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Plasma Branched-Chain Amino Acids and Incident Cardiovascular Disease in the PREDIMED Trial

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BACKGROUND: The role of branched-chain amino acids (BCAAs) in cardiovascular disease (CVD) remains poorly understood. We hypothesized that baseline BCAA concentrations predict future risk of CVD and that a Mediterranean diet (MedDiet) intervention may counteract this effect.

METHODS: We developed a case-cohort study within the Prevención con Dieta Mediterránea (PREDIMED), with 226 incident CVD cases and 744 noncases. We used LC-MS/MS to measure plasma BCAAs (leucine, isoleucine, and valine), both at baseline and after 1 year of follow-up. The primary outcome was a composite of incident stroke, myocardial infarction, or cardiovascular death.

RESULTS: After adjustment for potential confounders, baseline leucine and isoleucine concentrations were associated with higher CVD risk: the hazard ratios (HRs) for the highest vs lowest quartile were 1.70 (95% CI, 1.05–2.76) and 2.09 (1.27–3.44), respectively. Stronger associations were found for stroke. For both CVD and stroke, we found higher HRs across successive quartiles of BCAAs in the control group than in the MedDiet groups. With stroke as the outcome, a significant interaction ($P = 0.009$) between baseline BCAA score and intervention with MedDiet was observed. No significant effect of the intervention on 1-year changes in BCAAs or any association between 1-year changes in BCAAs and CVD were observed.

CONCLUSIONS: Higher concentrations of baseline BCAAs were associated with increased risk of CVD, especially stroke, in a high cardiovascular risk population. A Mediterranean-style diet had a negligible effect on 1-year changes in BCAAs, but it may counteract the harmful effects of BCAAs on stroke.

Although compelling evidence has demonstrated that adherence to the Mediterranean diet (MedDiet)[20] is effective for primary cardiovascular disease (CVD) prevention *(1)*, the biological mechanisms underpinning this effect are not well understood *(2)*. Recently, metabolomics applied to the field of nutrition has offered great potential to provide new insights into underlying mechanisms *(3)*. Several studies have found an association between the concentrations of selected small molecule metabolites in peripheral blood and CVD risk *(4–7)*. Among various metabolites, branched-chain amino acids (BCAAs) have been found to predict obesity, insulin resistance, diabetes, and CVD outcomes.

BCAAs, including leucine, isoleucine, and valine, are a subgroup of amino acids derived from the diet that are essential for normal growth and function at the cell and organism levels *(8)*. The relevance of the metabolism of these amino acids to coronary heart disease remains poorly understood *(8)*, but increased circulating concentrations of BCAAs may be explained by an obesity-related

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[20] Nonstandard abbreviations: MedDiet, Mediterranean diet; CVD, cardiovascular disease; BCAA, branched-chain amino acid; PREDIMED, Prevención con Dieta Mediterránea; EVOO, extra-virgin olive oil; HR, hazard ratio; MET, metabolic equivalent task; mTOR, mammalian target of rapamycin.

decline in their catabolism in adipose tissue *(9)*. By use of a case-cohort study conducted in older participants at high cardiovascular risk from the Prevención con Dieta Mediterránea (PREDIMED) trial, we sought to address (*a*) whether baseline BCAA concentrations predict future risk of CVD; (*b*) whether the MedDiet interventions counteract the presumed deleterious effects of BCAAs on CVD risk; and (*c*) whether the beneficial effect of the MedDiet on CVD is partially explained by its effects on plasma BCAA concentrations.

Methods

We designed an unstratified case-cohort study within the PREDIMED trial (www.predimed.es). The protocol, design, methods, and primary results of the PREDIMED trial have been reported in detail elsewhere *(1, 10)*. Briefly, 7447 participants were randomly assigned to a Mediterranean diet supplemented with 1 L/week extra-virgin olive oil (EVOO) for participants and their families (MedDiet + EVOO), a Mediterranean diet supplemented with 30 g/day mixed nuts (15 g walnuts, 7.5 g hazelnuts, and 7.5 g almonds) (MedDiet + nuts), or a control diet consisting of advice to reduce the intake of all types of fat (control group).

The present study comprises a random selection of 794 participants (approximately 10%) from the eligible PREDIMED cohort at baseline with available EDTA plasma samples, together with all incident cases of CVD during follow-up with available samples (samples were unavailable for 57 of the 288 incident CVD cases in the PREDIMED study). We excluded 18 participants because metabolite values were not available (n = 3) or because extreme outlying values of metabolite concentrations (baseline or after 1 year of follow-up) were detected in the initial multidimensional scaling analysis (n = 15). Of the 970 participants included in our analyses, 781 were in the subcohort (including 744 noncases and 37 overlapping cases), and 226 were CVD cases (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue4). Of the CVD cases, 177 experienced a stroke (112 ischemic and 5 hemorrhagic). In addition, 899 participants of the 970 also had a 1-year follow-up sample and were included in the 1-year change analyses. Medical conditions and risk factors were collected by use of a questionnaire during the first screening visit.

