Diabetes, Metabolic Syndrome and Obesity

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ORIGINAL RESEARCH

Targeted Metabolomics Analysis of Serum Amino Acids in T2DM Patients

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Purpose: Amino acids are the important metabolites in the body and play a crucial role in biological processes. The purpose of this study is to provide a profile of amino acids change in the serum of T2DM patients and identify potential biomarkers.

Patients and Methods: In this study, we quantitatively determined the serum amino acid profiles of 30 T2DM patients and 30 healthy volunteers. *T* test and multivariate statistical analysis were used to identify candidate biomarkers with GraphPad Prism 9.5 software and MetaboAnalyst 5.0 on-line platform.

Results: Thirty-four amino acids were quantified, and 19 amino acid levels differed significantly between T2DM and Healthy groups. Screened by the specific screening criteria (VIP>1.0; P<0.05; FC>1.5, or FC<0.67) in MetaboAnalyst 5.0 platform, 8 amino acids were identified as potential biomarkers. Pearson rank correlation test showed 14 differential amino acids were significantly correlated with T2DM-related physiological parameters.

Conclusion: The results of this study provide theoretical basis for the subsequent development of dietary supplements for the prevention or treatment of T2DM and its complications.

Keywords: diabetes mellitus type 2, T2DM, targeted metabolomics, amino acids, biomarker

Introduction

Diabetes mellitus type 2 (T2DM) is a group of clinical syndromes caused by genetic and environmental factors and characterized by glucose metabolism disorder. According to the statistical data from the International diabetes Alliance in 2021, there are about 537 million adults with T2DM in the world, and the incidence rate of T2DM in China is as high as 10.6%. In addition, once the blood sugar is not under controlled, complications are very likely to occur, which will seriously affect the quality of life and survival time of patients. Therefore, preventing and improving the occurrence and development of T2DM is of great significance for national health and economic development.

Early people's cognition of T2DM was pancreatic islet injury, insulin deficiency or insulin resistance, and abnormal elevation of blood sugar is the main clinical diagnostic indicator at present. However, several years before the appearance of elevated blood sugar, metabolites in the human body have quietly changed. With the development of metabolomics, people have recognized a series of biomarkers related to T2DM. The discovery of these markers will help us to understand the pathogenesis of T2DM, and also provide a new direction for the prevention and treatment of T2DM. At present, researchers have found that T2DM is related with abnormal metabolism of many substances in the body, such as sugar, lipids, amino acids, etc. Especially in recent years, people found that amino acids have a strong correlation with insulin resistance and T2DM. Amino acid is the basic unit of protein and the main repository of glucose production. It also affects the secretion of insulin and glucagon. A large number of cross-sectional or prospective studies show that amino acids (such as phenylalanine, tyrosine, proline, etc.) may play an important role in the occurrence and development of type 2 diabetes,[1] and branched chain amino acids and aromatic amino acids can increase the risk of insulin resistance and type 2 diabetes.[2] Yu found that a low isoleucine (Ile) diet can improve liver and adipose tissue metabolism

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by regulating FGF21-UCP1, increase liver sensitivity to insulin and ketone production, increase energy expenditure, and inhibit obesity.[3] There are also studies that show that the accumulation of branched chain amino acids can activate mammalian mTORC1, and then affect downstream target ribosomal protein S6 kinase I (S6K1), thus affecting the body's sensitivity to insulin.[4] The researcher from Tehran University reported that glutamine can effectively combat obesity and diabetes, and its mechanism needs further study.[5]

With the development of omics technology and molecular biology in recent years, researchers have more and more clear understanding of the relationship between amino acids and T2DM, and the important role of amino acids in the occurrence and development of T2DM. However, the human body is an organic whole, different levels of circulating amino acids have different correlations with the risk of major adverse outcomes of T2DM. Therefore, it cannot be said that amino acids are adverse or beneficial factors for the occurrence and development of diabetes. In this study, targeted ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS) was used to quantify 34 types of serum Amino acids. We explored the differences of serum amino acids in healthy volunteers and T2DM patients and analyzed the relationship between amino acids and T2DM. Then, several important amino acids were screened to provide theoretical basis for the subsequent development of dietary supplements for the prevention or treatment of T2DM and its complications.

