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ORIGINAL ARTICLE

A non-targeted metabolomics study on different glucose tolerance states

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Abstract
A non-targeted metabolomics method was employed to study metabolic characteristics in subjects with different glucose tolerance. Plasma samples of 120 participants with normal glucose tolerance (NGT), impaired glucose regulation (IGR), and type 2 diabetes (T2D) were collected. Gas chromatography/mass spectrometry (GC/MS) was used to profile and compare the plasma metabolome among the three groups. Through the use of multivariate statistical analysis, we found distinct metabolome change from NGT to IGR and to T2D. ANOVA found that the IGR and T2D groups had perturbations of monosaccharide and lipid metabolism, disorders of glucogenic amino acids, and branched-chain amino acid catabolism. Furthermore, we also found that the levels of 2-hydroxybutyrate and 2-ketoisocaproate were progressively increased with glucose tolerance severity. The results from this study help us better understand the relationship between plasma metabolism and glucose tolerance states and also suggest that 2-hydroxybutyrate and 2-ketoisocaproate may be closely associated with the development of T2D.

Keywords Type 2 diabetes · Impaired glucose regulation · Metabolomics · Gas chromatography/mass spectrometry

Introduction

Diabetes is one of the most common metabolic disorders worldwide, seriously impacting human health, labor force, and economic status. As living standards improve, population aging, and increasing incidence of obesity, the prevalence of diabetes, especially type 2 diabetes (T2D), is rapidly accelerating year by year. Impaired glucose regulation (IGR), also known as pre-diabetes, is an abnormal intermediate state that exists between normal glucose tolerance (NGT) and T2D. According to the International Diabetes Federation and the American Diabetes Association, patients with T2D almost always undergo the period of IGR. Therefore, it is warranted to perform the proper investigations on the different states of glucose toleration, in order to help us penetrate into the disease progress process of diabetes and obtain insightful clues of early diagnosis and effective interventions for preventing or delaying the course of diabetes.

Metabolomics, profiling the global state of metabolites in biological fluids and tissues, is emerging as a field with tremendous promise in extending "omics" from the gene to the small molecule [1]. It measures the dynamic metabolic responses to pathophysiological stimuli or genetic modifications [2]. Metabolomics investigations combined with multivariate analysis serve in characterizing the offset of the body metabolism caused by physiological and/or pathophysiological changes through abundant endogenous information, which can easily reveal the differences in metabolism among various groups. In addition, metabolomics has begun to play a more important role in discovering and identifying potential biomarkers discriminating normal from abnormal states. Recognition of the differential metabolites can provide insight into the underlying molecular mechanism and is helpful in clinical diagnosis.

Since type 2 diabetes is a typical metabolic disease with a chronic dysfunction of metabolic system, many researchers have successfully used the platform of metabolomics to investigate metabolic alterations of pre-diabetes and/or diabetes in

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the last decade [3–7]. To date, published findings suggest that amino acid (branched-chain amino acids and glucogenic amino acids), lipid (phospholipids, sphingomyelins, free fatty acids and acylcarnitines), carbohydrate (glucose, mannose, galactose and fructose), and bile acid (cholate and deoxycholate) metabolism present the complex abnormalities in individuals with pre-diabetes and diabetes compared with control subjects. More recently, metabolomics research based on diabetes has mainly focused on the identifying novel predictive biomarkers associated with pre-diabetes and diabetes by using prospective study designs. Current evidence revealed the close correlation of branched-chain and aromatic amino acids with insulin resistance and future development of diabetes [8, 9]. The hexose sugars (fructose, mannose, galactose, and inositol) were strongly associated with higher risk of pre-diabetes and diabetes in these prospective studies [10, 11]. Lipidomics has also revealed that a number of lipids may be predictive of type 2 diabetes [12, 13], but inconsistent results have been reported in different studies [14]. Additionally, a few studies also identified some novel predictors of type 2 diabetes, including 2-aminoadipicacid [15], 2-ketoisocaproate [16], α-hydroxybutyrate [17], and so on. However, T2D is rarely a static condition, but rather one that evolves and changes over time during the lifespan of an individual [18]. Therefore, more attention should be paid to find the relationship between plasma metabolome and different states of glucose tolerance and further look for the metabolites that may be changed gradually with the glucose tolerance progression from NGT to IGR and to T2D.

