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High plasma glutamate and low glutamine-to-glutamate ratio are associated with type 2 diabetes: Case-cohort study within the PREDIMED trial

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ABBREVIATIONS/ACRONYMS USED
EVOO;
Extra virgin olive oil;
MedDiet:

Abstract *Background and aims:* Glutamate, glutamine are involved in energy metabolism, and have been related to cardiometabolic disorders. However, their roles in the development of type-2 diabetes (T2D) remain unclear. The aim of this study was to examine the effects of Mediterranean diet on associations between glutamine, glutamate, glutamine-to-glutamate ratio, and risk of new-onset T2D in a Spanish population at high risk for cardiovascular disease (CVD).
Methods and results: The present study was built within the PREDIMED trial using a case-cohort design including 892 participants with 251 incident T2D cases and 641 non-cases. Participants (mean age 66.3 years; female 62.8%) were non diabetic and at high risk for CVD at baseline. Plasma levels of glutamine and glutamate were measured at baseline and after 1-year of intervention. Higher glutamate levels at baseline were associated with increased risk of T2D with a hazard ratio (HR) of 2.78 (95% CI, 1.43–5.41, *P* for trend = 0.0002). In contrast, baseline levels of glutamine (HR:

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mediterranean diet

0.64, 95% CI, 0.36–1.12; *P* for trend = 0.04) and glutamine-to-glutamate ratio (HR: 0.31, 95% CI, 0.16–0.57; *P* for trend = 0.0001) were inversely associated with T2D risk when comparing extreme quartiles. The two Mediterranean diets (MedDiet + EVOO and MedDiet + mixed nuts) did not alter levels of glutamine and glutamate after intervention for 1 year. However, MedDiet mitigated the positive association between higher baseline plasma glutamate and T2D risk (P for interaction = 0.01).

Conclusion: Higher levels of glutamate and lower levels of glutamine were associated with increased risk of T2D in a Spanish population at high risk for CVD. Mediterranean diet might mitigate the association between the imbalance of glutamine and glutamate and T2D risk.

This trial is registered at http://www.controlled-trials.com, ISRCTN35739639.

Introduction

Globally, the prevalence of Type-2 Diabetes (T2D) is projected to affect 591.9 million people by 2035, which is equivalent to a 55% increase from 2013 [1]. In the US, an estimate of 30.3 million people had T2D in 2015 [2].The pathophysiology of T2D is beyond simple disorders in carbohydrate metabolism [3]. Amino acid metabolites are also disturbed in conditions of insulin resistance and T2D. Unfavorable changes in energy metabolism accompanied by insulin resistance and dysfunctional pancreatic β-cell are present years before the disease is clinically diagnosed by elevated blood glucose levels [4,5]. Therefore, early diagnosis of T2D is extremely important as early intervention may delay or prevent the onset of complications.

Recent metabolomics technology provides new tools for identifying metabolites involved in metabolic pathways related to T2D. Imbalance of metabolic regulatory systems is the basis for many metabolic disorders, including diabetes. Elevated levels of branched chain amino acids (BCAAs) and aromatic amino acids (AAAs) have been associated with an increased risk of prediabetes and T2D [6,7].

A key function of the BCAAs is to provide nitrogen needed to maintain glutamate, alanine and glutamine pools in skeletal muscle [8]. Glutamine is the most abundant nonessential amino acid in human biology and is involved in regulation of pancreatic β-cell function and insulin secretion [9]. Previous studies have reported associations of glutamine and glutamate metabolites with T2D risk in healthy individuals and in subjects with metabolic syndrome [6,10]. Among participants who were free of diabetes and cardiovascular disease, plasmatic glutamate levels were associated with insulin resistance traits (glucose, insulin and HOMA), and with an increased risk of T2D incidence [6]. Glutamate was able to increase transamination of pyruvate to alanine and promote gluconeogenesis [11] which is commonly seen in obese individuals [12]. Glutamate is also a precursor to α-ketoglutarate, an intermediate in the Krebs cycle that has a crucial role in cellular energy metabolism [13].

The ability of glutamate and glutamine in predicting T2D risk in individuals at high risk of cardiovascular disease (CVD) remains uninvestigated [14]. This question needs to be addressed as these high-risk individuals are those who will benefit the most from early diagnosis and lifestyle interventions to delay the onset of T2D.

Mediterranean diet has demonstrated protective effects in T2D through modifying metabolites related to T2D risk [15]. However, the underlying mechanisms of how Mediterranean diet influences metabolites involved in energy metabolism is not fully understood. Therefore, in the present study, we examined the following hypotheses using a case-cohort study design nested within the PREvención con DIeta Mediterránea (PREDIMED) trial in non-diabetic participants at high risk for CVD 1) baseline plasma levels of glutamate were positively associated with increased risk of incident T2D whereas, glutamine level is inversed associated with risk of incident T2D; 2) increases in these amino acids at 1 year are associated with a higher subsequent risk of T2D; (3) a Mediterranean style diet (MedDiet) can attenuate the association between glutamate, glutamine-to-glutamate ratio and T2D.

