Plasma Amino Acids and Incident Type 2 Diabetes in Patients With Coronary Artery Disease

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OBJECTIVE

Altered plasma amino acid levels have been implicated as markers of risk for incident type 2 diabetes; however, amino acids are also related to established diabetes risk factors. Therefore, potential for confounding and the impact from competing risks require evaluation.

RESEARCH DESIGN AND METHODS

We prospectively followed 2,519 individuals with coronary artery disease but without diabetes. Mixed Gaussian modeling identified potential for confounding. Confounding, defined as a change in effect estimate (≥10%), was investigated by comparing amino acid–incident diabetes risk in a Cox model containing age and sex with that in models adjusted for potential confounders (BMI, estimated glomerular filtration rate, HDL cholesterol, triacylglycerol, C-reactive protein), which were further adjusted for plasma glucose, competing risks, and multiple comparisons (false discovery rate = 0.05, Benjamini-Hochberg method). Finally, component-wise likelihood-based boosting analysis including amino acids and confounders was performed and adjusted for competing risks in order to identify an optimal submodel for predicting incident diabetes.

RESULTS

The mean age of the source population was 61.9 years; 72% were men. During a median follow-up of 10.3 years, 267 incident cases of diabetes were identified. In age- and sex-adjusted models, several amino acids, including the branched-chain amino acids, significantly predicted incident diabetes. Adjustment for confounders, however, attenuated associations. Further adjustment for glucose and multiple comparisons rendered only arginine significant (hazard ratio/1 SD 1.21 [95% CI 1.07–1.37]). The optimal submodel included arginine and asparagine.

CONCLUSIONS

Adjustment for relevant clinical factors attenuated the amino acid–incident diabetes risk. Although these findings do not preclude the potential pathogenic role of other amino acids, they suggest that plasma arginine is independently associated with incident diabetes. Both arginine and asparagine were identified in an optimal model for predicting new-onset type 2 diabetes.

Identifying early metabolic alterations remains paramount in efforts to understand better the pathophysiology of type 2 diabetes and to develop preventive strategies (1). Type 2 diabetes is characterized by impaired insulin-mediated glucose homeostasis and compromised pancreatic insulin secretion capacity (2). Research investigating the

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etiology of type 2 diabetes has increasingly focused on the interaction between impaired glucose homeostasis, insulin resistance, lipid metabolism, and obesity (2–4). These factors are thought to induce the metabolic dysfunction reflected by abnormal circulating levels of glucose, lipids, proteins, and other classes of metabolites, including amino acids (5).

Metabolomics-based studies have proliferated in the past decade in an attempt to gain insight into the underlying pathophysiology of type 2 diabetes (6). Numerous studies using both targeted and untargeted approaches have reported alterations in circulating amino acid levels in patients with prevalent and incident diabetes. Studies suggest that elevated levels of branched-chain amino acids (BCAAs) (isoleucine, leucine, and valine), and to a lesser extent aromatic amino acids (AAAs) (phenylalanine and tyrosine), are associated with obesity and insulin resistance, as well as established and incident diabetes (6–11). Alanine (Ala), proline (Pro), glutamate (Glu), and aspartate (Asp) have also been positively associated with type 2 diabetes, whereas glycine (Gly), glutamine (Gln), and asparagine (Asn) seem to be inversely related to the disease (8,12).

Despite abundant research linking alterations in circulating amino acid levels to various obesity-related mechanisms and diabetes (10,11,13–18), their potential as independent biomarkers reflecting the etiology and pathogenesis of type 2 diabetes is yet to be fully realized. The inherent challenge of evaluating amino acids as independent risk markers can be explained in part by the interrelatedness of plasma amino acids and their association with several well-established risk factors, including elevated plasma glucose, dyslipidemia, and obesity. Such associations suggest that the relation between plasma amino acids (independent variable) and incident diabetes (outcome) is likely to be affected by confounders, defined simply as variables associated with both the independent variable and the outcome (19). Failure to adjust appropriately for confounders means the crude independent variable–outcome association will be a biased estimate of the true association (20).

Throughout the literature on amino acids and incident type 2 diabetes, however, lipid parameters are not consistently included as covariates (17,21). Studies also tend to focus on fasting cohorts only (17,21,22), despite limited evidence to do so. Several established diabetes risk markers, including lipids, BMI, and insulin sensitivity, have been shown to be correlated with plasma amino acid levels (23), fulfilling the definition of a potential confounder. Yet, their role as actual confounders is rarely evaluated. Finally, as several risk factors for mortality are also risk factors for type 2 diabetes, bias may occur from the competing risk of death in longitudinal studies.

We aimed to investigate amino acids as independent risk factors with various adjustments for potential confounders, assess whether the competing risk of death is a source of bias, and use a model-selection approach to identify the best-fitting model.

RESEARCH DESIGN AND METHODS

Study Population

As described in detail elsewhere (24), the source population for this study included 4,164 adults who underwent elective coronary angiography at two Norwegian university hospitals between 2000 and 2004 (clinical trial reg. no. NCT00354081, clinicaltrials.gov/). Collection of demographic, clinical, and biochemical characteristics at baseline has been described previously (24). Participants were diagnosed with coronary artery disease (CAD) if coronary angiography revealed at least one significant stenosis (defined as ≥50% luminal narrowing in the main coronary arteries or major side branches). Venous blood samples were obtained during a clinical examination before or immediately after coronary angiography. The study fulfilled the principles of the Declaration of Helsinki and was approved by the regional Committee for Medical and Health Research Ethics (approval no. 2010/1880) and the Norwegian Data Protection Authority. All participants provided written informed consent.

