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ABOUT COVER

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AIMS AND SCOPE

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WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

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ORIGINAL ARTICLE

Retrospective Study

Heterogeneously elevated branched-chain/aromatic amino acids among new-onset type-2 diabetes mellitus patients are potentially skewed diabetes predictors

Min Wang, Yang Ou, Xiang-Lian Yuan, Xiu-Fang Zhu, Ben Niu, Zhuang Kang, Bing Zhang, Anwar Ahmed, Guo-Qiang Xing, Heng Su

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Abstract

BACKGROUND

The lack of specific predictors for type-2 diabetes mellitus (T2DM) severely impacts early intervention/prevention efforts. Elevated branched-chain amino acids (BCAAs: Isoleucine, leucine, valine) and aromatic amino acids (AAAs: Tyrosine, tryptophan, phenylalanine)) show high sensitivity and specificity in predicting diabetes in animals and predict T2DM 10-19 years before T2DM onset in clinical studies. However, improvement is needed to support its clinical utility.

AIM

To evaluate the effects of body mass index (BMI) and sex on BCAAs/AAAs in new-onset T2DM individuals with varying body weight.

METHODS
Ninety-seven new-onset T2DM patients (< 12 mo) differing in BMI [normal weight (NW), n = 33, BMI = 22.23 ± 1.60; overweight, n = 42, BMI = 25.9 ± 1.07; obesity (OB), n = 22, BMI = 31.23 ± 2.31] from the First People's Hospital of Yunnan Province, Kunming, China, were studied. One-way and 2-way ANOVAs were conducted to determine the effects of BMI and sex on BCAAs/AAAs.

RESULTS
Fasting serum AAAs, BCAAs, glutamate, and alanine were greater and high-density lipoprotein (HDL) was lower (P < 0.05, each) in OB-T2DM patients than in NW-T2DM patients, especially in male OB-T2DM patients. Arginine, histidine, leucine, methionine, and lysine were greater in male patients than in female patients. Moreover, histidine, alanine, glutamate, lysine, valine, methionine, leucine, isoleucine, tyrosine, phenylalanine, and tryptophan were significantly correlated with abdominal adiposity, body weight and BMI, whereas isoleucine, leucine and phenylalanine were negatively correlated with HDL.

CONCLUSION
Heterogeneously elevated amino acids, especially BCAAs/AAAs, across new-onset T2DM patients in differing BMI categories revealed a potentially skewed prediction of T2DM development. The higher BCAA/AAA levels in obese T2DM patients would support T2DM prediction in obese individuals, whereas the lower levels of BCAAs/AAAs in NW-T2DM individuals may underestimate T2DM risk in NW individuals. This potentially skewed T2DM prediction should be considered when BCAAs/AAAs are to be used as the T2DM predictor.

Key Words: Hyperaminoacidemia; Branched-chain/aromatic amino acids; New-onset type-2 diabetes; Predictor; Obesity; Sex

Core Tip: Elevated branched-chain amino acids (BCAAs) and aromatic amino acids (AAAs) predict diabetes in animals with high sensitivity and specificity (both > 97%) and predict type-2 diabetes mellitus (T2DM) 10-20 years before T2DM onset. However, our results indicate that heterogeneously elevated BCAAs/AAAs among new-onset T2DM patients in differing BMI categories and sex may skew BCAA/AAA prediction of T2DM development among the general population: the greater BCAA/AAA elevation in obese individuals, especially males, would support T2DM prediction in these individuals, whereas the lack of or reduced BCAA/AAA elevation in NW and reproductive-aged females may compromise BCAA/AAA prediction of T2DM in these individuals. Potential nutritional, metabolic and molecular mechanisms are discussed.

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INTRODUCTION

According to the World Health Organization (WHO) (https://www.who.int/news-room/fact-sheets/detail/diabetes) and other global surveys[1], more than 465 million people worldwide will have type-2 diabetes mellitus (T2DM) in 2023, which is projected to double by 2050. T2DM was responsible for more than 4.2 million annual deaths in 2019. The global economic burden of T2DM in 2015 was 1.3 trillion United States dollars, which will double by 2030. Obesity (OB) is a major risk factor for T2DM and cardiovascular diseases (CVDs)[2]. More than 2.1 billion adults worldwide (39% of the adult population) are overweight (OW) or obese (700 million, 13% of the adult population) and are responsible for approximately 2.8 million deaths each year. China's prevalence of OW and OB in 2019 was 34.3% and 16.4% for adults (≥ 18 years), respectively[3]. The prevalence of diabetes among Chinese adults increased from 10.9% in 2013 to 12.4% in 2018 [3,4]. Moreover, 45.2% of the obese individuals had metabolically unhealthy OB (MUO) with comorbid diabetes (18.5%) or prediabetes (26.7%), whereas the other 55% of the obese individuals were metabolically healthy OW (MHO). Similarly, 32.7% of OW people have diabetes (12.8%) or prediabetes (19.9%), compared to 20.7% of normal weight (NW) people who have diabetes (7.6%) or prediabetes (13.1%)[3]. A 4-year follow-up study of 6748 nondiabetic middle-aged subjects (average 43 years old) showed that 55% of the population is metabolically healthy with a low risk of T2DM development, whereas 45% of the population is metabolically unhealthy and has a higher risk of diabetes development[5].

Early T2DM prediction could prevent or minimize the global impact of T2DM. T2DM onset can be delayed or prevented if intervened preclinically[6,7]. However, more than two-thirds of T2DM patients are not aware of having T2DM until their diagnosis. Elevated blood branched-chain amino acids (BCAAs: Leucine, isoleucine, valine) and aromatic amino acids (AAAs: Tyrosine, tryptophan and phenylalanine) are promising T2DM predictors, as their

elevations have successfully predicted prediabetes[7-9], homeostasis model assessment-insulin resistance[10-12], and T2DM 10-20 years ahead of their onset[13-16]. However, these findings have not been replicated in heterogeneous populations or with an established cutoff for standard diagnosis. In fact, most of the BCAAs/AAAs findings were tentative and reported as odds ratios, hazard ratios (HRs) or relative risks (RRs), or the results of comparing the highest quartile *vs* lowest quartile individuals were reported without mentioning the sensitivity, specificity, accuracy, and positive and negative predictive values of standardized diagnosis criteria.

Furthermore, most of the T2DM BCAAs/AAAs findings are based on obese individuals. Significant confounding effects of OB on BCAA/AAA elevation in T2DM were reported[17]. Greater baseline body mass index (BMI) and metabolic syndromes (MetS) often coexist in individuals who subsequently develop T2DM than in those without T2DM, suggesting a confounding effect of baseline BMI/OB and MetS on BCAA/AAA elevation, CVD and T2DM development [18-20]. In an 8.5-year follow-up study of 6134 nondiabetic individuals resulting in 306 new T2DM cases, the HR of the highest *vs* lowest quartile dropped from 12.07 to 3.20 (nearly 4-fold) after adjustment for BMI, family history of T2DM, alcohol consumption, and MetS[10]. Furthermore, T2DM prevalence/incident rates in these BCAAs/AAAs studies (3%-5%) were often much lower than the population-based epidemiological data (approximately 10% for diabetes and approximately 20% for prediabetes) (WHO: https://www.who.int/news-room/fact-sheets/detail/diabetes, CDC: https://www.cdc.gov/diabetes/prevention/about-prediabetes.html)[21].

Although the exact mechanisms remain unknown, publication bias, systematic exclusions of T2DM comorbid conditions such cardiovascular disorders, cancer or other diseases, or selective inclusion of healthier control individuals or male subjects may have caused the discrepancies. One 19-year follow-up study of 1279 nondiabetic European and 1007 nondiabetic South Asian male individuals reported a 35% prevalence of T2DM in South Asian men and a 14% prevalence of T2DM in European men without reporting women's data[14], which may compromise the results due to the sex-dependent elevation in BCAAs/AAAs[17,22,23].

BCAA/AAA elevation is further complicated by interactions of body composition, age, sex, genetics and dietary protein, fat, and energy intake. Higher dietary BCAA intake and elevated blood BCAAs are associated with increased risk of OB and IR in men but reduced risk in reproductive-aged women[24-26]. Higher animal protein but not plant protein intake is associated with higher longitudinal insulin resistance and risk of T2DM[27]. However, five years of consumption of a low-fat Mediterranean diet normalized BCAA levels and promoted T2DM remission[28].

The metabolic impact of BCAA supplementation is affected by BMI and/or adiposity status. A higher percentage intake of BCAAs in terms of total protein was associated with a significantly decreased risk of diabetes in lean/NW middle-aged Japanese men and women (BMI = 22)[29]. A higher dietary BCAA intake/ratio and elevated blood BCAAs were inversely associated with the risk/prevalence of OB in lean individuals (BMI < 24)[30,31]. Replacing animal protein with plant protein is associated with decreased T2DM risk in adult males[32]. Because a higher intake of animal protein is often associated with increased consumption of saturated fats and increased body fat/weight gain, a body fat/weight-dependent effect of BCAAs may be associated with T2DM risk/onset. In support of that, BCAA supplementation significantly increased hepatic gluconeogenesis, plasma lipid and muscular and renal lipid accumulation and reduced hepatic lipid accumulation in high-fat-diet-induced obese mice[33], whereas BCAA supplementation attenuated the severity of streptozotocin-induced diabetes in lean rats[34].

