD	C52_5_TG	↑
	C54_6_TG	↑
	C54_5_TG	↑
	C54_7_TG	↑
	C56_8_TG	↑
	C56_7_TG	↑
	C56_8_TG	↑
	C58_9_TG	↑
	1-methylhistidine % 10227\01.03 uk × 45*	↑
	C58_9_TG	↑
	C52_6_TG	↑
	C18_3_ChE	↑
	C16_0_ChE	↑
	C22_6_ChE	↑
	C20_3_ChE	↑
	Creatinine	↑
	1-palmitoyl-*L*-alpha-lysophosphatidic acid	↓
	1-stearoyl-*sn*-glycero-3-phosphocholine	↓
QYD_Stag vs. QYD	C22_5_ChE	↑
	C54_7_TG	↑
	C54_6_TG	↑
	C58_10_TG	↑
	C52_6_TG	↑
	C56_8_TG	↑
	C18_3_ChE	↑
	C52_5_TG	↑
	C22_6_ChE	↑
	C56_7_TG	↑
	C56_9_TG	↑
	C20_3_ChE	↑
	C54_5_TG	↑
	C58_9_TG	↑
	31944 uk 05	↓
QYD_Stag vs. QYD_Damp	Beta-alanine	↓
	6926 uk × 10*	↓
	1-methylhistidine % 10227\01.03 uk × 45*	↓
	31944 uk 05*	↓

The sequence of the metabolites indicates their importance for the contribution to the group separations.
↑: increase; ↓: decrease.
*: structural unidentified metabolites in GC–MS untargeted measurement.

(hypercholesterolemia and hypertriglyceridemia, respectively) are associated with fat accumulation, atherosclerosis, and cardiovascular disease [49,50]. Therefore, patients in the pre-T2DM subgroups, QYD_Damp and QYD_Stag, may have an increased risk of developing atherosclerosis and/or cardiovascular disease in a later disease stage.

Although TGs and ChEs were increased in both the QYD_Damp and QYD_Stag groups relative to the QYD group, these two groups had several metabolic differences (Table 1). The relatively lower levels of amine metabolites in the QYD_Stag group (and/or the relatively higher levels in the QYD_Damp group) may suggest that the difference between the QYD_Stag and QYD_Damp subtypes is based primarily on differences in the tricarboxylic acid (TCA) cycle and/or muscle catabolism processes [51]. In summary, 23 metabolites contribute to the stratification of pre-T2DM subtypes. Thus, different pre-T2DM subtypes may be discriminated based on differences in plasma metabolomics, including plasma lipids and amine metabolites.

Previously, Wei et al. [17] reported that urine metabolomics can be used to reflect changes in carbohydrate metabolism and renal function in patients with QYD_Stag syndrome; specifically, two of the three TCM-based subtypes could be stratified (between QYD and QYD_Stag or between QYD_Damp and QYD_Stag). In contrast, plasma metabolomics provides stratification among the three subgroups; to be specific, QYD and QYD_Damp could be stratified successfully using plasma metabolomics, probably due to the use of a lipidomics platform, which measures a class of compounds that cannot be measured using urine metabolomics. This finding suggests that measuring lipid metabolomics is important for accurately subtyping pre-T2DM. In the previous study [17], urine metabolomics was shown to provide additional biomarkers to understand the differences between QYD_Damp and QYD_Stag; this finding indicates that performing various metabolomics platforms using different body fluids may provide more comprehensive information to study TCM-based diagnostics.

3.2. Correlation between metabolomics and UPE

Analytical methods can only be performed using currently available analytical platforms, which may not necessarily cover the entire profiling. Therefore, complementary tools are still needed in order to further characterize TCM-based diagnostics at an integrated, dynamic systems level. Measuring UPE may approximate the organizational level of TCM-based diagnostics, since UPE has been proposed as a non-invasive indicator of the integrated states in living organisms [19]. UPE measurement has matured to be rapid and highly sensitive, and there is some evidence that measuring UPE from human hands is sufficient to reflect body states [19]. As a promising tool, UPE has been suggested for use in clinical research [21]. Therefore, we measured UPE in our cohort of subjects with pre-T2DM. Stratification of the three TCM-based syndrome subtypes using 16 UPE parameters has been studied previously [28]. However, the molecular interpretation of UPE parameters requires further study. One available strategy is to link metabolomics to UPE [23,52]. Given that both plasma metabolomics and UPE can stratify subjects into pre-T2DM subgroups, plasma metabolomics data may be used to obtain biochemical insight into UPE [53–55]. To explore the relationship between these two approaches, we used Spearman's rank correlation coefficient to establish a correlation-based metabolite-to-UPE network. Such a correlation network may provide additional information that may further stratify disease subtypes and provide a biochemical interpretation of UPE parameters.

