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Amino acid signature predictive of incident prediabetes: A case-control study nested within the longitudinal pathobiology of prediabetes in a biracial cohort

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ABSTRACT

Objective: Circulating branched-chain amino acids (BCAAs, isoleucine, leucine, valine) and aromatic amino acids (AAAs, tyrosine and phenylalanine) predicted type 2 diabetes mellitus (T2DM) risk in a Caucasian population. Here, we assessed amino acid levels in relation to incident prediabetes among initially normoglycemic African Americans (AA) and European Americans (EA).

Research design and methods: Using a nested case-control design, we studied 70 adults (35 AA, 35 EA) who developed prediabetes (progressors) and 70 matched participants who maintained normoglycemia (nonprogressors) during 5.5 years of follow-up in the Pathobiology of Prediabetes in a Biracial Cohort study. Assessments included plasma amino acid levels, insulin sensitivity, and beta-cell function.

Results: The total level of all 18 amino acid assayed was significantly associated with lean mass ($r = 0.36$, $P < 0.0001$), waist circumference ($r = 0.27$, $P = 0.001$), fasting plasma glucose ($r = 0.24$, $P = 0.005$), HOMA-IR ($r = 0.22$, $P = 0.01$) and HDL cholesterol ($r = -0.18$, $P = 0.03$). Individual amino acid levels were significantly associated with insulin sensitivity and insulin secretion. Compared with nonprogressors, progressors had higher baseline levels of asparagine and aspartic acid ($P < 0.0001$), glutamine/glutamic acid ($P = 0.005$) and phenylalanine ($P = 0.02$), and lower histidine ($P = 0.02$) levels. In fully-adjusted logistic regression models, aspartic acid/asparagine (OR 2.72 [95% CI 1.91–3.87]) and histidine (OR 0.90 [95% CI 0.85–0.96]) were the only amino acids that significantly predicted incident prediabetes.

Conclusions: Baseline plasma aspartic acid and asparagine levels predicted progression to prediabetes, whereas histidine levels were protective of prediabetes risk. Thus, the amino acid signature associated with prediabetes in a diverse population may be distinct from that previously linked to T2DM in Caucasians.

1. Introduction

Emerging data indicate that plasma levels of certain amino acids and other metabolites in baseline specimens can predict the risk of incident type 2 diabetes (T2DM) during follow-up of participants in longitudinal studies [1,2]. In a pivotal report, analyses of archived fasting plasma specimens collected at baseline in the Framingham Heart Study (a predominantly Caucasian cohort) showed that elevated levels of three branched-chain amino acids (BCAAs; isoleucine, leucine, valine) and two aromatic amino acids (AAAs; tyrosine and phenylalanine) significantly predicted incident T2DM 12 years later among study participants [1]. Those findings were replicated in another Caucasian cohort (Malmö Diet and Cancer study) [1]. Among participants in the multiethnic Insulin Resistance Atherosclerosis Study (IRAS), decreased baseline plasma levels of glycine and increased levels of valine, leucine, phenylalanine, and glutamine/glutamate characterized individuals with insulin resistance and predicted the development of T2DM [3].

In another multiethnic cohort (Diabetes Prevention Program, DPP) that followed prediabetic subjects for the outcome of incident T2DM, the association between previously reported diabetes risk predictors (branched-chain and aromatic amino acids) and incident T2DM were attenuated after adjustment for baseline variables, including age, sex, race/ethnicity, body mass index (BMI) and fasting plasma glucose (FPG) levels [4]. However, the report from the DPP cohort did identify

Abbreviations: AAA, aromatic amino acid; AIRg, acute insulin response to intravenous glucose; BCAA, branched-chain amino acid; BMI, body-mass index; DEXA, dual-energy x-ray absorptiometry; EDTA, ethylenediaminetetraacetic acid; FFM, fat-free mass; FPG, fasting plasma glucose; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; HOMA, homeostasis model assessment; OGTT, oral glucose tolerance test; Si-clamp, insulin sensitivity measured by hyperinsulinemic euglycemic clamp; T2DM, type 2 diabetes mellitus; 2hrPG, two hour value of plasma glucose during OGTT.

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glycine betaine, methionine sulfoxide, serine, and propionylcarnitine as significant predictors of incident diabetes even after adjustments for multiple baseline variables [4]. The IRAS investigators noted that ethnic-stratified results regarding the association of amino acids and insulin resistance/incident T2DM were strongest in European Americans (EA), compared with Hispanics and African Americans (AA) [3]. Other studies in populations of South Asian and African ancestry have found either no association between BCAA and diabetes risk [5] or a different pattern of association between amino acids and T2DM risk than was reported for Caucasian populations [6]. In a study of Chinese subjects, plasma levels of BCAAs (isoleucine, leucine, valine) and AAAs (tyrosine and phenylalanine) were significantly lower in patients with T2DM than those with normal glucose tolerance [7], which is the reverse of the pattern observed in Caucasians [1]. A similar pattern of lower fasting plasma levels of leucine, isoleucine, valine, phenylalanine, and tyrosine was reported in a multiethnic population of adolescents with T2DM compared with normal-weight healthy control subjects [8]. Thus, the signatures of amino acid metabolites associated with T2DM are still evolving and appear to differ across ethnic populations.