This study aimed to evaluate the effects of BMI and sex on BCAAs/AAAs in new-onset T2DM individuals in differing BMI categories.

MATERIALS AND METHODS

T2DM patients

New-onset T2DM patients were diagnosed at the Department of Endocrinology, the First People's Hospital of Yunnan Province, Kunming, China, from December 2016 to June 2018. Ninety-seven T2DM patients diagnosed with T2DM within 1 year were included in the analysis (53 male/44 female, 43.3 ± 11.2 years of age). The diagnosis and classification of T2DM were based on 1999 WHO standards[35]: (1) Fasting plasma glucose concentration ≥ 7.0 mmol (or ≥ 126 mg/dL); (2) ≥ 11.1 mmol (or ≥ 200 mg/dL) 2 h after a 75 g oral glucose load; and (3) HbA1c ≥ 6.5%. The exclusion criteria were as follows: (1) A history of diabetes that was diagnosed more than 12 mo prior; and (2) acute complications of diabetes and severe liver and/or kidney dysfunction or other serious health conditions. The study procedures were conducted in accordance with the Helsinki Declaration of 1975 and were approved by the Medical Ethics Review Committee of the First People's Hospital of Yunnan Province [No. 2016(001)].

Body weight classifications and abdominal fat area measures

Anthropometric measures of height, body weight and waist circumference (WC) were used to determine BMI and body weight status. The 2004 WHO classifications for the Asian/Chinese population were used for this study: (1) NW (BMI < 24); (2) OW (BMI ≥ 24 and < 28); and (3) OB (BMI ≥ 28), which differ from the standards of United States and European populations (NW: BMI < 25 kg/m^2, OW: BMI ≥ 25, OB: ≥ 30). Visceral adipose tissue (VAT) at the level of the umbilicus was measured *via* an abdominal dual BIA machine following the manufacturer's protocol (DUALSCAN HDS-2000, Omron Health care Co., Kyoto, Japan).

Blood sample collection and serum amino acid analysis

Fasting blood samples of the T2DM patients were collected and analyzed following the standard operation protocol. Briefly, overnight fasting (> 8 h) venous blood samples were collected and centrifuged immediately to separate the

serum.

The frozen serum samples were thawed on ice and at 4°C followed by deproteinization by the addition of acetonitrile (1:3 ratio of serum to acetonitrile). The sample was then vortexed for 2 min followed by centrifugation at 12000 rpm for 10 min at 4°C to remove any precipitate from the supernatant before ultra-performance liquid chromatography (UPLC)/triple stage quadrupole mass spectrometer (TSQ/MS) analysis. A quality control sample consisting of 6 reference standards (isoleucine, leucine, valine, tyrosine, tryptophan, phenylalanine) was prepared and run after each of the 15 serum samples.

A 5 μL aliquot of extracted serum sample was injected into the UPLC column (DionexUltiMate3000-UPLC, United States) TSQ/MS (Thermo TSQ Endura, United States). Separation was achieved on a Luna Omega Polar C18 column (2.1 mm × 100 mm, 1.6 μm, Phenomenex) held at 40°C. The samples were eluted with A (water with 0.1% formic acid) and B (acetonitrile), and the gradient program was 1% B over 0-0.5 min, 1%-20% B over 0.5-9 min, 20%-75% B over 9-11 min, and 75%-99% B over 11-16 min. The composition was held at 99% B for 0.5 min and finally returned to 1% B at 20 min. The flow rate was 0.3 mL/min. Mass spectrometry was performed in positive ion electrospray (ESI +) mode. The temperature for the ion transfer tube and vaporizer was set at 350°C and 300°C, respectively. The pressures for the sheath gas, aux gas and sweep gas were set at 40, 15 and 1 Arb, respectively. The positive ion voltage was set to 3.5 kV. All the compounds were detected in selective reaction monitoring mode.

Statistical analysis

The data are presented as the mean ± standard deviation or median [25% quartile range (QR), 75% QR]. The effects of BMI and sex were analyzed using ANOVA for quantitative variables with a normal distribution or using the Wilcoxon rank sum test for nonnormally distributed parameters (SPSS 24, IBM). Post hoc multiple comparisons were assessed using Bonferroni correction. Pearson's correlation coefficients were calculated among the variables. A 2-sided P≤0.05 was considered indicative of statistical significance.

RESULTS

Demographic and anthropometric characteristics

Ninety-seven (97) T2DM patients, including 57 males (59%) and 40 females (41%), all within 1 year of T2DM diagnosis, were identified and included in this study. Of them, 33 (34%) were NW-T2DM (BMI = 22.23 ± 1.60), 42 (43.2%) were OW-T2DM (BMI = 25.90 ± 1.08), and 22 (22.8%) were OB-T2DM (BMI = 30.96 ± 2.59), based on the WHO body weight classification (Table 1).

No group differences were found in T2DM diagnosis, sex distribution, height, medication, or systolic and diastolic blood pressures (Table 1). The OB-T2DM group was significantly younger (36.77 ± 10.32 years) than the NW-T2DM and OW-T2DM groups (43.64 ± 6.95 and 42.21 ± 9.76 years, respectively, $P < 0.05$, each). The OB-T2DM group also showed greater values than the NW-T2DM and OW-T2DM groups in body weight, WC, WC-to-height ratio (WHR), and BMI ($P < 0.01$, each, Table 1).

The male and female patients showed similarities in T2DM onset age (30.4 ± 77.0 *vs* 43.3 ± 91.7 d), BMI (25.9 ± 4.2 *vs* 25.8 ± 2.9), WHR (0.535 ± 0.05 *vs* 0.555 ± 0.05), systolic blood pressure (125.46 ± 16.4 *vs* 125.88 ± 16.1 mmHg), diastolic blood pressure (84.0 ± 14.3 *vs* 81.2 ± 7.3 mmHg), and antidiabetic medication. The males were, however, significantly younger (39.7 ± 8.8 *vs* 44.0 ± 9.6 year), heavier (76.7 ± 13.7 *vs* 64.9 ± 8.2 kg), and taller (171.6 ± 6.3 *vs* 159.2 ± 4.9 cm) with greater WC (91.7 ± 8.7 *vs* 88.3 ± 7.7 cm, $P < 0.05$, all) than the females.

Effects of BMI and sex on the serum amino acid profile

Two-way ANOVA of the effects of BMI and sex showed that among the 23 serum amino acids measured by using UPLC/TSQ-MS, 8 amino acids were significantly greater in obese than in NW and/or OW subjects (alanine, asparagine, carnosine, glutamate, hydroxyproline, proline, tyrosine, and tryptophan, $P < 0.05$, each) (Figure 1, Table 2). Valine, isoleucine, and phenylalanine were marginally greater in obese than in NW and/or OW subjects ($P \leq 0.1$, each). Ten amino acids were significantly greater in male subjects than in female subjects (serine, asparagine, glutamate, lysine, hydroxyproline, citrulline, isoleucine, leucine, tyrosine, and tryptophan, $P < 0.05$, each). Histidine, methionine, phenylalanine, and threonine were marginally greater in male subjects than in female subjects ($P < 0.1$, each). Significant BMI and sex interactions were found for proline only.

Further 1-way ANOVA of BMI effects largely confirmed the 2-way ANOVA results. Within-sex 1-way ANOVA showed that most of the BMI differences were present among the male subjects only (alanine, asparagine, glutamate, valine, isoleucine, leucine, tyrosine, and tryptophan) ($P \leq 0.05$, each) (Figure 1, Table 2), except that carnosine was significantly greater in the female obese group and proline was significantly lower in the female OW group than in the other two groups. Covariance analysis showed no difference in serum amino acids after controlling for day of T2DM diagnosis or controlling for metformin equivalent dose. of T2DM medicine. However, covariance analysis showed significant effects of BMI on taurine, citrulline and proline after controlling for age ($P \leq 0.05$, each, Table 2).