In our present work, we performed a non-targeted metabolomics study based on GC/MS to illustrate the plasma metabolome change in different developing states of diabetes and find the progressively changed metabolites related with glucose tolerance. This study will help us profoundly realize the development process of diabetes and explore the underlying molecular mechanism.

Material and methods

Chemicals

Methoxyamine hydrochloride, MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide), pyridine, tridecanoic acid, and methyl laurate were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol was purchased from Tedia (USA).

Sample collection

Plasma samples were collected by Datong NO.3 People's Hospital. Totally, 120 age-, gender- and body mass index-matched subjects were included for non-targeted metabolomic analysis. Thirty-nine subjects had NGT (fasting plasma glucose < 5.6 mmol L^{-1} and 2-h glucose < 7.8 mmol L^{-1}), 40 subjects had IGR (fasting plasma glucose between 5.6–7.0 mmol L^{-1} and/or 2-h glucose between 7.8 and 11.1 mmol L^{-1}), and 41 subjects had T2D (fasting plasma glucose > 7.0 mmol L^{-1} and/or 2-h glucose > 11.1 mmol L^{-1}). All of the T2D participants were newly diagnosed without any anti-diabetic drugs. Plasma samples were collected after an overnight fasting in the standard protocol and immediately stored frozen at − 80 °C until use. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Sample preparation

Prior to metabolomic analysis, the plasma samples were thawed at 4 °C, and 20 μL tridecanoic acid (250 μg mL^{-1} in methanol) was added to a 100-μL aliquot of sample as an internal standard. Subsequently, 400 μL of methanol was pipetted into the mixture for protein precipitation. After vortexing for 30 s and centrifuging at 15,000 rpm for 20 min, the supernatant (450 μL) was transferred to a glass sampler vial and lyophilized at 10 °C. The dried extract was oximated using 65 μL of methoxyamine hydrochloride (20 mg mL^{-1} in pyridine) at 40 °C for 90 min, and then trimethylsilylated using 65 μL of MSTFA for 60 min at 40 °C. The final solution was spiked with 20 μL external standard solution (0.9 mg mL^{-1} methyl laurate dissolved in pyridine).

Metabolomic analysis

Metabolomic analysis was performed using an Agilent 7890/5975C-GC/MSD system (Agilent Co.,USA). Separation was achieved on a fused-silica capillary column (30 m × 0.25 mm i.d.) chemically bonded with 0.25 μm DB-5 stationary phase (J&W Scientific, Folsom, CA, USA). The injection temperature was 300 °C and the split ratio was 10:1. Helium was used as the carrier gas with a constant velocity of 1.0 mL min^{-1}. The column temperature was initially kept at 70 °C for 2 min, changed to 90 °C at 3 °C min^{-1} and then increased to 200 °C at 2 °C min^{-1}, finally to 320 °C at a rate of 15 °C min^{-1}, and held for 5 min. The effluent was introduced into the electron ionization source. The following parameters were used: interface temperature, 280 °C; ion source temperature, 230 °C; and the detector voltage, 1.38 kV. Full scan mode was employed in the mass range of 33–500 amu at a rate of 3.1 spectra s^{-1}. The solvent delay time was set at 8.5 min.

Data analysis

GC/MS raw data were exported in the NetCDF format, and then preprocessed by using the XCMS toolbox [19]. The

parameters of retention time correction and peak alignment were set to default values except for full width at half-maximum (FWHM = 4) and group (bw = 5). The area of each variable was normalized to the internal standard in the same chromatogram. The resulting data were then exported into SIMCA-P software version 11.0 (Umetrics, Umea, Sweden) for multivariate statistical analysis. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were carried out to visualize the global metabolome change among the different glucose tolerance groups. Subsequently, ANOVA by the SPSS 13.0 software (SPSS, Chicago, IL) was used to find differential metabolites among the three groups. $p < 0.05$ was considered significant. Metabolites identification was performed by searching the NIST database installed in the equipment system, with a similarity threshold of 75%, with all of them verified by commercial standard samples.

Results

Clinical administration

The clinical practice data are shown in Table 1. Fasting plasma glucose and 2-h plasma glucose concentrations in T2D patients were significantly higher than those in both NGT and IGR subjects, fasting insulin, and C-reaction protein had similar changes although were not statistically significant. As for blood lipids, the levels of triglycerides, total cholesterol and LDL-c were all higher in T2D patients than those in the other two groups, while LDL-c level showed no statistical difference. Meanwhile, the concentration of HDL-c was lower in T2D patients than in the other two groups with statistical significance. The levels of fasting plasma glucose, 2-h plasma glucose, and triglycerides were also remarkably higher in IGR group than those in NGT group, as expected.

