

ORIGINAL ARTICLE

Circulating Branched-Chain Amino Acids and Incident Cardiovascular Disease in a Prospective Cohort of US Women

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BACKGROUND: Circulating branched-chain amino acids (BCAAs; isoleucine, leucine, and valine) are strong predictors of type 2 diabetes mellitus (T2D), but their association with cardiovascular disease (CVD) is uncertain. We hypothesized that plasma BCAAs are positively associated with CVD risk and evaluated whether this was dependent on an intermediate diagnosis of T2D.

METHODS: Participants in the Women's Health Study prospective cohort were eligible if free of CVD at baseline blood collection (n=27 041). Plasma metabolites were measured via nuclear magnetic resonance spectroscopy. Multivariable Cox regression models estimated hazard ratios (HRs) and 95% confidence intervals (CIs) for BCAAs with incident CVD (myocardial infarction, stroke, and coronary revascularization).

RESULTS: We confirmed 2207 CVD events over a mean 18.6 years of follow-up. Adjusting for age, body mass index, and other established CVD risk factors, total BCAAs were positively associated with CVD (per SD: HR, 1.13; 95% CI, 1.08–1.18), comparable to LDL-C (low-density lipoprotein cholesterol) with CVD (per SD: HR, 1.12; 95% CI, 1.07–1.17). BCAAs were associated with coronary events (myocardial infarction: HR, 1.16; 95% CI, 1.06–1.26; revascularization: HR, 1.17; 95% CI, 1.11–1.25), and borderline significant association with stroke (HR, 1.07; 95% CI, 0.99–1.15). The BCAA–CVD association was greater (*P* interaction=0.036) among women who developed T2D before CVD (HR, 1.20; 95% CI, 1.08–1.32) versus women without T2D (HR, 1.08; 95% CI, 1.03–1.14). Adjusting for LDL-C, an established CVD risk factor, did not attenuate these findings; however, adjusting for HbA1c and insulin resistance eliminated the associations of BCAAs with CVD.

CONCLUSIONS: Circulating plasma BCAAs were positively associated with incident CVD in women. Impaired BCAA metabolism may capture the long-term risk of the common cause underlying T2D and CVD.

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CLINICAL PERSPECTIVE

Rapidly evolving metabolomics technologies have identified several novel metabolites and metabolic pathways associated with type 2 diabetes mellitus (T2D). Circulating branched-chain amino acids (BCAAs; isoleucine, leucine, and valine) in particular have been consistently observed as strongly associated with T2D, and compelling evidence supports a causal role of dysfunctional BCAA metabolism in the development of insulin resistance and T2D. But few studies have evaluated the association of BCAAs with cardiovascular disease (CVD). We hypothesized that higher baseline levels of plasma BCAAs would be associated with an elevated risk of CVD events, and postulated that the BCAA–CVD relationship would be related to cardiometabolic risk pathways. Plasma BCAA metabolites were measured via nuclear magnetic resonance spectroscopy in 27 041 women and examined in relation to 2207 incident CVD events over a mean 18.6 years of follow-up. We found that a single random plasma measurement of baseline circulating plasma BCAAs was positively associated with incident CVD to a comparable degree as the association of LDL-C (low-density lipoprotein cholesterol) with CVD and was independent of multiple established risk factors. The association was stronger among women who developed T2D before CVD. Further adjustment for biomarkers of cardiometabolic risk suggested that the relationship between BCAAs and CVD could be mostly explained by more downstream biomarkers of T2D metabolism. Hence, impaired BCAA metabolism may represent a shared pathway of metabolic dysfunction that links T2D and CVD, and a one-time level of plasma BCAAs may capture the long-term cardiometabolic risks of this common cause.

Accumulating evidence implicates alterations in branched-chain amino acid (BCAA) metabolism as a feature of poor cardiometabolic health. BCAAs (isoleucine, leucine, and valine) have been associated with several cardiometabolic risk factors in cross-sectional studies, including anthropometric measures of excess body weight and adiposity, impaired fasting glucose, insulin resistance, elevated blood pressure, dyslipidemia, and indicators of coronary artery disease.^{1–5} Mechanisms underlying the role of BCAAs and disease risk have long been appreciated and may include their interruption in insulin signaling and chronic mTOR (mammalian target of rapamycin) activation.^{6–8} Alternatively, elevated circulating BCAA levels may be a result of

impaired catabolism or clearance, that is, a consequence of a disease process rather than an upstream cause.^{9–11} Only a prospective study design can address the limitations of previous cross-sectional evidence.

Although BCAAs have been consistently associated with an elevated risk of type 2 diabetes mellitus (T2D) in epidemiological studies,^{12–14} few prior studies have conducted targeted prospective analyses of BCAAs in relation to incident cardiovascular disease (CVD) events. Because elevations in BCAAs may represent a biochemical phenotype of cardiometabolic risk common to T2D and CVD (the common soil hypothesis),^{15–17} and given the compelling evidence for BCAAs as predictors of T2D and intermediate CVD phenotypes and risk factors, we hypothesized that higher baseline levels of plasma BCAAs would be associated with an elevated risk of incident CVD events. We postulated that the BCAA–CVD relationship would be related to shared cardiometabolic risk pathways and evaluated whether this relationship was dependent on an intermediate diagnosis of T2D. Therefore, we conducted a prospective targeted analysis of BCAAs in relation to incident CVD in 27 041 apparently healthy US women with >18 years of follow-up for incident CVD and T2D and examined whether BCAAs were associated with CVD independent of their association with incident T2D.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request. Use of the data via this mechanism is overseen by standard Women's Health Study (WHS) policies and procedures, which assure that participant consents are honored.