Materials and Methods

Reagents

UPLC-MS grade methanol and acetonitrile were obtained from Thermo Fisher Scientific (Fair Lawn, NJ, USA). UPLC-MS grade formic acid was purchased from Sigma-Aldrich (St. Luis, MO, USA). Ultrapure water (18.2 MΩ) was produced by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Analyte standards were purchased from Sigma-Aldrich (Gillingham, U.K.). Isotopically labeled amino acids for use as internal standards (IS) were from Cambridge Isotope Laboratories (MA, USA) or QMX Laboratories (Essex, U.K.), and the AccQTag Ultra reagent from Waters Corporation (Milford, MA, U.S.A.).

Human Serum Samples

Thirty T2DM patients (defined as FBG≥6.1 mmol/L) and 30 matched healthy individuals without chronic disease were included in our experiment, and we obtained blood from the vein for amino acids assay. All participants were from Beijing Shijitan Hospital, Capital Medical University (Beijing, China), who provided written informed consent and detail information including age, sex and clinical parameters. The study was approved by the Ethics Committee of Beijing Shijitan Hospital, Capital Medical University (permission No. sjtkyll-lx-2021(4)) and registered on the Chinese Clinical Trial Registry (ChiCTR2100042049), as well as conducted under the guidelines of the Helsinki Declaration.

Sample Collection and Pretreatment

To avoid any disturbance caused by diet, venous blood samples were taken at 6:30–7:30 AM after an overnight fast. After collection, the samples were centrifuged at 3000 rpm at 4°C for 10 min to obtain serum, which was stored in a refrigerator at −80°C before analysis.

Standard Curve, Quality Control, and the Preparation of Isotope Internal Standards

The amino acids were diluted 1:1 with acetonitrile aqueous solution to a mixed standard concentration of 1, 2, 4, 10, 20, 40, 100, 200, and 400 μM, respectively, or diluted to the desired concentration on its own as needed. Standard solutions were prepared at concentrations of 3, 30, and 300 μM for use as control samples. A Cambridge isotope internal standard was prepared in a volume of 1 mL (1:1 acetonitrile in water for complete dissolution) and diluted prior to use.

Sample Pre-Treated Method

In this study, 10 μL of each serum sample in the 1.5 mL Eppendorf tube was added to 10 μL of water and 5 μL of the internal standard (10 μg/mL), after mixed well, 40 μL of iso-propanol was added, and then shaken for 10 min to precipitate the protein. After centrifuged 12,000 rpm for 15min, 10 μL of the supernatant was placed in another 1.5 mL

Eppendorf tube for derivatization. About 1mL acetonitrile was added to the AccQTag Ultra reagent powder, vortex mixed, and dissolved by heating at 55 °C (less than 15 min) to get the derivated reagent solution. Then, 70 μL of borate buffer (pH8.6) and 20 μL of the prepared derivated reagent solution were added into the 10 μL sample supernatant, and shook rapidly for 10s, then heated in a heating device at 55 °C for 10 min. Upon completion of derivatization, the sample was diluted with 900 μL of water and injected for analysis.

Chromatographic and Mass Spectrometric Conditions

Chromatographic conditions: Chromatography was carried out with an ACQUITY UPLC HSS T3 column (150 mm × 2.1 mm, 1.8 μm) (Waters, USA) with the following conditions: column temperature, 55 °C; injector temperature, 4 °C; injection volume, 2 μL; mobile phase A, 0.1% (volume) aqueous formic acid; mobile phase B, 0.1% (volume) formic acid in acetonitrile; flow rate, 0.6 mL/min; gradient elution procedure, 0 ~ 0.5 min, 4% B; 0.5 ~ 2.5 min, 10% B; 2.5 ~ 5.0 min, 10% B ~ 28% B; 5.1 ~ 6.1 min, 95% B ~ 95% B; 6.2 ~ 7.5 min, 4% B.

Mass spectrometric conditions were as follows: capillary voltage, 1.5 kV; source offset, 50 V; desolvation temperature, 600 °C; source temperature, 150 °C, desolvation gas flow, 1000 L/h; cone gas flow, 150 L/h; nebulizer gas, 7.0 bar; collision gas, 0.15 mL/min.

Data Analysis

Raw data were collected using a Waters Masslynx data processing workstation (Waters Co., Ltd.). The concentration of each metabolite was then calculated by reference to a standard curve. The difference in the target substance concentration between samples was compared by the univariate *t*-test with GraphPad Prism 9.5 software (GraphPad Software, USA) and MetaboAnalyst 5.0 on-line platform.

Results

Study Population

Based on their Fasting plasma glucose (FBG) level, we collected 30 serum samples from T2DM patients, and 30 matched healthy volunteers were selected from the participants without cardiovascular disease or other chronic disease. The clinical parameters included HbA1c (glycated haemoglobin), BMI (body mass index), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) were recorded. There were no statistically significant differences in age between 2 groups, and detailed characteristics are provided in Table 1.