STUDY SAMPLES AND METABOLITE PROFILING

Fasting blood samples were collected at baseline and yearly thereafter during follow-up. After an overnight fast, plasma EDTA tubes were collected, and aliquots were coded and kept refrigerated until they were stored at −80 °C. In June 2014, pairs of samples (baseline and first-year visits from each participant) were randomly ordered and shipped on dry ice to the Broad Institute of Harvard and MIT for the metabolomics analyses.

We used LC-MS/MS techniques developed at the Broad Institute to quantitatively profile amino acids, biogenic amines, and other polar plasma metabolites *(6, 7)*. Pooled reference samples and standard reference samples were inserted in the analytical queue every 20 samples. The raw data were processed with MultiQuant software (AB SCIEX) to integrate chromatographic peaks, and the data were visually inspected to ensure the quality of signal integration.

CLINICAL ASSESSMENT

The PREDIMED primary end point was a composite of nonfatal acute myocardial infarction, nonfatal stroke, or cardiovascular death. Information on primary end points was collected by study physicians, who were blinded to the intervention, and from other sources of information, such as the National Death Index *(1)*. This anonymized information was sent to the Clinical Endpoint Committee, which adjudicated the events blinded to the intervention group.

STATISTICAL ANALYSIS

Individual BCAA values were normalized and scaled to multiples of 1 SD with the rank-based inverse normal transformation. We fitted weighted Cox regression models with Barlow weights to account for the overrepresentation of cases, as recommended for case-cohort designs *(11)*. We calculated hazard ratios (HRs) and their 95% CIs for the composite CVD end point, and also separately for stroke, which was the most frequently occurring event of the cardiovascular composite end point. Follow-up time was calculated from the date of enrollment to the date of diagnosis of CVD for cases, and to the date of the last visit or the end of the follow-up period (December 1, 2010) for noncases. We fitted crude models; age-, sex-, and intervention group–adjusted models; and multivariable models. Multivariate-adjusted models were additionally adjusted for smoking status (never/current/former), body mass index (kg/m^2), leisure-time physical activity [metabolic equivalent tasks (METs) min/day], and family history of premature coronary heart disease (yes/no). In a secondary analysis, we additionally adjusted for diabetes, hypertension, and dyslipidemia because these risk factors were likely to be intermediate biomarkers in the causal pathway between BCAAs and risk of CVD.

We calculated a baseline BCAA score as the weighted sum of normalized values of leucine, isoleucine, and valine by inverse normal transformation. The weights corresponded to the respective coefficients from the multivariable Cox regression model fitted with each individual metabolite *(7)*.

The individual BCAAs and the BCAA score were also analyzed according to quartiles of their distributions. Quartile cutpoints were generated on the basis of the distributions of BCAAs among noncases. We conducted tests of linear trend by examining an ordinal score on the basis of the median value in each quartile of BCAAs in the multivariable models.

We conducted joint analyses and interaction tests for the BCAA score and the intervention groups (MedDiet + EVOO and MedDiet + nuts vs control group). We additionally conducted stratified analyses by diabetes status at baseline, sex, and age group (<70 vs ≥70 years). The likelihood ratio test was used to assess the significance of interaction between stratifying variables and the BCAA score.

We examined the associations between 1-year changes in individual BCAAs and the overall BCAA score on CVD risk (using as outcome only cases occurring after 1 year of follow-up). With respect to individual metabolites, we first calculated the difference between baseline and 1-year concentrations, then normalized this difference with the inverse normal transformation. For 1-year changes in the BCAA score, we summed 1-year changes of the 3 metabolites and normalized the sum.

We conducted several sensitivity analyses. First, we additionally adjusted for total fat, protein, alcohol, and fiber (all grams per day) and total energy intake (kilocalories per day). Second, we adjusted for baseline adherence to the Mediterranean dietary pattern, by use of a previously validated 14-item score *(12)*. Finally, we adjusted for plasma concentrations of other amino acids that were correlated with the BCAA score.

All statistical analyses were performed with Stata version 13.1 (Stata Corp.).

Results

We included 970 participants (226 CVD cases and 744 noncases) who were followed for a median of 4.6 years. Table 1 shows characteristics of participants in the random subcohort and in all CVD cases (with 37 individuals overlapping). As expected, as a result of random selection, the characteristics of the subcohort were very similar to those obtained in the full PREDIMED cohort *(1)*. A higher intake of protein, fats, alcohol, and total energy but a lower intake of carbohydrates and fiber were found in participants with higher concentrations of BCAAs (see online Supplemental Table 1).