Study Population

The WHS is an on-going long-term prospective observational cohort of US women that started as a randomized placebo-controlled factorial trial of low-dose aspirin, β -carotene, and vitamin E for the primary prevention of CVD and cancer (URL: <https://www.clinicaltrials.gov>. Unique identifier: NCT00000479). The trial randomized 39 876 female US health professionals aged ≥ 45 years without a history of cancer (except nonmelanoma skin cancer) or CVD and ran from 1993 to 2004.^{18,19} The WHS continues to follow participants annually on an observational basis. At baseline, participants were also asked if they would be willing (but not required) to provide a baseline blood sample; 28 345 women did so, and blood samples were collected and shipped on ice via overnight courier to the central laboratory where they were processed and stored at -170°C in vapor liquid nitrogen until biomarker measurements were performed. Baseline characteristics of those contributing blood samples did not differ from those who did not, with the exception of current smoking status (11.7% versus 16.8%, respectively) and total physical activity energy expenditure (median 599 kcal/wk versus 526 kcal/wk).²⁰ Questionnaires captured information on demographics, health status, reproductive history,

and lifestyle characteristics. Written informed consent was obtained from all participants, and the study protocol was approved by the Institutional Review Board of the Brigham and Women's Hospital (Boston, MA).

BCAA Laboratory Methods

Targeted metabolites were previously quantified in the WHS baseline blood samples. Briefly, aliquots of the baseline blood samples were shipped on dry ice blinded to case status. Isoleucine, leucine, and valine were measured by proton nuclear magnetic resonance (¹H NMR) spectroscopy at LipoScience, Inc, now LabCorp, on EDTA plasma samples using an NMR Profiler, a 400 MHz NMR platform, as described for the NMR LipoProfile test.^{21,22} To quantify BCAAs, the NMR spectra were deconvoluted using proprietary software with models containing reference spectra from serum proteins, BCAAs, and lipoproteins. The derived amino acid signal amplitudes were converted to μmol/L using a factor that was empirically determined using dialyzed serum samples spiked with known amounts of each amino acid. The intra- and interassay coefficients of variation for the amino acid assays are isoleucine 5.9 to 6.1%, leucine 4.5 to 4.9%, and valine 1.5 to 2.1%.

Ascertainment of Incident CVD

We evaluated a composite end point of CVD defined as first confirmed nonfatal or fatal myocardial infarction (MI), stroke, or revascularization (coronary artery bypass surgery or percutaneous coronary intervention), and secondarily investigated these end point categories individually. Follow-up included the ascertainment of health outcomes every 6 months in the first year, and annually thereafter (through the end of 2016). Through written consent, medical records were obtained and reviewed by a committee of physicians blinded to treatment assignment. MI was confirmed if symptoms met the World Health Organization criteria in addition to elevated levels of cardiac enzymes or diagnostic electrocardiograms. The committee confirmed stroke events if there was documentation of a sudden onset of a new neurological deficit persisting for >24 hours or until death <24 hours.²³ Deaths because of cardiovascular causes were confirmed by autopsy reports, death certificates, medical records, and next of kin or family members. As previously described, we confirmed incident T2D cases identified through self-report on annual questionnaires via a physician-administered telephone interviews or supplemental questionnaire,²⁴ using the American Diabetes Association diagnostic criteria.^{25,26}

Assessment of Covariates

Participants self-reported several health-related characteristics, lifestyle, family history of premature (<60 years old) MI in either parent, and other risk factors via questionnaires at baseline. Diet was assessed via a validated 131-item semiquantitative food frequency questionnaire.^{27,28} The baseline validated²⁹ physical activity questionnaire³⁰ asked participants their average time per week spent engaged in recreational activity domains (eg, walking, running, and bicycling), flights of stairs climbed daily, and their usual walking pace. Participants reported whether they had any history of T2D, high cholesterol, or hypertension among the list of physician-diagnosed

illnesses on each questionnaire. Self-reported T2D was previously validated in the WHS with >91% positive predictive value.³¹ The baseline questionnaire also collected self-reported information on use of antihypertensive treatment, cholesterol-lowering medication, systolic and diastolic blood pressure, and cholesterol levels. In this study, hypertension is defined as self-report of a physician diagnosis, past or current antihypertensive treatment, systolic blood pressure ≥140 mmHg, or diastolic blood pressure ≥90 mmHg. Self-reported history of a hypertension diagnosis was previously validated among a similar cohort of US health professionals with excellent confirmation (100%) against medical records using these blood pressure cut points.³² Finally, high cholesterol is defined as self-report of a physician diagnosis or use of cholesterol-lowering medication. In addition, we also had measured baseline levels of standard lipids, inflammatory, and glucose metabolism biomarkers, including hemoglobin A1c (Hba1c),³³ lipoprotein insulin resistance score,^{34,35} GlycA (glycosylated acute phase proteins),³⁶ triglyceride levels, LDL-C (low-density lipoprotein cholesterol) concentration, HDL-C (high-density lipoprotein cholesterol) concentration, and hsCRP (high-sensitivity C-reactive protein).

Statistical Analysis

The metabolite concentrations were natural log (ln) transformed and standardized values to a mean of 0 and SD of 1. Total BCAAs were the sum of isoleucine, leucine, and valine metabolite levels. We compared the characteristics of participants at baseline across quintiles of total BCAA levels calculating tests for trend. The Alternative Health Eating Index 2010 dietary pattern score was calculated from individual food and nutrient components as previously described in detail,³⁷ ranging from 10 to 100 with a higher score indicating adherence to a higher quality diet.