Table 1 Baseline Characteristics of Healthy Controls and T2DM Patients

	Health Group (n=30)	T2DM Group (n=30)
Age (years)	48.53±12.29	54.37±13.04
Sex (%Male)	53.00% (16/30)	60.00% (18/30)
Course of disease (year)	–	10.6±7.54
BMI (Kg/m^2)	21.76±1.14	25.42±4.81***
Height (cm)	166.8±8.86	167.57±10.81
Weight (kg)	60.78±7.73	71.63±19.41*
Fasting plasma glucose (mmol/L)	5.29±0.35	8.07±3.32***
2h-post load glucose (mmol/L)	5.44±0.29	16.67±4.85***
HbA1c	–	9.21±2.27
Total cholesterol (mmol/L)	3.79±0.54	4.89±1.24***
HDL-cholesterol (mmol/L)	1.71±0.28	1.05±0.22***
LDL-cholesterol (mmol/L)	2.24±0.68	2.81±0.76**
Triglyceride (mmol/L)	1.01±0.25	2.04±1.16***

Notes: All data were presented with Means±SEM, *P<0.05, **P<0.01, ***P<0.001 compared with Health group.

Targeted Metabolomics Analysis of Amino Acids in Healthy Volunteers and T2DM Patients

We analyzed the serum amino acid concentration of healthy volunteers and T2DM patients using derivated method with 6-Aminoquinolyl-n-hydroxysuccinimidyl carbonate, and we can find the UPLC-MS spectra in Figure 1. Here, we quantitatively analyzed 34 amino acids (MS parameters were in Table S1), including 8 essential amino acids and other amino acids that play an important role in protein synthesis.

Screening and Identification of Differential Metabolites

The UPLC-MS data were all imported into Metaboanalyst 5.0 on-line platform for further analysis, as shown in the PCA score chart and heatmap (Figure 2A and B), serum samples of healthy volunteers and T2DM patients were distributed in different areas due to the different physiological states, which indicated that there were differences in the composition and concentration of amino acids. And then we obtained the variable importance in the projection (VIP) value (Figure S1), the P value and fold change (FC) value that were used to identify differential metabolites (Figure S2 and 3), according to the specific screening

