Changes in Plasma Tryptophan are Inversely Associated with Incident Cardiovascular Disease in the PREDIMED Study*

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ABSTRACT

BACKGROUND

During development of cardiovascular disease (CVD), interferon-γ-mediated inflammation accelerates degradation of tryptophan into downstream metabolites. A Mediterranean Diet (MedDiet) consisting of high intake of extra virgin olive oil, nuts, fruits, vegetables, and cereals has been demonstrated to lower risk of CVD. The longitudinal relationship between these metabolites and CVD in the context of a MedDiet is unstudied.

OBJECTIVE

We sought to investigate the relationship between metabolites in the tryptophan-kynurenine pathway and CVD in the context of diet.

METHODS

We employed a case-cohort design nested in the PREDIMED randomized controlled trial. There were 231 CVD cases (stroke, myocardial infarction, vascular death) among 985 participants over a median of 4.7 y of follow-up [mean ± SD age: 67.6 ± 6.1 y; 53.7% women; mean ± SD body mass index: 29.7 ± 3.7 kg/m^2]. We assessed plasma tryptophan, kynurenine, kynurenic acid, 3-hydroxyanthranilic acid, and quinolinic acid levels at baseline and after 1 year of intervention with MedDiet. We combined these metabolites in a kynurenine risk score (KRS) by weighting each metabolite by the

adjusted coefficient of its associations with CVD. Cox models were used in the primary analysis.

RESULTS

Changes in tryptophan after 1 year were associated with lower risk of composite CVD (hazard ratio [HR] per SD: 0.79; 95% CI: 0.63, 0.98). Baseline kynurenic acid was associated with higher risk of myocardial infarction and CHD death, but not stroke. A higher KRS was more strongly associated with CVD in the control group than in the 2 intervention groups (*P*-interaction: 0.003). Adjustment for tryptophan changes attenuated the inverse association between MedDiet+EVOO and CVD.

CONCLUSIONS

Changes in tryptophan were significantly associated with decreased risk of CVD. A MedDiet may counteract the deleterious effect of an unfavorable tryptophan-kynurenine metabolite profile.

KEYWORDS: Metabolomics, Cardiovascular disease, Mediterranean diet, tryptophan

ABBREVIATIONS

3-HAA, 3-hydroxyanthranilic acid

ANOVA, analysis of variance

BCAA, branched chain amino acids

BMI, body mass index

CHD, coronary heart disease

CI, confidence interval

CVD, cardiovascular disease

EVOO, extra-virgin olive oil

HR, hazard ratio

ICAM-1, Intercellular adhesion molecule 1

IDO, indoleamine 2,3-dioxygenase

IFN-γ, interferon-γ

IL-6, interleukin 6

KA, kynurenic acid

KRS, kynurenine risk score

Kyn, kynurenine

LDL, low density lipoprotein

MedDiet, Mediterranean Diet

NAD, nicotinamide adenine dinucleotide

P, probability

PREDIMED, Prevención con Dieta Mediterránea

Q, quartile

QA, quinolinic acid

SD, standard deviation

Tryp, tryptophan

VCAM-1, Vascular cell adhesion protein 1

INTRODUCTION

Inflammation has long been identified as an important component in the pathophysiology of cardiovascular disease (CVD) (1). Interferon-γ (IFN-γ) is a key molecule that orchestrates many pathways involved in inflammation by acting as a transcriptional regulator for immune-related genes (2). Its excessive release by activated T-lymphocytes has been implicated in the pathogenesis of chronic inflammation and autoimmune disorders (3). IFN-γ has also been shown to activate macrophages, which are intimately involved in plaque formation, and can also trigger the release of reactive oxygen species, a hallmark of subsequent atherogenesis and progression of CVD(4). Another well-known consequence of IFN-γ release is the activation of the enzyme indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan into kynurenine (5). The classic kynurenine pathway, involves the breakdown of tryptophan into downstream products such as kynurenine, kynurenic acid and 3-hydroxyanthranilic acid, the latter of which is eventually converted into quinolinic acid and picolinic acid (6). The final result is the production of one equivalent of nicotinamide adenine dinucleotide (NAD) for every processed tryptophan molecule (7). To date, most scientific literature regarding this pathway has focused on its relationship to neurological disorders such as Alzheimer's disease, Huntington's disease, and depression (8). These publications stem from the early finding that tryptophan is an essential precursor to brain serotonin and melatonin (9). Some studies relating tryptophan metabolites to CVD have reported inverse associations with tryptophan and the risk of CVD (10-16). However, to our knowledge, no studies have examined whether

changes in tryptophan levels are prospectively associated with the incidence of CVD. Furthermore, the effects of diet on metabolites of this pathway in relation to CVD have not been investigated.

In the present case-cohort study nested in the PREvención con DIeta MEDiterránea (PREDIMED) trial, we used repeated measurements of these metabolites to longitudinally assess: a) whether baseline and 1-year changes in tryptophan, kynurenine, kynurenic acid, 3-hydroxyanthranilic acid (3-HAA), and quinolinic acid were associated with future risk of CVD; b) whether these associations differed by the type of CVD events (stroke vs. non-stroke events [i.e. coronary heart disease and non-stroke related cardiac death]). In light of previous research on cognitive diseases, we hypothesized that a favorable tryptophan profile would be more beneficial in preventing stroke, as there may be common risk factors for both stroke and cognitive disorders, such as inflammation or preclinical cerebrovascular disease; c) whether significant associations could be counteracted by a Mediterranean diet (MedDiet); and d) whether the cardioprotective effect of a MedDiet is attenuated after adjusting for changes in these metabolites.

METHODS

Study population and design

The PREDIMED trial is a multicenter, randomized trial of dietary interventions with a MedDiet supplemented with either nuts or extra-virgin olive oil (EVOO) for the

primary prevention of CVD compared to a low-fat control group (17). Protocol, design, and primary results are detailed elsewhere (18, 19). Briefly, 7447 eligible men and women at high risk of CVD were randomly assigned to a MedDiet supplemented with a free provision for the family of 1 L/week EVOO (MedDiet + EVOO), a MedDiet 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 primary endpoint was a composite CVD, defined as stroke, myocardial infarction, or death from cardiovascular causes. Information on primary end points was collected by physicians and from other sources of information, such as the National Death Index. Only end points that were confirmed by the adjudication committee and that occurred between October 2003, and December 2010, were included in the analyses.

For the present study, we sampled approximately 10% of all PREDIMED participants in a case-cohort design. A random selection of 791 participants was chosen at baseline as subcohort. From the full cohort (including the subcohort), there were 231 CVD cases during follow-up and were included in the analysis. Of the CVD cases, 118 experienced a stroke (113 ischemic and 5 hemorrhagic), and 113 non-stroke events. We excluded individuals from specific analyses if they were missing metabolite values for that analysis. At baseline and at 1-year of follow-up, 896 and 806 participants had valid measurements for all five metabolites, respectively.

Quantification of Metabolites

Fasting plasma EDTA tubes were collected for all participants, and aliquots were coded and kept refrigerated until they were stored at −80 °C. Pairs of samples (baseline and first-year visits from each participant) were randomly ordered and shipped to the Broad Institute of Harvard and MIT (Cambridge, MA, USA) for metabolomics analyses. Liquid chromatography tandem mass spectrometry to quantitatively profile metabolites in fasting plasma collected at baseline and year 1 of the intervention. Reference standards were used to confirm metabolite identities. The details of this procedure are described elsewhere (20, 21).

Statistical Analyses

Rank-based inverse normal transformations were used to transform the non-normal distributions of the five metabolites under study (22). We calculated a kynurenine risk score (KRS) using as weights the adjusted coefficients obtained in the assessment of the association between the respective metabolite and the risk of CVD. We first regressed composite CVD on each individual metabolite in a fully adjusted Cox model, then multiplied the resulting beta by the normalized values for the corresponding metabolite. These five products were then summed to produce the KRS. If any of the individuals were missing any metabolite values, we did not calculate a KRS for that individual. Each normalized metabolite was also analyzed according to its quartile distribution. Quartile cutpoints were generated on the basis of the distributions of metabolites and score among non-cases. Scores for acylcarnitines (20), BCAAs (21), and ceramides were also calculated in this fashion. We conducted tests of linear trend

by examining an ordinal score on the basis of the median value in each quartile of metabolites and score in the multivariable models.

Baseline data by quartiles of the KRS were presented as means (± standard deviations) for continuous variables and N and percentages for categorical variables. Baseline characteristics were compared across quartiles of the KRS using chi-squared tests for categorical variables and one-way analysis of variance (ANOVA) for continuous variables.

We used weighted Cox regression models with non-cases up-weighted by their sampling fraction (22), and used robust variance to account for correlation between observations (23). We calculated hazard ratios (HRs) and their 95% confidence intervals (95% CIs) for the composite CVD end point, and also separately for stroke and non-stroke cases (myocardial infarction and non-stroke CVD mortality). 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 non-cases. In model 1, we adjusted for age, sex, family history of CHD, smoking status, and body mass index, and stratified by intervention group. In model 2, we additionally adjusted for model 1 plus baseline hypertension, dyslipidemia, diabetes, and scores for branched chain amino acids (BCAAs), acylcarnitines, and ceramides (previously reported as associated with CVD in our cohort). For models including 1-year change variables, we also adjusted for continuous baseline levels of that metabolite. We additionally conducted subgroup analyses by restricting the analysis to the group of interest. To test for effect modification, we used a likelihood ratio test to compare the

model without interaction terms vs. model with interaction terms (indicator variable for subgroup x KRS) among all participants.