We used Cox proportional hazards regression models to estimate the hazard ratios (HR) and 95% confidence intervals (CI) of baseline BCAA metabolite levels with incident CVD. We analyzed the relationship between total and individual BCAAs with CVD categorically (in quintiles) and continuously per SD of the continuous ln-transformed metabolite. In addition to the age- and randomized treatment group-adjusted model, we adjusted for CVD risk factors at baseline including menopausal status (pre, post, uncertain, and missing), current hormone therapy use, family history of premature MI, White race/ethnicity, smoking status (never, past, current <15 cigarettes/d, current 15+ cigarettes/d), Alternative Health Eating Index diet quality score (quintiles), fasting status, alcohol beverage intake (never/rarely, 1–3/mo, 1–6/wk, ≥1/d), total physical activity MET-h/wk (quintiles), history of high cholesterol, history of hypertension, and body mass index (BMI kg/m²; <18.5, 18.5–19.9, 20.0–22.4, 22.5–24.9, 25.0–27.4, 27.5–29.9, 30.0–32.4, 32.5–34.9, 35.0–39.9, 40.0+). T2D was not included as it is a potential intermediate of CVD risk. Missing indicator categories were used for missing covariate data. The Cox proportional hazard assumptions were tested through the inclusion of a cross-product term for BCAA and time in the risk model for overall composite incident CVD; this assumption was met, with no indication for an interaction by time.

In additional analyses, we further adjusted for CVD risk biomarkers previously measured from baseline blood samples to assess whether BCAAs were associated with CVD risk

independent of pathways of insulin resistance or dyslipidemia. For these, we adjusted individually and altogether for the following, as ln-transformed continuous variables: Hba1c, a marker of long-term hyperglycemia³³; lipoprotein insulin resistance, a lipid-based metabolomic score derived to reflect lipoprotein derangements of insulin resistance,³⁴ previously associated with T2D in our cohort³⁵; GlycA, a measure of glycosylated acute phase proteins³⁶; triglyceride level; LDL, HDL, and hsCRP.

We stratified by whether participants had a prevalent (reported at baseline) or confirmed incident diagnosis of T2D. For CVD cases, we considered only T2D diagnoses before the first CVD event. We also stratified by CVD risk factors at the time of blood draw, including BMI category (normal <25.0 versus overweight/obese ≥ 25.0 kg/m²), menopausal status (pre versus post), current postmenopausal hormone use (yes versus no), the presence of the metabolic syndrome, HbA1c <5.7% versus $\geq 5.7\%$, fasting status <8 hours since last meal (yes versus no), and follow-up time (<10 versus ≥ 10 years). Metabolic syndrome was previously defined in WHS as having at least 3 of the following traits: (1) BMI >25.0 ; (2) hypertension; (3) elevated triglycerides ≥ 150 mg/dL (1.69 mmol/L); (4) low HDL-C <50 mg/dL (1.29 mmol/L); and (5) Hba1c $\geq 5.7\%$ or T2D.³⁸ Statistical tests for interactions were performed with likelihood ratio tests comparing the multivariable models with and without inclusion of the multiplicative interaction term.

RESULTS

Baseline characteristics of the 27 041 eligible participants with baseline measurements of BCAA metabolites are presented in Table 1, according to quintiles of total BCAAs. Nearly all CVD risk factors differed across the BCAA quintiles indicating worse cardiometabolic profiles with higher BCAA levels. For example, the prevalence of overweight/obesity (BMI ≥ 25 kg/m²) in the highest quintile 5 was more than double that of the lowest quintile 1, with 68.8% and 30.2%, respectively. Never smokers, however, were more prevalent among the higher BCAA quintiles. The randomized treatment assignments were balanced across BCAA quintiles.

We confirmed 2207 incident CVD cases over a mean 18.6 years of follow-up, including 551 first cases of MI, 799 first cases of stroke, and 1178 revascularization events. We give the results of the age- and multivariable-adjusted models for the association between continuous total and individual BCAA metabolites at baseline with incident CVD events in Table 2. Total BCAAs were associated with a 23% greater risk of total CVD (per SD: HR, 1.23; 95% CI, 1.18–1.29; $P < 0.0001$) in age- and treatment-adjusted models. This was moderately attenuated in the multivariable model 2 after adjusting for several traditional CVD risk factors (per SD: HR, 1.16; 95% CI, 1.11–1.21; $P < 0.0001$), and slightly more so with additional adjustment for BMI in multivariable model 3 (per SD: HR, 1.13; 95% CI, 1.08–1.18; $P < 0.0001$). These associations were comparable when

evaluating LDL-C as the exposure of interest with risk of CVD events in multivariable model 3 (per SD of LDL-C: HR, 1.12; 95% CI, 1.07–1.17; $P < 0.0001$). Addition of BCAAs to the multivariable model increased the receiver operator curve from area under the curve=0.718 to area under the curve=0.721 ($P < 0.0001$), whereas the addition of LDL increased to area under the curve=0.719 ($P < 0.0001$). Similar trends were observed for individual BCAA metabolites.

Total BCAA levels were associated with individual CVD end points (Table 2), indicating higher risks per SD greater BCAA level for MI (multivariable model 3, per SD: HR, 1.16; 95% CI, 1.06–1.26) and coronary revascularization (multivariable model 3, per SD: HR, 1.17; 95% CI, 1.11–1.25). A borderline association with stroke did not reach statistical significance (multivariable model 3, per SD: HR, 1.07; 95% CI, 0.99–1.15). Isoleucine and valine were of similar magnitude to total BCAAs for individual end points, whereas leucine was generally weaker.

When we additionally adjusted individually for correlated CVD baseline biomarkers (Table I in the [Data Supplement](#)) from cardiometabolic pathways (Hba1c, lipoprotein insulin resistance score, triglycerides, GlycA, LDL-C, HDL-C, and hsCRP), the associations between BCAAs with total CVD and the individual CVD end points were variably attenuated (Table II in the [Data Supplement](#)). LDL-C, HDL-C, or hsCRP did not appreciably impact the results. Adjusting for biomarkers related to impaired glucose metabolism simultaneously, Hba1c, lipoprotein insulin resistance score, triglycerides, and GlycA, completely attenuated the relationship between total plasma BCAAs and risk of CVD and the individual components (multivariable model 3, per SD: HR, 1.02; 95% CI, 0.98–1.07).