From the source cohort of 4,164 adults, 496 individuals with medication-confirmed or a self-reported diagnosis of diabetes at baseline were excluded from these prospective analyses. In addition, 42 individuals with missing HbA_{1c} records and 1,107 individuals with HbA_{1c} ≥6.5% (≥48 mmol/mol), fasting plasma glucose (FPG) ≥7.0 mmol/L, or non-FPG ≥11.1 mmol/L were also excluded because of the possible presence of prediabetes or undiagnosed type 2 diabetes. Thus, 2,519 individuals were deemed eligible for the prospective follow-up analyses.

Identification of Subjects With Incident Type 2 Diabetes

Information on incident diabetes was collected until 31 December 2014. The majority of new cases of type 2 diabetes were identified through linkage to the Norwegian Prescription Database (www.norpd.no), a national registry containing data on all drugs dispensed at outpatient pharmacies in Norway. Participants were classified as having incident type 2 diabetes upon receiving their first prescription for an oral glucose-lowering drug or insulin (Anatomical Therapeutic Chemical Classification System code A10). Incident diabetes was also identified according to ICD-10 codes (specifically codes E11–E14; www.who.int/classifications/icd/en/) on participants' discharge summaries after admission to a Norwegian hospital. Hospital data were obtained from the Cardiovascular Disease in Norway (CVDNOR) project (https://cvdnor.w.uib.no) (25). We also obtained from self-reports additional information identifying cases of new-onset type 2 diabetes, which we verified using plasma glucose measurements during in-trial follow-up of the original source cohort (2000–2005) (24). The median (interquartile range [IQR]) follow-up time from blood sampling to incident diabetes diagnosis was 10.3 years (9.1–11.6).

Biochemical Analyses

Participants who reported no intake of food or beverages during the 6 h before sampling were considered to be fasting. All plasma and serum samples were stored at −80°C until analyses were performed at Bevital Laboratory (www.bevital.no). Plasma concentrations of all amino acids were measured by using gas chromatography–tandem mass spectrometry (26), with the exception of arginine (Arg), which was analyzed by using liquid chromatography–tandem mass spectrometry (27). The lower limits of detection and coefficients of variability have been reported elsewhere (26,27). Estimated glomerular filtration rate (eGFR), HbA_{1c}, serum lipoproteins, and C-reactive protein (CRP) were calculated or measured as previously described (28). We used the updated HOMA-2 to estimate both insulin resistance and β-cell function based on

Table 1—Baseline characteristics of the study population according to diabetes status at baseline and follow-up

Variable	Type 2 diabetes or prediabetes at baseline (confirmed or suspected)			Type 2 diabetes at follow-up*		
	No (*n* = 2,519)	Yes (*n* = 1,645)	*P*	No (*n* = 2,252)	Yes (*n* = 267)	*P*
Age (years)	61.3 (10.4)	62.4 (10.3)	0.001	61.4 (10.5)	60.3 (9.9)	0.08
Male sex, *n* (%)	1,841 (73.1)	1,147 (70.1)	0.04	1,630 (72.5)	210 (78.7)	0.03
Fasting, *n* (%)	642 (25.5)	493 (30.1)	0.001	582 (25.9)	59 (22.1)	0.22
Calorie intake/day (kcal)	2,024 (1,631–2,482)	2,059 (1,637–2,481)	0.92	2,023 (1,632–2,496)	2,062 (1,614–2,458)	0.74
Protein intake (%)	16.6 (15.1–18.3)	16.6 (15.1–18.4)	0.78	16.6 (15.1–18.3)	16.6 (15.1–18.4)	0.55
BMI (kg/m^2)	26.3 (3.6)	27.4 (4.4)	<0.001	26.1 (3.5)	28.7 (3.8)	<0.001
Current smoker, *n* (%)	581 (23.1)	390 (23.9)	0.54	522 (23.2)	58 (21.7)	0.58
Hypertension (mmHg), *n* (%)	1,115 (44.3)	827 (50.6)	<0.001	965 (42.9)	148 (55.4)	<0.001
Systolic BP	139 (125–152)	140 (129–157)	<0.001	139 (125–152)	140 (128–154)	0.11
Diastolic BP	80 (75–88)	80 (75–89)	0.53	80 (75–88)	83 (76–90)	0.001
Significant CAD†, *n* (%)	1,890 (75.0)	1,227 (75.0)	0.98	1,669 (74.2)	219 (82.0)	0.005
Renal function and inflammation						
Serum creatinine (μmol/L)	89 (81–98)	89 (80–99)	0.08	89 (81–98)	89 (82–99)	0.95
GFR (mL · min^{-1} · 1.73 m^2)	91 (79–99)	90 (76–99)	0.004	91 (79–99)	91 (80–100)	0.17
Serum CRP (mg/L)	1.7 (0.8–3.4)	1.9 (0.9–4.0)	0.04	1.7 (0.8–3.3)	2.2 (1.1–4.2)	0.41
Serum lipids						
ApoA1 (g/L)	1.31 (1.14–1.48)	1.28 (1.11–1.47)	0.02	1.31 (1.15–1.49)	1.24 (1.11–1.40)	<0.001
ApoB (g/L)	0.87 (0.73–1.05)	0.85 (0.73–1.03)	0.56	0.87 (0.73–1.04)	0.90 (0.76–1.08)	0.03
HDL (mmol/L)	1.26 (1.02–1.50)	1.20 (1.00–1.50)	<0.001	1.30 (1.10–1.50)	1.10 (1.00–1.30)	<0.001
LDL (mmol/L)	3.0 (1.6–4.4)	3.0 (1.7–4.3)	0.60	3.0 (2.4–3.8)	3.0 (2.4–3.6)	0.81
TAG (mmol/L)	1.44 (1.06–2.03)	1.59 (1.13–2.34)	<0.001	1.40 (1.03–1.95)	1.80 (1.30–2.65)	<0.001
Glucose homeostasis						
Plasma glucose (mmol/L)	5.4 (5.0–6.1)	6.1 (5.3–8.4)	<0.001	5.4 (4.9–5.9)	6.2 (5.6–7.4)	<0.001
HbA_{1c} (% [mmol/mol])	5.6 (5.0–6.0) [38 (31–42)]	7.1 (6.7–7.8) [54 (50–62)]	<0.001	5.6 (5.0–6.0) [38 (31–42)]	5.7 (5.0–6.1) [39 (31–43)]	0.22
Serum insulin (pmol/L)	21.8 (19.7–55.0)	36.3 (19.7–88.2)	<0.001	21.8 (19.7–54.0)	39.4 (19.7–105.0)	0.01
Serum C-peptide (pmol/L)	0.71 (0.53–0.98)	0.82 (0.60–1.16)	<0.001	0.70 (0.51–0.96)	0.90 (0.66–1.17)	0.001
HOMA-2 C-peptide‡						
β-Cell activity	112 (93–138)	104 (70–135)	0.45	112 (92–138)	118 (97–142)	0.77
Insulin resistance	1.6 (1.2–2.2)	1.9 (1.4–2.8)	<0.001	1.6 (1.1–2.2)	2.0 (1.5–2.7)	<0.001
Medication, *n* (%)						
Aspirin	2,084 (82.7)	1,309 (80.0)	0.03	1,846 (82.1)	236 (88.4)	0.01
Statins	2,020 (80.2)	1,311 (80.1)	0.94	1,791 (79.6)	227 (85.0)	0.04
β-Blockers	1,830 (72.6)	1,182 (72.2)	0.78	1,618 (71.9)	210 (78.7)	0.02
Loop diuretics	231 (9.2)	220 (13.4)	<0.001	195 (8.7)	36 (13.5)	0.01
ACE inhibitors	475 (18.9)	383 (23.4)	<0.001	403 (17.9)	71 (26.6)	0.001
Plasma amino acids (μmol/L)						
Glycine	214 (186–251)	202 (174–241)	<0.001	216 (188–254)	199 (176–226)	<0.001
Serine	104 (91–118)	102 (89–117)	0.013	104 (91–119)	102 (89–115)	0.005

