Plasma acylcarnitines and the risk of cardiovascular disease: effect of Mediterranean Diet interventions

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Abbreviations: CVD, cardiovascular disease; LC-MS/MS, liquid chromatography tandem mass spectrometry; MedDiet, Mediterranean Diet.

Clinical trial registry: The trial was registered at http://www.controlled-trials.com (ISRCTN35739639).

ABSTRACT

Background: Previous studies have suggested that metabolite profiles of elevated acylcarnitines were associated with an increased risk of cardiovascular disease (CVD) in populations with established coronary disease. This, however, has not been evaluated in the context of primary cardiovascular prevention.

Objectives: We evaluated the association between 28 plasma acylcarnitine species and the risk of incident CVD, and the potential modifying effect of Mediterranean Diet (MedDiet) interventions.

Methods: We measured plasma acylcarnitines, using high-throughput liquid chromatography tandem mass spectrometry, at baseline and after 1-year of follow-up, individually and classified into short-, medium- or long-chain scores; in a case-cohort study within the PREDIMED Study, a randomized Mediterranean dietary intervention for primary cardiovascular prevention. The present study included a randomly selected subcohort (*n*=751) and all available incident CVD cases (n=229) after 4.8-years of follow-up.

Results: After adjusting for age, sex, BMI and other cardiovascular risk factors, participants in the highest quartile of baseline short- and medium-chain acylcarnitines had higher risk of CVD compared to those in the lowest quartile [hazard ratio (HR) (95% Confidence Interval (CI)): 1.80 (1.11, 2.91), P for trend 0.01 and 1.55 (1.01, 2.48), P for trend=0.04, respectively]. Increased short-chain acylcarnitines after 1-yr were associated with higher risk of total CVD and stroke. Participants with higher baseline levels of short-, medium- and long-chain acylcarnitines and who were randomly

assigned to the control group had higher risk of CVD as compared to those with lower levels of acylcarnitines assigned to the MedDiet group.

Conclusions: Our data support that metabolite profiles characterized by elevated concentrations of acylcarnitines are independently associated with the risk of total CVD and stroke alone in participants at high cardiovascular risk. MedDiet interventions may mitigate the adverse associations found between higher concentrations of acylcarnitines and CVD.

Keywords: Acylcarnitines, metabolomics, Mediterranean diet, cardiovascular disease, PREDIMED.

BACKGROUND

Cardiovascular disease (CVD) is the leading cause of death and disability in industrialized countries (1). Although many cardiovascular risk factors, such as smoking, obesity, diabetes, and poor dietary habits have been identified (2), the understanding of CVD etiology and its mechanisms is still incomplete. More comprehensive approaches may help to deepen our knowledge of CVD physiopathology, and also identify patients at high risk —potentially years before diagnosis— that will benefit most from preventive strategies. Thus, emerging high-throughput metabolomics has been proposed as one of the novel and useful tools for the early diagnosis of metabolic diseases (3,4).

Among several metabolites identified as potential keys to metabolic diseases, acylcarnitines, which are intermediates of fatty acid oxidation, have been associated with CVD risk (5–8). Elevated concentrations of these metabolites may be indicative of impaired β-oxidation and mitochondrial dysfunction (5), and have been associated with higher risk of obesity, insulin resistance and type 2 diabetes (9,10), all recognized risk factors of CVD. To date, only few studies have examined the associations between acylcarnitines and CVD, and these studies had small sample sizes and were conducted only in patients with established coronary heart disease. Also, they included no or scarce information on diet and lifestyles (5–8). The association between acylcarnitines and CVD, and in particular changes in their concentrations, in the context of primary cardiovascular prevention remains therefore to be elucidated.

Importantly, a Western dietary pattern was associated with a specific metabolite signature characterized by increased levels of short-chain acylcarnitines (11), and some dietary components have been shown to change the acylcarnitine metabolite profile in

plasma or other fluids and tissues (12–15). However, whether the potential association between acylcarnitines and CVD risk is modified by an intervention with an overall healthy dietary pattern such as the Mediterranean diet has not yet been investigated.

In the present prospective nested case-cohort study, we performed quantitative profiling of 28 acylcarnitine species in plasma samples of elderly participants at high cardiovascular risk from the PREDIMED trial. We hypothesized that higher concentrations at baseline and 1-year changes in acylcarnitine concentrations would be associated with the risk of CVD and that these associations might be modified by a Mediterranean Diet (MedDiet) intervention. Our aim was to determine the association of baseline acylcarnitine profiles and 1-year changes in acylcarnitine profiles with the risk of incident CVD and stroke, and to examine whether these associations may be mitigated by MedDiet interventions.