Method

Study population

Design and protocol of the PREDIMED (www.predimed.es) study have been published in details elsewhere [16]. The PREDIMED study is an Spanish primary CVD prevention trial using a Mediterranean diet as the main intervention. In brief, 7447 participants were randomly allocated to three groups to examine the effects of two Mediterranean enriched diets with 1) extra virgin olive oil or 2) mixed nuts compared to a low-fat diet (control diet) on the primary prevention of CVD. Male and female participants between 55 and 80 years who were at high risk of CVD at baseline have been recruited and enrolled among 11 centers across Spain between 2003 and 2009. The study was stopped earlier in July 2011 when an interim analysis presented that there were significant benefits from to the two Mediterranean diets, Participants were considered to be at high CVD risk if they had either T2D or more than three of the following CVD risk factors including current smoking, hypertension, elevated low density lipoprotein

cholesterol (LDL-C), decreased high density lipoprotein cholesterol (HDL-C), overweight/obesity, or family history of premature coronary heart disease. The primary endpoint of the PREDIMED trial was a composite of CVD events (non-fatal MI, stroke, or death from CVD) assessed after a median of 4.8-year of follow-up. 3541 participants were free of T2D at baseline. We documented 273 incident T2D cases and plasma samples were available for 251 of those participants.

The present study was built within the PREDIMED trial using a case-cohort design [17]. Consistent with the case-cohort design, in addition to 251 cases of incident T2D, we included a random sample of 20% of PREDIMED participants who were free of diabetes at baseline known as the "subcohort participants" (n = 694). These "subcohort participants" which consists of 53 overlapping incident T2D cases and 641 "noncases" which were randomly selected using a computer-generated random number sequence. Together, 892 participants were included in the study (Supplemental Fig. 1). After 1 year of follow-up, 663 participants (505 noncases and 158 cases) had follow-up samples. With this sample size, we had 80% power to detect an OR of 1.25 at a false positive rate of 0.05. The case-cohort study design with random sampling of the subcohort permit to measure the metabolites in a subsample rather than the entire study population and allows us to generalize the findings to the full cohort.

The PREDIMED trial was approved by the Institutional Review Boards at participating institutions across Spain. The present study was approved by the Institutional Review Boards at Harvard T.H. Chan School of Public Health and all participating institutions across Spain.

Case ascertainment

The primary endpoint of the present study was incidence of T2D, which was a secondary endpoint of the PREDIMED trial. Diagnosis of T2D incidence was based on the American Diabetes Association criteria including fasting plasma glucose concentrations $\geq$ 7.0 mmol/L or 2 h plasma glucose concentrations $\geq$ 11.1 mmol/L after an oral dose of 75-g glucose or recent use of an oral/insulin medication. Participants' medical records were reviewed annually by physicians and investigators who were blinded to the intervention groups. When new-onset diabetes cases were identified on the basis of medical records or on a glucose test during routine biochemical analyses (conducted at least once per year), reports were sent to the PREDIMED Clinical Events Committee who were blind to the allocation group. The new diabetes cases would be confirmed only when a second test could be repeated.

Covariate assessment

Lifestyle variables, medical records, medication, and family history of disease were collected by questionnaires at baseline and yearly during the follow-up. Anthropometric measurements and blood pressure were measured on sites by trained personnel. Physical activity was assessed using the validated Spanish version of the Minnesota Leisure-Time Physical Activity questionnaire [18]. Diet was assessed using a 137-item validated semi-quantitative food frequency questionnaire during an in-person interview with trained dietitians [19]. Energy and nutrient intakes were estimated by the Spanish food composition tables [20]. Adherence to the Mediterranean diet was assessed by a 14-item food questionnaire [21].

Metabolites profiling

Prior each blood draw, participants were required to fast overnight. Fasting plasma samples were collected at baseline and year-1, using EDTA tubes and stored at −80 °C condition. Metabolites profiling was measured at the Broad Institute. 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) were used for metabolites profiling. Detailed mass spectrometry setting was published previously [22]. Authentic reference standards were used for metabolite identification.

Statistics

Baseline characteristics between cases and non-cases are presented as mean ± standard deviations (SDs) for continuous variables, and as n (%) for categorical variables. We used *t*-tests for comparing continuous variables and chi-squared tests for categorical variables.

