Metabolites of Glutamate Metabolism Are Associated With Incident Cardiovascular Events in the PREDIMED PREvención con DIeta MEDiterránea (PREDIMED) Trial

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Background—Glutamate metabolism may play a role in the pathophysiology of cardiometabolic disorders. However, there is limited evidence of an association between glutamate-related metabolites and, moreover, changes in these metabolites, and risk of cardiovascular disease (CVD).

Methods and Results—Plasma levels of glutamate and glutamine were measured at baseline and 1-year follow-up in a case-cohort study including 980 participants (mean age 68 years; 46% male) from the PREvención con DIeta MEDiterránea (PREDIMED) randomized trial, which assessed a Mediterranean diet intervention in the primary prevention of CVD. During median 4.8 years of follow-up, there were 229 incident CVD events (nonfatal stroke, nonfatal myocardial infarction, or CVD death). In fully adjusted models, per 1-SD, baseline glutamate was associated with 43% (95% CI: 16% to 76%) and 81% (39% to 137%) increased risk of composite CVD and stroke alone, respectively, and baseline glutamine-to-glutamate ratio with 25% (6% to 40%) and 44% (25% to 58%) decreased risk of composite CVD and stroke alone, respectively. Associations appeared linear for stroke (both $P_{linear\ trend} \leq 0.005$). Among participants with high baseline glutamate, the interventions lowered CVD risk by 37% compared to the control diet; the intervention effects were not significant when baseline glutamate was low ($P_{interaction}=0.02$). No significant effect of the intervention on year-1 changes in metabolites was observed, and no effect of changes themselves on CVD risk was apparent.

Conclusions—Baseline glutamate was associated with increased CVD risk, particularly stroke, and glutamine-to-glutamate ratio was associated with decreased risk. Participants with high glutamate levels may obtain greater benefits from the Mediterranean diet than those with low levels.

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Cardiovascular disease (CVD) is the leading cause of mortality in the world.[1] Although CVD is to a large degree both predictable and preventable through identification and modification of traditional risk factors, its metabolic and etiologic pathways are not completely understood. Metabolites of glutamate (Glu) metabolism are related to cardiometabolic factors. Previous studies have suggested that glutamine (Gln)—a conditionally essential amino acid synthesized from Glu and ammonia by glutamine synthetase and produced mainly by muscle tissue[2]—enhances cardiac recovery in mice and human.[3,4] In cross-sectional studies, plasma Glu levels were associated with higher body mass index (BMI), blood pressure, and insulin resistance, whereas Gln levels or glutamine-to-glutamate ratio (Gln:Glu) was inversely associated with these parameters.[5-8] Higher circulating Gln:Glu ratios have also reportedly been associated with a reduced risk of type 2 diabetes mellitus.[2] Gln supplementation in humans and animals improves glucose homeostasis.[2,9-11] These studies suggest that Glu metabolites may play an important role in the pathophysiological mechanisms of CVD. They also can represent early biomarkers of CVD. However, evidence that relates these metabolites directly to CVD risk is limited. Moreover, previous studies measured Glu metabolites on only a single occasion and did not assess the effect of a randomized intervention on these metabolites.

Diet is 1 main source of circulating Gln and Glu; diet as well is 1 of the key modifiable factors in CVD development and prevention. Various clinical trials have assessed dietary modifications in CVD prevention, and they have shown improvements of cardiometabolic risk factors and reductions in the rate of clinical events.[12-14] However, the underlying mechanisms, especially with respect to the involved metabolic pathways, are not completely understood. Understanding the evolution of cardiometabolic morbidity, especially the pathophysiological mechanisms involving metabolic effects of dietary exposures, may be important in addressing CVD prevention. In this context, interest in the traditional Mediterranean diet has recently grown because it may represent the ideal model for cardiovascular health. A better appraisal of the mechanisms involved in the observed prevention of CVD events by a Mediterranean diet is needed.[15]

The present research evaluates the hypothesis that circulating levels of products of Glu metabolism are related to CVD risk in a prospective case-cohort study within the PREDIMED ("PREvención con DIeta MEDiterránea") trial, a well-characterized, large, randomized, controlled dietary intervention trial for the primary prevention of CVD. Secondarily, because the human metabolome responds to[15] and may interact with changes in diet, we tested the hypotheses that Mediterranean diet interventions influence changes in products of Glu metabolism and that the effects of these metabolites can be modified by the cardioprotective effects of the Mediterranean diet interventions.

Methods

Study Population and Design

The PREDIMED trial was a parallel-group, multicenter, randomized trial of dietary interventions with a Mediterranean diet supplemented with either nuts or extra-virgin olive oil for the primary prevention of CVD. The design and primary results of the trial have been published in detail.[14,16] In brief, eligible participants were men and women (55-80 years old) free of CVD at enrollment, who had either type 2 diabetes mellitus or at least 3 of the following major CVD risk factors: smoking, hypertension, elevated low-density lipoprotein cholesterol levels, low high-density lipoprotein cholesterol levels, overweight or obesity, or a family history of premature coronary heart disease. Beginning on October 1, 2003, participants were randomly assigned, in a 1:1:1 ratio, to 1 of 3 dietary intervention groups: a Mediterranean diet (MedDiet) supplemented with extra-virgin olive oil (MedDiet+EVOO), a Mediterranean diet supplemented with mixed nuts (MedDiet+nuts), or a control diet in which the participants were advised to reduce all types of fat intake. The primary endpoint was composite CVD, defined as nonfatal stroke, nonfatal myocardial infarction, or death from cardiovascular causes. During a median follow-up time of 4.8 years (study end on December 1, 2010), 288 incident CVD events occurred.

