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Clinical Research Article

Sex Differences in Branched-chain Amino Acid and Tryptophan Metabolism and Pathogenesis of Youth-onset Type 2 Diabetes

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Abstract

Objectives: Insulin resistance is associated with elevations in plasma branched-chain amino acids (BCAAs). BCAAs compete with aromatic amino acids including tryptophan for uptake into β cells. To explore relationships between BCAAs and tryptophan metabolism, adiposity, and glucose tolerance, we compared urine metabolites in overweight/obese youth with type 2 diabetes (T2D) with those in nondiabetic overweight/obese and lean youth.

Methods: Metabolites were measured in 24-hour and first-morning urine samples of 56 nondiabetic adolescents with overweight/obesity, 42 adolescents with T2D, and 43 lean controls, aged 12 to 21 years. Group differences were assessed by Kruskal Wallis or ANOVA.

Results: Groups were comparable for age, pubertal status, and ethnicity. Youth with T2D were predominantly female and had highest percent body fat. BCAAs, branched-chain ketoacids (BCKAs), tryptophan, and kynurenine were higher in urine of subjects with T2D. There were no differences between lean controls and nondiabetic youth with overweight/obesity. T2D was associated with diversion of tryptophan from the serotonin to the kynurenine pathway, with higher urinary kynurenine/serotonin ratio and lower serotonin/tryptophan and 5-HIAA/kynurenine ratios. Urinary BCAAs, BCKAs, tryptophan, and ratios reflecting diversion to the kynurenine pathway correlated positively with metrics of body fat and hemoglobin A1c. Increases in these metabolites in the obese T2D group were more pronounced and statistically significant only in adolescent girls.

Conclusion: Increases in urinary BCAAs and BCKAs in adolescent females with T2D are accompanied by diversion of tryptophan metabolism from the serotonin to the kynurenine pathway. These adaptations associate with higher risks of T2D in obese adolescent females than adolescent males.

Key Words: BCAAs, BCKAs, tryptophan-kynurenine-serotonin pathway, youth-onset type 2 diabetes mellitus, obesity

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; BF%, percent body fat; BMI, body mass index; HbA1c, hemoglobin A1c; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; IR, insulin resistance; IRB, institutional review board; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate; MS/MS, tandem mass spectrometry; PHQ, Patient Health Questionnaire; T2D, type 2 diabetes.

Type 2 diabetes (T2D) has a more aggressive course in youth than in adults, with early and rapid deterioration of β-cell function, inadequate responses to oral hypoglycemic agents, rapid progression to insulin dependence, and higher prevalence and earlier presentation of diabetes complications (1). Obesity and insulin resistance (IR) correlate strongly with development of T2D in youth; however, only one-half of obese children are insulin resistant and a far lower percentage

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progress to T2D (2, 3). This suggests that factors in addition to obesity contribute to progression to overt glucose intolerance. It is essential to identify metabolic markers that can predict the development of IR and progression to T2D; this might enable us to reduce the incidence and prevent the complications of T2D in youth.

Emerging literature demonstrates that IR and T2D are associated with, and may be predicted by, derangements in amino acid metabolism (4-12). For example, we showed that IR in youth with obesity is associated with a sex-dependent metabolomic profile comprising increases in the fasting plasma concentrations of the branched-chain amino acids (BCAAs) and their catabolic by-products, including branched-chain keto acids (BCKAs), glutamate, and C3/C5 acylcarnitines (4-6). Plasma levels of these metabolites are higher in adolescent boys than girls of comparable body mass index (BMI) *z* score (4). Likewise, IR in adults is associated with increases in plasma BCAAs and related metabolites (13-20), as well as altered metabolism of tryptophan, an aromatic amino acid and the precursor of the neurotransmitter serotonin (21-24). Serotonin plays an important role in glucose homeostasis by increasing pancreatic β-cell replication, β-cell mass, and glucose-dependent insulin secretion (25-28). Serotonin production is regulated by the activity of tryptophan hydroxylase and limited by the availability of tryptophan (29). In peripheral tissues, tryptophan can be converted to kynurenine as well as serotonin (Fig. 1). In adults with obesity and IR, the metabolism of tryptophan is diverted preferentially to kynurenines, with a concomitant increase in the plasma kynurenine/tryptophan ratio (22-24). Thus, decreased serotonin production in obese, high-risk subjects might explain defects in β-cell function that lead to the development of overt hyperglycemia.

In this study, we analyzed 24-hour urine samples to identify factors associated with T2D among youth with overweight/obesity. In contrast to single point-in-time plasma analyses, urine metabolomic profiling integrates differences in metabolic status over a 24-hour period in a noninvasive manner (30). We hypothesized that: (1) urine excretion of BCAAs and BCKAs is higher in overweight/obese diabetic youth than in normal weight and overweight/obese, nondiabetic youth; (2) overweight/obesity and diabetes in youth are associated with decreases in the urinary ratio of 5-HIAA, the major metabolite of serotonin, to kynurenine; (3) the urinary concentrations of BCAAs and BCKAs and the ratio of 5-HIAA/kynurenine correlate with metrics of body fat and glycemic control; and (4) the urinary metabolites of BCAAs and tryptophan metabolism differ among adolescent boys and girls.

Methods

Participants and Anthropometric Measurements

To test these hypotheses, we recruited 56 adolescents with overweight/obesity without T2D ("obese"), 42 adolescents with overweight/obesity and T2D ("T2D"), and 43 normal weight controls ("lean"). The majority of the participants were recruited from Duke Children's primary care clinics, diabetes clinics at Duke's Lenox Baker Children's Hospital, and the Duke Children's Healthy Lifestyles program. The Healthy Lifestyles program is a comprehensive, interdisciplinary clinic for pediatric obesity treatment. The program uses motivational interviewing, as well as support from registered dieticians and pediatric physical therapists, to create individualized, evidence-based lifestyle modifications to help improve habits and reduce excess weight (5). Three participants with T2D were recruited from the Pediatric Endocrine and Diabetes Clinic at the University of North Carolina in Chapel Hill, NC. In addition, 2 participants with T2D were recruited from Pennington Biomedical Research Center in Baton Rouge, LA.

The participants were between ages 12 and 21 years. Weight was measured to the nearest .1 kg using the same calibrated scales. Normal weight was defined as a BMI between the 5th and 85th percentile, and overweight/obesity was defined as a BMI ≥ 85th percentile (18). Height was measured to the nearest .1 cm using wall-mounted stadiometers. Body composition was measured by a Tanita BC-418 segmental body composition analyzer, and pubertal status evaluated by the participants' general practitioner including pediatricians, nurse practitioners who received training in pubertal staging, and pediatric endocrinologists per standard of care. Diabetes was defined as hemoglobin A1c (HbA1c) ≥ 6.5%, fasting plasma glucose ≥126 mg/dL, 2-hour glucose ≥200 mg/dL during an oral glucose tolerance test, and/or random plasma glucose ≥200 mg/dL with symptoms of hyperglycemia (31). For diabetes management, participants with T2D were on metformin and/or an insulin regimen (Supplemental Table 1) (32). Additionally, 3 participants with T2D were taking liraglutide, 3 were taking dulaglutide, and 1 was taking sitagliptin. The duration from onset of T2D to date of urine

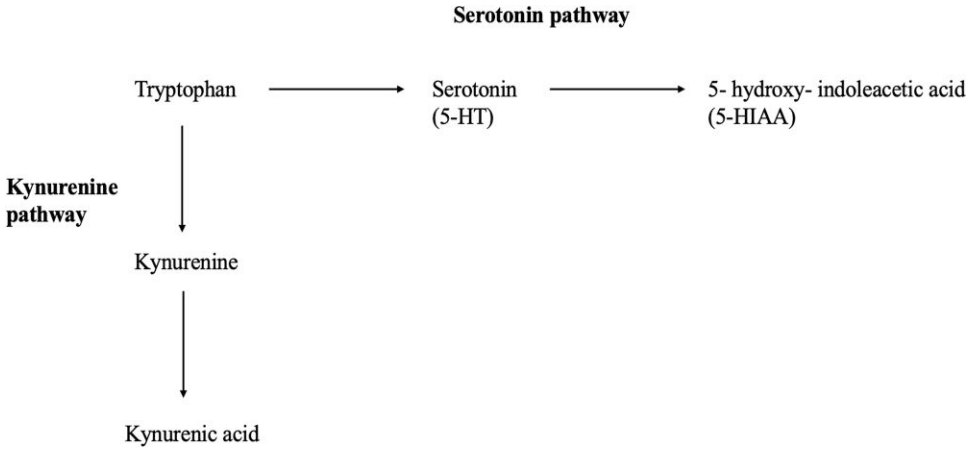

Figure 1. Tryptophan metabolism via kynurenine and serotonin metabolic routes.