Rank-based inverse normal transformations were applied to approximate a normal distribution of metabolites levels. General linear model was used to examine the associated between food intake and quartile of glutamine-to-glutamate ratio at baseline. Cox regression models with Barlow weights [to account for over-represented cases in the case-cohort study] were used to estimate Hazard Ratios (HRs) and their 95% Confidence Intervals (CIs) for T2D incidence. Non-cases in the sub-cohort were weighted inversely proportionally to the sampling fraction [17]. We calculated HR and their 95% CIs for T2D by quartiles of the amino acids and also for each SD as a continuous variable. Follow-up time was calculated from the date of enrollment to the date of diagnosis of T2D or the date of the last visit or the end of the follow-up period for participants without type 2 diabetes (December 1, 2010).

Model 1 was age-adjusted. All models were stratified by intervention group (MedDiet + EVOO, MedDiet + nuts, low-fat control) and recruitment center. Multivariable-adjusted models were additionally adjusted for sex (male, female), BMI (kg/m^2), smoking status (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia (yes, no), hypertension (yes, no). In a secondary analysis, we further adjusted for baseline levels of total BCAAs and fasting glucose levels as blood glucose was likely to be a confounder and/or an intermediate link in the causal

pathway between glutamine-cycling pathway and risk of T2D. Quartile cut-off point for amino acids were generated based on the distribution of amino acids among participants without diabetes. Median value of metabolite in each quartile was assigned and analyzed as a continuous variable to test the linear trend across quartiles. To examine the associations between 1-year changes in glutamate (GLU), glutamine (GLN), Gln/Glu and risk of T2D, we used the same multivariable adjusted Cox regression models with additional adjustment for baseline metabolites levels. We conducted joint analyses and interaction tests for glutamine, glutamate, Gln/Glu at baseline and the intervention groups (MedDiet + EVOO and MedDiet + nuts vs control group). Likelihood ratio test was used to assess the significance of interaction between levels of amino acids and intervention groups. In model 2, participants with lowest risk were set as reference group based on the direction of association between baseline plasma levels of amino acids and risk of T2D.

We also examined how changes in individual amino acid levels at 1 year were associated with risk of T2D. For each individual metabolite, we first calculated the changes in the individual amino acid level between baseline and 1 year and then normalized the differences using the inverses normal transformation. We used general linear model to assess the changes in glutamine and glutamate levels after one year in response to the dietary interventions. Models were adjusted for age (years), sex (male, female), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, and baseline metabolites levels. We used robust estimates of the variance to correct for potential intra-cluster correlation, and adjusted models for propensity scores that has been built with 30 baseline variables to estimate the probability of assignment to each of the intervention groups. All statistical analyses were performed by SAS (v9.4, SAS Institutes, Cary, NC).

Results

We examined associations of baseline glutamine, glutamate, and their ratio (Gln/Glu) with incident T2D in 892 participants from the PREDIMED study. Participants had a mean age of 66 years, BMI of 30 kg/m^2 and majority of them had dyslipidemia and hypertension (Table 1). Participants with T2D were more likely to be obese (30.8 ± 3.3), hypertensive (96%), current smoker (25.1%) with higher baseline glucose level (118.6 ± 18.0 mg/dL). The proportion of women was lower in diabetic participants. Individuals with T2D had a slightly greater energy intake and lower consumption of vegetables, fish, and legume when compared with participants without T2D. Higher Gln/Glu was associated with greater fruit

Table 1 Baseline participant characteristics by case status and quartile of glutamine-to-glutamate ratio.

