Different composition of serum amino acids and bile acids in patients with impaired fasting glucose and type 2 diabetes mellitus compared with normal controls

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Directed by Professor Jeong-Ho Kim

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ABSTRACT

Different composition of serum amino acids and bile acids in patients with impaired fasting glucose and type 2 diabetes mellitus compared with normal controls

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Background: Recent advance of metabolomics has made remarkable progress in discovery of novel biomarkers. Among these biomarkers, blood amino acids (AAs) profiles have produced a number of promising results. Elevated blood levels of AAs, especially branched chain amino acids (BCAAs) have been reported in Type 2 DM (T2DM), obesity, and cardiovascular disease. In addition, emerging researches shows the importance of bile acids (BAs) in glucose homoeostasis. Therefore, we performed AAs and BAs profiling in patients with impaired fasting glucose (IFG) and T2DM as well as healthy control to find specific alteration of these profile in patients with IFG and T2DM.

Methods: Twenty AAs and fifteen kinds of BAs were analyzed in samples from 72 T2DM patients without diabetic medications, 97 patients with IFG and 75

healthy control subjects using high performance liquid chromatography (HPLC) - tandem mass spectrometry. Serum TNF-α and IL-6 were measured using a commercially available human ELISA kits. The C2C12 mouse myoblast cell lines were used to examine the changes of MAFbx and MuRF1 expressions after development of insulin resistance induced by palmitate treatment.

Results: Fasting serum AAs, not only BCAAs but also glutamic acid, lysine, phenylalanine, arginine, alanine, tyrosine, aspartic acid are higher in patients with T2DM and intermediate in patients with IFG compared with normoglycemic controls. These serum AAs concentrations positively correlated with fasting glucose, HOMA-IR, and pro-inflammatory cytokines (TNF-α and IL-6). In addition, HOMA-IR and pro-inflammatory cytokines were two important independent predictor of blood AAs level. In vitro experiments showed that palmitate treatment in C2C12 myotubes induced insulin resistance, increased pro-inflammatory cytokine gene expression, and increased MAFbx gene and protein expression. Finally, we found that glycine conjugated BAs were decreased in the patients with IFG and T2DM.

Conclusion: Fasting blood AAs increase can be early manifestation of insulin resistance. Increased muscle proteolysis by insulin resistance and inflammatory cytokines is possible mechanism of these AAs elevation. Glycine conjugated BAs are decreased in the patients with IFG and T2DM suggesting control of glycine conjugation and deconjugation of BAs could affect glucose homeostasis.

Key words: amino acids profile, branched chain amino acids, inflammatory cytokines, bile acids profile, insulin resistance, type 2 diabetes mellitus

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I. INTRODUCTION

Diabetes mellitus (DM) is a complex endocrine and metabolic disorder characterized by hyperglycemia.[1] Type 2 DM (T2DM), the most common type of DM, is a heterogeneous and progressive disorder with variable degrees of insulin resistance and pancreatic beta cells dysfunction, which resulted from several genetic and environmental factors such as obesity, low exercise, high calorie intake and stress.[2] The prevalence of T2DM and prediabetes have been globally increasing in both developed and developing countries because of eating habit shift towards western style, inactivity, and the aging of the adult population.[3] In Korea about 9.9% of the population (2.8 million people) suffered from T2DM according to the KNHANES 2007-2009.[4] Pathophysiologic mechanism of metabolic diseases often begins for years before clinical symptoms become apparent.[5] For T2DM, considerable insulin resistance and pancreatic β cell insufficiency has already occurred by the time relative insulin deficiency manifests as hyperglycemia and a diagnosis of T2DM is made.[5]

In recent years, metabolomics has made remarkable progress with continually emerging novel biomarkers. Among these biomarkers, plasma amino acids (AAs) profiles have produced an overwhelming number of promising results.[3] A number of observational studies indicated that elevated blood levels of branched chain amino acids (BCAAs), which include leucine, isoleucine and valine, with or without aromatic AAs were associated with obesity, insulin resistance, and T2DM.[6-14] Furthermore, increased levels of BCAAs or aromatic AAs have been also linked to the metabolic syndrome[15] and cardiovascular disease.[16-18] Several longitudinal analysis in different cohorts confirmed that blood levels of AAs could predict the future development of insulin resistance or T2DM.[5,19-26] The Framingham Offspring Study over a 12-year period revealed that BCAAs and aromatic AAs concentrations had significant associations with the future development of diabetes, which has led to speculation about a potential causative role for BCAAs.[5] However, it is still unclear whether BCAAs are a causative factor in insulin resistance and T2DM or just an early manifestation of impaired insulin action.[27]

Unlike BCAAs, the change of aromatic AAs in insulin resistance and T2DM were inconsistent, some studies reported increased level but others showed no significant difference[28] Furthermore, the mechanism of increased aromatic AAs in insulin resistance and diabetes is unknown. For glycine, some studies presented decreased concentration and others did not.[28-30] With these inconsistent results regarding AAs except BCAAs, there have been few studies about an association between insulin resistance and AAs profiles in Asian populations.

In addition, emerging researches show the importance of bile acids (BAs) in glucose homoeostasis.[31,32] BAs are amphipathic steroids that are synthesized from cholesterol exclusively in the liver. These molecules are conjugated by either taurine or glycine to increase hydrophilicity, secreted into the bile and then released into the intestine.[31]

The compositional diversity of the BAs pool in the body could be increased by conversion of primary bile acids into secondary BAs by intestinal bacteria.

BAs are ligands of the nuclear farnesoid X receptor (FXR) that plays an important role in the regulation of synthesis of BAs, as well as lipid, glucose and energy metabolism.[33] BAs also act as signaling molecules through the cell surface G protein-coupled receptor, TGR5.[33] BAs mediated TGR5 signaling promotes glucagon-like peptide 1 (GLP-1) release by enteroendocrine cells, which induce insulin secretion form pancreatic β cells.[31]

The biological properties of BAs depend on their chemical structure.[33] Thus, not all BAs are equally effective in the activation of FXR and TGR5. The ranking of BAs in potency of their receptor activation is, from the highest to the lowest potency: chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA) and cholic acid (CA) for FXR[34]; LCA, DCA, CDCA and CA for TGR5.[35] This different activity among BAs implies that changes in the composition of BAs might induce altered regulation of glucose metabolism through altered FXR or TGR5 activity resulting in disease states, such as T2DM.

Blood BAs composition in T2DM has been assessed by several researchers, with inconsistent results.[36-41] Brufau et al. showed that patients with T2DM exhibited increased concentrations of DCA and decreased concentrations of CDCA.[40,41] Haeusler et al. supported this result with the finding that the ratio of 12α-OH/non-12α-OH BAs were significantly associated with insulin resistance.[37] A larger ratio of 12α-OH/non-12α-OH BAs implied relatively lower concentrations of CDCA, which is a major component of non-12α-OH BAs and the most potent FXR agonist. Thus, these results revealed that alterations in the BAs pool composition may play an important role in the pathophysiology of T2DM through changing FXR activity. However, recent studies regarding blood BAs composition in T2DM did not support

this hypothesis showing different alteration in BAs profile.

Therefore, we performed AAs and BAs profiling in patients with impaired fasting glucose (IFG) and T2DM and healthy controls to find specific alteration of these profile in Asian patients with insulin resistance and T2DM. Furthermore, we tried to find mechanisms of these alteration in the patients with insulin resistance and T2DM.

II. MATERIALS AND METHODS

1. Subject

From September 2015 to August 2016, we recruited 72 T2DM patients without diabetic medications, 97 patients with IFG, and 75 healthy control subjects by the Diabetes Center at the tertiary-level, university-affiliated Severance Hospital, Yonsei University College of Medicine, and stored the residual serums. The diagnosis of IFG and T2D was determined based on the American Diabetes Association guidelines.[42] Subjects who met the following criteria were excluded based on our protocol: (1) subjects who took medications known to affect glucose metabolism, (2) types of diabetes other than T2DM. Written informed consent was secured from all of the participants, and the protocol of this study was approved by the Institutional Review Board at Severance Hospital (No. 4-2015-0503).

2. Clinical and laboratory measurements

Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters. Serum levels of fasting glucose, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, uric acid, and total bilirubin levels were determined by Hitachi 7600 analyzer (Hitachi Co., Tokyo,

Japan). Glycosylated hemoglobin (HbA1c) was measured by immunoassay using Integra 800 (Roche Diagnostics GmbH, Penzberg, Germany). Fasting serum insulin and c-peptide levels were determined by electrochemiluminescent method using cobas e601 analyzer (Roche Diagnostics GmbH). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by using the following equation: fasting plasma glucose (mg/dL) × fasting insulin (μU/ml) / 405.[43]

3. Amino acids and cytokine analysis

Twenty major AAs including alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine were analyzed in samples stored at -70°C, using high performance liquid chromatography (HPLC) - tandem mass spectrometry and aTRAQ™ Kits (AB SCIEX, Foster City, CA, USA). Analysis was performed by first precipitating any proteins with sulfosalycylic acid, followed by labeling with amine-reactive isotope-coded tags according to the manufacturer's instructions. The sample was labeled with the Δ8 reagent and the internal standard was pre-labeled with the Δ0 reagent. The Agilent 1260 Infinity LC system (Agilent Technologies Inc., Santa Clara, CA, USA) with a C18 reverse-phase column (5 μm, 4.6 mm x 150 mm) was used to separate AAs. The AAs were monitored using API 3200 triple quadrupole tandem mass spectrometry (AB SCIEX) with positive electrospray ionization (ESI) in multiple-reaction monitoring (MRM) mode. Quantification of the individual AAs was calculated using a ratio of the sample to internal standard peak areas.

Serum TNF-alpha and IL-6 were measured using a commercially available human ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions.

4. Bile acids analysis

Fifteen kinds of BA including CA, CDCA, DCA, ursodeoxycholic acid (UDCA), LCA, and their respective taurine and glycine conjugates (T-CA, T-CDCA, T-DCA, T-UDCA, T-LCA, G-CA, G-CDCA, G-DCA, G-UDCA, and G-LCA) were analyzed using HPLC - tandem mass spectrometry and Biocrates Bile Acids Kits (BIOCRATES Life Sciences, Innsbruck, Austria) according to the manufacturers' instructions. All target isobaric BAs could be baseline separated under HPLC systems (Agilent 1260 Infinity LC system) with flow rate of 0.4 mL/min and runtime of 11 min. A proprietary reversed-phased HPLC column for Biocrates Bile Acids Kit (Biocrates Life Sciences) was used. Mass spectrometric detection was accomplished with negative ESI in MRM mode using 5500 QTrap (AB SCIEX).