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Table 1—Continued

Variable	Type 2 diabetes or prediabetes at baseline (confirmed or suspected)			Type 2 diabetes at follow-up*		
	No (n = 2,519)	Yes (n = 1,645)	P	No (n = 2,252)	Yes (n = 267)	P
Glutamine	510 (462–558)	492 (440–544)	<0.001	512 (465–560)	489 (437–542)	<0.001
Asparagine	50 (44–58)	49 (43–56)	<0.001	51 (45–58)	48 (43–55)	<0.001
Threonine	120 (104–138)	116 (100–134)	<0.001	120 (104–139)	120 (102–134)	0.29
Total cysteine	295 (272–320)	297 (274–325)	0.11	295 (271–319)	301 (278–326)	0.006
Methionine	27 (22–32)	26 (22–31)	0.14	26 (22–32)	28 (23–32)	0.05
Proline	244 (199–298)	241 (192–301)	0.59	243 (198–297)	251 (204–305)	0.63
Histidine	73 (67–79)	71 (65–78)	<0.001	73 (67–79)	73 (66–79)	0.65
Ornithine	72 (61–84)	71 (60–84)	0.66	72 (61–84)	71 (61–84)	0.84
Lysine	176 (154–202)	176 (154–201)	0.63	175 (154–201)	182 (157–205)	0.01
Arginine	79 (64–93)	77 (63–92)	0.009	78 (64–93)	83 (67–100)	0.005
Tryptophan	69 (61–79)	69 (59–79)	0.11	69 (61–78)	73 (65–84)	<0.001
Glutamic acid	71 (47–102)	77 (53–106)	0.001	68 (46–100)	89 (67–118)	<0.001
Aspartic acid	5.8 (4.6–7.5)	5.9 (4.7–7.4)	0.56	5.7 (4.5–7.4)	6.5 (5.1–8.5)	<0.001
Tyrosine	68 (58–81)	68 (58–82)	0.16	67 (57–80)	72 (60–87)	<0.001
Alanine	369 (310–431)	371 (312–436)	0.11	368 (306–430)	387 (339–438)	0.001
Phenylalanine	58 (52–66)	59 (52–67)	0.08	58 (52–66)	61 (55–69)	<0.001
Leucine	126 (108–151)	132 (112–160)	<0.001	125 (107–148)	141 (117–167)	<0.001
Valine	254 (226–290)	265 (233–306)	<0.001	252 (224–287)	280 (245–317)	<0.001
Isoleucine	68 (57–83)	72 (60–89)	<0.001	68 (57–82)	76 (63–92)	<0.001

Data are presented as mean (SD) or median (IQR) unless otherwise indicated. ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; BP, blood pressure. *Duration of follow-up: median 10.3 years (IQR 9.1–11.6). †At least one stenosis with ≥50% luminal narrowing in a main coronary artery or its major side branches, as identified on coronary angiography. ‡Sample size, n = 607.

serum C-peptide in a subgroup of fasting participants (n = 607) (29).