	Non-cases	Cases	*P* value	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P* value[a]
N	641	251		227	257	221	187	
Age (y)	66.6 (5.8)	66.4 (5.7)	0.88	66.3 ± 5.9	66.1 ± 5.7	66.6 ± 5.7	67.2 ± 5.7	0.18
BMI (kg/m^2)	29.8 (3.6)	30.8 (3.3)	0.24	30.8 ± 3.6	30.4 ± 3.3	30.0 ± 3.4	28.8 ± 3.6	<0.001
Sex (% women)	62.8	55.0	0.03	132 (58.1)	131 (51.0)	138 (62.4)	145 (81.5)	<0.001
Fasting glucose (mg/dL)	99.7 (15.2)	118.6 (18.0)	<0.01	102.(19.8)	107.9 (17.9)	104.1 (15.2)	97.0 (14.7)	<0.001
Hypertension, n (%)	577 (90)	241 (96)	<0.01	206 (90.7)	241 (93.8)	200 (90.5)	171 (91.4)	0.54
Dyslipidemia, n (%)	552 (86)	200 (80)	<0.01	184 (81.1)	216 (84.0)	191 (86.4)	161 (86.1)	0.39
Physical activity	238 (235)	249 (233)	0.90	223.7 (247.1)	242.8 (247.1)	262.2 (257.1)	233.4 (197.1)	0.35
Smoking (%)			0.06					
Never	61.5	52.6		141 (62.1)	130 (50.1)	128 (57.9)	127 (67.9)	0.01
Former	22.5	22.3		45 (19.8)	75 (29.2)	50 (22.6)	30 (16.0)	
Current	16.1	25.1		41 (18.0)	52 (20.2)	43 (19.5)	30 (16.0)	
Intervention group (%)								
MedDiet + EVOO	30.9	29.9		71 (31.3)	79 (30.7)	62 (28.1)	61 (32.6)	0.39
MedDiet + nuts	37.2	33.9		81 (35.7)	92 (35.8)	81 (36.6)	70 (37.5)	
Control	31.8	36.3		75 (33.0)	86 (33.5)	78 (35.3)	56 (29.9)	
Energy intake (kcal/day)	2289 (565)	2327 (622)	0.06	2315 (550)	2366 (596)	2277 (586)	2215 (585)	0.04
Food intake (g/day)								
Vegetables	333 (143)	294 (117)	0.0002	336 (145)	316 (133)	318 (128)	318 (144)	0.2
Fruits	360 (191)	336 (174)	0.09	319 (174)	367 (208)	373 (177)	356 (178)	0.03
Meat	133 (54)	135 (52)	0.42	140 (52)	138 (55)	129 (53)	126 (54)	0.003
Fish	101 (49)	97 (42)	0.005	101 (52)	102 (46)	98 (43)	98 (49)	0.36
Dairy	374 (222)	360 (229)	0.55	368 (221)	350 (211)	385 (224)	383 (242)	0.27
Nuts	11 (13)	12 (14)	0.24	11 (13)	12 (14)	11 (14)	10 (13)	0.47
Olive oil	38 (16)	39 (17)	0.28	37 (16)	39 (16)	39 (16)	37 (15)	0.69
Cereal	227 (101)	242 (110)	0.09	240 (101)	242 (100)	223 (108)	217 (106)	<0.001
Legume	21 (14)	18 (9)	<0.001	22 (13)	19 (11)	20 (9)	21 (17)	0.07

Values are mean (SD) or percentage.
BMI, body mass index; MET, metabolic equivalent; MedDiet, Mediterranean diet intervention group; EVOO, Extra-virgin olive oil.
[a] *P* value for the association of variables with quartile groups of glutamine-to-glutamate ratio.

consumption (*P* for trend, 0.03), and lower consumption of meat and cereal (*P* for trend < 0.05 for both).

Association of baseline metabolites with risk of incident T2D

Associations between glutamine, glutamate, Gln/Glu at baseline and risk of incident T2D are presented in Table 2. Higher glutamine levels at baseline were associated with lower risk of incident T2D when comparing extreme quantiles (model 2, HR: 0.64, 95% CI, 0.36−1.12, *P* for trend = 0.04). In contrast, higher glutamate levels were associated with increased T2D risk (model 2, *P* for trend = 0.0002). Participants who were in the top quartile of glutamate levels at baseline had significantly higher risk of incident T2D, with a HR of 2.78 (95% CI: 1.43−5.41) when compared to those who were in the bottom quartile. The Gln/Glu was strongly and inversely associated with risk of incident T2D [15]. Participants in the highest quartile had about 70% lower T2D risk (HR: 0.31, 95% CI: 0.16−0.57, *P* for trend <0.0001) compared with those in the bottom quartile.

After we further adjusted for baseline BCAAs levels in model 3, the associations between glutamine, glutamate, Gln/Glu and incident T2D risk remained statistically significant. The association between baseline glutamine and incident T2D risk was attenuated after model (model 2) was further adjusted for baseline glucose (model 4). Whereas associations between glutamate, Gln/Glu and risk of incident T2D remained statistical significant.

Fig. 1 shows the HRs for joint effects of the intervention and baseline plasma levels of Gln/Glu, glutamine, and glutamate (dichotomised at their median) on T2D risk. Lower level of baseline glutamine was associated with increased risk of T2D (Fig. 1a). Participants in upper quartiles of glutamate levels had higher risk of T2D in both of the control group (HR 2.68 for Q3-4 vs Q1−Q2, 95% CI: 1.57−4.61) and the MedDiet group (HR 1.65 for Q3-4 vs Q1−Q2, 95% CI: 1.07−2.56) (Fig. 1b). The MedDiet attenuated the positive association between higher baseline plasma glutamate levels and T2D risk (*P* for interaction = 0.01). We also observed a higher risk of T2D in participants in lower quantiles of baseline Gln/Glu in the control group (HR 2.5 for Q1-2 vs Q3−Q4, 95% CI: 1.44−4.33) and the MedDiet group (HR 1.86 for Q1-2 vs Q3−Q4, 95% CI: 1.20−2.89) (Fig. 1c). The association between baseline plasma Gln/Glu and risk of T2D was attenuated in response to the MedDiet. There was a significant interaction (*P* for interaction = 0.03) between the intervention (MedDiet) and baseline Gln/Glu.