For the mediation analysis of the MedDiet+EVOO, we report the coefficient for the MedDiet+EVOO vs. control (excluding individuals allocated to the MedDiet+nuts group) with and without adjustment for both baseline and change in tryptophan. We repeated this analyses for MedDiet+nuts excluding the MedDiet+EVOO participants.

To calculate 1-year changes in metabolites, we performed an ANOVA for each metabolites and calculated adjusted means of metabolite values at baseline and at 1 year, stratified by intervention group. We used paired t-tests to test if changes in metabolites were significant within each arm of the trial.

For correlation among metabolites and scores, we used Spearman correlation coefficients and p-values.

All statistical analyses were performed with SAS (v9.4, SAS Institute, Cary, NC) and R (v2.13.0, R Foundation, Vienna, Austria).

RESULTS

Descriptive Results

Demographic and behavioral characteristics of the participants in the subcohort with valid measurements of all five metabolites at baseline (n=724) are classified according to quartiles of KRS in **Table 1**. Increasing quartiles of KRS were significantly associated with age, % female sex, % hypertension, and % of current smokers. The median follow-up time was 4.7 years.

1-year changes in tryptophan metabolites according to intervention group are shown in **Supplemental Figure 1**. Metabolites of those randomized to the MedDiet with EVOO group uniformly decreased after 1-year. Using paired t-tests for the non-transformed metabolite quantifications at baseline and 1-year, we observed significant changes in: tryptophan and 3-HAA in the MedDiet with EVOO group, and kynurenic acid and quinolinic acid in the MedDiet with nuts group.

Spearman correlation coefficients of tryptophan metabolites, branched chain amino acids, ceramides, and acylcarnitine score are depicted in **Supplemental Table 1**. In general, tryptophan metabolites had low to moderate correlations with each other (r = 0.00 to 0.48), low to moderate correlation with branched chain amino acid score (r = 0.03 to 0.49), and modest correlations with ceramides and acylcarnitine scores (r = -0.29 to 0.14).

Individual Metabolites, Risk Score, and Composite CVD

Table 2 details the associations of baseline and 1-year changes of kynurenine pathway metabolites with composite CVD incidence. Only baseline kynurenic acid was associated with composite CVD risk in the fully adjusted model (HR per SD: 1.23; 95% CI: 1.02, 1.48; $P < 0.05$). Using 1-year changes, an increase in tryptophan was associated with a significantly reduced risk in the categorical analysis (HR for Q4 vs. Q1: 0.49; 95% CI: 0.26, 0.95; P-trend < 0.05) models, as well as in the continuous analysis (HR per SD: 0.79; 95% CI: 0.63, 0.98; $P < 0.05$)

Since the kynurenine pathway has been examined most often in relation to brain disorders, we hypothesized that associations of these metabolites would be stronger for stroke than for non-stroke events. Using stroke as the outcome in **Table 3**, we found no significant associations of any baseline metabolite concentration with stroke risk. Furthermore, the inverse association observed for 1-year changes in tryptophan was not present (HR per SD: 0.91; 95% CI: 0.70, 1.19; *P* = 0.49) when using stroke alone as the outcome. In **Table 3** when using only non-stroke events as the outcome, kynurenic acid (HR per SD: 1.46; 95% CI: 1.12, 1.92; *P* < 0.05) was associated with higher subsequent incidence of outcomes different from stroke (myocardial infarction or any other cause of vascular death). Furthermore, the relationship between 1-year increase in tryptophan and non-stroke outcomes was significantly associated with a lower risk of events in both the continuous models (HR per SD: 0.65; 95% CI: 0.46, 0.91; *P* < 0.05) and categorical models (HR for Q4 vs. Q1: 0.19; 95% CI: 0.06, 0.61; *P*-trend < 0.05). 1-year changes in kynurenine were also inversely associated with non-stroke event risk in the categorical analysis (HR for Q4 vs. Q1: 0.62; 95% CI: 0.28, 1.36; *P*-trend < 0.05).

In **Table 4**, using the KRS as exposure, we observed a similar pattern of elevated incidence of the composite CVD outcome (HR per SD: 1.41; 95% CI: 1.14, =1.75; *P* < 0.05), and a strengthened association with non-stroke events (HR per SD: 1.70; 95% CI: 1.27, 2.29; *P* < 0.001). We also considered the ratio of kynurenine to tryptophan in **Supplemental Table 2**, but found no significant associations of this ratio with any CVD outcome. Thus, we proceeded with the KRS to test effect modification by a Mediterranean diet.

Effect Modification by a Mediterranean Diet

In **Supplemental Table 3**, we sought to address whether several baseline variables potentially modified the positive association of the KRS with CVD. We only found significant interaction according to intervention group (*P*-interaction = 0.003), with those in the control group experiencing a greater elevation in risk per SD increase (HR per SD: 2.02; 95% CI: 1.31, 3.13) compared to the MedDiet + EVOO group (HR per SD: 1.27; 95% CI: 0.83, 1.94) and the MedDiet + nuts group (HR per SD: 1.23; 95% CI: 0.79, 1.92).

Figure 1 depicts the fully adjusted HRs stratified by intervention (combined MedDiet *vs.* control) and quartiles of the baseline KRS. Compared to those randomized to a MedDiet and in the lowest quartile of baseline KRS, we found a significant positive association for individuals randomized to the control group and quartiles 2 – 4 of the KRS, but not for those randomized to the MedDiet and quartiles 2 – 4 of the KRS.

Attenuation of the Benefits of a Mediterranean Diet by Tryptophan Changes

In **Supplemental Tables 4 and 5** we explore the strength of the protective association of a MedDiet before and after adjustment of tryptophan. When comparing MedDiet + EVOO vs. control, adjustment for baseline and 1-year changes in tryptophan attenuated the inverse association from HR per SD: 0.62; 95% CI: 0.38, 1.01 to HR per SD: 0.95; 95% CI: 0.66, 1.36. This attenuation appeared to be weaker for stroke and greater for non-stroke outcomes. However, when comparing MedDiet + nuts vs. control,

we did not observe these patterns of attenuation. After controlling for baseline and 1-year changes in tryptophan, the inverse association for the MedDiet + nuts only slightly changed from HR per SD: 0.61; 95% CI: 0.37, 1.00 to HR per SD: 0.60; 95% CI: 0.35, 1.02. Similarly, effect size was lower for stroke events and higher for non-stroke events.

DISCUSSION

In the present case-cohort study, we report that lower baseline kynurenic acid and higher baseline tryptophan, were associated with lower risk of non-stroke events, and 1-year increase in tryptophan levels was strongly associated with lower risk of CVD and non-stroke events. A combined score of five plasma metabolites in this pathway (tryptophan, kynurenine, kynurenic acid, 3-hydroxyanthranilic acid, and quinolinic acid), designed to summarize the pathway in a single parameter by maximizing predictiveness of outcome within the present study, was significantly associated with the risk of hard clinical events of CVD. Furthermore, consuming a MedDiet counteracted the harmful effect of an unfavorable metabolite profile in the tryptophan-kynurenine pathway. Lastly, changes in tryptophan may be involved in the cardioprotective benefits of the MedDiet.

The finding that tryptophan is inversely associated with CVD incidence is in line with previous publications (24-26). However, we report no significant associations of kynurenine/tryptophan ratio with any CVD outcome. Clinical studies have reported that tryptophan degradation, as operationalized by low blood tryptophan levels or a high kynurenine/tryptophan ratio, were predictive of coronary heart disease status (15), elevated oxidative stress in patients with kidney dysfunction (13), and greater mean

carotid artery intima-media thickness (27). Large scale epidemiologic studies have reported positive associations of tryptophan catabolism (i.e. lower levels of tryptophan and higher levels of downstream metabolites in the tryptophan-kynurenine pathway) with primary acute coronary events (28), worse prognosis after diagnosis of coronary artery disease (14), and greater risk of mortality from CVD(16).

Using repeated measurements of metabolites, our observation that baseline and 1-year increases in tryptophan are associated with lower risk of CVD events (especially when stroke was excluded) needs to be compared with the existing literature which has suggested that tryptophan levels are decreased in stroke patients compared to controls (12), and that lower blood tryptophan concentrations are related to greater infarct volume in stroke patients (11) and worse prognosis (29) and faster cognitive decline (30) after stroke. Our results are consistent with an inverse association between tryptophan and CVD, but they may differ from those of previous studies according to the subtype of CVD event, given that we have not found a stronger effect for stroke than for other events. A potential explanation might be that existing publications largely used cross-sectional designs examining stroke patients at baseline, whereas we used a longitudinal design for both the exposure and the outcome and followed disease-free individuals for repeated measurements of metabolites after an intervention and also for incident endpoints. Mangge et al. (31) suggested that decreases in tryptophan levels may be a consequence of chronic low-grade inflammation, rather than a cause of disease. Our findings of an inverse association between changes in tryptophan and non-stroke endpoints would support the inflammation hypothesis for non-stroke events, given the

anti-inflammatory properties of the MedDiet. However, further studies are needed to elucidate the differing effects of tryptophan on stroke vs. non-stroke CVD events.