Effects of BMI and sex on lipid profile

Two-way ANOVA showed no significant effects of BMI and sex on triglycerides, total cholesterol, and low-density lipoprotein. However, high-density lipoprotein (HDL) was significantly affected by BMI, with significantly lower HDL levels found in obese T2DM patients than in their NW and OW counterparts ($P < 0.01$, $P < 0.05$, respectively), especially

Table 1 Characteristics of 97 new-onset type 2 diabetes patients of normal weight, overweight and obesity

	NW (33)	OW (42)	OB (22)	*F* value	*P* value
Sex: M/F [*n* (%)]	20/13 (33)	24/18 (42)	13/9 (22)	0.095	0.954
Age (yr, mean ± SD)	43.64 ± 6.95	42.21 ± 9.76	36.77 ± 10.32[a]	4.054	0.020[a]
T2DM onset (d)	46.1 ± 96.8	41.8 ± 90.3	8.5 ± 20.2	1.569	0.214
Medication (Y/N)	13/20	13/29	6/16	1.016	0.602
Metformin equiv dose (g, mean ± SD)	176 ± 221	214 ± 225	546 ± 34	1.384	0.256
Weight (kg)	60.53 ± 6.87	73.49 ± 9.71[b,c]	85.64 ± 11.35[b,c]	49.593	0.000[b]
Height (cm)	165.3 ± 8.26	168.5 ± 9.05	164.4 ± 6.91	1.407	0.250
BMI index	22.23 ± 1.60	25.88 ± 1.07[b,c]	31.23 ± 2.31[b,c]	208.086	0.000[b]
Waist circumference (cm)	84.85 ± 6.21	89.76 ± 6.07[b,c]	99.61 ± 7.05[b,c]	35.920	0.000[b]
Waist: Height ratio	0.514 ± 0.042	0.535 ± 0.034[a]	0.604 ± 0.041[b,c]	37.279	0.000[b]
Systolic blood pressure (mmHg)	122.1 ± 17.6	125.6 ± 13.2	130.7 ± 18.2	12.68	0.07
Diastolic blood pressure (mmHg)	81.9 ± 12.8	83.8 ± 8.7	82.5 ± 15.7	0.509	0.602

[a]$P < 0.05$ compared with normal weight.
[b]$P < 0.01$, compared with normal weight.
[c]$P < 0.01$, compared with overweight.
Data are numbers (*n*) of individuals (%) unless otherwise indicated. NW: Normal weight (body mass index: 18.5-23.9); OB: Obesity (body mass index: ≥ 28.0); OW: overweight (body mass index: 24.0-27.9); BMI: Body mass index; M: Male; F: Female.

among male patients (Table 2).

Associations of serum amino acids with VAT, body weight, BMI, and lipid profile

Although multiple amino acids were significantly correlated with VAT, body weight and BMI and negatively correlated with HDL, in pooled samples (Table 3), the correlations with VAT were the strongest (tyrosine, $r = 0.524$, $P < 0.0001$; phenylalanine, $r = 0.508$, $P < 0.0001$; tryptophan $r = 0.373$, $P < 0.01$; isoleucine, $r = 0.443$, $P = 0.002$; leucine, $r = 0.396$, $P = 0.006$, valine, $r = 0.375$, $P < 0.01$; methionine, $r = 0.379$, $P < 0.01$; alanine, $r = 0.429$, $P = 0.003$; glutamate, $r = 0.398$, $P < 0.01$; lysine, $r = 0.39$, $P < 0.01$; hydroxyproline, $r = 0293$; $P < 0.05$, threonine, $r = 0.388$, $P < 0.01$; proline, $r = 0.314$, $P < 0.05$; and histidine, $r = 0.278$, $P < 0.06$), followed by body weight, BMI and HDL (Table 3). Taurine was negatively correlated with VAT ($r = -0.367$, $P = 0.01$), and HDL was negatively correlated with isoleucine ($r = -0.309$, $P < 0.01$), leucine ($r = -0.276$, $P < 0.01$) and phenylalanine ($r = -0.307$ $P < 0.01$). Within-BMI category correlation analysis showed no significant correlations in the NW group (Table 3). For the OW group, phenylalanine was highly correlated with VAT ($r = 0.607$, $P = 0.002$), and BCAAs and AAAs were significantly or marginally correlated with body weight (tyrosine, $r = 0.343$, $P < 0.05$; tryptophan $r = 0.339$, $P < 0.05$; isoleucine, $r = 0.289$, $P = 0.063$; leucine, $r = 0.32$, $P = 0.039$, valine, $r = 0.286$, $P = 0.067$, respectively), and HDL was negatively correlated with isoleucine ($r = -0.417$, $P < 0.01$), leucine ($r = -0.345$, $P < 0.05$) and phenylalanine ($r = -0.323$, $P < 0.05$) (Table 3). For the obese group, alanine was significantly correlated with VAT ($r = 0.64$, $P < 0.05$); homocysteine was significantly correlated with body weight ($r = 0.475$, $P < 0.05$) and BMI ($r = 0.43$, $P < 0.05$)); HDL was negatively correlated with asparagine ($r = -0.478$, $P < 0.01$) and hydroxyproline ($r = -0.436$, $P < 0.05$); and BCAAs and AAAs were negatively correlated with waist-to-height ratio (isoleucine, $r = -0.54$, $P < 0.01$; leucine, $r = -0.51$, $P < 0.05$; and tryptophan, $r = -0.512$, $P < 0.05$). These observations support VAT as the major source of elevated BCAAs/AAAs in obese T2DM patients[36].

DISCUSSION

The global T2DM epidemic could be better managed or even prevented if potential T2DM candidates were identified and treated at the preclinical stage. BCAAs and AAAs are promising T2DM predictors because of their high diagnostic sensitivity and specificity (both > 97%) in predicting and discriminating diabetic rats from nondiabetic rats[37]. Such diagnostic sensitivity and accuracy, however, have not been demonstrated in T2DM prediction, possibly due to the confounding effects of unknown factors. Both OB and sex are implicated, although the exact mechanism remains unknown.

In this study, fasting serum levels of alanine, asparagine, carnosine, glutamate, hydroxyproline, proline, tyrosine, and tryptophan were significantly greater, whereas HDL was significantly lower in obese T2DM patients than in normal T2DM patients. Valine, isoleucine, leucine, and phenylalanine trended toward higher levels in obese than in NW-T2DM patients. The amino acid levels of the OW-T2DM patients were intermediate between those of the OB and NW groups. Ten amino acids (serine, asparagine, glutamate, lysine, hydroxyproline, citrulline, isoleucine, leucine, tyrosine, and

Table 2 One-way/two-way ANOVA of serum amino acids in newly onset type-2 diabetes mellitus patients of normal weight, overweight and obesity (mean ± SD)

		NW	OW	OB	Obesity, F, P (1-way ANOVA)	Obesity, F, P (2-way ANOVA)	Sex, *F* value, *P* value	Obesity × sex interaction, *F* value, *P* value	Age-adjusted *F* value, *P* value	Onset-adjusted, *F* value, *P* value	Metf. dose-adjusted, *F* value, *P* value
HbA1c (%)	Pooled	10.96 ± 2.73	9.91 ± 2.69	9.09 ± 2.40[a]	3.471, 0.035[a]	2.888, 0.061[d]	1.603, 0.209	0.376, 0.688	NS	NS	NS
	Male	11.51 ± 2.93	10.07 ± 2.78	9.25 ± 2.16	3.012, 0.058[d]						
	Female	10.12 ± 2.24	9.70 ± 2.64	8.85 ± 2.82	0.661, 0.522						
TC (mmol/L)	Pooled	4.82 ± 1.36	4.68 ± 1.65	4.55 ± 1.10	0.229, 0.796	0.239, 0.788	1.020, 0.315	0.035, 0.965	NS	NS	NS
	Male	4.91 ± 1.24	4.80 ± 1.96	4.73 ± 1.14	0.053, 0.948						
	Female	4.68 ± 1.58	4.52 ± 1.15	4.29 ± 1.04	0.243, 0.786						
TG (mmol/L)	Pooled	3.12 ± 6.77	2.73 ± 3.18	4.66 ± 4.20	1.161, 0.318	0.992, 0.375	1.758, 0.188	0.253, 0.777	NS	NS	NS
	Male	3.85 ± 8.61	2.91 ± 3.94	5.45 ± 4.99	0.711, 0.496						
	Female	2.00 ± 1.64	2.49 ± 1.80	3.53 ± 2.54	1.683, 0.200						
HDL (mmol/L)	Pooled	1.07 ± 0.27	1.03 ± 0.27	0.86 ± 0.22	4.744, 0.011[a]	4.478, 0.014[a]	0.998, 0.320	0.337, 0.715	NS	NS	NS
	Male	1.07 ± 0.26	0.98 ± 0.23	0.84 ± 0.24	3.602, 0.034[a]						
	Female	1.08 ± 0.31	1.09 ± 0.32	0.88 ± 0.19	1.635, 0.209						
LDL (mmol/L)	Pooled	2.82 ± 1.10	2.81 ± 0.87	2.34 ± 1.02	1.922, 0.152	1.670, 0.194	0.009, 0.925	0.121, 0.887	NS	NS	NS
	Male	2.83 ± 0.96	2.87 ± 0.84	2.29 ± 0.99	1.870, 0.164						
Histidine (μg/L)	Pooled	188.42 ± 40.62	197.28 ± 57.63	219.62 ± 53.34	2.488, 0.089	2.010, 0.140	3.614, 0.060[d]	0.651, 0.524	NS	NS	NS
	Male	190.30 ± 45.33	205.95 ± 62.41	234.87 ± 49.68	2.680, 0.078[d]						
	Female	185.53 ± 33.68	185.72 ± 49.92	197.59 ± 53.32	0.234, 0.792						
Arginine (μg/L)	Pooled	458.53 ± 180.93	480.08 ± 198.77	507.06 ± 127.36	0.487, 0.616	0.423, 0.656	0.219, 0.641	0.044, 0.957	NS	NS	NS
	Male	459.85 ± 163.56	492.30 ± 231.02	516.31 ± 131.56	0.369, 0.693						
	Female	456.51 ± 211.98	463.80 ± 150.31	493.70 ± 127.59	0.140, 0.870						
β-Alanine (μg/L)	Pooled	436.45 ± 188.57	445.91, 188.70	394.88, 202.89	0.530, 0.591	0.402, 0.670	0.569, 0.452	0.942, 0.394	NS	NS	NS
	Male	454.86 ± 200.68	425.40 ± 177.60	357.16 ± 178.59	1.100, 0.340						
	Female	408.11 ± 172.11	473.25 ± 204.50	449.37 ± 233.59	0.395, 0.676						
Carnosine (μg/L)	Pooled	5.99 ± 1.37	5.98 ± 2.06	6.95 ± 1.50	2.671, 0.074	3.122, 0.049	0.013, 0.908	1.193, 0.308	NS	NS	NS