BASELINE BCAAs AND RISK OF CVD AND STROKE

Table 2 shows the associations between baseline individual BCAAs (leucine, isoleucine, and valine) and both CVD and stroke. Leucine and isoleucine, but not valine, were associated with CVD (model 2). Baseline concentrations of individual BCAAs were more strongly associated with stroke than with the composite CVD end point. Each 1-SD increase in baseline leucine, isoleucine, and valine was associated with 45% (95% CI, 13%–85%), 51% (17%–95%), and 37% (8%–73%) relative increases in the risk of stroke, respectively (Table 2). Similarly, we found a stronger association for the outcome limited to stroke than for the overall CVD composite outcome in the comparison between extreme quartiles of BCAAs: after multivariate adjustment, the estimated HR for incident stroke in the top vs the bottom quartile was 2.17 (95% CI, 1.17–4.03) for leucine, 2.85 (1.49–5.45) for isoleucine, and 1.91 (1.02–3.57) for valine. These associations were attenuated and no longer significant except for isoleucine after adjusting for diabetes, dyslipidemia, and hypertension (see online Supplemental Table 2).

Per 1-SD increase in the BCAA score, a 46% higher risk [HR 1.46 (95% CI, 1.05–2.01)] of incident CVD was observed in the multivariable model (Table 3). In stratified analyses by intervention groups, higher HRs across BCAA score quartiles were observed in the control group compared with the MedDiet groups, for both CVD and stroke. The direct association between baseline BCAA concentrations and CVD became weaker and no longer statistically significant after additionally adjusting for diabetes, dyslipidemia, and hypertension. The association with stroke was also attenuated after adjusting for these additional factors (see online Supplemental Table 3). Similarly, no association was found within the 2 subgroups after stratifying by diabetes status (see online Supplemental Table 4).

In a joint classification of BCAA score and intervention groups, the reference category corresponded to those participants in the MedDiet groups who were in the lowest quartile of the BCAA score. We found a positive and significantly higher risk of CVD in the higher quartiles of the BCAA score of the control group [HR 1.80 for Q1 vs Q2–Q4 (95% CI, 1.17–2.76); *P* for interaction = 0.058 between the BCAA score (Q1 vs. Q2–Q4) and the intervention group and *P* for interaction = 0.263 between the BCAA score (continuous) and the intervention group] (Fig. 1A) compared with the lowest quartile of the BCAA score of the MedDiet groups. The interaction was stronger in joint analyses limited to stroke (*P* for interaction = 0.009) (Fig. 1B). Specifically, after excluding participants in the MedDiet + EVOO group, for those in the MedDiet + nuts group, the increased risk of stroke associated with higher baseline concentrations of BCAA was attenuated (*P* for interaction = 0.014). We did not find any statistically significant interaction between MedDiet + EVOO and baseline concentrations of BCAA after excluding participants in the MedDiet + nuts group (*P* for interaction = 0.60).

Table 1. Baseline participant characteristics in the random subcohort and cases.[a]

Characteristic	Subcohort[b]	Cases	P[c]
n	781	226	
Age, years	67.2 (6)	69.5 (6.5)	<0.001
Women, %	56.7	40.3	<0.001
Intervention group, %			
MedDiet + EVOO	37.0	35.4	0.122
MedDiet + nuts	33.2	28.8	
Control	29.8	35.8	
Family history of premature CHD, %	24.8	19.5	0.064
Hypertension, %	83.5	82.3	0.627
Dyslipidemia, %	73.4	58.0	<0.001
Diabetes, %	47.1	64.6	<0.001
Smoking, %			
Never	62.1	46.0	<0.001
Former	25.5	33.6	
Current	12.4	20.4	
Waist circumference, cm	99.9 (9.9)	101.5 (10.7)	0.028
Body mass index, kg/m^2	29.7 (3.6)	29.6 (3.8)	0.704
Physical activity, METs min/d	259 (259)	237 (238)	0.164
Education, %			
Elementary or less	76.2	80.1	0.388
Secondary or more	23.8	19.9	
Total energy intake, kcal/d	2337 (618)	2363 (688)	0.572
Leucine intake, g/d	7.5 (2.0)	7.4 (2.2)	0.667
Isoleucine intake, g/d	4.6 (1.3)	4.6 (1.3)	0.940
Valine intake, g/d	5.1 (1.3)	5.1 (1.5)	0.746
Score for adherence to Mediterranean diet[d]	8.8 (1.9)	8.5 (1.8)	0.005

[a] Data are mean (SD) unless noted otherwise.
[b] The randomly selected subcohort included 37 cases.
[c] To avoid the problem inherent to some cases being included both in the subcohort and cases, we compared the observed means (or proportions) in the cases vs the expected values (expected = mean or proportion observed in the subcohort).
[d] Based on the 14-item dietary screener [Schroder et al. *(12)*].

Additional adjustment for dietary variables (energy-providing macronutrients and total energy intake) and baseline adherence to the Mediterranean diet did not materially change the results of the association between baseline BCAA score and CVD [HR per SD 1.49 (95% CI, 1.07–2.09) and 1.49 (1.07–2.07), respectively]. We observed stronger associations between BCAA and CVD after adjusting for baseline plasma concentrations of amino acids correlated with the BCAA score: HRs per SD were 1.85 (95% CI, 1.13–3.03), 1.67 (1.18–2.36), and 1.81 (1.22–2.72) when additionally adjusted for phenylalanine/tyrosine, arginine, and γ-aminobutyric acid, respectively.