5. C2C12 myoblasts culture and differentiation

C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Corning Cellgro, Manassas, VA, USA) and 1% antibiotic-antimycotic (Gibco-BRL, NY, USA). The cells were maintained at 37°C in a humidified incubator with a 5% CO_2 atmosphere. For C2C12 differentiation, C2C12 were seeded in six well plates (1 x 10^5 cells per well) with 10% FBS and 1% antibiotic-antimycotic DMEM medium. After 3 days, cell were 90%-100% of confluence, the medium was changed every day with 2% horse serum (HS) (Gibco-BRL) and 1% antibiotic-antimycotic (Gibco-BRL) until 5-7 days for differentiation.

6. Bovine Serum Albumin (BSA) -Conjugated Palmitate treatment

Palmitate (Sigma-Aldrich, St Louis, MO, USA) was dissolve in 99% ethanol (EtOH)

by heating at 70°C and the stock solutions were stored at -20°C. We filtrated 2% fatty acid free BSA (Gold biotechnology, St Louis, MO, USA)-serum free medium with 0.45μm pore syringe filter (Merck Millipore, Darmstadt, Germany) before use. For conjugating, the palmitate solution were diluted with 2% fatty acid free BSA-serum free medium and incubate at 37°C for one hour with inverting every 10 min. For BSA-conjugated palmitate treatment, we change the culture medium with serum free medium and then palmitate were treated for 24 hours. For insulin stimulation, 5 μg/mL insulin was treated for 15 min before cell harvest.

7. Cell viability assay

Cell viability was measured using water soluble tetrazolium (WST) based cell viability assay kit, EZ-Cytox (DoGen-bio, Seoul, Korea). C2C12 cells were differentiated and BSA-conjugated palmitate was treated for 24 hours. WST solution was added to each well, after one hour incubation the absorbance of formazan was measured on SpectraMAX 250 ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

8. Western blot analysis

C2C12 mycotubes were lysed in lysis buffer (20 mM Tris pH 7.5, 140 mM NaCl, 1 mM EDTA, and 0.1% NP-40) containing a Xpert protease inhibitor cocktail and phosphatase inhibitor cocktail (GenDEPOT, Barker, TX, USA). The cell lysate was centrifuged for 20 min at 13,200 rpm at 4°C and the supernatant was collected. Equal amounts of protein (10-20 μg) were heat-denatured in 4× sample buffer (2% sodium dodecyl sulfate, 62.5 mM Tris (pH 6.8), 0.01% bromophenol blue, 1.43 mM mercaptoethanol, and 0.1% glycerol), separated on 10% or 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto polyvinylidene

fluoride (PVDF) transfer membrane (Merck Millipore). After transfer, the membranes were blocked with 5% BSA (Bovagen biologicals, Victoria, Australia) or 5% skim milk (Becton Dickinson and company, Sparks, MD, USA) for one hour and incubated with primary antibody dissolved in 5% BSA overnight at 4°C. After that the membranes were incubated with anti-mouse or anti-rabbit secondary antibody conjugated with HRP (Bethyl Laboratories Inc, Montgomery, TX, USA) for 1 hour, followed by protein bands were detected by Clarity western ECL substrate (Bio-Rad, Hercules, CA, USA) using LAS 4000 image analyzer. The following antibodies were used: anti-Akt, anti-p-Akt (Ser 473) (Cell signaling, Danvers, MA, USA), anti-insulin receptor substrate-1 (IRS-1), anti-p-IRS-1 (Tyr632), anti-MAFbx (Muscle atrophy F-box/Atrogin-1), anti-MuRF1 (muscle RING Finger 1) antibodies (Santa Cruz, Dallas, CA, USA).

9. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the C2C12 mycotubes using Isol-RNA Lysis Reagent (5prime, Gaithersburg, MD, USA) according to the manufacturer's protocol. Single-stranded complementary DNA (cDNA) was synthesized using 1 μg of total RNA by ReverTra Ace® qPCR RT Master Mix (TOYOBO, Tokyo, Japan). The PCR for β-actin (used as an internal control) and target genes were performed on Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq ll (TaKaRa, Kyoto, Japan). Oligonucleotide primers for mouse genes used for PCR amplification were as follows:

MAFbx: 5'-CATCCCTGAGTGGCATCG-3' (sense)
5'- GAGTCTGGAGAAGTTCCCGTAT-3' (anti-sense);

MuRF1: 5'-ATCCTGCCCTGCCAACAC-3' (sense)
5'-CGGAAACGACCTCCAGACAT-3' (anti-sense);

TNF-α: 5'-CGTCAGCCGATTTGCTATCT-3' (sense)
5'-CGGACTCCGCAAAGTCTAAG-3' (anti-sense);
IL-6: 5'-GAGGATACCACTCCCAACAGACC-3' (sense)
5'-AAGTGCATCATCGTTGTTCATACA-3' (anti-sense);
Glut-4: 5'- TCTCAATGGTTGGGAAGGAAA-3' (sense)
5'-GAACCGTCCAAGAATGAGTATCTC-3' (anti-sense);
β-actin: 5'-CCTCTATGCCAACACAGTGC-3' (sense)
5'-CCTGCTTGCTGATCCACATC-3' (anti-sense)

10. Statistical analysis

Demographic characteristics were presented as median and first and third quartile or N (%). Each AAs and BAs concentrations were compared by groups using Kruskal-Wallis test. Jonckheere-Terpstra tests were used for trend analysis between groups. Spearman's rank correlation analysis were used to assess the relationship between individual AAs and metabolic parameters including BMI, triglycerides, total cholesterol, HDL-C, HbA1c, fasting glucose, fasting insulin, and HOMA-IR. We also performed correlation analyses to examine the relationship between each AA levels and TNF-α or IL-6 concentration. Multiple linear regression was used to identify independent predictors of increased AAs in the study participants. Significant differences in gene and protein expression between the control group and treatment group were determined using the t test. A P value of <0.05 was considered statistically significant. Statistical analyses were conducted using the PASW Statistics software (v18.0.0, SPSS Inc, Chicago, IL, USA) or the Analyze-it for Microsoft Excel 3.90.3 (Analyze-it Software Ltd., UK). All in vitro experiment results are presented as representative data from six experiments.

III. RESULTS

1. Participant characteristics according to diabetic status

General characteristics of study participants are presented in Table 1 according to diabetic status. Gradual increase of fasting glucose and HbA1c levels were observed in IFG and DM groups compared with normal control group. Significant differences in liver enzymes such as AST and ALT were also observed between groups. Greater levels of triglycerides, HOMA-IR, insulin, and c-peptides were found in DM, followed by IFG and normal group. In contrast, decreasing trend of HDL-C concentrations were observed between groups. In addition, significant differences in weight, BMI, and waist circumference were also observed between groups.

Table 1. Baseline characteristics according to diabetic status

Variables	Normal (n = 75)	IFG (n = 97)	T2DM (n = 72)	*P* value*
Sex (men, %)	37.3%	42.3%	61.1%	0.0090
Age (years)	33 (24 - 50)	55 (46 - 63)	54.5 (45 - 64)	<0.0001
Height (cm)	165 (160 - 173.3)	161.4 (156.1 - 170)	164.5 (158.6 - 172.2)	0.0500
Weight (kg)	59 (54.2 - 69.4)	64.5 (56.1 - 74.6)	70.7 (60.9 - 77.7)	0.0001
BMI	21.8 (20.5 - 24.0)	24.3 (22.6 - 26.5)	24.9 (22.9 - 28.1)	<0.0001
WC (cm)	76.2 (71.1 - 82.3)	86.5 (79.0 - 91.3)	90.0 (82.0 - 96.3)	<0.0001
Glucose (mg/dL)	91 (87 - 94)	107 (100 - 115)	129 (115.8 - 148.8)	<0.0001
HbA1c (%)	5.2 (5.0 - 5.4)	5.8 (5.5 - 6.0)	6.9 (6.2 - 8.2)	<0.0001
AST (IU/L)	18 (15 - 22)	20 (17 - 25)	21 (17 - 28.8)	0.0003
ALT (IU/L)	13 (10 - 20)	19 (14 - 27)	26.5 (16.3 - 35)	<0.0001
Total cholesterol (mg/dL)	183.7 (165.2 - 212.5)	197 (170 - 218)	184.5 (154.8 - 228)	0.4765
HDL cholesterol (mg/dL)	56 (48 - 63)	48 (42 - 57)	45.5 (39.3 - 53)	<0.0001
Triglycerides (mg/dL)	79 (53 - 127)	121.5 (84 - 172.5)	127.5 (92.5 - 179.5)	<0.0001
Creatinine (mg/dL)	0.73 (0.61 - 0.85)	0.73 (0.65 - 0.91)	0.80 (0.65 - 0.97)	0.0498
Uric acid (mg/dL)	4.6 (3.8 - 5.5)	4.8 (4.2 - 5.8)	5.3 (4.3 - 6.1)	0.0359
Bilirubin (mg/dL)	0.7 (0.51 - 0.9)	0.7 (0.5 - 0.9)	0.8 (0.6 - 1.0)	0.3181
Insulin (μU/dL)	5.99 (3.89 - 7.70)	7.25 (4.86 - 10.60)	8.95 (6.08 - 12.54)	<0.0001
HOMA-IR	1.34 (0.90 - 1.78)	1.83 (1.24 - 3.22)	2.84 (2.03 - 4.32)	<0.0001
C peptide (ng/dL)	1.49 (1.24 - 1.75)	2.24 (1.60 - 2.74)	2.53 (1.99 - 3.11)	<0.0001
WBC (/μL)	5755 (4415 - 6718)	5630 (4640 - 6940)	6655 (5520 - 7890)	0.0003
Hb (g/dL)	13.8 (12.8 - 14.9)	13.9 (13.0 - 15.0)	14.6 (13.7 - 15.9)	0.0005
Plt (10^3/μL)	239 (208 - 298)	229 (191 - 266)	245 (213 - 289)	0.0500

Values are median (inter-quartile range) or percentage.

*The median differences are compared by Kruskal-Wallis test.

Abbreviations: IFG, impaired fasting glucose; T2DM, type 2 diabetes mellitus; BMI,

body mass index; WC, waist circumference; HbA1c, hemoglobin A1c; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; WBC, white blood cell; Hb, hemoglobin; Plt, platelet.

2. Fasting plasma amino acids profiles according to the diabetic status

Table 2 shows differences in each fasting serum AA median concentration according to the diabetic status. Ten AAs levels, glutamic acid, lysine, phenylalanine, leucine, valine, arginine, alanine, isoleucine, tyrosine and aspartic acid had *P* values of less than 0.001 for the median differences among groups divided by diabetic status. Jonckheere-Terpstra test also showed increasing trends of these ten AAs among groups. Three of these AAs were BCAAs: leucine, valine, and isoleucine (all *P* values < 0.0001) (Fig. 1). Other two were aromatic AAs: phenylalanine and tyrosine. Tryptophan, a third aromatic AAs was not significantly different among groups (*P* value = 0.1448). The other five AAs did not belong to BCAAs and aromatic AAs (Fig. 2). For *P* values of less than 0.05, fourteen out of twenty AAs were significantly increased in patients with IFG and T2DM. Cystine was only AA, which was decreased in patients with IFG and T2DM, but decreasing trend was not observed.