	Male	6.27 ± 1.41	5.93 ± 2.36	6.65 ± 1.34	0.632, 0.535						
	Female	5.55 ± 1.22	6.04 ± 1.63	7.39 ± 1.69[a]	4.035, 0.026[a]						
Serine (μg/L)	Pooled	268.73 ± 91.46	239.17 ± 83.33	283.56 ± 79.43	2.258, 0.110	2.182, 0.119	7.451, 0.008[b]	0.567, 0.569	NS	NS	NS
	Male	297.99 ± 77.49	256.57 ± 87.30	295.74 ± 64.80	1.817, 0.172						
	Female	223.72 ± 95.80	215.97 ± 73.74	265.98 ± 98.35	1.038, 0.364						
Taurine (μg/L)	Pooled	71.97 ± 18.22	78.96 ± 30.38	75.98 ± 22.23	0.721, 0.489	1.054, 0.353	1.714, 0.194	0.685, 0.507	9.335, 0.003[b]	NS	NS
	Male	72.61 ± 17.85	74.52 ± 30.24	76.10 ± 21.65	0.083, 0.921						
	Female	71.00 ± 19.47	84.88 ± 30.39	75.81 ± 24.38	1.131, 0.334						
Alanine (μg/L)	Pooled	130.77 ± 49.32	139.06 ± 40.66	168.94 ± 56.03[a]	4.531, 0.013[a]	3.967, 0.022[a]	0.007, 0.934	0.812, 0.447	NS	NS	NS
	Male	123.80 ± 46.98	143.52 ± 40.18	170.88 ± 56.72[a]	4.013, 0.024[a]						
	Female	141.51 ± 52.77	133.10 ± 41.67	166.14 ± 58.32	1.357, 0.270						
Asparagine (μg/L)	Pooled	36.73 ± 13.82	30.17 ± 16.07	40.40 ± 13.67[c]	3.900, 0.024[a]	3.817, 0.026[a]	7.684, 0.007[b]	0.586, 0.559	NS	NS	NS
	Male	38.32 ± 15.37	34.05 ± 18.30	45.52 ± 11.83	2.161, 0.125						
	Female	34.28 ± 11.16	25.01 ± 10.95	33.01 ± 13.30	2.865, 0.070[d]						
Glutamine (μg/L)	Pooled	464.62 ± 156.05	450.21 ± 188.81	489.96 ± 120.18	0.422, 0.657	0.530, 0.590	0.652, 0.421	0.857, 0.428	NS	NS	NS
	Male	459.31 ± 156.09	486.60 ± 219.88	495.68 ± 135.66	0.192, 0.826						
	Female	472.80 ± 162.00	401.70 ± 127.29	481.70 ± 100.88	1.534, 0.229						
Glutamate (μg/L)	Pooled	2812.36 ± 1104.11	2768.39 ± 1153.78	3588.65 ± 1138.22[a,e]	4.259, 0.017[a]	4.028, 0.021[a]	5.188, 0.025[a]	0.176, 0.839	NS	NS	NS
	Male	2949.39 ± 1011.65	3028.47 ± 1234.29	3866.48 ± 1088.44	3.039, 0.056[d]						
	Female	2601.55 ± 1245.38	2421.62 ± 962.57	3187.33 ± 1147.67	1.470, 0.243						
Lysine (μg/L)	Pooled	341.06 ± 128.62	365.48 ± 152.71	423.72 ± 142.42	2.262, 0.110	2.087, 0.130	6.688, 0.011[a]	0.376, 0.687	NS	NS	NS
	Male	357.60 ± 124.07	406.28 ± 172.85	462.00 ± 157.70	1.831, 0.170						
	Female	315.62 ± 136.32	311.08 ± 101.65	368.42 ± 100.58	0.833, 0.443						
Hydroxyproline (μg/L)	Pooled	21.09 ± 11.77	14.33 ± 11.68[a]	21.41 ± 12.09	4.050, 0.021[a]	3.901, 0.024[a]	9.074, 0.003[b]	0.614, 0.543	NS	NS	NS
	Male	22.84 ± 12.69	17.00 ± 12.59	26.07 ± 12.48	2.472, 0.094						
	Female	18.39 ± 10.06	10.78 ± 9.55	14.67 ± 7.98	2.490, 0.097						
Threonine (μg/L)	Pooled	201.17 ± 88.38	208.93 ± 86.51	236.53 ± 82.51	1.173, 0.314	0.950, 0.390	3.035, 0.085[d]	0.293, 0.746	NS	NS	NS

	Male	209.66 ± 92.11	217.95 ± 89.86	258.54 ± 87.84	1.259, 0.292						
	Female	188.12 ± 84.22	196.90 ± 82.80	204.73 ± 66.12	0.118, 0.889						
Citrulline (μg/L)	Pooled	95.53 ± 32.87	90.68 ± 34.25	94.71 ± 27.50	0.237, 0.790	0.527, 0.592	6.414, 0.013[a]	0.001, 0.999	6.232, 0.014[a]	NS	NS
	Male	101.68 ± 34.73	96.78 ± 38.17	98.59 ± 30.95	0.105, 0.901						
	Female	86.06 ± 28.48	82.54 ± 27.12	89.11 ± 22.12	0.195, 0.824						
Proline (μg/L)	Pooled	2313.70 ± 644.63	2244.77 ± 716.36	2561.43 ± 555.47	1.707, 0.187	3.138, 0.048[a]	0.941, 0.335	3.279, 0.042[a]	6.754, 0.011[a]	NS	NS
	Male	2176.16 ± 572.03	2404.58 ± 839.03	2604.08 ± 534.61	1.560, 0.220						
	Female	2525.30 ± 714.13	2031.68 ± 448.42[a]	2499.81 ± 611.56	3.418, 0.043[a]						
Homocysteine (μg/L)	Pooled	2.50 ± 1.71	2.68 ± 1.64	2.27 ± 1.33	0.481, 0.620	0.800, 0.453	0.194, 0.661	1.812, 0.169	NS	NS	NS
	Male	2.26 ± 1.66	2.39 ± 1.43	2.61 ± 1.52	0.205, 0.815						
	Female	2.88 ± 1.78	3.06 ± 1.85	1.77 ± 0.82	1.916, 0.161						
Valine (μg/L)	Pooled	3839.99 ± 883.13	3718.96 ± 851.75	4259.02 ± 820.20	2.938, 0.058[d]	2.627, 0.078[d]	1.547, 0.217	1.385, 0.256	NS	NS	NS
	Male	3763.81 ± 926.69	3876.21 ± 837.88	4465.80 ± 583.75	3.144, 0.051[d]						
	Female	3957.18 ± 833.97	3509.28 ± 847.57	3960.35 ± 1041.61	1.264, 0.294						
Methionine (μg/L)	Pooled	2.44 ± 1.86	2.48 ± 1.97	3.21 ± 2.24	1.201, 0.306	0.754, 0.473	3.091, 0.082[d]	2.568, 0.082[d]	NS	NS	NS
	Male	2.23 ± 1.92	2.84 ± 2.11	3.99 ± 2.50	2.658, 0.079[d]						
	Female	2.76 ± 1.78	2.01 ± 1.70	2.09 ± 1.15	0.879, 0.424						
Isoleucine (μg/L)	Pooled	306.68 ± 75.83	326.23 ± 81.56	357.51 ± 75.72	2.778, 0.067	2.325, 0.104	6.977, 0.010[b]	0.786, 0.459	NS	NS	NS
	Male	313.67 ± 73.98	343.49 ± 87.07	386.75 ± 75.14[a]	3.283, 0.045[a]						
	Female	295.93 ± 80.37	303.21 ± 69.32	315.27 ± 56.43	0.200, 0.820						
Leucine(μg/L)	Pooled	492.26 ± 136.62	518.94 ± 147.01	577.40 ± 153.91	2.301, 0.106	1.909, 0.154	8.751, 0.004[b]	0.856, 0.428	NS	NS	NS
	Male	511.46 ± 137.96	548.74 ± 163.13	638.14 ± 137.20	2.896, 0.064[d]						
	Female	462.73 ± 134.45	479.21 ± 114.87	489.66 ± 138.87	0.129, 0.879						
Tyrosine (μg/L)	Pooled	155.13 ± 57.40	166.00 ± 63.14	203.68 ± 74.79[a]	3.998, 0.022[a]	3.370, 0.039[a]	5.255, 0.024[a]	1.448, 0.240	NS	NS	NS
	Male	154.79 ± 57.19	182.95 ± 70.70	225.47 ± 58.95[b]	4.857, 0.011[a]						
	Female	155.65 ± 60.07	143.39 ± 43.65	172.21 ± 87.10	0.685, 0.511						
Phenylalanine (μg/L)	Pooled	664.47 ± 163.17	699.89 ± 172.50	785.74 ± 197.37[a]	3.223, 0.044[a]	2.909, 0.060[d]	2.806, 0.097[d]	0.384, 0.682	NS	NS	NS
	Male	688.44 ±	709.87 ±	828.45 ±	2.534,						