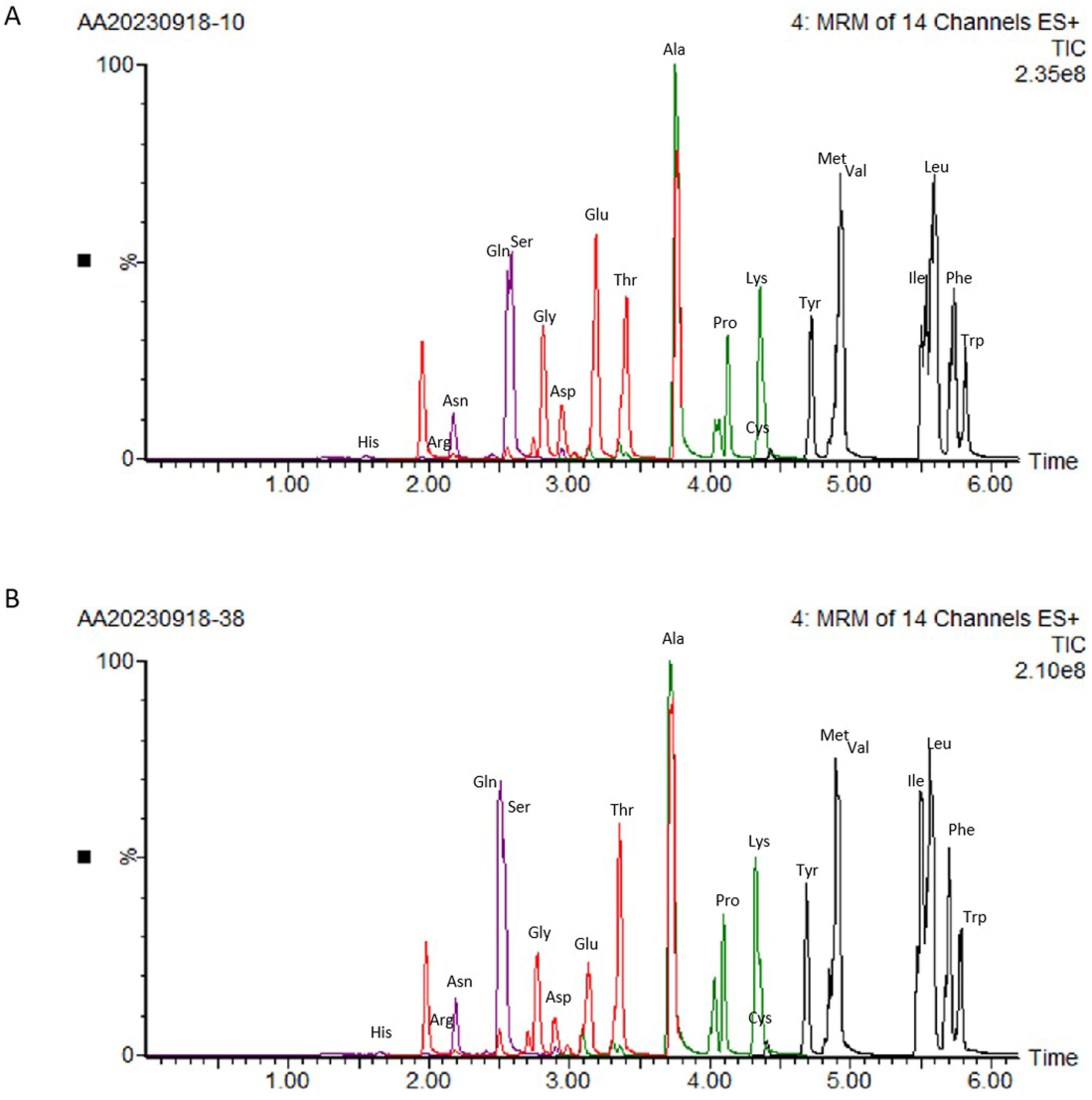

Figure 1 Representative MRM chromatography from amino acid quantification of Healthy group (**A**) and T2DM group (**B**).

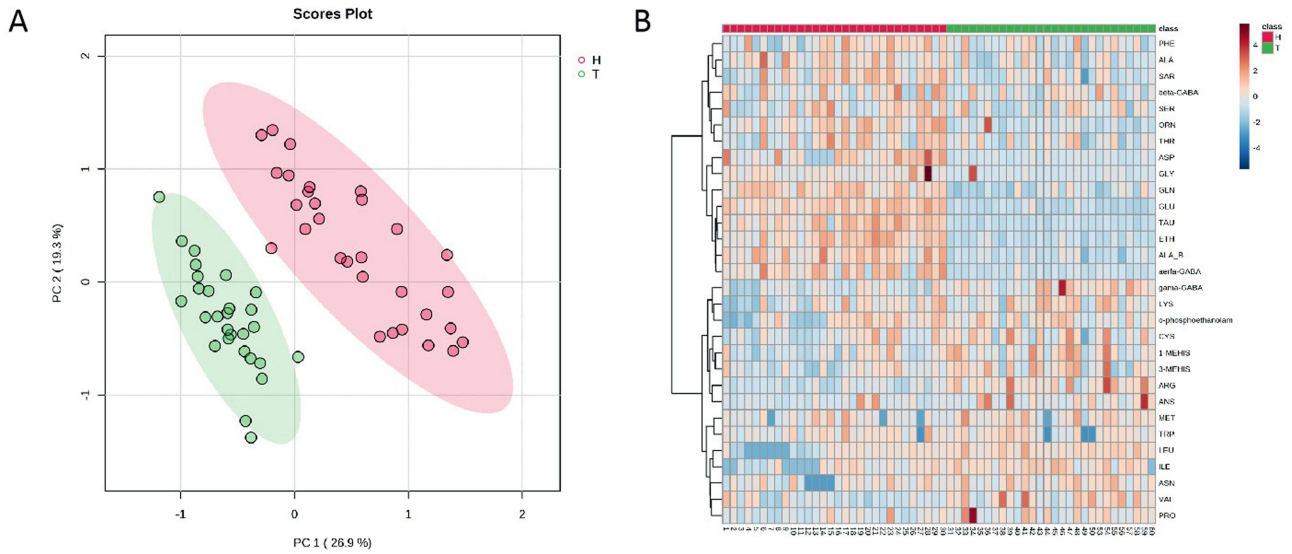

Figure 2 (**A**) PCA score plots (n = 30) of the healthy group and T2DM group. (**B**) Heat maps of normalized amino acids concentrations in serum sample. Columns represent the samples (Healthy and T2DM groups), and rows represent amino acids.

criteria (VIP>1.0; P<0.05; FC>1.5, or FC<0.67), 2 amino acids were significantly increased in T2DM patients (ARG, LEU), and 6 metabolites were significantly decreased in T2DM patients (TAU, GLU, ETH, β-ALA, α-GABA, GLN).