Metabolomic analysis

The plasma metabolic profile was analyzed by GC/MS. Figure 1 gives the total ion chromatogram (TIC) of a representative plasma sample. To verify the reproducibility and reliability of the method including sample preparation and instrument performance, quality control samples derived from the equal pooling of all samples were prepared and analyzed accompanying with real samples in the whole analytical workflow. The relative standard deviations (RSDs) of the retention time and peak area of the main peaks were less than 1 and 15%, respectively, which reflected acceptable levels of variability for overall process. In addition, methyl laurate as the external standard was utilized to further evaluate the stability of the analytical platform. The RSDs of the retention time and the peak area of methyl laurate in the quality control samples were 0.04 and 5.6%, respectively, indicating that the instrument performance was perfectly stable during the whole analytical process.

To learn whether or not we can distinguish the three groups (NGT, IGR, and T2D) using the GC/MS data and understand the relationship between plasma metabolome and different states of glucose tolerance, we first performed a principal component analysis (PCA) model. The score plot (Fig. 2a) using two components ($R^2X = 0.66$) shows a separation tendency from NGT to IGR, and further to T2D group, suggesting that, from the perspective of metabolomics, the metabolome differences indeed existed among the three groups and IGR was the intermediate status. A partial least squares discriminant analysis (PLS-DA) model with two components ($R^2Y = 0.48$, $Q^2Y = 0.30$) was also constructed in order to visualize the cluster more clearly. It is evident from Fig. 2b that a complete separation between NGT and T2D groups was achieved, demonstrating that the metabolic characteristics of T2D was quite different with NGT. The data points from IGR group mainly located in the middle, with less overlap with NGT but more overlap with T2D, indicating that the metabolic states of some subjects with IGR were already approaching T2D. Furthermore, the results of permutation test (intercepts $R^2 = 0.106$, $Q^2 = -0.18$) suggested that there was no overfitting and the model was reliable.

To better explore the different glucose tolerance-related changes in the metabolites, ANOVA and multiple comparison were employed to select significantly changed metabolites among the groups. As shown in Table 2, a total of 23 metabolites were obviously different. In detail, compared to the NGT, the metabolic state of T2D resulted in increased levels of 2-hydroxybutyrate, branched-chain amino acids (isoleucine, leucine, valine), 2-ketoisocaprate, inositol, monosaccharide (fructose, galactose, glucose), glycerol, glycerate, and free fatty acids (FFA C16:0, FFA C18:1, FFA C18:0), as well as decreased levels of citrate, malate and glucogenic amino acids including alanine, glycine, serine, threonine, phenylalanine, glutamine, and asparagine. Additionally, the IGR group had striking higher concentrations of 2-hydroxybutyrate, 2-ketoisocaprate, leucine, isoleucine, glucose, and FFA C16:0 together with lower levels of citrate, glycine, and asparagine compared to those in NGT group. Finally, when compared with IGR group, T2D patients had remarkably higher levels of 2-hydroxybutyrate, 2-ketoisocaprate, valine, inositol, glycerate, glycerol, FFA C18:0, fructose, galactose as well as glucose, but lower levels of alanine, phenylalanine, and glutamine. In addition to glucose, 2-hydroxybutyrate and 2-ketoisocaproate also distinctly gradually increased from NGT to IGR and further to T2D. The results of the current study are well in-line with our previous preliminary investigation in another diabetic population (unpublished data). Box plots of the two metabolites are shown in Fig. 3 to visually present the distribution of variables among the three groups.