		154.62	191.09	208.14	0.089[d]						
	Female	627.58 ± 175.24	686.60 ± 148.44	724.03 ± 173.28	1.002, 0.377						
Tryptophan(μg/L)	Pooled	658.00 ± 150.01	694.36 ± 188.43	790.15 ± 162.27[a]	4.082, 0.020[a]	4.277, 0.017[a]	13.121, 0.000[b]	0.051, 0.951	NS	NS	NS
	Male	702.38 ± 151.16	746.40 ± 193.72	847.94 ± 127.90	3.062, 0.055[d]						
	Female	589.72 ± 124.48	624.97 ± 161.06	706.66 ± 176.95	1.567, 0.222						

[a]$P < 0.05$ *vs* normal weight (or male *vs* female).
[b]$P < 0.01$, *vs* normal weight (or male *vs* female).
[c]$P < 0.1$ *vs* overweight.
[d]$P < 0.1$ *vs* normal weight.
[e]$P < 0.01$, *vs* overweight.
NS: Not significant, NW: Normal weight; OB: Obesity; OW: Overweight; HbA1c: Hemoglobin A1c; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

tryptophan) were significantly greater in male patients than in female patients. Histidine, methionine, phenylalanine, and threonine also trended toward being greater in male patients. Serum AAAs, BCAAs and several other amino acids were significantly correlated with abdominal adiposity and less so with body weight or BMI, whereas BCAAs/AAAs and other amino acids were negatively correlated with HDL.

Our results are in agreement with previous reports that BMI-/abdominal adiposity-dependent BCAA/AAA elevations are associated with BMI, insulin resistance and T2DM development[38-40]. A 15-year metabolomics follow-up study of 11896 non-T2DM Finnish individuals [baseline age 24-45 years, 392 incident T2DM cases identified (3.2% T2DM of the study population *vs* 8% T2DM of the general Finnish population)] showed that BCAAs/AAAs are the strongest predictor of diabetes along with triacylglycerol, linoleic n-6 fatty acid and HDL-cholesterol[41]. Simultaneous hyperaminoacidemia and dyslipidemia precede prediabetes and T2DM onset in MUO individuals[42], whereas amino acid and lipid homeostasis in MHO individuals is intermediate between lean health and MUO individuals[43,44]. A meta-analysis shows significant confounding effects of OB and metabolic health on T2DM development in that MUO individuals pose 10 times higher risk, metabolically unhealthy OW (MUOW) pose 7 times higher risk, metabolically unhealthy NW (MUNW) pose 4 times higher risk, MHO group pose 3 times higher risk ratio and MHO individuals (MHOW) pose 2 times higher risk ratio for T2DM development than metabolically healthy NW (MHNW) individuals, respectively[45]. However, MUO poses a 3.5 times higher risk, MUOW poses a 4 times higher risk and MUNW poses a 4 times higher risk for T2DM development than its metabolically healthy counterparts of the same BMI categories, *i.e.*, than MHO, MHOW, and MHNW, respectively[45]. Another 3-year follow-up study of 9623 non-T2DM Chinese adults showed decreased diabetes RR in the MHOW phenotype (0.65), no change in the MHO phenotype (0.99), and increased RR in the MUNW (1.81), MUOW (2.02) and MUO (2.48) phenotypes compared to MHNW[46].

The mechanism by which BCAAs/AAAs trigger T2DM development remains largely unknown and complex. Abnormal BCAA catabolism in muscle or loss of skeletal muscle mass may play a key role in the pathogenesis of elevated BCAAs in MetS, IR, liver cirrhosis, and T2DM, as skeletal muscle is the major site of BCAA catabolism due to the high activity of BCAA aminotransferase, which is absent in the liver[47-49]. Low skeletal muscle mass is associated with insulin resistance, diabetes, and MetS[50]. Furthermore, recent studies show that altered body composition, such as increased body fat percentage, abdominal fat mass and reduced lean muscle mass rather than BMI/body weight per se, could determine metabolic phenotypes in both obese and lean/NW children[51-54], adolescents and adults (including pre- and postmenopausal women)[55-59].

Both early-onset T2DM and typical T2DM show impaired expression of genes involved in branched-chain amino acid metabolism in muscle[60] but high circulating BCAAs and leucine treatment enhanced myotube lipid accumulation and oxidative stress in myotubes[61]. Increased BCAA catabolic flux may promote gluconeogenesis and glucose intolerance *via* glutamate transamination to alanine or trigger T2DM incidence by overstimulation of beta cell secretion and subsequent impairment of glucose-stimulated insulin secretion. Others show that 3-hydroxyisobutyrate, a catabolic intermediate of valine secreted from muscle cells, stimulates muscle fatty acid uptake and promotes lipid accumulation in muscle, causing insulin resistance in mice and in T2DM patients[62].

A direct association between tissue-specific alteration of BCAA-catabolizing enzymes and OB-related elevation in plasma BCAAs/branched-chain alpha-keto acids (BCKAs) has been demonstrated in rodent models of OB (OB/OB mice and Zucker rats) and in obese human subjects who underwent surgical weight loss intervention[63]. Plasma concentrations of BCAAs were significantly higher (56%-84%) in randomly fed obese mice and rats (OB/OB mice and Zucker rats) than in lean controls, and BCAA elevation diminished after overnight fasting due to reduced BCAA elevation (by 30%) in obese mice[63]. Therefore, fasting plasma BCAA levels did not differ between lean and obese animals. BCAA metabolism was altered in liver and adipose tissue but not in muscle in fed obese mice, which contributed to elevated plasma BCAA levels. In comparison with lean controls, obese rodents (OB/OB mice and Zucker rats) show decreased expression and activity of BCATm and BCKD E1α in liver and epididymal fat along with increased decreasing branched-chain α-keto acid dehydrogenase (BCKD) kinase (BCKDK) expression, whereas no such changes were found in skeletal

Table 3 Correlation coefficients between serum amino acids and adiposity, body weight and body mass index in 97 type 2 diabetes patients differing in body mass index categories

Amino acids	Pooled (*n* = 97)				Obese (*n* = 22)				Overweight (*n* = 42)				Normal weight (*n* = 33)			
	VAT	Body weight	BMI	HDL	VAT	Body weight	BMI	HDL	VAT	Body weight	BMI	HDL	VAT	Body weight	BMI	HDL
Valine	0.375[b]	0.236[a]	0.212[a]	NS	0.311	0.217	0.009	NS	0.140	0.286[c]	0.223	NS	0.150	0.008	0.167	NS
Isoleucine	0.443[b]	0.287[b]	0.255[a]	-0.309[b]	0.109	0.106	-0.095	NS	0.347[c]	0.289[c]	0.301[d]	-0.417[b]	0.351	0.047	0.114	NS
Leucine	0.396[b]	0.315[b]	0.249[a]	-.276[b]	0.120	0.201	-0.068	NS	0.312	0.320[a]	0.340[a]	-0.345[a]	0.133	0.159	0.173	NS
Tyrosine	0.524[b]	0.341[b]	0.301[b]	NS	0.447	0.136	0.002	NS	0.251	0.343[a]	0.094	NS	-0.036	0.154	0.347[a]	NS
Phenylalanine	0.508[b]	0.264[b]	0.262[a]	-0.307[b]	0.177	0.176	0.027	NS	0.607[b]	0.096	0.171	-0.323[a]	0.185	0.156	0.085	NS
Tryptophan	0.373[a]	0.362[b]	0.236[a]	NS	0.382	0.076	-0.230	NS	0.107	0.339[a]	0.114	NS	-0.042	0.280	-0.012	NS
Methionine	0.379[b]	0.251[a]	0.228[a]	NS	0.229	0.223	0.105	NS	0.377[c]	0.327[a]	0.357[a]	NS	-0.263	0.050	0.296[c]	NS
Taurine	-0.367[a]	-0.001	0.047	NS	-0.479[c]	-0.043	-0.164	NS	-0.451[a]	-0.153	0.142	NS	-0.345	0.083	-0.082	NS
Alanine	0.429[b]	0.194[d]	0.268[b]	NS	0.640[a]	0.178	0.078	NS	-0.027	-0.041	-0.095	NS	0.304	-0.171	0.043	NS
Asparagine	0.278[d]	0.218[a]	0.079	-0.222[a]	0.200	0.333	0.049	-0.478[a]	0.129	0.287[c]	0.008	NS	-0.192	0.206	-0.052	NS
Glutamine	0.253[c]	0.157	0.052	NS	0.422	0.075	0.042	NS	-0.021	0.282[c]	0.043	NS	0.000	0.070	-0.072	NS
Glutamate	0.398[b]	0.263[b]	0.234[a]	NS	0.147	0.172	0.133	NS	0.291	0.181	0.021	NS	-0.024	0.113	-0.053	NS
Lysine	0.390[b]	0.272[b]	0.203[a]	NS	0.372	0.124	0.053	NS	0.237	0.277[c]	0.090	NS	-0.246	0.059	-0.054	NS
Hydroxyproline	0.293[a]	0.025	-0.008	NS	-0.428	0.080	0.015	-0.436[a]	0.276	0.172	0.056	NS	0.192	-0.105	-0.171	NS
Threonine	0.388[b]	0.237[a]	0.190[c]	NS	0.573[a]	0.322	0.233	NS	0.108	0.190	0.177	NS	0.012	0.088	0.005	NS
Citrulline	0.118	0.035	-0.046	NS	-0.146	-0.114	-0.043	NS	0.068	0.231	0.015	NS	-0.120	-0.052	-0.233	NS
Proline	0.314[a]	0.099	0.104	NS	0.295	-0.015	-0.162	NS	0.059	0.205	-0.061	NS	0.018	-0.310[c]	0.047	NS
Homocysteine	-0.132	0.007	-0.011	NS	0.232	0.475[a]	0.431	NS	-0.201	-0.109	-0.061	NS	0.251	-0.015	-0.020	NS
Histidine	0.278[d]	0.331[b]	0.250[a]	NS	0.316	0.358	0.123	NS	0.085	0.275[c]	0.231	NS	-0.274	0.100	0.012	NS
Arginine	0.167	0.042	0.042	NS	0.097	-0.189	-0.226	NS	0.122	0.078	-0.100	NS	-0.215	-0.182	-0.088	NS
β-Alanine	-0.134	-0.073	-0.108	NS	0.241	-0.108	0.069	NS	-0.360[c]	-0.175	-0.329[a]	NS	0.193	0.306[c]	-0.037	NS
Carnosine	0.278[d]	0.162	0.157	NS	0.113	-0.121	-0.143	NS	0.056	0.062	0.054	NS	-0.649[a]	0.200	-0.156	NS
Serine	0.149	0.151	0.039	-0.240[a]	-0.157	-0.031	-0.154	NS	0.121	0.159	-0.121	NS	-0.220	0.465[b]	0.111	NS