We designed a case-cohort study[17] within the PREDIMED trial. Consistent with the case-cohort design, we included a random, nonstratified sample of ~10% of PREDIMED participants at baseline ("subcohort members," N=788, including 751 "noncases" and 37 "incident internal cases") in addition to all other incident CVD cases ("incident external cases" who were not "subcohort members," N=192), for whom blood samples were available (see Figure S1). Given the random sampling of the study design, results of case-cohort studies are generalizable to the entire cohort without a need to ascertain the whole trial population.[18]

Our protocol included the specific assessment of the following hypotheses on a potential relationship with CVD for the following metabolites: (1) branched-chain and aromatic amino acids; (2) the glutamine-cycling pathway; (3) small- and medium-chain acylcarnitines; (4) gut flora metabolites such as choline and betaine; (5) urea-cycle metabolites; and (6) lipids. The associations with branched-chain amino acids (BCAAs) and acylcarnitines have already been published.[19,20] Here, we present the results for the second hypothesis, which relates to the glutamine-cycling pathway. The trial protocol was approved by the Institutional Review Boards at participating institutions across Spain; the present study

protocol was approved by the Institutional Review Boards at all study locations involved (eg, at institutions across Spain and at the Harvard T. H. Chan School of Public Health in Boston, MA). All study participants provided written, informed consent.

Measurement of Metabolites

Fasting blood samples were collected by PREDIMED nurses 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. Pairs of samples (baseline and first-year visit samples from each participant) were randomly ordered and shipped on dry ice to the Broad Institute for metabolomics analyses.

Amino acids, including Glu and Gln, were profiled using liquid chromatography–tandem mass spectrometry (LC-MS) on a system comprised of a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp, Marlborough, MA) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Metabolite identities were confirmed using authentic reference standards. Raw data were processed using TraceFinder software (Thermo Fisher Scientific, Waltham, MA) and Progenesis QI (Nonlinear Dynamics, Newcastle upon Tyne, UK). The detailed mass spectrometry setting was previously described.[20] The analytical coefficients of variation for glutamine and glutamate measurements were both 0.04.

Case Ascertainment

The PREDIMED primary endpoint was the incidence of composite CVD, consisting of nonfatal stroke, nonfatal myocardial infarction, or cardiovascular death. Information on primary endpoints was collected by study physicians who were blinded to the intervention and from other sources of information, such as the National Death Index. This anonymized information was sent to the Clinical Endpoint Committee, which adjudicated the events blinded to the intervention groups. The primary outcome in the present study was composite CVD as defined above, and our secondary outcome was limited to the incidence of nonfatal stroke, because stroke was the most common CVD endpoint (48%) in PREDIMED participants.

Covariate Assessment

Medical conditions and risk factors were collected using a questionnaire during the first screening visit. Weight, height, other anthropometric measures, and blood pressure were directly measured by the PREDIMED nurses. A general medical questionnaire was administered on a yearly basis regarding participant lifestyle factors such as smoking, family history of CVD, incident diseases, and dietary intake.[14] Diet was assessed by trained dietitians who administered a 137-item validated semiquantitative food frequency questionnaire in a face-to-face interview.[21,22] Energy and nutrient intakes were computed using food composition tables.[23-25] Participants also completed a 14-item dietary screener to assess adherence to the MedDiet.[26]

Statistical Analysis

Rank-based inverse-normal transformations were used to transform the markedly nonnormal distributions of our primary exposures: circulating levels of Gln and Glu.[27] We also examined Gln:Glu as a metabolite trait (by dividing the raw values and then taking inverse normal transformations) because the ratio has been related to metabolic risk,[2] and Gln and Glu are in a precursor-product relationship.

Baseline data by case status were presented as means (±standard deviations) for continuous variables and as N and percentages for categorical variables. Baseline characteristics were compared between cases and noncases using chi-squared tests for categorical variables and t tests for continuous variables.

Our statistical approach sought to answer several questions: (1) Are baseline levels of these metabolites (Gln, Glu, and their ratio) or their year-1 changes associated with the risk of CVD clinical events? (2) Do the MedDiet interventions change metabolite levels after 1 year? (3) Do metabolites modify the effect of the MedDiet interventions on CVD risk? To examine the first question—the relationship of baseline metabolites and first-year changes in metabolite levels with the risk of CVD events—we developed Cox proportional hazards models adjusted for demographic and clinical characteristics: in model 1, we adjusted for age (years), sex (male/female), family history of CVD (yes/no), smoking status (never, former, or current smoker), and body mass index (BMI, kg/m^2) and stratified by intervention group (MedDiet+EVOO, MedDiet+nuts, and control diet); in model 2, we further adjusted for baseline disease status, specifically baseline hypertension, dyslipidemia, and diabetes mellitus (yes/no for all). Because previous findings have suggested that circulating BCAAs are highly related to cardiometabolic risk,[28] we considered an additional model that further adjusted model 2 for total circulating BCAAs (as the sum of untransformed levels of leucine, isoleucine, and valine, followed by inverse normal transformation). There were moderate correlations at baseline between plasma BCAAs and Glu metabolites among subcohort members (Pearson partial correlation coefficients with adjustment for age, sex, and BMI ranged from −0.32 to 0.36).

The Glu metabolites were analyzed both as continuous variables and in quartile categories (using cut-points defined from the quartile values among noncases). We conducted tests of linear trend across increasing quartiles by using the median value for each quartile and fitting this as a continuous variable in the models.

To assess the second question, we compared the mean changes in metabolites from baseline to 1 year in the MedDiet intervention groups with changes in the control group. For the third question, we assessed whether baseline levels of metabolites modified the cardioprotective effects of MedDiets by introducing in the fully adjusted Cox models (as described above) a multiplicative interaction term between the baseline metabolite level (continuous) and the MedDiet intervention groups (combined intervention groups vs control).