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collection was 1.6 ± 1.5 years. No participants were ill or metabolically unstable at the time of urine collection.

Exclusion criteria included use of systemic corticosteroids, antipsychotics, medications for weight loss, topiramate, recent start of oral contraceptives (within the past 3 months), or medroxy-progesterone acetate either currently or within the month before urine collection, as well as use of over-the-counter medications such as acetaminophen or aspirin, either chronically or within the week before urine collection. In addition, those with a genetic syndrome causing obesity, Cushing syndrome, untreated hypothyroidism, persistent hyperprolactinemia, proteinuria, or chronic kidney dysfunction were also excluded.

At least 1 parent/guardian provided informed consent for all children aged younger than 18 years. Informed consent was obtained from the participant if older than age 18 years and assent was obtained for those younger than age 18 years. The protocol was approved by the Duke University Health System institutional review board (IRB), Pediatrics Clinical Research Unit. The other sites utilized IRB reliance on the Duke University Health System IRB for their approvals.

Because serotonin metabolism is highly affected by stress and depression, we extracted data from the Patient Health Questionnaire (PHQ)-2/PHQ-9 Modified for Teens from medical charts and the initial research visits (33). The PHQ-2/PHQ-9 Modified for Teens questionnaire is a reliable and valid method to detect depression and assess severity of depression; thus, all youth receive it as part of standard care for routine annual well-child checks at Duke's primary clinics. Youth who are obese/overweight or have diabetes also receive the same surveys as standard of care at their first visits and annually at Duke's diabetes clinics and the Healthy Lifestyles program.

Urine Collection and Metabolomic Profiling

All participants collected urine for 24 hours in a container, provided by the study team, at home under standardized conditions. The first specimen was discarded, and all subsequent urine was saved, stored, and kept cold, either on ice or in a refrigerator for the next 24 hours. A urine log was kept during collection to record the urine volume and voiding times. Subjects also provided a first morning urine in a urine cup at completion of the 24-hour period to reflect metabolic changes during fasting. The container and the urine cup were returned cold to the recruitment site in an US Food and Drug Administration-compliant thermal bag. The samples were aliquoted, frozen immediately, and stored at −80 °C until processing. All participants taking metformin were asked to stop the drug 24 hours before the start of collection of urine samples. Because of safety concerns, we did not stop insulin and/or liraglutide, dulaglutide, or sitagliptin.

BCAAs (5-1000 µmol/L, < 15%) were analyzed by tandem mass spectrometry (MS/MS) using a Waters TQD instrument, as described previously (34-38). BCKAs including the alpha-keto acids of leucine (α-keto-isocaproate [KIC]), isoleucine (α-keto-β-methylvalerate [KMV]), and valine (α-keto-valerate [KIV]) were analyzed by liquid chromatography MS/MS, as previously described (6, 39, 40). Urine concentrations of tryptophan, kynurenine, and kynurenic acid were measured by liquid chromatography MS/MS as previously described (41, 42). Briefly, 10 µL of urine was diluted 10-fold with water and spiked with the heavy isotope-labeled internal standards tryptophan-d5, kynurenine-d6 (Cambridge Isotope Laboratories) and kynurenic acid-d5 (CDN Isotopes). Metabolites were analyzed on a Waters Acquity UPLC system coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Milford, MA). The analytical column (Waters Acquity UPLC HSS T3 Column, 1.8 µm, 2.1 × 100 mm) equipped with a guard column (Agilent Rapid Resolution cartridge, ZORBAX SB-C8, 3.5 m, 2.1 30 mm) was maintained at 30 °C and the flow rate was set at .3 mL/min. The gradient began with 100% eluent A (.1% formic acid in water) and was then programmed as follows: 0 to 2 minutes 0% eluent B (95:5 acetonitrile-water, .1% formic acid); 2 to 10 minutes gradient to 40% eluent B; 10 to 11 minute gradient to 100% eluent B followed by a 2-minute wash and 2-minute equilibration. Mass transitions of m/z 205 > 146 for tryptophan, 210 > 150 for tryptophan-d5, m/z 209 > 146 for kynurenine, m/z 215 > 151 for kynurenine-d6, m/z 190 > 116 for kynurenic acid, and 195 > 121 for kynurenic acid-d5 were monitored in a positive ion electrospray ionization mode. Metabolite concentrations were computed using Waters TargetLynx Quantitative Analysis and an external calibration constructed from a serial dilution of the tryptophan, kynurenine, and kynurenic acid standards (Sigma, St. Louis, MO) in dialyzed fetal bovine serum.

Quantitative measurements of 5-HIAA were analyzed on a Thermo ARIA TX4 LC system coupled to a SCIEX API5000 mass spectrometer with HPLC/MS-MS. Serotonin in spot and 24-hour urine samples were measured using an immunoassay kit (DLD Diagnostika Cat# EA602/96, RRID:AB_3073757) on a Molecular Devices M2e plate reader (Mountain View, CA). To adjust for variation in dilution effects, sample volume, and rate of urine production, the metabolite levels were normalized to urinary creatinine; the rate of urinary creatinine excretion is fairly constant (43). Concentration values were normalized by creatinine, as measured using reagents from Beckman (Brea, CA) on a DxC 600 clinical analyzer.

HbA1c levels were measured in youth with overweight/obesity and/or T2D at each clinic visit and were also extracted from the participants' medical charts.

Data Analysis

Chi-square test was used to assess differences in ethnicity, self-reported race, sex, and Tanner stage across 3 groups. Kruskal-Wallis test or ANOVA was used to assess differences across 3 groups for age, BMI%, BMI *z*-score, body fat percent (BF%), blood pressure, PHQ2/9 scores, HbA1c, metabolites related to BCAA catabolism (BCKAs) and tryptophan-kynurenine pathway. For the same comparisons between 2 groups, *t* tests or Wilcoxon rank sum tests were performed. We used a conservative *P* value threshold ($P \leq .01$) when interpreting results. To address and explore the influences of sex as a biological variable (44, 45), we performed sex-stratified analyses of all data. Additionally, 2-sample *t* tests were performed to compare the anthropometric and demographic measurements between males and females within the T2D group. To determine if differences in urinary metabolites among the 3 groups were different between males and females, we calculated interaction *P* values from linear regression models with group, sex, and group by sex interaction as covariates. Interaction $P < .05$ was considered statistically significant. To evaluate the association between urinary metabolites of interest with glycemic control, Spearman Rho correlation coefficients were calculated. Metabolites related to BCAAs, BCKAs, and tryptophan-kynurenine-serotonin pathway

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were also assessed as dependent variables using multivariate linear regression models adjusting for Tanner stage, sex, BMI, age, and self-reported race. Among obese and T2D groups, we also performed analyses in which the models were additionally adjusted for glycemic control because HbA1c data were available. All analyses were performed in R version 4.1.2 (2021-11-01) and SAS v.9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

Anthropometric and Demographic Characteristics

Anthropometric and demographic comparisons for the overall cohort are shown in Table 1, for males only in Table 2, and females only in Table 3 (17, 30). A total of 141 participants were studied, of which 43 (30%) had normal weight, 56 (40%) had overweight/obesity without T2D, and 42 (30%) had obesity with T2D. In the overall cohort, lean, obese, and T2D groups were comparable for age, ethnicity, and pubertal status. Obese youth with and without T2D were predominantly female and Black. Youth with T2D had higher BMI-*z*, BMI%, and BF% than lean and nondiabetic obese youth. Youth with T2D also had the highest systolic blood pressures ($P = 5.49e^{-10}$), with comparable diastolic blood pressures across 3 groups. Compared with obese youth without T2D, youth with T2D had higher levels of HbA1c (T2D, 8.38 ± 2.70%; obese, 5.54 ± .37%; $P = 2.70e^{-10}$). The PHQ-2 and PHQ-9 scores were comparable across groups.

Urinary Metabolites of BCAA Metabolism and Tryptophan-kynurenine-serotonin Pathway

Metabolites related to BCAA catabolism and the tryptophan-kynurenine-serotonin pathways in the overall cohort are shown in Table 4 and Supplemental Table 2 (32).