The exact mechanisms linking elevations in circulating levels of BCAAs and certain other amino acids to increased risk of T2DM are not entirely clear. Plasma levels of amino acids reflect the balance among dietary consumption, protein synthesis, proteolysis, and catabolism of excess amino acids [9]. The accumulation of amino acids in plasma, thus, indicates dominance of amino acid production (from diet and proteolysis) over clearance (through utilization in protein synthesis or catabolism and conversion to Krebs' cycle intermediates). The well-known actions of insulin to promote protein synthesis and inhibit proteolysis predict that states of insulin deficiency or insulin resistance would be associated with hyperaminoacidemia. Furthermore, decreased activity of key enzymes involved in the catabolism of BCAAs has been reported in insulin-resistant states [10]. Thus, elevated fasting plasma levels of BCAAs and other amino acids reflect insulin resistance, relative insulin deficiency or decreased amino acid catabolism, all being processes that could alter glucose homeostasis. In addition, increased plasma levels of BCAAs and other amino acids can induce insulin resistance via activation of the molecular target of rapamycin (mTOR), AMPK, and other pathways [11]. The biological significance of the link between BCAAs and T2DM is suggested by reports that gastric bypass surgery, which leads to early improvement in glucose tolerance (independently of weight loss), decreases plasma BCAA levels in obese subjects [10,12]. Clearly, further studies are needed to elucidate the exact mechanisms linking elevated BCAAs and other amino acids to increased risk of diabetes.

Prediabetes is an intermediate stage between normal glucose regulation and T2DM, and it is unknown whether the same amino acids associated with incident T2DM [1–3] also predict the initial transition from normoglycemia to incident prediabetes. In contrast to T2DM, reports of associations between amino acids and prediabetes are limited to cross-sectional studies that do not permit inferences as to whether the observed amino acid patterns preceded the development of prediabetes [13,14,15]. Moreover, the latter studies were conducted in mostly Caucasian populations. Given the limitations of cross-sectional studies, prospective studies are needed to confirm whether specific amino acid signatures are predictive of incident prediabetes risk, and to discern any ethnic differences in the expression of such signatures. In the Pathobiology of Prediabetes in a Biracial Cohort (POP-ABC) study, we enrolled normoglycemic AA and EA offspring of parents with T2DM and followed them for 5.5 years, the primary outcome being the occurrence of prediabetes (impaired fasting glucose {IFG} or impaired glucose tolerance {IGT}) [16–18]. The POP-ABC study has identified several behavioral, clinical, and biochemical predictors of incident prediabetes among our high-risk AA and EA individuals [19–23]. In the present report, we performed an exhaustive analysis of plasma amino acids in relation to ethnicity, sex, cardiometabolic profile, and the risk of incident prediabetes during longitudinal follow-up of our biracial cohort. Specifically, we determined whether the three BCAAs (isoleucine, leucine, valine) and two AAAs (tyrosine and phenylalanine), known to predict T2DM risk, also are significant predictors of prediabetes risk in a diverse population.

2. Research design and methods

2.1. Study subjects

Using a nested case-control design, we selected 70 participants (35 AA, 35 EA) in the Pathobiology of Prediabetes in a Biracial Cohort (POP-ABC) study [12–14] who developed incident prediabetes (Progressors) during a follow-up period of 5.5 years (mean 2.62 years) and 70 participants who maintained normoglycemia during the same period (Nonprogressors). The two groups were age-, sex- and ethnicity-matched according to the distribution of these three variables. Eligible for enrollment in POP-ABC study were individuals aged 18–65 years, of self-reported non-Hispanic white (EA) or non-Hispanic black (AA) race/ethnicity status, who had one or both biological parents with T2DM. Enrollees had normal fasting plasma glucose (FPG) (≪100 mg/dl{5.6 mmol/l}) and/or normal glucose tolerance (NGT) (2-h plasma glucose {2hrPG} < 140 mg/dl {7.8 mmol/l} during a screening 75-g oral glucose tolerance test {OGTT}), as previously described [16–18]. Excluded from participation were individuals using any medications or interventions known to alter body weight, glucose or lipid metabolism, or energy balance [16–18]. The University of Tennessee Institutional Review Board approved the POP-ABC protocol. Written informed consent was given by all participants before initiation of the study, which was conducted at the General Clinical Research Center (GCRC) in accordance with the World Medical Association's Declaration of Helsinki.

2.2. Assessments

Participants were studied at the GCRC after an overnight fast for baseline assessments, which included a medical history, physical examination, anthropometry, and a 75-gram OGTT [12,14,15]. Dietary information was obtained from all participants, using the validated Food Habits Questionnaire, as previously described [19,24]. The body-mass index (BMI) was calculated as the weight in kilogram divided by the height in meter squared. Additional baseline measurements included HbA1c and fasting lipid profile. Thereafter, participants made quarterly visits to the GCRC for scheduled, staggered assessments: FPG quarterly; OGTT and insulin secretion annually; body composition analysis with DEXA annually; and insulin sensitivity in years 1, 3 and 5, as previously described [16–18]. The primary outcome was the occurrence of prediabetes, as indicated by IFG (FPG 100–125 mg/dl or 5.6–6.9 mmol/l) and/or IGT (2hrPG 140–199 mg/dl or 7.8–11.0 mmol/l) [16,17]. Each endpoint occurrence was confirmed with a standard 75-gram OGTT within 6 weeks. The Institutional Data and Safety Officer (Murray Heimberg, MD, PhD) independently adjudicated each endpoint occurrence. At the end of the 5.5-year follow-up period, participants with incident prediabetes were classified as progressors and those who maintained normoglycemia were classified as nonprogressors.