In all Cox models, Barlow weights were used to account for oversampling of cases in the study design.[17] Noncases in the subcohort were weighted inversely proportionally to the sampling fraction (ie, in our study, the weight was 10). For noncases, observation time began on the date of their randomization and ended on their date of death (from noncardiovascular causes), the study end date (December 1, 2010), or the date of the last medical visit (if they had stopped attending scheduled intervention appointments), whichever came first. Cases had a weight of 1 at the instant the individual experienced an endpoint regardless of subcohort membership, and observation time was the instant when the participant developed CVD. "Incident internal cases" were treated as noncases (observation time began on the date that they were randomized and ended the instant the individual failed) until they became cases, and at such instant their weight changed. We used a risk-set approach and a robust variance estimator for the multivariate modeling.[29] We examined the possibly nonlinear relation between metabolites and CVD risk nonparametrically with restricted cubic splines in a multivariable logistic regression model. Tests for nonlinearity used the likelihood ratio test, comparing the model with only the linear term to the model with the linear and the cubic spline terms.

For the secondary outcome of incident stroke alone, we conducted analyses both including and excluding the non-stroke CVD cases (ie, keeping the 113 nonstroke CVD cases and treating them as noncases or deleting them from analyses) and found the results were similar. Therefore, we present the stroke results only after deleting the nonstroke CVD cases.

A *P*<0.05 was considered statistically significant. All statistical analyses were performed in SAS (v9.4, SAS Institute, Cary, NC) and R (v2.13.0, R Foundation, Vienna, Austria).

Results

Characteristics of the participants at baseline by quartiles of Gln:Glu are shown in Table 1. The participants in our study were, by design, middle-aged to elderly, generally overweight, and most had hypertension, dyslipidemia, or diabetes mellitus at baseline. Participants who had a lower baseline level of Gln:Glu were more likely to be male, diabetic, current smokers, and they tended to weigh more, compared to noncases.

Association of Baseline Metabolites and Changes in Metabolites With Incident CVD

Table 2 shows the association of baseline levels of metabolites with incident CVD. In the unadjusted model, baseline levels of Glu were significantly associated with increased risk; that of Gln:Glu was associated with decreased CVD risk, whether metabolites were modeled continuously (per 1 SD) or as quartiles; Gln as a continuous variable was not associated with CVD risk and as quartiles related to CVD risk. Adjustment of baseline demographic factors (model 1) attenuated the results somehow. After additional adjustment for baseline disease status (model 2), per 1 SD, baseline Glu was associated with 43% increased CVD risk (*P*=0.0008); participants in the highest quartile of baseline Glu exhibited a nonsignificantly increased risk of CVD compared to those in the lowest quartile (HR Q4 vs Q1 [95% CI] 1.39 [0.84-2.31], P_{trend}=0.06). Per 1 SD, baseline Gln:Glu was significantly associated with 25% decreased risk of CVD (*P*=0.01); participants in the highest quartile of the baseline Gln:Glu ratio showed a nonsignificant 26% decreased risk of CVD compared to those in the lowest quartile (HR Q4 vs Q1 [95% CI] 0.74 [0.44-1.22], P_{trend}=0.16). The associations of the metabolites with the composite CVD endpoint appeared nonlinear (model 2 in Table 2, *P* values for linear trend across quartiles of Gln, Glu, and Gln:Glu=0.93, 0.06, and 0.16, respectively). Spline analysis (Figure S2) suggested nonlinear and significant associations between these metabolites and CVD risk (*P* value for nonlinearity with CVD risk of Gln was 0.002, of Glu was 0.001, and of Gln:Glu <0.001, and *P* value for overall association of Gln was 0.003, of Glu <0.001, and of Gln:Glu <0.001).

There was no evidence of interaction between sex and the baseline metabolites on incident CVD (*P* values for interaction >0.05). Additional adjustment of model 2 for circulating BCAAs did not materially change the results (Table S1).

Baseline levels of metabolites were more strongly and linearly associated with the secondary stroke endpoint alone than with the composite CVD endpoint (Table 3). In model 1, baseline Glu levels were significantly associated with

Table 1. Participant Characteristics at Baseline by Quartiles of Glutamine-to-Glutamate Ratio Among 980 Spanish Participants of the PREDIMED Trial

	Q1 (Low)	Q2	Q3	Q4 (High)*	P Value[†]
N	277	231	240	232	
Age, y	67.03±6.2	67.55±6.0	68.34±6.0	67.51±6.1	0.16
Male, n (%)	148 (53.4)	113 (48.9)	110 (45.8)	81 (34.9)	<0.001
BMI, kg/m^2	30.67±3.6	29.77±3.5	29.59±3.5	28.74±3.7	<0.001
Hypertension, n (%)	228 (82.3)	187 (81.0)	202 (84.2)	200 (86.2)	0.45
Dyslipidemia, n (%)	186 (67.2)	169 (73.2)	174 (72.5)	163 (70.3)	0.43
Diabetes mellitus, n (%)	164 (59.2)	131 (56.7)	110 (45.8)	89 (38.4)	<0.001
Family history of premature CHD, n (%)	64 (23.1)	57 (24.7)	56 (23.3)	60 (25.9)	0.88
Current smoker, n (%)	41 (14.8)	36 (15.6)	39 (16.3)	23 (9.9)	0.01
Intervention group					
MedDiet+EVOO	106 (38.3)	79 (34.2)	80 (33.3)	98 (42.2)	0.32
MedDiet+nuts	88 (31.8)	77 (33.3)	87 (36.3)	62 (26.7)	
Control	83 (30.0)	75 (32.5)	73 (30.4)	72 (31.0)	

For continuous variables, means±standard deviations are shown. For categorical variables, N and percentages are shown. BMI indicates body mass index; CHD, coronary heart disease; CVD, cardiovascular disease; EVOO, extra-virgin olive oil; MedDiet, Mediterranean diet intervention group; PREDIMED, Prevención con Dieta Mediterránea.