Quantitative Analysis of Amino Acid Concentration

A total of 34 amino acids were detected and quantified in this study, the standard curves were found to be linear over the range measured 1–100, 200 or 400uM, with correlation coefficients (R2) of 0.980 or better (Table 2). In Table 3, we obtained the concentration of 34 amino acids in serum of healthy volunteers and T2DM patients according to the linear equation. Firstly, we observed three of the eight essential amino acids (VAL, ILE, LEU) were significantly increased in T2DM group compared with healthy group (228.98 μmol/L vs 198.85 μmol/L, 128.00 μmol/L vs 104.36 μmol/L and

Table 2 Standard Curve Linearity Data Obtained for the Analyses

Amino Acid	Abbreviation	Linear Equation	r^2	Linearity Range (μmol/L)
Threonine	THR	Y=9189.81+9722.12	0.996720	1–400
Leucine	LEU	Y=0.0271421X+0.0136813	0.996680	1–100
Isoleucine	ILE	Y=25807.5931x+2085.8635	0.996254	1–100
Valine	VAL	Y=0.0152271X+0.00292939	0.997734	1–200
Phenylalanine	PHE	Y=0.0388753X+0.00787353	0.990535	1–100
Methionine	MET	Y=0.130294X+0.0220339	0.991864	1–200
Tryptophan	TRP	Y=0.132323X+0.0249838	0.992632	1–100
Lysine	LYS	Y=0.0881003X+0.0275966	0.994684	1–200
δ-Hydroxylysine	OH-LYS	Y=5395.27X+794.596	0.996051	1–100
Histidine	HIS	Y=471.025X-27.9178	0.987669	1–400
1-Methylhistidine	1-MEHIS	Y=298.254X-52.7013	0.985188	1–400
3-Methylhistidine	3-MEHIS	Y=218.769X-35.5509	0.981883	1–400
Glycine	GLY	Y=0.00250766X+0.144617	0.991037	2–200
Sarcosine	SAR	Y=17037.8X+10485	0.994777	1–100
Tyrosine	TYR	Y=0.100472X+0.0230359	0.995043	1–100

(*Continued*)

Table 2 (Continued).

Amino Acid	Abbreviation	Linear Equation	r^2	Linearity Range (μmol/L)
β-Alanine	ALA_B	Y=2700.95X+7954.14	0.989546	1–100
α-Alanine	ALA	Y=0.00126224X+0.000715378	0.994383	1–200
Cysteine	CYS	Y=3843.73X+1982.53	0.990827	1–400
Aspartic acid	ASP	Y=0.0654146X+0.0304666	0.990312	1–200
Proline	PRO	Y=0.00709628X+0.00134925	0.998382	1–200
Serine	SER	Y=0.0309731X+0.0540789	0.984065	1–200
Glutamic acid	GLU	Y=0.0162808X+0.00381015	0.991416	1–200
Arginine	ARG	Y=224.395X-14.4734	0.984827	1–400
Citrulline	CIT	Y=3457.03X+2110.79	0.980604	1–200
Ornithine	ORN	Y=2182.65+430.424	0.994599	1–100
Asparagine	ASN	Y=0.0216939X+0.0201025	0.993572	1–200
Glutamine	GLN	Y=5534.14X-2200.6258	0.996794	1–400
α-Aminobutyric acid	α-GABA	Y=24399.5X+4298.92	0.992687	1–100
β-Aminobutyric acid	β-GABA	Y=27933.8X+2842.25	0.989107	1–100
γ-Aminobutyric acid	γ-GABA	Y=23135.9X+3004.45	0.992405	1–100
Anserine	ANS	Y=460.044X-12.3337	0.980254	1–200
Taurine	TAU	Y=48.1016X-14.2997	0.996904	1–400
Ethanolamine	ETH	Y=8821.76X+6399.15	0.983612	1–200
α-Aminoadipic acid	AMADP	Y=16775.6X+3103.56	0.986693	1–200

Table 3 Amino Acid Concentration in Serum of Healthy Volunteers and T2DM Patients

