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Characteristic changes in plasma glutamate levels and free amino acid profiles in Japanese patients with type 1 diabetes mellitus

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ABSTRACT

Aims/Introduction: In addition to absolute insulin deficiency, dysregulated glucagon in type 1 diabetes is considered pathophysiologically important. Previously, we confirmed the presence of dysregulated glucagon in Japanese patients with type 1 diabetes, and found a significant correlation between plasma glucagon and blood urea nitrogen levels, suggesting an association between glucagon and amino acid metabolism. In this study, we evaluated plasma amino acid levels in Japanese patients with type 1 diabetes in the context of their functional relationship with glucagon.

Materials and Methods: We assessed plasma free amino acid levels using liquid chromatography–mass spectrometry in 77 Japanese patients with type 1 diabetes, and statistically analyzed their characteristics and relationships with clinical parameters, including glucagon.

Results: Participants with type 1 diabetes showed a large decrease in glutamate levels together with a characteristic change in plasma free amino acid profiles. The network structural prediction analyses showed correlations between each amino acid and glucagon in type 1 diabetes.

Conclusions: Participants with type 1 diabetes showed characteristic changes in plasma glutamate levels and free amino acid profiles compared with controls and type 2 diabetes patients. Glucagon showed a closer correlation with amino acids than with parameters of glucose metabolism, suggesting that type 1 diabetes includes dysregulation in amino acids through dysregulated glucagon from remaining pancreatic α-cells, together with that in glucose by insulin deficiency.

INTRODUCTION

In type 1 diabetes mellitus, recent advances in insulin treatment, such as agents, devices and various related technologies, have made it possible to achieve improved glycemic control and decrease the prevalence of complications. However, in contrast to the decline in classical diabetes complications, such as diabetic retinopathy, nephropathy and neuropathy, other disorders, including sarcopenia and fatty liver diseases, possibly due to inadequate energy and nutrient metabolism, become evident in patients with type 1 diabetes. Thus, regarding these "new" complications, an understanding of the underlying mechanisms and development of new therapeutic strategies that are beyond glucose and insulin is warranted.

The systemic glucose status is not controlled exclusively by insulin; another important pancreatic hormone, glucagon, also plays a significant role in the maintenance of fundamental glucose supply from energy-storing tissues to energy-consuming organs. Thus, an adequate balance between the two essential hormones, insulin and glucagon, is important for the proper maintenance of systemic glucose circulation.

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However, it has been widely recognized that the regulation of glucagon is also impaired in diabetes, both types 1 and 2, and the dysregulated glucagon is involved in the exacerbation of both hyper- and hypoglycemia[1]. Indeed, we recently reconfirmed dysregulated plasma glucagon levels in Japanese patients with type 1 diabetes in response to plasma glucose levels[2,3] using the newly-developed dual antibodies "sandwich" enzyme-linked immunosorbent assay, which improved its accuracy compared with that of conventional single antibody radioimmunoassay developed in the 1970s[4], possibly due to a lack of intra-islet regulation by neighboring β-cells, mainly by insulin[5]. Plasma glucagon levels were not correlated with other clinical parameters for glucose and lipid metabolism, liver and renal function, and diabetic complications. Interestingly, plasma glucagon levels were significantly and strongly correlated with serum blood urea nitrogen (BUN) levels, suggesting a functional relationship with nitrogen metabolism[2]. Recent advances in basic medical research on glucagon have shown a new functional link between glucagon and amino acids[6,7]. Amino acids are known to acutely stimulate glucagon secretion. In addition, amino acids have been reported to trigger proliferation signals in glucagon-secreting α-cells[8,9]. In contrast, a new glucagon function in the liver and promotion of amino acid metabolism has been reported[4]. Taken together, these findings from our clinical and recent basic research propose that dysregulated glucagon in type 1 diabetes contributes to disorders in not only glucose metabolism, but also in protein-amino acid metabolism. Indeed, it is reported that Japanese patients with type 1 diabetes show a high prevalence of dynapenia and sarcopenia[10], suggesting possible metabolic changes of amino acids in skeletal muscles.

These changes in concepts, the pathophysiology and treatment goals of type 1 diabetes, and the significance of glucagon in multiple-nutrient regulation should be verified in real-world settings, but there are no recent studies reported. In particular, it is also important to assess amino acids together with the glucagon status for two reasons: (i) its newly revealed role in amino acid metabolism; and (ii) the necessity of glucagon re-evaluation by the novel enzyme-linked immunosorbent assay. Here, we comprehensively assessed plasma glucagon levels and plasma free amino acid (PFAA) levels in 77 Japanese patients with type 1 diabetes using state-of-art techniques, and statistically analyzed their characteristics and relationships with clinical parameters. Our participants showed a characteristic change in their PFAA profiles, especially a large decrease in glutamate levels, which contradicts the increased plasma glutamate levels in type 2 diabetes. The correlation analyses between parameters and network structural prediction analyses both show a closer relationship of glucagon with amino acids than with markers of glucose metabolism. These results suggest that type 1 diabetes shows multiple-nutrition dysregulation in not only glucose, but also amino acids, through dysregulated glucagon from remaining pancreatic α-cells, leading to protein-related complications.

MATERIALS AND METHODS

Clinical examination

For selection of patients with type 1 diabetes, a total of 77 Japanese patients with type 1 diabetes who underwent an annual checkup in 2018 were enrolled in the present study after providing written informed consent. All participants were diagnosed with type 1 diabetes by diabetes specialists at the time of presenting hyperglycemia and/or ketosis in their clinical history, and thereafter, all had undergone treatment with insulin therapy. Most participants were considered to have autoimmune, acute-onset type 1 diabetes; however, the inclusion of fulminant-type patients could not be completely excluded when they were diagnosed before the establishment of the concept and diagnostic criteria of fulminant type 1 diabetes. It is less possible that the slowly-progressive-type diabetes patients with preserved endogenous insulin secretion were included in the participants. Their acute onset and diagnosis of diabetes was confirmed by medical interviews, and patients possibly showing this subtype (e.g., treated with oral medications) were excluded from the study. Patients with neoplasms and those undergoing treatment with oral antidiabetic agents were excluded. The study protocol was approved by the local ethics committee of Osaka University Hospital (no. 12372), and was carried out in accordance with the tenets of the Declaration of Helsinki. Regarding clinical examination of patients with type 1 diabetes, the detailed methodology was the same as that previously reported[2]. The clinical assessments included a medical history interview, physical examination, blood sampling, and evaluation of micro- and macrovascular complications, as previously reported[11–13]. Venous blood samples were collected at the time of the visit without any restrictions on food intake, insulin treatment or daily activities, and laboratory analyses were carried out at SRL Inc. (Tokyo, Japan). All data and blood samples were collected on July 28 and August 18 at Osaka University Hospital in 2018. Plasma glucagon concentration was measured using a specific dual-antibody sandwich enzyme-linked immunosorbent assay (Mercodia AB, Uppsala, Sweden/Cosmic Corporation Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions.