*Q1 to Q4: quartiles (using cut-points defined from the quartile values among noncases).

[†]P value is derived from either linear regression or chi-squared test for the association of characteristics with quartile groups of metabolite.

increased stroke risk, and baseline levels of Gln and Gln:Glu were significantly associated with decreased stroke risk. Further adjustment for disease status attenuated the association of Gln with stroke risk to nonsignificance, but the associations of Glu and Gln:Glu with stroke risk remained. Per 1 SD, baseline Glu was associated with 81% increased risk of stroke ($P=1.4\times10^{-5}$); participants in the highest quartile of baseline Glu had 3 times the risk of stroke of those in the lowest quartile (HR Q4 vs Q1 [95% CI] 2.95 [1.44–6.03], $P_{trend}=0.0008$). Per 1-SD increment in baseline Gln:Glu, there was a significant 44% decreased risk of stroke ($P=9.5\times10^{-5}$); participants in the highest quartile of the Gln:Glu ratio had less than half the risk of those in the lowest quartile (HR Q4 vs Q1 [95% CI] 0.37 [0.18–0.76], $P_{trend}=0.005$).

There were no statistically significant associations of first-year changes in metabolites, adjusted for baseline levels, with the risk of either composite CVD or stroke alone (Table S2). Therefore, there was no basis to assess the hypothesis that changes in these metabolites were potential mediators for the effect of the intervention.

Effects of Dietary Interventions on Changes in Metabolite Levels

There were no statistically significant differences in mean first-year changes of metabolites between intervention and control groups (Figure).

Modifying Effects of Metabolite Levels on Dietary Interventions in Incident CVD

Effect modification by baseline Glu of the combined MedDiet interventions was statistically significant in model 2 (P for interaction=0.02) when we used the metabolites as continuous variables in the interaction product term (1 *df*). This suggests a greater protection against CVD by the MedDiet among participants with higher baseline Glu levels than among those with lower Glu levels. The combined MedDiet interventions lowered CVD risk by 37% (HR [95% CI] 0.63 [0.40–0.99]) compared to the control diet among participants with baseline Glu levels above the median; whereas the interventions' cardioprotective effects among participants with baseline Glu levels below the median were not significant (HR [95% CI] 0.65 [0.37–1.12]). Figure S3 shows the joint association of baseline Glu level and dietary intervention with incident CVD risk. The common reference category in this figure included participants in the control group with baseline Glu levels above the median. The strongest reduction in risk was observed for participants in both MedDiet groups (merged together) with low baseline levels of Glu. The other 2 categories (control group and low Glu levels; MedDiet and high Glu levels) presented an intermediate risk. Results were consistent after further adjusting for 1-year changes in Glu and when we limited the analyses to the secondary outcome including only stroke cases. For the other 2 metabolite traits (baseline Gln and the Gln:Glu ratio), there were no statistically

Table 2. Relative Risk of Incident Composite CVD by Baseline Glutamate-Related Metabolites: Hazard Ratios (95% CI) for Metabolite Traits as Continuous Variables and by Quartile Levels of Metabolite Traits (N=980)

	Glutamine	Glutamate	Glutamine-to-Glutamate Ratio
Unadjusted model			
Metabolite as continuous variable			
Hazard ratio (95% CI) per SD*	0.92 (0.76–1.10)	1.56 (1.30–1.86)	0.68 (0.57–0.83)
P value	0.36	<0.0001	0.0001
Metabolite in quartile categories,† as compared to Q1 (reference)			
Q2	0.78 (0.52–1.19)	1.09 (0.68–1.74)	0.51 (0.34–0.77)
Q3	0.93 (0.62–1.40)	1.31 (0.83–2.06)	0.58 (0.39–0.86)
Q4	0.93 (0.62–1.39)	2.09 (1.36–3.23)	0.51 (0.34–0.78)
P for trend	0.04	0.0004	0.002
Model 1			
Metabolite as continuous variable			
Hazard ratio (95% CI) per SD*	0.90 (0.74–1.09)	1.48 (1.22–1.81)	0.71 (0.57–0.88)
P value	0.27	0.0001	0.002
Metabolite in quartile categories,† as compared to Q1 (reference)			
Q2	0.64 (0.41–1.00)	0.83 (0.50–1.37)	0.51 (0.33–0.79)
Q3	0.85 (0.55–1.32)	1.04 (0.64–1.69)	0.55 (0.36–0.84)
Q4	0.85 (0.55–1.31)	1.60 (1.00–2.60)	0.64 (0.40–1.03)
P for trend	0.69	0.01	0.03
Model 2			
Metabolite as continuous variable			
Hazard ratio (95% CI) per SD*	0.94 (0.77–1.14)	1.43 (1.16–1.76)	0.75 (0.60–0.94)
P value	0.51	0.0008	0.01
Metabolite in quartile categories,† as compared to Q1 (reference)			
Q2	0.74 (0.47–1.17)	0.78 (0.47–1.30)	0.56 (0.36–0.89)
Q3	1.03 (0.65–1.64)	0.97 (0.59–1.59)	0.65 (0.42–1.00)
Q4	0.94 (0.60–1.48)	1.39 (0.84–2.31)	0.74 (0.44–1.22)
P for trend	0.93	0.06	0.16

Q1 to Q4: quartiles. Model 1 was adjusted for age, sex, family history of CHD, smoking status, and body mass index and was stratified by intervention group. Model 2 was adjusted as for model 1 plus baseline hypertension, dyslipidemia, and diabetes mellitus.

*The SD for glutamine was 202 µmol/L, and that for glutamate was 90 µmol/L in our study population.