BCAA and Their Catabolic Byproducts

In 24-hour urine samples, total BCAAs, BCKAs, KIV, KMV, and KIC were markedly increased in the T2D group (unadjusted 3-group comparison: $P = .0005$, $P = .0002054$, $P = .000004572$, $P = .0008751$, and $P = .00005518$, respectively) (Table 4). In pairwise group comparisons, the 24-hour urinary levels of BCAAs, BCKAs, KIV, KMV, and KIC were significantly higher in subjects with obesity and T2D than in subjects with obesity without T2D ($P = .0004$, $P = .0002$, $P < .0001$, $P = .0004$, and $P < .0001$, respectively) as well as lean subjects ($P = .0009$, $P = .0008$, $P < .0001$, $P = .0039$, and $P = .0002$, respectively). On the other hand, there were no significant differences between the obese and lean groups ($P = .8495$, $P = .7781$, $P = .5916$, $P = .5394$, and $P = .5863$, respectively; Supplemental Table 3) (32). The findings were similar in first morning urine samples: total BCAAs, BCKAs, KIV, and KIC were elevated in the T2D group (unadjusted 3-group comparisons $P = .0045$, $P = .00151$, $P = .000000438$, and $P = .0000744$, respectively) (Supplemental Table 2 and Supplemental Table 4)(32), whereas BCAA and BCKA levels were comparable between the lean group and obese without T2D group (Supplemental Table 4) (32).

When models were adjusted for multiple variables including Tanner stage, sex, BMI, age, and race, the levels of BCAAs ($P < .0001$), BCKAs ($P < .0001$), KIV ($P < .0001$), KMV ($P < .0001$), and KIC ($P = .0018$) in 24-hour urine samples were still significantly different across the 3 groups. Results from the adjusted models showed that the differences were driven by higher levels in the obese with T2D group compared with the obese without T2D group (least square mean differences between obese with and without T2D: BCAA 4.97, $P < .0001$; BCKA 2.47, $P < .0001$; KIV .58, $P < .0001$; KMV 1.37, $P < .0001$; KIC .52, $P = .0004$). Because HbA1c levels were available only among obese and T2D subjects, we also adjusted the models for HbA1c in addition to

Table 1. Anthropometric and demographic comparisons across 3 groups in overall cohort

	Lean (n = 43)	Obese without T2D (n = 56)	Obese with T2D (n = 42)	*P*
Age (y)	14.93 ± 1.87	14.77 ± 1.93	15.68 ± 2.09	.067
Sex (females/males)	17/26	33/23	28/14	.030
Race	AA = 21 (48.8%), Asian = 1 (2.3%), More than 1 = 5 (11.6%), NA = 1 (2.3%), White = 15 (34.9%)	AA = 37 (66.1%), Asian = 1 (1.8%), More than 1 = 2 (3.6%), NA = 8 (14.3%), White = 8 (14.3%)	AA = 31 (73.8%), Asian = 0, More than 1 = 2 (4.8%), NH = 1 (2.4%), NA = 3 (7.1%), White = 5 (11.9%)	.030
Ethnicity	Hispanic/Latino = 2, Not Hispanic/Latino = 40, NA = 1	Hispanic/Latino = 11, Not Hispanic/Latino = 43, NA = 2	Hispanic/Latino = 6, Not Hispanic/Latino = 36, NA = 0	.18
Tanner staging (early/mid, late)	5/35 (NA = 3)	12/44	3/35 (NA = 4)	.171
BMI-*z*	.06 ± .63	2 ± .49	2.36 ± .41	4.22e-45
BMI%	52.41 ± 21.92	96.37 ± 3.68	98.51 ± 1.91	5.89e-40
BF%	20.11 ± 6.28	37.25 ± 9.5	42.9 ± 9.87	2.58e-22
Systolic blood pressure	111.65 ± 9.13	115.36 ± 11.54	127.83 ± 12.08	5.49e-10
Diastolic blood pressure	67.91 ± 7.54	69.11 ± 8.98	71.51 ± 10.53	.182
PHQ2 score	.3 ± .62	.54 ± 1.12	1.06 ± 1.56	.053
PHQ9 score	1.09 ± 3.04	3.55 ± 5.12	4.53 ± 6.19	.064
HbA1c	—	5.54 ± .37	8.38 ± 2.70	<.0001

Abbreviations: AA, African American; BF%, percent body fat; BMI, body mass index; HbA1c, hemoglobin A1c; NA, not answered/not available; NH, Native Hawaiian/Pacific Islander; PHQ, Patient Health Questionnaire; T2D, type 2 diabetes.

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Table 2. Anthropometric and demographic comparisons across 3 groups in males

	Lean (n = 26)	Obese without T2D (n = 23)	Obese with T2D (n = 14)	*P*
Age (y)	15.44+/−1.79	14.53+/−1.55	15.68+/−1.61	.076
Race	Black = 13, Asian = 1, More than 1 = 1, NA = 0, White = 11	Black = 17, Asian = 1, More than 1 = 1, NA = 1, White = 3	Black = 11, Asian = 0, More than 1 = 0, NA = 1, White = 2	.314
Ethnicity	Hispanic/Latino = 0 Not Hispanic/ Latino = 26	Hispanic/Latino = 2 Not Hispanic/ Latino = 21	Hispanic/Latino = 0 Not Hispanic/ Latino = 14	.166
Tanner staging (early/mid, late)	Early/mid = 2 Late = 21 (NA = 3)	Early/mid = 7, Late = 16 (NA = 0)	Early = 3, Late = 10 (NA = 0)	.18
BMI-*z*	.3 ± .67	1.98 ± .54	2.45 ± .51	1.33e-10
BMI%	51.51 ± 23.71	96.53 ± 3.46	98.32 ± 2.77	7.8e-16
BF%	16.04 ± 3.37	32.08 ± 8.22	36.11 ± 10.32	4.4e-12
Systolic BP	115.08 ± 7.9	115.65 ± 11.62	132.14 ± 9.86	2.8e-6
Diastolic BP	68.5 ± 8	67.91 ± 8.3	70.93 ± 8.07	.53
PHQ2 score	.17 ± .58	.11 ± .32	1.17 ± 1.17	.002
PHQ9 score	.29 ± .73	1.28 ± 1.81	4.67 ± 7.37	.022
HbA1c	—	5.69 ± .36	8.59 ± 3.76	.050

Abbreviations: BF%, percent body fat; BMI, body mass index; BP, blood pressure; NA, not answered; NH, Native Hawaiian/Pacific Islander; PHQ, Patient Health Questionnaire: T2D, type 2 diabetes.

Table 3. Anthropometric and demographic comparisons across 3 groups in females

	Lean (n = 17)	Obese without T2D (n = 33)	Obese with T2D (n = 28)	*P*
Age, y	14.16 ± 1.76	14.93 ± 2.16	15.67 ± 2.32	.074
Race	Black = 8, More than 1 = 4, NA = 1, White = 4	Black = 20, More than 1 = 1, NA = 7, White = 5	Black = 20, More than 1 = 2, NH = 1, NA = 2, White = 3	.130
Ethnicity	Hispanic/Latino = 2, not Hispanic/ Latino = 14, NA = 1	Hispanic/Latino = 9, not Hispanic/ Latino = 22, NA = 2	Hispanic/Latino = 6, not Hispanic/ Latino = 22, NA = 0	.494
Tanner staging (early/mid, late)	early/mid = 3, Late = 14 (NA = 0)	Early/mid = 5, Late = 28 (NA = 0)	Early/mid = 0, late =25 (NA = 3)	= 103
BMI-*z*	.11+/−.57	2.05+/−.46	2.3+/−.35	2.83e-10
BMI%	53.73+/−19.62	96.26+/−3.88	98.61+/−1.27	2.83e-10
BF%	25.86+/−4.71	40.86+/−8.72	46.58+/−7.52	5e-12
Systolic BP	106.41+/−8.55	115.15+/−11.66	125.59+/−12.67	2.7e-6
Diastolic BP	67+/−6.91	69.94+/−9.46	71.81+/−11.73	.3
PHQ2 score	.5 +/− .65	.9 +/− 1.41	1 +/− 1.79	.59
PHQ9 score	2.33 +/− 4.66	5.12 +/− 6.05	4.45 +/− 5.84	.47
HbA1c	—	5.45 +/− .35	8.28 +/− 2.06	<.0001

Abbreviations: BF%, percent body fat; BMI, body mass index; BP, blood pressure; NA, not answered; NH, Native Hawaiian/Pacific Islander; PHQ, Patient Health Questionnaire: T2D, type 2 diabetes.

Tanner stage, sex, BMI, age, and race. After adjustment for these variables, the major determinant of BCKAs, KIV, KMV, and KIC was HbA1c ($P = .0013$, $P = .0004$, $P = .0056$, and $P = .0009$, respectively), with higher HbA1c levels associated with higher BCKAs, KIV, KMV, and KIC. The BCAA levels were significantly higher in subjects in the obese with T2D group compared with those in the obese without T2D group ($P = .0056$), whereas HbA1c approached nominal significance ($P = .0684$). After adjustment for HbA1c, levels of BCKA, KIV, KMV, and KIC were not significantly different between obese with and without T2D ($P = .18$, $P = .23$, $P = .11$, $P = .54$, respectively).