We did not observe any statistically significant interaction between baseline BCAAs and sex or age on composite CVD or stroke. In sensitivity analyses, we assessed the effect of the MedDiet intervention on the overall CVD composite outcome and also limited to stroke, stratified by baseline concentrations of BCAA score (see online Supplemental Table 5). The cardioprotective effect of the MedDiet interventions appeared more pronounced among participants with lower baseline BCAA scores, although the interaction was not statistically significant.

ASSOCIATIONS BETWEEN 1-YEAR CHANGES IN BCAAs AND THE EFFECT OF MedDiet INTERVENTION ON THE RISK OF CVD AND STROKE

Fig. 2 shows adjusted mean values (and 95% CIs) for each metabolite at baseline and after 1 year of follow-up

Table 2. Incident composite CVD and stroke by baseline plasma BCAA concentrations[a] (leucine, isoleucine, and valine) in the PREDIMED Trial, 2003–2010: observed event rates and HRs.

	Leucine		Isoleucine		Valine	
Incident cases	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Composite CVD (226 cases, 744 noncases)						
Crude, per SD	1.25 (1.07–1.45)	0.004	1.36 (1.17–1.58)	<0.001	1.18 (1.00–1.37)	0.043
Model 1[b]						
Per SD	1.19 (1.01–1.41)	0.039	1.29 (1.09–1.53)	0.004	1.13 (0.96–1.34)	0.140
Across quartiles						
Q1	1 (reference)		1 (reference)		1 (reference)	
Q2	1.55 (0.96–2.50)		1.44 (0.86–2.41)		1.42 (0.89–2.25)	
Q3	1.32 (0.82–2.14)		1.72 (1.05–2.81)		1.22 (0.76–1.95)	
Q4	1.71 (1.06–2.75)		2.11 (1.29–3.45)		1.42 (0.90–2.23)	
P for trend		0.060		0.002		0.238
Model 2						
Per SD	1.19 (1.00–1.42)	0.044	1.29 (1.08–1.54)	0.005	1.12 (0.95–1.33)	0.183
Across quartiles						
Q1	1 (reference)		1 (reference)		1 (reference)	
Q2	1.57 (0.97–2.55)		1.44 (0.85–2.42)		1.42 (0.88–2.28)	
Q3	1.31 (0.80–2.14)		1.71 (1.04–2.83)		1.18 (0.73–1.91)	
Q4	1.70 (1.05–2.76)		2.09 (1.27–3.44)		1.40 (0.88–2.24)	
P for trend		0.072		0.003		0.290
Stroke (117 cases, 744 noncases)						
Crude, per SD	1.38 (1.11–1.72)	0.003	1.45 (1.17–1.81)	0.001	1.34 (1.08–1.66)	0.008
Model 1						
Per SD	1.44 (1.13–1.84)	0.004	1.51 (1.17–1.94)	0.001	1.36 (1.08–1.72)	0.009
Across quartiles						
Q1	1 (reference)		1 (reference)		1 (reference)	
Q2	1.36 (0.72–2.57)		1.59 (0.81–3.14)		1.69 (0.91–3.14)	
Q3	1.41 (0.75–2.64)		1.61 (0.82–3.18)		1.67 (0.89–3.14)	
Q4	2.17 (1.17–4.03)		2.84 (1.48–5.47)		1.90 (1.04–3.49)	
P for trend		0.010		0.001		0.065
Model 2						
Per SD	1.45 (1.13–1.85)	0.003	1.51 (1.17–1.95)	0.001	1.37 (1.08–1.73)	0.009
Across quartiles						
Q1	1 (reference)		1 (reference)		1 (reference)	
Q2	1.35 (0.71–2.57)		1.60 (0.82–3.13)		1.69 (0.91–3.16)	
Q3	1.39 (0.73–2.63)		1.60 (0.81–3.17)		1.66 (0.87–3.17)	
Q4	2.17 (1.17–4.03)		2.85 (1.49–5.45)		1.91 (1.02–3.57)	
P for trend		0.011		0.001		0.071

[a] An inverse normal transformation was applied to raw values.

[b] Model 1 was adjusted for age (years), sex (male, female), and intervention group (MedDiet + EVOO, MedDiet + nuts). Model 2 was adjusted as for model 1, plus body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (METs min/day), and family history of premature coronary heart disease (yes, no).

by intervention group. A nonsignificant decrease in individual BCAAs was observed only in the MedDiet + EVOO group.

Changes in individual BCAAs were not associated with the risk of CVD (see online Supplemental Table 6). Analyses limited to stroke yielded similar results. Similarly, we did not observe any significant association between 1-year changes in BCAA score and risk of CVD or stroke (see online Supplemental Table 7), nor did we observe any association between the intervention

Table 3. Incident composite CVD and stroke by baseline plasma BCAA score[a] in the PREDIMED trial, 2003–2010: observed event rates and HRs.