The causal role of tryptophan and kynurenine pathway metabolites in CVD remains poorly understood. It is thought that IFN-γ plays a central role in the activation of IDO and subsequent degradation of tryptophan (4); however, activation of the kynurenine pathway has also been shown to have anti-inflammatory effects (3). Treatment of human peripheral blood mononuclear cells and monocyte-derived macrophages with IFN-γ attenuated the extent of low density lipoprotein (LDL) oxidation, and tryptophan degradation in concert with 3-HAA formation was instrumental in this inhibitory effect (32). 3-HAA has also been independently identified as having anti-atherogenic properties by regulating lipid metabolism and inflammation (33, 34). Other experimental studies suggest a beneficial effect of IDO on the vasculature. IDO-deficient mice fed high-fat diets showed marked increases in F4/80 and TNF mRNA levels, as well as greater hepatic inflammation compared to controls (35). IDO inhibition also blunted the protective effects of eicosapentaenoic acid in $LDLr^{-/-}$ mice (31). In light of the prevailing theme in experimental studies that IDO-mediated degradation of tryptophan is beneficial, we speculate that activation of the tryptophan-kynurenine pathway may be a compensatory mechanism to, rather than a cause of, inflammation and cardiovascular dysfunction. Furthermore, since most individuals consume adequate amounts of tryptophan (i.e. do not suffer from tryptophan deficiency), the protection conferred by the MedDiet is unlikely to be related to greater availability of tryptophan (36).

The novel finding that a MedDiet may offset the deleterious effects of a high-risk profile in metabolites of the tryptophan-kynurenine pathway also warrants discussion. To our knowledge, this the first report of an association of tryptophan metabolites with CVD in the context of a nutritional intervention in a large randomized trial. Previous clinical trials have concluded that close adherence to a MedDiet has a beneficial effect on inflammatory markers (37, 38), and other PREDIMED reports have noted that participants randomized to the MedDiet interventions had lower incidence of CVD events compared to those in the control group, even among those with comparable levels of plasma branched chain amino acids (21) or acylcarnitines (20). These publications, in addition to support the favorable interaction of the MedDiet with various CVD biomarkers also point to the need of future experimental studies to clarify the biological mechanisms underlying this effect.

The randomization of dietary interventions at baseline allowed us to study possible mediating effects of tryptophan in the association between MedDiet and the risk of CVD. We found that among the MedDiet + EVOO arm, adjustment for changes in tryptophan attenuated the HRs for CVD. These results suggest a possible role of tryptophan degradation (or preservation) as a mediator in the causal pathway of EVOO consumption and CVD prevention. We acknowledge that our statistical power for detecting interactions is limited. Consumption of both EVOO (39) and nuts (40, 41) has been associated with reduced circulating levels of inflammatory biomarkers, although the specific roles of IFN-γ and tryptophan degradation in diets enriched with these foods are not well characterized. Primary results from the PREDIMED trial also reported no striking differences between the two MedDiet groups in relation to composite CVD or

secondary outcomes (18). However, dietary exposure to walnuts has been characterized by changes in various metabolites, including intermediate metabolites of the tryptophan pathway (42). Future lines of inquiry should investigate the biological roles of IFN-γ, tryptophan, and related kynurenine metabolites in diets involving EVOO or nuts.

Strengths of the present study include blood draws at repeated intervals to assess changes in metabolites, the prospective design of the cohort, as well as adjustment for potential confounders related to CVD. Our study also has limitations. First, we did not measure all metabolites on interest in the tryptophan-kynurenine pathway, such as picolinic acid or 3-hydroxykynurenine. Second, we cannot rule out the possibility that concentrations of the metabolites were different between missing and non-missing cases. Lastly, results from our study among a population of high-risk participants living in the Mediterranean region may not be generalizable to individuals of different demographics.

Our results indicate that 1-year changes in tryptophan are predictive of lower CVD incidence and, especially, non-stroke incidence and that a score combining five metabolites in the tryptophan-kynurenine pathway is also prospectively associated with clinical cases of CVD. The harmful effects of an unfavorable tryptophan metabolite profile were in part mitigated by consuming a MedDiet. The cardioprotective effect of MedDiets supplemented with EVOO or nuts could be mediated in part by processes associated with changes in tryptophan.

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Author Contributions

EY conducted the analysis and wrote the manuscript. MRC, MGF, YZ, and DDW provided code and guidance needed to complete the analysis. ET, CBC, JSS, LL, DC, MF, EGG, JL, RE, ER, MC, FA, DR, LSM, JVS, FBH, and MAM designed the research plan and oversaw the study. All authors read and approved the final version of the manuscript.

REFERENCES

1. Willerson JT, Ridker PM. Inflammation as a Cardiovascular Risk Factor. Circulation 2004;109(21 suppl 1):II-2-II-10. doi: 10.1161/01.cir.0000129535.04194.38.
2. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. J Leukocyte Biol 2004;75(2):163-89. doi: 10.1189/jlb.0603252.
3. Muhl H, Pfeilschifter J. Anti-inflammatory properties of pro-inflammatory interferon-gamma. International immunopharmacology 2003;3(9):1247-55. doi: 10.1016/s1567-5769(03)00131-0.
4. Schroecksnadel K, Frick B, Winkler C, Fuchs D. Crucial role of interferon-gamma and stimulated macrophages in cardiovascular disease. Current vascular pharmacology 2006;4(3):205-13.
5. Taylor MW, Feng GS. Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 1991;5(11):2516-22.
6. Schwarcz R. The kynurenine pathway of tryptophan degradation as a drug target. Curr Opin Pharmacol 2004;4(1):12-7. doi: http://dx.doi.org/10.1016/j.coph.2003.10.006.
7. Nishizuka Y, Hayaishi O. Studies on the Biosynthesis of Nicotinamide Adenine Dinucleotide: I. Enzymic synthesis of niacin ribonucleotides from 3-hydroxyanthranilic acid in mammalian tissues. J Biol Chem 1963;238(10):3369-77.
8. Chen Y, Guillemin GJ. Kynurenine Pathway Metabolites in Humans: Disease and Healthy States. Int J Tryptophan Res 2009;2:1-19.
9. Fernstrom JD, Wurtman RJ. Brain Serotonin Content: Physiological Dependence on Plasma Tryptophan Levels. Science 1971;173(3992):149-52. doi: 10.1126/science.173.3992.149.
10. Lovelace MD, Varney B, Sundaram G, Lennon MJ, Lim CK, Jacobs K, Guillemin GJ, Brew BJ. Recent evidence for an expanded role of the kynurenine pathway of tryptophan metabolism in neurological diseases. Neuropharmacology 2016. doi: 10.1016/j.neuropharm.2016.03.024.
11. Darlington LG, Mackay GM, Forrest CM, Stoy N, George C, Stone TW. Altered kynurenine metabolism correlates with infarct volume in stroke. Eur J Neurol 2007;26(8):2211-21. doi: 10.1111/j.1460-9568.2007.05838.x.
12. Ormstad H, Verkerk R, Aass HC, Amthor KF, Sandvik L. Inflammation-induced catabolism of tryptophan and tyrosine in acute ischemic stroke. Journal of molecular neuroscience : MN 2013;51(3):893-902. doi: 10.1007/s12031-013-0097-2.
13. Pawlak K, Domaniewski T, Mysliwiec M, Pawlak D. The kynurenines are associated with oxidative stress, inflammation and the prevalence of