We also give the results of the age- and multivariable-adjusted models between quintiles of BCAA metabolites and incident CVD events (Table III in the [Data Supplement](#)). In the multivariable model 3 adjusting for CVD risk factors, including BMI, comparing quintile 5 to quintile 1, total BCAAs, isoleucine, leucine, and valine were associated with 31%, 37%, 18%, and 29% greater CVD risks, respectively. All P values for linear trend were statistically significant except for leucine (P trend=0.07).

There was significant effect modification for BCAAs with CVD risk according to T2D status (P interaction=0.036; Figure). P values for interaction were significant for revascularization end points (P interaction=0.019), and of borderline statistical significance for MI (P interaction=0.059) and stroke (P interaction=0.066). The relationship between total BCAAs with CVD risk was significantly greater when T2D preceded the CVD event (multivariable model 3, per SD: HR, 1.20; 95% CI, 1.08–1.32). The association between BCAAs and CVD was weaker among women without

Table 1. Baseline Characteristics According to Quintiles of Plasma Total Branched-Chain Amino Acid Metabolite Levels

Participants, n	Total Plasma Branched-Chain Amino Acid Quintiles Q1 (Bottom) 5443	Q2 5409	Q3 5648	Q4 5429	Q5 (Top) 5472	P Trend*
Age, y	54.4 (7.2)	54.8 (7.3)	54.9 (7.1)	54.9 (7.1)	54.6 (6.9)	0.12
Body mass index, kg/m ²	24.0 (3.9)	24.7 (4.1)	25.6 (4.6)	26.7 (5.0)	28.3 (5.7)	<0.0001
Normal weight (<25)	69.8	62.7	53.5	43.4	31.3	<0.0001
Overweight/obese (≥25)	30.2	37.3	46.5	56.6	68.8	
Menopausal status						
Premenopausal	31.7	27.8	26.2	26.4	26.2	<0.0001
Postmenopausal	51.5	54.6	56.3	56.0	53.1	0.04
Uncertain	12.7	13.8	13.5	14.0	16.3	<0.0001
Postmenopausal hormone use	43.4	44.8	45.5	43.7	40.7	0.001
Family history of premature MI	11.1	10.9	10.9	11.8	12.7	0.003
White race/ethnicity	95.2	95.4	94.9	94.1	93.0	<0.0001
Smoking status						
Never	48.6	51.6	52.1	53.3	52.8	<0.0001
Past	38.3	37.2	37.0	35.6	35.7	0.0009
Current <15 cig/d	4.7	4.5	4.1	3.8	4.4	0.1
Current ≥15 cig/d	8.3	6.5	6.6	7.2	7.0	0.07
Alcohol						
Rarely/never	38.6	40.7	42.7	45.5	51.9	<0.0001
1–3 drinks/mo	12.5	14.0	13.3	13.1	13.4	0.6
1–6 drinks/wk	35.2	33.2	33.6	32.4	27.7	<0.0001
≥1 drink/d	13.7	12.0	10.4	9.1	7.1	<0.0001
AHEI diet quality score (10–100)	54.5 (10.3)	53.9 (10.2)	53.7 (10.1)	53.1 (10.1)	52.4 (10.1)	<0.0001
Total physical activity, MET-h/wk (median, interquartile range)	10.3 (19.5)	10.0 (18.2)	9.2 (17.5)	8.0 (17.4)	7.0 (15.1)	<0.0001
History of high cholesterol	23.5	26.9	29.8	32.3	34.1	<0.0001
History of hypertension	18.2	21.0	22.9	27.6	34.2	<0.0001
History of type 2 diabetes mellitus	0.6	0.9	1.2	2.1	7.9	<0.0001
Randomized treatment assignment						
Aspirin	49.7	50.0	49.8	49.6	51.2	0.2
β carotene	49.6	50.6	49.7	49.7	49.4	0.6
Vitamin E	50.3	49.8	49.8	50.8	49.8	0.9
Fasting at blood draw	72.9	76.1	75.4	74.0	62.1	<0.0001
Plasma metabolite levels, μmol/L (median, interquartile range)						
Total BCAAs	308 (41)	360 (22)	104 (20)	447 (27)	528 (78)	<0.0001
Isoleucine	35 (15)	43 (16)	50 (17)	58 (18)	76 (26)	<0.0001
Leucine	96 (25)	117 (22)	132 (22)	148 (23)	177 (34)	<0.0001
Valine	172 (27)	199 (21)	219 (23)	242 (24)	284 (43)	<0.0001
Other biomarkers						
Hba1c, %	5.0 (0.3)	5.0 (0.4)	5.0 (0.4)	5.1 (0.5)	5.3 (1.0)	<0.0001
LPIR score	30.8 (20.5)	35.4 (21.7)	39.7 (22.8)	46.2 (23.2)	55.5 (23.5)	<0.0001
Triglycerides, mg/dL	115.6 (69.2)	123.5 (69.5)	133.1 (71.5)	145.9 (77.7)	172.9 (89.5)	<0.0001
LDL-C, mg/dL	117.5 (32.7)	122.3 (33.1)	125.1 (34.1)	127.6 (33.9)	128.7 (34.9)	<0.0001
GlycA, μmol/L	357.8 (63.4)	374.7 (64.0)	385.4 (64.6)	399.4 (66.1)	416.2 (69.3)	<0.0001

Values are means (SD) or % unless otherwise specified. AHEI indicates Alternative Healthy Eating Index; BCAA, branched-chain amino acids; GlycA, glycoprotein N-acetyl methyl groups; Hba1c, glycated hemoglobin; LDL-C, low-density lipoprotein cholesterol; LPIR score, lipoprotein insulin resistance score that reflects lipoprotein derangements of insulin resistance; MET, metabolic equivalent of tasks; MI, myocardial infarction; and Q, quintile.