For selection of controls and patients with type 2 diabetes, the study was carried out in accordance with the Declaration of Helsinki, and the protocol was approved by the ethical committee of Mitsui Memorial Hospital. A total of 1,000 Japanese individuals who had undergone the Ningen Dock Comprehensive Medical Check-up[14] in 2017 at the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital, were enrolled. The main inclusion criteria were as follows: individuals who had undergone the Ningen Dock Comprehensive Medical Check-up for Periodic Health Examination, were aged ≥20 years and provided informed consent to participate in the study. Patients with hepatitis C were excluded from the study. For clinical examination of healthy controls and patients with type 2 diabetes, blood samples were collected from the participants after an overnight fast. Serum levels of total cholesterol,

triglycerides, high-density-lipoprotein cholesterol and low-density-lipoprotein cholesterol were determined enzymatically. Plasma glucose levels were measured using the hexokinase method, and glycated hemoglobin A1c was determined using a latex agglutination immunoassay. All other variables were assessed as previously described[15,16]. For extraction of healthy controls, three exclusion criteria were used to screen healthy controls from the 1,000 individuals. The first exclusion criterion was the use of regular drug therapy for chronic diseases, such as cancer, cerebrovascular disease, diabetes, hypertension, hyperlipidemia, hepatitis, pancreatitis, chronic kidney disease, intestinal disease, gout and depression. The second criterion was any abnormality in ordinary clinical laboratory tests: total protein ≤6.3 and ≥8.4 g/dL, albumin (Alb) ≤ 3.7 and Alb ≥ 5.3 g/dL, total bilirubin ≥2.0 mg/dL, white blood cell count ≤1.5 × 10^3/μL, red blood cell count ≤330 × 10^4/μL, hemoglobin ≤10 g/dL, mean corpuscular volume ≤ 70 fl, uric acid ≤1.5, ≥ 9.0 mg/dL, triglycerides ≥300 mg/dL, total cholesterol ≥300 mg/dL, fasting plasma glucose ≥121 mg/dL, γ-glutamyltransferase ≥ 100 U/L, glutamate oxaloacetate transaminase ≥60 U/L and C-reactive protein ≥0.8 mg/dL, in accordance with a previous report on reference intervals for various biochemical parameters in the Japanese population[17–20]. The remaining exclusion criteria were as follows: body mass index ≤14 and ≥30 kg/m^2, in accordance with the criteria of the Japan Society of Ningen Dock[21,22]. Type 2 diabetes was defined as a fasting plasma glucose ≥126 mg/dL, glycated hemoglobin A1c ≥6.5% or treatment with medication for diabetes.

PFAA analysis

PFAA was evaluated using liquid chromatography–mass spectrometry. Briefly, 5-mL whole blood samples were collected from forearm veins into sample tubes containing ethylenediaminetetraacetic acid disodium salt (Termo, Tokyo, Japan) and immediately cooled in an iced water bath for >15 min. The plasma was separated using a 1,500 *g* desktop centrifuge for 20 min at 4°C and subsequently stored in a −80°C deep freezer until measurements. The amino acids in the plasma samples were derivatized with 3-aminopyridyl-N-hydroxysuccinimidyl carbamate (Wako Pure Chemical Industries Ltd., Osaka, Japan)[23] and were measured using liquid chromatography–mass spectrometry using a Shimadzu UF-Amino Station (Shimadzu Corporation, Kyoto, Japan). The details of sample preparation, derivatization and amino acid measurement have also been described[24]. The following 19 amino acids were measured: alanine, arginine, asparagine, citrulline, glutamine, glycine, histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), ornithine, phenylalanine (Phe), proline, serine, threonine (Thr), tryptophan (Trp), tyrosine and valine (Val).

Recently, we developed a novel, multivariate regression model of PFAAs, which is referred to as "AminoIndex technology", and utilized to compress the multidimensional information from PFAA profiles into a single score to maximize the differences between case and control populations[25–27]. By using this technology, we successfully obtained a model (AminoIndex™ LifeStyle disease [AILS].DM) for evaluating the risk of diabetes, consisting of Val, Ala, Tyr, Asn, Trp and Gly as objective variables[25].

Statistical analysis

Statistical analyses were carried out using JMP Pro (version 13.2.0; SAS Institute, Cary, NC, USA) and R (version 4.1.0; R Development Core Team, 2021; R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria). Data are expressed as the mean ± standard deviation.

A correlation analysis between each of the single PFAA levels, AILS values and clinical parameters of the patients with type 1 diabetes was carried out using the Pearson product–moment correlation coefficient (red for positive; blue for negative). In addition, a two-dimensional hierarchical cluster analysis based on the correlation coefficient matrix between each of the single PFAA levels, AILS values and clinical parameters was carried out. A correlation network was constructed using Pearson correlation coefficients ($|R| > 0.25$). The thickness of the edge reflects the strength of the correlation between the nodes.

RESULTS

Patient characteristics

The clinical characteristics of the 77 patients with type 1 diabetes (Table 1), demographic and clinical characteristics (Table 2), and the plasma levels of various free amino acids (Table 3) of the study groups are presented. The significant (Pearson's correlation coefficient $r = 0.325$, $P = 0.004$) and independent ($P = 0.022$ in a multivariate linear regression analysis) correlation of plasma glucagon levels with serum BUN levels in the type 1 diabetes group as reported[3] was confirmed. In addition, their endogenous insulin secretion capacity was severely exhausted, as almost all participants showed undetectable urine C-peptide levels, whereas a few patients showed relatively remaining urine C-peptide levels (10–20 μg/g·Cr).

Closer correlation of glucagon with amino acid groups than with parameters of glucose metabolism in type 1 diabetes

Thereafter, to assess the clinical and pathophysiological significance of PFAA in type 1 diabetes, we analyzed the correlations between clinical parameters and estimated the structure of the correlations (Figure 1). Clustering of functionally related parameters, such as amino acids, glucose and lipid metabolism appeared. Various amino acids were also clustered with each other, together with glucagon (Figure 1, red arrow). We also estimated the correlation network structure of clinical parameters in the participants with type 1 diabetes (Figure 2). The network visualized correlations of functionally related parameters in lipid metabolism and liver function. Glycated hemoglobin A1c and GA levels were correlated with lipid levels. Parameters of renal function, including BUN, creatinine and estimated glomerular filtration

Table 1 | Clinical characteristics of the patients with type 1 diabetes group (n = 77)

	Mean ± SD		Mean ± SD
Age (years)	34.9 ± 6.4	AST (U/L)	20.8 ± 8.6
Female, *n* (%)	52 (67.5)	ALT (U/L)	17.4 ± 11.5
Duration of diabetes (years)	25.8 ± 7.7	GGT (U/L)	22.8 ± 28.1
BMI (kg/m^2)	23.7 ± 3.6		
		BUN (mg/dL)	13.1 ± 3.3
HbA1c (%)	7.6 ± 1.0	UA (mg/dL)	4.2 ± 1.2
GA (%)	22.9 ± 4.0	Cr (mg/dL)	0.68 ± 0.11
Plasma glucose (mg/dL)	177 ± 82		
Plasma glucagon (pg/mL)	16.0 ± 9.8	LDL-C (mg/dL)	105 ± 22
Daily total insulin dose (U/day)	40.6 ± 15.0	HDL-C (mg/dL)	73 ± 16
Insulin dose (U/kg bodyweight)	0.63 ± 0.19	TG (mg/dL)	97 ± 91
		TC (mg/dL)	195 ± 26
max-IMT (mm)	0.77 ± 0.12		
ABI	1.05 ± 0.08	Proliferative retinopathy (%)	9 (11)
ba-PWV (cm/s)	1,329 ± 215	CV_{R-R} (%)	4.79 ± 3.8

Data are expressed as mean ± standard deviation or number (percentage). ABI, ankle-brachial index; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; CV_{R-R}, coefficient of variation of R-R intervals; GA, glycoalbumin; GGT, γ-glutamyltransferase; HbA1c, glycated hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; IMT, intima media thickness; LDL-C, low-density lipoprotein cholesterol; PWV, pulse wave velocity; TC, total cholesterol; TG, triglyceride; UA, uric acid.