	Overall			Both MedDiet groups			Control group		
Incident cases	**n**	**HR (95% CI)**	***P***	**n**	**HR (95% CI)**	***P***	**n**	**HR (95% CI)**	***P***
Subcohort, n	781			548			233		
Cases, n	226			145			81		
Composite CVD									
Crude, per SD		1.60 (1.21–2.13)	0.001		1.66 (1.16–2.37)	0.006		1.48 (0.93–2.35)	0.101
Model 1[b]									
Per SD		1.46 (1.07–2.01)	0.018		1.52 (1.02–2.27)	0.038		1.32 (0.80–2.19)	0.278
Across quartiles									
Q1		1 (reference)			1 (reference)			1 (reference)	
Q2		1.19 (0.73–1.92)			0.72 (0.40–1.30)			2.54 (1.04–6.19)	
Q3		1.24 (0.78–1.97)			0.86 (0.49–1.51)			2.41 (0.99–5.88)	
Q4		1.45 (0.91–2.32)			1.44 (0.84–2.46)			1.45 (0.57–3.70)	
P for trend			0.114			0.114			0.828
Model 2									
Per SD		1.46 (1.05–2.01)	0.024		1.58 (1.05–2.37)	0.029		1.28 (0.77–2.14)	0.346
Across quartiles									
Q1		1 (reference)			1 (reference)			1 (reference)	
Q2		1.20 (0.74–1.94)			0.75 (0.41–1.37)			2.73 (1.11–6.76)	
Q3		1.22 (0.76–1.97)			0.86 (0.47–1.55)			2.44 (0.98–6.08)	
Q4		1.43 (0.89–2.31)			1.53 (0.88–2.67)			1.40 (0.53–3.67)	
P for trend			0.145			0.082			0.966
Stroke									
Subcohort, n	767			539			228		
Cases, n	117			71			46		
Crude, per SD		1.38 (1.12–1.69)	0.002		1.31 (1.16–2.37)	0.051		1.50 (1.08–2.08)	0.015
Model 1[b]									
Per SD		1.42 (1.13–1.79)	0.003		1.37 (1.01–1.86)	0.043		1.52 (1.05–2.18)	0.025
Across quartiles									
Q1		1 (reference)			1 (reference)			1 (reference)	
Q2		0.99 (0.52–1.88)			0.80 (0.37–1.72)			1.67 (0.53–5.23)	
Q3		1.39 (0.77–2.50)			0.93 (0.44–1.98)			2.37 (0.78–7.22)	
Q4		1.70 (0.94–3.09)			1.68 (0.82–3.43)			1.97 (0.64–6.12)	
P for trend			0.048			0.146			0.227
Model 2									
Per SD		1.43 (1.13–1.80)	0.003		1.40 (1.03–1.90)	0.034		1.51 (1.06–2.16)	0.022
Across quartiles									
Q1		1 (reference)			1 (reference)			1 (reference)	
Q2		0.97 (0.51–1.85)			0.81 (0.37–1.78)			1.69 (0.54–5.27)	
Q3		1.37 (0.75–2.49)			0.94 (0.42–2.10)			2.46 (0.8–7.56)	
Q4		1.69 (0.92–3.10)			1.77 (0.84–3.72)			2.03 (0.65–6.36)	
P for trend			0.053			0.120			0.211

[a] An inverse normal transformation was applied to raw values of leucine, isoleucine, and valine, and a weighted sum of these 3 values was computed to calculate the BCAA score.
[b] Model 1 was adjusted for age (years), sex (male, female), and intervention group (except in analyses by intervention group). Model 2 was adjusted as for model 1, plus body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (METs min/day), and family history of premature coronary heart disease (yes, no).