*We performed tests for trend across BCAA quintiles.

Table 2. Prospective Association Between Baseline Circulating BCAA Metabolites in Relation to Incident Total Cardiovascular Disease, MI, Stroke, and Revascularization Risk in 27 401 US Women

	Total CVD	MI	Stroke	Revascularization
	HR per SD (95% CI)			
No. of events	2207	551	799	1178
Total BCAAs*				
Model 1: age, treatment	1.23 (1.18–1.29)	1.30 (1.19–1.41)	1.11 (1.03–1.19)	1.33 (1.26–1.41)
Model 2: multivariable	1.16 (1.11–1.21)	1.19 (1.09–1.29)	1.06 (0.99–1.14)	1.23 (1.16–1.30)
Model 3: +BMI	1.13 (1.08–1.18)	1.16 (1.06–1.26)	1.07 (0.99–1.15)	1.17 (1.11–1.25)
Isoleucine				
Model 1: age, treatment	1.24 (1.19–1.30)	1.34 (1.22–1.47)	1.09 (1.02–1.18)	1.39 (1.30–1.48)
Model 2: multivariable	1.17 (1.12–1.22)	1.22 (1.12–1.34)	1.05 (0.97–1.13)	1.28 (1.20–1.37)
Model 3: +BMI	1.14 (1.09–1.19)	1.19 (1.09–1.31)	1.05 (0.97–1.13)	1.24 (1.16–1.32)
Leucine				
Model 1: age, treatment	1.14 (1.09–1.19)	1.16 (1.06–1.26)	1.07 (1.00–1.15)	1.20 (1.13–1.28)
Model 2: multivariable	1.08 (1.04–1.13)	1.08 (0.99–1.18)	1.04 (0.97–1.12)	1.12 (1.05–1.19)
Model 3: +BMI	1.06 (1.02–1.11)	1.05 (0.97–1.15)	1.04 (0.97–1.12)	1.08 (1.02–1.14)
Valine				
Model 1: age, treatment	1.23 (1.18–1.28)	1.29 (1.19–1.40)	1.10 (1.03–1.18)	1.31 (1.24–1.39)
Model 2: multivariable	1.17 (1.12–1.22)	1.20 (1.10–1.30)	1.06 (0.99–1.14)	1.22 (1.15–1.30)
Model 3: +BMI	1.13 (1.08–1.18)	1.16 (1.06–1.27)	1.06 (0.99–1.15)	1.16 (1.09–1.24)

Model 2 includes the following: age (continuous), randomized treatment assignments, fasting status at blood draw, menopausal status (pre, post, uncertain, and missing), current hormone therapy use, family history of MI, White race/ethnicity, smoking status (never, past, current <15 c/d, current 15+ c/d), AHEI diet quality score (quintiles), alcohol intake (4 categories), total physical activity MET-h/wk (quintiles), history of high cholesterol, history of hypertension. Model 3 includes the covariates adjusted for in model 2 plus BMI (adjusted for in 10 categories). AHEI indicates Alternative Healthy Eating Index; BCAA, branched-chain amino acids; BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; HR, hazard ratio; MET, metabolic equivalent of tasks; and MI, myocardial infarction.

*Metabolites were ln-transformed and standardized; SDs are the following: total BCAAs=0.22; isoleucine=0.42; leucine=0.28; and valine=0.20.

T2D, although still statistically significant (multivariable model 3, per SD: HR, 1.08; 95% CI, 1.03–1.14). We observed the same trends of effect modification by T2D status for MI, stroke, and revascularization (Figure). Stratification by baseline CVD risk factors, including by baseline BMI, postmenopausal status, postmenopausal hormone use, or metabolic syndrome, did not significantly modify the association between total BCAAs and CVD risk (Table IV in the [Data Supplement](#)). Further, we did not observe a difference in the association with longer follow-up time (P interaction=0.24), indicating that baseline BCAA levels are a significant predictor of CVD risk for both shorter-term (<10 years: HR, 1.18; 95% CI, 1.10–1.26) and longer-term follow-up (\geq 10 years: HR, 1.11; 95% CI, 1.04–1.18).

DISCUSSION

In this longitudinal cohort of US women with >18 years of follow-up, we observed a significant positive association between baseline plasma levels of BCAAs with incident CVD events, after adjusting for numerous traditional CVD risk factors, including BMI. The BCAA–CVD association was much more pronounced for the

women who developed T2D prior to a CVD event, in particular for coronary events. Further adjustment for biomarkers of cardiometabolic risk suggested that the relationship between BCAAs and CVD could be mostly explained by more downstream biomarkers of T2D metabolism. Hence, impaired BCAA metabolism may represent a shared pathway of the metabolic pathophysiology that links the risks of T2D and CVD.

Prior prospective analyses of BCAA metabolites and incident CVD risk are sparse. In a case-cohort analysis within the PREDIMED (PREvención con Dieta MEDiterránea) Mediterranean diet trial population, 970 men and women at high CVD risk had BCAA measures available from baseline blood samples. After a median 4.6 years of follow-up, there were 226 CVD events (nonfatal MI or stroke, CV death).¹³ Isoleucine, leucine, and valine were associated with 2.9-fold, 2.2-fold, and 1.9-fold greater risks of CVD in BMI-adjusted models comparing the fourth and first quartiles, respectively. In contrast to the present study, however, the PREDIMED findings did not appreciably differ according to baseline T2D status. Further, PREDIMED investigators reported a positive association between BCAA metabolites with stroke risk, which was not statistically significant in our

WHS cohort after additional adjustment for baseline diet quality score, alcohol intake, menopausal status, and other risk factors, despite our many stroke events.

