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Circulating metabolites difference in type 2 diabetes patients from regions: a cross-sectional study

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Author contributions

Jiang-Lan Long and Dan Yan were in charge of manuscript conception, design, and writing. Jiang-Lan Long, Zeng-Hui Miao, Ling-Wei Kong, Hui-Jing Cui and Na-Na Zhang contributed to recruit participants and collect samples. Jiang-Lan Long and Zeng-Hui Miao contributed to acquisition, analysis or interpretation of data. All authors contributed to the article and approved the submitted version.

Competing interests

The authors declare no conflicts of interest.

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Peer review information

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Abbreviations

T2D, type 2 diabetes; UHPLC-Q-Orbitrap HRMS, ultra-high performance liquid chromatography coupled to a Q Exactive-Orbitrap high-resolution mass spectrometer; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; BMI, body mass index; ESI, electrospray ionization; QC, quality control; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; VIP, variable importance in the projection; $T2D_B$, type 2 diabetes patients from Beijing; $T2D_K$, type 2 diabetes patients from Kaifeng; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine.

Citation

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Abstract

Background: Type 2 diabetes (T2D) has already become a global pandemic. As its simple, rapid, economical, and relatively non-invasive, metabolic markers have become a method for T2D diagnosis. However, region, race, and diet all affect the metabolism of the body. The purpose of current study is to explore the differences of metabolites in T2D patients from regions. **Methods:** We recruited 103 T2D patients in two clinical centers, including 52 T2D patients from Beijing ($T2D_B$) and 51 T2D patients from Kaifeng ($T2D_K$). The serum samples from T2D patients were analyzed using high-resolution mass spectrometer. After screened using univariate and multivariate analysis, the differential metabolites were identified. Moreover, to reveal biological information, we performed pathway analysis with the differential metabolites. **Results:** Thirty-six differential metabolites were identified, including 16 metabolites were higher concentrations while 20 metabolites were lower concentrations in the serum of $T2D_B$ patients than $T2D_K$ patients. There were higher serum concentrations of L-phenylalanine, 4-methyl-2-oxovaleric acid, L-carnitine, decanoylcarnitine, 9-decenoylcarnitine and sphinganine in $T2D_B$ patients, in which decanoylcarnitine in $T2D_B$ patients was up to 35-fold higher than $T2D_K$ patients. While there were lower concentrations of L-valine, L-isoleucine, arachidonic acid, oleic acid, 16-hydroxyhexadecanoic acid, lysophosphatidylcholine (18:0) and 1-Phenylethylamine in $T2D_B$ patients, in which 1-phenylethylamine in $T2D_B$ patients was decreased to 0.45-fold lower than $T2D_K$ patients. The reason for the differences might be that phosphatidylethanolamine biosynthesis, phosphatidylcholine biosynthesis, valine, leucine and isoleucine degradation, and beta-oxidation of very long chain fatty acids were different in $T2D_B$ patients and in $T2D_K$ patients. **Conclusion:** Metabolites from different pathways are independently related to regions, providing valuable insight and potential for the diagnosis and treatment of T2D.

Keywords: type 2 diabetes; metabolomics; region; high-resolution mass spectrometer

Background

As a metabolic disease characterized by hyperglycemia, diabetes has become one of the main causes of death in the world [1], of which type 2 diabetes (T2D) exceeds 90% [2]. As a large-scale population and severe aging, China has the largest number of T2D patients in the world [3]. Without blood glucose control, T2D will lead to serious complications with the development of the disease, which can involve various organ systems of the whole body, including the brain, cardiovascular system, kidney, etc. [4, 5]. It is estimated that in 2021, about 6.7 million adults in the world died of diabetes or its complications, which indicates that one person dies of diabetes every five seconds on average [6]. Therefore, early diagnosis and treatment of T2D is extremely important.

Understanding of T2D is fundamental to disease prevention and treatment. There are a lot of studies on the characteristics of genes, proteins and metabolites before diagnosis/onset related to T2D [7–9]. As it is simple, rapid, economical and relatively non-invasive, metabolic markers are advantageous for research of diagnosis/onset related to T2D. However, region, race, and diet all affect the metabolism of the body [10, 11]. Due to the influence of climate, diet, lifestyle, etc., there are cultural differences in different regions in China. And metabolic markers with low reproducibility might be less to reflect the clinical application value. The study of T2D-associated metabolomic profiles from regions in the Chinese population hence is needed.

In the current study, a metabolomics method based on a high-resolution mass spectrometer is adopted to explore the differences in metabolites in serum samples of T2D patients from two clinical centers in China. In addition, we explore the reasons for the differences in metabolites, so as to provide valuable insight and potential for the diagnosis and treatment of T2D.

Materials and methods

Participants

The participants aged 30–79 years were recruited at two clinical centers in China: Beijing Jiaotong University Community Health Center (Beijing, China) and Kaifeng Hospital of Traditional Chinese Medicine (Kaifeng, China) (Ethical approval number: 2022-P2-215-01). This study was a cross-sectional study. The baseline survey collected detailed information on medical history and physical measurements. The diagnostic criteria: fasting blood glucose (FBG) ≥ 7 mmol/L, and/or hemoglobin A1c (HbA1c) ≥ 6.5% [12]. The exclusion criteria were participants with severe infectious diseases, human immunodeficiency virus, autoimmune disorders, acquired immunodeficiency syndrome, malignant tumors, severe renal dysfunction, liver dysfunction, hepatitis B, hepatitis C, gestational diabetes or diabetic complications. To exclude interference, we controlled matched variables that affected the participants' metabolism, such as age, body mass index (BMI), etc. As a cross-sectional study, at least 50 patients were recruited in each group after sample estimation [13, 14]. All participants provided written informed consent.

Sample collection and pretreatment

After the consent of the participants, the serum samples were collected, which was used for the detection of FBG, HbA1c, blood lipid, liver function and renal function indexes. Then, serum samples were immediately subpackaged and stored with −80 ˚C until analysis.

After thawed on ice for 30 min, 50 μL of each serum were mixed with 150 μL methanol solution, in which methanol solution included the internal standards: 2-chloro-L-phenylalanine (50 ng/mL, J&K Chemical, Beijing, China) and ketoprofen (500 ng/mL, Sigma-Aldrich, Burlington, MA, USA) [15]. After vortexing, the mixture was centrifuged to precipitate the protein. Then, 100 μL supernatant was used for analysis in positive and negative electrospray ionization (ESI+ and ESI–) modes, respectively. Moreover, 10 μL of each serum sample was mixed to form mixed serum as the quality control (QC) sample, which was used to evaluate the stability of the detection system [16]. The QC sample and serum samples used the same treatment method. Detection of QC sample was inserted in serum samples sequence.