Tryptophan, Kynurenine, Serotonin, and 5-HIAA

In 24-hour urine samples, urinary concentrations of kynurenine increased ($P = .00008695$) and the ratio of kynurenine/serotonin was higher in the obese with T2D group than in the lean and obese without T2D groups ($P < .0001$). The ratio of kynurenine/tryptophan also trended higher in the T2D group ($P = .0012$). In contrast, the ratios of serotonin/tryptophan ($P = .0036$) and 5-HIAA/kynurenine ($P = .0067$) were lower in the obese with T2D group (Table 4). In pairwise group comparisons, the 24-hour urinary concentrations of tryptophan and kynurenine and the ratios of kynurenine/tryptophan and kynurenine/serotonin were significantly higher in

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Table 4. Metabolites related to BCAA metabolism and tryptophan-kynurenine- serotonin- pathways in 24-hour urine samples across 3 groups in overall cohort, normalized for urine Cr (mmol/mol Cr)

	Lean (n = 43)	Obese without T2D (n = 56)	Obese with T2D (n = 42)	*P*, unadjusted	*P*, adjusted[a]
BCAAs mmol/mol Cr	9.82 ± 4.38	9.47 ± 3.83	14.07 ± 6.9	.0005	<.0001
BCKAs mmol/mol Cr	1.97 ± .94	1.92 ± 1.05	4.39 ± 4.05	.0002054	<.0001
KIV mmol/mol Cr	.32 ± .15	.35 ± .21	.96 ± .95	.000004572	<.0001
KMV mmol/mol Cr	1.32 ± .69	1.22 ± .68	2.56 ± 2.15	.0008751	<.0001
KIC mmol/mol Cr	.33 ± .13	.35 ± .25	.88 ± 1.08	.00005518	.0018
Tryptophan mmol/mol Cr	6.63 ± 3.36	6.53 ± 3.12	8.58 ± 3.48	.004464	.0037
Kynurenine mmol/mol Cr	.32 ± .21	.28 ± .17	.52 ± .34	.00008695	<.0001
Kynurenic acid mmol/mol Cr	1.08 ± .44	1.13 ± .48	1.08 ± .46	.794	.9599
Serotonin nmol/mmol Cr	78.06 ± 30.63	81.61 ± 33.04	78.9 ± 25.26	.8843	.9714
5-HIAA mg/g Cr	2.94 ± 1.45	2.75 ± 1.35	3.41 ± 3.55	.5353	.0244
Kynurenine/tryptophan	.05 ± .02	.04 ± .02	.06 ± .03	.0112	.0139
5-HIAA/tryptophan	.51 ± .22	.48 ± .19	.44 ± .36	.0272	.7559
5-HIAA/kynurenine	14.04 ± 12.84	13.03 ± 7.75	9.59 ± 9.24	.0067	.6504
Kynurenine/serotonin	.0043 ± .0029	.0035 ± .0018	.0070 ± .0046	<.0001	.0001
Serotonin/tryptophan	13.13 ± 4.87	13.53 ± 4.71	10.84 ± 5.11	.0036	.0649

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; Cr, creatinine; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate; T2D, type 2 diabetes.
[a]Model adjusted for Tanner stage, sex, body mass index, age, self-reported race.

subjects in the obese with T2D group than in subjects in the obesity without T2D group ($P = .0027$, $P < .0001$, and $P = .0033$, respectively). Likewise, the urinary levels of tryptophan and kynurenine and the ratio of kynurenine/serotonin were significantly higher in the obese with T2D group than the lean group ($P = .0068$, $P = .0021$, and $P = .0021$, respectively), but there were no differences between the obese and lean groups ($P = .8560$, $P = .4110$, $P = .2713$, and $P = .3246$, respectively) (Supplemental Table 3) (32). Analysis of first morning urine yielded similar results: the ratios of 5-HIAA/tryptophan ($P = .001$) and 5-HIAA/kynurenine ($P = .0036$) in spot morning urines were lowest in T2D (Supplemental Table 2 and Supplemental Table 4)(32). All tryptophan, kynurenine, and serotonin metabolites and calculated ratios were comparable between the lean group and obese without T2D group (Table 4 and Supplemental Table 2) (32).

When models were adjusted for multiple variables including Tanner stage, sex, BMI, age, and race, the levels of tryptophan ($P = .0037$), kynurenine ($P < .0001$), and kynurenine/serotonin ratio ($P = .0001$) in 24-hour urine samples remained significantly different across 3 groups. Again, these differences were driven by differences associated with T2D; the obese with T2D group had higher levels of kynurenine, tryptophan, and kynurenine/serotonin ratio than the obese group without T2D (least square mean differences between obese with and without T2D: kynurenine .25, $P < .0001$; tryptophan 2.34, $P = .0011$; kynurenine/serotonin ratio .0033, $P < .0001$). Because HbA1c levels were available only among obese and T2D subjects, we also adjusted the models for HbA1c in addition to Tanner stage, sex, BMI, age, and race. After adjustment for all those variables, the levels of kynurenine ($P = .0008$) and the ratios of kynurenine/tryptophan ($P = .0100$) and kynurenine/serotonin ($P = .0093$) remained higher among the T2D group compared with the obese group. Tryptophan levels also trended higher ($P = .0222$) in the T2D group. These findings were similar to those for the BCAAs but differed from those for the BCKAs in that HbA1c was not a primary driver of the differences in tryptophan metabolism.

Associations Between BCAA, BCAA Catabolic Byproducts, Tryptophan and its Byproducts, and Metrics of Body Fat and Glycemic Control

Bivariate Associations Between BCAA Metabolism, Tryptophan-Kynurenine-Serotonin Pathway, and Metrics of Body Fat

In 24-hour urine samples, the concentrations of BCAAs ($r = .294$, $P = .0009$), total BCKAs ($r = .276$, $P = .0016$), KIV ($r = .315$, $P = .0003$), KMV ($r = .251$, $P = .0043$), KIC ($r = .259$, $P = .0031$), tryptophan ($r = .248$, $P = .0047$), and kynurenine ($r = .126$, $P = .0143$) correlated positively with BF%. The ratios of 5-HIAA/tryptophan, 5-HIAA/kynurenine, and serotonin/tryptophan correlated negatively with BMI ($r = -.321$, $P = .0002$; $r = -.245$, $P = .0044$; and $r = -.229$, $P = .0089$, respectively) and BF% ($r = -.272$, $P = .0019$; $r = -.188$, $P = .0340$; and $r = -.237$, $P = .0081$, respectively) (Table 5).

In first morning urine samples, the concentrations of BCAAs, total BCKAs, KIV, KMV, and KIC correlated positively with BMI-related metrics, with BF% being the strongest association. Similarly, tryptophan and kynurenine correlated positively with BMI-related metrics including BF%, whereas the ratios of 5-HIAA/tryptophan and 5-HIAA/kynurenine associated negatively with BMI-related metrics and BF% (Supplemental Table 5) (32). When data were stratified by sex, these correlations were driven primarily by females (Table 6, Supplemental Table 6, Table 7, and Supplemental Table7)(32).

Bivariate Associations Between BCAA Metabolism, Tryptophan-Kynurenine-Serotonin Pathway, and HbA1c

In the 24-hour urine samples of the overall cohort, the concentrations of BCAAs, total BCKAs, KIV, KMV, and KIC

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Table 5. Spearman correlations in 24-hour urine samples in the overall cohort

Metabolite	HbA1c	BMI	BMI percent	BMI-*z* score	BF%
BCAAs mmol/mol Cr, 24 h	.389, $P = .0006$	.179, $P = .0418$	.192, $P = .0296$	.19, $P = .0308$	.294, $P = .0009$
BCKAs mmol/mol Cr, 24 h	.401, $P = .0002$	.136, $P = .1163$	.128, $P = .1438$	.128, $P = .1444$	.276, $P = .0016$
KIV mmol/mol Cr, 24 h	.457, $P < .0001$	.222, $P = .0096$	.208, $P = .0173$	.208, $P = .0174$	.315, $P = .0003$
KMV mmol/mol Cr, 24 h	.387, $P = .0004$	.114, $P = .1871$	.108, $P = .2174$	.108, $P = .2178$	.251, $P = .0043$
KIC mmol/mol Cr, 24 h	.439, $P < .0001$	.124, $P = .1504$	.103, $P = .2424$	.102, $P = .2466$	.259, $P = .0031$
Tryptophan mmol/mol Cr, 24 h	.381, $P = .0004$	.179, $P = .0374$	.196, $P = .0248$	.196, $P = .0251$	.248, $P = .0047$
Kynurenine mmol/mol Cr, 24 h	.377, $P = .0005$	.202, $P = .0191$	.215, $P = .0138$	.214, $P = .0140$	.216, $P = .0143$
Kynurenic acid mmol/mol Cr, 24 h	−.259, $P = .0195$	−.005, $P = .9580$	.017, $P = .8467$	.016, $P = .8568$	.079, $P = .3757$
5 HIAA mg/g Cr, 24 h	.005, $P = .9662$	−.161, $P = .0635$	−.152, $P = .0823$	−.152, $P = .0828$	.032, $P = .7177$
Serotonin nmol/mmol Cr, 24 h	−.014, $P = .9036$	−.011, $P = .9019$	.03, $P = .7359$	.03, $P = .7353$	.083, $P = .3584$
Kynurenine/tryptophan, 24 h	.196, $P = .0795$	.135, $P = .1191$	.123, $P = .1607$	.123, $P = .1601$	.084, $P = .3464$
5-HIAA/tryptophan, 24 h	−.406, $P = .0002$	−.321, $P = .0002$	−.327, $P = .0001$	−.327, $P = .0001$	−.272, $P = .0019$
5-HIAA/kynurenine, 24 h	−.391, $P = .0003$	−.245, $P = .0044$	−.251, $P = .0039$	−.251, $P = .0039$	−.188, $P = .0340$
Kynurenine/serotonin, 24 h	.443, $P < .0001$	.212, $P = .0157$	.199, $P = .0240$	.199, $P = .0243$	.193, $P = .0319$
Serotonin/tryptophan, 24 h	−.423, $P = .0001$	−.229, $P = .0089$	−.206, $P = .0198$	−.205, $P = .0203$	−.237, $P = .0081$