Studies of broader metabolomic profiling in relation to incident CVD have been mixed in identifying BCAAs among metabolites significantly associated with elevated risk.^{39–41} Baseline concentrations of isoleucine and leucine, but not valine, were among 33 other metabolites significantly associated with CVD risk (stroke, MI, revascularizations, and angina) after correction for multiple comparisons in a discovery cohort.³⁹ These associations were not replicated in separate cohorts, however, and were attenuated after adjusting for traditional lipid markers. This result differs from our analyses whereby adjusting for measured LDL-C, HDL-C, or for the history of high cholesterol did not impact the positive associations between BCAAs with CVD risk. Isoleucine and leucine were identified from a panel of metabolites measured in stored plasma samples of patients undergoing cardiac catheterization and were significantly related to incident MI in multivariable models, including adjusting for prevalent T2D.⁴¹ An agnostic BCAA-related metabolite factor was also significantly associated with MI risk, and this finding was replicated in an external case–control sample. A pattern of metabo-

lites strongly predictive of T2D, including isoleucine, was positively related to incident CVD as well as prevalent moderate-to-severe atherosclerosis.⁵

The relationship between BCAAs with incident T2D is striking and consistent across study populations, duration of follow-up, and methods of metabolomic profiling.¹² It is possible that elevated BCAA metabolites, reflecting impaired catabolism and accumulation in circulation,⁴² may increase CVD risk through the promotion of insulin resistance-mediated atherosclerosis.² There is accumulating evidence in support of a BCAA catabolism impairment being upstream of insulin resistance, although we acknowledge the reverse may be true. A Mendelian randomization study observed that circulating BCAA metabolite-related polymorphisms were significantly associated with an elevated risk of T2D.⁴² Further, a randomized trial of metformin did not lead to reductions in BCAAs, despite significant improvements in insulin sensitivity and changes to other circulating metabolites.⁴³ In the present article, the relationship between BCAAs with incident CVD was stronger for those participants who also developed T2D before CVD. Cross-sectional analyses of CVD predictors and intermediate phenotypes are consistent with our findings, and the hypothesis that is circulating BCAA

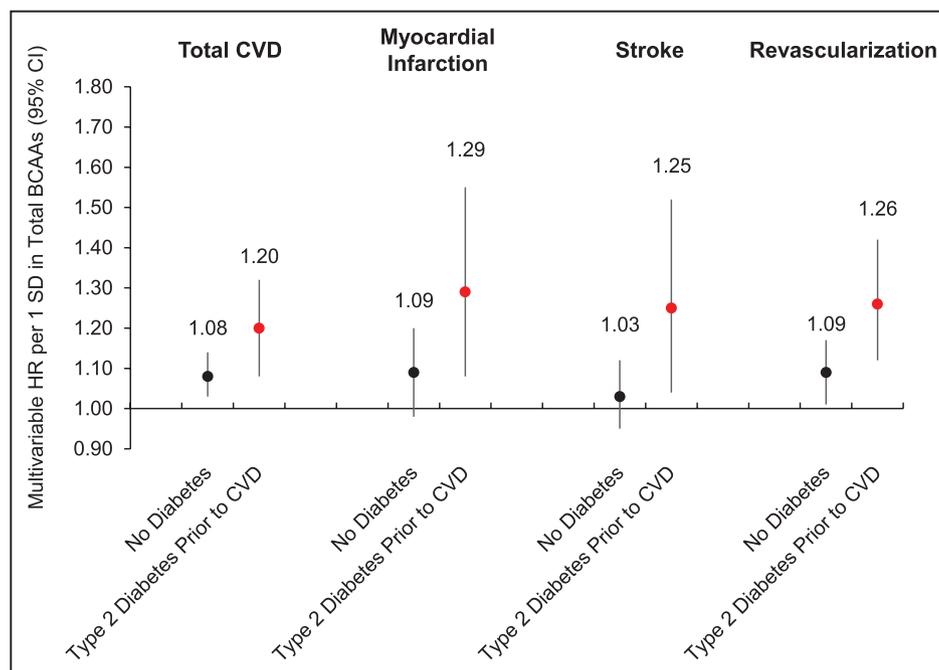


Figure. Prospective association between baseline circulating total branched-chain amino acids (BCAAs) in relation to incident total cardiovascular disease (CVD), myocardial infarction (MI), stroke, and revascularization risk in 27401 US women, according to type 2 diabetes mellitus diagnosis before CVD end point.

Multivariable-adjusted model includes the following: age (continuous), randomized treatment assignments (aspirin, beta-carotene, and vitamin E), fasting status at blood draw, menopausal status (pre, post, uncertain, and missing), current hormone therapy use, family history of MI, White race/ethnicity, smoking status (never, past, current <15 c/d, current 15+ c/d), Alternative Healthy Eating Index (AHEI) diet quality score (quintiles), alcohol intake (4 categories), total physical activity metabolic equivalent of tasks (MET)-h/wk (quintiles), history of high cholesterol, history of hypertension, and body mass index (10 categories). *P* values for interaction: total CVD, *P*=0.036; MI, *P*=0.059; stroke, *P*=0.066; and revascularization, *P*=0.019. CI indicates confidence interval; and HR, hazard ratio.

metabolites are associated with CVD predominantly through T2D-related pathways related to impaired metabolic and inflammatory disarray. For example, plasma BCAAs were positively correlated with carotid intima-media thickness after multivariable adjustment among adult subjects with HbA1c values $\geq 5.6\%$, whereas no correlation between BCAAs and carotid intima-media thickness persisted among the subgroup of individuals with HbA1c $< 5.6\%$.⁴ Thus, elevated BCAAs may be a marker proximal to excess adiposity, and an upstream predictor of insulin resistance, T2D, and CVD.