Metabonomics analysis

The non-targeted metabolomics was analyzed using a Dionex Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) coupled to a Q Exactive-Orbitrap high-resolution mass spectrometer (UHPLC-Q-Orbitrap HRMS) (Thermo Fisher Scientific Co., Ltd., Waltham, MA, USA). To separate the metabolites, an Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 μm) (Waters, Milford, MA, USA) was used with a temperature of 40 ˚C. Mobile phases A was 0.1% formic acid aqueous solution, and mobile phases B was acetonitrile. The elution gradient started with 5% B. After holding for 1 min, 5% B increased linearly to 100% B at 9 min. After holding for 3 min with 100% B, the elution gradient decreased to 5% among 0.1 min, then kept for 2.9 min. The injected volume was 5 μL with the flow rate of 0.35 mL/min, and the auto-sampler was conditioned at 10 ˚C.

The HRMS was equipped with a heat ESI, which operated in the full scan mode with m/z 80 to 1200. The auxiliary gas was at a flow rate of 10 psi. The temperatures of the capillary and probe heater were kept at 320 ˚C and 300 ˚C, respectively. The spray voltage in ESI+ and ESI– were 3.5 kV and 2.8 kV, the sheath gas flow velocity was 40 psi and 38 psi, respectively. The remaining variables are as previously reported [15].

Statistical analysis

A statistical comparison of clinical characteristics and metabolites of the different groups was performed using unpaired t test or Mann-Whitney U test. $P < 0.05$ was considered to represent statistical significance.

The data collected in positive and negative ion mode was processed using the Compound Discoverer software (version 3, Thermo Fisher Scientific Co., Ltd., Waltham, MA, USA). After searching the online database mzCloud, ChemSpider (Databases: BioCyc; Human Metabolome Database; Kyoto Encyclopedia of Genes and Genomes), mzCloud and mzVault, the resulting matrix data concerning metabolites was derived for subsequent analysis. Multivariate analysis including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) was analyzed using SIMCA 14.0 (Umetrics AB, Umea, Sweden). PCA was used to evaluate changes in metabolites between groups and the stability of the detection system by QC sample [17]. OPLS-DA with Pareto scaling was performed to identify significant discriminating metabolites between groups based on their variable importance in the projection (VIP) [15], and 200 permutation tests were conducted to evaluate the risk of over-fitting the model. The differential metabolites ($P < 0.05$ and VIP > 1) were identified by searching the Human Metabolome Database (https://hmdb.ca/) to match the MS/MS fragment ions with reference substances [18]. The enrichment analysis and pathway analysis were performed by MetaboAnalyst 5.0 Web service (www.metaboanalyst.ca/) [19].

Results

Participants

According to the diagnostic criteria and exclusion criteria, 103 T2D patients were included in this study, of which 52 T2D patients were from Beijing Jiaotong University Community Health Center ($T2D_B$) and 51 T2D patients were from Kaifeng Hospital of Traditional Chinese Medicine ($T2D_K$). By controlling the matched variables, there were no differences in age, FBG, HbA1c, BMI, triglyceride, total cholesterol, low-density lipoprotein cholesterol, alanine aminotransferase, and aspartate transaminase between $T2D_B$ and $T2D_K$ patients (Table 1). Although the concentrations of high-density lipoprotein cholesterol and Creatinine in $T2D_B$ patients were significantly higher than that of $T2D_K$ patients, they were within the

normal range, which did not affect the metabolism of patients. According to the results, the liver function and renal function of patients in the two groups were normal. A total of 103 serum samples were analyzed by UHPLC-Q-Orbitrap HRMS.

Quality control evaluation

A brief scheme of the analysis method is shown in Figure 1. In process of Compound Discoverer software, were after aligned and normalized with 12 QC samples, the peak in the analytical samples with missing value was filled with the detection rate of > 50%. After excluded peaks with coefficient of variation in the QC samples of more than 30%, there were 2193 and 1236 ion peaks captured in ESI+ and ESI–, respectively. Further, the detection system was stable and the data was credible, as the QC samples gathered together in ESI+ and ESI– (Figure 2A, 2B).

Table 1 Clinical characteristics of study participants

	$T2D_B$ subjects (N = 51)	$T2D_K$ subjects (N = 52)	*P* value
Females, N (%)	24 (46.15%)	21 (41.18%)	-
Age (years)	50.35 ± 9.87	48.86 ± 10.37	0.5035
Fasting blood glucose (mmol/L)	9.8 ± 3.09	10.09 ± 2.06	0.0784
Hemoglobin A1c (%)	7.24 ± 1.11	7.36 ± 1.48	0.9869
Body mass index (kg/m^2)	25.93 ± 3.91	26.29 ± 2.89	0.2374
Low-density lipoprotein cholesterol (mmol/L)	3.06 ± 0.83	3.15 ± 0.87	0.3362
High-density lipoprotein cholesterol (mmol/L)	1.42 ± 0.4	1.23 ± 0.37	0.0007
Triglyceride (mmol/L)	2.32 ± 1.48	2.42 ± 2.06	0.4606
Total cholesterol (mmol/L)	5.18 ± 1.04	5.11 ± 1.26	0.7799
Alanine transaminase (U/L)	29.02 ± 17.47	29.06 ± 16.23	0.7562
Aspartate transaminase (U/L)	21.77 ± 8.54	24.51 ± 9.39	0.0815
γ-Glutamyl transpeptidase (U/L)	32.35 ± 16.04	29.98 ± 21.21	0.1410
Creatinine (μmol/L)	65.9 ± 14.68	51.17 ± 11.99	< 0.0001

Data presented as mean ± SD, unless otherwise indicated. The differences in groups were analyzed using unpaired *t* test or Mann-Whitney U test. $T2D_B$, type 2 diabetes patients from Beijing; $T2D_K$, type 2 diabetes patients from Kaifeng; SD, standard deviation.