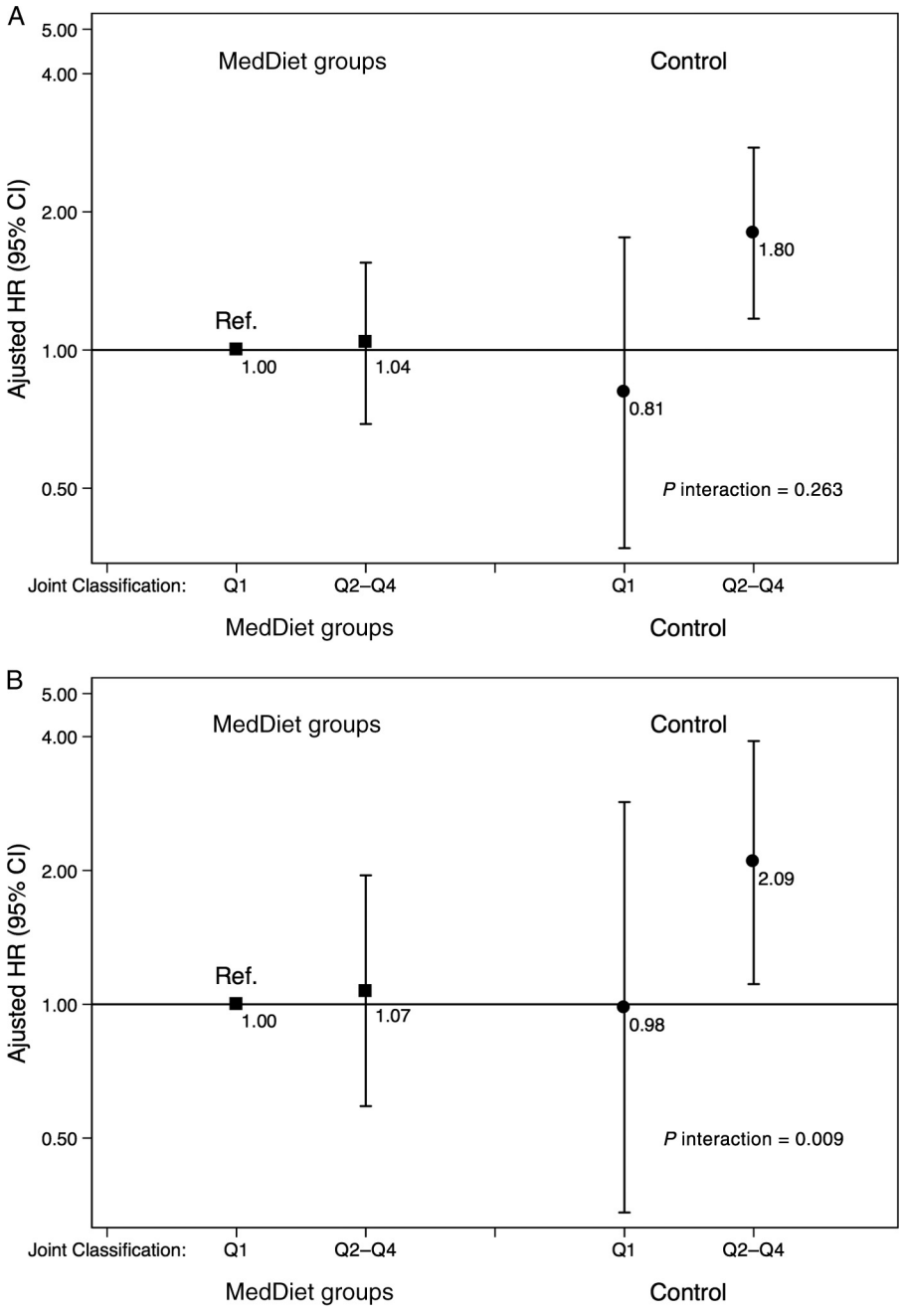

Fig. 1. Multivariate-adjusted HRs (95% CIs) of incident CVD and quartiles of BCAA score at baseline, stratified by intervention group (MedDiet versus control group).

(A), Composite CVD. (B), Stroke. An inverse normal transformation was applied to raw values of leucine, isoleucine, and valine, and a weighted sum of these 3 values was computed to calculate the BCAA score. HRs are adjusted for age (years), sex (male/female), intervention group, body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (METs min/day), and family history of premature coronary heart disease. *P* for interaction with 2 degrees of freedom between each MedDiet intervention group (EVOO and nuts) (binary, yes/no) and the BCAA score (continuous), with 2 cross-product terms (EVOO × BCAA and nuts × BCAA).