2.3. Insulin sensitivity and insulin secretion

Insulin sensitivity and insulin secretion were assessed, as previously described [16,25,26]. In brief, the hyperinsulinemic euglycemic clamp procedure was utilized to measure insulin sensitivity in subjects who had fasted overnight. A primed, continuous i.v. infusion of regular human insulin (2 $mU \cdot kg^{-1} \cdot min^{-1}$; 14.4 $pmol \cdot kg^{-1} \cdot min^{-1}$) was administered for 180 min along with a variable-rate infusion of dextrose (20%) to maintain blood glucose at ~100 mg/dl (5.6 mmol/l). Blood specimens (arterialized by warming participants' forearm) for

measurement of glucose and insulin levels were obtained every 10 min. During steady state (final 60 min of insulin infusion), the rate of total insulin-stimulated glucose disposal (M) was calculated and corrected for the steady-state plasma insulin levels, to derive the insulin sensitivity index (Si-clamp) [25].

Acute insulin secretory response to glucose (AIRg) was measured using the frequently sampled intravenous glucose tolerance test (FSIVGTT), as previously described [16–18]. Briefly, subjects who had fasted overnight received an i.v. bolus of dextrose (25 g). Arterialized blood samples for the measurement of glucose and insulin levels were collected 30 min before and at 2, 3, 4, 5, 7, and 10 min following the i.v. dextrose bolus [16–18]. The AIRg was calculated as the mean incremental insulin level from 3 to 5 min after the dextrose bolus [16–18].

Additionally, fasting glucose and insulin levels were used to derive values for the homeostasis model assessment (HOMA) of insulin resistance (HOMA-IR), an estimate of hepatic insulin sensitivity, and (HOMA-B), an estimate of beta-cell function [27].

2.4. Biochemical measurements

Plasma glucose was measured at the bedside using the YSI glucose analyzer (Yellow Spring Instruments Co., Inc., Yellow Spring, OH). Plasma insulin levels were measured immunochemically in our Endocrine Research Laboratory, using commercial ELISA kits. Hemoglobin A1c (HbA1c) and fasting plasma lipid profiles were measured in a contract clinical laboratory. The within-batch coefficients of variation for the plasma glucose, insulin, and HbA1c assays were all <5%.

2.5. Mass spectrometry profiling of plasma amino acids

As already stated, the present report is a case-control comparison of fasting plasma amino acid levels in specimens obtained at baseline from POP-ABC participants who subsequently developed prediabetes (progressors) or remained normoglycemic (nonprogressors) during follow-up. Blood specimens were collected in same-size EDTA tubes, processed, and stored at -80° C until time of assay. Amino acids were analyzed, under contract, at the Duke University Metabolomics Laboratory (Durham, NC), using stable isotope dilution techniques. The measurements were made by flow injection tandem mass spectrometry, as described previously [28,29]. The data were acquired using a Waters triple quadrupole detector equipped with Acquity™ UPLC system and controlled by MassLynx 4.1 software platform (Waters, Milford, MA). Leucine and isoleucine, being isobaric, appeared together at the same mass and their levels were combined in the present report. During hot, acidic esterification, some or all asparagine molecules were converted to aspartic acid, so the combined asparagine and aspartate concentration was reported. Similarly, glutamine molecules are converted to glutamic acid during hot, acidic esterification, so we have reported their combined concentration as well.

2.6. Statistical analysis

Data are reported as means ± SD unless SEM is specified. We compared baseline characteristics between cases (progressors) and controls (nonprogressors), and between other defined groups (AA vs. EA; Women vs. Men), using unpaired *t*-tests. The associations between plasma amino acid levels and cardiometabolic risk markers, insulin sensitivity, and insulin secretion, were analyzed using linear regression models and Pearson correlation coefficients. We identified those plasma amino acids that showed differences at the $P < 0.15$ level in univariate analysis as potential predictors of incident prediabetes and entered them into stepwise logistic regression models, before and after adjustments for age, sex, race/ethnicity, and BMI. All analyses were conducted using SAS (version 9.2, Cary, N.C., USA). $P < 0.05$ was accepted as significant.

3. Results

3.1. Cohort characteristics

Table 1 shows the characteristics, at enrollment in the POP-ABC study, of the 70 participants who subsequently developed prediabetes (progressors) and a control group of 70 nonprogressors. The groups were similar except for higher BMI, FPG and HOMA-IR in progressors compared with nonprogressors. Table 2 shows values for total and individual fasting plasma amino acids by race/ethnicity. The level of total amino acids was lower in AA than EA subjects (1668 ± 184 μM vs. 1749 ± 199 μM, $P = 0.01$). Values for 12 of the 18 individual amino acids assayed were similar in AA and EA subjects. Tyrosine ($P = 0.04$) and methionine ($P = 0.05$) levels were higher in AA than EA participants, whereas alanine ($P = 0.03$), citrulline ($P < 0.0001$), glycine ($P = 0.005$) and ornithine ($P = 0.003$) levels were higher in EA than AA participants (Table 2). Supplemental Table 1 shows values for total and individual fasting plasma amino acids by gender. The level of total fasting plasma amino acids was lower in women than men (1672 ± 188 μM vs. 1775 ± 192 μM, $P = 0.003$). The gender difference was explained by higher male levels of glutamine/glutamic acid ($P < 0.0001$), isoleucine/leucine ($P < 0.0001$), methionine ($P < 0.0001$), proline ($P = 0.0002$) and valine ($P < 0.0001$). The levels of the 11 other amino acids assayed were similar in men and women (Supplemental Table 1). Analyzed by gender, total plasma amino acid levels were similar in men (AA vs. EA: 1734 ± 182 μM vs. 1810 ± 198 μM, $P = 0.17$) but lower in AA women than EA women (1635 ± 177 μM vs. 1712 ± 193 μM, $P = 0.045$).