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; BF%, percent body fat; BMI, body mass index; Cr, creatinine; HbA1c, hemoglobin A1c; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate.

Table 6. Spearman correlations in 24-hour urine samples in males

Metabolite	HbA1c	BMI	BMI percent	BMI-*z* score	BF%
BCAAs mmol/mol Cr, 24 h	.265, $P = .1736$	.149, $P = .2517$	.234, $P = .0721$	.234, $P = .0713$	.231, $P = .0779$
BCKAs mmol/mol Cr, 24 h	.281, $P = .1326$	.067, $P = .6040$	.144, $P = .2688$	.144, $P = .2679$	.142, $P = .2839$
KIV mmol/mol Cr, 24 h	.312, $P = .0928$	.148, $P = .2508$	.219, $P = .0896$	.219, $P = .0894$	.204, $P = .1212$
KMV mmol/mol Cr, 24 h	.317, $P = .0878$	.047, $P = .7187$	.123, $P = .3465$	.123, $P = .3444$	.123, $P = .3536$
KIC mmol/mol Cr, 24 h	.322, $P = .0832$	.046, $P = .7254$	.102, $P = .4363$	.101, $P = .4379$	.1, $P = .4495$
Tryptophan mmol/mol Cr, 24 h	.37, $P = .0440$	.176, $P = .1707$	.242, $P = .0606$	.243, $P = .0588$	.285, $P = .0289$
Kynurenine mmol/mol Cr, 24 h	.328, $P = .0768$	.063, $P = .6259$	.129, $P = .3225$	.13, $P = .3167$	.07, $P = .5958$
Kynurenic acid mmol/mol Cr, 24 h	−.242, $P = .1968$	−.064, $P = .6196$	.013, $P = .9238$	.011, $P = .9313$	.021, $P = .8766$
5-HIAA mg/g Cr, 24 h	−.341, $P = .0654$	−.248, $P = .0523$	−.177, $P = .1725$	−.177, $P = .1731$	−.037, $P = .7810$
Serotonin nmol/mmol Cr, 24 h	−.207, $P = .2824$	−.002, $P = .9906$	.066, $P = .6212$	.066, $P = .6198$	.063, $P = .6407$
Kynurenine/tryptophan, 24 h	.236, $P = .2089$	−.055, $P = .6707$	−.029, $P = .8233$	−.028, $P = .8304$	−.136, $P = .3046$
5-HIAA/tryptophan, 24 h	−.567, $P = .0011$	−.315, $P = .0126$	−.333, $P = .0087$	−.334, $P = .0085$	−.285, $P = .0284$
5-HIAA/kynurenine, 24 h	−.484, $P = .0067$	−.1, $P = .4412$	−.133, $P = .3053$	−.135, $P = .3011$	−.034, $P = .7980$
Kynurenine/serotonin, 24 h	.404, $P = .0297$	.064, $P = .6261$	.086, $P = .5166$	.088, $P = .5092$	.025, $P = .8538$
Serotonin/tryptophan, 24 h	−.489, $P = .0071$	−.197, $P = .1304$	−.185, $P = .1609$	−.186, $P = .1584$	−.231, $P = .0839$

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; BF%, percent body fat; BMI, body mass index; Cr, creatinine; HbA1c, hemoglobin A1c; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate.

correlated positively with HbA1c ($r = .389$, $P = .0006$; $r = .401$, $P = .0002$; $r = .457$, $P < .0001$; $r = .387$, $P = .0004$; and $r = .439$, $P < .0001$, respectively; Table 5). Likewise, the concentrations of tryptophan and kynurenine correlated positively with HbA1c ($r = .381$, $P = .0004$; $r = .377$, $P = .0005$, respectively). The ratios of serotonin/tryptophan ($r = -.423$, $P = .0001$), 5-HIAA/tryptophan ($r = -.406$, $P = .0002$), and 5-HIAA/kynurenine ($r = -.391$, $P = .0003$) correlated negatively with hemoglobin A1C, whereas the ratio of kynurenine/serotonin ($r = .443$, $P < .0001$) correlated positively with HbA1c (Table 5).

Similarly, in the spot morning urine samples of the overall cohort, BCAAs ($r = .462$, $P = .0001$), total BCKAs ($r = .458$, $P < .0001$), KIV ($r = .578$, $P < .0001$), KMV ($r = .369$, $P = .0021$), KIC ($r = .561$, $P < .0001$), and tryptophan ($r = .409$, $P = .0006$) correlated positively with HbA1c, whereas the ratios of 5-HIAA/tryptophan and 5-HIAA/kynurenine correlated negatively with HbA1c ($r = -.423$, $P = .0004$; $r = -.331$, $P = .0063$, respectively) (Supplemental Table 5)(32). When data were stratified by sex, these correlations were driven primarily by females (Table 6, Supplemental Table 6, Table 6, and Supplemental Table 7)(32).

Correlations Between Urinary Metabolites of BCAAs and BCKAs and the Byproducts of Tryptophan Metabolism

In 24-hour urine samples, the concentrations of tryptophan, kynurenine, and kynurenic acid correlated positively with

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Table 7. Spearman correlations in 24-hour urine samples in females

Metabolite	HbA1c	BMI	BMI percent	BMI-*z* score	BF%
BCAAs mmol/mol Cr, 24 h	.456, $P = .0013$	.09, $P = .4610$	.083, $P = .4985$	.078, $P = .5259$	.049, $P = .6918$
BCKAs mmol/mol Cr, 24 h	.468, $P = .0005$	.105, $P = .3757$	.086, $P = .4772$	.085, $P = .4867$	.124, $P = .3104$
KIV mmol/mol Cr, 24 h	.544, $P < .0001$	.213, $P = .0704$	.188, $P = .1189$	.187, $P = .1218$	.2, $P = .1002$
KMV mmol/mol Cr, 24 h	.438, $P = .0013$	.087, $P = .4636$	.07, $P = .5634$	.068, $P = .5749$	.108, $P = .3771$
KIC mmol/mol Cr, 24 h	.52, $P < .0001$	.111, $P = .3482$	.082, $P = .5018$	.079, $P = .5135$	.127, $P = .2995$
Tryptophan mmol/mol Cr, 24 h	.418, $P = .0023$	.077, $P = .5184$	.108, $P = .3737$	.105, $P = .3876$	.035, $P = .7737$
Kynurenine mmol/mol Cr, 24 h	.389, $P = .0048$	.217, $P = .0647$	.229, $P = .0565$	.226, $P = .0605$	.155, $P = .2048$
Kynurenic acid mmol/mol Cr, 24 h	−.26, $P = .0650$	−.057, $P = .6328$	−.074, $P = .5450$	−.078, $P = .5236$	−.086, $P = .4817$
5-HIAA mg/g Cr, 24 h	.168, $P = .2433$	−.24, $P = .0426$	−.241, $P = .0449$	−.242, $P = .0437$	−.289, $P = .0162$
Serotonin nmol/mmol Cr, 24 h	.139, $P = .3518$	−.044, $P = .7187$	−.006, $P = .9605$	−.007, $P = .9541$	−.043, $P = .7311$
Kynurenine/tryptophan, 24 h	.166, $P = .2453$	.248, $P = .0344$	.236, $P = .0497$	.234, $P = .0509$	.193, $P = .1117$
5-HIAA/tryptophan, 24 h	−.336, $P = .0172$	−.29, $P = .0134$	−.302, $P = .0110$	−.301, $P = .0114$	−.264, $P = .0281$
5-HIAA/kynurenine, 24 h	−.363, $P = .0096$	−.33, $P = .0047$	−.345, $P = .0034$	−.344, $P = .0036$	−.293, $P = .0145$
Kynurenine/serotonin, 24 h	.498, $P = .0004$	.253, $P = .0362$	.242, $P = .0449$	.239, $P = .0481$	.213, $P = .0828$
Serotonin/tryptophan, 24 h	−.413, $P = .0039$	−.189, $P = .1199$	−.16, $P = .1881$	−.156, $P = .1995$	−.171, $P = .1670$

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; BF%, percent body fat; BMI, body mass index; Cr, creatinine; HbA1c, hemoglobin A1c; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate.