	Amino Acid	Healthy	T2DM
1	THR	369.59±16.81	328.47± 14.60
2	LEU	50.59±2.59	63.86±1.82***
3	ILE	104.36±5.78	128.00±4.90**
4	VAL	198.85±4.32	228.98±6.68***
5	PHE	69.64±1.73	66.49±1.51
6	MET	24.61±0.95	26.72±1.16
7	TRP	53.83±2.53	50.52±3.57
8	LYS	198.80±11.04	217.76±10.27
9	OH-LYS	7.72±0.69	6.65±0.51
10	GLY	425.86±48.08	262.97±33.98**
11	TYR	66.16±2.11	62.91±3.79
12	HIS	96.62±14.29	102.75±4.17
13	1-MEHIS	3.82±0.24	4.94±0.33**
14	3-MEHIS	4.21±0.37	5.64±0.54*
15	ALA_B	21.41±0.75*	14.00±0.50***
16	ALA	668.50±28.21	537.93±26.95**
17	CYS	52.85±3.59	60.57±4.98
18	ASP	9.57±0.85	3.99±0.23***
19	PRO	206.09±10.69	257.78±19.73*
20	SER	163.42±5.33	153.32±5.02
21	GLU	90.98±2.84	37.25±2.11***
22	ARG	16.52±2.08	47.50±5.06***
23	ASN	63.03±2.42	68.81±2.47
24	GLN	837.11±21.89	537.57±28.35***

(*Continued*)

Table 3 (Continued).

	Amino Acid	Healthy	T2DM
25	α-GABA	0.81±0.06	0.16±0.03***
26	β-GABA	1.74±0.22	1.55±0.17
27	γ-GABA	27.21±1.70	39.77±2.69***
28	ANS	0.096±0.02	0.15±0.03
29	TAU	1105.616±63.42	188.80±16.46***
30	ETH	38.20±1.78	17.50±0.89***
31	CIT	38.13±2.40	36.14±2.79
32	SAR	489.45±8.74	454.24±7.55**
33	AMADP	1.04±0.12	1.31±0.13
34	ORN	1190.06±59.90	769.36±63.53***

Notes: All data were presented with Means±SEM, *P<0.05, **P<0.01, ***P<0.001 compared with Health group.

63.86 μmol/L vs 50.59 μmol/L, Figure S4 and Table 3). Meanwhile, we also observed that the other important amino acids were also changed in the state of diabetes, such as GLY, ALA, ASP, PRO, GLU, GLN and ARG (Figure S5).

According to the specific screening criteria (VIP>1.0; P<0.05; FC>1.5, or FC<0.67), 8 amino acids were screened as biomarker of T2DM, and we also obtained the quantitative results of these important amino acids in Table 3 and Figure 3, ARG and LEU were significantly increased in T2DM group (47.50 μmol/L vs 16.52 μmol/L, and 63.86 μmol/L vs 50.59 μmol/L, Figure 3A and B), and the other 6 biomarkers were significantly decreased in T2DM patients (Figure 3C–H).

Correlation of T2DM-Related Amino Acids and Physiological Parameters

To explore the association between the amino acids and physiological parameters, we performed a Pearson rank correlation test. Totally, 14 differential amino acids were significantly correlated with at least 1 of the diabetes-related physiological