METHODS

Study population

The design and protocol of the PREDIMED study (http://www.predimed.es) have been described in detail elsewhere (16,17). In brief, the PREDIMED study was a large, multicenter, parallel-group, randomized controlled trial, evaluating the effect of MedDiet on the primary prevention of CVD, conducted in Spain from 2003 until 2010. Participants were men (aged 55–80 years) and women (aged 60–80 years) who were free of CVD at baseline but at high cardiovascular risk because they had either type 2 diabetes mellitus or at least three of the following cardiovascular risk factors: current smoking, hypertension, hypercholesterolemia, low high-density lipoprotein cholesterol, overweight/obesity, or family history of premature coronary heart disease. Exclusion criteria were the presence of any severe chronic illness, alcohol or drug abuse, body mass index (BMI) ≥40 kg/m^2, and allergy or intolerance to olive oil or nuts (16). Participants were randomly assigned to receive one of these three interventions: a MedDiet supplemented with extra-virgin olive oil, a MedDiet supplemented with mixed nuts, or advice on a low-fat diet (control group). The primary end-point of the PREDIMED trial was a composite of cardiovascular events (myocardial infarction, stroke, or death from cardiovascular causes); 288 incident CVD cases occurred during 4.8 years of follow-up. We designed a case-cohort study in the framework of the PREDIMED trial (18). A case-cohort study design dictates that all cases and a randomly selected percentage of the original cohort (referred to as the “subcohort,” which by its random selection may therefore include some cases) are selected as participants. In the context of a trial, the case-cohort study design allows us to: 1) maintain the trial’s original randomization

scheme; 2) limit the randomly selected subcohort to fewer participants than the entire cohort for cost savings; and 3) extrapolate the results to all the participants in the study. In keeping with the study design, from the eligible cohort participants with available plasma samples at baseline, we selected a random, non-stratified subsample of 10% of all PREDIMED participants at baseline, plus all incident CVD cases with available blood samples occurring during follow-up. Of 980 participants included in the analyses, 751 were non-cases and 229 were cases (there were 37 overlapping cases between the subcohort and the total cases). Of these, 923 participants out of the 980 had available samples after 1-year of follow-up and were included in the 1-year change analyses (See **Supplemental Figure 1**). We defined “cases” as the participants who developed a cardiovascular event (stroke, myocardial infarction or cardiovascular death) during follow-up. We defined “subcohort” as the random sample selected from the full roster of the PREDIMED study (including some incident CVD cases). We defined “internal cases” as the cases that were randomly included in this random subcohort. We defined “external cases” as the cases that weren’t randomly included in the subcohort. All participants provided written informed consent according to a protocol approved by the institutional review boards prior to inclusion in the study.

Metabolite profiling

At baseline and at yearly follow-up visits, trained nurses collected fasting blood samples from the PREDIMED participants. After an overnight fast, tubes for EDTA plasma were collected and aliquots were coded and kept refrigerated until they were

stored at -80°C freezers. Pairs of samples (baseline and first-year visit) were randomly ordered and shipped on dry ice to the Broad Institute for the metabolomics analysis. Amino acids, acylcarnitines, and other polar plasma metabolites were profiled using liquid chromatography-tandem mass spectrometry (LC-MS) on a system comprised of a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp.; Marlborough, MA) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA) (19). LC, through the application of a number of distinct stationary phase chemistries, affords reproducible separation of metabolites in complex mixtures on the basis of their physical properties. MS enables further resolution of metabolites on the basis of mass-to-charge ratio (m/z) and quantification over a wide linear dynamic range. Metabolite extracts were prepared from plasma samples (10 µL) via protein precipitation with the addition of nine volumes of 74.9:24.9:0.2 v/v/v acetonitrile/methanol/formic acid containing stable isotope-labeled internal standards (valine-d8, Sigma-Aldrich; St. Louis, MO; and phenylalanine-d8, Cambridge Isotope Laboratories; Andover, MA). The samples were centrifuged (10 min, 9,000 x g, 4°C), and the supernatants were injected directly onto a 150 x 2 mm, 3 µm Atlantis HILIC column (Waters; Milford, MA). The column was eluted isocratically at a flow rate of 250 µL/min with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 0.5 minute followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 minutes. MS analyses were carried out using electrospray ionization in the positive ion mode using full scan analysis over 70-800 m/z at 70,000 resolution and 3 Hz data acquisition rate. Other MS settings were: sheath gas 40, sweep gas 2, spray voltage 3.5 kV, capillary temperature 350°C, S-lens RF 40, heater temperature 300°C, microscans