cardiovascular disease in patients with end-stage renal disease. Atherosclerosis 2009;204(1):309-14. doi: 10.1016/j.atherosclerosis.2008.08.014.
14. Pedersen ER, Svingen GF, Schartum-Hansen H, Ueland PM, Ebbing M, Nordrehaug JE, Igland J, Seifert R, Nilsen RM, Nygard O. Urinary excretion of kynurenine and tryptophan, cardiovascular events, and mortality after elective coronary angiography. European heart journal 2013;34(34):2689-96. doi: 10.1093/eurheartj/eht264.
15. Wirleitner B, Rudzite V, Neurauter G, Murr C, Kalnins U, Erglis A, Trusinskis K, Fuchs D. Immune activation and degradation of tryptophan in coronary heart disease. Eur J Clin Invest 2003;33(7):550-4. doi: 10.1046/j.1365-2362.2003.01186.x.
16. Zuo H, Ueland PM, Ulvik A, Eussen SJPM, Vollset SE, Nygård O, Midttun Ø, Theofylaktopoulou D, Meyer K, Tell GS. Plasma Biomarkers of Inflammation, the Kynurenine Pathway, and Risks of All-Cause, Cancer, and Cardiovascular Disease Mortality: The Hordaland Health Study. Am J Epidemiol 2016. doi: 10.1093/aje/kwv242.
17. PREDIMED. Internet: www.predimed.es (accessed November 24 2016).
18. Estruch R, Ros E, Salas-Salvadó J, Covas M-I, Corella D, Arós F, Gómez-Gracia E, Ruiz-Gutiérrez V, Fiol M, Lapetra J, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. New Engl J Med 2013;368(14):1279-90. doi: doi:10.1056/NEJMoa1200303.
19. Martinez-Gonzalez MA, Corella D, Salas-Salvado J, Ros E, Covas MI, Fiol M, Warnberg J, Aros F, Ruiz-Gutierrez V, Lamuela-Raventos RM, et al. Cohort profile: design and methods of the PREDIMED study. Int J Epidemiol 2012;41(2):377-85. doi: 10.1093/ije/dyq250.
20. Guasch-Ferré M, Zheng Y, Ruiz-Canela M, Hruby A, Martínez-González MA, Clish CB, Corella D, Estruch R, Ros E, Fitó M, et al. Plasma acylcarnitines and risk of cardiovascular disease: effect of Mediterranean diet interventions. Am J Clin Nutr 2016;103(6):1408-16. doi: 10.3945/ajcn.116.130492.
21. Ruiz-Canela M, Toledo E, Clish CB, Hruby A, Liang L, Salas-Salvadó J, Razquin C, Corella D, Estruch R, Ros E, et al. Plasma Branched-Chain Amino Acids and Incident Cardiovascular Disease in the PREDIMED Trial. Clin Chem 2016;62(4):582-92. doi: 10.1373/clinchem.2015.251710.
22. Barlow WE, Ichikawa L, Rosner D, Izumi S. Analysis of case-cohort designs. Journal of clinical epidemiology 1999;52(12):1165-72.
23. Lee EW, Wei LJ, Amato DA, Leurgans S. Cox-Type Regression Analysis for Large Numbers of Small Groups of Correlated Failure Time Observations. Edtion ed. In: Klein JP, Goel PK, eds. Survival Analysis: State of the Art. Dordrecht: Springer Netherlands, 1992:237-47.
24. Polyzos KA, Ketelhuth DF. The role of the kynurenine pathway of tryptophan metabolism in cardiovascular disease. An emerging field. Hamostaseologie 2015;35(2):128-36. doi: 10.5482/hamo-14-10-0052.
25. Murr C, Grammer TB, Kleber ME, Meinitzer A, Marz W, Fuchs D. Low serum tryptophan predicts higher mortality in cardiovascular disease. Eur J Clin Invest 2015;45(3):247-54. doi: 10.1111/eci.12402.

26. Mangge H, Reininghaus E, Fuchs D. Role of Kynurenine Pathway in Cardiovascular Diseases. Edtion ed. In: Mittal S, ed. Targeting the Broadly Pathogenic Kynurenine Pathway. Cham: Springer International Publishing, 2015:133-43.
27. Kato A, Suzuki Y, Suda T, Suzuki M, Fujie M, Takita T, Furuhashi M, Maruyama Y, Chida K, Hishida A. Relationship between an increased serum kynurenine/tryptophan ratio and atherosclerotic parameters in hemodialysis patients. Hemodialysis international International Symposium on Home Hemodialysis 2010;14(4):418-24. doi: 10.1111/j.1542-4758.2010.00464.x.
28. Sulo G, Vollset SE, Nygard O, Midttun O, Ueland PM, Eussen SJ, Pedersen ER, Tell GS. Neopterin and kynurenine-tryptophan ratio as predictors of coronary events in older adults, the Hordaland Health Study. International journal of cardiology 2013;168(2):1435-40. doi: 10.1016/j.ijcard.2012.12.090.
29. Brouns R, Verkerk R, Aerts T, De Surgeloose D, Wauters A, Scharpe S, De Deyn PP. The role of tryptophan catabolism along the kynurenine pathway in acute ischemic stroke. Neurochemical research 2010;35(9):1315-22. doi: 10.1007/s11064-010-0187-2.
30. Stone TW, Forrest CM, Stoy N, Darlington LG. Involvement of kynurenines in Huntington's disease and stroke-induced brain damage. J Neutral Transm 2012;119(2):261-74. doi: 10.1007/s00702-011-0676-8.
31. Nakajima K, Yamashita T, Kita T, Takeda M, Sasaki N, Kasahara K, Shinohara M, Rikitake Y, Ishida T, Yokoyama M, et al. Orally administered eicosapentaenoic acid induces rapid regression of atherosclerosis via modulating the phenotype of dendritic cells in LDL receptor-deficient mice. Arteriosclerosis, thrombosis, and vascular biology 2011;31(9):1963-72. doi: 10.1161/atvbaha.111.229443.
32. Christen S, Thomas SR, Garner B, Stocker R. Inhibition by interferon-gamma of human mononuclear cell-mediated low density lipoprotein oxidation. Participation of tryptophan metabolism along the kynurenine pathway. The Journal of clinical investigation 1994;93(5):2149-58. doi: 10.1172/jci117211.
33. Zhang L, Ovchinnikova O, Jonsson A, Lundberg AM, Berg M, Hansson GK, Ketelhuth DF. The tryptophan metabolite 3-hydroxyanthranilic acid lowers plasma lipids and decreases atherosclerosis in hypercholesterolaemic mice. European heart journal 2012;33(16):2025-34. doi: 10.1093/eurheartj/ehs175.
34. Krause D, Suh H-S, Tarassishin L, Cui QL, Durafourt BA, Choi N, Bauman A, Cosenza-Nashat M, Antel JP, Zhao M-L, et al. The Tryptophan Metabolite 3-Hydroxyanthranilic Acid Plays Anti-Inflammatory and Neuroprotective Roles During Inflammation: Role of Hemeoxygenase-1. Am J Pathol 2011;179(3):1360-72. doi: 10.1016/j.ajpath.2011.05.048.
35. Nagano J, Shimizu M, Hara T, Shirakami Y, Kochi T, Nakamura N, Ohtaki H, Ito H, Tanaka T, Tsurumi H, et al. Effects of indoleamine 2,3-dioxygenase deficiency on high-fat diet-induced hepatic inflammation. PloS one 2013;8(9):e73404. doi: 10.1371/journal.pone.0073404.
36. Moehn S, Pencharz PB, Ball RO. Lessons Learned Regarding Symptoms of Tryptophan Deficiency and Excess from Animal Requirement Studies. The Journal of Nutrition 2012;142(12):2231S-5S. doi: 10.3945/jn.112.159061.

37. Chrysohoou C, Panagiotakos DB, Pitsavos C, Das UN, Stefanadis C. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adultsThe Attica study. J Am Coll Cardiol 2004;44(1):152-8. doi: 10.1016/j.jacc.2004.03.039.
38. Esposito K, Marfella R, Ciotola M, et al. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: A randomized trial. JAMA 2004;292(12):1440-6. doi: 10.1001/jama.292.12.1440.
39. Cicerale S, Lucas LJ, Keast RSJ. Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. Curr Opin Biotech 2012;23(2):129-35. doi: http://dx.doi.org/10.1016/j.copbio.2011.09.006.
40. Salas-Salvado J, Casas-Agustench P, Murphy MM, Lopez-Uriarte P, Bullo M. The effect of nuts on inflammation. Asia Pacific journal of clinical nutrition 2008;17 Suppl 1:333-6.
41. Yu Z, Malik VS, Keum N, Hu FB, Giovannucci EL, Stampfer MJ, Willett WC, Fuchs CS, Bao Y. Associations between nut consumption and inflammatory biomarkers. The American Journal of Clinical Nutrition 2016. doi: 10.3945/ajcn.116.134205.
42. Garcia-Aloy M, Llorach R, Urpi-Sarda M, Tulipani S, Estruch R, Martinez-Gonzalez MA, Corella D, Fito M, Ros E, Salas-Salvado J, et al. Novel multimetabolite prediction of walnut consumption by a urinary biomarker model in a free-living population: the PREDIMED study. Journal of proteome research 2014;13(7):3476-83. doi: 10.1021/pr500425r.

Table 1. Baseline characteristics of the full sub-cohort according to quartiles of kynurenine risk score.

	Kynurenine risk score				
	Q1	Q2	Q3	Q4	*P* value
n	180	181	179	188	
Median Score	-0.43	-0.14	0.08	0.41	
Range	-1.06 to -0.24	-0.24 to -0.03	-0.03 to 0.18	0.18 to 1.08	
Age (years)[1]	66.2 ± 5.8	66.8 ± 6.0	66.8 ± 5.8	69.0 ± 5.7	<0.0001
Sex (% Women)	46.1	57.5	60.3	62.2	0.01
Body mass index, kg/m^2	29.5 ± 3.6	29.5 ± 3.4	29.8 ± 3.7	30.3 ± 3.5	0.09
Intervention group, %					0.21
MedDiet+EVOO	33.3	32.6	40.2	44.2	
MedDiet+nuts	34.4	37.0	29.1	30.3	
Control	32.2	30.4	30.7	25.5	
Family history of CHD, %	21.7	27.6	22.9	27.7	0.41
Hypertension, %	78.3	84.5	82.1	87.8	0.10
Dyslipidemia, %	71.1	74.6	73.2	73.4	0.90
Diabetes, %	47.2	40.3	53.6	47.9	0.09
Obesity (BMI ≥ 30), %	43.3	40.3	46.9	53.2	0.08
Smoking, %					<0.0001
Never	52.2	65.8	62.6	69.7	
Former	22.2	23.2	26.8	26.6	
Current	25.6	11.1	10.6	3.7	

[1]*Data are expressed as means ± SD or percentage. P value for comparisons between cases and controls across quartiles (Pearson X^2 test for categorical variables or one way ANOVA for continuous variables) as appropriate.*

Table 2. Hazard ratios (95% CI) for composite CVD (stroke, non-stroke, death from vascular causes) by baseline and 1-year changes in plasma metabolites (tryptophan, kynurenine, kynurenic acid, 3-hydroxyanthranilic acid, quinolinic acid) as continuous and categorical variables. Number of participants will vary according to availability of plasma metabolite quantifications.