Table 2. Median differences of each amino acid according to diabetic status

Amino acids (μmol/L)	Normal (N = 73)	IFG (N = 69)	T2DM (N = 56)	Kruskal-Wallis, *P* value	Jonckheere-Terpstra, *P* value
Glutamic acid	53.5 (41.5 – 72.1)	79.3 (61.8 – 104.7)	99.7 (72.8 – 133.5)	<0.0001	<0.0001
Lysine	178.2 (158.4 – 218.5)	218.2 (193.2 – 251.4)	239.6 (202.9 – 280.4)	<0.0001	<0.0001
Phenylalanine	79 (70.3 – 91.6)	95.5 (81.9 – 110.7)	104.1 (86.3 – 121.4)	<0.0001	<0.0001
Leucine	129.7 (113.8 – 160.9)	154.4 (135.3 – 172.6)	181 (151.1 – 202.9)	<0.0001	<0.0001
Valine	250.9 (229.1 – 307.9)	297.8 (275.9 – 324)	331.6 (286.2 – 381.3)	<0.0001	<0.0001
Arginine	132.6 (119.1 – 148.5)	154.6 (137.7 – 179.9)	150.9 (134.7 – 170)	<0.0001	<0.0001
Alanine	468.4 (403.9 – 543.6)	540.5 (460.8 – 641.8)	588.1 (502.8 – 670.3)	<0.0001	<0.0001
Isoleucine	67 (53.3 – 83)	79.3 (64.6 – 87.1)	85.9 (75.1 – 101.9)	<0.0001	<0.0001
Tyrosine	63.5 (57.2 – 73.7)	73.1 (62.4 – 81.2)	77.3 (65.5 – 89.6)	<0.0001	<0.0001
Aspartic acid	21.9 (17.7 – 27.1)	26.6 (23 – 30.2)	27.2 (22.9 – 31.3)	0.0001	<0.0001
Cystine	18.2 (13.3 – 24)	11.9 (6.8 – 18.6)	14.4 (9.7 – 21.1)	0.0011	0.0592
Histidine	88.8 (82.3 – 100.1)	94.3 (86.5 – 106.3)	103.3 (84.3 – 113.4)	0.0020	0.0003
Proline	150.2 (122.9 – 195.6)	172 (142.7 – 204.6)	185.6 (159.6 – 213)	0.0078	0.0018
Asparagine	59.3 (53.2 – 66.6)	64.8 (56.9 – 69.4)	64.3 (57 – 73.4)	0.0405	0.0176
Serine	177.4 (151.9 – 201.3)	188.6 (163.9 – 217.2)	189.4 (172.6 – 230.6)	0.0489	0.0146
Glycine	313.5 (278.4 – 365.3)	325.6 (281.9 – 364.3)	300.7 (263.2 – 328.9)	0.0553	0.0708
Threonine	144.7 (129.2 – 170.7)	154.6 (130.4 – 184.4)	141.6 (127.5 – 161.4)	0.1415	0.9191
Tryptophan	57.3 (50.8 – 64.9)	59.3 (53.4 – 65.8)	62.2 (53.8 – 68.9)	0.1448	0.0511
Glutamine	669.1 (614.8 – 722.2)	682.7 (637.6 – 768.1)	685.8 (580.2 – 771.5)	0.3659	0.2757
Methionine	31.9 (26.9 – 37.7)	32.1 (26 – 38.4)	32 (26.9 – 40.3)	0.7910	0.5760

Values are median (inter-quartile range).

The median differences are compared by Kruskal-Wallis test and trend analyses across groups are performed by Jonckheere-Terpstra test.

Abbreviations: IFG, impaired fasting glucose; T2DM, type 2 diabetes mellitus.

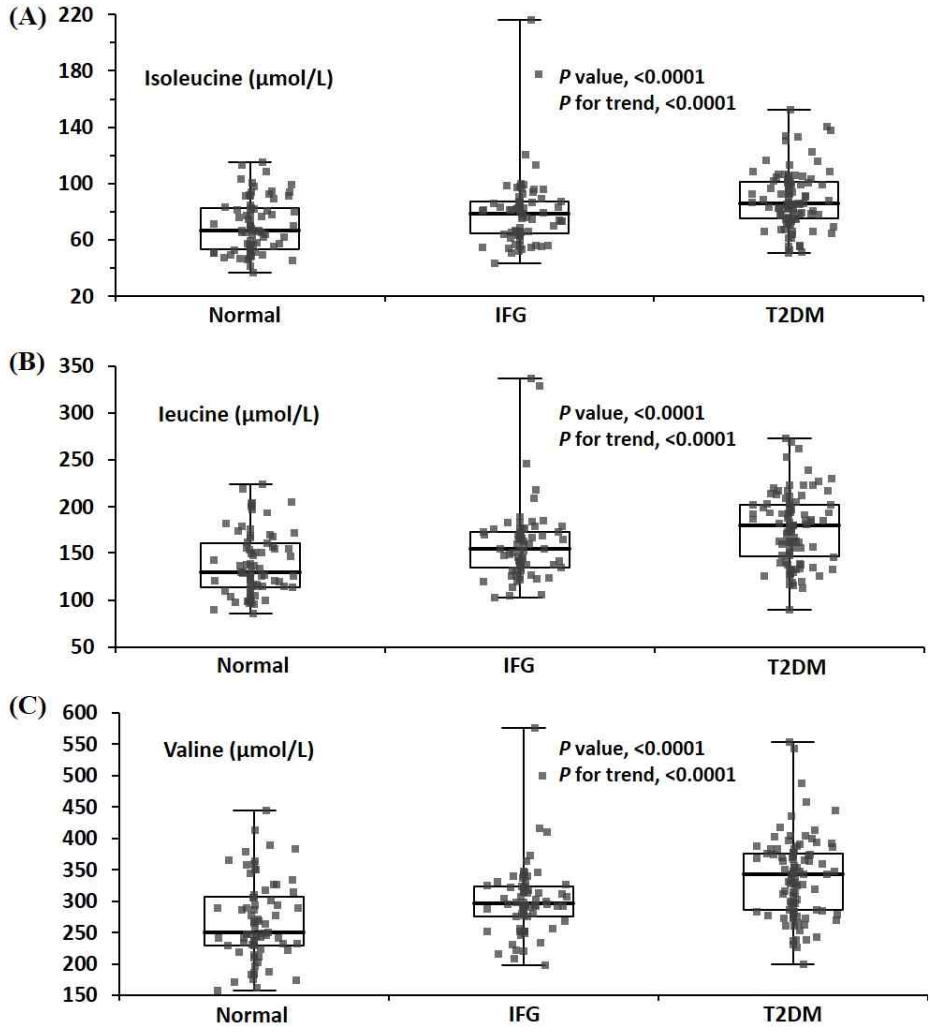

Figure 1. Box plot comparisons of BCAAs concentration. The box denotes the inter-

quartile range (25th to 75th percentile) and the horizontal line in the box indicates the median. The vertical line with horizontal end line indicated the range. The Kruskal-Wallis test and Jonckheere-Terpstra test showed a significant difference between the median values of 3 groups with increasing trend for isoleucine (A), leucine (B), and valine (C).

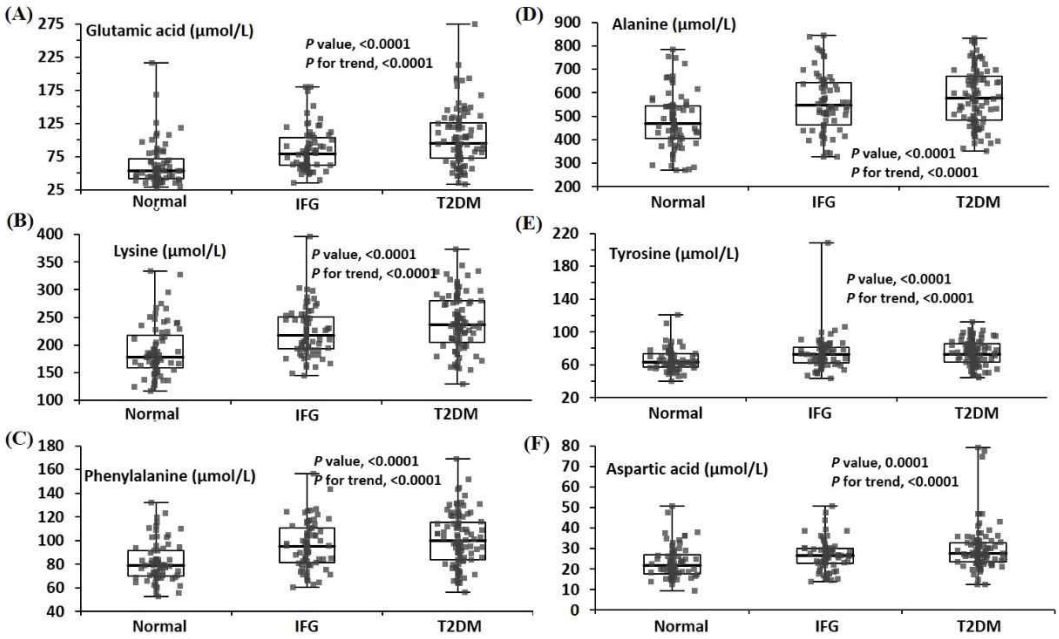

Figure 2. Box plot comparisons of AAs concentration other than BCAAs. The box denotes the inter-quartile range (25th to 75th percentile) and the horizontal line in the box indicates the median. The vertical line with horizontal end line indicated the range. The Kruskal-Wallis test and Jonckheere-Terpstra test showed a significant difference between the median values of 3 groups with increasing trend for glutamic acid (A), lysine (B), phenylalanine (C), alanine (D), tyrosine (E), and aspartic acid (F).

3. Fasting serum bile acids profiles according to the diabetic status

When we compared each serum BA median concentration according to the diabetic status, G-CDCA had *P* value of less than 0.05 (Table 3). Jonckheere-Terpstra test also showed decreasing trends of G-CDCA with progression to IFG, and T2DM. Other glycine conjugated BAs, G-CA, G-DCA, and G-UDCA also showed decreasing trends with progression to IFG and T2DM. In addition, total glycine conjugated BAs had statistically significant differences among groups divided by diabetic status.