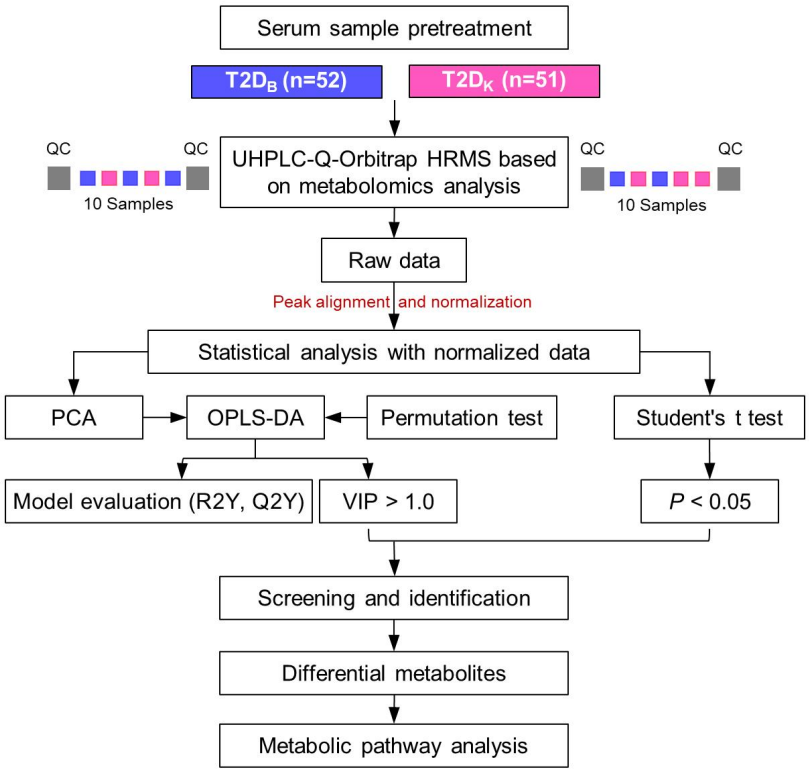

Figure 1 Scheme of analytical method. $T2D_B$, type 2 diabetes patients from Beijing; $T2D_K$, type 2 diabetes patients from Kaifeng; QC, quality control; UHPLC-Q-Orbitrap HRMS, ultra-high performance liquid chromatography coupled to a Q Exactive-Orbitrap high-resolution mass spectrometer; VIP, variable importance in the projection; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis.

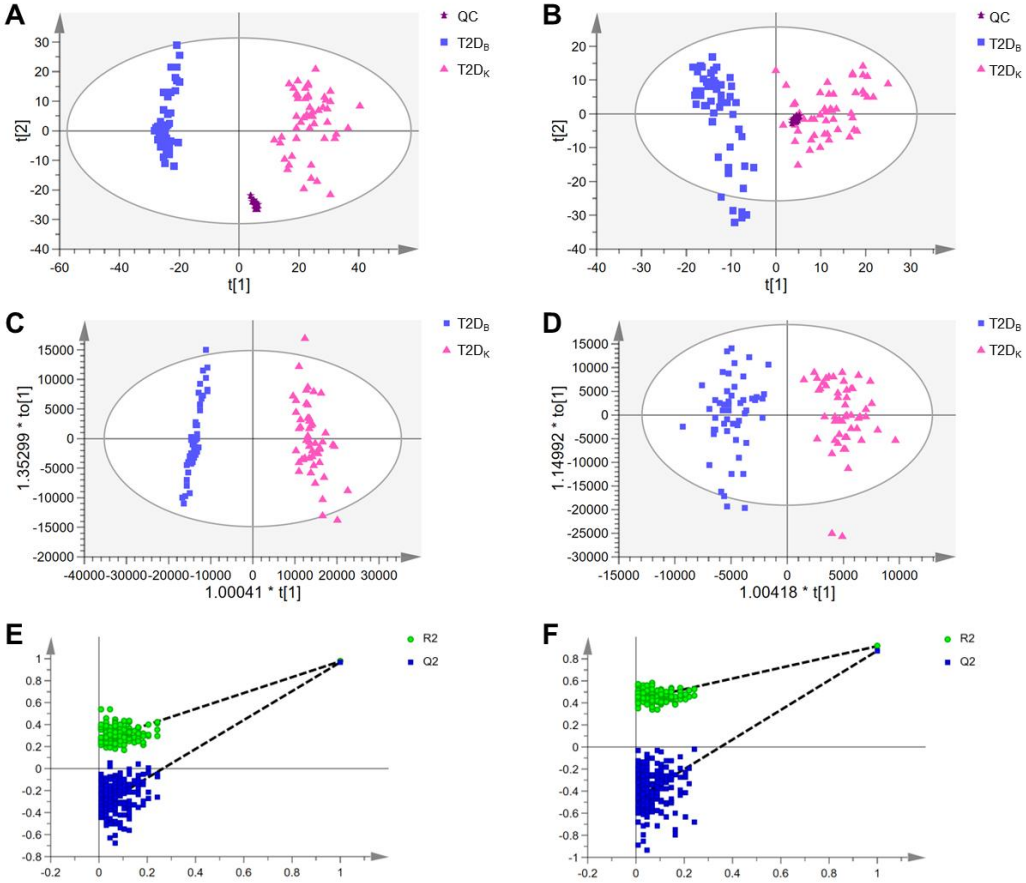

Figure 2 Metabonomics characteristics. (A & B) The PCA score plot in positive and negative ion modes. (C & D) The OPLS-DA score plot in positive and negative ion modes. The interpretation rate (R2Y) of the OPLS-DA model in positive ion mode is 0.977, and the prediction rate (Q2) is 0.968; in negative ion mode, the interpretation rate (R2Y) of the OPLS-DA model is 0.915, and the prediction rate (Q2) is 0.873. (E & F) Cross-validation plot with a permutation test repeated 200 times in positive and negative ion modes. In positive ion mode, intercepts: R2 = (0, 0.254), Q2 = (0, –0.347); in negative ion mode, intercepts: R2 = (0, 0.426), Q2 = (0, –0.469). PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; $T2D_B$, type 2 diabetes patients from Beijing; $T2D_K$, type 2 diabetes patients from Kaifeng.