	Baseline[1]				
	Tryptophan	Kynurenine	Kynurenic Acid	3-HAA	Quinolinic Acid
Model 1, Metabolite as continuous variable, per SD					
HR (95% CI)	0.88 (0.74 – 1.04)	1.00 (0.86 – 1.16)	1.14 (0.99 – 1.31)	0.82 (0.69 – 0.98)	1.03 (0.86 – 1.22)
P	0.13	0.99	0.08	0.03	0.77
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	0.91 (0.59 – 1.40)	0.86 (0.55 – 1.35)	1.49 (0.91 – 2.42)	0.83 (0.53 – 1.30)	0.93 (0.59 – 1.47)
Q3	0.67 (0.42 – 1.05)	0.93 (0.61 – 1.44)	1.39 (0.86 – 2.24)	0.62 (0.38 – 1.03)	0.99 (0.64 – 1.54)
Q4	0.75 (0.48 – 1.19)	0.91 (0.59 – 1.40)	1.49 (0.92 – 2.40)	0.69 (0.43 – 1.11)	1.17 (0.75 – 1.83)
P-trend	0.12	0.76	0.14	0.07	0.46
Model 2, Metabolite as continuous variable, per SD					
HR (95% CI)	0.87 (0.67 – 1.11)	1.06 (0.89 – 1.26)	1.23 (1.02 – 1.48)	0.82 (0.67 – 1.01)	1.15 (0.94 – 1.41)
P	0.28	0.52	0.03	0.06	0.19
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	0.95 (0.58 – 1.57)	1.27 (0.75 – 2.13)	1.58 (0.91 – 2.76)	0.72 (0.42 – 1.23)	1.00 (0.60 – 1.67)
Q3	0.58 (0.33 – 1.01)	1.17 (0.72 – 1.92)	1.62 (0.92 – 2.85)	0.84 (0.46 – 1.50)	1.08 (0.65 – 1.82)
Q4	0.75 (0.40 – 1.44)	1.21 (0.73 – 1.99)	1.73 (0.97 – 3.10)	0.64 (0.36 – 1.16)	1.53 (0.91 – 2.57)
P-trend	0.21	0.55	0.08	0.22	0.11
	1-Year Changes[2]				
	Tryptophan	Kynurenine	Kynurenic Acid	3-HAA	Quinolinic Acid
Model 1, Metabolite as continuous variable, per SD					
HR (95% CI)	0.77 (0.64 – 0.94)	0.86 (0.69 – 1.06)	0.87 (0.73 – 1.04)	1.15 (0.91 – 1.45)	0.96 (0.80 – 1.15)
P	0.01	0.15	0.13	0.26	0.64
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	1.18 (0.72 – 1.94)	0.79 (0.48 – 1.30)	0.95 (0.56 – 1.61)	1.59 (0.79 – 3.19)	0.84 (0.48 – 1.47)
Q3	0.75 (0.44 – 1.27)	0.37 (0.20 – 0.70)	0.88 (0.50 – 1.53)	1.91 (0.91 – 3.99)	0.95 (0.54 – 1.66)

Q4	0.49 (0.27 – 0.87)	0.68 (0.41 – 1.13)	0.69 (0.40 – 1.20)	1.46 (0.70 – 3.05)	0.89 (0.52 – 1.51)
P-trend	0.005	0.02	0.18	0.27	0.78
Model 2, Metabolite as continuous variable, per SD					
HR (95% CI)	0.79 (0.63 – 0.98)	0.85 (0.67 – 1.06)	0.93 (0.75 – 1.15)	1.15 (0.90 – 1.49)	0.92 (0.75 – 1.13)
P	0.03	0.15	0.52	0.27	0.42
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	1.19 (0.70 – 2.02)	0.78 (0.45 – 1.34)	1.00 (0.53 – 1.87)	1.26 (0.56 – 2.84)	0.82 (0.43 – 1.57)
Q3	0.72 (0.40 – 1.28)	0.39 (0.20 – 0.77)	1.00 (0.52 – 1.93)	1.37 (0.55 – 3.43)	0.97 (0.51 – 1.85)
Q4	0.49 (0.26 – 0.95)	0.60 (0.34 – 1.07)	0.84 (0.44 – 1.58)	1.32 (0.57 – 3.03)	0.78 (0.43 – 1.42)
P-trend	0.01	0.02	0.60	0.50	0.55

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 covariates in model 1, plus baseline hypertension, dyslipidemia, diabetes, and scores for BCAAs, acylcarnitines, and ceramides. For change analyses, metabolites were adjusted for baseline values.
[1]*Baseline analysis consisted of n = 986 participants and n = 231 cases for tryptophan, kynurenine, KA, and quinolinic acid, and n = 904 participants and n = 204 cases for 3-HAA.*
[2]*1-year change analysis consisted of n = 908 participants and n = 162 cases for tryptophan, kynurenine, KA, and quinolinic acid, and n = 750 participants and n = 122 cases for 3-HAA.*

Table 3. Hazard ratios (95% CI) for stroke and non-stroke cases by baseline and 1-year changes in plasma metabolites (tryptophan, kynurenine, kynurenic acid, 3-hydroxyanthranilic acid, quinolinic acid) as continuous and categorical variables. Number of participants will vary according to availability of plasma metabolite quantifications.

STROKE					
Baseline[1]					
	Tryptophan	Kynurenine	Kynurenic Acid	3-HAA	Quinolinic Acid
Model 1, Metabolite as continuous variable, per SD					
HR (95% CI)	0.97 (0.76 – 1.24)	0.97 (0.79 – 1.20)	1.05 (0.87 – 1.26)	0.78 (0.61 – 0.98)	0.91 (0.72 – 1.15)
P	0.81	0.79	0.65	0.04	0.42
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	0.68 (0.38 – 1.22)	0.87 (0.49 – 1.54)	1.43 (0.79 – 2.61)	0.67 (0.37 – 1.21)	0.81 (0.45 – 1.46)
Q3	0.61 (0.34 – 1.10)	0.80 (0.46 – 1.40)	1.00 (0.54 – 1.86)	0.69 (0.37 – 1.30)	0.95 (0.55 – 1.65)
Q4	0.95 (0.54 – 1.66)	0.89 (0.50 – 1.57)	1.16 (0.61 – 2.18)	0.63 (0.34 – 1.17)	0.96 (0.54 – 1.72)
P-trend	0.77	0.62	0.94	0.18	0.97
Model 2, Metabolite as continuous variable, per SD					
HR (95% CI)	0.98 (0.71 – 1.35)	1.02 (0.82 – 1.26)	1.06 (0.83 – 1.37)	0.76 (0.57 – 1.01)	1.05 (0.81 – 1.37)
P	0.89	0.89	0.63	0.06	0.72
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	0.73 (0.38 – 1.41)	1.22 (0.65 – 2.30)	1.43 (0.72 – 2.82)	0.56 (0.28 – 1.12)	0.93 (0.49 – 1.75)
Q3	0.52 (0.25 – 1.09)	0.90 (0.48 – 1.67)	1.07 (0.52 – 2.18)	0.87 (0.42 – 1.80)	1.09 (0.58 – 2.05)
Q4	0.96 (0.43 – 2.16)	1.13 (0.60 – 2.12)	1.19 (0.55 – 2.61)	0.52 (0.24 – 1.12)	1.26 (0.65 – 2.47)
P-trend	0.74	0.96	0.85	0.23	0.43
1-Year Changes[2]					
	Tryptophan	Kynurenine	Kynurenic Acid	3-HAA	Quinolinic Acid
Model 1, Metabolite as continuous variable, per SD					
HR (95% CI)	0.89 (0.70 – 1.12)	0.88 (0.66 – 1.17)	0.90 (0.71 – 1.15)	1.27 (0.93 – 1.72)	0.87 (0.69 – 1.10)
P	0.32	0.37	0.41	0.14	0.24
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	1.51 (0.79 – 2.90)	0.64 (0.33 – 1.26)	0.46 (0.22 – 0.99)	1.65 (0.63 – 4.31)	1.11 (0.54 – 2.27)