Table 1 Biochemical parameters for NGT, IGR, and T2D groups

	NGT ($n = 39$)	IGR ($n = 40$)	T2D ($n = 41$)
No. (male/female)	20/19	19/21	20/21
Age (year)	60 ± 1[a]	60 ± 1	60 ± 1
Current smokers	10	9	12
Alcohol use	7	8	8
Body mass index (kg/m^2)	24.9 ± 3.3	25.2 ± 2.3	26.0 ± 3.2
Fasting plasma glucose (mmol/L)	5.34 ± 0.56	5.93 ± 0.44[b]	7.88 ± 1.67[c,d]
Fasting plasma insulin (μIU/mL)	7.32 ± 5.65	7.44 ± 5.37	26.9 ± 99.1
2 h plasma glucose (mmol/L)	6.16 ± 0.55	8.25 ± 1.41[b]	14.9 ± 6.20[c,d]
Uric acid (μmol/L)	269.4 ± 63.7	321.5 ± 115.8[b]	301.1 ± 88.8
Total cholesterol (mmol/L)	4.75 ± 0.85	4.80 ± 0.95	6.33 ± 0.71[c,d]
Triglyceride (mmol/L)	1.17 ± 0.62	1.62 ± 0.81[b]	1.95 ± 0.89[c,d]
HDL cholesterol (mmol/L)	1.42 ± 0.37	1.30 ± 0.24	0.95 ± 0.26[c,d]
LDL cholesterol (mmol/L)	2.82 ± 0.67	2.96 ± 0.76	3.15 ± 0.68
C-reaction protein (mg/L)	2.88 ± 2.83	4.44 ± 5.29	6.15 ± 12.1

[a] Mean ± SD

[b] $p < 0.05$ for IGR vs. NGT

[c] $p < 0.05$ for T2D vs. IGR

[d] $p < 0.05$ for T2D vs. NGT

Discussion

In order to investigate the complex perturbations of the metabolism related to the glucose tolerance states, we carried out a GC/MS-based non-targeted metabolomics study to investigate plasma samples from NGT, IGR, and T2D subjects. Our results showed a distinct metabonome change from NGT to IGR and to T2D. Moreover, differential metabolites revealed disturbances of various biological pathways in IGR and T2D groups. In addition to glucose, we found that the levels of 2-hydroxybutyrate and 2-ketoisocaproate progressively increased with impaired glucose tolerance severity. Although blood glucose level is routinely used to screen and assess diabetes, our results suggested that glucose measurement alone is not comprehensive and the metabolites associated with the development of T2D should be considered.

In carbohydrate metabolism, we observed the elevation of glucose, fructose, and galactose in IGR and/or T2D groups

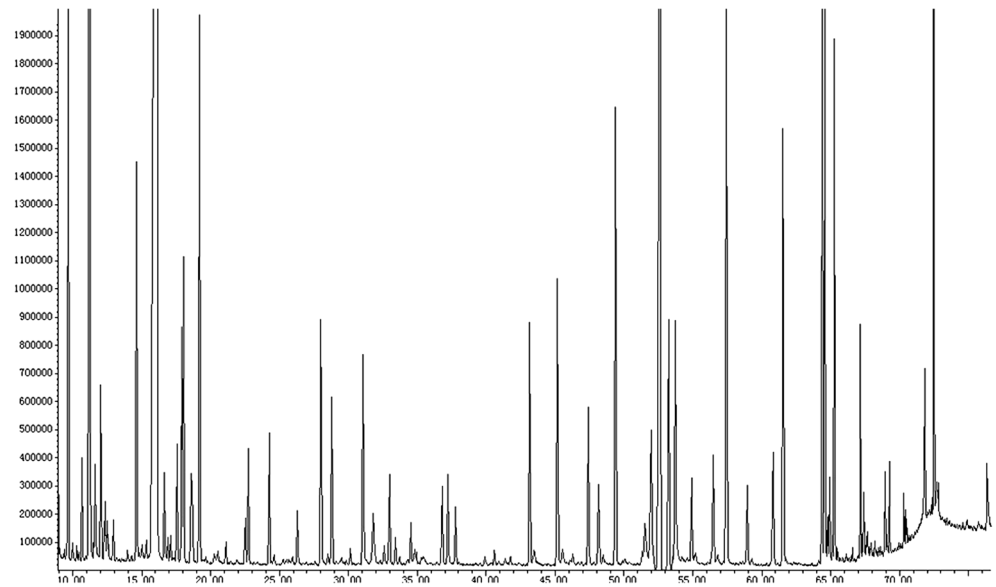

Fig. 1 Representative total ion chromatograms (TIC) of plasma sample from a participant