Table 2 Incident type 2 diabetes by baseline plasma amino acid levels in the PREDIMED trial, 2003−2010: Observed event rates and hazard ratios.[a]

Levels at baseline	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P* for trend
	Incidence Cases: 251 cases, 641 non-cases				
Glutamine	HR (95% CI)				
Cases	59	85	54	53	
Model 1	1.00 (ref)	1.25 (0.84, 1.86)	0.94 (0.61, 1.44)	0.85 (0.55, 1.30)	0.27
Model 2	1.00 (ref)	1.14 (0.68, 1.93)	0.80 (0.47, 1.36)	0.64 (0.36, 1.12)	0.04
Model 3	1.00 (ref)	1.02 (0.60, 1.73)	0.69 (0.41, 1.19)	0.48 (0.26, 0.87)	0.004
Model 4	1.00 (ref)	1.02 (0.51,2.02)	1.02 (0.54, 1.92)	0.77 (0.41,1.48)	0.43
Glutamate	HR (95% CI)				
Cases	25	62	108	56	
Model 1	1.00 (ref)	2.35 (1.40, 3.95)	4.13 (2.59, 6.98)	2.45 (1.44, 4.16)	<0.0001
Model 2	1.00 (ref)	2.24 (1.22, 4.11)	4.34 (2.42, 7.78)	2.78 (1.43, 5.41)	0.0002
Model 3	1.00 (ref)	2.06 (1.10, 3.85)	3.91 (2.15, 7.08)	2.32 (1.17, 4.61)	0.003
Model 4	1.00 (ref)	4.07 (1.50, 11.1)	8.31 (3.40, 20.3)	6.26 (2.21, 17.7)	<0.0001
Glutamine-to-glutamate ratio	HR (95% CI)				
Cases	67	98	59	27	
Model 1	1.00 (ref)	1.40 (0.95, 2.06)	0.78 (0.51, 1.18)	0.39 (0.23, 0.64)	<0.0001
Model 2	1.00 (ref)	1.18 (0.72, 1.93)	0.69 (0.40, 1.20)	0.31 (0.16, 0.57)	0.001
Model 3	1.00 (ref)	1.22 (0.74, 1.99)	0.72 (0.41, 1.25)	0.34 (0.18, 0.64)	0.0006
Model 4	1.00 (ref)	0.99 (0.54, 1.82)	0.52 (0.26, 1.04)	0.26 (0.10, 0.66)	0.001

Model 1: adjusted for age.
Model 2:Model 1 + sex (male, female), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia (yes, no) and hypertension (yes, no). Analysis was stratified by recruitment center and intervention group (MedDiet + EVOO, MedDiet + nuts, low-fat control).
Model 3: Model 2 + baseline levels of BCAAs. Analysis was stratified by recruitment center and intervention group (MedDiet + EVOO, MedDiet + nuts, low fat control).
Model 4: Model 2 + baseline levels of glucose. Analysis was stratified by recruitment center and intervention group (MedDiet + EVOO, MedDiet + nuts, low fat control).
[a] Inverse normal transformation was applied to raw values.