A Priori Identification of Potential Confounders

We identified potential confounders linked to plasma amino acid levels in the literature (7–9,11,17,21). The identified confounders were largely established, clinically relevant risk factors for type 2 diabetes (BMI, eGFR, HDL cholesterol, triacylglycerol [TAG], CRP) or surrogate measures of type 2 diabetes (e.g., hyperglycemia, which indicates cellular insulin resistance).

Statistical Definition of Confounding

For this study, we empirically confirmed potential confounders using the change-in-estimate (CIE) criterion (30). The CIE criterion defines confounders as variables for which the percentage difference between the values of the regression estimate before and after adjustment is equal to or larger than a prespecified value.

Statistical Analyses

Variables were reported as count (percentage), mean (SD), or median (IQR), as appropriate. Skewed variables were transformed according to the Tukey ladder of powers (square root, ln, 1/square root, and inverse transformations). The aim was to linearize relationships, reduce heteroscedasticity, maintain power, and reduce the type I error rate. Missing amino acid data (all <5%) were determined through mean imputation. We used the R packages mixed Gaussian model and qgraph to visualize the relations between amino acids and potential confounders as weighted adjacencies. The amino acid–associated hazard for incident diabetes was assessed in a crude Cox model (with age and sex), to which potential confounders and then plasma glucose were added. All models included an indicator variable for fasting. Confounding was defined as a 10% CIE. Estimates from Cox regression were compared with estimates from the method described by Fine and Gray (31) in order to assess whether the competing risk of death affected the estimates (32). The P values were adjusted for multiple comparisons at a false discovery rate (FDR) of 0.05 (Benjamini-Hochberg method). Finally, we applied a variable selection model (CoxBoost package in R) with all amino acids and potential confounders using component-wise likelihood-based

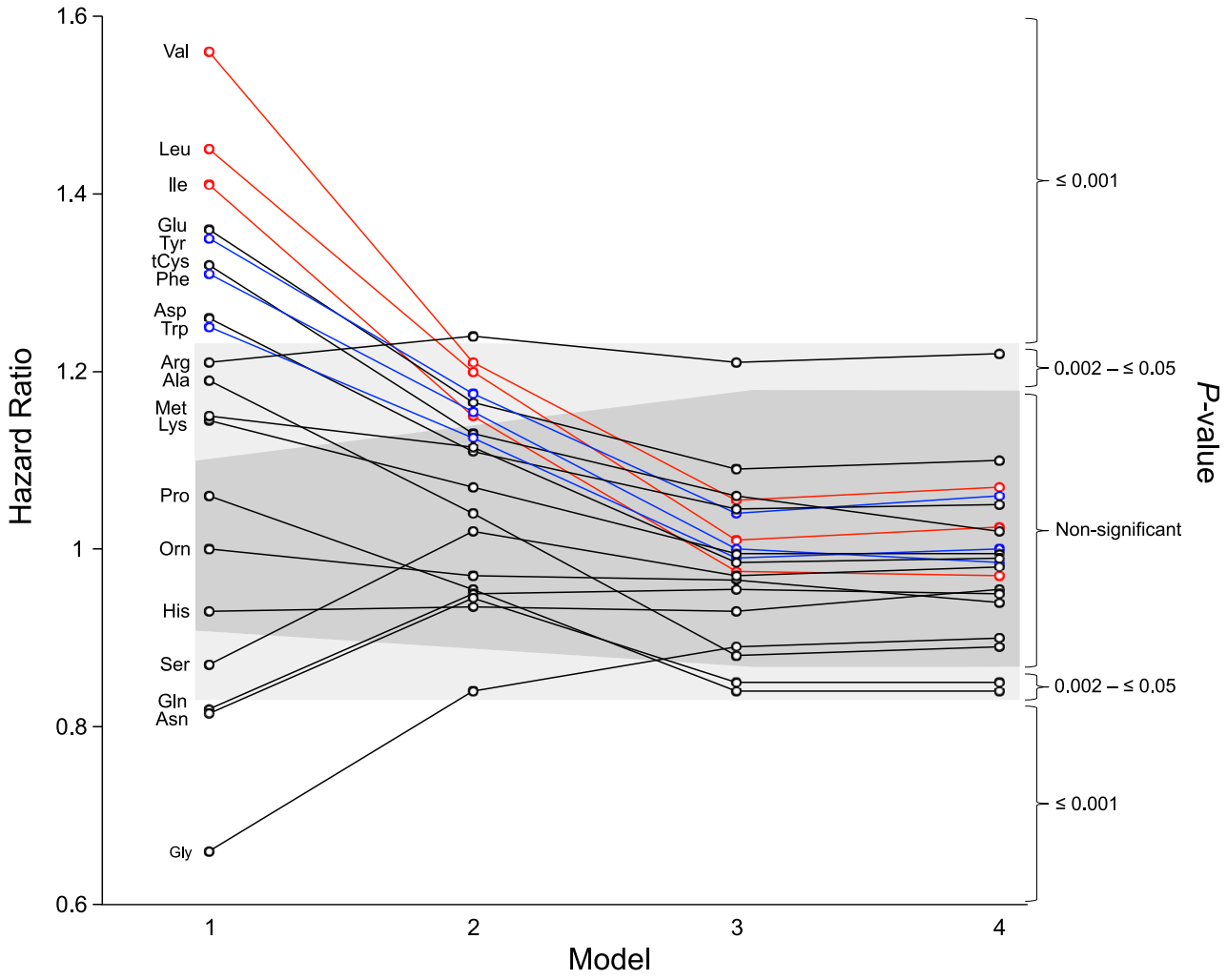

Figure 1—Association of baseline plasma amino acid concentrations with incident type 2 diabetes. Observations for 2,519 individuals, 267 of whom had incident diabetes; 464 mortality events occurred. Hazard ratios were obtained by using Cox regression and adjusting for age and sex (model 1); age, sex, eGFR, BMI, HDL cholesterol, TAG, and CRP (model 2); model 2 factors plus plasma glucose (model 3); and model 3 factors plus mortality (model 4). Red circles and lines indicate BCAAs; blue circles and lines indicate AAAs; black circles and lines indicate other amino acids. Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; tCys, total cysteine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