Strengths of this analysis include the large sample size and prospective design with consideration for antecedent T2D. The US study population is limited by its relative homogeneity, limiting our ability to generalize these results to men and non-White racial/ethnic groups. We carefully adjusted for numerous potential CVD risk factors, although it is possible residual or unmeasured confounding remains. Despite the potential limitations of using a single baseline measurement of the metabolites, we observed a relationship between BCAAs with elevated CVD risk over the duration of follow-up (>18 years). Rigorous large-scale metabolomics studies are still needed to quantify the expected variability for most metabolites over time. A longer-term reproducibility study reported intra-class correlations ≥ 0.40 for BCAA metabolites in repeated plasma samples 1 to 2 years apart.⁴⁴ Similarly, in a subset (n=27) of the EPIC (European Prospective Investigation into Cancer and Nutrition) study, BCAA intra-class correlations ranged 0.3 to 0.4 for serum collected 2 years apart.⁴⁵ This same cohort reported intra-class correlations of 0.5 for triglycerides.⁴⁶ Collectively, these pilot data suggest acceptable reproducibility for BCAAs with variation similar to other biomarkers.

The current study is the largest prospective analysis of BCAA metabolites with incident CVD risk to date. We observed significant positive associations between BCAA metabolites with long-term CVD risk, comparable in magnitude to (and independent of) LDL-C, an established CVD biomarker. Notably, this relationship was prominent among women developing T2D before CVD. Despite being the main cause of death in the US, the mechanisms linking behavioral and environmental risk factors with CVD and T2D risk remain incompletely understood. Identifying novel biomarkers downstream of risk factors such as excess adiposity may allow for intervention with preventive strategies years before CVD symptom onset, for example at the time of T2D diagnosis or even earlier. BCAAs may represent an early excess adiposity-related perturbation in the etiologic pathway linking T2D with excess CVD risk. Future research investigating the modifiability of BCAA metabolism with reduction of subsequent disease risk is warranted to understand the role these metabolites may play in clinical practice.

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Disclosures

Dr Ridker, listed as a coinventor on patents held by the Brigham and Women's Hospital related to the use of inflammatory biomarkers in cardiovascular disease (licensed to AstraZeneca and Siemens); Dr Mora has a patent application about the use of GlycA in relation to colorectal cancer risk.

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**Circulating Branched-Chain Amino Acids and Incident Cardiovascular Disease in a
Prospective Cohort of US Women**

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SUPPLEMENTAL MATERIAL

Supplement Table 1. Correlation between baseline circulating BCAA metabolites and cardiometabolic biomarkers in WHS.

	BCAA	Isoleucine	Leucine	Valine	Hba1c%	LPIR	Triglycerides	LDL-C	GlycA
BCAA	1	0.74 <.0001	0.86 <.0001	0.93 <.0001	0.23 <.0001	0.31 <.0001	0.28 <.0001	0.11 <.0001	0.30 <.0001
Isoleucine		1	0.51 <.0001	0.60 <.0001	0.18 <.0001	0.31 <.0001	0.28 <.0001	0.07 <.0001	0.20 <.0001
Leucine			1	0.68 <.0001	0.16 <.0001	0.18 <.0001	0.14 <.0001	0.12 <.0001	0.26 <.0001
Valine				1	0.24 <.0001	0.32 <.0001	0.30 <.0001	0.09 <.0001	0.27 <.0001
Hba1c%					1	0.17 <.0001	0.18 <.0001	0.11 <.0001	0.20 <.0001
LPIR						1	0.71 <.0001	0.13 <.0001	0.46 <.0001
Triglycerides							1	0.29 <.0001	0.46 <.0001
LDL-C								1	0.17 <.0001

Metabolite values are ln-transformed and standardized; other biomarkers are ln-transformed for analysis.

BCAAs=branched-chain amino acids, Hba1c=glycated haemoglobin, LPIR score=lipoprotein insulin resistance score that reflects lipoprotein derangements of insulin resistance, LDL-C=low density lipoprotein cholesterol, GlycA=glycoprotein N-acetyl methyl groups

Supplement Table 2. Prospective association between baseline circulating total BCAAs in relation to incident total cardiovascular disease, MI, stroke, and revascularization risk in 27,401 US women, with further adjustment for individual ln-transformed biomarkers of CVD risk.

	Total CVD
	HR per SD
	(95% CI)
Model 3*	
Total BCAAs	1.13 (1.08, 1.18)
+ Hba1c%	1.07 (1.02, 1.12)
+ triglycerides	1.09 (1.04, 1.14)
+ LPIR Score	1.10 (1.05, 1.15)
+ GlycA	1.10 (1.05, 1.15)
+ hsCRP	1.11 (1.06, 1.16)
+ LDL cholesterol	1.12 (1.08, 1.18)
+ HDL cholesterol	1.12 (1.08, 1.18)
+ Hba1c%, triglycerides, LPIR Score, and GlycA	1.02 (0.98, 1.07)

*Multivariable adjusted model 3 includes the following: age (continuous), randomized treatment assignments (ASA, BC, VE), fasting status at blood draw, menopausal status (pre, post, uncertain, missing), current hormone therapy use, family history of MI, Caucasian race/ethnicity, smoking status (never, past, current <15 c/d, current 15+ c/d), AHEI diet quality score (quintiles), alcohol intake (4 categories), total physical activity MET-hrs/wk (quintiles), history of high cholesterol, history of hypertension, and BMI (10 categories), plus the biomarkers as indicated above.

BCAAs=branched-chain amino acids, Hba1c=glycated haemoglobin, LPIR score=lipoprotein insulin resistance score that reflects lipoprotein derangements of insulin resistance, GlycA=glycoprotein N-acetyl methyl groups, hsCRP=high sensitivity C-reactive protein, LDL-C=low density lipoprotein cholesterol, HR=hazard ratio, CI=confidence interval, SD=standard deviation

Supplement Table 3. Cox proportional hazards regression models of quintiles of baseline circulating BCAAs in relation to incident total cardiovascular disease (MI, stroke, revascularization) risk in 27,401 US women.