1, automatic gain control target 1e6, and maximum ion time 250 ms. Metabolite identities were confirmed using authentic reference standards. Free carnitine and 27 acylcarnitine subtypes were analyzed using this approach. Raw data were processed using TraceFinder software (Thermo Fisher Scientific; Waltham, MA) and Progenesis QI (Nonlinear Dynamics; Newcastle upon Tyne, UK)

Case ascertainment

For the present analysis, the primary end-point was a composite of cardiovascular events (myocardial infarction, stroke, or death from cardiovascular causes), and as a secondary end-point we separately analyzed incident stroke, as this was the most common element included in the definition of the composite primary end-point in the PREDIMED. The end-point adjudication committee, whose members were blinded to treatment allocation and dietary information, updated information on these endpoints once a year. The committee used different sources of information: 1) yearly questionnaires and examinations for all participants, 2) primary care physicians, 3) comprehensive yearly review of medical records of all participants, and 4) yearly consultation of the National Death Index. Medical records of participants were requested, and the end-point committee adjudicated major events and determined the cause of death.

Covariate assessment

At baseline and at yearly follow-up visits, a questionnaire about lifestyle variables, educational achievement, history of illnesses, medication use, and family history of disease was administered. Physical activity was assessed using the validated Spanish

158 version of the Minnesota Leisure-Time Physical Activity questionnaire (20). Participants
159 were considered to have diabetes, hypercholesterolemia, or hypertension if they had
160 previously been diagnosed, and/or they were being treated with antidiabetic,
161 cholesterol-lowering, or antihypertensive agents, respectively. Trained dietitians
162 completed a 137-item validated semi-quantitative food frequency questionnaire in face-
163 to-face interviews with participants (21). We used Spanish food composition tables to
164 estimate energy and nutrient intake (22). Trained personnel took anthropometric and
165 blood pressure measurements.

166 **Statistics**

167 We applied an inverse normal transformation to approximate a normal
168 distribution of metabolite levels (23). Baseline characteristics of the participants are
169 presented according to case status as the mean (SD) for quantitative traits and n (%) for
170 categorical variables. Baseline characteristics were compared between cases and non-
171 cases using t-tests for continuous variables and chi-squared tests for categorical
172 variables.

173 We used Cox proportional hazard models, with Barlow weights (to account for
174 oversampling of cases in the study design (18)), to estimate hazard ratios (HRs) and
175 their 95% confidence intervals (CIs) for the primary combined end-point of CVD and,
176 separately, for non-fatal stroke. Follow-up time was calculated as the interval between
177 the date of randomization and the date of cardiovascular event, death, or end of follow-
178 up, whichever came first. Cox models were adjusted for age (years), sex (men/women),
179 family history of premature heart disease (yes/no), smoking (never, former, or current),
180 and BMI (kg/m^2), and were stratified by intervention group (both MedDiet interventions,

and low-fat control group) (Model 1). Model 2 was additionally adjusted for leisure-time physical activity (metabolic equivalent task in minutes/day), baseline hypertension (yes/no), dyslipidemia (yes/no), and diabetes (yes/no). Baseline 28 individual acylcarnitine species were analyzed as both continuous variables and using quartiles (using cut-points defined among non-cases). To account for multiple testing in individual analysis of the 28 acylcarnitines, we used a corrected p-value according to Benjamini-Hochberg method. We calculated three acylcarnitine scores. An inverse normal transformation was applied to raw values of baseline acylcarnitines and a weighted sum (see below) of these values was computed to calculate the scores: a) short-chain acylcarnitines (10 acylcarnitines species): C2carnitine–C7carnitine; b) medium-chain acylcarnitines (9 species): C8carnitine–C14:2carnitine; and c) long-chain acylcarnitines (8 species): C16carnitine–C26carnitine. The weights correspond to the respective coefficients from the multivariable Cox regression model fitted with each individual metabolite (19). We conducted additional models adjusted for total energy intake (Kcal/day) and a branched-chain amino acids (valine, isoleucine and leucine) score calculated using the same method as the carnitine scores (24). To test for the linear trend across quartiles, the median of each quartile was assigned and analyzed as a continuous variable. To compare the predictive ability of a classical model (multivariable model 2 described above without acylcarnitines) and the same model including the acylcarnitine scores we have additionally calculated the Area Under the ROC (Receiver Operating Characteristic) curve (AUC).