3.2. Association of amino acids with cardiometabolic risk markers

We examined the association between the level of total fasting plasma amino acids and measures of adiposity, lipid profile, blood pressure, glycemia and glucoregulatory function (including insulin sensitivity and secretion). As shown in Table 3, the level of total amino acids was significantly associated with weight ($r = 0.24$, $P = 0.004$) but not total body fat mass. The latter finding is explained by the strong correlation between total amino acids and lean mass ($r = 0.36$, $P < 0.0001$). The lean mass was similar among men (54.0 ± 11.5 kg in AA vs. 51.2 ± 11.0 kg in EA, $P = 0.14$), but higher in AA women than EA women (49.7 ± 6.66 kg vs.45.1 ± 7.58 kg, $P = 0.0036$). However, the association between lean mass and plasma amino acids was consistent by race and sex. Circulating total amino acids also were significantly associated with waist circumference ($r = 0.27$, $P = 0.001$), FPG ($r = 0.24$, $P0.005$), HOMA-IR ($r = 0.22$, $P = 0.01$) and HDL cholesterol level ($r = -0.18$, $P = 0.03$) (Table 3). There was no significant association between fasting plasma total amino acids and systolic or diastolic blood pressure, insulin secretion (HOMA-B and AIRg), or serum triglycerides

Table 1
Baseline characteristics of participants with incident prediabetes (progressors) and those with normoglycemia (nonprogressors) during follow-up.

Characteristics	All	Nonprogressors	Progressors	*P*-value
Number	140	70	70	
Age (yr)	48.1 ± 8.69	47.6 ± 9.16	48.5 ± 8.23	0.55
BMI (kg/m^2)	30.1 ± 5.78	29.03 ± 5.22	31.3 ± 6.12	0.02
FPG (mg/dl)	92.7 ± 5.84	91.3 ± 6.18	94.03 ± 5.17	0.006
2hrPG (mg/dl)	121 ± 23.3	119 ± 25.02	123 ± 21.4	0.37
HOMA-IR	1.87 ± 1.61	1.58 ± 1.53	2.14 ± 1.64	0.04
Si-clamp	0.127 ± 0.067	0.138 ± 0.068	0.118 ± 0.065	0.14
AIR (μU/ml)	84.1 ± 75.4	88.8 ± 89.7	79.6 ± 59.6	0.49
Food habits score	2.56 ± 0.49	2.51 ± 0.49	2.56 ± 0.50	0.52

Data are means ± SD. AIRg, acute insulin response to i.v. glucose; BMI, body-mass index; FPG, fasting plasma glucose; 2hrPG, 2-h plasma glucose during 75-g oral glucose tolerance test; HOMA-IR, homeostasis model of insulin resistance; Si-clamp, insulin sensitivity by euglycemic clamp; To convert the values for glucose to millimoles per liter, multiply by 0.0555. To convert the values for insulin (AIRg) to picomoles per liter, multiply by 7.175.

Table 2
Comparison of fasting plasma amino acid levels (μM) in normoglycemic African Americans and European Americans.

	African Americans	European Americans	*P* value
Total amino acids	1668 ± 184	1749 ± 199	0.01
Alanine	321 ± 67.8	349 ± 81.7	0.03
Arginine	81.3 ± 18.3	79.1 ± 20.5	0.52
Asparagine + Aspartic acid	11.8 ± 3.57	12.5 ± 3.91	0.24
Citrulline	27.3 ± 6.03	32.3 ± 5.80	<0.0001
Glutamic acid + glutamine	94.8 ± 21.0	94.3 ± 17.7	0.86
Glycine	247 ± 57.8	280 ± 79.7	0.005
Histidine	77.9 ± 11.1	77.8 ± 10.5	0.98
Isoleucine + Leucine	151 ± 27.9	148 ± 27.3	0.55
Methionine	26.5 ± 4.53	25.1 ± 3.71	0.05
Ornithine	52.6 ± 13.7	59.4 ± 13.3	0.003
Phenylalanine	54.1 ± 7.91	54.4 ± 6.42	0.82
Proline	152 ± 37.9	162 ± 42.7	0.14
Serine	100 ± 15.2	101 ± 20.5	0.84
Tyrosine	61.9 ± 12.5	57.8 ± 11.1	0.04
Valine	210 ± 38.5	217 ± 38.6	0.27

Data are means ± SD.

(Table 3). The observed cardiometabolic correlates of total plasma amino acids were broadly consistent in AA and EA participants, with a few exceptions. Notably, AA participants showed stronger association between total amino acids and measures of glycemia (FPG, 2-hrPG, Glucose-AUC) than EA subjects, whereas the opposite was observed regarding the association between total amino acids and lean mass and insulin sensitivity (Si-clamp) (Table 3).