Identification of differential metabolites

For multivariate analysis, the results from PCA showed that there were systematic differences between $T2D_B$ and $T2D_K$ patients (Figure 2A, 2B). As shown in Figure 2C and Figure 2D, the results from OPLS-DA suggested an apparent separations between $T2D_B$ and $T2D_K$ groups. Moreover, the results from 200 times permutation indicated that the OPLS-DA model had not been fitted (Figure 2E, 2F). Further, the VIP of metabolites based on the OPLS-DA model was used to screen differential metabolites.

After univariate analysis and multivariate analysis, the differential metabolites ($P < 0.05$ and VIP > 1) were screened. By matching the MS/MS fragment ions with reference substances, we identified 36 differential metabolites in the serum of $T2D_B$ and $T2D_K$ patients, in which 16 metabolites were higher concentrations while 20 metabolites were lower concentrations in the serum of $T2D_B$ than $T2D_K$. Particularly, the concentrations of decanoylcarnitine, pyroglutamic acid, 9-decenoylcarnitine, 4-methyl-2-oxovaleric acid, L-lactic acid, glycoursodeoxycholic acid, thiomorpholine 3-carboxylate, 3,4,5-trimethoxyhydrocinnamic acid, 2-hydroxy-L-methionine and 3-oxopentanoic acid in the serum of $T2D_B$ patients were 35.03, 12.53, 6.19, 4.71, 3.43, 3.35, 2.78, 2.61, 2.54 and 2.18 times higher than those in $T2D_K$ patients, respectively. While, the concentrations of monomenthyl succinate, camphoric acid, 1-phenylethylamine, 16-hydroxyhexadecanoic acid, 3-hydroxybutyric acid, 3-methyl-2-oxovaleric acid, 4-oxopentanoic acid, arabinosylhypoxanthine, oleic acid, arachidonic acid, diethylpyrocarbonate and threonic acid in the serum of $T2D_B$ patients were 0.02, 0.02, 0.45, 0.56, 0.59, 0.61, 0.62, 0.65, 0.66, 0.68, 0.7 and 0.77 times lower than those in $T2D_K$ patients, respectively (Table 2). Amino acid metabolism and lipid metabolism of diabetic patients in the two centers were different. The serum concentrations of hexose, L-valine, L-isoleucine, L-arginine, and citric acid in $T2D_B$ patients were lower than those in $T2D_K$ patients (Figure 3A–3C). Contrary, the serum concentrations of L-phenylalanine, hippuric acid, and L-carnitine in $T2D_B$ patients were higher than those in $T2D_K$ patients (Figure 3D–3F). The serum concentrations of lysophosphatidylcholine (LysoPC) (18:0), LysoPC (20:4), and lysophosphatidylethanolamine (LysoPE) (20:4) in $T2D_B$ patients were significantly lower than those in $T2D_K$ patients (Figure 3G–3I). Moreover, the serum concentrations of choline, sphinganine, and uric acid in $T2D_B$ patients were higher than those in $T2D_K$ patients (Figure 3J–3L).

Pathway analysis

To explore the differences in metabolic pathways in T2D patients from regions, we performed the enrichment analysis and pathway analysis. The results showed that phosphatidylethanolamine biosynthesis, phosphatidylcholine biosynthesis, valine, leucine, and isoleucine degradation, and beta-oxidation of very long chain fatty acids were obviously different in $T2D_B$ patients and in $T2D_K$ patients (Figure 4). In addition, biosynthesis of phenylalanine, tyrosine and tryptophan, arachidonic acid metabolism, and sphingolipid metabolism were different between $T2D_B$ patients and $T2D_K$ patients (Figure 5).

Table 2 Differential metabolites in type 2 diabetes patients from regions

No.	ESI mode	RT (min)	*m/z*	Formula	BMDB ID	Metabolites	VIP	Fold change[#]	*P* value
1	+	0.823	174.11145	$C_6H_{14}N_4O_2$	HMDB0000517	L-Arginine	1.07	0.86	2.87E-04
2	+	0.870	161.10495	$C_7H_{15}NO_3$	HMDB0000062	L-Carnitine	2.08	1.11	5.73E-03
3	+	0.901	129.04323	$C_5H_7NO_3$	HMDB0000267	Pyroglutamic acid	1.20	12.53	1.42E-15
4	–	0.912	136.03687	$C_4H_8O_5$	HMDB0000943	Threonic acid	1.04	0.77	2.23E-06
5	–	0.939	168.02799	$C_5H_4N_4O_3$	HMDB0000289	Uric acid	4.14	1.22	3.41E-04
6	–	0.948	162.05255	$C_6H_{10}O_5$	HMDB0032873	Diethylpyrocarbonate	1.57	0.70	6.98E-10
7	–	0.996	180.06305	$C_6H_{12}O_6$	HMDB0242534	Hexose	11.09	0.82	4.04E-05
8	–	1.113	90.03146	$C_3H_6O_3$	HMDB0000190	L-Lactic acid	8.95	3.43	2.13E-10
9	+	1.116	117.07872	$C_5H_{11}NO_2$	HMDB0000883	L-Valine	2.09	0.80	2.77E-08
10	+	1.118	147.03521	$C_5H_9NO_2S$	HMDB0059611	Thiomorpholine3-carboxylate	1.06	2.78	6.21E-22
11	+	1.119	165.04581	$C_5H_{11}NO_3S$	HMDB0246915	2-Hydroxy-L-methionine	1.02	2.54	4.30E-20
12	–	1.174	192.02672	$C_6H_8O_7$	HMDB0000094	Citric acid	1.39	0.82	1.04E-02
13	–	1.222	268.08045	$C_{10}H_{12}N_4O_5$	HMDB0003040	Arabinosylhypoxanthine	1.06	0.65	1.07E-04
14	+	1.280	131.09447	$C_6H_{13}NO_2$	HMDB0000172	L-Isoleucine	4.28	0.85	8.41E-06
15	–	1.540	104.04712	$C_4H_8O_3$	HMDB0000357	3-Hydroxybutyric acid	3.81	0.59	1.59E-07
16	–	1.576	116.04699	$C_5H_8O_3$	HMDB0245963	3-Oxopentanoic acid	1.15	2.18	2.10E-05
17	+	1.710	165.07881	$C_9H_{11}NO_2$	HMDB0000159	L-Phenylalanine	1.66	1.27	1.04E-02
18	–	1.995	116.04706	$C_5H_8O_3$	HMDB0000720	4-Oxopentanoic acid	2.51	0.62	2.00E-07
19	–	3.368	130.06266	$C_6H_{10}O_3$	HMDB0000695	4-Methyl-2-oxovaleric acid	5.79	4.71	2.58E-09
20	–	3.709	179.05794	$C_9H_9NO_3$	HMDB0000714	Hippuric acid	1.22	1.65	4.63E-02
21	–	3.741	130.06274	$C_6H_{10}O_3$	HMDB0000491	3-Methyl-2-oxovaleric acid	7.06	0.61	9.11E-07
22	+	5.682	313.22489	$C_{17}H_{31}NO_4$	HMDB0013205	9-Decenoylcarnitine	1.21	6.19	1.96E-14
23	+	6.003	315.24058	$C_{17}H_{33}NO_4$	HMDB0000651	Decanoylcarnitine	1.46	35.03	2.44E-17
24	+	6.007	240.09932	$C_{12}H_{16}O_5$	HMDB0030254	3,4,5-trimethoxyhydrocinnamic acid	1.11	2.61	4.32E-05
25	+	6.131	200.10445	$C_{10}H_{16}O_4$	HMDB0034491	Camphoric acid	1.00	0.02	5.69E-65
26	–	6.848	449.31381	$C_{26}H_{43}NO_5$	HMDB0000708	Glycoursodeoxycholic acid	1.07	3.35	4.21E-04
27	+	7.062	301.2975	$C_{18}H_{39}NO_2$	HMDB0000269	Sphinganine	1.30	1.29	2.53E-10
28	+	8.028	543.33115	$C_{28}H_{50}NO_7P$	HMDB0010395	LysoPC (20:4)	1.16	0.85	1.70E-02
29	–	8.048	501.28515	$C_{25}H_{44}NO_7P$	HMDB0011517	LysoPE (20:4)	1.30	0.80	9.90E-05
30	+	8.558	103.09951	$C_5H_{13}NO$	HMDB0000097	Choline	1.22	1.63	6.57E-15
31	+	8.594	256.16677	$C_{14}H_{24}O_4$	HMDB0036143	Monomenthyl succinate	2.78	0.02	3.05E-55
32	+	9.277	523.36302	$C_{26}H_{54}NO_7P$	HMDB0010384	LysoPC (18:0)	1.29	0.87	7.52E-03
33	–	9.619	272.23479	$C_{16}H_{32}O_3$	HMDB0006294	16-Hydroxyhexadecanoic acid	1.01	0.56	1.61E-16
34	–	9.761	304.23977	$C_{20}H_{32}O_2$	HMDB0001043	Arachidonic acid	1.30	0.68	1.59E-04
35	–	10.389	282.25548	$C_{18}H_{34}O_2$	HMDB0000207	Oleic acid	1.13	0.66	2.46E-04
36	+	10.898	121.08874	$C_8H_{11}N$	HMDB0002017	1-Phenylethylamine	1.06	0.45	1.36E-10