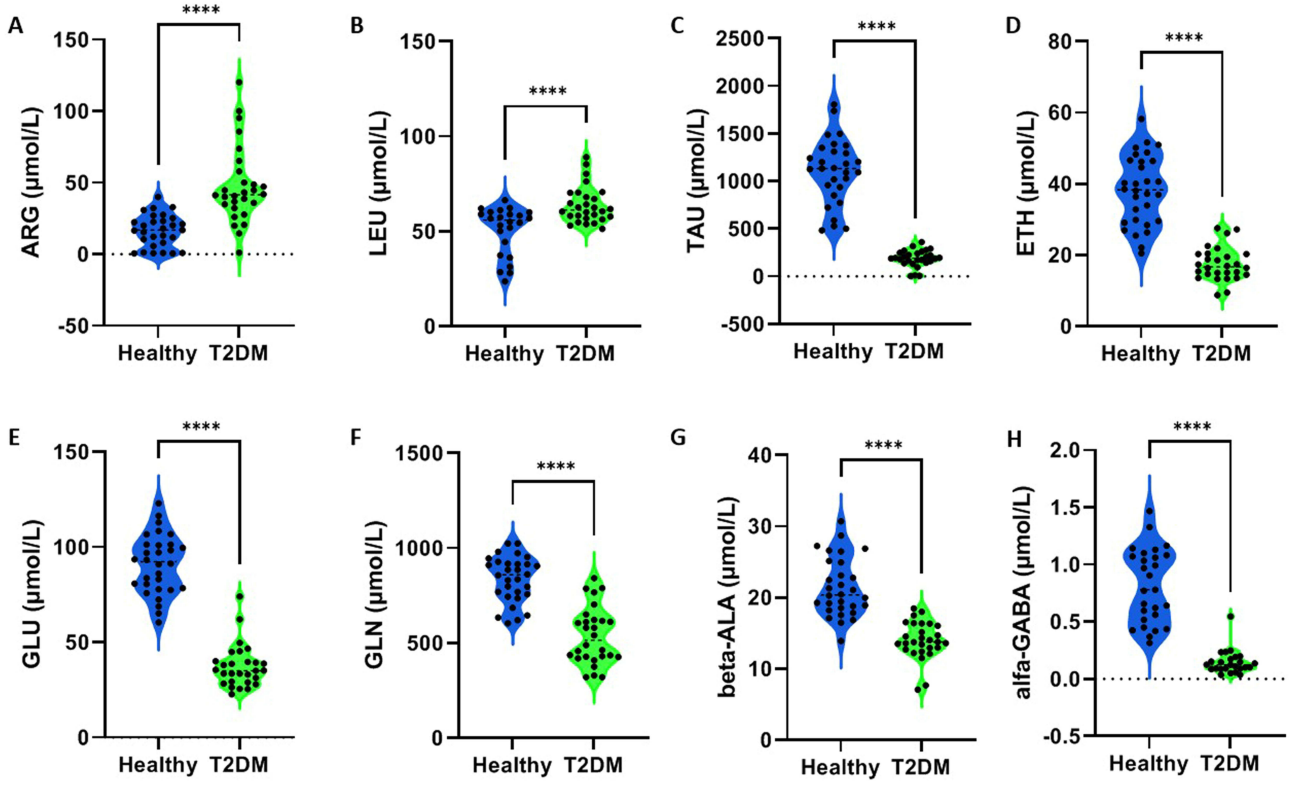

Figure 3 Quantitative analysis of 8 significant changed amino acids. A two-tailed Mann–Whitney *U*-test was applied to test for statistical significance; The asterisk denotes a significance, ****p < 0.0001. The content of ARG (**A**), LEU (**B**), TAU (**C**), ETH (**D**), GLU (**E**), GLN (**F**), beta-ALA (**G**) AND alfa-GABA (**H**) in the serum of healthy volunteers and T2DM patients.

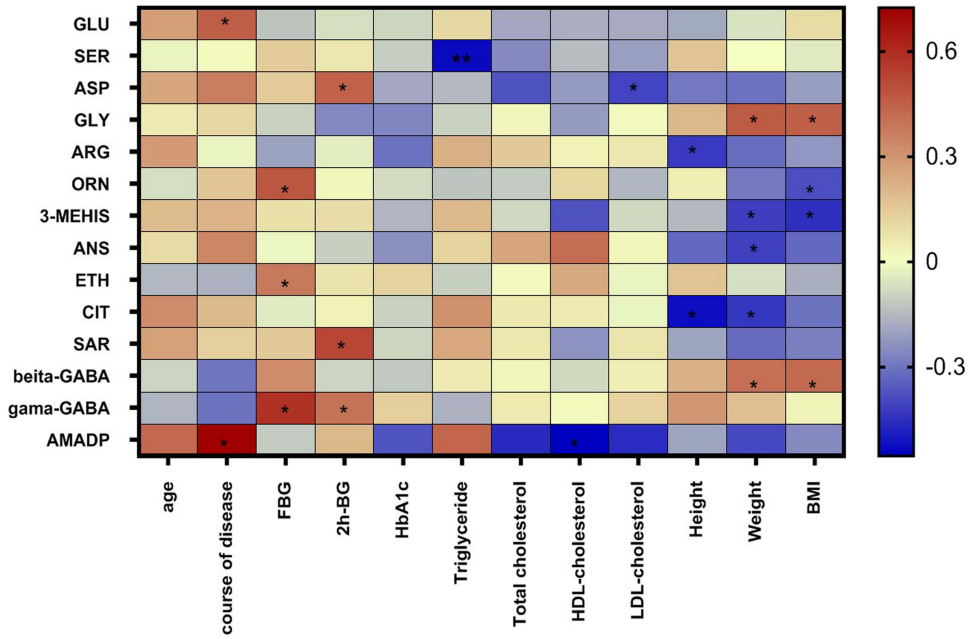

Figure 4 The heatmap of association between physiological parameters and 14 significantly changed amino acids. Red indicates positive correlations, and blue indicates negative correlations. The asterisk denotes a significance, *p < 0.05, **p < 0.01.

parameters (eg, FBG, 2h-BG, TG, HDL-C, LDL-C, etc., Figure 4). Among these 14 amino acids, we observed ORN, ETH and γ-GABA showed higher positive correlations with FBG (R>0.37, P<0.05), ASP, SAR and γ-GABA showed positive correlations with FBG (R>0.39, P<0.05), while SER was found significantly negatively correlated with TG (R=−0.53, P=0.009).