We found associations between individual amino acids and insulin sensitivity and acute insulin secretion (AIRg). The fasting plasma levels of 10 amino acids (alanine, glutamine/glutamic acid, glycine, isoleucine/leucine, phenylalanine, proline, tyrosine and valine) exhibited significant associations with insulin sensitivity ($P = 0.03$–<0.0001) (Table 4). Glycine levels were positively associated with insulin sensitivity, whereas plasma levels of the other nine amino acids were inversely related to insulin sensitivity. Furthermore, insulin secretion was positively associated with the fasting plasma levels of four amino acids (citrulline, glutamine/glutamic acid, and tyrosine) and inversely associated with glycine levels. The levels of glycine, tyrosine, glutamine and glutamic acid were significantly associated with both insulin sensitivity and insulin secretion in our biracial cohort (Table 4, Fig. 1).

Table 3
Association of total plasma amino acid levels with cardiometabolic risk markers in normoglycemic African Americans and European Americans.

	All subjects		African Americans		European Americans	
	r	*P*	*r*	*P*	*r*	*P*
Age (yr)	0.05	0.53	0.01	0.95	0.04	0.74
Weight (kg)	0.24	0.004	0.25	0.03	0.38	0.001
Total fat mass (kg)	0.06	0.46	0.06	0.66	0.16	0.19
Lean mass (kg)	0.36	<0.0001	0.28	0.02	0.51	<0.0001
Waist circ. (cm)	0.27	0.001	0.33	0.006	0.30	0.01
SBP (mmHg)	0.002	0.98	0.063	0.61	0.067	0.58
DBP (mmHg)	0.053	0.54	0.08	0.63	0.012	0.92
FPG (mg/dl)	0.24	0.005	0.25	0.03	0.18	0.13
2hrPG (mg/dl)	0.10	0.26	0.30	0.01	0.11	0.37
Glucose-AUC	0.14	0.10	0.32	0.007	0.10	0.44
HbA1c	0.03	0.76	0.02	0.88	0.21	0.08
HOMA-IR	0.22	0.01	0.21	0.09	0.20	0.10
Si-Clamp	−0.19	0.06	−0.16	0.29	−0.34	0.01
HOMA-B (%)	0.13	0.14	0.14	0.29	0.11	0.36
AIRg (pmol/l)	0.01	0.89	0.16	0.21	0.02	0.88
LDL (mg/dl)	0.02	0.78	0.15	0.23	0.11	0.35
HDL (mg/dl)	−0.18	0.03	0.15	0.22	−0.17	0.15
Trig. (mg/dl)	0.16	0.06	0.19	0.11	0.06	0.61

Table 4
Association of plasma amino acid levels with insulin sensitivity and insulin secretion.

Amino acid (μM)	Insulin sensitivity (Si-clamp)		Insulin secretion (AIR)	
	r	*P*	*r*	*P*
Alanine	−0.22	0.03	0.03	0.77
Arginine	−0.08	0.63	0.09	0.47
Asparagine + Aspartic acid	−0.06	0.55	0.05	0.77
Citrulline	0.07	0.49	0.24	0.006
Glutamine + Glutamic acid	−0.26	0.009	0.26	0.002
Glycine	0.30	0.002	0.26	0.003
Histidine	−0.10	0.34	0.04	0.65
Isoleucine + Leucine	−0.26	0.01	0.08	0.38
Methionine	−0.11	0.27	0.09	0.30
Ornithine	−0.15	0.13	0.06	0.47
Phenylalanine	−0.25	0.01	0.11	0.21
Proline	−0.23	0.02	0.05	0.62
Serine	0.17	0.09	0.05	0.58
Tyrosine	−0.39	<0.0001	0.40	<0.0001
Valine	−0.25	0.01	0.09	0.29

3.3. Amino acid levels and incident prediabetes

We analyzed baseline fasting plasma levels of our panel of 18 amino acids in participants who experienced glycemic progression to prediabetes (progressors) and those who maintained normoglycemia (nonprogressors) during a total follow-up period of 5 years (mean 2.62 years).

Progressors and nonprogressors were concordant in the fasting levels of 12 amino acids and discordant in the levels of six amino acids (glutamic acid, glutamine, histidine, phenylalanine, aspartic acid and asparagine) (Table 5 and Fig. 2). Compared to nonprogressors, the progressors had higher baseline levels of four polar amino acids (aspartic acid/asparagine and glutamine/glutamic acid) and the aromatic amino acid phenylalanine, and lower levels of histidine, another aromatic amino acid (Table 5 and Fig. 2).

In stepwise logistic regression models, adjusted for age, sex and race, the only amino acids whose baseline levels significantly predicted incident prediabetes were aspartic acid, asparagine, phenylalanine, and histidine (Table 6). After additional adjustment for BMI, phenylalanine no longer was predictive, leaving aspartic acid, asparagine and histidine as the only significant predictors of incident prediabetes. Each 1-standard deviation increase in baseline aspartic acid and asparagine levels predicted a 2.72-fold higher risk of incident prediabetes, whereas a 1-standard deviation increase in baseline histidine levels predicted an 10% lower risk of incident prediabetes (Table 6). In separate stepwise logistic regression analyses (adjusted for age, sex and BMI), aspartic acid, asparagine and histidine remained consistent predictors of incident prediabetes in AA and EA subjects. The odds ratios for risk reduction associated with histidine were 0.92 [95% CI 0.86–0.98], $P = 0.00118$ in AA subjects and 0.85 [0.75–0.97], $P = 0.0133$ in EA subjects. The odds ratio for increased prediabetes risk associated with aspartic acid + asparagine was 4-fold greater in white subjects (9.57 {95%CI 2.56–35.7}, $P = 0.0008$) than black subjects (2.05 {95% CI 1.41–2.98}, $P = 0.0002$) (Table 6b).