Table 3. Median differences of each bile acid according to diabetic status

Bile acids (μmol/L)	Normal (N = 75)	IFG (N = 95)	T2DM (N = 71)	Kruskal-Wallis, *P* value	Jonckheere-Terpstra, *P* value
CA	0.0987 (0.0348 – 0.197)	0.0905 (0.05 – 0.276)	0.0717 (0.021 – 0.248)	0.2953	0.4295
G-CA	0.15 (0.0981 – 0.232)	0.113 (0.0595 – 0.205)	0.102 (0.0566 – 0.202)	0.0741	0.0405*
T-CA	0.0195 (0.0125 – 0.0339)	0.0199 (0.0115 – 0.043)	0.0228 (0.0118 – 0.0391)	0.9199	0.8235
TOTAL CA	0.3213 (0.1828 – 0.5455)	0.3212 (0.156 – 0.7131)	0.2648 (0.1493 – 0.5939)	0.6372	0.3894
CDCA	0.266 (0.0728 – 0.545)	0.204 (0.0998 – 0.559)	0.17 (0.0523 – 0.499)	0.2642	0.1708
G-CDCA	0.77 (0.392 – 1.17)	0.488 (0.266 – 0.999)	0.487 (0.233 – 0.964)	0.0309*	0.0119*
T-CDCA	0.0625 (0.0337 – 0.124)	0.0506 (0.03 – 0.0912)	0.0583 (0.0271 – 0.118)	0.5596	0.4330
Total CDCA	1.2653 (0.7435 – 1.7561)	1.0042 (0.5159 – 1.953)	0.8624 (0.4152 – 1.597)	0.0932	0.0294*
DCA	0.283 (0.15 – 0.457)	0.292 (0.145 – 0.487)	0.274 (0.127 – 0.442)	0.7476	0.6125

G-DCA	0.21 (0.139 – 0.342)	0.165 (0.0773 – 0.377)	0.119 (0.059 – 0.337)	0.0797	0.0269*
T-DCA	0.0229 (0.0128 – 0.0425)	0.0182 (0.0092 – 0.0426)	0.0218 (0.0082 – 0.0413)	0.3927	0.1965
Total DCA	0.5397 (0.3336 – 0.9908)	0.5267 (0.2801 – 0.8727)	0.4691 (0.2243 – 0.8079)	0.4091	0.1814
UDCA	0.0562 (0.0175 – 0.113)	0.0465 (0.021 – 0.124)	0.0388 (0.0182 – 0.104)	0.5398	0.5719
G-UDCA	0.0815 (0.0507 – 0.156)	0.0654 (0.0333 – 0.13)	0.058 (0.0326 – 0.123)	0.0588	0.0248*
T-UDCA	0.0095 (0.0062 – 0.0133)	0.0105 (0.0063 – 0.0168)	0.0092 (0.0063 – 0.0142)	0.6809	0.9837
Total UDCA	0.1484 (0.081 – 0.2965)	0.1391 (0.0749 – 0.2921)	0.1179 (0.0653 – 0.2149)	0.2782	0.1137
LCA	0.0218 (0.0138 – 0.0295)	0.0154 (0.0008 – 0.0343)	0.0211 (0.0067 – 0.0345)	0.2120	0.9111
G-LCA	0.0089 (0.0043 – 0.0137)	0.0076 (0.003 – 0.0157)	0.0071 (0.0042 – 0.0161)	0.8405	0.9749
T-LCA	0.0037 (0.0031 – 0.0047)	0.0035 (0.0026 – 0.0044)	0.0038 (0.0028 – 0.0045)	0.5870	0.6665
Total LCA	0.0335 (0.022 – 0.0498)	0.0325 (0.0095 – 0.0521)	0.0318 (0.0207 – 0.0532)	0.4670	0.8997
Glycine conjugated BAs	1.361 (0.4202 – 0.6761)	0.9347 (0.3548 – 0.6346)	0.7696 (0.329 – 0.6594)	0.0320*	0.0116*
Taurine conjugated BAs	0.1243 (0.0331 – 0.0905)	0.1145 (0.0341 – 0.0848)	0.1093 (0.0397 – 0.1139)	0.7274	0.5287
Conjugated BAs	1.5342 (0.8624 – 2.1136)	1.0496 (0.5901 – 2.107)	0.9184 (0.5337 – 1.8117)	0.0606	0.0233*
Total BAs	2.4722 (1.8021 – 3.6489)	2.0604 (1.1445 – 4.1452)	2.0644 (0.9504 – 3.4337)	0.1856	0.0699

Values are median (inter-quartile range).

The median differences are compared by Kruskal-Wallis test and trend analyses across groups are performed by Jonckheere-Terpstra test. *P <0.05.

Abbreviations: IFG, impaired fasting glucose; T2DM, type 2 diabetes mellitus; CA, cholic acid; G-CA, glycine conjugated CA; T-CA, taurine conjugated CA; CDCA,

chenodeoycholic acid; G-CDCA, glycine conjugated CDCA; T-CDCA, taurine conjugated CDCA; DCA, deoxycholic acid; G-DCA, glycine conjugated DCA; T-DCA, taurine conjugated DCA; UDCA, ursodeoxycholic acid; G-UDCA, glycine conjugated UDCA; T-UDCA, taurine conjugated UDCA; LCA, lithocholic acid; G-LDA, glycine conjugated LCA; T-LCA, taurine conjugated LCA; BAs, bile acids.

4. Correlations between serum amino acids and clinical profile

Spearman's rank correlation coefficients between the fasting serum AAs and the variables related to glycemia/insulin resistance and obesity were shown in Table 4. The glucose-related variables such as fasting serum glucose and HbA1c, which are widely used for the diagnosis of diabetes, showed strong positive correlation with glutamic acid and phenylalanine and moderate positive correlation with alanine, aspartic acid, isoleulcine, leucine, lysine, tyrosine, valine, BCAAs, and total AAs. Triglycerides was strongly correlated with alanine, glutamic acid, leucine, valine, BCAAs, and total AAs. HOMA-IR and c-peptide showed strong positive correlations with alanine, glutamic acid, and phenylalanine and moderate correlations with isoleucine, leucine, lysine, tyrosine, valine, BCAAs, total AAs. These AAs also showed significant positive correlations with BMI, weight, and waist circumference. In addition, glutamic acid and phenylalanine have strong positive correlation with ALT. HDL-C showed negative correlations with glutamic acid, isoleucine, leucine, lysine, phenylalanine, proline, valine, BCAAs, and total AAs. Creatinine was positively correlated with isoleucine, leucine, lysine, proline, tryptophan, valine, BCAAs, and total AAs.

Table 4. Correlations between fasting serum amino acids and biochemical and clinical variables

Variable	Glucose	HbA1c	ALT	AST	TC	HDL	TG	Cr	Insulin	HOMA-IR	C-peptide	Weight	BMI	WC	IL-6	TNF-α
Alanine	0.360**	0.445**	0.444**	0.316**	0.076	-0.291**	0.509**	0.230**	0.488**	0.538**	0.569**	0.362**	0.362**	0.425**	0.334**	0.364**
Arginine	0.270**	0.322**	0.174*	0.076	0.263**	-0.212**	0.305**	0.149*	0.160*	0.228**	0.231**	0.118	0.190*	0.285**	0.328**	0.341**
Asparagine	0.176*	0.075	0.138	0.067	-0.011	-0.158*	0.103	0.242**	0.038	0.085	0.128	0.167*	0.072	0.089	0.075	0.128
Aspartic acid	0.304**	0.444**	0.297**	0.249**	0.232**	-0.215**	0.290**	-0.040	0.214**	0.276**	0.384**	0.044	0.220**	0.264**	0.345**	0.255**
Cystine	-0.158*	-0.178*	-0.020	0.008	-0.217**	-0.049	-0.085	0.267**	-0.014	-0.056	-0.058	0.145	0.087	0.036	-0.178*	-0.079
Glutamic acid	0.508**	0.636**	0.613**	0.470**	0.181*	-0.430**	0.504**	0.216**	0.462**	0.562**	0.641**	0.426**	0.509**	0.593**	0.520**	0.504**
Glutamine	0.050	0.047	0.109	0.079	-0.013	-0.127	0.027	0.230**	-0.015	-0.005	-0.019	0.096	0.035	0.149	0.013	0.078
Glycine	-0.141*	-0.048	0.012	0.004	-0.066	-0.031	-0.011	0.039	-0.073	-0.093	-0.080	-0.142	-0.049	-0.124	0.038	0.059
Histidine	0.195**	0.304**	0.295**	0.167*	0.191**	-0.267**	0.354**	0.284**	0.215**	0.261**	0.282**	0.274**	0.213**	0.284**	0.277**	0.362**
Isoleucine	0.347**	0.359**	0.389**	0.187**	0.061	-0.408**	0.493**	0.454**	0.374**	0.461**	0.423**	0.575**	0.468**	0.485**	0.334**	0.449**
Leucine	0.429**	0.473**	0.459**	0.238**	0.142*	-0.396**	0.544**	0.421**	0.361**	0.476**	0.425**	0.549**	0.480**	0.537**	0.403**	0.479**
Lysine	0.434**	0.460**	0.371**	0.260**	0.113	-0.372**	0.421**	0.303**	0.251**	0.370**	0.345**	0.334**	0.355**	0.385**	0.331**	0.429**
Methionine	0.003	0.144	0.156*	0.041	-0.063	-0.150*	0.127	0.248**	0.167*	0.173*	0.065	0.372**	0.191*	0.253**	0.071	0.208**
Phenylalanine	0.432**	0.537**	0.568**	0.427**	0.123	-0.398**	0.449**	0.254**	0.401**	0.489**	0.522**	0.349**	0.417**	0.500**	0.538**	0.476**

Proline	0.239**	0.202**	0.220**	0.105	0.047	-0.304**	0.377**	0.325**	0.269**	0.316**	0.358**	0.362**	0.333**	0.363**	0.259**	0.268**
Serine	0.132	0.287**	0.190**	0.198**	0.021	-0.175*	0.151*	-0.056	0.123	0.159*	0.200*	0.053	0.204**	0.176*	0.312**	0.279**
Threonine	-0.009	-0.015	0.107	0.031	-0.084	-0.159*	0.137	0.171*	0.100	0.086	0.087	0.218**	0.159*	0.130	0.133	0.247**
Tryptophan	0.141*	0.204**	0.325**	0.214**	0.001	-0.272**	0.403**	0.360**	0.288**	0.305**	0.244**	0.452**	0.305**	0.281**	0.132	0.274**
Tyrosine	0.323**	0.381**	0.443**	0.359**	0.105	-0.230**	0.420**	0.221**	0.439**	0.464**	0.449**	0.435**	0.413**	0.461**	0.334**	0.325**
Valine	0.437**	0.475**	0.468**	0.257**	0.153*	-0.392**	0.549**	0.406**	0.382**	0.492**	0.490**	0.549**	0.476**	0.552**	0.397**	0.430**
BCAAs	0.428**	0.470**	0.464**	0.247**	0.137	-0.410**	0.557**	0.431**	0.384**	0.494**	0.477**	0.567**	0.490**	0.549**	0.400**	0.464**
AAs	0.356**	0.433**	0.445**	0.293**	0.115	-0.390**	0.506**	0.348**	0.368**	0.444**	0.467**	0.420**	0.427**	0.484**	0.416**	0.470**
IL-6	0.395**	0.477**	0.370**	0.285**	0.042	-0.416**	0.361**	0.063	0.291**	0.397**	0.434**	0.147	0.327**	0.416**	1.000	0.619**
TNF-α	0.316**	0.446**	0.338**	0.242**	0.068	-0.426**	0.419**	0.234**	0.244**	0.345**	0.402**	0.335**	0.292**	0.417**	0.619**	1.000

Correlation coefficients and corresponding *P*-value were estimated by Spearman′s rank correlation analysis. **P* <0.05, ***P* <0.01.