Fold change[#] indicates the fold change value of $T2D_B$ group compared with $T2D_K$ group. ESI, electrospray ionization; RT, retention time; VIP, variable importance in the projection; $T2D_B$, type 2 diabetes patients from Beijing; $T2D_K$, type 2 diabetes patients from Kaifeng; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine.

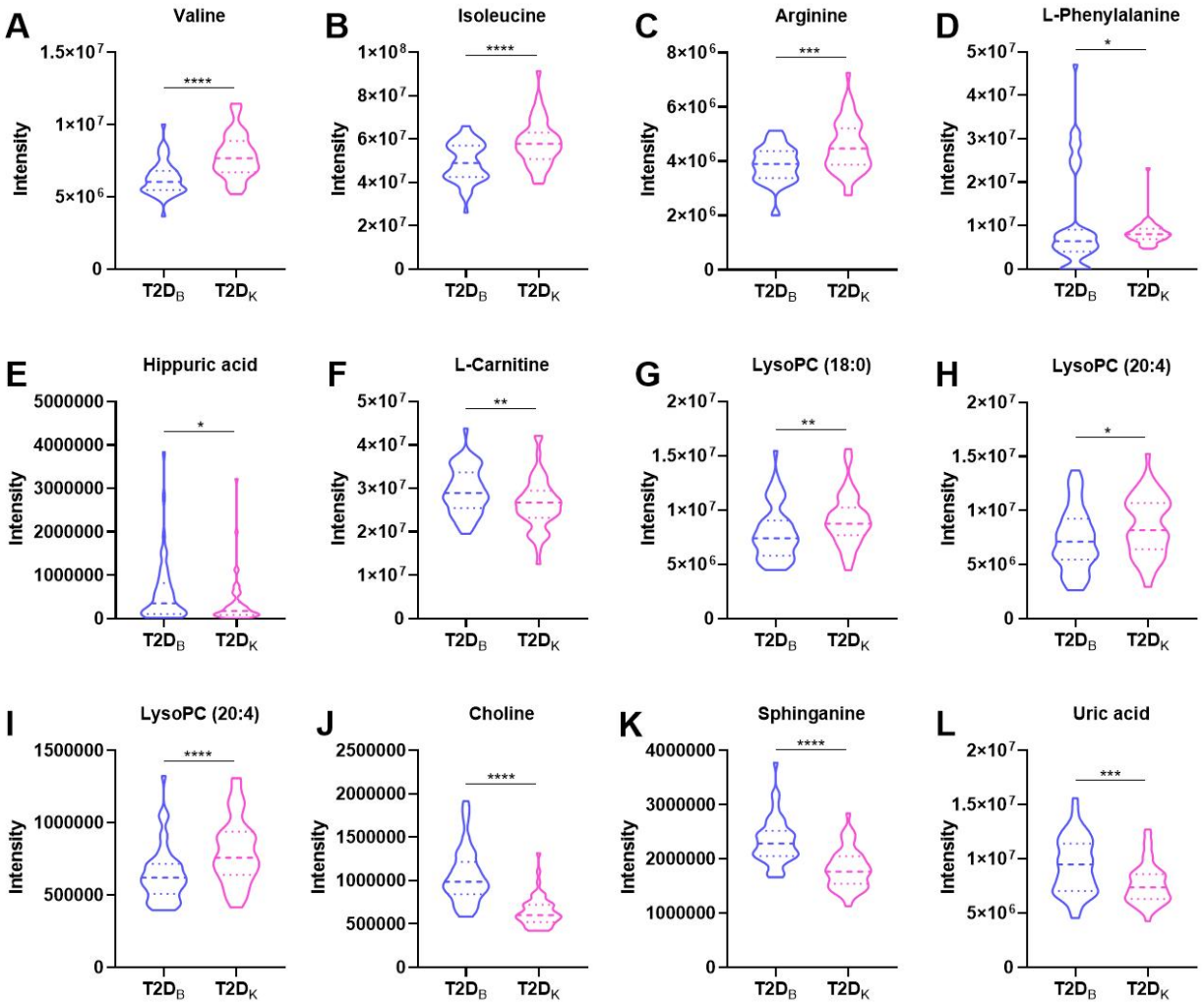

Figure 3 Differential metabolites. The intensity of differential metabolites (A) L-valine, (B) L-isoleucine, (C) L-arginine, (D) L-phenylalanine, (E) hippuric acid, (F) L-carnitine, (G) LysoPC (18:0), (H) LysoPC (20:4), (I) LysoPE (20:4), (J) choline, (K) sphinganine and (L) uric acid. The differences in groups were analyzed using unpaired *t* test or Mann-Whitney U test. LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; $T2D_B$, type 2 diabetes patients from Beijing; $T2D_K$, type 2 diabetes patients from Kaifeng.

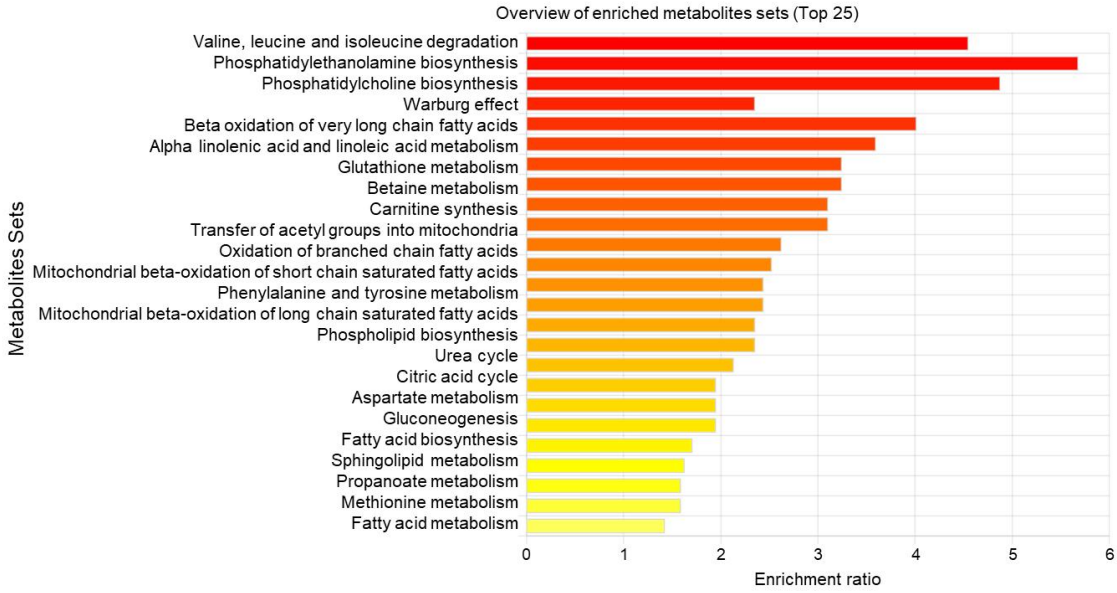

Figure 4 The enrichment analysis of differential metabolites

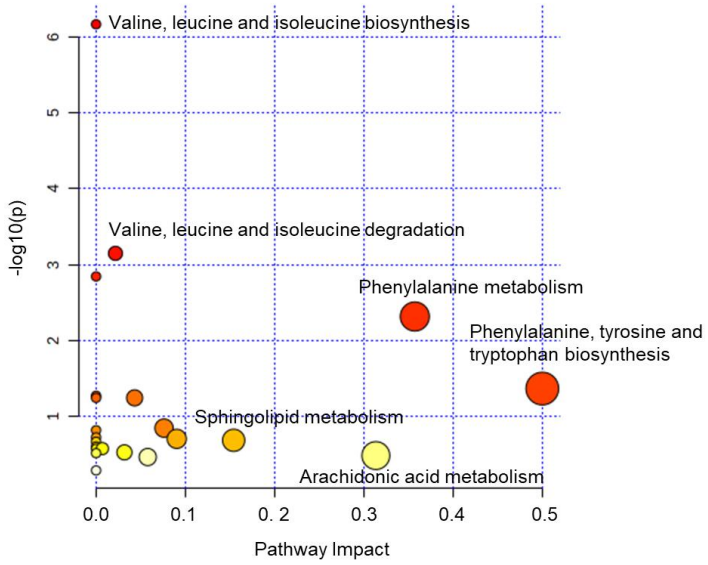

Figure 5 The pathway analysis of differential metabolites

Discussion

It was a sectional study in two regions of China of diverse circulating metabolites associated with T2D. The results showed that metabolites across varied pathways were independently related to regions. At present, a large number of metabolites were found to be independently related to the risk of T2D. However, there were few metabolites with clinical application value. The possible reason was that metabolites change inconsistently in different centers.