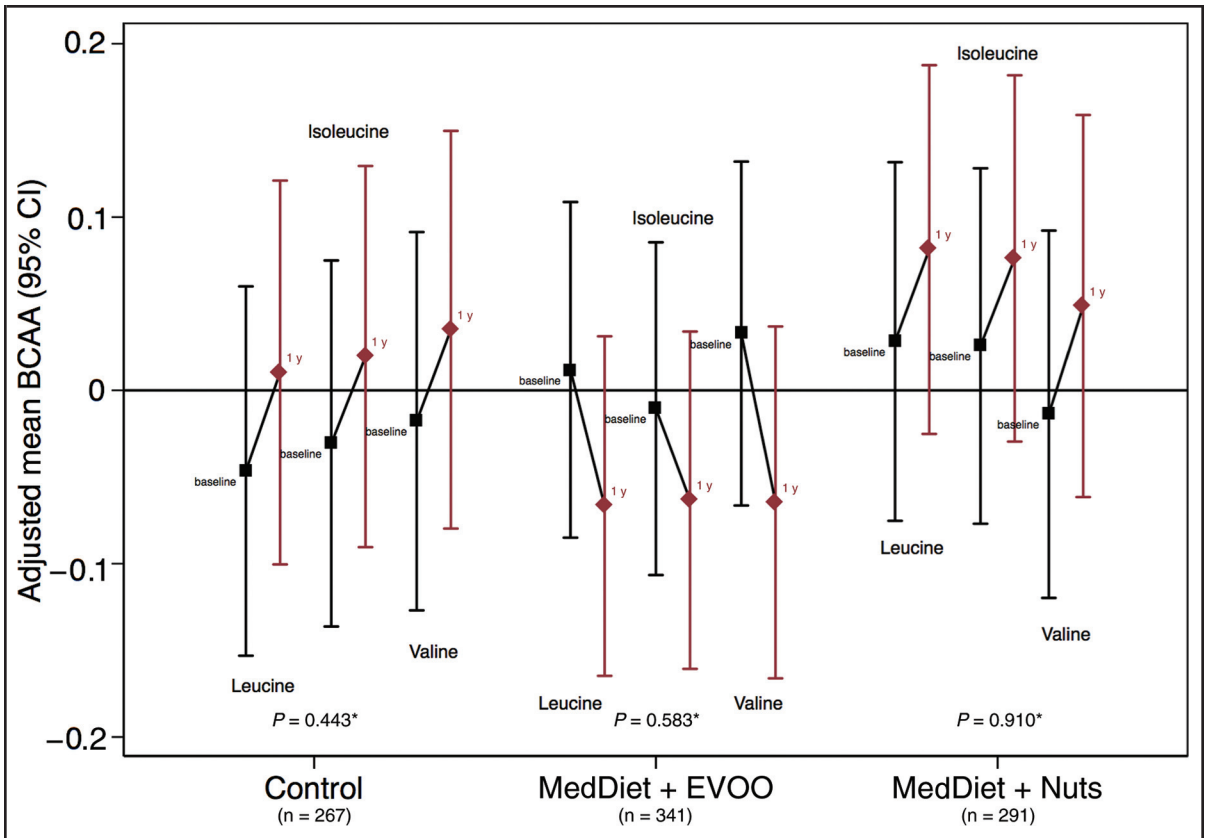

Fig. 2. Changes in leucine, isoleucine, and valine after 1 year of intervention, by intervention group.
Changes are adjusted for age (years), sex (male, female), and body mass index (kg/m^2). *Baseline means comparison between intervention groups.

and 1-year changes in the BCAA score ($P = 0.101$ and 0.729 for MedDiet + EVOO and MedDiet + nuts, respectively).

The magnitude of effects of the MedDiet intervention on CVD or stroke was unchanged after adjusting for baseline and 1-year change in BCAA score (see online Supplemental Table 8).

Discussion

In this case-cohort study of 970 participants from the PREDIMED trial, we found that baseline circulating BCAAs were positively associated with CVD and even more strongly associated with stroke. Our results suggest that the MedDiet interventions may counteract the deleterious associations between baseline BCAAs and risk of CVD and especially of stroke.

Our results are consistent with several previous studies of baseline BCAAs and CVD *(4, 6, 13)*; however, our study represents the first longitudinal assessment with repeated measurements of BCAA concentrations. Shah et al. *(4)* found a direct cross-sectional association between a principal component-derived factor with BCAAs and related catabolites and prevalent myocardial infarction. They also showed an association between this BCAA-related factor and coronary artery disease, an association that was also replicated in a nested case-control study *(13)*. Another case-control study observed a positive association between a score of 3 amino acids at baseline (tyrosine, phenylalanine, and isoleucine) and the risk of CVD *(6)*.

Interestingly, despite the increased CVD risk observed for baseline BCAAs, we found no association between 1-year changes in BCAAs and CVD risk. Further, our study was able to assess the interaction with a dietary intervention, and our results suggest that the reduction in CVD risk in participants receiving a MedDiet intervention was not explained by 1-year changes in BCAAs.

The health effect of BCAAs remains controversial. On the one hand, BCAA-rich diets or diets supplemented with BCAAs could improve metabolic health *(14)*, and they could have benefits in patients with heart

failure *(15)*. On the other hand, several studies have shown detrimental associations between high plasma values of BCAAs and cardiometabolic diseases *(16)*, especially obesity *(17)*, insulin resistance *(18)*, diabetes *(7)*, and carotid intima-media thickness *(19)*. Potential mechanisms underlying the effects of BCAAs are yet to be elucidated. High concentrations of BCAAs could lead to insulin resistance, but alternatively these high values could also represent a biomarker of a metabolic dysregulated status rather than an initiating event in the causal chain leading from dietary exposures to insulin resistance *(14)*. Current research suggests that the rise in circulating BCAAs is driven in part by an obesity-related decline in their catabolism in adipose tissue *(9)*. This defective BCAA catabolism leads to an accumulation of BCAAs and their intermediate products, inducing oxidative stress *(20)*. Specifically, leucine may have a role in the inhibition of NO synthesis in endothelial cells, and this reduced bioavailability contributes to the development of endothelial dysfunction *(21)*. Moreover, higher concentrations of BCAAs can lead to increased activity of mammalian target of rapamycin (mTOR) and, consequently, induce alterations in protein turnover, lipid/glucose/nucleotide metabolism, and autophagy regulation in the heart *(20)*.

With the exception of isoleucine, the associations became attenuated and nonsignificant when we additionally adjusted for diabetes or stratified by diabetic status. A similar change was observed when adjusting for hypertension and dyslipidemia (see online Supplemental Tables 2 and 3). However, we did not adjust our main estimates for type 2 diabetes because this would probably represent an overadjustment, given that type 2 diabetes may represent an intermediate step in the causal pathway.