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; Cr, creatinine; UA, uric acid; HOMA-IR, homeostatic model assessment of insulin resistance; BMI, body mass index; WC, waist circumference; IL-6, interleukin-6 ; TNF-α , tumor necrosis factor-α.

5. Correlations between serum amino acids and pro-inflammatory cytokines

TNF-α showed strong positive correlation with glutamic acid and moderate positive correlation with alanine, arginine, histidine, isoleucine, leucine, lysine, phenylalanine, tyrosine, valine, BCAAs, and total AAs (Table 4, Fig. 3 and 4). IL-6 also strongly correlated with glutamic acid and phenylalanine, and moderately correlated with alanine, arginine, aspartic acid, isoleucine, leucine, lysine, phenylalanine, serine, tyrosine, valine, BCAAs, and total AAs (Table 4, Fig. 5 and 6). In addition, TNF-α and IL-6 showed positive correlations with glucose, HbA1c, ALT, triglycerides, HOMA-IR, C-peptide, and waist circumference and negative correlation with HDL-C. When we compared median concentration of TNF-α and IL-6 according to the diabetic status, stepwise increase of these cytokines with progression to IFG and T2DM were found (Table 5).

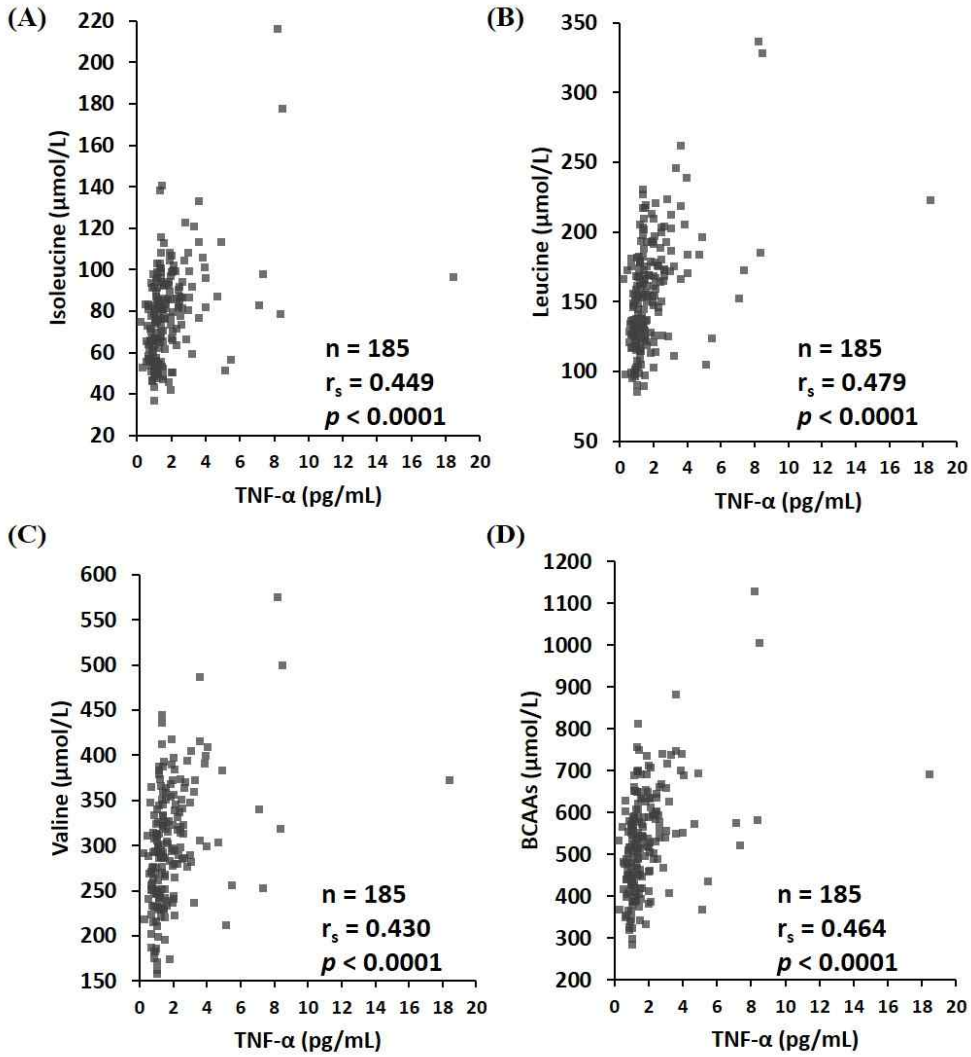

Figure 3. Correlations between TNF-α and BCAAs. Correlation coefficients and corresponding *P*-value were estimated by Spearman's rank correlation analysis for isoleucine (A), leucine (B), valine (C), and total BCAAs (D).

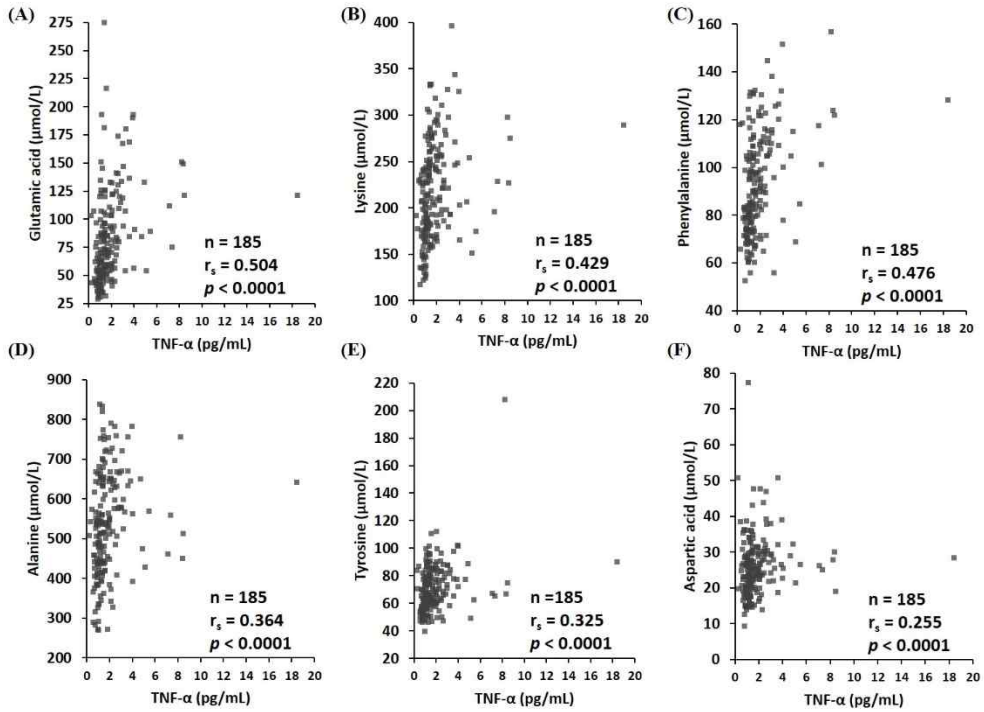

Figure 4. Correlations between TNF-α and other AAs concentration. Correlation coefficients and corresponding *P*-value were estimated by Spearman's rank correlation analysis for glutamic acid (A), lysine (B), phenylalanine (C), alanine (D), tyrosine (E), and aspartic acid (F).

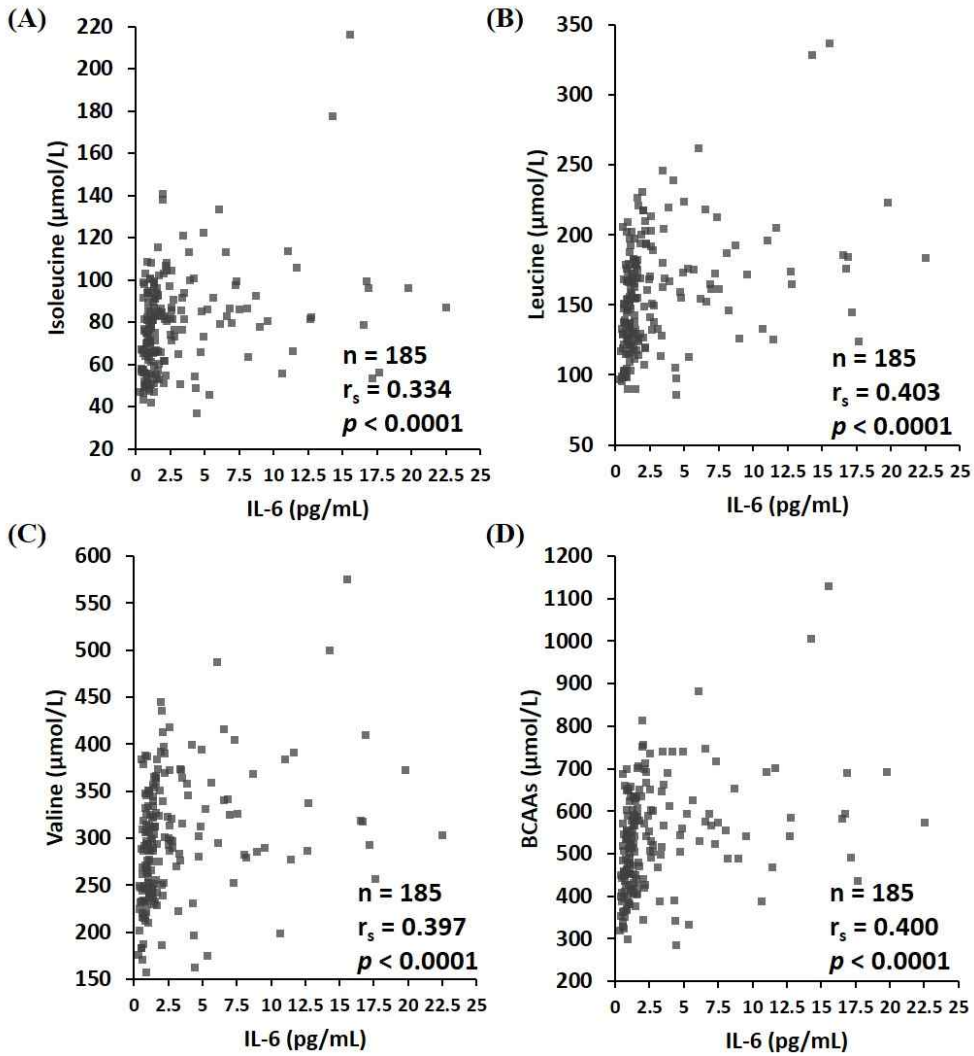

Figure 5. Correlations between IL-6 and BCAAs. Correlation coefficients and corresponding *P*-value were estimated by Spearman′s rank correlation analysis for isoleucine (A), leucine (B), valine (C), and total BCAAs (D).