Phenylalanine, which was a weak predictor in the combined cohort, no longer was a significant predictor in the fully adjusted separate analysis.

Baseline levels of the branched chain amino acids isoleucine, leucine, and valine and the aromatic amino acid tyrosine did not predict incident prediabetes in the unadjusted or adjusted models.

4. Discussion

The primary focus of this study was to determine whether the fasting plasma signatures of elevated amino acid associated with

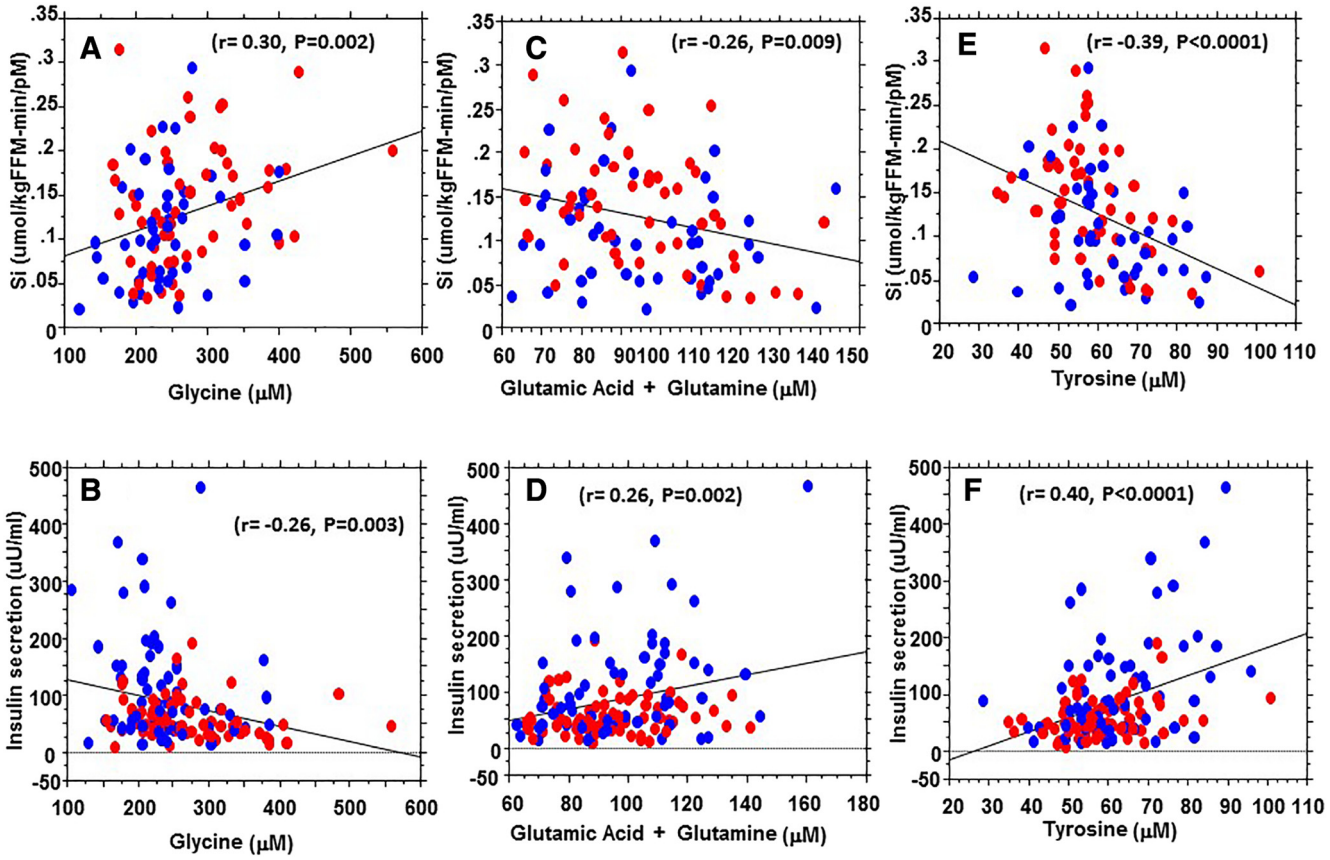

Fig. 1. Association of fasting plasma levels of glycine (A,B), glutamic acid and glutamine (C,D) and tyrosine (E,F) with insulin sensitivity (upper panels) and insulin secretion (lower panels) in normoglycemic African Americans (blue symbols) and European Americans (red symbols).

incident T2DM in a predominantly Caucasian population [1,2] also were predictive of the risk of progression from normoglycemia to prediabetes in a biracial cohort. Pioneering reports from the Framingham Heart Study cohort and the Malmo study specifically identified elevated levels of three BCAAs (isoleucine, leucine, valine) and two AAAs (tyrosine and phenylalanine) as significant predictors of future T2DM risk [1,2]. Cross-sectional studies have reported associations between prediabetes and an extensive range of amino acids (reviewed in reference [30]), including BCAAs (valine, leucine, and isoleucine) [9,10,30–34], AAAs (histidine, tyrosine and phenylalanine) [7,34,35], hydrophobic amino acids (alanine, glycine and proline) [30,33], polar amino acids (serine, glutamine and glutamic acid, glutamate) [32,34,35] and the basic amino acid lysine [31,32].