Q3	0.88 (0.43 – 1.80)	0.44 (0.20 – 0.98)	0.83 (0.41 – 1.68)	1.90 (0.68 – 5.31)	1.10 (0.54 – 2.25)
Q4	0.84 (0.41 – 1.70)	0.67 (0.34 – 1.31)	0.59 (0.28 – 1.23)	1.79 (0.65 – 4.92)	0.85 (0.43 – 1.70)
P-trend	0.35	0.16	0.40	0.25	0.66
Model 2, Metabolite as continuous variable, per SD					
HR (95% CI)	0.91 (0.70 – 1.19)	0.85 (0.63 – 1.16)	0.97 (0.72 – 1.29)	1.24 (0.90 – 1.72)	0.85 (0.66 – 1.09)
P	0.49	0.31	0.81	0.19	0.20
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	1.60 (0.82 – 3.16)	0.59 (0.28 – 1.25)	0.44 (0.18 – 1.04)	1.30 (0.46 – 3.74)	1.03 (0.46 – 2.29)
Q3	0.90 (0.42 – 1.92)	0.51 (0.23 – 1.14)	0.86 (0.38 – 1.92)	1.42 (0.41 – 4.85)	1.08 (0.49 – 2.36)
Q4	0.89 (0.42 – 1.92)	0.56 (0.26 – 1.18)	0.71 (0.31 – 1.58)	1.55 (0.53 – 4.56)	0.78 (0.37 – 1.63)
P-trend	0.46	0.12	0.78	0.43	0.54
NON-STROKE					
Baseline[3]					
	Tryptophan	Kynurenine	Kynurenic Acid	3-HAA	Quinolinic Acid
Model 1[2], Metabolite as continuous variable, per SD					
HR (95% CI)	0.78 (0.63 – 0.97)	1.03 (0.85 – 1.26)	1.24 (1.02 – 1.51)	0.85 (0.69 – 1.06)	1.17 (0.92 – 1.47)
P	0.03	0.74	0.03	0.14	0.20
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	1.20 (0.67 – 2.16)	0.80 (0.43 – 1.50)	1.50 (0.73 – 3.09)	1.04 (0.56 – 1.91)	1.05 (0.57 – 1.95)
Q3	0.78 (0.42 – 1.44)	1.03 (0.58 – 1.85)	1.90 (0.96 – 3.76)	0.53 (0.26 – 1.10)	1.00 (0.54 – 1.86)
Q4	0.58 (0.30 – 1.11)	0.94 (0.52 – 1.71)	2.02 (1.02 – 3.97)	0.74 (0.40 – 1.40)	1.48 (0.80 – 2.73)
P-trend	0.05	0.94	0.03	0.13	0.26
Model 2, Metabolite as continuous variable, per SD					
HR (95% CI)	0.76 (0.54 – 1.05)	1.10 (0.87 – 1.39)	1.46 (1.12 – 1.92)	0.85 (0.66 – 1.10)	1.30 (0.97 – 1.73)
P	0.09	0.42	0.01	0.22	0.08
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	1.16 (0.60 – 2.26)	1.26 (0.60 – 2.65)	1.77 (0.77 – 4.08)	0.99 (0.49 – 2.00)	1.12 (0.54 – 2.35)
Q3	0.69 (0.33 – 1.43)	1.49 (0.75 – 2.94)	2.49 (1.10 – 5.66)	0.81 (0.36 – 1.82)	1.10 (0.53 – 2.30)
Q4	0.52 (0.21 – 1.29)	1.33 (0.65 – 2.74)	2.89 (1.26 – 6.65)	0.74 (0.35 – 1.60)	2.05 (1.00 – 4.21)
P-trend	0.10	0.39	0.01	0.39	0.07
1-Year Changes[4]					

	Tryptophan	Kynurenine	Kynurenic Acid	3-HAA	Quinolinic Acid
Model 1, Metabolite as continuous variable, per SD					
HR (95% CI)	0.64 (0.48 – 0.86)	0.82 (0.62 – 1.07)	0.82 (0.65 – 1.05)	1.04 (0.76 – 1.42)	1.03 (0.79 – 1.33)
P	0.003	0.15	0.11	0.82	0.84
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	0.93 (0.47 – 1.84)	0.94 (0.48 – 1.81)	1.69 (0.85 – 3.36)	1.59 (0.62 – 4.11)	0.59 (0.26 – 1.37)
Q3	0.63 (0.31 – 1.30)	0.27 (0.11 – 0.68)	0.79 (0.34 – 1.86)	2.03 (0.76 – 5.45)	0.81 (0.36 – 1.83)
Q4	0.22 (0.08 – 0.56)	0.64 (0.32 – 1.28)	0.79 (0.37 – 1.70)	1.19 (0.44 – 3.21)	0.90 (0.43 – 1.88)
P-trend	0.001	0.03	0.24	0.64	0.99
Model 2, Metabolite as continuous variable, per SD					
HR (95% CI)	0.65 (0.46 – 0.90)	0.81 (0.60 – 1.09)	0.85 (0.64 – 1.14)	1.09 (0.78 – 1.53)	0.96 (0.71 – 1.30)
P	0.01	0.17	0.29	0.61	0.81
Metabolite in quartile categories, as compared to Q1 (reference)					
Q2	0.84 (0.39 – 1.84)	0.98 (0.48 – 2.00)	2.14 (0.91 – 5.04)	1.30 (0.39 – 4.36)	0.59 (0.22 – 1.57)
Q3	0.55 (0.24 – 1.27)	0.22 (0.07 – 0.64)	1.08 (0.39 – 2.97)	1.45 (0.40 – 5.28)	0.84 (0.30 – 2.35)
Q4	0.19 (0.06 – 0.61)	0.62 (0.28 – 1.36)	0.98 (0.38 – 2.51)	1.20 (0.37 – 3.88)	0.74 (0.31 – 1.77)
P-trend	0.003	0.02	0.62	0.73	0.70

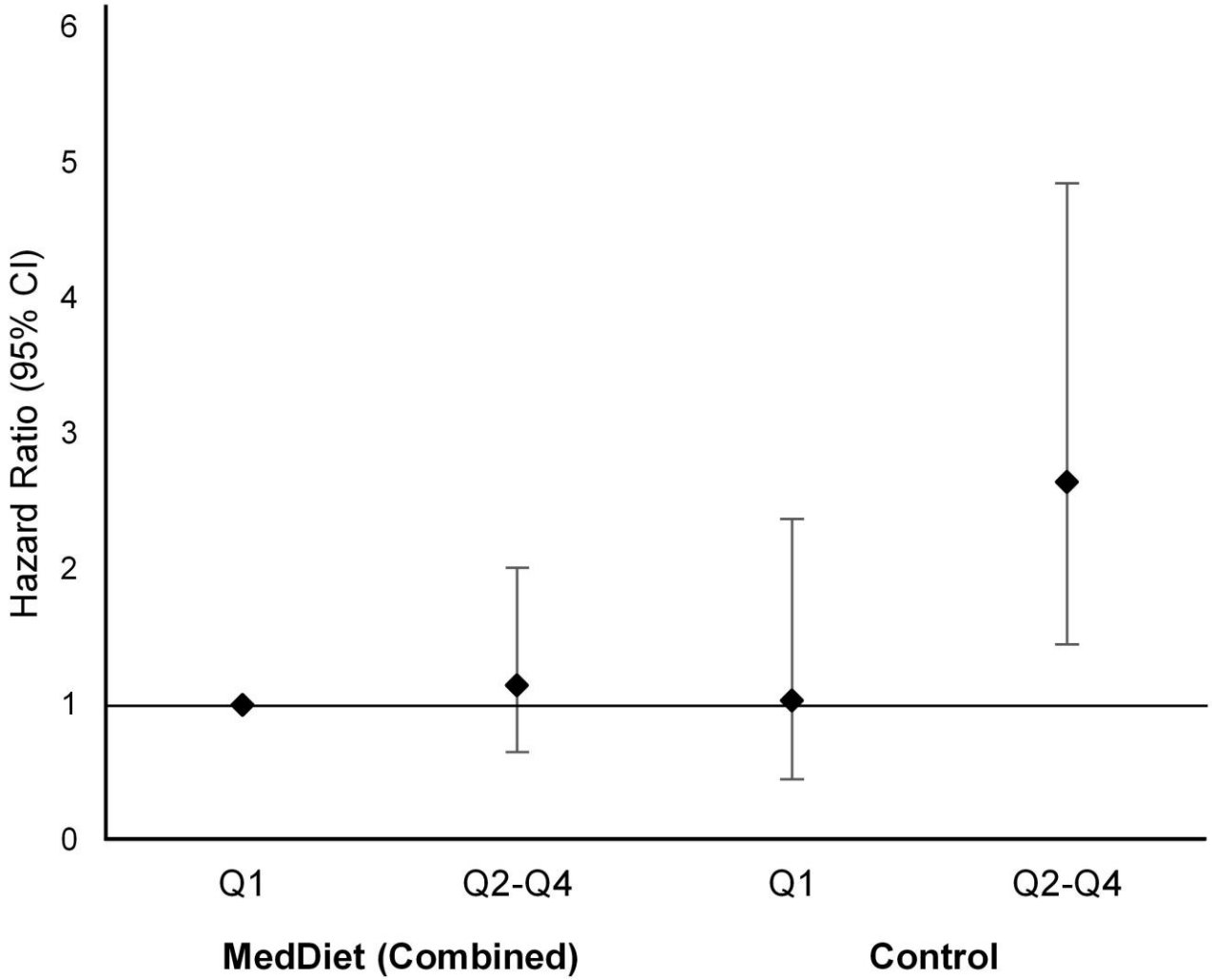
Model 1 was adjusted for age, sex, family history of CHD, smoking status, and body mass index, and was stratified by intervention group.
Model 2 was adjusted as for model 1, plus baseline hypertension, dyslipidemia, diabetes, and scores for BCAAs, acylcarnitines, and ceramides. For change analyses, metabolites were adjusted for baseline values.
[1]*Baseline analysis for stroke consisted of n = 872 participants and n = 118 cases for tryptophan, kynurenine, KA and quinolinic acid, and n = 804 participants and n = 103 cases for 3-HAA. Non-stroke cases are excluded.*
[2]*1-year change analysis for stroke consisted of n = 835 participants and n = 86 cases for tryptophan, kynurenine, KA, and quinolinic acid, and n = 692 participants and n = 63 cases for 3-HAA. Non-stroke cases are excluded.*
[3]*Baseline analysis for non-stroke consisted of n = 868 participants and n = 113 cases for tryptophan, kynurenine, KA and quinolinic acid, and n = 800 participants and n = 100 cases for 3-HAA. Stroke cases are excluded.*
[4]*1-year change analysis for non-stroke consisted of n = 825 participants and n = 76 cases for tryptophan, kynurenine, KA and quinolinic acid, n = 687 participants and n = 59 cases for 3-HAA. Stroke cases are excluded.*

Table 4. Hazard ratios (95% CI) for composite CVD, stroke only, and non-stroke only by baseline kynurenine risk score among participants with available data for all five metabolites under study.