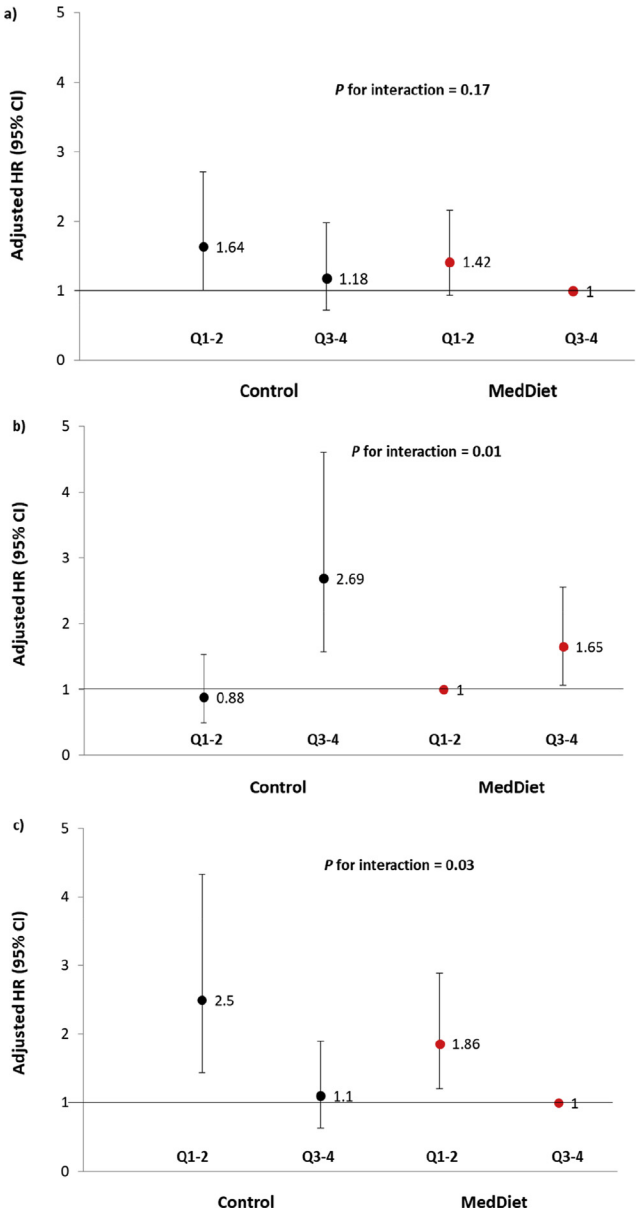

Figure 1 Multivariate-adjusted HRs (95% CIs) of incident T2D and quartiles of **a)** glutamine, **b)** glutamate, **c)** glutamine-to-glutamate ratio (GLN/GLU) at baseline stratified by intervention group (MedDiet versus control group). Control group is in solid black. MedDiet group is in solid red. An inverse normal transformation was applied to raw values of glutamine, glutamate and their ratio. Model was adjusted for age (years), sex (male/female), smoking status (never, former, or current smoker), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (METs-min/day), dyslipidemia (yes, no), hypertension (yes, no), metabolites (continuous) and stratified by intervention group (MedDiet + EVOO, MedDiet + nuts, and control diet); *P* for interaction with 2 degrees of freedom between MedDiet intervention groups (combined intervention groups vs. control: binary, yes/no) and baseline glutamine-to-glutamate ratio. Based on the association between metabolites and T2D risk, participants who were on the MedDiet and with higher quantiles (Q3-4) of baseline glutamine and Gln/Glu were set as the reference. Participants who were on MedDiet and with lower quantiles (Q1-2) of glutamate at baseline were set as the reference. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

One year changes in metabolites and the effect of dietary intervention on risk of incident T2D

Glutamine, glutamate, and Gln/Glu did not change significantly in response to the intervention diets after one year (Fig. 2). Overall, the associations of 1-year changes in glutamine, glutamate with risk of incident T2D were non-significant (Table 3). In contrast with our findings for baseline levels, changes in Glu/Gln were positively associated with the risk of incident T2D in age-adjusted model. However, the association was attenuated after further adjustment for additional anthropometric, lifestyle variables and baseline levels of Gln/Glu. We did not find any association between changes in these metabolites and risk of T2D nor did we find any effect of the intervention on these metabolites after one year.

Discussion

In this case-cohort study, we found that higher baseline plasma glutamate levels were associated with increased risk of incident T2D in a population at high risk of CVD. An imbalance between glutamine and glutamate may contribute to the development of T2D. We propose that abnormal glutamate homeostasis, could cause elevated extracellular glutamate concentrations that may participate in β-cell death, possibly in combination with increased FFA and glucose concentrations [13]. The two Mediterranean diets did not significantly change the levels of these metabolites after intervention for 1 year. However, our findings suggest that MedDiet could mitigate the adverse effects of T2D that is associated with imbalance between glutamine and glutamate. A unique aspect of our study is that we observed these prospective associations in a population at high risk for CVD in the context of a nutritional primary prevention study. In fact, these high-risk individuals are those who will benefit the most from early diagnosis and lifestyle interventions to delay the onset of T2D. These associations further support that glutamine-cycling pathways are prominently involved in the pathophysiology of T2D. The results reported herein add to the evidence base of using metabolomics signatures of glutamine and glutamate as possible future predictive tools of T2D risk in asymptomatic patients.[7]The metabolomics approach may enable timely and effective identification of T2D risk.

In a prospective cohort of 9369 Finish men, glutamine levels were inversely associated with risk of T2D after a follow-up of 4.7 years [10]. In 601 participants from the Framingham Heart Study (FHS) who were free of T2D at baseline, glutamine levels were also inversely associated with incident disease and other cardiometabolic risk factors over 12 years.[6]Evidence from clinical trials showed that supplementation of glutamine was beneficial in lowering blood glucose and improving other cardiometabolic risk factors in patients with T2D [23]. The proposed underlying mechanism from previous studies include that glutamine can stimulate insulin secretion by increasing the release of glucagon-like peptide (GLP-1),