Table 2 | Demographic and clinical characteristics of the study groups

	HC (n = 526)	T1D (n = 77)	T2D (n = 49)	ROC_AUC (95% CI)		
				HC vs T1D	HC vs T2D	T2DM vs T1D
Clinical characteristics						
Plasma glucose (mg/dL)	97.4 ± 8.2	176.7 ± 82.5	154.1 ± 33.8	**0.82 (0.74 to 0.90)**	**1.00 (1.00 to 1.00)**	0.54 (0.44 to 0.64)
HbA1c (%)	5.6 ± 0.3	7.6 ± 1.0	7.4 ± 1.1	**0.98 (0.96 to 1.00)**	**1.00 (0.99 to 1.00)**	0.59 (0.49 to 0.70)
BMI (kg/m^2)	22.5 ± 2.8	23.8 ± 3.6	25.8 ± 5.1	**0.58 (0.51 to 0.65)**	**0.72 (0.63 to 0.80)**	**0.64 (0.54 to 0.74)**
Waist circumference (cm)	81.6 ± 8.2	78.1 ± 10.5	91.1 ± 11.9	**0.64 (0.56 to 0.71)**	**0.75 (0.67 to 0.83)**	**0.81 (0.74 to 0.89)**
TC (mg/dL)	209.4 ± 31.8	195.5 ± 26.6	201.5 ± 38.1	**0.64 (0.58 to 0.70)**	0.57 (0.49 to 0.66)	0.56 (0.46 to 0.67)
TG (mg/dL)	92.4 ± 45.6	96.5 ± 90.8	152.0 ± 80.5	0.56 (0.49 to 0.64)	**0.77 (0.70 to 0.83)**	**0.80 (0.73 to 0.88)**
HDL-C (mg/dL)	69.4 ± 18.7	72.6 ± 15.7	54.6 ± 13.1	**0.57 (0.51 to 0.63)**	**0.74 (0.67 to 0.81)**	**0.82 (0.74 to 0.90)**
LDL-C (mg/dL)	125.2 ± 28.3	105.4 ± 22.3	123.1 ± 32.2	**0.71 (0.66 to 0.77)**	0.54 (0.45 to 0.62)	**0.67 (0.57 to 0.77)**
SBP (mmHg)	118.9 ± 15.4	119.5 ± 15.3	130.1 ± 15.1	0.50 (0.44 to 0.57)	**0.70 (0.63 to 0.77)**	**0.70 (0.61 to 0.79)**
DBP (mmHg)	75.7 ± 10.2	71.0 ± 11.1	79.1 ± 8.9	**0.64 (0.57 to 0.71)**	**0.60 (0.52 to 0.67)**	**0.73 (0.64 to 0.82)**
TP (g/dL)	7.0 ± 0.3	7.6 ± 0.4	7.1 ± 0.4	**0.81 (0.76 to 0.87)**	0.58 (0.49 to 0.66)	**0.77 (0.68 to 0.85)**
Alb (g/dL)	4.4 ± 0.2	4.5 ± 0.3	4.4 ± 0.2	**0.64 (0.57 to 0.72)**	0.52 (0.43 to 0.61)	**0.62 (0.52 to 0.71)**
T-bil (mg/dL)	0.8 ± 0.3	0.7 ± 0.3	0.8 ± 0.2	**0.66 (0.59 to 0.73)**	0.56 (0.48 to 0.65)	**0.60 (0.50 to 0.71)**
AST (U/L)	19.8 ± 5.0	17.4 ± 11.5	24.8 ± 14.4	**0.72 (0.64 to 0.80)**	**0.64 (0.55 to 0.73)**	**0.77 (0.69 to 0.86)**
ALT (U/L)	18.7 ± 9.3	20.8 ± 8.6	29.0 ± 19.5	**0.61 (0.55 to 0.67)**	**0.72 (0.64 to 0.80)**	**0.66 (0.55 to 0.76)**
gGTP (U/L)	29.7 ± 18.2	22.8 ± 28.1	63.4 ± 63.6	**0.72 (0.65 to 0.78)**	**0.71 (0.63 to 0.79)**	**0.85 (0.78 to 0.92)**
UA (mg/dL)	5.7 ± 1.3	4.2 ± 1.1	5.7 ± 1.1	**0.80 (0.75 to 0.85)**	0.50 (0.43 to 0.58)	**0.83 (0.76 to 0.90)**
Cre (mg/dL)	0.8 ± 0.2	0.7 ± 0.1	0.8 ± 0.3	**0.68 (0.62 to 0.73)**	0.51 (0.42 to 0.60)	**0.66 (0.56 to 0.76)**
eGFR (mL/min/1.73 m^2)	74.0 ± 12.9	88.9 ± 16.5	78.2 ± 21.0	**0.78 (0.73 to 0.84)**	0.59 (0.49 to 0.68)	**0.68 (0.58 to 0.78)**
Age (years)	56.2 ± 10.1	35.3 ± 6.4	62.3 ± 7.8	**0.96 (0.95 to 0.97)**	**0.67 (0.61 to 0.74)**	**1.00 (1.00 to 1.00)**

The area under the curve (AUC) of the receiver operating characteristic (ROC) with 95% confidence intervals (CI) for each clinical variable for discriminating type 1 diabetes patients (T1D) from healthy controls (HC), type 2 diabetes patients (T2D) from HC, and T1D from T2D. Significant AUC of ROC is highlighted in bold type. The continuous variables are summarized as the means ± standard deviations (SD). ABI, ankle-brachial index; Alb, albumin; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; BUN, blood urea nitrogen; Cre, creatinine; CV_{R-R}, coefficient of variation of R-R intervals; eGFR, estimated glomerular filtration rate; GA, glycoalbumin; gGTP, γ-glutamyltransferase;; HbA1c, glycated hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; IMT, intima media thickness; LDL-C, low-density lipoprotein cholesterol; PWV, pulse wave velocity; T-bil, total bilirubin; TC, total cholesterol; TG, triglyceride; UA, uric acid.