We also examined the associations between 1-year changes in the 28 individual acylcarnitines species and 1-year changes in acylcarnitine scores (short-, medium- and

long-chain acylcarnitines) and CVD risk. We used the same models as in the baseline analyses but we further adjusted for baseline levels and an interaction term between acylcarnitines at baseline and 1-yr change of acylcarnitines (both as continuous variables).

We repeated the analysis using incident stroke as the outcome, both including and excluding the non-stroke CVD cases (i.e., treating the 113 non-stroke CVD cases as non-cases, or removing them from analyses), and found similar results. Therefore, we present stroke results only after removing the non-stroke CVD cases.

To assess the effect of the MedDiet interventions, we introduced a multiplicative interaction term with one degree of freedom between each of the 3 baseline acylcarnitine scores (continuous) and the MedDiet intervention groups (the two MedDiet group as a single group vs. the control group). We stratified the analysis described above by intervention group (both MedDiet interventions vs. control). We conducted joint analyses for both the baseline and 1-year changes in acylcarnitine scores and the intervention group using as the reference group participants assigned to the MedDiet group and with lower baseline levels of acylcarnitines (quartile 1 of the three scores). Finally, we compared the adjusted mean changes in the individual metabolites from baseline to 1 year by intervention group, using the same models as described above.

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

RESULTS

Baseline characteristics of the 980 individuals (229 cases and 751 non-cases) included in the present case-cohort study are described in **Table 1**. The mean age of participants at baseline was 68 years and the mean BMI was 29.7 (3.7) kg/m^2. Compared to non-cases, those participants who developed CVD were more likely to be older, men, current smokers, and to have diabetes and less likely to have dyslipidemia and a family history of coronary heart disease (Table 1).

Baseline acylcarnitines and the effect of dietary interventions on the risk of CVD and stroke

The associations between baseline acylcarnitine scores and the risk of CVD are presented in **Table 2**. Across all models in the overall study population, higher risk of CVD was observed per SD increase in short-chain, medium-chain and long-chain acylcarnitine scores. After adjusting for age, sex, BMI, family history of premature heart disease, and smoking (model 1), participants in the top quartile of short-chain and medium-chain acylcarnitine scores had significantly higher CVD risk. In the second model (further adjusted for physical activity, baseline hypertension, diabetes and dyslipidemia), HRs (95% CIs) for CVD in the top versus bottom quartiles were 1.80 (1.11, 2.91), P for trend = 0.01, for short-chain acylcarnitine score and 1.55 (1.01, 2.48), P for trend = 0.04, for medium-chain acylcarnitine score. In analyses stratified by intervention group, those participants who were assigned to the control group and in the top baseline quartile of short-chain and medium-chain acylcarnitines had significantly higher risk of CVD compared to those in the bottom baseline quartile [3.19 (1.34, 7.56), P for trend =0.02, 2.17 (1.02, 4.63), P for trend =0.03, respectively]. In contrast, no

248 significant associations between acylcarnitine scores and CVD were found for
249 participants who were assigned to the MedDiet interventions. When analyzing baseline
250 quartiles of long-chain acylcarnitine scores, the results were non-significant in both the
251 overall and stratified analyses. The associations between baseline concentrations of
252 each of the 28 acylcarnitine species and the risk of CVD are presented in
253 **Supplemental Table 1**. In the second model and after applying a multiple testing
254 correction, no significant associations were found between individual acylcarnitines at
255 baseline and CVD risk.

256 **Table 3** shows the associations between baseline acylcarnitine scores and the
257 risk of stroke. A total of 869 individuals (118 stroke cases and 751 non-cases) were
258 included in these analyses (participants who experienced myocardial infarction or
259 cardiovascular death, but not stroke were removed from these analyses). Short-chain
260 acylcarnitines were strongly associated with a higher stroke risk [HR (95% CI) for Q4 vs.
261 Q1: 2.53 (1.24, 5.18), P for trend <0.01]. Medium-chain and long-chain acylcarnitines
262 were not significantly associated with stroke in the adjusted models. Similar to CVD
263 results, participants in the control group and in the highest quartile of baseline short-
264 chain, had higher risk of stroke compared to those in the lowest quartile [HR (95% CI):
265 4.37 (1.24, 15.38), P for trend = 0.01]. No significant associations between acylcarnitine
266 scores and stroke were found for those individuals assigned to MedDiet interventions.

267 Results for further adjustment of multivariable model 2 for total energy intake and a
268 branched-chain amino acids score were consistent with the primary analysis and
269 resulted in higher risk of CVD for short, medium and long-chain acylcarnitine scores.
270 Per SD increase in short-chain acylcarnitines the HR (95% CI) was 2.43 (1.49, 3.97), for

medium-chain acylcarnitines: 1.51 (1.02, 2.24), and for long-chain acylcarnitine score: 1.34 (1.01, 1.81). The AUC for the model without including acylcarnitine scores was 0.70 and including acylcarnitine scores the AUC increased to 0.72, but there were no significant differences between both AUCs.