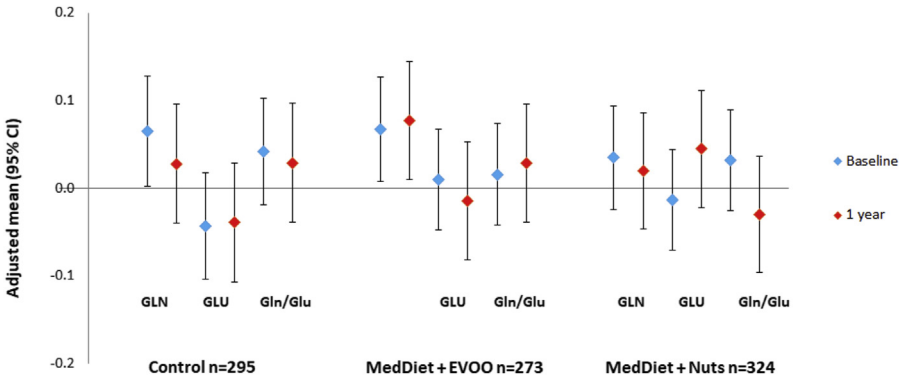

Figure 2 Glutamine (GLN), glutamate (GLU), glutamine-to-glutamate ratio (Gln/Glu) at baseline and changes after 1 year of intervention. Values were adjusted for age (years), sex (male, female), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, and baseline metabolites levels. Blue diamond: baseline metabolites levels, red diamond: metabolites levels at 1 year Sample size was from baseline. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3 Incident T2D by 1-year changes in plasma amino acid levels in the PREDIMED trial, 2003–2010: Observed event rates and hazard ratios.[a]

Changes at year 1	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P* for trend
	Incidence Cases: 158 cases, 505 non-cases				
Glutamine	HR (95% CI)				
Cases	31	58	44	48	
Model 1	1.00 (ref)	1.33 (0.80,2.20)	1.06 (0.62,1.79)	1.17 (0.71,1.95)	0.82
Model 2	1.00 (ref)	1.12 (0.59,2.13)	0.82 (0.41,1.62)	0.94 (0.47,1.87)	0.64
Model 3	1.00 (ref)	0.94 (0.48, 1.85)	0.73 (0.36, 1.48)	0.90 (0.45, 1.83)	0.66
Model 4	1.00 (ref)	0.96 (0.47, 1.97)	1.02 (0.52, 2.01)	1.29 (0.65, 2.68)	0.44
Glutamate	HR (95% CI)				
Cases	54	47	55	25	
Model 1	1.00 (ref)	0.81 (0.52,1.29)	1.00 (0.64,1.56)	0.59 (0.34,1.02)	0.14
Model 2	1.00 (ref)	1.00 (0.49,2.04)	1.68 (0.84,3.34)	0.68 (0.31,1.47)	0.84
Model 3	1.00 (ref)	0.94 (0.45, 1.94)	1.69 (0.84, 3.4)	0.76 (0.35, 1.64)	0.90
Model 4	1.00 (ref)	1.28 (0.59, 2.77)	1.79 (0.80, 4.01)	1.05 (0.41, 2.68)	0.52
Glutamine-to-glutamate ratio	HR (95% CI)				
Cases	12	69	47	53	
Model 1	1.00 (ref)	4.88 (2.51,9.47)	3.30 (1.66,6.58)	3.63 (1.84,7.13)	0.009
Model 2	1.00 (ref)	3.03 (1.37,6.70)	1.26 (0.50,3.20)	2.30 (0.99,5.33)	0.41
Model 3	1.00 (ref)	3.07 (1.33, 6.78)	1.08 (0.41, 2.84)	2.38 (1.01, 5.63)	0.38
Model 4	1.00 (ref)	3.67 (1.19, 11.3)	1.83 (0.56, 5.97)	2.37 (0.73, 7.68)	0.70

Model 1: adjusted for age.
Model 2:Model 1 + sex (male, female), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia (yes, no), hypertension (yes, no) and baseline metabolites levels. Analysis was stratified by recruitment center and intervention group (MedDiet + EVOO, MedDiet + nuts, low fat control).
Model 3: Model 2 + baseline levels of BCAAs. Analysis was stratified by recruitment center and intervention group (MedDiet + EVOO, MedDiet + nuts, low fat control).
Model 4: Model 2 + baseline levels of glucose. Analysis was stratified by recruitment center and intervention group (MedDiet + EVOO, MedDiet + nuts, low fat control.
[a] Inverse normal transformation was applied to raw values of 1-year changes in plasma metabolites.

thus lowering blood glucose levels [24] and reducing postprandial insulin response [25]. In our study there was no significant association between glutamine levels and T2D risk. Differences in studies' design and study populations can explain these discrepancies.

In contrast to the favorable effects of glutamine, abnormal glutamate homeostasis is often associated with increased oxidative stress and inflammation, which are commonly observed in people with obesity or diabetes [11]. Evidence from animal studies and clinical trials showed that elevated glutamate levels is likely to 1) increase the susceptibility of pancreatic β-cells to oxidative damage [26,27], 2) aggravate β-cell dysfunction and apoptosis by activating the glutamate receptor (*N*-methyl-D-aspartate receptor) in β-cells [28], and 3) increase the activity of GAD65, one of the major antigens that triggers

autoimmunity accelerating cell dysfunction and apoptosis [29]. Along with our findings, these data suggest that a dysregulation in glutamate homeostasis may contribute to the development of T2D. Glutamate is commonly present in protein-rich foods e.g. meats, poultry, seafood, dairy and offers a meaty savory taste known as "UMAMI" [30]. Previous studies showed that elderly tend to increase intake of glutamate enhanced foods [31,32]. Disregard of age, subjects with relatively poorer biochemical status preferred higher glutamate concentrations [33]. It is plausible that the differences in plasma glutamate levels may partially reflect a dietary pattern with higher glutamate levels associated with a higher meat, poultry consumption and/or increased energy intake which may contribute to a greater T2D risk.