boosting (33), with death as a competing risk. We did not penalize confounders and reestimated the model to check for adjustment from unselected key confounders; we estimated amino acids with penalized partial likelihood. We first identified the optimal penalty, then we identified the optimal number of steps through k-fold cross-validation (k = 10). All tests were two-tailed, and the significance level was set to 0.05. Statistical analyses were performed by using STATA version 15 (StataCorp LLC; www.stata.com) and R version 3.3.0 for Mac (www.R-project.org).

RESULTS

Participants With and Without Type 2 Diabetes at Baseline

Among the source population (n = 4,164), 1,645 participants had confirmed or suspected prediabetes or undiagnosed type 2 diabetes. We refer to them here as the prevalent diabetes group. Compared to participants identified as diabetes-free at baseline, participants in the prevalent diabetes group had higher BMI, lower HDL cholesterol levels, and elevated TAG and plasma glucose levels (Table 1). Participants in the prevalent diabetes group also had a higher prevalence of diagnosed hypertension and used more loop diuretics and ACE inhibitors. The baseline amino acid profile of those in the prevalent diabetes group also differed from the profile of those in the diabetes-free group, including lower plasma concentrations of Gly and Gln and higher concentrations of Glu and the BCAAs (Table 1).

Diabetes-Free Participants and Incident Diabetes

Of the 2,519 diabetes-free participants at baseline, 1,841 (73.1%) were men; their mean age was 61.3 years (SD 10.4) and mean BMI was 26.3 kg/m^2 (SD 3.6). During a median of 10.3 years of follow-up (IQR 9.1–11.6), a total of 267 cases of incident diabetes (10.6%) were identified. The group with incident diabetes had higher BMI, lower HDL cholesterol levels, and higher TAG and plasma glucose levels at baseline than the group who remained diabetes-free during follow-up (Table 1). They also had a higher prevalence of CAD and hypertension and a higher rate of medication use, including aspirin, statins, β-blockers, loop diuretics, and ACE inhibitors. The amino acid profile of those with incident

Table 2—Optimal model for risk of incident type 2 diabetes obtained by CoxBoost with competing risk*

Variable	SHR	95% CI Lower	95% CI Upper	*z*	*P* > *z*
Age	1.00	0.84	1.21	0.04	0.97
Sex	0.93	0.67	1.29	−0.43	0.67
eGFR	1.16	0.98	1.38	1.71	0.09
Plasma glucose	1.96	1.75	2.19	11.90	≥0.001
Age∗plasma glucose	1.20	1.08	1.33	3.39	0.001
BMI	1.64	1.45	1.85	7.99	≥0.001
HDL	0.84	0.72	0.98	−2.25	0.02
TAG	1.27	1.10	1.47	3.19	0.001
Arg	1.30	1.14	1.49	3.99	≥0.001
Asn	0.80	0.70	0.91	−3.42	0.001

Competing-risk regression was based on 2,519 observations, with incident diabetes as a failure to event (PE = 1; *n* = 267) and mortality incidence as a competing event (CR = 2; *n* = 464) (Wald χ^2[10] = 311.42; log pseudolikelihood = −1,842.1068; probability > χ^2 = 0.0000). CR, competing risk (mortality incidence); PE, primary end point (incident diabetes); SHR, standardized subdistribution hazard (risk of incident diabetes related to selected variable, given that participant has not died). *Optimal steps (*n* = 7) with optimal penalty of 86 and the unpenalized obligatory predictors of age and sex.

diabetes also differed significantly from that of those who remained diabetes-free at follow-up and included lower concentrations of Gly, Gln and Asn and higher concentrations of Glu, Asp, BCAAs, and AAAs (Table 1).

Potential Confounders and Amino Acids

The widespread associations between the amino acids and factors related to diabetes risk demonstrated the potential for confounding, which we visualized through the use of a network plot (Supplementary Fig. 1). Other potential confounders, such as prevalence of CAD, hypertension, and prescribed medications, displayed no association with baseline plasma amino acid concentrations (data not shown) and were excluded from the network plot. Associations between amino acids and glucose homeostasis parameters (FPG, HbA_{1c}, serum insulin and C-peptide, β-cell function, and insulin resistance) for a subset of participants are presented in Supplementary Table 1. The strongest overall associations with glucose homeostasis parameters were observed for the BCAAs and AAAs.

Amino Acids, Incident Type 2 Diabetes, and Confounding

We identified numerous amino acids as significant predictors of type 2 diabetes in age- and sex-adjusted Cox analyses (model 1) (Fig. 1). Additional adjustment for eGFR, BMI, HDL cholesterol, TAG, and CRP (model 2) attenuated most associations. Further adjustment for plasma glucose (model 3) left only Arg, Asn, and Pro as significant predictors of incident diabetes. The competing risk of death (model 4) did not affect the estimates. Adjustment for multiple tests with the FDR at 0.05 left only Arg as a significant independent predictor of type 2 diabetes risk. Effect size estimates for each amino acid in relation to incident diabetes across the four models are provided in Supplementary Table 2.