Figure 1 shows the risk of incident CVD using a joint classification of the baseline acylcarnitine scores and intervention groups, in which the reference category is composed of participants in the bottom quartile of scores and assigned to MedDiet interventions (both MedDiet groups merged together). Participants in the control group and in the top quartiles of short-chain, medium-chain and long-chain acylcarnitines had higher risk of CVD as compared to the reference group [HR (95% CI): 2.11 (1.26, 3.25), 1.88 (1.09, 3.23), and 2.12 (1.19, 3.79), respectively].

1-year change in acylcarnitines and the effect of dietary interventions on the risk of CVD and stroke

Associations between 1-year changes in acylcarnitine scores and risk of CVD are presented in **Supplemental Table 2**. Per each SD increase in 1-year change in the short-chain acylcarnitine score, the risk of CVD was 36% higher [HR (95% CI): 1.36 (1.01, 1.83)] and the corresponding risk per each additional SD of 1-year change in the long-chain acylcarnitine score was 71% higher [HR (95% CI): 1.71 (1.04, 2.81)]. For the score of medium-chain acylcarnitines, an increase of 1 SD after 1-year was associated with an increased risk of CVD in model 1 [HR (95% CI): 1.47 (1.04, 1.09)] but results were attenuated and no longer statistically significant after further adjustment. The associations between 1-year changes in acylcarnitine scores and the risk of stroke were

similar to those of CVD (**Supplemental Table 3**). One-year changes in short-chain and long-chain acylcarnitine scores were associated with an increased risk of stroke in both analyses. Consistent with the CVD analyses, 1-year changes in medium-chain acylcarnitine score were associated with increased stroke risk in the first model but attenuated in the second model. Joint classification of 1-year changes in acylcarnitine scores and intervention groups, and the risk of CVD are presented in **Supplemental Figure 2**. Although the P for interaction was not significant, those participants in the control group and who had higher increases in long-chain had higher risk of CVD as compared to the reference group (those in the MedDiet and lower quartile of the scores) [HR (95% CI): 1.93 (1.05, 3.53)]. **Supplemental Figure 3** shows adjusted means for 1-year changes in acylcarnitine scores by intervention group. No significant differences between control and intervention groups were observed.

DISCUSSION

In this prospective case-cohort study of individuals at high cardiovascular risk, we found that a baseline profile of increased short-chain and medium-chain plasma acylcarnitines was associated with higher risk of CVD independent of established cardiovascular risk factors. We also identified elevated concentrations of short-chain acylcarnitines as potential biomarkers of future stroke risk. These metabolites were altered up to 3-4 years before the onset of CVD. We also observed that 1-year changes in short-chain and long-chain acylcarnitine scores were directly associated with an increased risk of both total CVD and stroke alone, independent of baseline levels of these metabolites and recognized risk factors of CVD. Participants with higher levels of short- and medium-chain acylcarnitines at baseline and who were assigned to the control group had a higher risk of both total CVD and stroke alone, suggesting that MedDiet interventions can mitigate, at least in part, the harmful effects of elevated acylcarnitines on CVD risk. These alterations in acylcarnitine concentrations provide pathway hypotheses on their implications in the development of CVD. However, no effect of MedDiet was observed for 1-year changes in acylcarnitine scores.

Our observations, which revealed a metabolomic signature of increased baseline acylcarnitines being related to higher cardiovascular risk, are consistent with previous studies, but our findings are novel and go beyond those previous studies because we also assessed 1-year changes in the context of a dietary intervention for primary cardiovascular prevention (5–8). In a prospective cohort study of 2,023 patients undergoing cardiac catheterization, a metabolic panel composed of short-, medium-, and long-chain acylcarnitines was associated with 14–24% higher risk of myocardial

infarction and death after 3.1 years of follow-up (6). A previous case-control study conducted by the same authors revealed that a factor composed of dicarboxylacylcarnitines was predictive of cardiovascular or death events in individuals with coronary artery disease (5). Similarly, a report of two independent nested case-control studies demonstrated that increased plasma levels of long-chain acylcarnitines were associated with 1-year cardiovascular mortality in dialysis patients (7). Finally, results from a principal component analysis conducted in Italian elderly patients, with a high rate of previous CVD, showed that medium- and long-chain acylcarnitines were associated with early cardiovascular events, independently of recognized cardiovascular risk predictors (8). Adding to this evidence, our study is the first to describe the association between plasma acylcarnitines, and CVD risk in individuals at high cardiovascular risk after controlling for well-established cardiovascular risk factors and with repeated longitudinal measurements of acylcarnitines. Our findings are noteworthy in the context of the primary prevention of CVD since it is important to identify individuals who are more likely to progress in their disease to target preventive strategies, thus, reducing future clinical and public health impact. Consequently, it is expected that this type of research will lead towards more personalized and precise medicine, widely considered to be the medical future.