Table 5
Comparison of fasting plasma amino acid levels (μM) in progressors to prediabetes and nonprogressors.

	Progressors	Nonprogressors	*P* value
Total amino acids	1730 ± 194	1687 ± 195	0.20
Alanine	335± 81.2	335 ± 71.2	0.99
Arginine	83.0 ± 19.8	77.5 ± 18.7	0.10
Aspartic acid + Asparagine	14.2 ± 4.03	10.1 ± 3.91	<0.0001
Citrulline	29.8 ± 6.53	29.8 ± 6.33	0.99
Glutamic acid + glutamine	99.1 ± 19.1	90.0 ± 18.6	0.005
Glycine	261 ± 68.1	265 ± 74.6	0.73
Histidine	75.8 ± 7.94	79.8 ± 12.7	0.02
Isoleucine + Leucine	154 ± 24.3	145 ± 29.9	0.07
Methionine	25.8 ± 4.02	25.8 ± 4.38	0.93
Ornithine	57.6 ± 13.2	54.3 ± 14.4	0.16
Phenylalanine	55.7 ± 7.13	52.9 ± 7.02	0.02
Proline	158 ± 37.1	155 ± 43.8	0.64
Serine	102 ± 18.2	99.2 ± 17.7	0.36
Tyrosine	61.3 ± 11.2	58.4 ± 12.6	0.15
Valine	218 ± 34.7	208 ± 41.8	0.17

Data are means ± SD.

In our prospective, diverse POP-ABC cohort, with equal representation of white and black subjects, progressors to prediabetes had higher baseline levels of four polar amino acids (aspartic acid, asparagine, glutamic acid, and glutamine) and one aromatic amino acid (phenylalanine), and lower levels of another aromatic amino acid (histidine) compared with nonprogressors. Elevated baseline levels of asparagine and aspartic acid emerged as the strongest risk predictors for prediabetes, whereas histidine levels predicted decreased prediabetes risk. Phenylalanine was the only one among the five T2DM-associated amino acids that emerged as a significant predictor of incident prediabetes, albeit in the partially adjusted model. Our findings suggest that the expression of amino acid signatures predictive of early glucose abnormalities (incident prediabetes) in a diverse cohort may be quite different from the pattern associated with diabetes risk in non-diverse populations [1].

In the mechanistic part of the present study, we demonstrated significant associations between total and individual plasma amino acid levels and the key glucoregulatory processes of insulin sensitivity and insulin secretion, broadly consistent with previous reports [36,37]. Surprisingly, the strongest associations we observed were not with BCAAs. The hydrophobic amino acid glycine was unique in its positive association with insulin sensitivity and inverse association with insulin secretion, whereas the aromatic amino acids (phenylalanine and tyrosine) and the polar amino acids (glutamine and glutamic acid) were inversely

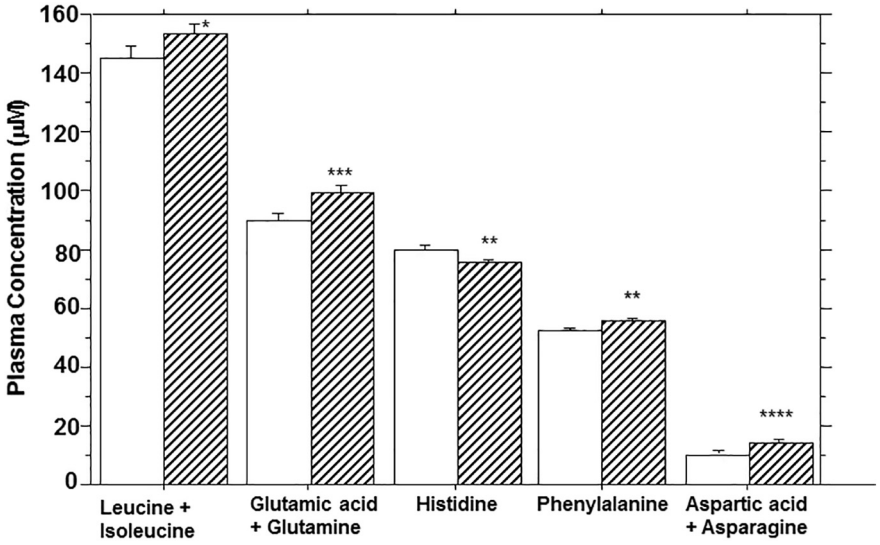

Fig. 2. Discordant baseline fasting plasma amino acids in study subjects who developed incident prediabetes (progressors, striped bars) compared with those who maintained normoglycemia (nonprogressors, open bars) during 5.5 years of follow-up. * $P = 0.07$, ** $P = 0.02$, *** $P = 0.005$, **** $P < 0.0001$.

associated with insulin sensitivity and positively associated with insulin secretion. These findings indicate that phenylalanine, glutamine, and glutamic acid are markers of insulin resistance (associated with compensatory hyperinsulinemia), whereas glycine is a marker of insulin sensitivity (associated with lower insulinemia). Our findings are in accord with reports of beneficial association between higher glycine levels and decreased cardiometabolic risk [38–40]. Although the exact mechanisms of its pro-metabolic attributes are unclear, glycine (a nonessential amino acid) is a precursor for creatine, purine, and collagen synthesis, and has been associated with beneficial anti-inflammatory, immunomodulatory, and anti-oxidant properties. Glycine receptors are expressed on pancreatic beta cells [41], and dietary glycine supplementation has been shown to improve anti-oxidant and metabolic functions in animal models and humans [42,43].