Identifying an Optimal Multivariable Model

We identified the optimal submodel as a competing risk model with plasma glucose, glucose∗age interaction, BMI, HDL cholesterol, TAG, Arg, and Asn (Table 2). Sex and eGFR were reintroduced because they were considered obligate, but they did not alter the estimates. Plasma Arg showed a positive but plasma Asn a negative association with the cumulative incidence of type 2 diabetes, as shown in Fig. 2.

CONCLUSIONS

In this study, interrelations between plasma amino acids and established risk factors for type 2 diabetes seemed to confound the prediction of incident type 2 diabetes developing over a median follow-up of 10.3 years. Our observations underscore the need to include these covariates when investigating amino acids as independent predictors of incident diabetes. Using a multivariable, competing risk–boosting analysis (33), we identified an optimal model for incident type 2 diabetes risk as one that included Arg and Asn along with plasma glucose, glucose∗age interaction, BMI, HDL cholesterol, and TAG.

Cross-sectional evaluation of the source population at baseline demonstrated significant differences in plasma amino acid levels between participants with confirmed or suspected type 2 diabetes or prediabetes and those who do not have diabetes. Our observations are consistent with those of others who demonstrated alterations in amino acid levels among overweight and insulin-resistant subjects, and those with prediabetes and overt diabetes (7,10,11,13). Network analysis demonstrated extensive associations between plasma amino acids and factors related to diabetes risk, indicating that potential for confounding was the rule rather than the exception. Like previous observations (7–9,21), our age- and sex-adjusted Cox regression model demonstrated that numerous amino acids, including BCAAs, AAAs, Arg, Ala, Gln, Asn, Glu, Asp, and Gly, were significant predictors of incident type 2 diabetes. After adjusting for eGFR, BMI, HDL cholesterol, TAG, and CRP, however, most associations were rendered statistically nonsignificant. Further adjustment for plasma glucose identified only Arg as an independent predictor of new-onset diabetes, above the expected FDR.

Our optimal model for predicting incident diabetes retained Arg and Asn along with plasma glucose, glucose∗age interaction, BMI, HDL cholesterol, and TAG. Although Arg is positively related to incident type 2 diabetes, we observed its inverse relation to prevalent diabetes (data not shown). Previous studies demonstrated that oral antidiabetes medications such as metformin are associated with a reduction of urea cycle metabolites, including Arg (34). We observed the same trend among individuals with established type 2 diabetes who were taking metformin (Supplementary Fig. 2). The biologically plausible mechanisms linking Arg and Asn to diabetes risk are less known. Isolated studies have suggested that Asn is inversely associated with future diabetes (35). A meta-analysis by Guasch-Ferré et al. (21) demonstrated that Arg was positively associated with risk for