†The corresponding medians of quartiles for glutamate were 71.8, 98.2, 114.6, and 150.2 µmol/L, respectively; for glutamine 656.7, 744.1, 795.0, and 891.3 µmol/L, respectively; and for glutamine-to-glutamate ratio 4.9, 6.6, 8.3, 10.8, respectively.

significant interactions with the MedDiet interventions on risk of CVD or stroke alone (*P* values for interaction=0.42 and 0.18 for CVD, respectively; 0.78 and 0.08 for stroke, respectively).

Discussion

In this case-cohort study embedded in a randomized, controlled dietary intervention trial, we found that Glu levels were associated with higher risk and that Gln:Glu was associated with lower risk of CVD. These associations were more pronounced and linear when assessed against the risk of stroke alone as opposed to all CVD events combined. Our results suggest that participants with higher circulating glutamate levels might especially benefit from Mediterranean diets. However, the intervention diets did not significantly change these metabolite levels.

The present research adds cardiovascular endpoints to the evidence from prior smaller studies, which have primarily investigated cardiometabolic risk factors.[2] A recent study investigated the metabolomic profiling in the Framingham Heart Study (FHS) (n=1015) and replicated the findings in the

Table 3. Relative Risk of Incident Stroke by Baseline Glutamate-Related Metabolites: Hazard Ratios (95% CI) for Metabolites as Continuous Variables and by Quartile Levels of Metabolites (N=869)

	Glutamine	Glutamate	Glutamine-to-Glutamate Ratio
Unadjusted model			
Metabolite as continuous variable			
Hazard ratio (95% CI) per SD*	0.76 (0.59-0.97)	1.80 (1.42-2.29)	0.56 (0.44-0.72)
P value	0.03	1.2×10^{-6}	6.2×10^{-6}
Metabolite in quartile categories,[†] as compared to Q1 (reference)			
Q2	0.59 (0.34-1.02)	1.70 (0.85-3.39)	0.51 (0.30-0.86)
Q3	0.83 (0.50-1.37)	2.12 (1.09-4.15)	0.53 (0.32-0.88)
Q4	0.53 (0.30-0.94)	3.43 (1.81-6.50)	0.32 (0.18-0.59)
P for trend	0.06	<0.0001	0.0002
Model 1			
Metabolite as continuous variable			
Hazard ratio (95% CI) per SD*	0.73 (0.57-0.95)	1.87 (1.45-2.43)	0.53 (0.41-0.70)
P value	0.02	2.2×10^{-6}	7.6×10^{-6}
Metabolite in quartile categories,[†] as compared to Q1 (reference)			
Q2	0.51 (0.28-0.91)	1.54 (0.76-3.13)	0.48 (0.27-0.84)
Q3	0.77 (0.46-1.31)	2.06 (1.04-4.09)	0.46 (0.27-0.79)
Q4	0.48 (0.26-0.86)	3.37 (1.69-6.72)	0.32 (0.16-0.63)
P for trend	0.04	0.0001	0.0005
Model 2			
Metabolite as continuous variable			
Hazard ratio (95% CI) per SD*	0.79 (0.62-1.01)	1.81 (1.39-2.37)	0.56 (0.42-0.75)
P value	0.06	1.4×10^{-5}	9.5×10^{-5}
Metabolite in quartile categories,[†] as compared to Q1 (reference)			
Q2	0.60 (0.33-1.11)	1.40 (0.67-2.89)	0.51 (0.28-0.92)
Q3	0.97 (0.56-1.70)	1.92 (0.95-3.87)	0.54 (0.31-0.93)
Q4	0.55 (0.30-1.02)	2.95 (1.44-6.03)	0.37 (0.18-0.76)
P for trend	0.14	0.0008	0.005

Nonstroke cases were excluded. Q1 to Q4: quartiles. Model 1 was adjusted for age, sex, family history of CHD, smoking status, and body mass index and was stratified by intervention group. Model 2 was adjusted as for model 1 plus baseline hypertension, dyslipidemia, and diabetes mellitus.

*The SD for glutamine was 202 μmol/L, and that for glutamate was 90 μmol/L in our study population.

[†]The corresponding medians of quartiles for glutamate were 71.8, 98.2, 114.6, and 150.2 μmol/L, respectively; for glutamine 656.7, 744.1, 795.0, and 891.3 μmol/L, respectively; and for glutamine-to-glutamate ratio 4.9, 6.6, 8.3, 10.8, respectively.

Malmö Diet and Cancer Study (MDC) (n=746). The main replicated findings included that circulating Glu levels were positively related to a wide range of cardiometabolic risk factors including BMI, waist circumference, fasting glucose, insulin, insulin resistance, and triglycerides and inversely related to high-density lipoprotein cholesterol, whereas circulating levels of Gln and the Gln:Glu exhibited opposite associations with these cardiometabolic risk factors.[2] In addition, in a subsample of the Framingham Heart Study Offspring cohort, the Gln:Glu ratio was associated with lower risk of incident type 2 diabetes mellitus.[2] Our population is comparable to those from the abovementioned studies, even though both FHS and MDC metabolomics studies were nested case-control studies whereas ours is a case-cohort design. That our results were more significant for stroke as compared to CVD was not unexpected; stroke is a more homogeneous endpoint than composite CVD, and among the PREDIMED cases, stroke was the single most common cardiovascular event.