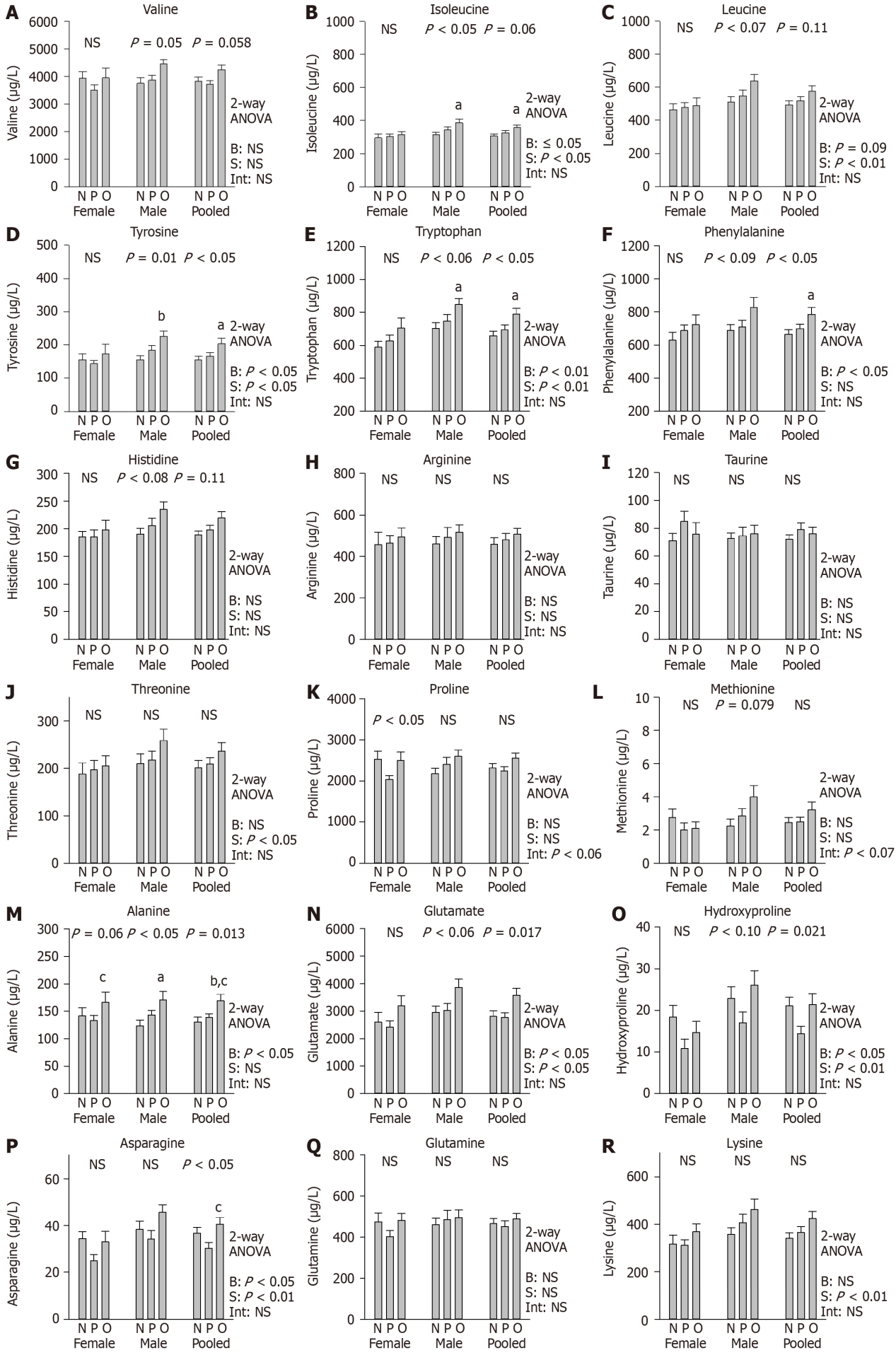
[a]$P < 0.05$.

[b]$P < 0.01$.
[c]$P < 0.1$.
[d]$P < 0.06$.
BMI: Body mass index; HDL: High density lipoprotein; VAT: Visceral adipose tissue; NS: Not significant.

muscle[63]. Because the postprandial elevation in BCAAs was more sensitive to fasting-induced BCAA reduction in obese rodents than in lean controls, this dynamic change in serum BCAAs suggests that postprandial samples rather than fasting samples could be better for analysis of potential T2DM predictors. The greater alteration in postprandial BCAAs/BCKAs in OB also indicates deficient BCAA/BCKA metabolic or disposal capabilities in the obese population. Furthermore, BCKAs are a more sensitive metabolic marker than BCAAs for OB[64]. Compared to wild-type animals, young male obese Zucker rats (with mutated leptin) show decreased BCKD activity in the kidney, heart, gastrocnemius and liver (-66% to -47%), increased plasma BCAAs (45%-69%) and BCKAs (100%) and hepatic BCKAs (193%-418%), leucine oxidation (23%), proteolysis (35%), urinary marker of proteolysis (183%-766%), increased dietary intake (23%), whole body protein synthesis (23%-29%), body weight (53%), liver weight (107%) and adiposity (300%)[64].

Among our findings, the male subjects exhibited higher levels of serum amino acids (including BCAAs and AAAs) and greater differences in amino acids between the obese and NW T2DM subjects than the female subjects. These sex differences are consistent with previous reports[65,66]. Although the underlying mechanism is unknown, animal studies have shown that the female steroid hormone 17β-estradiol stimulates BCAA catabolism by increasing the activity of the BCKD and by BCKDK, which is an inhibitor of BCKD activity[67]. As most of the female subjects in our study were of reproductive age, a potential confounding effect of estrogen may account for the sex difference in serum BCAA. Nevertheless, the moderate BMI-dependent BCAA elevation in the female subjects in our study may become significant if a large sample size or more postmenopausal women were included. Indeed, one cohort study of 2204 (most postmenopausal) women, including 115 T2DM patients, 192 individuals with impaired fasting glucose (IFG) and 1897 control individuals, who differed significantly in BMI (30.6 *vs* 27.9, *vs* 25.4, respectively) and age (63 *vs* 60 *vs* 50 years, respectively), showed significant BCAA elevation in subjects with T2DM and IFG compared with normal control individuals[68]. Moreover, among the elevated BCAA/BCKA metabolites, 3-methyl-2-oxovalerate was the strongest predictive biomarker for IFG with moderate heritability (h^2 = 0.20), based on the single-nucleotide polymorphism rs1440581 of the gene encoding for protein phosphatase (PP2Cm), which is needed for maintaining the activity of BCKD, the rate-limiting enzyme for BCKA catabolism[68]. However, that study may be confounded by a potentially higher estrogen level in the younger peri-postmenopausal control women (< 50 years) than in the postmenopausal women of the T2DM and IFG groups (both ≥ 60 years). Another study showed that higher diet and plasma BCAA concentrations were associated with increased T2DM risk among women with gestational diabetes, independent of BMI and other risk factors [69].