Besides their significant correlations with cardiometabolic risk markers, the levels of several amino acids displayed significant differences between progressors and nonprogressors to prediabetes. The group differences in circulating amino acid levels observed by us as well as their correlations with metabolic variables are of similar magnitude as those reported by other workers who compared healthy subjects with obese or prediabetic subjects or those with the metabolic syndrome [8,44]. The modest correlations between amino acids and the various metabolic measures suggest that circulating amino acids explain only part of the variances in those endpoints. Nonetheless, given that plasma contains only a very small fraction of the total amino acid pool (approximately 0.2 to 6% for individual amino acids) [45], the finding of significant correlations with cardiometabolic endpoints is remarkable. The fasting plasma levels of amino acids measured in the present study reflect proteolysis and amino acid catabolism during the post-absorptive period of starvation. As proteolysis is restrained by insulin action, the observed association between amino acid levels and markers of insulin sensitivity and insulin secretion is physiologically congruent. However, the exact mechanisms linking elevations in circulating levels of certain amino acids to increased risk of dysglycemia are unclear. Nonetheless, the decrease in plasma levels of amino acids and early improvement in glucose tolerance (independently of weight loss), following gastric bypass surgery, suggest a pathophysiological interaction between amino acids and glucoregulation [10,12].

Notably, baseline glycine levels were not significantly different in progressors and non-progressors to prediabetes among our POP-ABC study participants. Instead, we found a novel signature of higher baseline levels of asparagine and aspartic acid, and lower levels of histidine, as significant predictors of incident prediabetes. We did not observe associations between baseline levels of aspartic acid, asparagine, or histidine with insulin sensitivity or insulin secretion, indicating that the traditional glucoregulatory mechanisms may not explain the association between the aforementioned amino acids and risk of incident prediabetes. Thus, the exact mechanisms linking prediabetes risk to higher levels of aspartic acid and asparagine, and lower levels of histidine, remain to be determined. Supplementation with aspartic acid and asparagine decreased glucose transport in a rodent model [46]. Aspartic acid and asparagine are metabolized by skeletal muscle via anaplerotic reactions that lead to increased glutamine concentrations [47]. In the present study, glutamine and glutamic acid levels emerged as markers of insulin resistance, thereby providing an indirect link between aspartic acid/asparagine and insulin resistance/dysglycemia. Our finding that higher histidine levels predicted decreased risk of incident prediabetes is probably explained by the well-documented ameliorative effects of

Table 6
Stepwise logistic regression: significant predictors of incident prediabetes.

	Amino acid (μM)	Odds ratio	95% CI	*P*-value
Model 1	Aspartic acid + Asparagine	2.39	1.74–3.29	<0.0001
	Phenylalanine	1.08	1.01–1.16	0.0279
	Histidine	0.89	0.84–0.95	0.0002
Model 2	Aspartic acid + Asparagine	2.63	1.85–3.74	<0.0001
	Phenylalanine	1.08	1.00–1.17	0.0416
	Histidine	0.89	0.83–0.94	0.0001
Model 3	Aspartic acid + Asparagine	2.72	1.91–3.87	<0.0001
	Histidine	0.9	0.85–0.96	0.0007

Model 1, unadjusted; Model 2, adjusted for age, sex, and race; Model 3, adjusted for age, sex, race and BMI.

Table 6b
Significant predictors of incident prediabetes by race/ethnicity.

Amino acid (μM)	Odds ratio	95% CI	*P*-value
Aspartic acid + Asparagine			
African American	2.05	1.41–2.98	0.0002
European American	9.57	2.56–35.7	0.0008
Histidine			
African American	0.92	0.86–0.98	0.0118
European American	0.85	0.75–0.97	0.0133

Adjusted for age, sex and BMI.

histidine on systemic and adipose tissue inflammation, oxidative stress, adiposity, and ingestive behavior [48–50].

The strengths of our present report include the prospective case-control design, enrollment of a diverse cohort, rigorous adjudication of prediabetes endpoints and the use of robust measures of insulin sensitivity and insulin secretion. Among its weaknesses are the relatively small study sample and the restriction of enrollment to offspring of parents with T2DM. The latter does limit the generalizability of our findings. Nonetheless, the findings in such a high-risk group should prove useful for future studies aimed at risk prediction and early preventive intervention.

In conclusion, higher baseline fasting plasma levels of aspartic acid and asparagine, and lower levels of histidine, significantly predicted incident prediabetes during 5.5-year follow-up of initially normoglycemic African-American and European-American adults with parental diabetes. Our present findings suggest differential association of different sets of amino acids with progression to prediabetes versus established T2DM. Should this differentiation be maintained in future work, it would suggest that the evolution of dysglycemia across the spectrum from normal glucose regulation to T2DM might be reflected in changes in amino acid signatures. Thus, future studies in which amino acid profiles are measured repeatedly during longitudinal follow-up of initially normoglycemic individuals would be most informative.

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Statement of author contributions and acknowledgments

All authors materially participated in the research and article preparation, and gave final approval for the version submitted. SD-J conceived of and designed the study, analyzed data, wrote manuscript; IO, NU, FS collected data, reviewed and revised manuscript; JW performed statistical analysis, reviewed and revised manuscript.

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Declaration of Competing Interest

The authors have no conflict of interest to disclose regarding the content of this manuscript.

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