We all know that region, race, and diet have effects on metabolism. However, where are the effects of non-specific diets and lifestyles from different regions on the metabolism of T2D patients? The results of UHPLC-Q-Orbitrap HRMS analysis showed that amino acids, fatty acids, carnitines, and lysophospholipids profile were associated with regions, including higher concentrations of L-phenylalanine, pyroglutamic acid, 2-hydroxy-L-methionine, hippuric acid, 4-methyl-2-oxovaleric acid, decanoylcarnitine, 9-decenoylcarnitine, L-carnitine and L-lactic acid, while lower concentrations of L-valine, L-isoleucine, L-arginine, 3-methyl-2-oxovaleric acid, 4-oxopentanoic acid, 3-hydroxybutyric acid, arachidonic acid, oleic acid, 16-hydroxyhexadecanoic acid, LysoPC (18:0), LysoPC (20:4) and LysoPE (20:4) in serum of $T2D_B$ patients in the current study, which were reported to be closely related to T2D risk [20, 21]. Obviously, the current concentrations were evidently more extreme than those previously observed. For example, the concentrations of decanoylcarnitine and pyroglutamic acid in $T2D_B$ patients were up to 35-fold and 12.5-fold higher than $T2D_K$ patients (Table 2). The previous study showed that decanoylcarnitine was a risk factor for cardiovascular disease in T2D [22]. It was reported that the concentration of pyroglutamic acid was decreased in T2D [23, 24], and it was proved that pyroglutamic acid had an anti-diabetic effect [25]. However, there was a great difference between decanoylcarnitine and pyroglutamic acid in T2D from regions.