In the present study, BCAAs and AAAs were more closely correlated with abdominal adiposity than with body weight or BMI, indicating an important role of adiposity in hyperaminoacidemia/BCAA/AAA elevation. Other studies showed that the association of dietary BCAAs with T2DM risk/remission was dependent on the type of dietary fat, baseline triglycerides and body weight, as a Mediterranean diet rich in extravirgin olive oil (Med-diet) significantly reduced blood BCAAs and attenuated the association between plasma BCAA levels and T2DM incidence after a 3.8-year follow-up of 945 people compared to a control low-fat diet (LF)[70], and the Med-diet was associated with T2DM remission and BCAA reduction measured after an oral glucose tolerance test[28]. In addition, baseline plasma BCAAs indicated whether the LF or Med diet was capable of inducing T2DM remission. In diet-induced obese mice, high dietary fat increased the circulating BCAA pool, BCAA catabolism and OB by altering the gut microbiota[71,72]. BCAA supplementation to a


A
Valine
Valine (μg/L)
NS P = 0.05 P = 0.058
2-way ANOVA
B: NS
S: NS
Int: NS
B
Isoleucine
Isoleucine (μg/L)
NS P < 0.05 P = 0.06
a
a
2-way ANOVA
B: ≤ 0.05
S: P < 0.05
Int: NS
C
Leucine
Leucine (μg/L)
NS P < 0.07 P = 0.11
2-way ANOVA
B: P = 0.09
S: P < 0.01
Int: NS
D
Tyrosine
Tyrosine (μg/L)
NS P = 0.01 P < 0.05
b
a
2-way ANOVA
B: P < 0.05
S: P < 0.05
Int: NS
E
Tryptophan
Tryptophan (μg/L)
NS P < 0.06 P < 0.05
a
a
2-way ANOVA
B: P < 0.01
S: P < 0.01
Int: NS
F
Phenylalanine
Phenylalanine (μg/L)
NS P < 0.09 P < 0.05
a
2-way ANOVA
B: P < 0.05
S: NS
Int: NS
G
Histidine
Histidine (μg/L)
NS P < 0.08 P = 0.11
2-way ANOVA
B: NS
S: NS
Int: NS
H
Arginine
Arginine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: NS
Int: NS
I
Taurine
Taurine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: NS
Int: NS
J
Threonine
Threonine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: P < 0.05
Int: NS
K
Proline
Proline (μg/L)
P < 0.05 NS NS
2-way ANOVA
B: NS
S: NS
Int: P < 0.06
L
Methionine
Methionine (μg/L)
NS P = 0.079 NS
2-way ANOVA
B: NS
S: NS
Int: P < 0.07
M
Alanine
Alanine (μg/L)
P = 0.06 P < 0.05 P = 0.013
c
a
b,c
2-way ANOVA
B: P < 0.05
S: NS
Int: NS
N
Glutamate
Glutamate (μg/L)
NS P < 0.06 P = 0.017
2-way ANOVA
B: P < 0.05
S: P < 0.05
Int: NS
O
Hydroxyproline
Hydroxyproline (μg/L)
NS P < 0.10 P = 0.021
2-way ANOVA
B: P < 0.05
S: P < 0.01
Int: NS
P
Asparagine
Asparagine (μg/L)
NS NS P < 0.05
c
2-way ANOVA
B: P < 0.05
S: P < 0.01
Int: NS
Q
Glutamine
Glutamine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: NS
Int: NS
R
Lysine
Lysine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: P < 0.01
Int: NS
N P O
Female
Male
Pooled

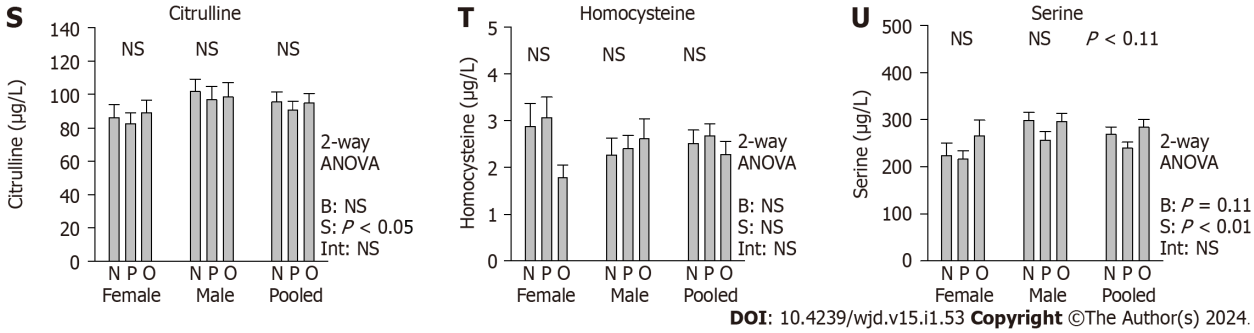


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Figure 1 One-way and two-way ANOVA of the effects of body mass index and sex on serum amino acid concentrations in male and female new-onset type-2 diabetes patients in differing body mass index categories. A: Valine; B: Isoleucine; C: Leucine; D: Tyrosine; E: Tryptophan; F: Phenylalanine; G: Histidine; H: Arginine; I: Taurine; J: Threonine; K: Proline; L: Methionine; M: Alanine; N: Glutamate; O: Hydroxyproline; P: Asparagine; Q: Glutamine; R: Lysine; S: Citrulline; T: Homocysteine; U: Serine. N: Normal weight; P: Overweight; O: Obese group. [a]$P < 0.05$ *vs* normal weight; [b]$P < 0.01$ *vs* normal weight; [c]$P < 0.05$ *vs* overweight; NS: Not significantly different ($P > 0.05$); B: Body mass index; S: Sex/sex; Int: BMI × sex interaction.

high-fat diet increased mTOR activity, whereas BCAA supplementation to a normal diet did not affect mTOR activity in animals[73].

Limitations and prospective use of BCAAs/AAAs in predicting different subtypes of T2DM

While elevated BCAAs/BCKAs may induce and interact with FFA accumulation in obese T2DM candidates, T2DM development in lean/NW populations may arise from different mechanisms. Unlike the gold standard for predicting different subtypes of T2DM diagnosis (*i.e.*, fasting glucose and HbA1c), so far, there is no established standard for T2DM prediction. Although BCAAs/AAAs is a promising predictor, its utility may be affected by a range of factors including race/ethnicity, age, sex, body weight/BMI, and subtypes of T2DM. The heterogeneous elevation of BCAAs/AAAs among new-onset T2DM patients indicates limitations and restricted utility of BCAAs/AAAs as the predictor for different subtypes of T2DM. While the greater BCAAs/AAAs elevation in obese T2DM patients would support its' prediction in individuals with OB(-propensity) which account for a large portion of T2DM population, the lower level, or a lack of BCAAs/AAAs elevation in normal-weight and reproductive-aged females would diminish its' predicting power in these individuals.

A longitudinal 12-year follow up study of old adults (56 ± 8 years) showed that when BMI-matched obese non-T2DM individuals (n = 189/group, BMI = 30) were compared, the highest quartile of individual with elevated baseline BCAAs/AAAs had a 2- to 3.5-fold higher odds of risk of developing diabetes per SD increment over a 12-year follow-up period based on individual BCAAs/AAAs, or a 5- to 7-fold higher odds of developing diabetes if all BCAAs/AAAs were combined, in comparison with those individuals whose plasma amino acid levels were in the lowest quartile[16]. However, such increments in odds of risk were reduced to 1.3 and 2.0, respectively if the obese T2D candidates were compared with NW controls (n = 400, BMI = 25) randomly selected form a larger pool. Thus, how the controls are selected can lead to very different outcomes.

Similarly, a meta-analysis show that MUO, MUOW and MUNW individuals would show similar 4-fold risk increase of developing T2DM if each of them is compared with healthy counterparts of their corresponding BMI category (*vs* MHO, MHOW, and MHNW, respectively), but MUO and MUOW would have 2-3-fold higher risks than the MU-NW group if each of them is compared with MH-NW[45]. Thus, BCAAs/AAAs elevation would be greater in obese T2DM candidates than in NW T2DM candidates if all were compared with NW controls, but similar BCAAs/AAAs elevations across different BMI categories if obese, OW and NW T2DM candidates were compared with same BMI categories controls, respectively.

T2DM is a highly heterogeneous disease that include latent autoimmune diabetes in adults (LADA, defined by the presence of glutamic acid decarboxylase antibodies (GADA), maturity onset diabetes of the young (MODY, defined by gene mutations that disrupt insulin production) and neonatal diabetes, in addition to insulin resistant and BMI-related subgroups.

A recent data-driven cluster analysis of 14755 European T2DM patients using six variables (GADA, age at diagnosis, BMI, HbA1c, β-cell function and insulin resistance) resulted in 5 well-separated novel subgroups of adult-onset diabetes with distinct outcomes: A cluster of more severe insulin resistant individuals associated with higher risk of diabetic kidney disease; insulin deficiency cluster associated with highest risk of retinopathy; relatively young insulin deficient individuals with poor glycemic control (high HbA1c) and; a larger group of elderly patients with benign disease course [74]. That finding has been confirmed and extended by another cluster analysis of 2316 Chinese T2D patients and 685 United States T2D patients using five variables (age at diagnosis, BMI, HbA1c/glucose, β-cell function and insulin resistance) that resulted in 4 clusters: Half of the patients were elders with milder metabolic derangements; 25% of the patients had the highest BMI values but average blood glucose, β-cell function and insulin resistance; 14% of the patients had severe insulin deficiency and highest blood glucose; 8% of the patients were elders with severe insulin resistance and β-cell dysfunction[75]. Similar results of cluster analysis were reported in 55777 individuals with prediabetes[76]. However, none of these studies included BCAAs/AAAs as the study variable. Thus, the contributions of BCAAs/AAAs to different clusters or subtypes of T2DM remain unknown.