	Outcome		
	Composite CVD	Stroke Only	Non-Stroke Only
n	896	797	793
cases	202	103	99
Model 1, Metabolite as continuous variable, per SD			
HR (95% CI)	1.33 (1.11 – 1.60)	1.17 (0.91 – 1.50)	1.56 (1.22 – 2.00)
P	0.002	0.22	<0.001
Metabolite in quartile categories, as compared to Q1 (reference)			
Q2	1.49 (0.88 – 2.50)	1.17 (0.62 – 2.20)	1.97 (0.92 – 4.22)
Q3	1.03 (0.60 – 1.78)	0.75 (0.38 – 1.47)	1.48 (0.68 – 3.23)
Q4	2.11 (1.26 – 3.52)	1.37 (0.70 – 2.65)	3.72 (1.80 – 7.71)
P-trend	0.02	0.64	0.002
Model 2, Metabolite as continuous variable, per SD			
HR (95% CI)	1.41 (1.14 – 1.75)	1.27 (0.94 – 1.71)	1.70 (1.27 – 2.29)
P	0.002	0.12	<0.001
Metabolite in quartile categories, as compared to Q1 (reference)			
Q2	1.60 (0.89 – 2.86)	1.34 (0.66 – 2.76)	2.07 (0.86 – 5.01)
Q3	1.00 (0.55 – 1.82)	0.71 (0.33 – 1.55)	1.59 (0.67 – 3.78)
Q4	2.41 (1.36 – 4.27)	1.76 (0.80 – 3.88)	4.11 (1.84 – 9.19)
P-trend	0.02	0.40	0.002

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 covariates in model 1, plus baseline hypertension, dyslipidemia, diabetes, and scores for BCAAs, acylcarnitines, and ceramides.
Kynurenine risk score was built by multiplying normalized individual metabolites (tryptophan, kynurenine, KA, 3-HAA, quinolinic acid) by their beta coefficient in a fully adjusted model with that metabolite alone and then summing up the products for each metabolite x coefficient value. The weights were -0.13474 for tryptophan, 0.0558 for kynurenine, 0.20359 for kynurenic acid, -0.20066 for 3-hydroxyanthranilic acid, and 0.13657 for quinolinic acid.

Figure 1. Multivariate adjusted HRs (95% CI) of composite CVD by quartiles of baseline kynurenine risk score stratified by intervention group (Mediterranean interventions combined versus control group) among participants with available data for all five metabolites under study (n=896).



Online Supporting Material

Supplemental Table 1. Spearman correlation heatmap of baseline plasma metabolites under study (tryptophan, kynurenine, kynurenic acid, 3-hydroxyanthranilic acid, quinolinic acid) and scores of plasma metabolites related to CVD (branched chain amino acid, ceramides, and acylcarnitine score).

	Tryp	KA	3-HAA	Kyn	QA	CS	SAS	MAS	LAS	BCAAS
Tryp	1	0.13***	0.29***	0.22***	0.00	-0.04	-0.17***	-0.08**	0.13***	0.49***
KA	0.13***	1	0.28***	0.29***	0.31***	-0.09**	-0.24***	0.14***	0.13***	0.30***
3-HAA	0.29***	0.28***	1	0.20***	0.18***	0.00	-0.18***	-0.05	0.01	0.41***
Kyn	0.22***	0.29***	0.20***	1	0.48***	0.10**	-0.16***	0.14***	0.07*	0.06
QA	0.00	0.31***	0.18***	0.48***	1	0.00	-0.29***	0.06*	-0.04	0.03
CS	-0.04	-0.09**	0.00	0.10**	0.00	1	0.04	0.07*	0.18***	0.04
SAS	-0.17***	-0.24***	-0.18***	-0.16***	-0.29***	0.04	1	0.08*	0.05	-0.14***
MAS	-0.08**	0.14***	-0.05	0.14***	0.06*	0.07*	0.08*	1	0.49***	0.07*
LAS	0.13***	0.13***	0.01	0.07*	-0.04	0.18***	0.05	0.49***	1	0.17***
BCAAS	0.49***	0.30***	0.41***	0.06	0.03	0.04	-0.14***	0.07*	0.17***	1

**P < 0.05; **P < 0.01; ***P < 0.001*

Abbreviations: 3-HAA, 3-hydroxyanthranilic acid; BCAAS, branched chain amino acid score; CS, ceramide score; KA, kynurenic acid; Kyn, kynurenine; LAS, long acylcarnitine score; MAS, medium acylcarnitine score; QA, quinolinic acid; SAS, short acylcarnitine score; Tryp, tryptophan.

Supplemental Table 2. Association of baseline plasma kynurenine/tryptophan ratio on composite CVD, stroke only, and non-stroke only among participants with available data for both kynurenine and tryptophan under study.

	Baseline		
	Composite CVD	Stroke Only	Non-Stroke Only
n	984	866	871
cases	231	118	113
Model 1, Metabolite as continuous variable, per SD			
HR (95% CI)	1.07 (0.91 – 1.26)	0.99 (0.79 – 1.24)	1.17 (0.94 – 1.45)
P	0.44	0.89	0.15
Metabolite in quartile categories, as compared to Q1 (reference)			
Q2	1.39 (0.89 – 2.19)	1.13 (0.64 – 1.99)	1.70 (0.91 – 3.17)
Q3	1.12 (0.71 – 1.76)	0.96 (0.54 – 1.68)	1.34 (0.70 – 2.57)
Q4	1.32 (0.84 – 2.07)	1.02 (0.57 – 1.81)	1.80 (0.97 – 3.37)
P-trend	0.42	0.90	0.14
Model 2, Metabolite as continuous variable, per SD			
HR (95% CI)	1.10 (0.91 – 1.37)	1.01 (0.79 – 1.30)	1.22 (0.95 – 1.57)
P	0.32	0.93	0.11
Metabolite in quartile categories, as compared to Q1 (reference)			
Q2	1.67 (0.81 – 3.41)	1.16 (0.48 – 2.82)	2.60 (0.95 – 7.12)
Q3	1.39 (0.54 – 3.55)	1.14 (0.36 – 3.65)	2.07 (0.56 – 7.60)
Q4	1.57 (0.41 – 5.93)	1.10 (0.20 – 6.10)	2.96 (0.50 – 17.47)
P-trend	0.60	0.93	0.30
	1-Year Change		
	Composite CVD	Stroke Only	Non-Stroke Only
n	904	829	818
cases	161	86	75
Model 1, Metabolite as continuous variable, per SD			
HR (95% CI)	1.05 (0.86 – 1.29)	0.89 (0.67 – 1.17)	1.27 (0.96 – 1.68)

P	0.64	0.38	0.10
Metabolite in quartile categories, as compared to Q1 (reference)			
Q2	0.75 (0.44 – 1.28)	0.61 (0.32 – 1.20)	1.05 (0.47 – 2.37)
Q3	0.89 (0.52 – 1.52)	0.44 (0.21 – 0.93)	1.87 (0.88 – 3.96)
Q4	1.03 (0.61 – 1.74)	0.69 (0.36 – 1.31)	1.77 (0.80 – 3.93)
P-trend	0.77	0.17	0.07
Model 2, Metabolite as continuous variable, per SD			
HR (95% CI)	1.03 (0.83 – 1.30)	0.85 (0.63 – 1.15)	1.28 (0.92 – 1.77)
P	0.79	0.30	0.14
Metabolite in quartile categories, as compared to Q1 (reference)			
Q2	0.71 (0.39 – 1.29)	0.57 (0.28 – 1.18)	0.95 (0.38 – 2.41)
Q3	0.76 (0.42 – 1.38)	0.34 (0.15 – 0.80)	1.69 (0.73 – 3.95)
Q4	1.00 (0.56 – 1.80)	0.63 (0.31 – 1.30)	1.78 (0.71 – 4.42)
P-trend	0.95	0.12	0.10

Cases occurring within one year of follow-up were excluded. For analyses using stroke as the endpoint, non-stroke cases were excluded, and for analyses using non-stroke as the endpoint, stroke cases were excluded.
Model 1 was adjusted for age, sex, family history of CHD, smoking status, and body mass index.
Model 2 was adjusted as for model 1, plus baseline hypertension, dyslipidemia, diabetes, and scores for BCAAs, acylcarnitines, and ceramides.

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Supplemental Table 3. Subgroup analysis for the associations between baseline kynurenine risk score and risk of composite CVD among participants with available data for all five metabolites under study (n=896).