Carnitine is one of the key substances in the beta-oxidation of long-chain fatty acids [26]. There was higher concentration of L-carnitine while lower 16-hydroxyhexadecanoic acid, oleic acid, and arachidonic acid in $T2D_B$ patients than $T2D_K$ patients, which was suggestive of there was lower beta-oxidation of very long chain fatty acids in $T2D_B$ patients than $T2D_K$ patients [27]. Arachidonic acid is a strong inducer of insulin secretion, which was also decreased in $T2D_B$ patients, suggesting that there was lower insulin sensitivity in $T2D_B$ patients than in $T2D_K$ patients [28, 29]. As arachidonic acid upstream metabolites [30], the serum concentrations of LysoPC (18:0), LysoPC (20:4), and LysoPE (20:4) also were decreased in $T2D_B$ patients. These results proved that phosphatidylcholine biosynthesis in T2D patients from regions was different (Figure 4).

From the diverse metabolites associated with T2D, branched-chain amino acids (including valine and isoleucine) were the strongest association observed [21, 31], as it was proved that higher branched-chain amino acid concentrations resulted from insulin resistance, causing T2D [32, 33]. In this study, the concentrations of L-valine, L-isoleucine, and 3-methyl-2-oxovaleric acid in $T2D_B$ patients were lower than in $T2D_K$ patients, the reason for the differences might be that valine, leucine, and isoleucine degradation in $T2D_B$ patients were lower than $T2D_K$ patients (Figure 4). Further, phenylalanine, tyrosine and tryptophan biosynthesis in $T2D_B$ patients might be lower than that of $T2D_K$ patients (Figure 5), resulting in the lower concentration of L-phenylalanine in $T2D_B$ patients.

To sum up, because of the great differences in metabolites caused by different regions, it is best to use metabolites as biomarkers for the diagnosis and treatment of T2D by screening in a single center before multi-center validation. Further targeted quantification of the biomarkers would be a great choice [24]. To avoid the judgment of the overall diagnosis affected by the bias of single or isolated biomarker, the use of biomarkers should be constructed to the integrated biomarker profiling of disease that could objectively reflect the metabolite profile of the disease [15, 24].

Our study is one of the few investigating the independent impact of different regions on associations of T2D with metabolite, identified 36 differential metabolites using UHPLC-Q-Orbitrap HRMS. Nonetheless, the study has several limitations, including limited sample size and case-sectional design. It should be taken into consideration that the evaluation of harder end points for the application of metabolic markers requires even larger and prospective data sets. Compensately, to identify the metabolites, high sensitivity, and high-resolution mass spectrometry platform was used based on metabolomics analysis. In addition, lack of information on socioeconomic status, diet, smoking habits and education impeded the investigation of potential confounding variables. Therefore, in order to reduce the influence of interference factors, we controlled matching variables, such as gender, age, BMI, complications, liver function, renal function, etc.

Conclusion

After UHPLC-Q-Orbitrap HRMS analysis, there are 36 differential metabolites identified in the serum of $T2D_B$ and $T2D_K$ patients. The

reason for the differences may be that phosphatidylethanolamine biosynthesis, phosphatidylcholine biosynthesis, valine, leucine, and isoleucine degradation, and beta-oxidation of very long chain fatty acids were different in $T2D_B$ patients and in $T2D_K$ patients. In conclusion, metabolites difference in T2D patients from different regions, providing valuable insight and potential for the diagnosis and treatment of T2D.