We generated correlation networks between the 23 metabolites and 16 UPE parameters that contributed to the stratification of subtypes in order to visualize the most relevant correlations related to the three subtypes (Fig. 3). These networks revealed clearly distinct distributions of UPE-to-metabolite correlations between the three subtypes. Specifically, the QYD_Damp subtype contained relatively few correlations, whereas the QYD_Stag subtypes contained relatively more positive and negative correlations compared with the QYD group.

First, in TCM, these three subtypes share a common, basic, cross-biological background syndrome—QYD—and the additional pathological factor (dampness or stagnation) reflects different symptoms that should be treated using different interventions [28]. Therefore, the different distributions of UPE-to-metabolite correlations in QYD_Damp and QYD_Stag may indicate the different dynamic changes in different directions in comparison with QYD.

Second, the principle source of UPE is closely related to the generation of reactive oxygen species (ROS) during metabolic processes [19]. The cellular damage inflicted by ROS has been associated with many diseases; in particular, the overproduction of ROS will result in pathogenesis of diabetes mellitus [4]. A major source of ROS is a family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. Increased lipid-derived signaling molecules may lead to activation of NADPH oxidase, hence resulting in cellular oxidative damage [56]. We found that lipid metabolites were present in higher levels in the QYD_Damp group

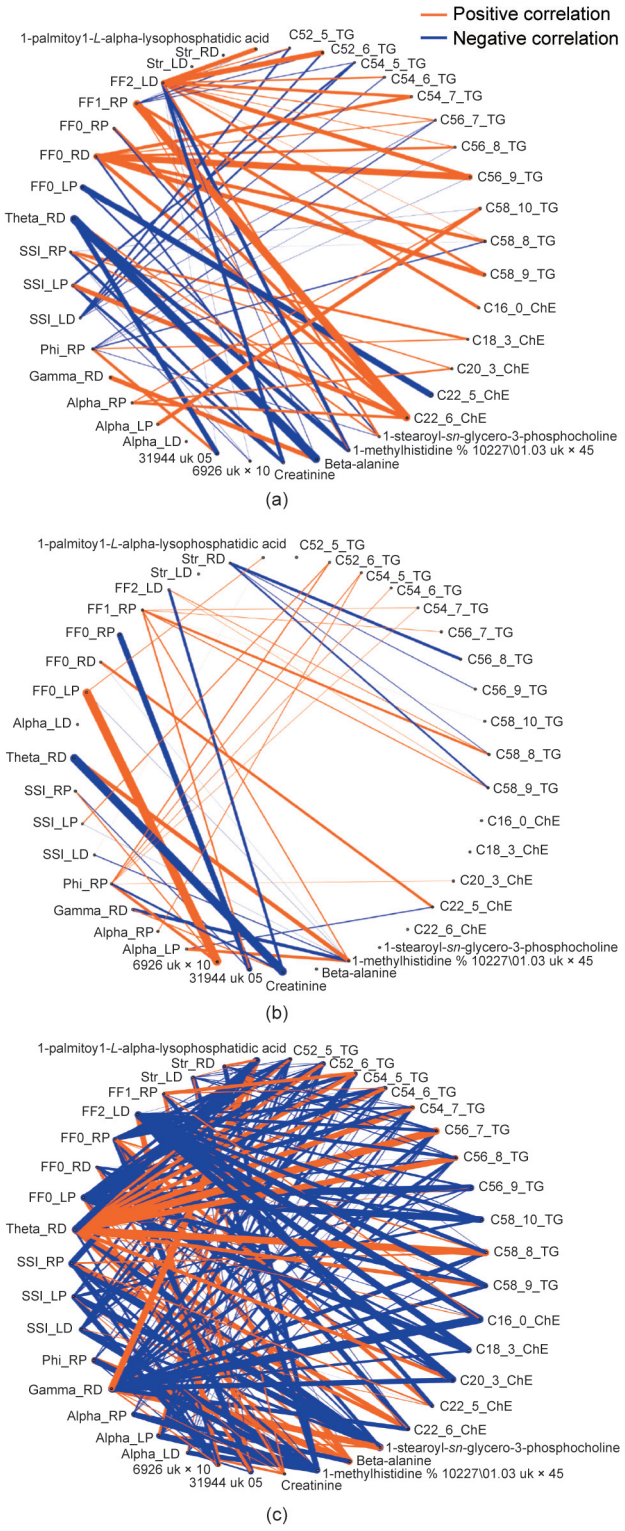

Fig. 3. Correlation networks between metabolic variables and UPE parameters for the indicated pre-T2DM subtypes: (a) QYD; (b): QYD_Damp; and (c) QYD_stag. Only the correlations between metabolites and UPE parameters with $|r| > 0.3$ are plotted.

and QYD_Stag group in comparison with the QYD group in plasma metabolomics. This finding might provide a potential interpretation of the different UPE-to-metabolite correlation distributions of the QYD_Damp group and QYD_Stag group compared with the QYD group. On the other hand, we found that the major differences in plasma metabolites were in the amine metabolites between the QYD_Damp group and QYD_Stag group; furthermore, the previous urine metabolites showed us that the QYD_Stag group had higher sugars levels than the QYD_Damp group [17]. Reducing sugars can react with a free amino group to produce a Schiff base, which can further lead to the accumulation of the Amadori products. In contrast, the Amadori glycation product tends to accumulate in proteins, leading to advanced glycation that results in the generation of ROS [4]. This information may help us to understand why the UPE-to-metabolite correlation is distributed differently in the QYD_Damp group and QYD_Stag group.