Nevertheless, BCAAs/AAAs elevation, if standardized based on different age and BMI sub-groups, could be useful in screening future prediabetes and OB-related diabetes in infants and adolescents as blood BCAAs/AAAs were found significantly correlated with BMI standard deviation score, fasting glucose, HbA1c, triglycerides, cystatin C and creatinine in 2191 healthy participants aged 3 months to 18 years[77]. A 7.5-year longitudinal study of 396 nondiabetic Finnish girls showed that serum BCAA profile in childhood (11.2 ± 0.8 years at baseline) were associated with insulin resistance during pubertal development (significant both before and after menarche) independent of adiposity, and it predicted dysregulated glycemic and triglyceride levels in adulthood[78-80]. Blood BCAA/AAA were also found significantly elevated in OW and obese prepubertal children than in healthy controls[39-41,81].

The global prevalence of prediabetes in children and adults have reached alarming levels[81], with an annualized diabetes conversion rate of 5%–10%[82]. And 500 million adults in China (50% of China's adult population) and 98 million adult Americans (38.0% of the United States adult population) have prediabetes (https://www.cdc.gov/diabetes/data/statistics-report/index.html#anchor_23827), the world T2DM population could double by 2050. In the United States, the total cost of diagnosed diabetes in 2022 was $413 billion, including $106 billion in indirect costs (https://www.cdc.gov/diabetes/health-equity/diabetes-by-the-numbers.html). Because preventive lifestyle modification can reduce the risk of diabetes by up to 70%[83], identification of "at-risk" individuals 10-20 years prior to T2DM onset based on BCAAs/AAAs elevation would offer plenty time for lifestyle modifications.

While BCAAs/AAAs elevation alone may not predict all subtypes of T2DM, its combined use with other anthropogenic, metabolic, and genetic biomarkers such as visceral adiposity index, muscle mass index[47-50], fasting glucose, GADA (associated with LADA), genetic polymorphisms (associated with MODY), and metabolic parameters associated with insulin deficiency, diabetic kidney disease and retinopathy should be evaluated in future studies.

Study limitations

This study has limitations. The lack of BMI-matched healthy control individuals makes it impossible to evaluate a potential low grade hyperaminoacidemia/BCAA/AAA elevation that may exist in NW-T2DM patients. Only fasting samples, not postprandial/oral glucose tolerance test samples or BCKAs, were studied, which may have missed the dynamic changes in BCAA catabolism. The moderate sample size of this cross-sectional study could not discern the causality of the findings. The lack of lifestyle data limits exploration of the influences of diet and social, psychological, and physical activities on BCAA/AAA. Anti-diabetic medication taken by some T2DM patients may have compromised the results[84,85]. In addition, no genetic, race/ ethnicity influences can be derived from this study, although sex differences and significant correlations between BCAAs/AAAs and anthropometric parameters were demonstrated.

Future studies should overcome the above limitations of this study. To date, most of the published BCAAs/AAAs findings were based on comparisons between obese T2DM patients and nonobese individuals, whereas the BCAAs/AAAs data of the lean/NW-T2DM groups were largely unreported. This publication bias should be addressed by stratified analyses for age, BMI, adiposity, sex, and genotype as discussed above and by comparisons between metabolically healthy *vs* metabolically unhealthy individuals of different BMI categories.

CONCLUSION

In summary, heterogeneous elevation of BCAAs/AAAs is found in new-onset T2DM patients. While the greater BCAA/AAA elevation in obese and male T2DM patients would support BCAA/AAA prediction of T2DM development in these individuals, the lower or lack of elevated BCAAs/AAAs in NW and reproductive-aged female T2DM patients could compromise its prediction in these people. This potentially skewed T2DM prediction should be considered when BCAAs/AAAs are to be used as the T2DM predictor. As BCAAs/AAAs can predict T2DM as early as from childhood to early adulthood and 1-2 decades prior to T2DM onset[86-89], and because BCAA/BCKA elevation can be effectively normalized through diet, exercise, weight control interventions and pharmacogenetic therapy[84-86], study and normalization of BCAA/AAA elevation could provide a novel opportunity for curbing global T2DM epidemic.

ARTICLE HIGHLIGHTS

Research background

Type-2 diabetes mellitus (T2DM) is a major cause of comorbidity and mortality in society and was responsible for more than 4.2 million annual deaths in 2019 alone. The current world population of T2DM (approximately 450 million) is expected to double to 1 billion soon after 2050. This T2DM pandemic, however, can be curbed or prevented if the population at risk of T2DM can be identified and prophylactic actions be taken long before the onset of T2DM.

Research motivation

Research over the past decades has indicated that elevated branched-chain amino acids (BCAAs: Isoleucine, leucine, valine) and aromatic amino acids (AAAs: Tyrosine, tryptophan, phenylalanine) show high sensitivity and specificity (both > 97%) in predicting diabetes in animals and can successfully predict T2DM nearly 20 years before T2DM onset in select human populations. However, these findings have not been widely translated into clinical utilization due to unidentified factors.

Research objectives

We hypothesized that body weight and sex are potential confounding factors that could affect BCAAs/AAAs as general T2DM predictors. As the first step, the aim of our study was to determine the effects of body weight and sex on BCAAs/AAAs in new-onset T2DM individuals.

Research methods

Fasting blood samples were collected from 97 new-onset T2DM patients (53 male/44 female, 43.3 ± 11.2 years of age, differing in body mass index (BMI): Normal weight (NW), n = 33, BMI = 22.23 ± 1.60; overweight, n = 42, BMI = 25.9 ± 1.07; and obesity, n = 22, BMI = 31.23 ± 2.31). All T2DM cases were diagnosed within 12 mo at the First People's Hospital of Yunnan Province, Kunming, China. Serum amino acids were analyzed using ultra-performance liquid chromatography/triple stage quadrupole mass spectrometry.

Research results

Fasting serum AAAs, BCAAs, glutamate, and alanine levels were significantly greater and high-density lipoprotein levels were significantly lower in obese T2DM patients than in NW-T2DM patients, especially among male patients. Arginine, histidine, leucine, methionine, and lysine were greater in male patients than in female patients. Moreover, histidine, alanine, glutamate, lysine, valine, methionine, leucine, isoleucine, tyrosine, phenylalanine, and tryptophan were significantly correlated with abdominal adiposity, body weight and BMI, respectively.

Research conclusions

Heterogeneously elevated amino acids, especially BCAAs/AAAs, are found in new-onset T2DM patients in differing BMI categories, which may indicate a potentially skewed prediction of T2DM development by BCAA/AAAs, *i.e.*, more accurate and reliable prediction in obese and male individuals than in NW individuals and females. This skewness may limit the universal application of this T2DM predictor.

Research perspectives

This study has limitations. The lack of BMI-matched healthy control individuals makes it impossible to determine whether a potential low grade hyperaminoacidemia/BCAA/AAA elevation exists in normal-weight T2DM patients. The moderate sample size and lack of lifestyle and genetic data of this cross-sectional study could not discern the causality and/or mechanisms of the heterogeneity. Further studies should include both metabolically healthy and metabolically unhealthy individuals in differing BMI categories to overcome the abovementioned limitations.

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FOOTNOTES

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Co-corresponding authors: Guo-Qiang Xing and Heng Su.

Author contributions: Wang M, Xing GQ and Su H conceptualized and designed the research; Ou Y, Niu B and Kang Z screened patients and acquired clinical data; Wang M, Yuan XL, Zhang B and Zhu XF collected blood specimen and performed laboratory analysis; Wang M, Ahmed A, Yuan XL and Xing GQ performed Data analysis; Wang M, Su H and Xing GQ wrote the paper. All the authors have read and approved the final manuscript. Wang M proposed, designed and conducted serum amino acids analysis, performed data analysis and prepared the first draft of the manuscript. Ou Y was responsible for patient screening, enrollment, collection of clinical data and blood specimens. Both authors have made crucial and indispensable contributions towards the completion of the project and thus qualified as the co-first authors of the paper. Both Su H and Xing GQ have played important and indispensable roles in the experimental design, data interpretation and manuscript preparation as the co-corresponding authors. Su H applied for and obtained the funds for this research project. Su H conceptualized, designed, and supervised the whole process of the project. He searched the literature, revised and submitted the early version of the manuscript with the focus on the association between visceral adipose tissue (VAT) and BCAA/AAA. Xing GQ was instrumental and responsible for data re-analysis and re-interpretation, figure plotting, comprehensive literature search, preparation and submission of the current version of the manuscript with a new focus on BCAAs/AAAs as the predictors of diabetes and on potential underlying mechanisms. This collaboration between Su H and Xing GQ is crucial for the publication of this manuscript and other manuscripts still in preparation.

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