A novel finding of our study is that a Mediterranean diet may offset the increased risk of CVD, and especially stroke, associated with high baseline concentrations of BCAAs. Given that the PREDIMED participants were at high risk of CVD at baseline, it seems plausible that BCAA concentrations were already abnormally high. Previous studies reported beneficial effects of a Mediterranean-style diet on obesity *(22)*, diabetes *(23)*, and cardiovascular risk factors *(24)*. In our study, the MedDiet interventions did not appear to significantly change BCAA concentrations, but participants with higher baseline BCAA scores had higher CVD risk, notably in the control group. These data and previous findings provide additional evidence to support the hypothesis that an intervention with a Mediterranean diet may counteract disease risk through changes in classic cardiovascular risk factors, but not directly on the potentially detrimental metabolic effects of increased concentrations of BCAAs. The benefits of a Mediterranean dietary pattern on stroke, and more specifically the potential protective effect of EVOO and nuts, are well known *(25–27)*. Our interpretation is that the overall Mediterranean pattern is more relevant to explain the risk reduction than just one of its components *(2)*.

Both basic science and epidemiologic studies are needed to increase our knowledge about the biological processes affected by altered BCAA homeostasis and its consequences on the development of CVD *(8)*. It is largely unknown how both systemic and local BCAA catabolism is impaired when the heart is under pathological stressors or during the acute phase of a CVD event *(20)*. In line with the stronger association found in our analyses limited to stroke, BCAAs are known to have a unique role in the brain. One hypothesis is that plasma BCAAs may provoke an increase in brain BCAAs and a decline in aromatic amino acids, and this may have functional consequences such as altered hormonal function and blood pressure *(28)*. Moreover, nuts are rich in arginine, and their antiatherogenic effect is in part connected with the arginine-NO pathway *(29)*. This effect may counteract the inhibition of leucine on NO synthesis from L-arginine *(21)*. However, a recent study found that diminished concentrations of BCAAs are present in patients with ischemic stroke *(30)*, and therefore, more research is needed to fully understand these complex biological mechanisms.

In both the control and the MedDiet + nuts intervention arms, we observed increased BCAA concentrations after 1 year of dietary intervention, in contrast to the MedDiet + EVOO group, in which a reduction was observed. However, none of these changes were statistically significant. This may seem counterintuitive (given that a reduced risk of CVD was obtained with the 2 MedDiet interventions) and therefore cast doubt on the use of changes in BCAAs as a relevant biomarker of the dietary interventions. On the other hand, given that the PREDIMED intervention was not specifically designed to modify the profile of amino acid intake, this result is not unexpected. The most likely explanation for our findings is that the MedDiet interventions did not appear to directly affect changes in BCAA concentrations, but they may nevertheless counteract the deleterious associations between baseline BCAAs and risk of CVD and especially of stroke, potentially via downstream pathways or alternative protective mechanisms *(2)*.

Several studies have assessed the association between dietary intakes and BCAA concentrations *(31–37)*. Two studies reported an immediate increase of plasma BCAA concentrations after consuming a Western-style diet *(31, 32)*. However, another study found increased BCAA content in a dietary pattern rich in fish (e.g., tuna), soybeans, cheese, chicken, and turkey *(33)*. Further research is needed to know the optimal quantity and quality of protein and amino acid intake to improve cardiometabolic health, especially among older people *(34)*. In contrast to previous findings, a recent study found no

correlation between long-term dietary intakes (estimated both with food frequency questionnaire and dietary records) and plasma concentrations of several amino acids, including BCAAs *(35)*. Those authors suggested that dietary intakes are probably not a major determinant of plasma BCAA concentrations *(35)*. However, because dietary protein from both animals and plants is an important source of BCAAs, dietary intake of protein quality and quantity should still be considered *(36)*. Our results may suggest that high baseline concentrations of BCAAs associated with higher cardiovascular risk might be mainly a biomarker of the underlying metabolic dysfunctions that can be partially independent of the quantity of BCAAs ingested with the diet.

Strengths of our study include the use of major cardiovascular events as main outcome, the prospective evaluation of the association between BCAA plasma concentrations and incident CVD, and the adjustment for multiple potential confounders associated with CVD within a well-characterized primary prevention trial. Several limitations should also be considered. First, we cannot rule out that BCAA concentrations were not equally distributed between missing and nonmissing samples in cases, but this number was relatively low (we assessed 226 of the original 288 cases). Second, although we included only 10% of noncases from the PREDIMED study, the case-cohort design retains randomization, maximizes the efficiency of a high-throughput metabolomic profiling, and enables the extension of our results to the full cohort. Third, our results may not be generalizable to other populations because all the study participants lived in a Mediterranean country and were at high cardiovascular risk.

In conclusion, our results indicate a direct association between higher concentrations of BCAAs at baseline and increased risk of CVD. This association was even stronger with stroke, but a Mediterranean-style diet appeared to offset the risk associated with increased BCAAs, especially when the diet was enriched with nuts. Our findings also suggest that the Mediterranean diet has a negligible effect of on 1-year changes in BCAAs; therefore, it likely exerts its cardioprotective effects via alternative pathophysiological processes.

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