	Q1	Q2	Q3	Q4	Q5	
		HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)	p for trend*
Total BCAAs						
CVD Events	358	395	421	454	579	
Model 1: Age, treatment	[ref]	1.07 (0.93, 1.24)	1.09 (0.95, 1.26)	1.24 (1.08, 1.42)	1.67 (1.47, 1.91)	<0.0001
Model 2: Multivariable	[ref]	1.07 (0.93, 1.24)	1.06 (0.92, 1.22)	1.13 (0.99, 1.30)	1.43 (1.25, 1.64)	<0.0001
Model 3: + BMI	[ref]	1.05 (0.91, 1.21)	1.01 (0.88, 1.17)	1.06 (0.92, 1.23)	1.31 (1.14, 1.50)	0.0001
Isoleucine						
CVD Events	400	352	415	449	591	
Model 1: Age, treatment	[ref]	0.99 (0.85, 1.14)	1.02 (0.89, 1.17)	1.16 (1.01, 1.33)	1.74 (1.53, 1.98)	<0.0001
Model 2: Multivariable	[ref]	0.95 (0.83, 1.10)	0.97 (0.84, 1.11)	1.06 (0.93, 1.22)	1.47 (1.29, 1.67)	<0.0001
Model 3: + BMI	[ref]	0.95 (0.82, 1.09)	0.94 (0.82, 1.08)	1.02 (0.89, 1.17)	1.37 (1.20, 1.57)	<0.0001
Leucine						
CVD Events	399	417	444	403	544	
Model 1: Age, treatment	[ref]	1.09 (0.95, 1.25)	1.10 (0.96, 1.26)	1.09 (0.95, 1.25)	1.43 (1.26, 1.63)	<0.0001
Model 2: Multivariable	[ref]	1.10 (0.96, 1.26)	1.08 (0.94, 1.23)	1.03 (0.90, 1.18)	1.26 (1.10, 1.43)	0.003
Model 3: + BMI	[ref]	1.09 (0.95, 1.25)	1.05 (0.92, 1.21)	0.98 (0.86, 1.13)	1.18 (1.03, 1.34)	0.07

Valine

CVD Events	357	387	440	454	569	
Model 1: Age, treatment	[ref]	1.07 (0.92, 1.23)	1.17 (1.01, 1.34)	1.28 (1.11, 1.47)	1.62 (1.42, 1.85)	<0.0001
Model 2: Multivariable	[ref]	1.08 (0.93, 1.25)	1.13 (0.99, 1.30)	1.19 (1.03, 1.36)	1.42 (1.24, 1.63)	<0.0001
Model 3: + BMI	[ref]	1.05 (0.91, 1.22)	1.08 (0.94, 1.25)	1.11 (0.96, 1.28)	1.29 (1.12, 1.49)	0.0002

BCAAs=branched chain amino acids, BMI=body mass index, Q=quintile, HR=hazard ratio, CI=confidence interval

Model 2 includes the following: age (continuous), randomized treatment assignments, fasting status at blood draw, menopausal status (pre, post, uncertain, missing), current hormone therapy use, family history of MI, Caucasian race/ethnicity, smoking status (never, past, current <15 c/d, current 15+ c/d), AHEI diet quality score (quintiles), alcohol intake (4 categories), total physical activity MET-hrs/wk (quintiles), history of high cholesterol, history of hypertension; Model 3 includes the covariates adjusted for in Model 2 plus BMI (adjusted for in 10 categories).

*P-values for tests for trend were estimated by modeling the median values across quintiles as a continuous variable.

Supplement Table 4. Prospective association between baseline circulating total BCAAs (continuous, per SD) in relation to incident total cardiovascular disease, MI, stroke, and revascularization risk in 27,401 US women, stratified by risk factors at time of baseline blood draw.

		Total CVD	
		HR per SD*	
Total BCAAs	N events	(95% CI)	
BMI, kg/m²			
<25	920	1.08 (1.01, 1.16)	
≥25	1,227	1.15 (1.09, 1.22)	
<i>P</i> -interaction		0.14	
Menopausal status			
Pre	668	1.13 (1.04, 1.23)	
Post	1,539	1.12 (1.07, 1.19)	
<i>P</i> -interaction		0.32	
Current hormone therapy use			
No	1,222	1.15 (1.08, 1.22)	
Yes	985	1.11 (1.03, 1.18)	
<i>P</i> -interaction		0.19	
Metabolic syndrome			
No	1,251	1.07 (1.01, 1.14)	
Yes	902	1.12 (1.05, 1.20)	
<i>P</i> -interaction		0.14	
Baseline HbA1c			
<5.7%	1,836	1.08 (1.02, 1.13)	
≥5.7%	359	1.17 (1.06, 1.30)	
<i>P</i> -interaction		0.023	

Fasting at blood draw

No	471	1.11 (1.02, 1.20)
Yes	1,611	1.14 (1.07, 1.20)
<i>P</i> -interaction		0.70

Follow-up time, years

<10	951	1.18 (1.10, 1.26)
≥10	1,056	1.11 (1.04, 1.18)
<i>P</i> -interaction		0.24

BCAAs=branched-chain amino acids, HR=hazard ratio, CI=confidence interval, SD=standard deviation
*Multivariable adjusted model includes the following: age (continuous), randomized treatment assignments (ASA, BC, VE), fasting status at blood draw, menopausal status (pre, post, uncertain, missing), current hormone therapy use, family history of MI, Caucasian race/ethnicity, smoking status (never, past, current <15 c/d, current 15+ c/d), AHEI diet quality score (quintiles), alcohol intake (4 categories), total physical activity MET-hrs/wk (quintiles), history of high cholesterol, history of hypertension, and BMI (10 categories).