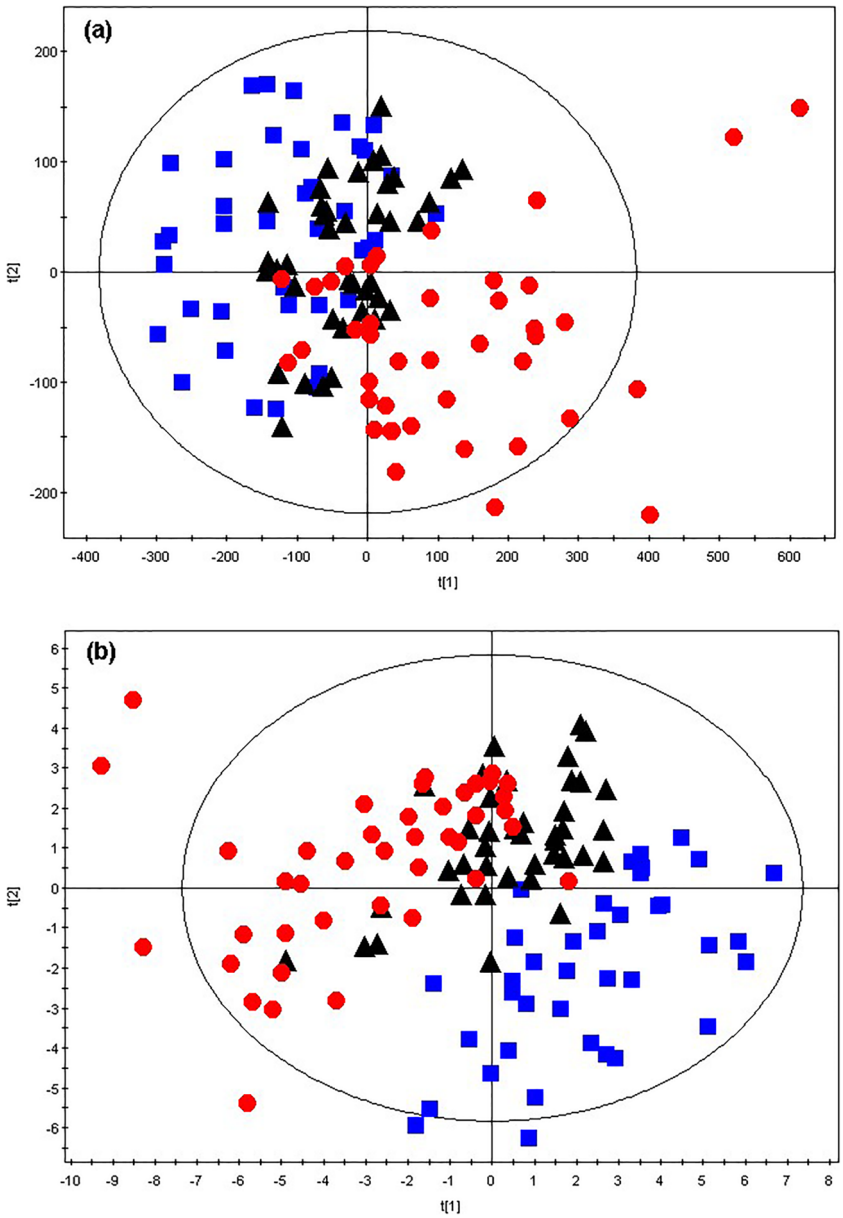

Fig. 2 **a** PCA and **b** PLS-DA score plots for NGT (blue squares), IGR (black triangles), and T2D (red dots) groups

compared to the NGT group. These data suggested that the perturbations of glycolysis in IGR and T2D groups results in the accumulation of monosaccharide, which causes harm to the metabolism homeostasis. It has been noted that long-term high level of fructose can stimulate lipogenesis and induce hepatic insulin resistance [20, 21], and galactose is associated with retinopathy [22, 23].

In addition, those with IGR and/or T2D had elevated levels of branched-chain amino acids and reduced glucogenic amino acids, when compared to NGT. In the states of IGR and T2D, the rate of utilization of glucose is reduced relatively, resulting in the disturbance of energy metabolism. Therefore, the body needs other fuel molecules to enter TCA cycle to supply sufficient amount of energy. The reduced levels of glucogenic amino acids indicate that a large number of amino acids are catabolized and ultimately form many intermediates of TCA cycle. The levels of branched-chain amino acids, in contrast, increased in IGR and/or T2D groups. It has been documented that the activity of branched-chain α-keto acid dehydrogenase, an enzyme in branched-chain amino acids catabolism, is downregulated in diabetes mellitus [24, 25]. Therefore, under the control of this key enzyme, the catabolism of branched-chain amino acids in patients is blocked, and consequently, the concentrations of branched-chain amino acids in blood increased.