Table 8. Correlations between BCKAs, BCAAs, and tryptophan, kynurenine, kynurenic acid, 5-HIAA, serotonin, and the ratios in 24-hour urine samples

Variable	BCKAs (mmol/mol Cr), 24 h	BCAAs (mmol/mol Cr), 24 h
Tryptophan mmol/mol Cr, 24 h	.59, $P < .0001$	.806, $P < .0001$
Kynurenine mmol/mol Cr, 24 h	.596, $P < .0001$	.649, $P < .0001$
Kynurenic acid mmol/mol Cr, 24 h	.216, $P = .0119$	.505, $P < .0001$
5-HIAA mg/g Cr, 24 h	.313, $P = .0002$	.526, $P < .0001$
Serotonin nmol/mmol Cr, 24 h	.337, $P < .0001$	.522, $P < .0001$
Kynurenine/tryptophan, 24 h	.277, $P = .0012$	.132, $P = .1370$
5-HIAA/tryptophan, 24 h	−.338, $P < .0001$	−.426, $P < .0001$
5-HIAA/kynurenine, 24 h	−.408, $P < .0001$	−.361, $P < .0001$
Kynurenine/serotonin, 24 h	.495, $P < .0001$	.402, $P < .0001$
Serotonin/tryptophan, 24 h	−.438, $P < .0001$	−.501, $P < .0001$

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; Cr, creatinine.

both BCAAs ($r = .806$, $P < .0001$; $r = .649$, $P < .0001$; $r = .505$, $P < .0001$, respectively) and BCKAs ($r = .59$, $P < .0001$; $r = .596$, $P < .0001$; $r = .216$, $P = .01$, respectively; Table 8). 5-HIAA and serotonin also correlated positively with BCAAs ($r = .526$, $P < .0001$ and $r = .522$, $P < .0001$) and BCKAs ($r = .313$, $P = .0002$ and $r = .337$, $P < .0001$). However, the ratios of 5-HIAA/tryptophan, 5-HIAA/kynurenine, and serotonin/tryptophan all correlated negatively with both BCAAs ($r = -.426$, $P < .0001$; $r = -.361$, $P < .0001$; and $r = -.501$, $P < .0001$) and BCKAs ($r = -.338$, $P < .0001$; $r = -.408$, $P < .0001$; $r = -.438$, $P < .0001$). Conversely, the ratio of kynurenine/serotonin correlated positively with BCAAs (.402, $P < .0001$) and BCKAs ($r = .495$, $P < .0001$) (Table 8).

In first morning urine samples, tryptophan correlated positively with both BCAAs ($r = .773$, $P < .0001$) and BCKAs ($r = .559$, $P < .0001$). Kynurenine also correlated positively with BCAAs ($r = .52$, $P < .0001$) and BCKAs ($r = .527$, $P < .0001$). Kynurenic acid and 5-HIAA both correlated positively with BCAAs ($r = .372$, $P < .0001$ and $r = .353$, $P = .0001$, respectively). The kynurenine/serotonin ratio correlated positively with BCAAs ($r = .343$, $P = .0002$) and BCKAs ($r = .443$, $P < .0001$). In contrast, the ratios of 5-HIAA/tryptophan, 5-HIAA/kynurenine, and serotonin/tryptophan correlated negatively with BCAAs ($r = -.442$, $P < .0001$; $r = -.337$, $P = .0003$; and $r = -.541$, $P < .0001$, respectively) and BCKAs ($r = -.352$, $P = .0001$; $r = -.337$, $P = .0002$; and $r = -.44$, $P < .0001$) (Supplemental Table 8) (32).

Also of note, correlations of all metabolites in spot and 24-hour urine samples were positive and statistically different from zero (Table 9).

Table 9. Correlations between spot and 24-hour urine metabolites

Metabolite	Correlation	*P*
KIV mmol/mol Cr	.792	<.0001
KMV mmol/mol Cr	.733	<.0001
KIC mmol/mol Cr	.727	<.0001
BCKAs mmol/mol Cr	.735	<.0001
BCAAs mmol/mol Cr	.687	<.0001
Tryptophan mmol/mol Cr	.786	<.0001
Kynurenine mmol/mol Cr	.791	<.0001
Kynurenic acid mmol/mol Cr	.675	<.0001
5-HIAA mg/g Cr	.575	<.0001
Serotonin nmol/mmol Cr	.597	<.0001

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; Cr, creatinine; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate.

Sex Differences in Urinary Metabolites of the BCAA Metabolism and Tryptophan-Kynurenine-Serotonin Pathway

We stratified the data to identify sex differences in the urinary BCAA and tryptophan metabolites. In males alone, there were

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Table 10. Metabolites related to BCAA metabolism and tryptophan-kynurenine-serotonin pathways in 24-hour urine samples across 3 groups in males, normalized for urine Cr (mmol/mol Cr)

	Lean (n = 26)	Obese without T2D (n = 23)	Obese with T2D (n = 14)	*P*
BCAAs mmol/mol Cr	8.17 ± 2.5	9.16 ± 3.96	11.1 ± 6.24	.2610
BCKAs mmol/mol Cr	1.72 ± .81	1.97 ± 1.30	2.59 ± 2.05	.49
KIV mmol/mol Cr	.29 ± .14	.36 ± .26	.53 ± .49	.25
KMV mmol/mol Cr	1.13 ± .58	1.24 ± .80	1.63 ± 1.31	.6
KIC mmol/mol Cr	.30 ± .11	.36 ± .34	.44 ± .32	.36
Tryptophan mmol/mol Cr	5.75 ± 2.49	6.64 ± 3.34	7.38 ± 4.12	.34
Kynurenine mmol/mol Cr	.31 ± .19	.26 ± .16	.41 ± .29	.26
Kynurenic acid mmol/mol Cr	.99 ± .33	.96 ± .39	1.01 ± .43	.91
5-HIAA mg/g Cr	2.459 ± .757	2.705 ± 1.783	2.501 ± 1.279	.73
Serotonin nmol/mmol Cr	73.20 ± 35.94	81.72 ± 30.03	68.25 ± 24.40	.2197
Kynurenine/tryptophan	.052 ± .025	.042 ± .017	.046 ± .019	.47
5-HIAA/tryptophan	.49 ± .18	.45 ± .20	.42 ± .26	.2884
5-HIAA/kynurenine	11.29 ± 7.75	13.44 ± 7.68	11.47 ± 10.81	.2656
Kynurenine/serotonin	.0045 ± .0029	.0035 ± .0022	.0062 ± .0044	.1818
Serotonin/tryptophan	13.17 ± 4.37	13.38 ± 5.41	11.05 ± 4.93	.3490

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; Cr, creatinine; HbA1c, hemoglobin A1c; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate; T2D, type 2 diabetes.

no significant differences among groups in concentrations of BCAAs, BCKAs, or any of the metabolites related to the serotonin-tryptophan pathway (Table 10 and Supplemental Table 9) (32). Rather, the differences observed in the overall cohort were driven by females, as seen in Table 11 and Supplemental Table 10 (32). In 24-hour urine samples of females, the levels of BCAAs, BCKAs, KIV, KMV, KIC, tryptophan, and kynurenine were higher in the T2D group (3-group comparison: $P = .0027$, $P = .00019$, $P = 6.4e\text{-}06$, $P = .00062$, $P = .00011$, $P = .011$, and $P = .00066$). The ratio of kynurenine/serotonin ($P < .0001$) was higher in T2D, whereas the ratios of serotonin/tryptophan and 5-HIAA/kynurenine were lower ($P = .013$ and $P = .0084$, respectively), suggesting a diversion of tryptophan metabolism toward production of kynurenine rather than serotonin (Table 11). In pairwise group comparisons, the 24-hour levels of BCAAs, BCKAs, KIV, KMV, KIC, tryptophan, and kynurenine were significantly higher in the obese with T2D females than in females with obesity without T2D ($P = .0008$, $P < .0001$, $P < .0001$, $P = .0002$, $P < .0001$, $P = .0020$, and $P = .0001$ respectively; Supplemental Table 11)(32). The ratio of 24-hour kynurenine/serotonin was also significantly higher in the obese females with T2D ($P < .0001$, Supplemental Table 11)(32). Similarly, in spot morning urine samples of females, the concentrations of total BCKAs, KIV, and KIC were all significantly higher in obese females with T2D group compared with the lean and obese without T2D groups (3-group comparison $P = .0086$, $P = 8.3e^{-06}$, and $P = .00052$, respectively). Conversely, 5-HIAA levels were lower ($P = .0038$) in obese females with T2D, with concomitant decreases in the ratios of 5-HIAA/kynurenine ($P = .0003$) and 5-HIAA/tryptophan ($P = .0016$) (Supplemental Table 10 and Supplemental Table 12 for pairwise group comparisons among females in spot urine samples)(32). Because 3-way comparisons were not different among males, we did not conduct pairwise group comparisons. In 24-hour urine samples, the differences among the 3 groups in total BCKAs, KIV, KMV, KIC, and kynurenine/tryptophan ratio were statistically different between males and females (Table 12, sex by group interaction $P = .02$, $P = .012$, $P = .037$, $P = .028$, and $P = .028$, respectively). In spot morning urine samples, only the differences among the 3 groups in kynurenine/tryptophan ratio were statistically different between males and females (Supplemental Table 13, sex by group interaction $P = .031$) (32).