References

1. Saeedi P, Petersohn I, Salpea P, et al. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. *Diabetes Res Clin Pract.* 2019;157:107843. Available at: http://doi.org/10.1016/j.diabres.2019.107843
2. Yan F, Li N, Shi J, et al. Lactobacillus acidophilus alleviates type 2 diabetes by regulating hepatic glucose, lipid metabolism and gut microbiota in mice. *Food Funct.* 2019;10(9):5804–5815. Available at: http://doi.org/10.1039/c9fo01062a
3. Li Y, Teng D, Shi X, et al. Prevalence of diabetes recorded in mainland China using 2018 diagnostic criteria from the American Diabetes Association: national cross sectional study. *BMJ.* 2020;369:m997. Available at: http://doi.org/10.1136/bmj.m997
4. Harding JL, Pavkov ME, Magliano DJ, Shaw JE, Gregg EW. Global trends in diabetes complications: a review of current evidence. *Diabetologia.* 2018;62(1):3–16. Available at: http://doi.org/10.1007/s00125-018-4711-2
5. Sabanayagam C, Banu R, Chee ML, et al. Incidence and progression of diabetic retinopathy: a systematic review. *Lancet Diabetes Endocrinol.* 2019;7(2):140–149. Available at: http://doi.org/10.1016/s2213-8587(18)30128-1
6. Sun H, Saeedi P, Karuranga S, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract.* 2022;183:109119. Available at: http://doi.org/10.1016/j.diabres.2021.109119
7. García-Calzón S, Perfilyev A, Martinell M, et al. Epigenetic markers associated with metformin response and intolerance in drug-naïve patients with type 2 diabetes. *Sci Transl Med.* 2020;12(561):eaaz1803. Available at: http://doi.org/doi:10.1126/scitranslmed.aaz1803
8. Zhao Y, Wang M, Meng B, et al. Identification of dysregulated complement activation pathways driven by N-glycosylation alterations in T2D patients. *Front Chem.* 2021;9:677621. Available at: http://doi.org/10.3389/fchem.2021.677621
9. Wigger L, Barovic M, Brunner A-D, et al. Multi-omics profiling of living human pancreatic islet donors reveals heterogeneous beta cell trajectories towards type 2 diabetes. *Nat Metab.* 2021;3(7):1017–1031. Available at: http://doi.org/10.1038/s42255-021-00420-9
10. Folsom LJ, Hannon TS. Collaboration is key for successful treatment of youth-onset type 2 diabetes. *J Adolesc Health.* 2017;60(4):360–362. Available at: http://doi.org/10.1016/j.jadohealth.2016.12.022
11. G Duarte F, da Silva Moreira S, Almeida M da CC, et al. Sex differences and correlates of poor glycaemic control in type 2 diabetes: a cross-sectional study in Brazil and Venezuela. *BMJ Open.* 2019;9(3):e023401. Available at: http://doi.org/10.1136/bmjopen-2018-023401
12. Hu C, Jia W. Diabetes in China: epidemiology and genetic risk factors and their clinical utility in personalized medication. *Diabetes.* 2017;67(1):3–11. Available at: http://doi.org/10.2337/dbi17-0013
13. Suresh K, Chandrashekara S. Sample size estimation and power analysis for clinical research studies. *J Hum Reprod Sci.* 2012;5(1):7. Available at: http://doi.org/10.4103/0974-1208.97779
14. Li X, Sun C, Hu HX, Zhao YJ, Liu CX. The current situation and influencing factors of cognitive function of older people in 30 nursing homes. *Chin J Nurs.* 2023;58(4):446–451. Available at: https://doi.org/10.3761/j.issn.0254-1769.2023.04.009
15. Long J, Liu L, Jia Q, et al. Integrated biomarker for type 2 diabetes mellitus and impaired fasting glucose based on metabolomics analysis using ultra-high performance liquid chromatography quadrupole-Orbitrap high-resolution accurate mass spectrometry. *Rapid Commun Mass Spectrom.* 2020;34(12):e8779. Available at: http://doi.org/10.1002/rcm.8779
16. Luo P, Yin P, Hua R, et al. A Large-scale, multicenter serum metabolite biomarker identification study for the early detection of hepatocellular carcinoma. *Hepatology.* 2018;67(2):662–675. Available at: http://doi.org/10.1002/hep.29561
17. Giuliani A. The application of principal component analysis to drug discovery and biomedical data. *Drug Discov Today.* 2017;22(7):1069–1076. Available at: http://doi.org/10.1016/j.drudis.2017.01.005
18. Wishart DS, Guo A, Oler E, et al. HMDB 5.0: the Human Metabolome Database for 2022. *Nucleic Acids Res.* 2022;50(D1):D622–D631. Available at: https://doi.org/10.1093/nar/gkab1062
19. Pang Z, Zhou G, Ewald J, et al. Using MetaboAnalyst 5.0 for LC-HRMS spectra processing, multi-omics integration and covariate adjustment of global metabolomics data. *Nat Protoc.* 2022;17(8):1735–1761. Available at: https://doi.org/10.1038/s41596-022-00710-w
20. Bertran L, Capellades J, Abelló S, et al. Untargeted lipidomics analysis in women with morbid obesity and type 2 diabetes mellitus: a comprehensive study. *PLoS One.* 2024;19(5):e0303569. Available at: http://doi.org/10.1371/journal.pone.0303569
21. Neinast M, Murashige D, Arany Z. Branched chain amino acids. *Annu Rev Physiol.* 2019;81(1):139–164. Available at: http://doi.org/10.1146/annurev-physiol-020518-114455
22. Zhao S, Feng X-F, Huang T, et al. The association between acylcarnitine metabolites and cardiovascular disease in Chinese patients with type 2 diabetes mellitus. *Front Endocrinol.* 2020;11:212. Available at: http://doi.org/10.3389/fendo.2020.00212
23. Chou J, Liu R, Yu J, et al. Fasting serum α-hydroxybutyrate and pyroglutamic acid as important metabolites for detecting isolated post-challenge diabetes based on organic acid profiles. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2018;1100–1101:6–16. Available at: http://doi.org/10.1016/j.jchromb.2018.09.004
24. Long J, Yang H, Yang Z, et al. Integrated biomarker profiling of the metabolome associated with impaired fasting glucose and type 2 diabetes mellitus in large-scale Chinese patients. *Clin Transl Med.* 2021;11(6):e432. Available at: http://doi.org/10.1002/ctm2.432
25. Yoshinari O, Igarashi K. Anti-diabetic effect of pyroglutamic acid in type 2 diabetic Goto-Kakizaki rats and KK-Aymice. *Br J Nutr.* 2011;106(7):995–1004. Available at: http://doi.org/10.1017/s0007114511001279
26. Hammoud S, Ivanova A, Osaki Y, et al. Tubular CPT1A deletion minimally affects aging and chronic kidney injury. *JCI Insight.* 2024;9(6):e171961. Available at: http://doi.org/10.1172/jci.insight.171961
27. Fedorenko A, Lishko PV, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell.* 2012;151(2):400–413. Available at: http://doi.org/10.1016/j.cell.2012.09.010
28. Sonnweber T, Pizzini A, Nairz M, Weiss G, Tancevski I. Arachidonic acid metabolites in cardiovascular and metabolic

diseases. *Int J Mol Sci.* 2018;19(11):3285. Available at: http://doi.org/10.3390/ijms19113285
29. Xie P, Xie JB, Xiao MY, et al. Liver lipidomics analysis reveals the anti-obesity and lipid-lowering effects of gypnosides from heat-processed Gynostemma pentaphyllum in high-fat diet fed mice. *Phytomedicine.* 2023;115:154834. Available at: http://doi.org/10.1016/j.phymed.2023.154834
30. Hu Y, Li W, Cheng X, et al. Emerging roles and therapeutic applications of arachidonic acid pathways in cardiometabolic diseases. *Circ Res.* 2024;135(1):222–260. Available at: http://doi.org/10.1161/circresaha.124.324383
31. Guasch-Ferré M, Hruby A, Toledo E, et al. Metabolomics in prediabetes and diabetes: a systematic review and meta-analysis. *Diabetes Care.* 2016;39(5):833–846. Available at: http://doi.org/10.2337/dc15-2251
32. Mahendran Y, Jonsson A, Have CT, et al. Genetic evidence of a causal effect of insulin resistance on branched-chain amino acid levels. *Diabetologia.* 2017;60(5):873–878. Available at: http://doi.org/10.1007/s00125-017-4222-6
33. Sawicki KT, Ning H, Allen NB, et al. Longitudinal trajectories of branched chain amino acids through young adulthood and diabetes in later life. *JCI Insight.* 2024;9(11):e181901. Available at: http://doi.org/10.1172/jci.insight.181901