Furthermore, higher concentration of free fatty acids as well as glycerol in IGR and/or T2D patients was observed. It is due to the effects of antilipolytic and promoting fat storage

Table 2 Identified significantly changed metabolites in NGT, IGR, and T2D groups

Metabolite	Fold change[a] In IGR(vs. NGT)/ *p* value[b]	Fold change In T2D(vs. IGR)/ *p* value	Fold change In T2D(vs. NGT)/ *p* value	Biological pathways
Valine	1.1↑/0.323	1.3↑/0.031	1.4↑/0.001	Valine metabolism
Leucine	1.2↑/0.025	1.0/0.511	1.2↑/0.023	Leucine metabolism
Isoleucine	1.1↑/0.037	1.1↑/0.414	1.2↑/0.010	Isoleucine metabolism
2-ketoisocaprate	1.2↑/0.009	1.2↑/0.015	$1.4↑/1.3 \times 10^{-4}$	Isoleucine metabolism
2-hydroxybutyrate	1.3↑/0.001	1.2↑/0.026	$1.5↑/1.6 \times 10^{-4}$	Propanoate metabolism
Alanine	1.0/0.412	0.8↓/0.025	0.8↓/0.013	Alanine metabolism
Glycine	0.7↓/0.003	1.0/0.627	0.7↓/0.009	Glycine metabolism
Serine	0.9↓/0.067	0.9↓/0.236	0.8↓/0.008	Serine metabolism
Threonine	0.9↓/0.295	0.9↓/0.291	0.8↓/0.031	Threonine metabolism
Phenylalanine	1.0/0.355	0.7↓/0.002	0.7↓/0.003	Phenylalanine metabolism
Glutamine	0.9↓/0.203	0.8↓/0.036	0.7↓/0.001	Glutamine metabolism
Asparagine	$0.7↓/1.1 \times 10^{-4}$	1.0/0.632	$0.7↓/3.9 \times 10^{-5}$	Asparagine metabolism
Malate	0.8↓/0.072	0.9↓/0.132	0.7↓/0.014	TCA cycle
Citrate	0.8↓/0.022	1.0/0.279	0.8↓/0.030	TCA cycle
Inositol	1.1↑/0.329	1.1↑/0.041	1.2↑/0.035	Inositol phosphate metabolism
Fructose	1.1↑/0.336	1.3↑/0.002	1.4↑/0.001	Fructose and mannose metabolism
Galactose	1.1↑/0.224	1.2↑/0.010	1.3↑/0.002	Galactose metabolism
Glucose	1.1↑/0.030	$1.4↑/2.5 \times 10^{-5}$	$1.5↑/5.1 \times 10^{-6}$	Glucose metabolism
Glycerate	1.2↑/0.374	1.2↑/0.035	1.4↑/0.001	Pentose phosphate metabolism
Glycerol	1.1↑/0.505	1.3↑/0.019	1.4↑/0.002	Lipid metabolism
FFA C16:0	1.1↑/0.043	1.1↑/0.150	1.2↑/0.014	Lipid metabolism
FFA C18:0	1.1↑/0.241	1.2↑/0.034	1.3↑/0.001	Lipid metabolism
FFA C18:1	1.1↑/0.378	1.1↑/0.092	1.2↑/0.025	Lipid metabolism

(↑) upregulated, (↓) downregulated

[a] Fold change was calculated from the mean values of each group

[b] *p* value was calculated from multiple comparison. (↑) upregulated, (↓) downregulated