Discussion

As obesity and its complications become increasingly more common in youth, it is important to develop new tools to identify those who are at highest risk of developing T2D. Studies in adolescents and adults with obesity suggest that plasma BCAAs and BCKAs are associated with IR and T2D and predict future risk of metabolic syndrome and hypertriglyceridemia (4-12, 46-48). Recent studies in adults also highlight the altered metabolism of tryptophan in obesity, whereby diversion to the kynurenine pathway is associated with IR and T2D (22-24).

In this study, we analyzed 24-hour urine samples to identify factors associated with progression from IR to T2D among youth with obesity. In contrast to single point-in-time plasma analyses, urine metabolomic profiling integrates differences in metabolic status over a 24-hour period in a noninvasive manner (17). We hypothesized that: (1) urine excretion of BCAAs and BCKAs are higher in obese diabetic youth than in normal weight and obese, nondiabetic youth; (2) obesity and diabetes in youth are associated with decreases in the urinary ratio of 5-HIAA, the major metabolite of serotonin, to kynurenine; (3) the urinary concentrations of BCAAs and BCKA and the ratio of 5HIAA/kynurenine correlate with metrics of body fat and glycemic control; and (4) the urinary metabolites of BCAAs and tryptophan metabolism differ among adolescent boys and girls.

Our findings include 4 novel observations. First, urine excretion of BCAAs and BCKAs are higher in obese diabetic youth than in normal weight and obese, nondiabetic youth. Second, youth with T2D exhibit differences in urinary

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Table 11. Metabolites related to BCAA metabolism and tryptophan-kynurenine-serotonin pathways in 24-hour urine samples across 3 groups in females, normalized for urine Cr (mmol/mol Cr)

	Lean (n = 17)	Obese without T2d (n = 33)	Obese with T2D (n = 28)	*P*
BCAAs mmol/mol Cr	12.34 ± 5.42	9.73 ± 3.77	15.55 ± 6.86	.0027
BCKAs mmol/mol Cr	2.34 ± 1.02	1.89 ± .81	5.29 ± 4.51	.00019
KIV mmol/mol Cr	.36 ± .16	.34 ± .15	1.17 ± 1.06	6.4e-06
KMV mmol/mol Cr	1.6 ± .76	1.21 ± .57	3.02 ± 2.35	.00062
KIC mmol/mol Cr	.38 ± .14	.34 ± .14	1.1 ± 1.26	.00011
Tryptophan mmol/mol Cr	7.93 ± 4.07	6.44 ± 2.99	9.17 ± 3.02	.011
Kynurenine mmol/mol Cr	.34 ± .25	.29 ± .17	.57 ± .36	.00066
Kynurenic acid mmol/mol Cr	1.22 ± .55	1.27 ± .51	1.11 ± .47	.47
Serotonin nmol/mmol Cr	84.94 ± 20.05	81.53 ± 35.77	85.11 ± 24.10	.4726
5-HIAA mg/g Cr	3.65 ± 1.89	2.78 ± .88	3.88 ± 4.23	.15
Kynurenine/tryptophan	.034 ± .012	.047 ± .022	.058 ± .036	.043
5-HIAA/tryptophan	.54 ± .26	.50 ± .19	.46 ± .41	.1131
5-HIAA/kynurenine	18.10 ± 17.41	12.69 ± 7.93	8.61 ± 8.37	.0084
Kynurenine/serotonin	.0040 ± .0030	.0036 ± .0015	.0075 ± .0048	<.0001
Serotonin/tryptophan	13.08 ± 5.65	13.66 ± 4.18	10.71 ± 5.31	.0129

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; Cr, creatinine; HbA1c, hemoglobin A1c; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate; T2D, type 2 diabetes.

Table 12. Metabolites related to BCAA metabolism and tryptophan-kynurenine- serotonin pathways: *P* values from testing if differences among the 3 groups are different between males and females in 24-hour urine samples

	24-h urine
BCAAs mmol/mol Cr	.11
BCKAs mmol/mol Cr	.020
KIV mmol/mol Cr	.012
KMV mmol/mol Cr	.037
KIC mmol/mol Cr	.028
Tryptophan mmol/mol Cr	.17
Kynurenine mmol/mol Cr	.41
Kynurenic acid mmol/mol Cr	.58
Serotonin nmol/mmol Cr	.40
5-HIAA mg/g Cr	.33
Kynurenine/tryptophan	.028
5-HIAA/tryptophan	.99
5-HIAA/kynurenine	.077
Kynurenine/serotonin	.50
Serotonin/tryptophan	.96

Abbreviations: BCAA, branched-chain amino acid; BCKA, branched-chain ketoacid; Cr, creatinine; KIC, α-keto-isocaproate; KIV, α-keto-valerate; KMV, α-keto-β-methylvalerate.

metabolites that are associated with diversion of tryptophan to the kynurenine pathway rather than the serotonin pathway; this is evident by higher urinary kynurenine concentrations, higher ratio of kynurenine/serotonin, lower ratios of serotonin/tryptophan, and 5-HIAA/kynurenine. Third, the urinary concentrations of BCAAs and BCKAs, and metabolites reflecting diversion to the kynurenine pathway were positively associated with metrics of body fat and HbA1c. In contrast, metabolites and ratios related to the serotonin pathway were negatively associated with metrics of body fat and HbA1c. Fourth, urinary BCAAs and BCKAs and byproducts of tryptophan metabolism differ strikingly among adolescent boys and girls.

These findings are of interest given that serotonin increases pancreatic β-cell replication, β-cell mass, and glucose-dependent insulin secretion (25-29). The higher urinary concentrations of kynurenine, higher ratios of kynurenine/tryptophan and kynurenine/serotonin, and lower ratios of serotonin/tryptophan and 5-HIAA/kynurenine in T2D may reflect a relative decrease in cellular serotonin availability. It is unclear why tryptophan metabolism is diverted toward production of kynurenine rather than serotonin; this may be related in part to inflammatory cytokine expression in obesity and T2D (25-28, 49). It should also be noted that elevations in BCAAs may inhibit cellular uptake of serotonin via their competition with tryptophan, the precursor of serotonin, for uptake into β cells and other tissues (42, 50, 51).

Similar observations have been described in prior investigations of tryptophan metabolism in states of obesity, impaired glucose tolerance, and T2D in adults. Studies by Oxenkrug et al (23) and others suggest that inflammation and/or stress-induced upregulation of tryptophan-kynurenine metabolism is a factor predisposing to IR (21, 22, 24). In a study of adults with obesity, the kynurenine/tryptophan ratio was elevated in plasma samples, similar to our findings in urine samples of youth with T2D. In addition, the levels of serotonin were decreased, consistent with a shift of tryptophan metabolism favoring kynurenine production over serotonin (49). In an integrative analysis of host genetics, diet, gut microbiome, and circulating metabolites, the levels of tryptophan, 4 kynurenine-pathway metabolites (kynurenine, kynurenate, xanthurenate, and quinolinate), and indolelactate were positively associated with T2D risk, whereas indolepropionate was inversely associated with T2D risk (52). Another study showed that the host and microbiota may play a significant dual role in either the production or regulation of kynurenine

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metabolites (53). In a recent investigation, the plasma levels of kynurenine, xanthurenic acid, and kynurenic acid were higher in patients with T2D (54). Similar findings were replicated in the PREDIMED Trial, with baseline tryptophan and 1-year increases in quinolinic acid positively associated with incident T2D (55). Finally, a recent study showed that bariatric surgery reduces serum levels of tryptophan and its downstream kynurenine metabolites; their reduction after surgery was associated with improvement in glycemic control (HbA1c) (56).