Additional novel findings of the present study include the apparent mitigating effect of the MedDiet intervention on the association between baseline acylcarnitine scores and incident CVD, as well as the direct associations between 1-year changes in acylcarnitine scores, and the risk of stroke and CVD. Participants with relatively high baseline acylcarnitines who were assigned to the control group showed higher risk of

CVD as compared to those individuals with lower concentrations of acylcarnitines who were assigned to the MedDiet interventions. These results were more pronounced among short-chain and medium-chain acylcarnitines, suggesting that these metabolites could have a role in the pathophysiology of CVD (6). These findings indicate that pathways related to acylcarnitine metabolism may be influenced by the diet (12–14) and MedDiet may potentially counteract the harmful effects of elevated acylcarnitines in blood. In fact, compared to the prudent diet, the Western dietary pattern was associated with a specific metabolite signature characterized by increased levels of short chain acylcarnitines (11); in addition, meat eaters tended to have increased concentrations of acylcarnitines and other metabolites (15) indicating that acylcarnitines metabolite profile can be affected by dietary components (12–14). In the context of previous cross-sectional studies, our study provides novel and useful longitudinal information with repeated measurements of acylcarnitines after 1-year intervention. However, contrary to our expectations, no significant differences in 1-year changes in acylcarnitines between control and intervention groups were observed in our study, perhaps because only 1-year might be a short period to observe these effects or because other mechanisms and pathways instead of the changes in acylcarnitines underlie the observed beneficial effect of the MedDiet on CVD. Gut microbiota, that can be modified by diet, may also play a role in the relation between acylcarnitines and CVD (25). Various short- and long-chain acyl carnitines possess a trimethylamine moiety and consequently they are likely to be involved in gut microbe–dependent pathways contributing to the formation of TMA and trimethylamine-N-oxide (TMAO), which may increase the risk of atherosclerosis and consequently CVD (26).

Since acylcarnitine concentrations have been associated with increased risk of insulin resistance and type 2 diabetes (27), our results are biologically plausible and consistent with previous cross-sectional assessments of acylcarnitines. Our study also showed for the first time, direct associations between 1-year changes in short- and long-chain acylcarnitines and the risk of CVD, reinforcing the hypothesis that pathways related to acylcarnitines may play an important role in CVD development. However, medium-chain acylcarnitines were not significantly related to total CVD or to stroke alone in the present study. In addition, we found strong associations between acylcarnitines and CVD and the AUC from the classical risk factor model showed a reasonable predictive ability, but adding acylcarnitine scores to the classical risk factor model did not add much to the C-statistic from the classical model. Although the acylcarnitines do not add the predictive power of CVD beyond traditional risk factors, our findings improve our understanding of the metabolic pathways related to MedDiet and CVD.

Acylcarnitines are derived from both fatty acid and amino acid β-oxidation (27). They can be formed from almost any CoA ester. Acylcarnitines can also be derived from other intermediates such as ketone bodies, degradation products of lysine, tryptophan, valine, leucine, isoleucine and carbon atoms from glucose (28). The main function of L-carnitine is to transport fatty acids from the cytosol to the mitochondrial matrix, where β-oxidation takes place, this process results in the esterification of L-carnitine to form acylcarnitine derivatives (29). Acylcarnitines can be measured in plasma after being transported across cell membranes (27). Accumulation of acylcarnitines may be indicative of inefficient β-oxidation and altered mitochondrial metabolism (8). These

399 processes are enhanced in the aging process, in particular, advanced age leads to
400 impaired flux of carnitines through the mitochondrial pathway, reflecting mitochondrial
401 dysfunction (8). Elderly individuals also have increased mitochondrial production of
402 reactive oxygen species that enhances vascular inflammation and contributes to
403 alterations in the composition of plaque and to its rupture (8). In addition, increased
404 acylcarnitines have been associated with higher risk of insulin resistance and type 2
405 diabetes (9,30), strong risk factors for CVD.