These consistent associations between metabolites in the Glu metabolism pathway further support the hypothesis that Glu-cycling pathways are prominently involved in the development of cardiometabolic risk and, in our study, in CVD and especially stroke. Our results also add evidence for the consideration of these metabolites as early markers of
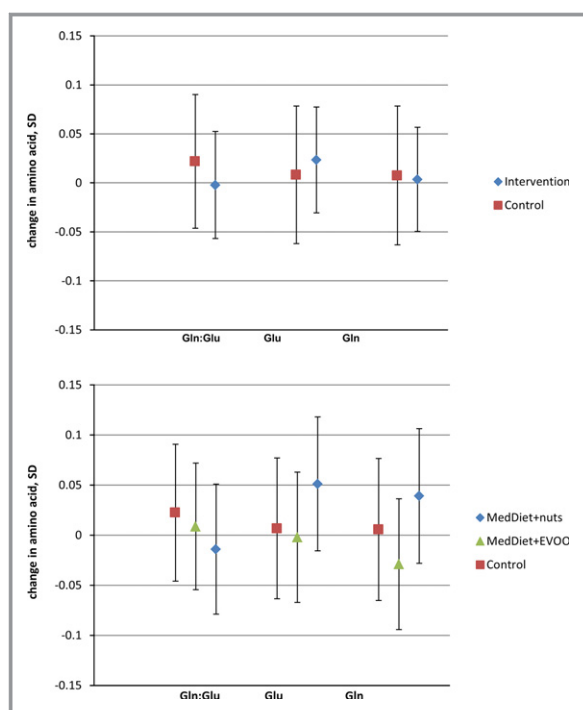
Figure. Mean changes in metabolites from baseline to 1 year of intervention, by intervention group. No significant difference was observed in least-squares means of 1-year changes of metabolite or ratio levels between intervention groups, combined or separately, and the control group (all *P*>0.1). One-year change in a given metabolite trait was calculated as the inverse-normal transformed difference between the year-1 raw value and the baseline raw value, among all participants with baseline and year-1 values (N=927). The SD for glutamine change was 173 μmol/L, and that for glutamate change was 78 μmol/L, in our study population. EVOO indicates extra-virgin olive oil.

disease. Gln and Glu are transformed into each other as part of numerous physiological processes. Cycling between Gln and Glu is regulated by the activity of glutamine synthetase and glutaminase, enzymes that have wide tissue distribution.[30] The proposed mechanisms underlying the beneficial effects of Gln include a wide range of metabolic pathways, such as stimulation of insulin secretion via enhancing release of glucagon-like peptide 1 and improvement in insulin sensitivity.[9,31,32] Furthermore, Gln has a regulatory capacity in immune cell modulation and has antiobesity and antidiabetic effects.[33] However, the potential mechanisms of the adverse effects of Glu on cardiometabolic factors are less clear. Both Glu and Gln are nonessential amino acids, and the association of dietary intakes of these amino acids with CVD risk is less studied.

To our knowledge, no study has previously reported on changes in circulating Gln or Glu in relation to incident CVD. Given that our study population already had high baseline risk for CVD, the high and low levels of Glu and Gln, respectively, likely already reflected an unfavorable metabolic state, which

may be 1 reason why changes in metabolite levels were not significantly associated with CVD risk when adjusted for the baseline levels.

In addition to the repeated measurements of metabolites, another important strength of our study is the assessment of potential effect modification by dietary intervention. Our results suggest that participants with higher baseline Glu levels might particularly benefit from MedDiets in preventing incident CVD compared to their lower-Glu counterparts. In other words, the greater the dysfunction in Glu metabolism, the more prominent the effects of changing the diet will be on downstream disease events. This statistically significant effect modification is important, and it may have a relevant biological meaning. It is known that the type and amount of fat in the diet not only modify blood lipid concentrations but also change the cell membrane lipid composition. Therefore, we suspect that the mechanism of such effect modification could be through changes in the composition and physical properties of the cell membranes by the intervention with the Mediterranean diets, which are dietary patterns high in total fat and in unsaturated fat. This dietary intervention might have led to alterations in the activities of membrane-bound enzymes and carriers for glutamate metabolism.

Several limitations of the present study warrant mention. First, our case-cohort study shares the limitations of the original PREDIMED study, such as the limited generalizability of the study's conclusion. However, the present results can be extended to all trial participants owing to the efficiency of the case-cohort design; direct biomarker measurements of the entire population would be cost-prohibitive. In addition, the LC-MS-based metabolite measurements together with the inverse-normal transformation may not have a direct clinical translation for each metabolite trait. Third, we used a single measure of metabolites at each of 2 time points, at baseline and at year-1 visits. Using a single measurement relies on the assumption that individual metabolite measurements are consistent over a medium-term period, which may not hold in reality. However, at least 1 previous study reported that both circulating Glu and Gln were reproducible within individuals over 1 to 2 years (intraclass correlations=0.6) using the same LC-MS platform as in the present study.[34]

In summary, in this prospective study of older men and women with high cardiovascular risk, our results suggest that baseline levels of circulating Glu are related to increased cardiovascular risk and that the ratio Gln:Glu is related to decreased risk, especially with regard to stroke risk.

Acknowledgments

Disclosures

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SUPPLEMENTAL MATERIAL

Table S1. Relative risk of incident composite CVD by baseline glutamate-related metabolites: hazard ratios (95% CI) for metabolite traits as continuous variables and by quartile levels of metabolite traits (N=980) with adjustment for branch-chained amino acids.

	Glutamine	Glutamate	Glutamine-to-Glutamate ratio
Metabolite as continuous variable			
Hazard Ratio (95%CI) per S.D.*	0.93 (0.77–1.14)	1.44 (1.17–1.79)	0.75 (0.60–0.94)
P	0.49	0.0008	0.01
Metabolite in quartile categories, as compared to Q1 (reference)**			
Q2	0.74 (0.47–1.17)	0.77 (0.46–1.30)	0.57 (0.36–0.90)
Q3	1.02 (0.65–1.63)	0.95 (0.56–1.59)	0.66 (0.43–1.03)
Q4	0.94 (0.60–1.48)	1.35 (0.80–2.29)	0.78 (0.46–1.31)
P for trend	0.95	0.10	0.23

Q1 to Q4: quartiles.