Despite these studies in adults, the literature in youth is limited and newly emerging. A study of targeted metabolomic profiling in plasma samples of youth with overweight and/or obesity found a strong positive association between Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), BCAAs, and kynurenine levels (57). In a recent prospective cohort study of subjects aged 9 to 19 years with severe obesity (BMI ≥ 97th percentile), the plasma concentrations of kynurenine correlated positively with BMI *z*-score and body fat mass, whereas concentrations of serotonin correlated negatively with metrics of adiposity. Tryptophan and kynurenine, but not serotonin, also correlated with IR as assessed by HOMA-IR. In addition, plasma tryptophan was higher in children with prediabetes (58). The current investigation showed that the urinary concentrations of BCAAs, BCKAs, tryptophan, and kynurenine and the ratios reflecting diversion from the serotonin to the kynurenine pathway were positively associated with metrics of body fat and glycemic control assessed by HbA1c. In contrast, urinary metabolites and ratios related to the serotonin pathway were negatively associated with body fat and HbA1c.

Finally, the significant differences in urinary metabolites related to BCAA and tryptophan metabolism and their associations with body fat and glycemic control were largely driven by the female participants. Diversion of tryptophan metabolism to the kynurenine pathway has been characterized in other studies of adult women with obesity, IR, gestational diabetes, and T2D. For example, a recent study of adult women with polycystic ovary syndrome also found abnormal activation of the tryptophan-kynurenine pathway, as indicated by a decrease in plasma tryptophan/kynurenine and an increase in tryptophan/serotonin (59). The increased levels of kynurenine and tryptophan were positively associated with HOMA-IR. Similarly, a longitudinal metabolomics study of pregnant Chinese women found that tryptophan and purine metabolites were consistently upregulated in the urinary metabolome of patients diagnosed with gestational diabetes mellitus throughout pregnancy (60). Another study found that adult women with obesity and T2D had higher serum tryptophan and kynurenine levels than obese, normoglycemic women (61). In the aggregate, these findings suggest that glucose intolerance, rather than, or in addition to, obesity is a critical determinant of tryptophan, serotonin, and kynurenine metabolism.

Our models were adjusted for BMI-related metrics because subjects in the T2D group had higher BMI and BR% than subjects in the nondiabetic obese group. Even after adjustment for BMI-related metrics, Tanner stage, sex, age, and race, subjects in the T2D group had the highest urinary levels of BCKAs, BCAAs, tryptophan, and kynurenine. Because measurement of HbA1c was available only among obese and T2D subjects, we also adjusted the models for HbA1c in addition to Tanner stage, sex, BMI, age, and race. The major exploratory variable or correlate of BCKAs, KIV, KMV, and KIC was HbA1c. BCAAs, kynurenine, kynurenine/tryptophan, and kynurenine levels were significantly elevated among the obese with T2D group compared with the obese without T2D group. We speculate that the chronic hyperglycemia in the diabetic state may suppress the expression of the enzyme branch chain ketoacid dehydrogenase, which catalyzes the oxidative decarboxylation of BCKAs. Suppression of branch chain ketoacid dehydrogenase activity could explain in part the elevated levels of BCKAs as well as BCAAs in T2D. We cannot however, exclude the possibility that increases in BCAAs and/or BCKAs impair insulin action or secretion and thereby contribute to the development of T2D (62, 63).

Interestingly, our previous studies in youth found that plasma BCAAs and products of BCAA catabolism were higher in overweight and obese adolescent males than females (4-6). The previous studies did not include participants with T2D; it is possible that lower BCAA levels in nondiabetic females with obesity are overshadowed by more striking elevations in obese females with T2D.

Our study has a number of important strengths. The participants were well matched for age, pubertal status, and ethnicity. The T2D group comprised predominantly females and contained more Black participants, which is consistent with our clinical experience. Similarly, the T2D group also had higher body fat percentage and BMI, which is also consistent with general clinical practice.

There are certain limitations. Female participants were not studied at standard phases of the menstrual cycle, though none was actively menstruating during urine collection. We did not calculate measures of insulin sensitivity, insulin secretion, or insulin secretion adjusted for insulin sensitivity because our cohort included subjects with overt T2D, whose relative β-cell function is severely diminished. Moreover, participants with T2D were taking metformin, insulin, and/or other medications (liraglutide, dulaglutide, sitagliptin), all of which modify insulin secretion and action and make interpretation of fasting or postprandial insulin levels difficult or impossible. Insulin and GLP-1 analogs are known to stimulate BCAA catabolism and would therefore be expected to lower the BCAA and BCAA-related catabolic byproducts (64). Thus, use of these medications in selected patients cannot explain the higher urinary excretion of BCAAs and BCKAs in youth-onset T2D. The effects of the medications on tryptophan metabolism have not yet been characterized, but the very strong correlations between BCAA and kynurenine ($r = .649$, $P < .001$) in the cohort as a whole make it less likely that treatment of a minority of subjects had a major influence on the overall results. The cohort of diabetic subjects was too small to conduct a rigorous statistical analysis correlating metabolomic data with specific medication use. Finally, to minimize the potential for spurious conclusions resulting from multiple statistical comparisons, we used a threshold of $P < .01$ to indicate statistical significance. Nevertheless, the exploratory nature of our study requires that our findings be interpreted with caution.

The use of urine as a source for metabolic biomarkers comes with its own strengths and limitations. Currently in the literature, there has been little investigation of urine as a source for biomarkers in youth. Urine is useful because it is collected noninvasively and available in large quantities. Furthermore, 24-hour urine samples integrate differences in metabolic status over time and are not affected by level of protein intake, circadian rhythm, or elapsed time since the subject's last meal (65). In our study, urine was preferable to plasma

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because of the challenges associated with measuring circulating serotonin levels. Serotonin circulates in very low concentrations, with the vast majority contained in platelets. Human free plasma serotonin in platelet-poor plasma has also proven difficult to measure, with a wide range of reference values reported (66).

However, urine can pose several analytical challenges because urine volume can vary widely depending on physiological status, hydration, and kidney function; sex, age, and BMI can also contribute to variations in the urine metabolome. As a biological waste material, it has broad chemical diversity that makes data analysis and interpretation challenging (67). We addressed these challenges by normalizing all measurements to urine creatinine. Our study is limited by lack of plasma samples to correlate with urine results; however, our findings in urine regarding the tryptophan/kynurenine pathway are consistent with findings in plasma in studies of adults and a handful of studies in children.

In summary, we showed that increased urinary excretion of BCAAs and BCKAs in youth-onset T2D is accompanied by diversion of tryptophan metabolism from the serotonin pathway to the kynurenine pathway. We hypothesize that these metabolic adaptations may associate with and/or contribute to β-cell dysfunction and glucose intolerance. Future studies investigating the cellular mechanisms by which BCAAs and serotonin interact in the control of glucose-stimulated insulin secretion could test this hypothesis. Urinary metabolites of BCAA catabolism are selectively higher in females with T2D than in lean and obese females without diabetes, and tryptophan metabolism is diverted from the serotonin to kynurenine pathway preferentially in females. These adaptations associate with, and may explain in part, the higher risks of T2D in obese adolescent females compared with males (68, 69).

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Author Contributions

N.H. was responsible for interpreting the data and writing the manuscript. Y.L. and M.E.R. were responsible for advanced statistical analysis. K.B. was responsible for interpretation of the data and critical review of the manuscript. M.L.C. and R.P.G. were responsible for mass spectrometry analysis of samples and critical review of the manuscript. J.R.B. and M.M. performed biochemical analysis and critical review of the manuscript. N.J., D.S.H., and S.A. contributed to data collection and critical review of the manuscript. C.B.N. and M.F. were responsible for development of the research question, conception and design of the research project, interpretation of the data, and critical review of the manuscript. P.G.B. was responsible for development of the research question, conception and design of the research project, obtaining funding, acquisition of data, statistical analysis and interpretation of the data, and writing the manuscript.

Disclosures

N.H., Y.L., M.E.R., O.I., M.J.M., D.S.H., N.J., S.A., C.B.N., and P.G.B. have no conflicts of interest to declare. M.F. is a co-investigator on a grant from the American Heart Association that deals with the pathogenesis and treatment of childhood obesity. M.F. is also the local principal investigator on a Rhythm-sponsored study of identification and treatment of children and adults with monogenic obesity and was previously a member of a Data Safety Monitoring Board for a separate Rhythm-sponsored study of treatment of patients with syndromic obesity. M.L.C. and R.P.G. own stock and are paid salary by Laboratory Corporation of America. J.R.B. has nonfinancial ties to Agilent Technologies, Inc.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Clinical Trial Information

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