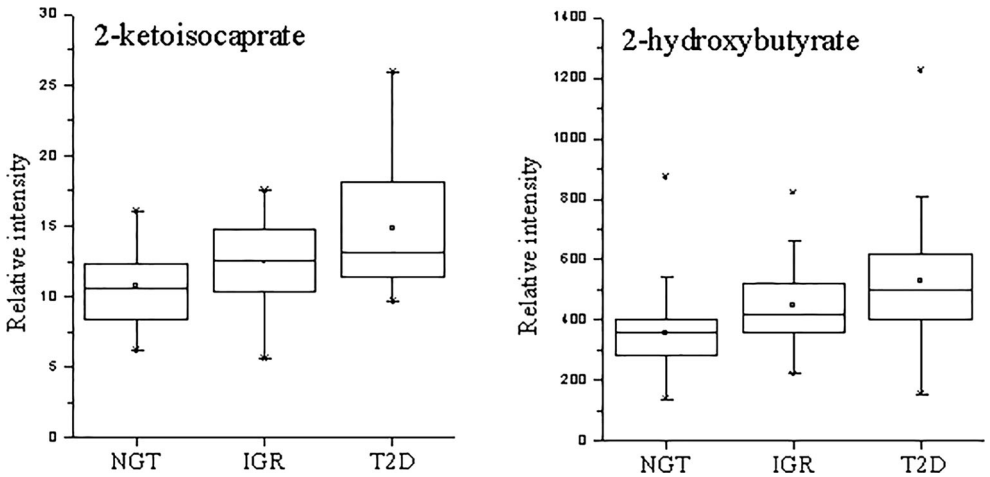

Fig. 3 Box plots of 2-ketoisocaprate and 2-hydroxybutyrate among the three groups

are weakened in patients with the impaired islet function [26, 27]. High levels of circulating fatty acids may, in turn, induce or exacerbate insulin resistance, thereby accelerating the onset of T2D [28, 29].

On the basis of the results, we can understand the metabolic dysregulations in the states of IGR and T2D. Reduction of glycolysis induces the manifold utilization of glucogenic amino acids, while in the meantime, the burden of glucose could be aggravated as a result of glucogenic amino acids transforming into glucose through gluconeogenesis. An abnormal high level of glucose can decrease the sensitivity of insulin and contribute to hyperlipidemia. Conversely, hyperlipidemia is the basis for the pathophysiology of insulin resistance and consequently exacerbates blood glucose, both as cause and effect, a vicious circle. Ultimately, TCA cycle as the center of metabolism is also disturbed, manifested as decreased levels of malate and citrate in our study.

Moreover, we also found the progressively changed metabolites related with different glucose tolerance besides glucose. Distinctly, the levels of 2-ketoisocaprate and 2-hydroxybutyrate were increased in IGR and T2D groups compared with those in the NGT group, and their concentrations in the T2D group were also higher than those in the IGR group. 2-Ketoisocaprate, derived from the deamination of isoleucine, is the substrate of branched-chain α-keto acid dehydrogenase. The gradual accumulation of 2-ketoisocaprate from NGT to IGR and to T2D indicates that isoleucine metabolism, particularly 2-ketoisocaprate, may be correlated with the pathological process of T2D. 2-Hydroxybutyrate is an organic acid and is produced during the pathway of threonine catabolism or glutathione anabolism. The accumulation of 2-hydroxybutyrate may be due to the disorders of upstream metabolism. Firstly, many researches have demonstrated that oxidative stress plays a critical role in the pathogenesis of diabetes mellitus [30–32]. Under dramatically increased oxidative stress states in IGR and T2D, large amounts of cystine are converted to cysteine to form glutathione [33, 34]; meanwhile, more 2-hydroxybutyrate is released as a by-product during this process. Secondly, 2-hydroxybutyrate is also formed from threonine catalyzed by serine-threonine dehydratase. In our study, the decrease of threonine level together with the increase of 2-hydroxybutyrate concentration in IGR and T2D patients suggest that the enhancement of threonine catabolism may be another reason for the elevation of 2-hydroxybutyrate. In other prospective studies, 2-ketoisocaprate [16] and 2-hydroxybutyrate [17] were found as the predictor of diabetes, respectively. Overall, from the data obtained in our study and others, we speculate that the two metabolites including 2-ketoisocaprate and 2-hydroxybutyrate may be not only good predictors for diabetes but also closely associated with the dynamic development of diabetes. Certainly, a lot of experiments are needed to verify the hypothesis and further explore the underlying molecular mechanisms of the two metabolites in future.

In the current study, a non-targeted metabolomics approach based on the combination of GC/MS and statistical analysis was employed to study the plasma metabolic patterns of subjects with NGT, IGR, and T2D. The metabolic changes in IGR and T2D groups included perturbations of monosaccharide and lipid metabolism, disorders of glucogenic amino acids, and branched-chain amino acid catabolism. At the same time, metabolites associated with the development of diabetes were also successfully obtained, manifested by 2-ketoisocaprate and 2-hydroxybutyrate as highlighted above. In conclusion, the metabolome alterations are the mirror image of different glucose tolerance states, and recognition of the relationships of 2-ketoisocaprate and 2-hydroxybutyrate with diabetes will help us deeply comprehend the pathological process of diabetes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Patient consent All the procedures were approved by institutional ethics review board of Datong NO.3 People's Hospital, and written informed consent was obtained from each participant.

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