Model was adjusted for age, sex, family history of CHD, smoking status, body mass index plus baseline hypertension, dyslipidemia, and diabetes, and baseline levels of branch-chained amino acids, and was stratified by intervention group.

* The S.D. for glutamine was 202 μmol/L, and for glutamate was 90 μmol/L in our study population.

** The corresponding medians of quartiles for glutamate were 71.8, 98.2, 114.6, and 150.2 μmol/L, respectively; for glutamine 656.7, 744.1, 795.0 and 891.3 μmol/L, respectively; and for Glutamine-to-Glutamate ratio 4.9, 6.6, 8.3, 10.8, respectively.
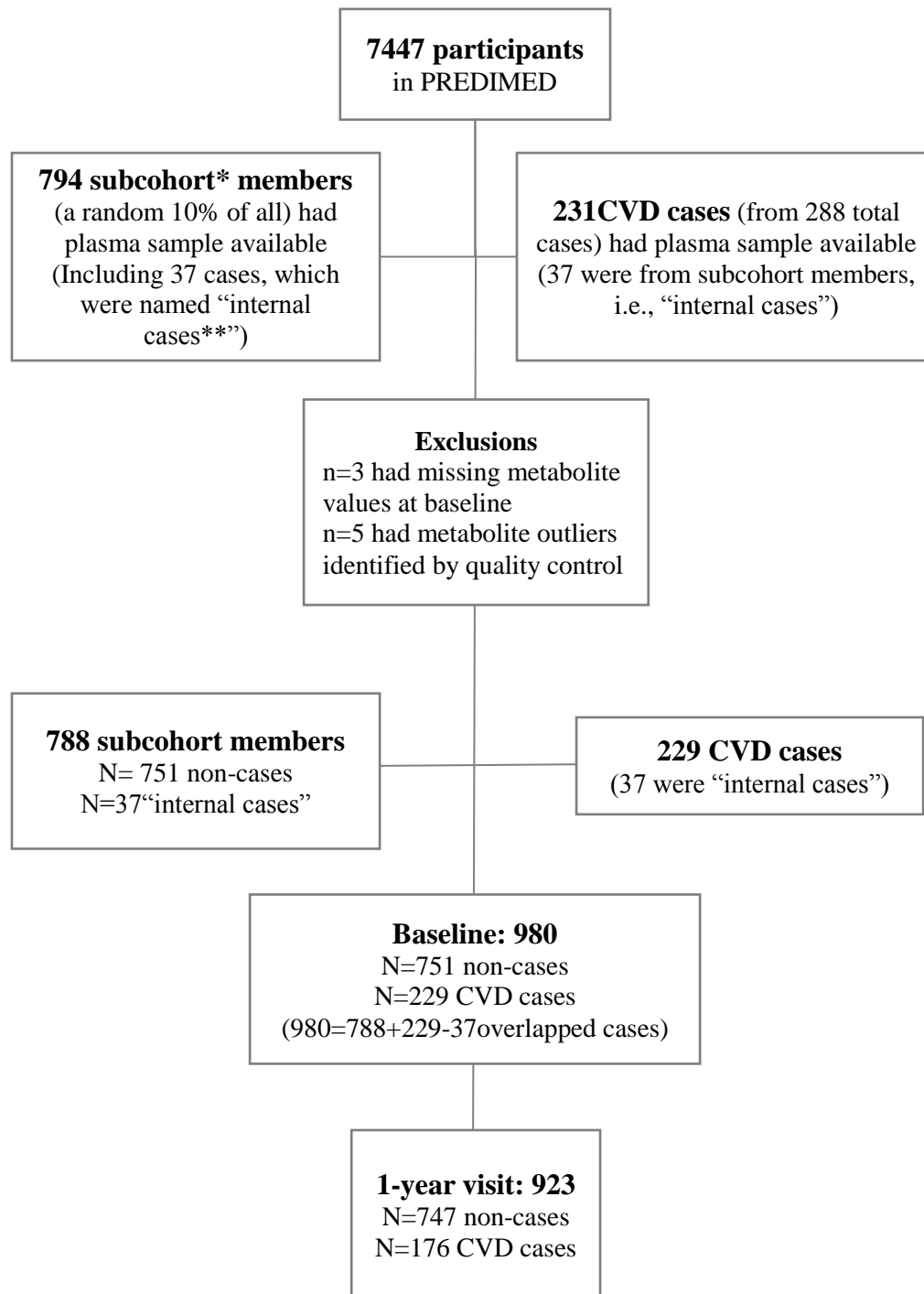
Table S2. Relationship between changes in metabolites from baseline to 1-year follow-up and

the risk of composite CVD and stroke, The PREDIMED trial.

	Y1 change in Glutamine	Y1 change in Glutamate	Y1 change in Glutamine-to-Glutamate ratio
Incident CVD			
Hazard Ratio per S.D.	0.96 (0.74 – 1.24)	0.97 (0.78 – 1.21)	1.09 (0.86 – 1.38)
P	0.72	0.80	0.49
Incident stroke			
Hazard Ratio per S.D.	1.04 (0.75 – 1.42)	0.91 (0.71 – 1.17)	1.14 (0.85– 1.54)
P	0.83	0.45	0.38

*The changes in metabolites were the exposure variables. Models were adjusted for age, sex,

family history of CHD, smoking status, body mass index, baseline hypertension, dyslipidemia,

diabetes, and respective baseline metabolite levels; stratified by intervention group. The analysis

protocols were the same as that in Tables 2 and 3.