Table 3 | Plasma free amino acid concentrations and AminoIndex™ LifeStyle disease for diabetes mellitus values

	HC (n = 526)	T1D (n = 77)	T2D (n = 49)	ROC_AUC (95% CI)		
				HC vs T1D	HC vs T2D	T2DM vs T1D
Plasma free amino acid concentrations (μmol/L)						
Glu	45.1 ± 18.9	17.6 ± 10.0	65.4 ± 23.6	**0.92 (0.88 to 0.95)**	**0.75 (0.68 to 0.82)**	**0.98 (0.96 to 1.00)**
Ser	116.2 ± 17.6	132.2 ± 29.8	114.5 ± 18.9	**0.66 (0.59 to 0.74)**	0.54 (0.45 to 0.63)	**0.69 (0.60 to 0.78)**
Asn	46.1 ± 6.6	50 ± 13.2	45.2 ± 6.6	0.55 (0.47 to 0.64)	0.53 (0.44 to 0.62)	0.58 (0.48 to 0.68)
Gly	215 ± 45.5	260 ± 65.8	195.7 ± 42.0	**0.73 (0.66 to 0.79)**	**0.64 (0.55 to 0.73)**	**0.81 (0.73 to 0.89)**
Gln	573.7 ± 62.2	573.5 ± 88.1	563.8 ± 89.3	0.53 (0.45 to 0.61)	0.54 (0.44 to 0.63)	0.49 (0.39 to 0.60)
His	82.2 ± 9.5	79.3 ± 12.9	82.9 ± 9.7	**0.61 (0.53 to 0.69)**	0.53 (0.43 to 0.62)	**0.62 (0.52 to 0.72)**
Thr	121.7 ± 24.6	132.9 ± 37.0	121.9 ± 21.7	**0.59 (0.51 to 0.67)**	0.51 (0.43 to 0.59)	0.58 (0.48 to 0.68)
Ala	323.6 ± 70.1	376.8 ± 101.0	371.1 ± 77.7	**0.65 (0.58 to 0.73)**	**0.68 (0.60 to 0.77)**	0.50 (0.40 to 0.61)
Cit	30.4 ± 6.0	31.6 ± 7.0	31.8 ± 10.2	0.55 (0.47 to 0.62)	0.50 (0.40 to 0.06)	0.53 (0.42 to 0.64)
Arg	89.3 ± 15.4	94.9 ± 23.6	91.8 ± 24.6	0.56 (0.48 to 0.64)	0.51 (0.40 to 0.61)	0.56 (0.45 to 0.66)
Pro	132.6 ± 37.2	186.6 ± 65.8	167.7 ± 71.2	**0.79 (0.74 to 0.84)**	**0.73 (0.66 to 0.80)**	**0.60 (0.50 to 0.70)**
ABA	21.5 ± 5.9	17.8 ± 5.2	24.4 ± 7.2	**0.69 (0.62 to 0.75)**	**0.62 (0.53 to 0.71)**	**0.77 (0.68 to 0.85)**
Tyr	61.7 ± 10.1	61.4 ± 15.5	66.9 ± 12.5	0.54 (0.46 to 0.62)	**0.62 (0.53 to 0.71)**	**0.64 (0.54 to 0.73)**
Val	218.2 ± 36.4	208.9 ± 45.7	264.2 ± 41.3	**0.59 (0.51 to 0.66)**	**0.79 (0.73 to 0.86)**	**0.82 (0.75 to 0.89)**
Met	25.1 ± 3.9	26 ± 8.5	25.9 ± 4.1	0.54 (0.45 to 0.62)	0.57 (0.48 to 0.65)	0.58 (0.48 to 0.68)
Orn	47.7 ± 9.7	50.8 ± 15.1	54.8 ± 12.0	0.56 (0.48 to 0.63)	**0.67 (0.58 to 0.76)**	**0.60 (0.50 to 0.70)**
Lys	187.9 ± 28.0	175.6 ± 36.9	200.5 ± 30.6	**0.62 (0.54 to 0.70)**	**0.62 (0.53 to 0.70)**	**0.70 (0.61 to 0.79)**
Ile	58.9 ± 12.7	62.9 ± 20.2	75.4 ± 15.9	0.54 (0.46 to 0.62)	**0.79 (0.72 to 0.86)**	**0.71 (0.62 to 0.80)**
Leu	118.6 ± 21.4	107 ± 32.3	143.4 ± 23.6	**0.64 (0.57 to 0.72)**	**0.78 (0.71 to 0.84)**	**0.83 (0.76 to 0.90)**
Phe	57.9 ± 9.3	56.9 ± 10.8	60.2 ± 6.7	0.55 (0.47 to 0.63)	**0.61 (0.53 to 0.69)**	**0.63 (0.53 to 0.73)**
Trp	56.1 ± 7.2	55.0 ± 11.5	56.3 ± 8.2	0.57 (0.49 to 0.65)	0.53 (0.44 to 0.62)	0.58 (0.48 to 0.68)
PFAA index values						
AILS.DM	5.2 ± 2.3	4.4 ± 2.5	8.0 ± 2.1	**0.59 (0.52 to 0.66)**	**0.81 (0.75 to 0.88)**	**0.86 (0.8 to 0.93)**

The area under the curve (AUC) of the receiver operating characteristic (ROC) with 95% confidence intervals (CI) for each amino acid and AminoIndex™ LifeStyle disease for diabetes mellitus (AILS) for discriminating type 1 diabetes (T1D) from healthy controls (HC), type 2 diabetes (T2D) from HC, and T1D from T2D. Significant AUC of ROC is highlighted in bold type. The HC participants (526 participants), the participants with T1D (77 participants) and the participants with T2D (46 participants) were included, respectively. The plasma free amino acid (PFAA) concentrations (μmol/L) and AILS values are summarized as means ± standard deviations. ABA, alpha-aminobutyric acid; ABI, ankle-brachial index; AILS.DM, AminoIndex™ LifeStyle disease for diabetes mellitus; Ala, alanine; Alb, albumin; ALT, alanine transaminase; Arg, arginine; Asn, asparagine; AST; aspartate transaminase; BMI, body mass index; BUN, blood urea nitrogen; Cit, citrulline; Cre, creatinine; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GA, glycol albumin; Gcg, glucagon; Gln, glutamine; Glu, glutamate; Gly, glycine; HbA1c, glycated hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; His, histidine; Ile, isoleucine; LDL-C, low-density lipoprotein cholesterol; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; PG, plasma glucose; Phe, phenylalanine; Pro, proline; SBP, systolic blood pressure; Ser, serine; TC, total cholesterol; TG, triglyceride; Trp, tryptophan; Tyr, tyrosine; Val, valine.

rate, were correlated with each other, as well as with some amino acids. Interestingly, glucagon showed a closer correlation with amino acids (Figure 2, arrowhead), consistent with previous results of this correlation analyses (Figure 1).