Discussion

Previous studies have shown that the levels of many amino acids and their metabolites have changed significantly in T2DM patients.[6,7] In this study, we measured the levels of 34 amino acids in the serum of T2DM, and the results showed that 19 amino acids had significant changes (Table 3), including essential and non-essential amino acids. Valine (VAL), isoleucine (ILE) and leucine (LEU) are branched chain amino acids (BCAA), it has been reported their high concentrations are closely related to diabetes and obesity,[8] and the overall high levels of these three amino acids can serve as reference indicators for the occurrence and prognosis of ischemic heart disease events.[9] Our results also show that these 3 essential amino acids in the serum of T2DM patients are significantly higher than those in healthy volunteers, which is similar to the literature reports. Glycine (GLY) is a "non essential" amino acid, and Yan's research suggests that low levels of glycine are not beneficial to insulin secretion,[10] therefore, glycine is negatively correlated with the occurrence of T2DM, and its decrease may be an early marker of T2DM. In our experiment, we also observed the decrease of serum glycine level in T2DM patients.

We further selected 8 significant amino acids that were screened by the specific screening criteria (VIP>1.0; P<0.05; FC>1.5, or FC<0.67) in metaboAnalyst 5.0 platform. Among the 8 amino acids, ARG and LEU are significantly increased in the serum of T2DM patients (Figure 3A and B), while TAU, GLU, GLN, ETH, β-ALA, and α-GABA are decreased in the serum of T2DM patients (Figure 3C–H). GABA is reported to play an important role in the treatment of diabetes by biphasic regulation, which increases insulin secretion by inducing cell membrane depolarization in pancreatic islet β Cells, and inhibits glucagon secretion by inducing cell membrane hyper-polarization in pancreatic islet α cells.[11] However, most of the studies focused on γ-GABA and diabetes, and the study of α-GABA is rare, our research may provide new clues for the treatment and prevention of T2DM. In our results, we also observed decreased serum TAU level in T2DM patients. TAU is a semi-essential micronutrient, the latest research shows that taurine can improve healthy life and reduce the prevalence of type 2 diabetes by reducing cell aging, preventing telomerase deficiency, inhibiting mitochondrial dysfunction, and reducing DNA damage.[12,13] β-ALA is a non-essential β-amino

acid with different biological functions and it is an amino acid naturally occurring in the human central nervous system.[14] β-ALA can improve cognitive ability and reduce anxiety and depression symptoms related to aging, neurological disorders.[15] To the best of our knowledge, there are few studies on β-ALA and diabetes, our results show that β-ALA levels are significantly lower in T2DM patients than that in healthy individuals.

In the correlated study of T2DM-related amino acids and physiological parameters, we found 14 differential amino acids were significantly correlated with T2DM-related physiological parameters (eg, FBG, 2h-BG, TG, HDL-C, LDL-C, etc., Figure 4), such as ORN, ETH and γ-GABA showed higher positive correlations with FBG, ASP, SAR and γ-GABA showed positive correlations with 2h-BG, while SER and ASP showed negative correlations with lipid metabolism. This discovery can help T2DM patients to improve their blood glucose status and prevent complications in the early stage through the changes of plasma amino acids, and achieve personalized and precise medication.

In this study, we used the targeted amino acid metabolome to detect the serum amino acid level of T2DM patients. Combined with metabioanalysis 5.0 platform analysis, most of our results are consistent with previous reports, and we also found amino acids that have not been reported to be related to diabetes. However, T2DM is a disease with complex etiology, in this project, our sample size is limited, which can only reflect the situation of T2DM. Next, we will continue to recruit more volunteers, further obtain more samples of patients with different stages of T2DM and complications, more clearly understand the role of amino acids in T2DM. Meanwhile, we will perform animal experiments in vivo to verify the accuracy and applicability of potential biomarkers.

Conclusion

In this study, the serum levels of 34 amino acids were detected in T2DM patients, and 19 amino acids were significantly changed in T2DM group. Further verification showed that 8 amino acids could be greatest associated with T2DM. This study will extend our knowledge of the pathophysiology of T2DM, and identified appropriate biomarkers can be used for the treatment and prognosis of T2DM.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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