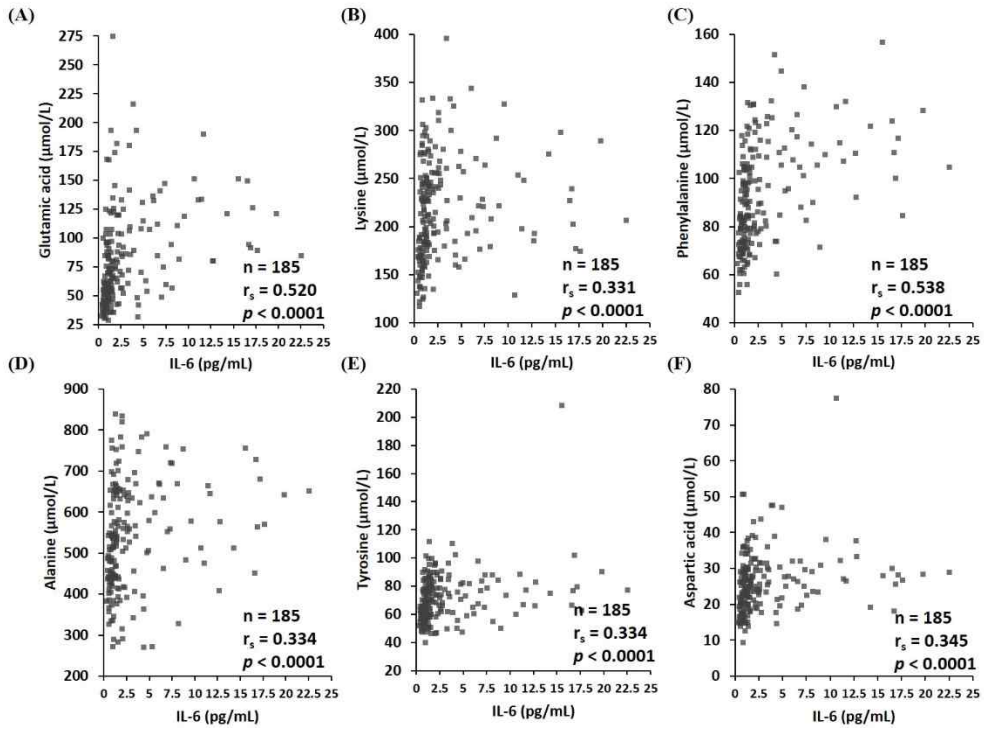

Figure 6. Correlations between IL-6 and other AAs concentration. Correlation coefficients and corresponding *P*-value were estimated by Spearman′s rank correlation analysis for glutamic acid (A), lysine (B), phenylalanine (C), alanine (D), tyrosine (E), and aspartic acid (F).

Table 5. Median differences of pro-inflammatory cytokines according to diabetic status

Cytokines (pg/mL)	Normal (N = 73)	IFG (N = 69)	T2DM (N = 56)	Kruskal-Wallis, *P* value	Jonckheere-Terpstra, *P* value
TNF-α	1.15 (0.88 - 1.67)	1.41 (1.08 - 2.28)	1.73 (1.35 - 2.62)	<0.0001	<0.0001
IL-6	0.97 (0.65 - 1.50)	1.79 (1.16 - 5.15)	2.20 (1.37 - 5.03)	<0.0001	<0.0001

Values are median (interquartile range).

The median differences are compared by Kruskal-Wallis test and trend analyses across groups are performed by Jonckheere-Terpstra test.

Abbreviations: IFG, impaired fasting glucose; T2DM, type 2 diabetes mellitus; TNF-α , tumor necrosis factor-α; IL-6, interleukin-6.

6. Independent predictors of serum AAs

Multiple linear regression analysis was used to identify independent predictors of serum BCAAs, glutamic acid, phenylalanine, alanine and total AAs (Table 6). After adjusting for sex, age, and weight, we found that TNF-α, IL-6, and HOMA-IR were independent predictors for serum BCAAs. TNF-α, IL-6, and HOMA-IR were also independent predictors for glutamic acid, phenylalanine, and total AAs after adjusting for sex, age, and weight.

Table 6. Association of fasting blood amino acids with pro-inflammatory cytokines and insulin resistance

Model	BCAAs	Glutamic acid	Phenylalanine	Total AAs
Model adjusting for age, sex, weight, HOMA-IR, TNF-α				
Sex (men=1, women=2)	-97.1 (-138.6 ~-55.6)	-3.4 (-19.0 ~ 12.3)	-4.4 (-12.1 ~ 3.3)	-244.9 (-421.8 ~ -68.1)
Age (years)	0.9 (-0.2 ~ 1.9)	0.6 (0.2 ~ 1.0)	0.3 (0.1 ~ 0.5)	7.8 (3.5 ~ 12.1)
Weight (kg)	1.2 (-0.6 ~ 2.9)	0.8 (0.1 ~ 1.4)	0.2 (-0.1 ~ 0.5)	50. (-2.5 ~ 12.5)
HOMA-IR	13.0 (6.0 ~ 20.1)	3.4 (0.7 ~6.0)	2.3 (1.0 ~ 3.6)	38.3 (8.3 ~ 68.3)
TNF-α (pg/mL)	16.1 (8.4 ~ 23.9)	4.6 (1.6 ~ 7.5)	3.3 (1.8 ~ 4.7)	51.8 (18.6 ~ 84.9)
Model adjusting for age, sex, weight, HOMA-IR, IL-6				
Sex (men=1, women=2)	-105.1 (-147.1 ~ -63.2)	-5.3 (-20.7 ~ 10.0)	-5.9 (-13.4 ~ 1.6)	-270.4 (-447.7 ~ -93.2)
Age (years)	1.0 (-0.1 ~ 2.0)	0.6 (0.2 ~ 1.0)	0.3 (0.1 ~ 0.5)	8.1 (3.8 ~ 12.5)
Weight (kg)	1.2 (-0.6 ~ 3.0)	0.8 (0.1 ~ 1.4)	0.2 (-0.1 ~ 0.5)	4.9 (-2.6 ~ 12.5)
HOMA-IR	12.8 (5.6 ~ 20.0)	3.1 (0.5 ~ 5.8)	2.2 (0.9 ~ 3.4)	37.6 (7.1 ~ 68.0)
IL-6 (pg/mL)	6.2 (2.5 ~ 9.8)	2.5 (1.1 ~ 3.8)	1.7 (1.0 ~ 2.3)	20.1 (4.6 ~ 35.7)

Values are β coefficients (95% confidence intervals) for amino acids from multiple linear regressions.

Abbreviations: BCAAs, branched chain amino acids; HOMA-IR, homeostatic model assessment of insulin resistance; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

7. Discriminative ability of amino acid profiles for IFG and T2DM

The ability of the glutamic acid, phenylalanine, BCAAs, AAs, and HOMA-IR to discriminate between normoglycemia, and IFG or T2DM determined by the receiver operating characteristic (ROC) curves (Fig. 7). The ROC-area under curve (AUC)

values were 0.79 for glutamic acid (95% confidence interval [CI], 0.72–0.86), 0.75 for phenylalanine (95% CI, 0.68–0.82), 0.72 for BCAAs (95% CI, 0.64–0.80), 0.72 for AAs (95% CI, 0.64–0.80), and 0.77 for HOMA-IR (95% CI, 0.71–0.83). The AUC values of these four AAs markers were not significantly different from standard HOMA-IR (glutamic acid versus HOMR-IR, P = 0.525; phenylalanine versus HOMA-IR, P = 0.715; BCAAs versus HOMA-IR, P = 0.266; and AAs versus HOMA-IR, P = 0.254).

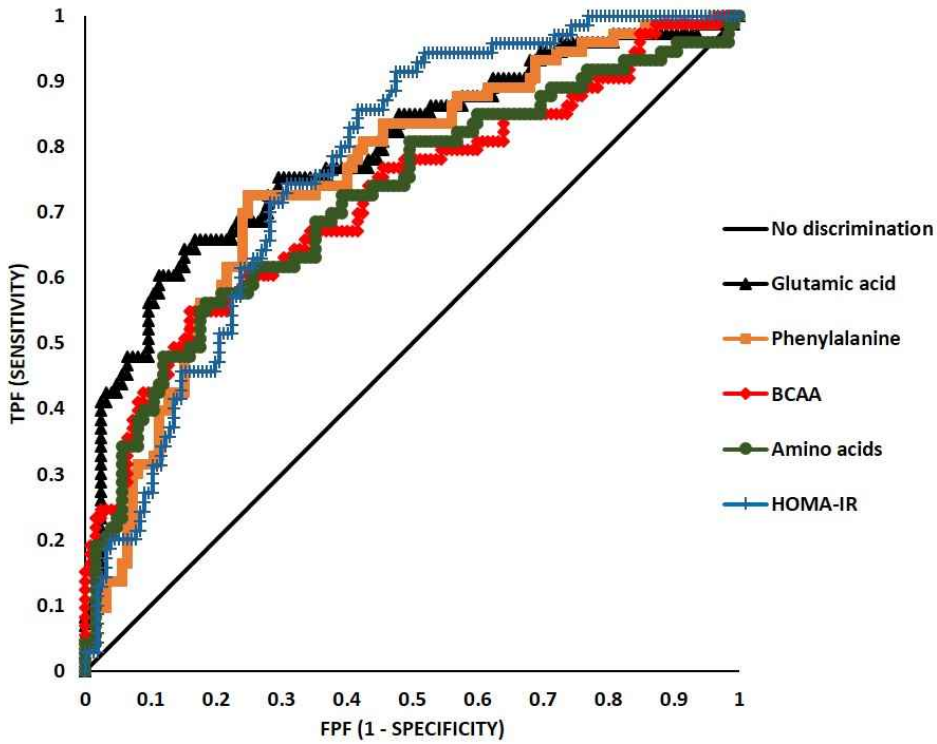

Figure 7. Receiver operating characteristic (ROC) curves of glutamic acid, phenylalanine, BCAAs and total amino acids for differentiating IFG and T2DM from normal control.

8. Development of insulin resistance in C2C12 myotubes by palmitate treatment

The toxic effect of palmitate treatment on cell viability of C2C12 myotubes was tested at first. The result of MTT assay showed that lower than 0.8 mM of palmitate did not suppress the cell viability of C2C12 myotubes below 60%. So, we selected 0.8 mM and 0.6 mM of palmitate for next experiments. To examine whether insulin resistance is developed after palmitate treatment, we performed immunoblot analysis for phosphorylation of Akt (Ser473) and IRS-1 (Try632). The palmitate treatment of 0.6 mM and 0.8 mM suppressed insulin-stimulated phosphorylation of Akt (Ser473) and IRS-1 (Try632) (Figure 8A). These concentrations of palmitate also reduced the expression of mRNA for glucose transporter 4 (Glut4) gene (Figure 8B). These immunoblot and qRT-PCR analyses suggested that 0.6 to 0.8 mM of palmitate decreased the insulin sensitivity of C2C12 myotubes.