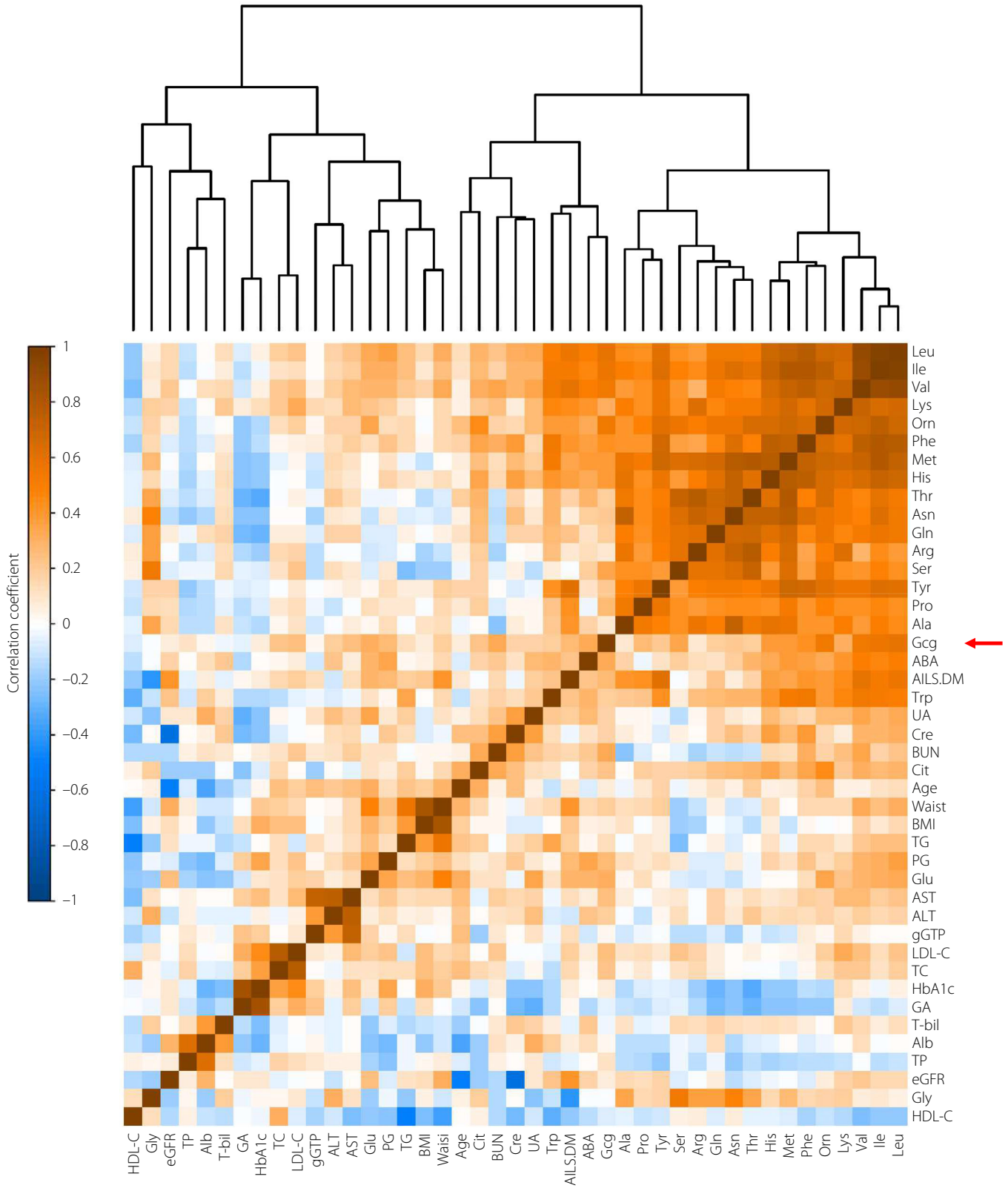
More positive-correlation clustering of PFAA in type 1 diabetes patients than in healthy controls and type 2 diabetes

We analyzed the correlations between clinical parameters, including PFAA, and compared them between the type 1 diabetes, control and type 2 diabetes groups (Figure 3, see details in Figures S1–S3). The type 1 diabetes group showed a characteristic pattern of correlations with a more positive-correlation clustering of PFAA than the healthy control and type 2 diabetes groups. In contrast, the correlation pattern in type 2 diabetes was more diverse than that in the controls.

Characteristic changes in plasma amino acid profiles in patients with type 1 diabetes

To determine the PFAA profile in the participants with type 1 diabetes, we evaluated the AILS[25,26]. The amino acid profile pattern in participants with type 1 diabetes was different from that in participants with type 2 diabetes (Table 3). In particular, glutamate showed a significant decrease in type 1 diabetes (Figure 4), contradicting that in type 2 diabetes, in which glutamate showed the highest increase.

As glutamate levels were significantly decreased in participants with type 1 diabetes, we analyzed the correlations between glutamate levels and various clinical parameters, including each amino acid value. In participants with type 1 diabetes, various parameters, including glucagon and amino acids, were found to have significant correlations; however,



fewer showed significant standardized partial regression coefficients in multiple linear regression analyses of glutamic acid (Tables S1 and S2). In contrast, in healthy controls, various clinical and amino acid parameters showed significant correlations with glutamate in both univariate and multivariate correlation analyses of glutamic acid (Tables S3 and S4). In addition,

FIGURE 1 | Correlation clustering using plasma free amino acid profiles, AminoIndex™ LifeStyle disease for diabetes mellitus (AILS) values and clinical variables. Pearson's correlation coefficients were calculated, and hierarchical clustering was carried out. The red arrow shows glucagon (Gcg). ABA, alpha-aminobutyric acid; ABI, ankle-brachial index; AILS.DM, AminoIndex™ LifeStyle disease for diabetes mellitus; Ala, alanine; Alb, albumin; ALT, alanine transaminase; Arg, arginine; Asn, asparagine; AST; aspartate transaminase; BMI, body mass index; BUN, blood urea nitrogen; Cit, citrulline; Cre, creatinine; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GA, glycol albumin; Gcg, glucagon; gGTP, γ-glutamyltransferase; Gln, glutamine; Glu, glutamate; Gly, glycine; HbA1c, glycated hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; His, histidine; Ile, isoleucine; LDL-C, low-density lipoprotein cholesterol; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; PG, plasma glucose; Phe, phenylalanine; Pro, proline; SBP, systolic blood pressure; Ser, serine; TC, total cholesterol; TG, triglyceride; Trp, tryptophan; Tyr, tyrosine; Val, valine; Waist, waist circumference.