Characteristic		Continuous HR[1] per 1 SD (95% CI)	*P*-interaction
Sex	Men (n=419)	1.59 (1.19 – 2.13)	0.23
	Women (n=477)	1.26 (0.90 – 1.75)	
Age	< 65 years (n=312)	1.58 (0.91 – 2.77)	0.73
	≥ 65 years (n=584)	1.39 (1.09 – 1.79)	
Intervention group	Control group (n=281)	2.02 (1.31 – 3.13)	0.003
	Mediterranean diet + EVOO (n=330)	1.27 (0.83 – 1.94)	
	Mediterranean diet + nuts (n=285)	1.23 (0.79 – 1.92)	
Obesity	≥ 30 kg/m^2 (n=406)	1.32 (0.93 – 1.88)	0.07
	< 30 kg/m^2 (n=490)	1.68 (1.18 – 2.39)	
Smoking status	Current/former smoking (n=365)	1.79 (1.21 – 2.64)	0.81
	Never smoking (n=531)	1.46 (1.05 – 2.02)	
Family history of CHD	Yes (n=215)	1.18 (0.72 – 1.93)	0.68
	No (n=681)	1.56 (1.21 – 2.02)	
Baseline T2D	Yes (n=450)	1.26 (0.95 – 1.66)	0.06
	No (n=446)	1.83 (1.26 – 2.67)	
Baseline Hypertension	Yes (n=749)	1.40 (1.10 – 1.79)	0.88
	No (n=147)	1.42 (0.64 – 3.16)	
Baseline Dyslipidemia	Yes (n=631)	1.50 (1.14 – 1.98)	0.51
	No (n=265)	1.40 (0.91 – 2.16)	

[1]*Hazard ratios were adjusted for age, sex, family history of CHD, smoking status, body mass index, baseline hypertension, dyslipidemia, diabetes, and scores for BCAAs, acylcarnitines, and ceramides and were stratified by intervention group.*

Supplemental Table 4. Analysis for mediation by plasma tryptophan change for the Mediterranean diet with extra virgin olive oil vs. control.

	Outcome		
	Composite CVD	Stroke Only	Non-Stroke Only
n	630	574	556
cases	130	74	56
Model 1			
MedDiet+EVOO vs. Control			
HR (95% CI)	0.66 (0.44 – 1.00)	0.63 (0.38 – 1.06)	0.69 (0.37 – 1.26)
P	0.05	0.08	0.22
Adjusted for 1-year tryptophan change and baseline tryptophan			
HR (95% CI)	0.91 (0.72 – 1.15)	0.68 (0.39 – 1.19)	0.88 (0.43 – 1.83)
P	0.42	0.18	0.74
Model 2			
MedDiet+EVOO vs. Control			
HR (95% CI)	0.62 (0.38 – 1.01)	0.56 (0.30 – 1.02)	0.69 (0.33 – 1.44)
P	0.05	0.06	0.33
Adjusted for 1-year tryptophan change			
HR (95% CI)	0.95 (0.66 – 1.36)	0.62 (0.68 – 1.26)	0.93 (0.40 – 2.17)
P	0.77	0.60	0.86

Cases occurring within one year of follow-up were excluded. For analyses using stroke as the endpoint, non-stroke cases were excluded, and for analyses using non-stroke as the endpoint, stroke cases were excluded.
Model 1 was adjusted for age, sex, family history of CHD, smoking status, and body mass index.
Model 2 was adjusted as for model 1, plus baseline hypertension, dyslipidemia, diabetes, and scores for BCAAs, acylcarnitines, and ceramides.

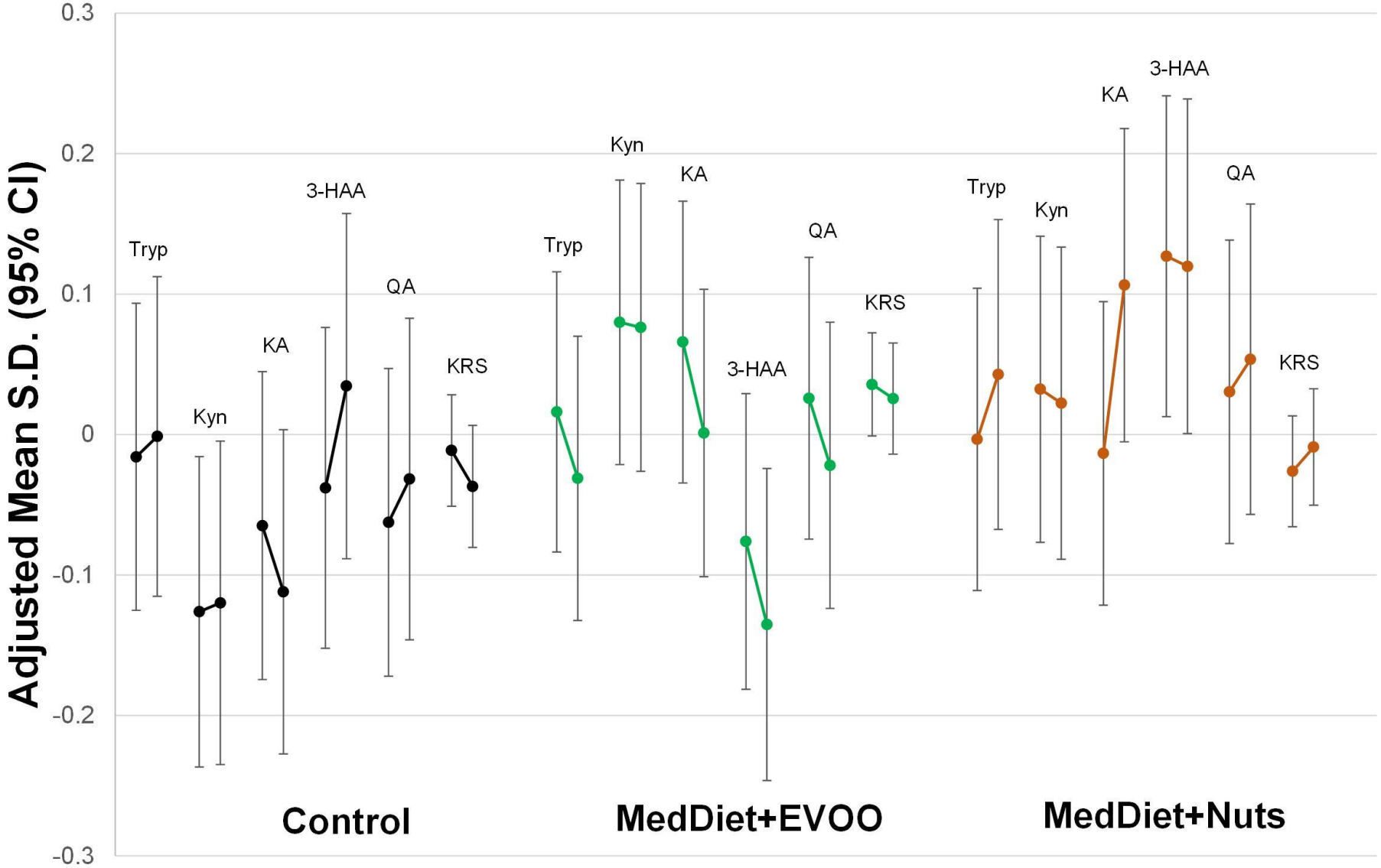
Supplemental Table 5. Analysis for mediation by plasma tryptophan change for the Mediterranean diet with nuts vs. control.

	Baseline		
	Composite CVD	Stroke Only	Non-Stroke Only
n	575	520	523
cases	107	55	52
Model 1			
MedDiet+nuts vs. Control			
HR (95% CI)	0.62 (0.40 – 0.95)	0.48 (0.27 – 0.86)	0.78 (0.43 – 1.40)
P	0.03	0.01	0.40
Adjusted for 1-year tryptophan change and baseline tryptophan			
HR (95% CI)	0.57 (0.35 – 0.93)	0.47 (0.24 – 0.90)	0.70 (0.34 – 1.44)
P	0.02	0.02	0.33
Model 2			
MedDiet+nuts vs. Control			
HR (95% CI)	0.61 (0.37 – 1.00)	0.43 (0.22 – 0.84)	0.89 (0.45 – 1.77)
P	0.05	0.01	0.73
Adjusted for 1-year tryptophan change			
HR (95% CI)	0.60 (0.35 – 1.02)	0.47 (0.23 – 0.98)	0.83 (0.36 – 1.88)
P	0.06	0.04	0.65

Cases occurring within one year of follow-up were excluded. For analyses using stroke as the endpoint, non-stroke cases were excluded, and for analyses using non-stroke as the endpoint, stroke cases were excluded.
Model 1 was adjusted for age, sex, family history of CHD, smoking status, and body mass index.
Model 2 was adjusted as for model 1, plus baseline hypertension, dyslipidemia, diabetes, and scores for BCAAs, acylcarnitines, and ceramides.

Online Supporting Material

Supplemental Figure 1. Changes in plasma tryptophan, kynurenine, kynurenic acid, 3-hydroxyanthranilic acid, quinolinic acid, and kynurenine risk score from baseline to 1 year (of intervention), stratified by intervention group.



**Abbreviations: 3-HAA, 3-hydroxyanthranilic acid; KA, kynurenic acid; KRS, kynurenine risk score; Kyn, kynurenine; QA, quinolinic acid; Tryp, tryptophan.*