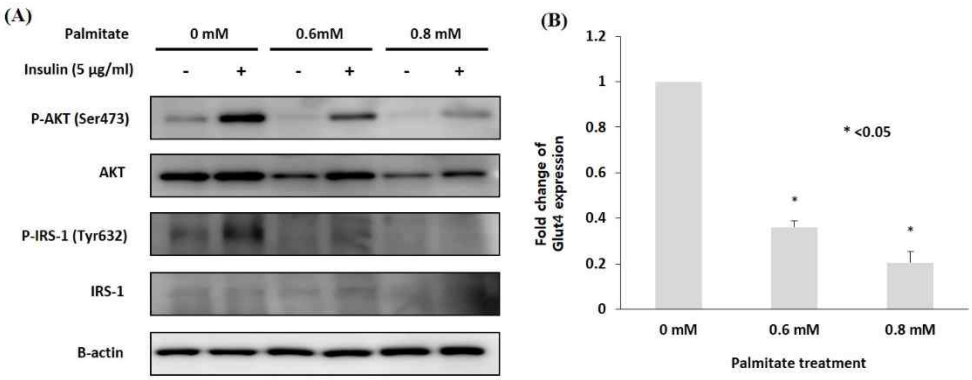

Figure 8. Palmitate induced insulin resistance in C2C12 myotubes. Immunoblot analysis for phosphorylation of Akt (Ser473) and IRS-1 (Try632) (A) and qRT-PCR result for Glut4 gene compared to the control group (0 mM) (B). Data are presented as the mean ± SE of three independent experiments. **P* <0.05 vs. compared to the cells with 0 mM palmitate treatment (control). Data are presented as the mean ± SE of six independent experiments.

9. Palmitate induced insulin resistance and ubiqutine-proteasome system

To examine whether palmitate induced insulin resistance was associated with increased proteolysis, we measured the transcription of two muscle specific E3 ligase genes of ubiqutine-proteasome pathway, MAFbx and MuRF1. The palmitate treatment increased the expression of MAFbx gene (Figure 9A), but reduced the expression of MuRF1 gene (Figure 9B). Immunoblot analysis for protein levels of MAFbx and MuRF1 showed similar results with qRT-PCR analyses, increased expression of MAFbx and decreased expression of MuRF1 (Figure 9C). To evaluate an association between insulin resistance induced by palmitate and pro-inflammatory cytokines expression, qRT-PCR analysis for TNF-α and IL-6 genes was performed. After palmitate treatment, the transcription of TNF-α and IL-6 genes was increased (Figure 9D and E).

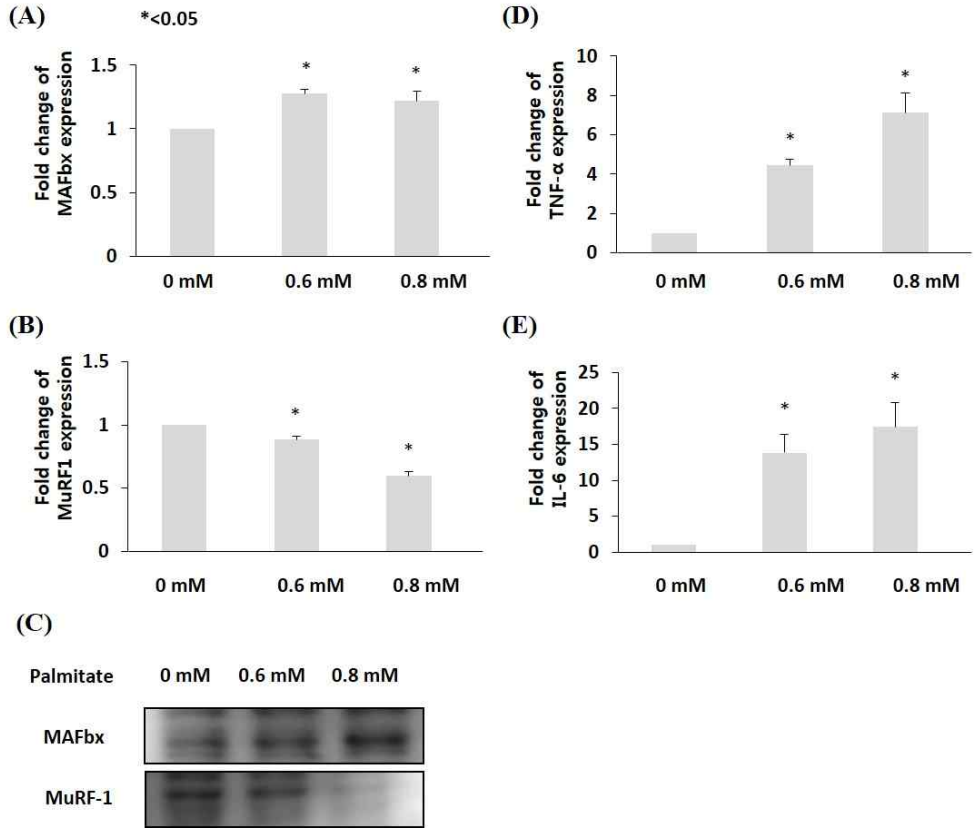

Figure 9. Differences in mRNA and protein expression for MAFbx, MuRF1, and cytokine after palmitate treatment of 0, 0.6, and 0.8 mM. qRT-PCR analysis for MAFbx (A) and MuRF1 (B), immunoblot analysis for for MAFbx and MuRF1 (C), and qRT-PCR analysis for TNF-α (D) and IL-6 genes (E). Expression of mRNAs were compared to the control group (0 mM). Data are presented as the mean ± SE of three independent experiments. *P <0.05 vs. compared to the cells with 0 mM palmitate treatment (control). Data are presented as the mean ± SE of six independent experiments.

IV. DISCUSSION

Metabolite profiling of individuals, or metabolomics, has shown to be a promising technique to investigate changes in metabolic status in patients with T2DM, because it represents the endpoints of many diseases as actual biochemical phenotype.[44,45] Several studies have already been performed to find metabolomic signature for T2DM.[28,46] Despite large variations in these predictive biomarkers, concentrations of blood BCAAs have been consistently associated with the risk of developing T2DM, both in cross-sectional and longitudinal studies.[28,44]

Here, we showed that beside BCAAs, other fasting blood AAs also associated with IFG and T2DM. These AAs were gradually increased with worsening glycemic control. These altered blood AAs levels significantly correlated with various clinical parameters for insulin resistance and glucose homeostasis, such as weight, BMI, waist circumference, HOMA-IR, HbA1c, and fasting blood glucose. In addition, these blood AAs concentrations positively correlated with pro-inflammatory cytokines (TNF-α and IL-6). The in vitro experiment using C2C12 mytoblast cells showed that insulin resistance induced by palmitate could increase the expression of MAFbx, but not MuRF1. The difference in target muscle proteins and ubiquitinization activity between MAFbx and MuRF1 should further studied to conclude whether muscle protein proteolysis by ubiquitine-proteasome system is overall increased or not in insulin resistance induced by palmitate.

Although recent clinical studies have reported that elevated fasting blood levels of the BCAAs were associated with IFG and T2DM, it remains to be shown whether or not the association is causal.[27] Several interesting studies of BCAAs supplementation or deprivation in mice[6,47-49] and in humans[14,50,51] indicated that BCAAs may directly induce insulin resistance, possibly via disruption of insulin signaling in skeletal muscle. The underlying cellular mechanisms may include activation of the

mammalian target of rapamycin complex 1 (mTORC1) and insulin receptor substrate-1 (IRS-1) signaling pathways. Persistent activation of mTORC1 and downstream ribosomal protein S6 kinase 1 (S6K1) by BCAAs promote insulin resistance through serine phosphorylation of IRS-1 which lead to an attenuation of insulin sensitivity.[52,53] However, many previous studies reported that supplementation of BCAAs seem to result in health benefits in diabetes,[54,55] obesity,[56,57] and liver diseases.[58] As a consequence, supplementation with BCAAs or a BCAA-rich diet has been believed to improve metabolic health.[27] Therefore, whether circulating BCAAs directly promote insulin resistance or not should be further evaluated.

A recent clinical study to directly evaluate the effects of acute exposure to increased BCAA levels reported that short-term exposure of young healthy subjects to increased plasma BCAA concentrations did not alter the insulin sensitivity of glucose metabolism.[59] Another recent experiment showed the reductions in fasting plasma AAs concentrations in response to three months of insulin sensitizer therapy.[60] The research clarified that the improvement in insulin sensitivity induced by the insulin sensitizer normalized the plasma AAs concentrations. These findings suggest that the elevation of BCAAs concentrations is early manifestation of insulin resistance rather than causative factor of insulin resistance. Furthermore the mechanism underlying the increase of other blood AAs except BCAAs, such as glutamic acid, lysine, phenylalanine, arginine, alanine, and aspartic acid, were poorly investigated in T2DM. The overall elevation of circulating AAs might be caused by the increased muscle protein degradation in skeletal muscles.[27] Therefore we hypothesized that these AAs alterations in insulin resistance and T2DM are caused by accelerated protein breakdown in muscle which is induced by a combination of insulin resistance and inflammation. The difference in the degree of increase for each AA could be explained by the proportion of each AA in skeletal muscle protein and difference in

metabolism pathway in muscle.