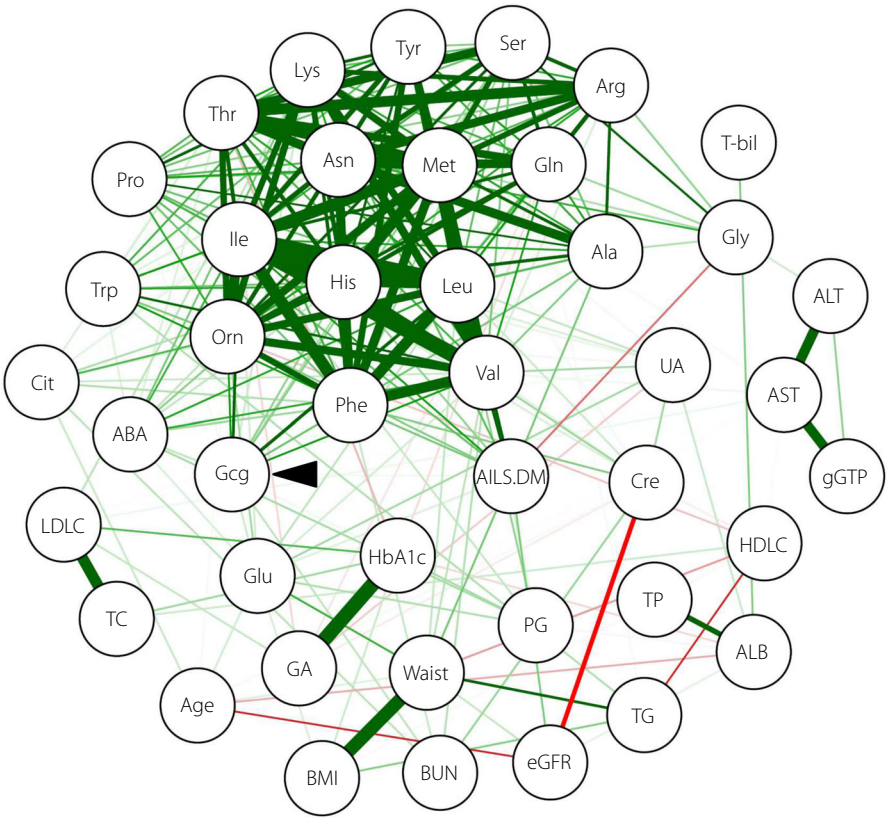


FIGURE 2 | Correlation network of plasma free amino acid profiles, AminoIndex™ LifeStyle disease for diabetes mellitus (AILS) values and clinical variables. A correlation network was constructed using Pearson's correlation coefficients ($|R| > 0.25$). The thickness of the edge reflects the strength of the correlation between the nodes. The arrowhead indicates glucagon (Gcg). ABA, alpha-aminobutyric acid; ABI, ankle-brachial index; AILS.DM, AminoIndex™ LifeStyle disease for diabetes mellitus; Ala, alanine; ALB, albumin; ALT, alanine transaminase; Arg, arginine; Asn, asparagine; AST; aspartate transaminase; BMI, body mass index; BUN, blood urea nitrogen; Cit, citrulline; Cre, creatinine; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GA, glycol albumin; Gcg, glucagon; gGTP, γ-glutamyltransferase; Gln, glutamine; Glu, glutamate; Gly, glycine; HbA1c, glycated hemoglobin A1c; HDLC, high-density lipoprotein cholesterol; His, histidine; Ile, isoleucine; LDLC, low-density lipoprotein cholesterol; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; PG, plasma glucose; Phe, phenylalanine; Pro, proline; SBP, systolic blood pressure; Ser, serine; TC, total cholesterol; TG, triglyceride; Trp, tryptophan; Tyr, tyrosine; Val, valine; Waist, waist circumference.

various parameters, including glutamate and other amino acids, were extracted, with significant correlations with glucagon in participants with type 1 diabetes; nevertheless, only lysine, ornithine and BUN yielded significant standardized partial regression coefficients in multiple linear regression analyses (Tables S5 and S6).

DISCUSSION

In our previous studies, we showed a significant and independent correlation between plasma glucagon and serum BUN levels in patients with type 1 diabetes[2,3], suggesting an altered amino acid metabolism in association with dysregulated glucagon. In the current study, we evaluated the PFAA levels in the

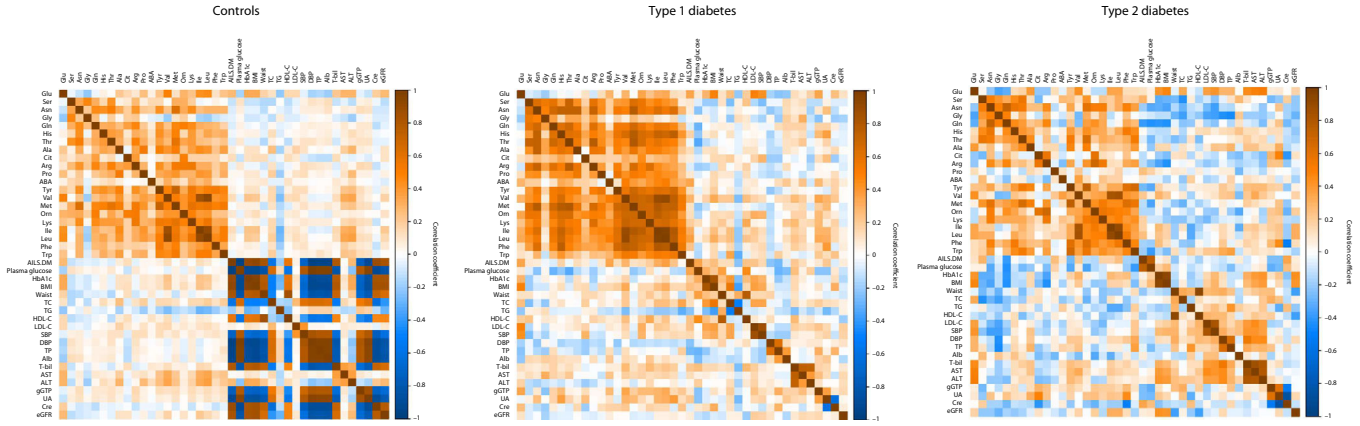


FIGURE 3 | Comparison of correlation clustering among the control, type 1 diabetes, and type 2 diabetes groups. Pearson's correlation coefficients were calculated, and hierarchical clustering was conducted. The detailed graphics are presented in Figures S1–S3.

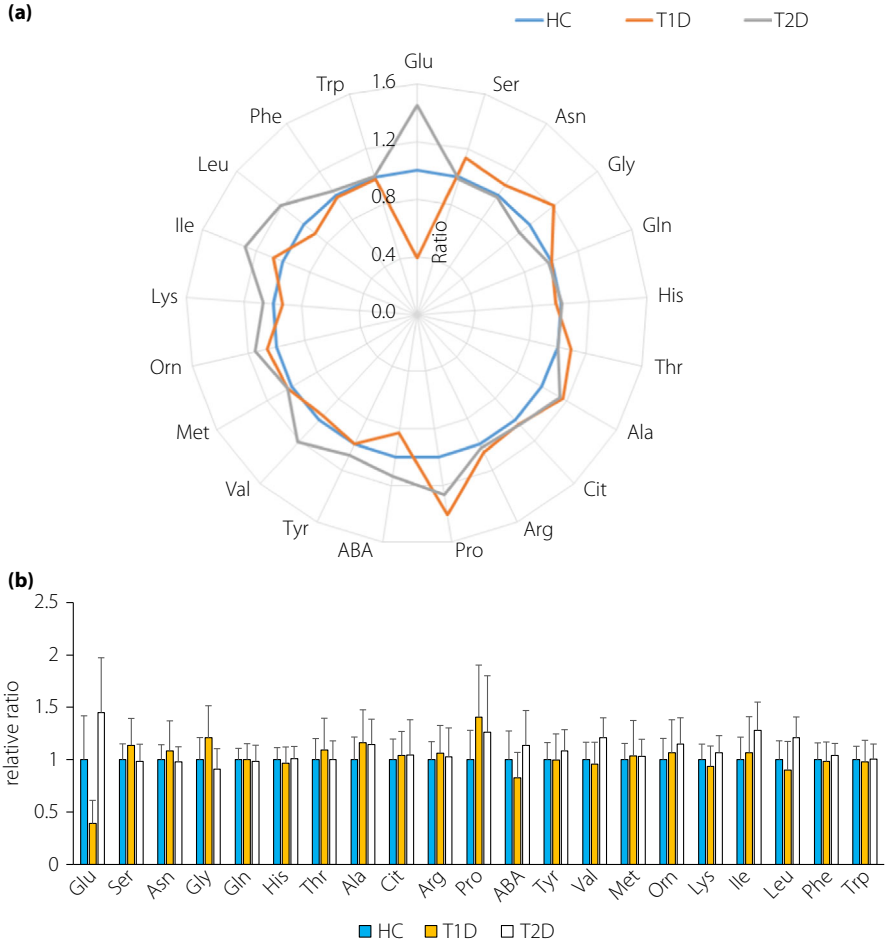


Figure 4 | Plasma free amino acid profiles of patients with types 1 and 2 diabetes and controls. (a) The ratio of each amino acid in type 1 diabetes (red bold line) and type 2 diabetes (gray bold line) divided by healthy control (HC) values. The blue bold lines indicate the HC point where the ratio = 1.0. (b) Relative levels of plasma free amino acids of patients with types 1 (T1D; red) and 2 diabetes (T2D; blank) and controls (HC; blue). Data are expressed by means ± standard deviations.