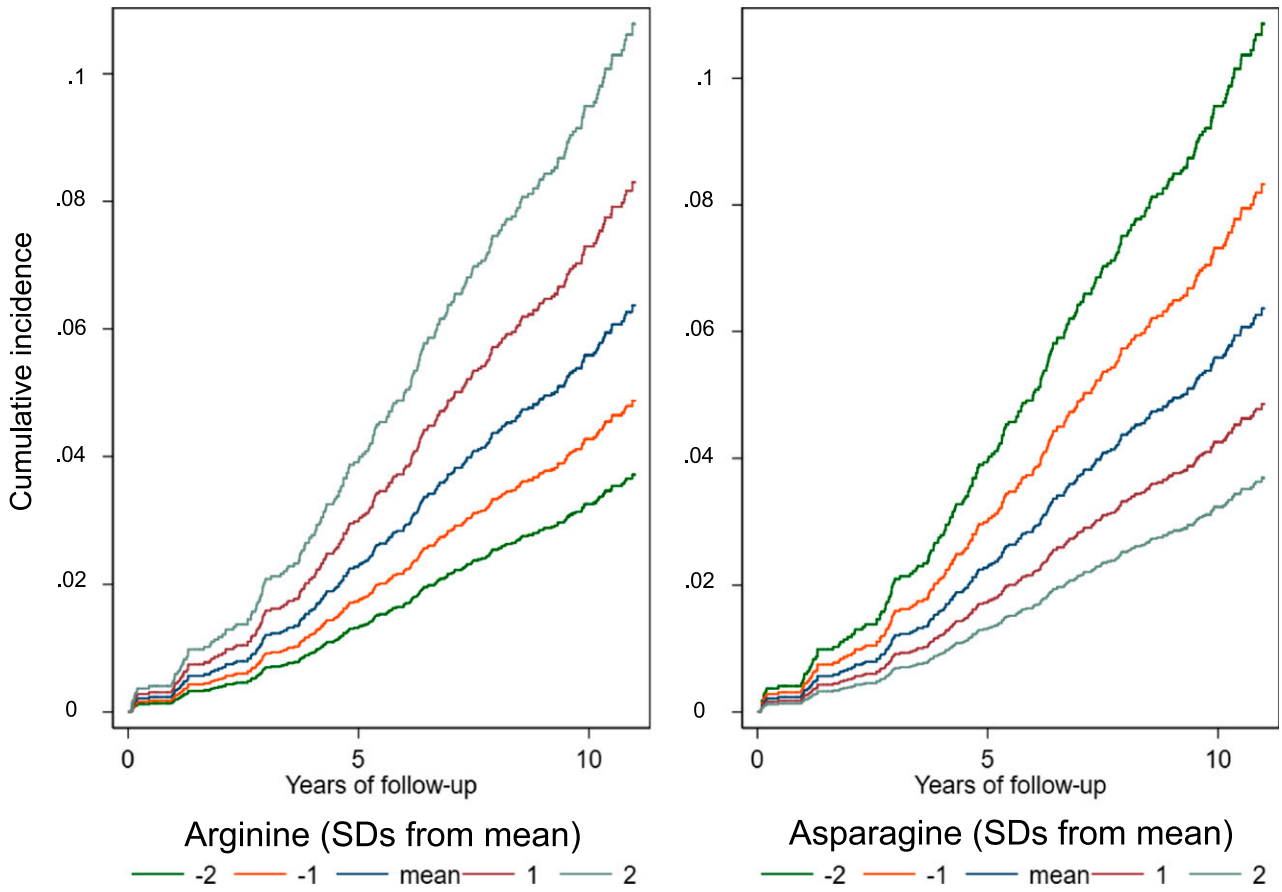

Figure 2—Association of plasma Arg (left) and plasma Asn (right) with cumulative incidence of type 2 diabetes. Data represent observations from 2,519 participants. The model included plasma glucose, competing risks, and fasted status.

type 2 diabetes. In vitro experiments suggest a role for Arg in aggregating insulin. Investigation of heat-induced aggregation of protein solutions showed that Arg may have a potentially promoting effect (36), whereas other investigations of bovine insulin suggest an inhibitory role (37). Our finding that Arg was independently related to risk for type 2 diabetes might reflect an effect promoting insulin aggregation. This may support hypotheses linking diabetes development to peptide aggregation and the formation of toxic amyloid fibrils at pancreatic islet β-cells (2,38). However, we did not observe associations between Arg or Asn and serum insulin, C-peptide, β-cell function, or other clinically relevant parameters such as inflammation (CRP). Although the mechanisms linking Arg and Asn to incident diabetes are not clear, our findings suggest that these amino acids could be involved in the early development of type 2 diabetes, independent of established risk factors.

Multivariable regression analyses confirmed that all amino acids except Arg were subject to confounding by established risk factors. These confounding effects are not surprising, given that risk markers such as BMI and lipids correlate with most plasma amino acids (23). Indeed, it is these associations that probably reduce the potential for amino acids to predict diabetes and may explain why including amino acids has not substantially improved clinical models (8,11,12). The nonstandardized approach to adjusting for potential confounders may undermine the validity and reliability of previous research linking amino acids to diabetes. Although age, sex, and BMI seem to be adjusted for regularly (17,21), adjustment for lipid and glucose parameters is much less consistent (17,21). Acknowledging the extensive interplay between amino acids, established risk factors, and underlying physiological processes, our findings suggest that it is imperative to consistently include confounders when evaluating amino acids as independent predictors of type 2 diabetes. We did not identify an impact of death as a competing risk. In our opinion, however, this cannot be generalized because of differences between cohorts, particularly with regard to duration of follow-up and mortality rates. As with any observational study, it is also important to acknowledge that causality cannot be inferred from statistical methods alone. Nor do our observations allow for conclusions precluding a possible role of other amino acids such as BCAAs in the pathogenesis type 2 diabetes (39).

This work has a number of strengths, including the large sample size, prospective design, and long follow-up. Data allowing incident type 2 diabetes to be confirmed was collected from national health registries to which reporting is mandatory for all drug prescriptions and hospital admissions in Norway. It is possible, however, that some cases of new-onset type 2 diabetes may have been missed during follow-up. We adopted a data-driven approach to confounders. Further, we applied objective criteria to identify the optimal submodel. Taken together, these steps should significantly

reduce bias, although we cannot rule out influence from residual confounding. Unfortunately, our study design did not allow us to identify individuals who had incident type 1 diabetes; the low prevalence of this condition, however, probably minimizes any potential effect on our results (40). The study participants were all referred to a hospital for elective coronary angiography, and the majority had CAD, limiting the generalizability of our findings. Last, the original source study was not designed to investigate incident diabetes, and samples were obtained from the majority of participants when they were in a nonfasting state.

In conclusion, after adjusted analyses, the associated hazard for type 2 diabetes was severely attenuated for most amino acids, including BCAAs. Only Arg was an independent predictor of future diabetes after adjusting for multiple comparisons, but Arg and Asn were selected for inclusion in an optimal predictive model. Adjusting for established metabolic and clinical risk factors was crucial for reaching a conclusion about the independence of the associations.