Third, the 16 UPE parameters indicate not only the photon intensity (Str) but also the photon-counting statistic properties (FF, SSI, alpha, etc.) [32,33]. The photon-counting statistic properties describe the photon emission distribution within a certain measurement time. That means that these properties are based on the timing of photon signals. This is crucial in order to understand the link between UPE and metabolic procedures from a systematic view. Although both QYD_Damp and QYD_Stag show increased levels of long-chain non-polar lipids compared with QYD, Fig. 3 reveals that the QYD_Stag group shows many strong correlations between these lipids and the UPE parameters, whereas the QYD_Damp group does not. This finding demonstrates that the UPE parameters are not only dependent on the quantity of ROS-producing molecules. The differences between the QYD_Damp and QYD_Stag groups lie in their amine metabolites and sugar levels. Both sugar and amine metabolisms belong to the tightly coupled metabolic network system in cells. The deficiency of a single metabolism may influence the whole metabolic network and further change the UPE in both the intensity (quantity of ROS) and timing of UPE emission (when the ROS are produced).

In brief, although clear links are visible between UPE parameters and specific classes of metabolites (e.g., TGs and ChEs), the correlations differ among the subtypes. The differences between the three networks provide a clear distinction between the subgroups and might serve as an additional diagnostic tool. However, further clinical trials must be performed using larger pre-diabetic and diabetic subject cohorts in order to investigate detailed biological mechanisms for both UPE and TCM-based diagnostics. This will not only provide new insight into biochemical interpretation for UPE to support its clinical application, but also provide more evidence-based data for TCM-based diagnostics.

4. Conclusions and perspectives

In this paper, we report that plasma metabolomics can be used to stratify the three TCM-based subtypes of early-stage type 2 diabetes, and provide better stratification than urine metabolomics. To be specific, increased levels of plasma lipids such as TGs and ChEs may indicate a relatively higher risk of developing cardiovascular disease among patients with specific subtypes. In addition, we used UPE as a non-invasive method for subtyping pre-T2DM; the UPE parameters were correlated with specific plasma metabolites—primarily lipid metabolites—and these correlations differed among the three subtypes. Thus, combining UPE with plasma metabolomics provides additional insight into the diagnosis of disease and the underlying biochemistry of UPE from a systems biology perspective.

The ability to identify the pre-T2DM syndrome subtype based on TCM is essential for achieving a personalized treatment plan, which will significantly improve patient care. These results provide a window of opportunity for combining metabolomics with UPE in order to achieve personalized medicine and improve the early diagnosis of disease. Nevertheless, metabolomics platforms do not necessarily cover the entire metabolome, and choices must be made based on the metabolomics platforms that are currently available. Given the difficulties associated with obtaining compre-

hensive information regarding the dynamic changes reflected by measuring metabolomics, linking metabolomics to UPE under the guidance of TCM-based diagnostics is particularly attractive for promoting the early diagnosis of T2DM. Additional research is needed in order to expand the correlation networks between metabolites and UPE parameters. In addition, current approaches for stratifying T2DM are based on varied criteria, which must be consistent for further clinical diagnosis. Therefore, additional research is needed in order to understand TCM-based concepts such as disease syndromes and subtypes.

Acknowledgements

Min He is financially supported by the China Scholarship Council (CSC) under the Chinese Government Graduate Student Overseas Study Program during her four-year PhD study at Leiden University in the Netherlands (20108220166) and National Key Basic Research Program of China (2014CB543100). The authors thank Herman van Wietmarschen for critical reading of the manuscript.

Author contributions

Min He, Eduard Van Wijk, Mei Wang, Slavik Koval, Thomas Hankemeier, and Jan Van der Greef gave substantial contributions to the design of the work. Min He, Mengmeng Sun, Eduard Van Wijk, Roeland Van Wijk, and Jan Van der Greef wrote the manuscript. All authors reviewed the manuscript and approved it for publishing.

Compliance with ethics guidelines

Min He, Mengmeng Sun, Slavik Koval, Roeland Van Wijk, Thomas Hankemeier, Jan Van der Greef, Eduard P.A. Van Wijk, and Mei Wang declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2019.03.011.

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