same patients with type 1 diabetes and found a characteristic change in their PFAA profiles. In addition, the PFAA-profile pattern in type 1 diabetes was considerably different from that in other diseases, including type 2 diabetes. In particular, plasma glutamate levels were significantly decreased in type 1 diabetes patients regardless of total PFAA levels, and this decrease was in contrast to the significant increase in glutamate levels in type 2 diabetes patients.

In the current study, we found a characteristic change in the PFAA profile of type 1 diabetes together with a notable decrease in plasma glutamate levels, despite obtaining the blood samples in randomly fed states. As meals include various protein ingredients that are potentially efficient sources of amino acids, PFAA levels are expected to increase after meals. Indeed, we also observed a mild yet significant increase in various amino acid levels in the participants with type 1 diabetes. Thus, we compared the plasma levels of essential amino acids in the 77 participants with type 1 diabetes between participants evaluated in fasted (n = 11) and random-fed (n = 66) states, but no significant differences in the parameters (Figure S4), suggesting less influence of fed or fasted states. These changes suggest that type 1 diabetes shows a different nutritional disorder, not simply because of insulin shortage. Thus, the current study is valuable, as we could show the detailed alteration in the PFAA profile using state-of-the-art technologies in a clinical setting.

Glutamate is a multifunctional amino acid that plays various physiological roles as an excitatory neurotransmitter, a tasty (umami) signal transmitter and a substrate for the mitochondrial tricarboxylic acid cycle. Thus, in the liver, which is the chief organ responsible for systemic energy supply, glutamate also plays an important role in energy metabolism. Interestingly, the decrease in plasma glutamate suggests a corresponding decrease in the liver[28]. In contrast, dietary glutamate intake potentially has minimal effects on plasma glutamate levels, as an animal study showed that dietary glutamate is exclusively utilized in the intestine[29]. Taken together, the significant decrease in plasma glutamate in individuals with type 1 diabetes suggests an intrahepatic decrease in glutamate. Type 1 diabetes involves two disorders of critical energy controlling hormones: insulin deficiency and glucagon dysregulation. In particular, excessive glucagon promotes excessive amino acid metabolism[6,7], thus accelerating glutamate metabolism. As the activity of α-ketoglutarate dehydrogenase, which catalyzes a metabolic step from glutamate to α-ketoglutarate, is suppressed by insulin, the insulin shortage in type 1 diabetes, especially hepatic deficiency of insulin action due to lack of endogenous insulin from pancreatic islets through the portal vein, potentially exacerbates the excessive glutamate metabolism. Excessive amino acid metabolism can induce excessive hepatic glucose supply due to upregulated gluconeogenesis from amino acids and subsequent hyperglycemia, and also lead to hypoaminoacidemia. Thus, it is highly possible that dysregulated glucagon in type 1 diabetes patients is responsible for the dysregulation of nutrient metabolism, including that of both glucose and protein–amino acids, and insulin deficiency exacerbates this.

However, the clinical relevance or mechanism underlying the significant decrease in plasma glutamate remains unclear. In participants with type 1 diabetes, only estimated glomerular filtration rate and UA levels showed significant correlations with glutamate in multiple linear regression analyses (Table S2), although we extracted various clinical parameters, including glucagon and amino acids, showing significant correlations with plasma glutamate levels (Table S1). These findings suggest the involvement of renal functions in the systemic regulation of amino acids. In the participants with type 1 diabetes, only a small number (6/77) showed diabetic nephropathy (urine albumin-creatinine ratio >30 mg/g □ Cre), suggesting limited influences of renal function/kidney diseases on amino acid status, but further detailed analyses are required. In addition, the factors that showed significant correlations with glutamate largely differed between participants with type 1 diabetes and healthy controls (Tables S1–S4). This might have been due to a statistical reason regarding the number of participants analyzed, especially the weaker extraction power due to the small number of participants with type 1 diabetes. Nevertheless, the differences in correlated factors between groups suggest that the decrease in plasma glutamate levels in type 1 diabetes is more related to the pathological state and is also independent of glucose metabolism.

As discussed in our previous reports[2,3], the current study had certain limitations. First, participants had type 1 diabetes; hence, the restriction of meals and insulin in order to standardize plasma glucose levels was not carried out due to medical and ethical reasons. In addition, blood samples were obtained randomly with respect to patient food intake; therefore, we cannot exclude the possible influence of meal ingredients, especially proteins and amino acids, on PFAA levels. Thus, it is necessary to carefully analyze amino acid intake and composition to reach definite conclusions in the future. Second, although it is reported that Japanese patients with type 1 diabetes show a high prevalence of dynapenia and sarcopenia[10], we could not obtain any data related to muscle power nor physical status in the current study using data of regular health checkups, thus could not incorporate these into the analyses with amino acid levels. In addition, the glucagon data were obtained only from individuals with type 1 diabetes, so it was difficult to compare the associations of amino acids and glucagon between participant groups. Now, measurement of plasma glucagon levels is limited to clinical or investigative purposes, but not commonly carried out in general health checkups, where we obtained the data of type 2 diabetes and healthy control groups. Third, it was a study with a small sample size, predominantly due to clinical limitations. However, this small size enabled us to collect a certain number of samples within a month in a relatively standardized condition, potentially increasing the reliability of clinical parameters in the subsequent analyses. For the controls, we used existing healthy control data from our database.

Fourth, the current study was cross-sectional, but not prospective. It is necessary to validate our current results at different time points in the same setting. We obtained a preliminary result using the dataset of the subsequent year's (2019) checkup and found the same tendency in PFAA profiles (data not shown). The clinical and technical limitations raised here should be considered to correctly interpret the results.

We evaluated the PFAA levels in 77 Japanese patients with type 1 diabetes in the context of dysregulated glucagon, and subsequently showed the characteristic changes in PFAA levels and profiles, especially large decreases in glutamate levels. The findings suggest that type 1 diabetes shows a comprehensive nutritional disorder that leads to various complications related to amino acid metabolism in addition to glucose metabolism.

DISCLOSURE

DK, YI, SH, NK and IS declare no conflict of interest. YK and TT are employees of Ajinomoto, Co. Inc., Japan.

Approval of the research protocol: The study protocol was approved by the local ethics committee of Osaka University Hospital (no. 12372) or Mitsui Memorial Hospital, and was carried out in accordance with the tenets of the Declaration of Helsinki.

Informed consent: All participants provided informed consent to participate in the study.