Skeletal muscle wasting occurs systemically in fasting and a variety of diseases (e.g., cancer, diabetes mellitus, and sepsis) and in muscle denervation or disuse.[61,62] Ubiquitin-proteasome-system is the predominating proteolysis system that is activated during skeletal muscle wasting.[63,64] Proteins are selectively targeted to the ubiquitin proteasome system by several ubiquitin E3 ligases. MAFbx and MuRF1 are two important E3 ligases found exclusively in skeletal muscles, acting as major mediators of skeletal muscle wasting.[65,66] The increased expression of MAFbx and MuRF1 in skeletal muscles is observed during immobilization, denervation, and glucocorticoid (dexamethasone) treatment.[63] Cachectic cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) are also shown to be associated with increased MAFbx and MuRF1 in the muscle.[67,68] Another potential proteolytic trigger of muscle protein breakdown is an insulin resistance. The previously studies showed that the accelerated muscle proteolysis in a model of insulin deficiency was caused by a decrease in the activity of phosphatidylinositol 3-kinase (PI3K).[62,69-71] The decrease in PI3K activity reduced the level of phosphorylated Akt (p-Akt), and subsequently decreased phosphorylation of the Forkhead box (FOXO) class of transcription factors, rendering them enter the nucleus to promote the expression of MAFbx and MuRF1 in muscle. However, inflammation that is linked to insulin resistance might also partially contribute to increased MAFbx and MuRF1 because high levels of circulating TNF-α and other pro-inflammatory cytokines can cause insulin resistance.[72,73]

Interestingly, although the concentrations of most AAs increased in the patients with IFG and T2DM, the concentrations of a few AAs, such as cystine, glycine, threonine, tryptophan, glutamine and methionine, did not change or even decreased. The precise

mechanism underlying the different change of circulating AAs in insulin resistance and T2DM has not yet been elucidated. We thought that in addition to increased muscle protein degradation in skeletal muscles, several distinctive modifications of the AAs metabolism pathway might affect the concentration of each AA. For BCAAs, specific mechanisms that dysregulate BCAAs metabolism have been intensively studied. Several studies have shown that increased circulating concentrations of BCAAs can be caused by decreased BCAAs metabolism in individuals with insulin-resistant obesity or untreated T2DM.[27]

The first step in the metabolism of BCAAs is catalyzed by the mitochondrial branched chain amino acid transaminase (BCAT) encoded by the BCAT2 gene.[27] After BCAT, the next step is the first irreversible step in BCAAs metabolism, which is catalyzed by the multienzyme, mitochondrial branched chain α-ketoacid dehydrogenase complex (BCKDC). BCKDC activity is inhibited or activated according to phosphorylation state by branched chain α-ketoacid dehydrogenase kinase (BCKDK) or the mitochondrial isoform of protein phosphatase 1K (PPM1K, also known as PP2CM), respectively. The activity and expression of these enzymes are affected by many metabolic factors changed in obesity, insulin resistance, and T2TM. Reduced enzyme activity and expression of BCKDC in obesity and T2DM has been demonstrated in animal and clinical studies[74-79] Such observations can support that suppression of BCAAs catabolism by insulin resistance is also a possible cause of elevated BCAAs concentrations in insulin resistance and T2DM in addition to increased proteolysis in skeletal muscle. Further studies for modifications of the AAs metabolism other than BCAAs in insulin resistance and T2DM are also needed.

As a possible cause of glutamic acid elevation in the patients with IFG and T2DM, the increased first step reaction of BCAAs catabolism mediated by BCAT could be considered, which result in accumulation of glutamic acid.[80] The accumulation of

glutamic acid may consecutively increase transamination of pyruvate to alanine.[80] Alanine, a highly gluconeogenic AA, is transported from muscle to liver and used for gluconeogenesis in liver. Therefore, alanine concentration can be increased in the patient with IFG and T2DM. The increase of aromatic AAs, phenylalanine and tyrosine may be partially explained by the report that tryptophan, phenylalanine, tyrosine, leucine, isoleucine, and valine compete for transport into mammalian cells by the large neutral AA transporter (LAT1).[80,81] However, exact mechanism of these AA elevation should be further studied.

Whether there is difference in profile of BAs in patients with T2D remains incompletely understood. In this study we found that the glycine-conjugated BAs were lower in patients with T2DM and intermediate in patients with IFG compared with normoglycemic controls. Total glycine conjugated BAs and G-CDCA were the only two parameters that were statistically significantly different among three groups. The gut microbial flora strongly impacts BAs metabolism by performing structural modifications through deconjugation, oxidation, or hydroxylation in the intestine.[82] The decreased glycine conjugated BAs could possibly be caused by higher rates of glycine deconjugation in the gut. The initial reaction in the bacterial metabolism of conjugated BAs is mediated by bile salt hydrolase (BSH), which catalyzes the deconjugation of conjugated BAs to liberate free BAs.[83] Some BSH specifically degraded taurine conjugated BAs and could not degrade glycine conjugated BAs.[83] For gut associated *Bacteroidetes*, there was difference in glycine deconjugation activity according to clone.[83] Therefore, population shifts of gut microbial flora may lead to fluctuations in capacity for BAs modification during the lifetime of the host.[83] Further studies are required to determine potential relationships between serum glycine conjugated BAs levels and gut microflora and glycemia. Interestingly, Patti et al. reported increased total BAs concentrations, especially G-CDCA and G-DCA,

were higher in patients with previous Roux-en-Y gastric bypass surgery, positively correlated with GLP-1, and inversely associated with post-meal glucose.[84] It is possible that anatomic, dietary, and microflora changes after a gastric bypass may impact greatly on total and relative proportions of BAs, yielding distinct metabolic effects.[36,84]

There has been no previous studies that reported the difference in the circulating concentration of fasting glycine-conjugated BAs according to diabetic status. A similar recent study assessing serum BAs composition in patients with impaired glucose tolerant, patients with T2D, and normal control reported that fasting taurine conjugated BAs are higher in patients with T2D compared with normoglycemic controls and the taurine conjugated BAs were associated with fasting and post-load glucose, HbA1c, fasting insulin, and measures of insulin sensitivity.[36] However, there was no significant difference in taurine conjugated BAs in our study population. These inconsistent results between studies could be potentially induced by large intra- and inter-individual variability of BAs concentration. The intra- and inter-individual variability of BAs concentration was high[38] and may be confounded by differences in dietary composition and the interval between nutrient intake and sampling.

Although our cross-sectional analysis cannot determine whether association between glycine conjugated BAs and glucose homeostasis is causal in nature, we find fasting serum glycine conjugated BA are lower in patients with T2D, and intermediate in patients with IFG, compared with healthy control with normal fasting glucose. Further study is needed to elucidate whether differences in levels of glycine conjugated BAs play a causal role in dysregulation of metabolic control in T2DM and to determine potential relationships between serum glycine conjugated BAs levels and gut microflora.

In the present study, we showed overall but various degree of increase of fasting blood

AAs in patients with IFG and T2DM. These increase was stepwise with progression to IFG and T2DM. In addition, levels of the inflammatory cytokines TNF-α and IL-6 were positively correlated with these increased AAs. We also found HOMA-IR and TNF-α or IL-6 were two important independent predictor of blood AAs level. Furthermore, palmitate treatment in C2C12 myotubes induced insulin resistance, increased pro-inflammatory cytokine gene expression, and increased MAFbx gene and protein expression. From these results, we can assume a cause-effect relationship between insulin resistance and the increased blood AAs, which might induced by the increased muscle proteolysis due to insulin resistance. Finally, we found that glycine conjugated BAs were decreased in the patients with IFG and T2DM.

V. CONCLUSION

The present study demonstrated that fasting blood AAs, not only BCAAs but also glutamic acid, lysine, phenylalanine, arginine, alanine, tyrosine, aspartic acid are increased in patients with IFG and T2DM suggesting fasting blood AAs increase can be early manifestation of insulin resistance. The ability to discriminate between normoglycemia and IFG or T2DM of these AAs were not significantly different from standard HOMA-IR suggesting use of these AAs as possible indicator of insulin resistance. The inflammatory cytokines and insulin resistance are independently associated with these AAs increase. The possible mechanism of elevated fasting blood AAs in insulin resistance and T2DM could be increase of skeletal muscle proteolysis. The insulin resistance induced by palmitate treatment in C2C12 muscle cell line increased the expression of MAFbx but reduced the expression of MuRF1. The priority and specificity of these two muscle specific E3 ligases need to be further evaluated. Elucidation of the underlying mechanisms of increased blood AAs in

insulin resistance and T2DM may broaden the knowledge of the complex pathophysiology of T2DM. Furthermore, glycine conjugated BAs are decreased in the patients with IFG and T2DM suggesting control of glycine conjugation and deconjugation of BAs, which is influenced by microbiome alteration, could affect glucose homeostasis.

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ABSTRACT(IN KOREAN)

정상인과 구별되는 공복혈당장애 및 제2형 당뇨병 환자의 특이적인 혈중 아미노산과 담즙산의 구성 변화

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배경 : 최근 메타볼로믹스 분석기법의 발달로 새로운 바이오마커의 개발에 지속적인 진보가 있어왔다. 새로운 바이오마커들 중 혈중 아미노산의 구성은 여러 질환들에서 유망한 바이오마커로써의 가능성을 보여주었다. 특히 분지사슬 아미노산(branched chain amino acid)의 증가가 제2형 당뇨병, 비만, 심장혈관질환에서 일관되게 보고되고 있다. 뿐만 아니라, 최근의 연구들에서는 담즙산이 포도당 대사에서 중요한 역할을 한다는 사실이 보고되었다. 따라서 공복혈당장애나 제2형 당뇨병을 가진 환자들에서의 특징적인 공복 혈중 아미노산과 담즙산 구성의 차이를 찾아내기 위해 건강한 대조군과 공복혈당장애 또는 제2형 당뇨병을 가진 환자들에서 혈중 아미노산과 담즙산의 구성을 분석하고자 하였다.

방법 : 당뇨약제를 복용하지 않은 제2형 당뇨병으로 최초 진단된 72명, 공복혈당장애 환자 97명, 정상인 75명에서 총 20종의 아미노산과 15종의 담즙산의 농도를 고성능액체크로마토그래피-질량분석기 법으로 측정하였다. 혈청 TNF-α와 IL-6의 농도는 상품화된 ELISA 키트를 이용하여 측정하였다. C2C12 마우스 근원세포를 사용하여 팔미테이트 처리를 통해 인슐린 저항성을 유발하고 MAFbx와 MuRF1의 발현의 변화를 평가하였다.

결과 : 정상인에 비해 공복혈당장애와 제2형 당뇨병 환자에서 공복 혈청 분지사슬 아미노산뿐만 아니라 글루탐산, 리신, 페닐알라닌, 아르기닌, 알라닌, 티로신, 아스파르트산이 함께 증가하는 것을 발견하였다. 이러한 아미노산의 증가는 공복혈당장애보다 제2형 당뇨병 환자에서 더 크게 나타났다. 증가된 아미노산들의 농도는 공복혈당, HOMA-IR, 염증성 사이토카인과 우수한 양의 상관성을 보였다. 그리고 HOMA-IR과 염증성 사이토카인은 공복 혈중 아미노산 농도를 결정하는 중요한 두 가지 독립적인 요소였다. C2C12 세포를 이용한 체외실험결과, 팔미테이트의 처리는 인슐린 저항성과 염증성 사이토카인 유전자의 발현을 촉진하였고 MAFbx 유전자 및 단백질의 발현 증가를 확인하였다. 마지막으로 글리신 포합 담즙산의 경우 정상인에 비해 공복혈당장내나 제2형 당뇨병 환자에서 감소함을 확인하였다.

결론 : 공복 혈중 아미노산의 상승은 인슐린 저항성의 원인요소이기 보다 인슐린 저항성의 조기증후일 가능성이 높다. 이러한 혈중 아미노산 상승의 기전으로는 골격근에서의 근육 단백질의 가수분해 증가가 유력하다. 당뇨병 환자에서의 글리신 포합 담즙산의 감소된 결과를 통해

장에서의 담즙산 포합조절이 포도당 대사에서 중요한 역할을 수행할 가능성을 확인하였다.

핵심되는 말 : 아미노산 구성, 분지사슬 아미노산, 염증성 사이토카인, 담즙산 구성, 인슐린 저항성, 제2형 당뇨병