Our findings on glutamine-to-glutamate ratio highlight the importance of the balance between these two gluconeogenic amino acids in relation to T2D risk. Glutamine and glutamate provide carbon for glucose production in kidney and liver [8] and glutamate is identified as one of the metabolic coupling factors that synergistically promote insulin secretion from pancreatic β-cell [34]. As the metabolism of glutamine and glutamate is exquisitely related to energy metabolism, the glutamine-to-glutamate ratio may reflect the overall status of energy metabolism. Glutamine, glutamate and BCAAs are correlated in metabolism. The branched-chain amino acid transaminase 1 (BCAT1) initiates the catabolism of BCAAs and uses glutamine as a nitrogen receiver; and leucine allosterically activating glutamate dehydrogenase that catalyzes the oxidative deamination of glutamate, which is present at a high concentration in the pancreatic β-cell [9]. The present study, demonstrating that low glutamine levels and higher glutamate were associated with subsequent onset of T2D in addition to previous established metabolites markers such as BCAAs, suggests that the chronically imbalance of amino acids metabolism contributes to the progression of T2D. It has been recently proposed β-cell neogenesis from α-cell can be a new pathway of potential therapeutic significance [35]. Amino acids, especially glutamine can serve as an energy source and play a major role in the regulation of α-cell mass in animal models [36,37]. Therefore we hypothesize that imbalance between glutamine and glutamate may potentially hinder α-cell proliferation thus limit the regeneration of β-cell.

The two Mediterranean diets did not significantly change the metabolite levels after intervention for 1 year. Given that study participants were at high risk for CVD at baseline, which presumably already had an unfavorable metabolite profile, this could contribute to the lack of associations observed between 1-year changes in metabolites and T2D risk after adjusting for baseline metabolites levels. Also, the time period of 1 year may not be sufficient to have a significant change in metabolites to influence the risk of T2D. Considering that the PREDIMED intervention was not designed to modify amino acid intake, these results are not unexpected. These results suggest that the MedDiet interventions may not directly influence glutamate levels at least in a short-time period, but may counteract their adverse effect on risk of T2D through other protective pathways and mechanisms [38]. The effects of olive oil and nuts on changes of glutamate may vary due to the different bioactive compounds in these foods.

One limitation of the present study is that these results may not be generalized to other populations, as the study subjects lived in a Mediterranean area and were at high risk of CVD. Another limitation is that we used LC-MS platform for metabolite profiling and applied inverse-normal transformation to express the results, and this approach may hamper a direct clinical translation for each metabolite trait. The strengths of our study include the control of potential confounders by comprehensive data recording and monitoring of intervention compliance; using repeated measure of metabolites to examine the changes of metabolites in response to dietary intervention. In addition, the case-cohort design maximized the efficiency of metabolite profiling, allowing us to generalize these findings to all PREDIMED participants.

Conclusion

In conclusion, we provide evidence of associations between glutamate and the glutamine-to-glutamate ratio and risk of incident T2D in a population at high-risk for CVD. Our findings support a role for these metabolites in the pathophysiology of T2D and the need to explore their potential predictive ability of disease risk. A Mediterranean-style dietary pattern appeared to modify risk of T2D associated with glutamate and the overall balance between glutamine and glutamate.

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Conflicts of interest

Dr. Emilio Ros has received funds for research through his institution and personal fees for lecture presentation from the California Walnut Commission and is a nonpaid member of its Scientific Advisory Committee.Dr. Jordi Salas-Salvadó has have received grants from the International Nut and Dried Fruit Foundation and is a non-paid member of the scientific advisory board of the International Nut and Dried Fruit Foundation.

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Authors' contributions to manuscript

Conception or design of the work: JSS, FBH, MAM-G.

Coordinators of subject recruitment and clinical data collection: JSS, MAM-G, DC, RE, MF, ER, FA, EG-G, JL, MF, LS-M.

Metabolomics data analysis: CC, LL.

Statistical analysis: LX, YZ, MG-F.

Data interpretation: JSS, FBH, MAM-G, LX, YZ, MG-F, MR-C, ET, CR.

Drafting the paper: LX, YZ.

JSS takes full responsibility for the work as a whole, the study design, access to data, and the decision to submit and publish the manuscript.

All authors have read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2019.06.005.

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