Approval date of Registry and the Registration No. of the study/trial: N/A.

Animal studies: N/A.

REFERENCES

1. Unger RH. Role of glucagon in the pathogenesis of diabetes: the status of the controversy. *Metabolism* 1978; 27: 1691–1709.
2. Kawamori D, Katakami N, Takahara M, *et al*. Dysregulated plasma glucagon levels in Japanese young adult type 1 diabetes patients. *J Diabetes Investig* 2019; 10: 62–66.
3. Kawamori D, Katakami N, Takahara M, *et al*. Consistency of plasma glucagon levels in patients with type 1 diabetes after a 1-year period. *J Diabetes Investig* 2020; 11: 337–340.
4. Miyachi A, Kobayashi M, Mieno E, *et al*. Accurate analytical method for human plasma glucagon levels using liquid chromatography-high resolution mass spectrometry: comparison with commercially available immunoassays. *Anal Bioanal Chem* 2017; 409: 5911–5918.
5. Kawamori D, Kurpad AJ, Hu J, *et al*. Insulin signaling in alpha cells modulates glucagon secretion in vivo. *Cell Metab* 2009; 9: 350–361.
6. Holst JJ, Wewer Albrechtsen NJ, Pedersen J, *et al*. Glucagon and amino acids are linked in a mutual feedback cycle: the liver-alpha-cell Axis. *Diabetes* 2017; 66: 235–240.
7. Hayashi Y, Seino Y. Regulation of amino acid metabolism and alpha-cell proliferation by glucagon. *J Diabetes Investig* 2018; 9: 464–472.
8. Dean ED, Li M, Prasad N, *et al*. Interrupted glucagon signaling reveals hepatic alpha cell Axis and role for L-glutamine in alpha cell proliferation. *Cell Metab* 2017; 25: e1365.
9. Kim J, Okamoto H, Huang Z, *et al*. Amino acid transporter Slc38a5 controls glucagon receptor inhibition-induced pancreatic alpha cell hyperplasia in mice. *Cell Metab* 2017; 25: e1348.
10. Mori H, Kuroda A, Yoshida S, *et al*. High prevalence and clinical impact of dynapenia and sarcopenia in Japanese patients with type 1 and type 2 diabetes: findings from the impact of diabetes mellitus on Dynapenia study. *J Diabetes Investig*. 2021; 12: 1050–1059.
11. Kawamori R, Yamasaki Y, Matsushima H, *et al*. Prevalence of carotid atherosclerosis in diabetic patients. Ultrasound high-resolution B-mode imaging on carotid arteries. *Diabetes Care* 1992; 15: 1290–1294.
12. Yamashina A, Tomiyama H, Takeda K, *et al*. Validity, reproducibility, and clinical significance of noninvasive brachial-ankle pulse wave velocity measurement. *Hyperten Res* 2002; 25: 359–364.
13. Osawa S, Kawamori D, Katakami N, *et al*. Significant elevation of serum dipeptidyl peptidase-4 activity in young-adult type 1 diabetes. *Diabetes Res Clin Pract* 2016; 113: 135–142.
14. Ikeda N, Saito E, Kondo N, *et al*. What has made the population of Japan healthy? *Lancet* 2011; 378: 1094–1105.
15. Yamakado M, Tanaka T, Nagao K, *et al*. Plasma amino acid profile is associated with visceral fat accumulation in obese Japanese subjects. *Clin Obes* 2012; 2: 29–40.
16. Nakamura H, Jinzu H, Nagao K, *et al*. Plasma amino acid profiles are associated with insulin, C-peptide and adiponectin levels in type 2 diabetic patients. *Nutr Diabetes* 2014; 4: e133.
17. Ichihara K, Itoh Y, Lam CW, *et al*. Sources of variation of commonly measured serum analytes in 6 Asian cities and consideration of common reference intervals. *Clin Chem* 2008; 54: 356–365.
18. Ichihara K, Boyd JC, Intervals ICoR, *et al*. An appraisal of statistical procedures used in derivation of reference intervals. *Clin Chem Lab Med* 2010; 48: 1537–1551.
19. Yamamoto Y, Hosogaya S. Session for clinical laboratory standards of JAMT; JAMT project fo the derivation of reference intervals in Japan. *J Anal Bio-Sci (Seibutsu Shiryo Bunseki)* 2011; 34: 199–210.
20. Yamamoto Y, Hosogaya S, Osawa S, *et al*. Nationwide multicenter study aimed at the establishment of common reference intervals for standardized clinical laboratory tests in Japan. *Clin Chem Lab Med* 2013; 51: 1663–1672.
21. Yoda T, Nakayama K, Kawai T. A report of small committee for draw-up the guide-line on determination of multiple health screening tests. No. II reference values. *Ningen Dock* 1998; 13: 162–183.

22. Japan Society of Ningen-Dock. wwwningen-dockjp/wp/common/data/other/inspection/2014/hantei_26-2pdf (in Japanese); 2014.
23. Shimbo K, Kubo S, Harada Y, *et al*. Automated precolumn derivatization system for analyzing physiological amino acids by liquid chromatography/mass spectrometry. *Biomed Chromatogr* 2010; 24: 683–691.
24. Yoshida H, Kondo K, Yamamoto H, *et al*. Validation of an analytical method for human plasma free amino acids by high-performance liquid chromatography ionization mass spectrometry using automated precolumn derivatization. *J Chromatogr B Analyt Technol Biomed Life Sci* 2015; 998-999: 88–96.
25. Yamakado M. "AminoIndex technology" for lifestyle-related disease risk screening. *Ningen Dock Int* 2018; 5: 3–14.
26. Yamakado M, Nagao K, Imaizumi A, *et al*. Plasma free amino acid profiles predict four-year risk of developing diabetes, metabolic syndrome, dyslipidemia, and hypertension in Japanese population. *Sci Rep* 2015; 5: 11918.
27. Miyagi Y, Higashiyama M, Gochi A, *et al*. Plasma free amino acid profiling of five types of cancer patients and its application for early detection. *PLoS One* 2011; 6: e24143.
28. Nakamura H, Kawamata Y, Kuwahara T, *et al*. The nitrogen moieties of dietary nonessential amino acids are distinctively metabolized in the gut and distributed to the circulation in rats. *J Nutr* 2017; 147: 1537–1545.
29. Nakamura H, Kawamata Y, Kuwahara T, *et al*. Nitrogen in dietary glutamate is utilized exclusively for the synthesis of amino acids in the rat intestine. *Am J Physiol Endocrinol Metab* 2013; 304: E100–E108.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Correlation clustering in controls.

Figure S2 | Correlation clustering in type 1 diabetes.

Figure S3 | Correlation clustering in type 2 diabetes.

Figure S4 | Comparison of high and low plasma free amino acid concentrations.

Table S1 | Correlation analysis for glutamate and variables in type 1 diabetes.

Table S2 | Multiple linear regression analysis for glutamate and independent variables in type 1 diabetes.

Table S3 | Correlation analysis for glutamate and variables in healthy controls.

Table S4 | Multiple linear regression analysis for glutamate and independent variables in healthy controls.

Table S5 | Correlation analysis for glucagon and variables in type 1 diabetes.

Table S6 | Multiple linear regression analysis for glucagon and independent variables in type 1 diabetes.