Both baseline values and year-1 changes were inverse-normal transformed and treated as

continuous variables. Tests of interaction between the baseline and year-1 values of the

metabolites were not significant, and not retained in the models. The presented results were

therefore based on models that excluded the multiplicative interaction term. All analyses

excluded participants who developed CVD before the first year follow-up visit.

Figure S1. Flowchart of the selection process of study participants.

```
                        ┌─────────────────────┐
                        │  7447 participants  │
                        │   in PREDIMED       │
                        └─────────────────────┘
```

7447 participants
in PREDIMED

794 subcohort* members
(a random 10% of all) had
plasma sample available
(Including 37 cases, which
were named "internal
cases**")

231CVD cases (from 288 total
cases) had plasma sample available
(37 were from subcohort members,
i.e., "internal cases")

Exclusions
n=3 had missing metabolite
values at baseline
n=5 had metabolite outliers
identified by quality control

788 subcohort members
N= 751 non-cases
N=37"internal cases"

229 CVD cases
(37 were "internal cases")

Baseline: 980
N=751 non-cases
N=229 CVD cases
(980=788+229-37overlapped cases)

1-year visit: 923
N=747 non-cases
N=176 CVD cases

*subcohort: a random, non-stratified sample of approximately 10% of PREDIMED participants at
baseline.
** incident cases which were subcohort members.

Figure S2. Spline of levels of glutamate-related metabolites and incident CVD. Dotted lines are 95% confidence intervals of the spline; horizon line is the reference. We used logistic regression to analyze the spline analysis of the association between metabolites and CVD, because results from logistic regression approximate those from Cox proportional hazards regression in general,[1,2] and the use of a Cox model for spline analysis in the setting of a case-cohort design is not well established.

Glutamine level. SD

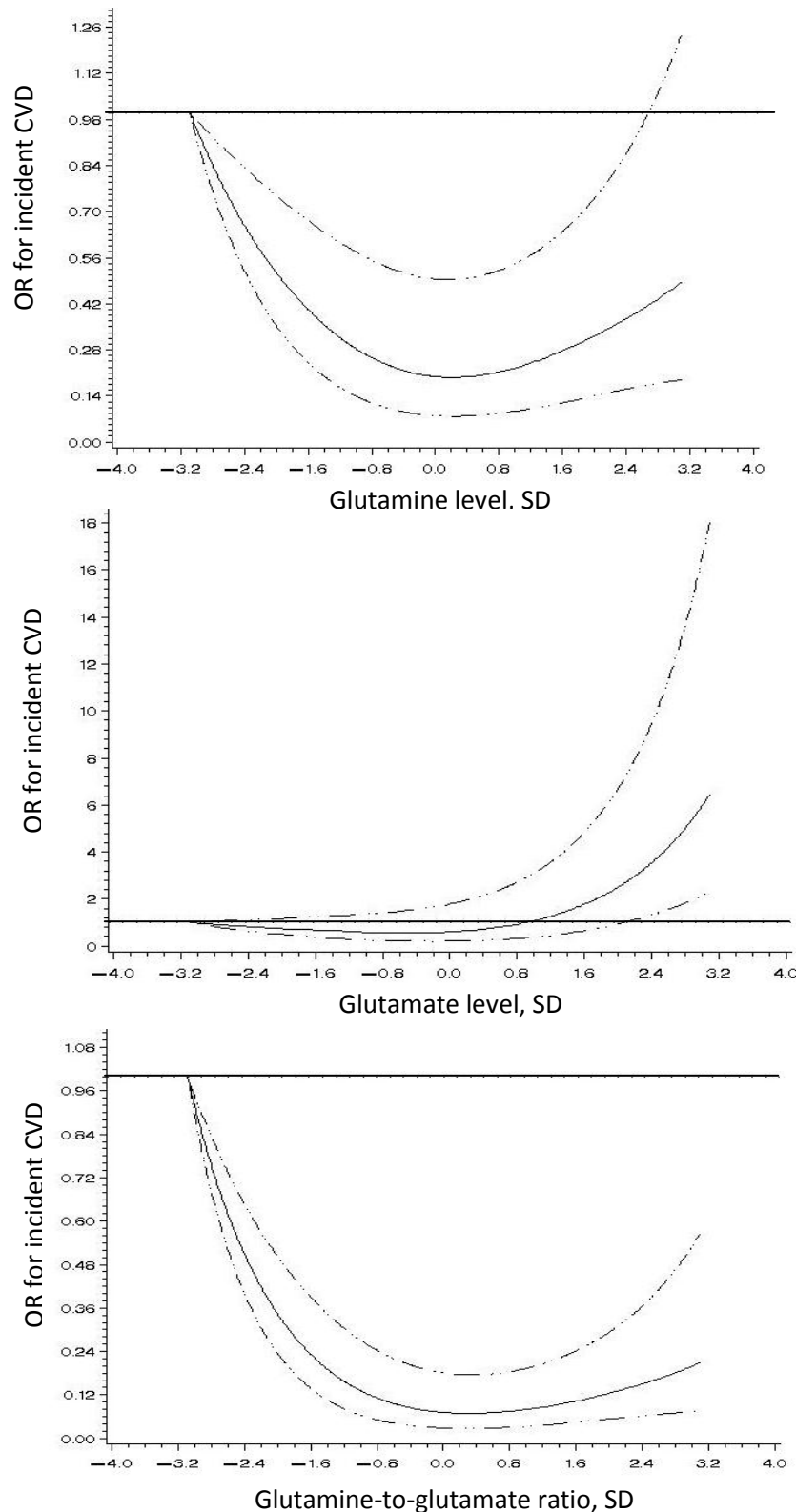Glutamate level, SD

Glutamine-to-glutamate ratio, SD

Figure S3. Hazard Ratios of cardiovascular risk by baseline high vs. low glutamate levels and intervention vs. control groups.