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References

1. Cho NH, Shaw JE, Karuranga S, et al. IDF Diabetes Atlas: global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res Clin Pract 2018;138:271–281
2. Kahn SE, Cooper ME, Del Prato S. Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. Lancet 2014;383:1068–1083
3. Savage DB, Petersen KF, Shulman GI. Disordered lipid metabolism and the pathogenesis of insulin resistance. Physiol Rev 2007;87:507–520
4. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444:840–846
5. Muoio DM, Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat Rev Mol Cell Biol 2008;9:193–205
6. Roberts LD, Koulman A, Griffin JL. Towards metabolic biomarkers of insulin resistance and type 2 diabetes: progress from the metabolome. Lancet Diabetes Endocrinol 2014;2:65–75
7. Newgard CB, An J, Bain JR, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 2009;9:311–326
8. Wang TJ, Larson MG, Vasan RS, et al. Metabolite profiles and the risk of developing diabetes. Nat Med 2011;17:448–453
9. Magnusson M, Lewis GD, Ericson U, et al. A diabetes-predictive amino acid score and future cardiovascular disease. Eur Heart J 2013;34:1982–1989
10. Wang-Sattler R, Yu Z, Herder C, et al. Novel biomarkers for pre-diabetes identified by metabolomics. Mol Syst Biol 2012;8:615
11. Yamakado M, Nagao K, Imaizumi A, et al. Plasma free amino acid profiles predict four-year risk of developing diabetes, metabolic syndrome, dyslipidemia, and hypertension in Japanese population. Sci Rep 2015;5:11918
12. Floegel A, Stefan N, Yu Z, et al. Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. Diabetes 2013;62:639–648
13. Martin FP, Montoliu I, Collino S, et al. Topographical body fat distribution links to amino acid and lipid metabolism in healthy obese women [corrected] [published correction appears in PLoS One 2013;8]. PLoS One 2013;8:e73445
14. Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. J Biol Chem 2010;285:11348–11356
15. Lackey DE, Lynch CJ, Olson KC, et al. Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity. Am J Physiol Endocrinol Metab 2013;304:E1175–E1187
16. Lerin C, Goldfine AB, Boes T, et al. Defects in muscle branched-chain amino acid oxidation contribute to impaired lipid metabolism. Mol Metab 2016;5:926–936
17. Bi X, Henry CJ. Plasma-free amino acid profiles are predictors of cancer and diabetes development. Nutr Diabetes 2017;7:e249
18. Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. Cell Metab 2012;15:606–614
19. Greenland S, Morgenstern H. Confounding in health research. Annu Rev Public Health 2001;22:189–212
20. Skelly AC, Dettori JR, Brodt ED. Assessing bias: the importance of considering confounding. Evid Based Spine Care J 2012;3:9–12
21. Guasch-Ferré M, Hruby A, Toledo E, et al. Metabolomics in prediabetes and diabetes: a systematic review and meta-analysis. Diabetes Care 2016;39:833–846
22. Tzoulaki I, Ebbels TM, Valdes A, Elliott P, Ioannidis JP. Design and analysis of metabolomics studies in epidemiologic research: a primer on -omic technologies. Am J Epidemiol 2014;180:129–139
23. Gar C, Rottenkolber M, Prehn C, Adamski J, Seissler J, Lechner A. Serum and plasma amino acids as markers of prediabetes, insulin resistance, and incident diabetes. Crit Rev Clin Lab Sci 2018;55:21–32
24. Ebbing M, Bleie Ø, Ueland PM, et al. Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. JAMA 2008;300:795–804
25. Sulo GIJ, Vollset SE, Nygård O, Øyen N, Tell GS. Cardiovascular disease and diabetes mellitus in Norway during 1994-2009 CVDNOR—a nationwide research project. Nor Epidemiol 2013;21:101–107
26. Midttun Ø, McCann A, Aarseth O, et al. Combined measurement of 6 fat-soluble vitamins and 26 water-soluble functional vitamin markers and amino acids in 50 μl of serum or plasma by high-throughput mass spectrometry. Anal Chem 2016;88:10427–10436
27. Midttun Ø, Kvalheim G, Ueland PM. High-throughput, low-volume, multianalyte quantification of plasma metabolites related to one-carbon metabolism using HPLC-MS/MS. Anal Bioanal Chem 2013;405:2009–2017
28. Rebnord EW, Pedersen ER, Strand E, et al. Glycated hemoglobin and long-term prognosis in patients with suspected stable angina pectoris without diabetes mellitus: a prospective cohort study. Atherosclerosis 2015;240:115–120
29. Levy JC, Matthews DR, Hermans MP. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. Diabetes Care 1998;21:2191–2192
30. McNamee R. Confounding and confounders. Occup Environ Med 2003;60:227–234; quiz 164, 234
31. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc 1999;94:496–509
32. Haneuse S, Lee KH. Semi-competing risks data analysis: accounting for death as a competing risk when the outcome of interest is nonterminal. Circ Cardiovasc Qual Outcomes 2016;9:322–331
33. Binder H, Allignol A, Schumacher M, Beyersmann J. Boosting for high-dimensional time-to-event data with competing risks. Bioinformatics 2009;25:890–896
34. Irving BA, Spielmann G. Does citrulline sit at the nexus of metformin's pleotropic effects on metabolism and mediate its salutatory effects in individuals with type 2 diabetes? Diabetes 2016;65:3537–3540
35. Palmer ND, Stevens RD, Antinozzi PA, et al. Metabolomic profile associated with insulin resistance and conversion to diabetes in the Insulin Resistance Atherosclerosis Study. J Clin Endocrinol Metab 2015;100:E463–E468
36. Shah D, Shaikh AR, Peng X, Rajagopalan R. Effects of arginine on heat-induced aggregation of concentrated protein solutions. Biotechnol Prog 2011;27:513–520

37. Varughese MM, Newman J. Inhibitory effects of arginine on the aggregation of bovine insulin. J Biophys 2012;2012:434289
38. Jurgens CA, Toukatly MN, Fligner CL, et al. β-Cell loss and β-cell apoptosis in human type 2 diabetes are related to islet amyloid deposition. Am J Pathol 2011;178:2632–2640
39. Arany Z, Neinast M. Branched chain amino acids in metabolic disease. Curr Diab Rep 2018; 18:76
40. Zinman B, Kahn SE, Haffner SM, O'Neill MC, Heise MA, Freed MI; ADOPT Study Group. Phenotypic characteristics of GAD antibody-positive recently diagnosed patients with type 2 diabetes in North America and Europe. Diabetes 2004;53:3193–3200