406 The main limitation of the present study is that our results may not be
407 generalized to other populations. However, the case-cohort design maximizes the
408 efficiency of the high-throughput metabolomics profiling, allowing us to extend the
409 results to all PREDIMED participants. Other limitations include that the LC-MS-based
410 metabolite measurements may not have a direct clinical translation for each metabolite
411 trait. Several strengths such as the prospective design, the ability to control for potential
412 confounders due to recording of comprehensive data, and the accurate and blind
413 assessment of incident cases also deserve mention.

414 In summary, our data strongly support that metabolite profiles composed of
415 elevated acylcarnitines are associated with the risk of stroke and CVD in individuals at
416 high cardiovascular risk. MedDiet interventions may partially mitigate the deleterious
417 effects of increased acylcarnitines on CVD risk.

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Authors Contribution: MAM-G and FBH designed research; MG-F, MR-C, MAM-G, DC, RE, ER, MF, FA, MF, JL, LS-M, JS-S conducted research; MAM-G, DC, ER, MF, RE, JL, LS-M, and JS-S were the coordinators of subject recruitment at the outpatient clinics; M-GF and YZ analyzed the data; MG-F, YZ, MAM-G, FBH, JS-S interpreted statistical analysis and data; CBB analyzed metabolomics data; MG-F drafted the paper; MG-F, YZ, MR-C, AH, MAM-G, FBH and JS-S supervised the study and MG-F, FBH and JS-S had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors revised the manuscript for important intellectual content, and read and approved the final manuscript.

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Table 1. Baseline characteristics of the PREDIMED nested case-cohort study population

	Total	Cases	Non-cases	*P* value
n	980	229	751	
Age (years)	67.6 (6)	69.4 (6.5)	67.0 (6)	<0.01
Sex (% Women)	528 (53.9)	91 (39.7)	437 (58.1)	<0.01
Body mass index, kg/m^2	29.7 (3.7)	29.6 (3.7)	29.7 (3.6)	0.67
Intervention group, %				
MedDiet+EVOO	363 (37.0)	82 (35.8)	281 (37.4)	0.15
MedDiet+Nuts	314 (32.0)	65 (28.4)	249 (33.1)	
Control group	303 (30.9)	82 (35.8)	221 (29.4)	
Family history of CHD, %	237 (24.2)	44 (19.2)	193 (25.7)	0.04
Hypertension, %	817 (83.3)	189 (82.5)	628 (83.6)	0.69
Dyslipidaemia, %	692 (70.6)	134 (58.5)	558 (74.3)	<0.01
Diabetes, %	494 (50.4)	147 (64.2)	347 (46.2)	<0.01
Smoking, %				
Never	139 (58.9)	104 (45.4)	474 (63.1)	<0.01
Former	263 (26.8)	79 (34.5)	184 (24.5)	
Current	139 (14.2)	46 (20.1)	93 (12.4)	

Data are expressed as means ± SD or percentage (n). P value for comparisons between cases and controls (Pearson X^2 test for categorical variables or one-way analysis of variance for continuous variables).

Abbreviations: CHD, coronary heart disease; EVOO, extra-virgin olive oil; MedDiet, Mediterranean Diet.

Table 2. Associations between baseline acylcarnitine scores and the risk of incident cardiovascular disease						
COMPOSITE CARDIOVASCULAR DISEASE						
	Overall		**Both MedDiet groups**		**Control group**	
Non-cases, n	751		530		221	
Cases, n	229		147		82	
Short-chain acylcarnitines (C2-C7)						
	HR (95%CI)	P	HR (95%CI)	P	HR (95%CI)	P
Crude per s.d.	1.70 (1.14, 2.52)	<0.01	1.33 (0.85, 2.10)	0.21	3.00 (1.33, 6.78)	<0.01
Model 1						
Per s.d.	2.09 (1.36, 3.21)	<0.01	1.57 (0.96, 2.57)	0.07	4.00 (1.68, 9.53)	<0.01
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	1.11 (0.69, 1.78)		1.18 (0.67, 2.08)		1.08 (0.45, 2.60)	
Q3	1.33 (0.86, 2.08)		1.53 (0.89, 2.64)		1.06 (0.48, 2.31)	
Q4	1.77 (1.12, 2.78)		1.43 (0.81, 2.51)		2.67 (1.22, 5.87)	
p for trend		<0.01		0.13		0.02
Model 2						
Per s.d.	2.08 (1.33, 3.25)	<0.01	1.50 (0.90, 2.49)	0.11	5.22 (1.87, 14.50)	<0.01
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	1.18 (0.72, 1.95)		1.31 (0.70, 2.43)		1.08 (0.42, 2.74)	
Q3	1.35 (0.85, 2.14)		1.65 (0.92, 2.95)		1.07 (0.47, 2.44)	
Q4	1.80 (1.11, 2.91)		1.39 (0.76, 2.54)		3.19 (1.34, 7.56)	
p for trend		0.01		0.19		0.02
Medium-chain acylcarnitines (C8-C14)						
Crude per s.d.	1.67 (1.17, 2.40)	<0.01	1.41 (0.89, 2.21)	0.13	2.79 (1.49, 5.24)	<0.01
Model 1						
Per s.d.	1.65 (1.12, 2.43)	0.01	1.32 (0.83, 2.10)	0.23	2.75 (1.36, 5.57)	<0.01
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	0.88 (0.54, 1.44)		1.01 (0.54, 1.86)		0.70 (0.31, 1.59)	
Q3	1.25 (0.78, 1.98)		1.24 (0.68, 2.25)		1.27 (0.60, 2.68)	
Q4	1.60 (1.02, 2.53)		1.36 (0.76, 2.43)		2.35 (1.12, 4.93)	
p for trend		0.02		0.21		0.01
Model 2						
Per s.d.	1.53 (1.04, 2.26)	0.03	1.23 (0.76, 2.00)	0.38	2.36 (1.17, 4.76)	0.02

Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	0.92 (0.55, 1.51)		0.90 (0.48, 1.68)		0.74 (0.31, 1.78)	
Q3	1.19 (0.74, 1.92)		1.10 (0.59, 2.07)		1.39 (0.63, 3.02)	
Q4	1.55 (1.01, 2.48)		1.30 (0.70, 2.39)		2.17 (1.02, 4.63)	
p for trend		0.04		0.28		0.03
Long-chain acylcarnitines (C16-C26)						
Crude per s.d.	1.54 (1.19, 1.99)	<0.01	1.42 (1.04, 1.94)	0.02	1.94 (1.20, 3.13)	<0.01
Model 1						
Per s.d.	1.33 (1.00, 1.76)	0.04	1.19 (0.86, 1.67)	0.28	1.66 (0.95, 2.90)	0.07
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	1.01 (0.61, 1.65)		1.33 (0.71, 2.47)		0.55 (0.21, 1.44)	
Q3	1.11 (0.68, 1.78)		1.38 (0.74, 2.57)		0.81 (0.36, 1.81)	
Q4	1.44 (0.90, 2.29)		1.56 (0.85, 2.87)		1.26 (0.56, 2.84)	
p for trend		0.09		0.16		0.38
Model 2						
Per s.d.	1.31 (0.98, 1.75)	0.07	1.20 (0.85, 1.69)	0.30	1.59 (0.87, 2.91)	0.12
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	1.02 (0.61, 1.71)		1.29 (0.68, 2.47)		0.59 (0.21, 1.63)	
Q3	1.14 (0.69, 1.88)		1.45 (0.75, 2.80)		0.87 (0.37, 2.02)	
Q4	1.47 (0.91, 2.40)		1.61 (0.85, 3.03)		1.29 (0.54, 3.10)	
p for trend		0.08		0.14		0.43

*An inverse normal transformation was applied to raw values of carnitines and a weighted sum of these values was computed to calculate the scores (**short-chain acylcarnitines**: C2carnitine, C3DCCH3carnitine, C3carnitine, C4carnitine, C4OHcarnitine, C5carnitine, C5:1carnitine, C5:DCcarnitine, C6carnitine, C7carnitine; **medium-chain acylcarnitines**: C8carnitine, C9carnitine, C10carnitine, C10:2carnitine, C12carnitine, C12:1carnitine, C14carnitine, C14:1carnitine, C14:2carnitine*; ***long-chain acylcarnitines:** C16carnitine, C18carnitine, C18:1carnitine, C18:1OHcarnitine, C18:2carnitine, C20carnitine, C20:4carnitine, C26carnitine). Model 1: Adjusted for age, sex, BMI, family history of premature heart disease, smoking, and stratified by intervention group (only in overall analyses). Model 2: additionally adjusted for physical activity (metabolic equivalent task minutes/day), hypertension, dyslipidemia and diabetes.*

Table 3. Associations between baseline acylcarnitine scores and the risk of incident stroke

	STROKE					
	Overall		**Both MedDiet groups**		**Control group**	
Non-cases, n	751		530		221	
Cases, n	118		72		46	
	Short-chain acylcarnitines (C2-C7)					
	HR (95%CI)	P	HR (95%CI)	P	HR (95%CI)	P
Crude per s.d.	1.64 (1.18, 2.28)	<0.01	1.43 (0.97, 2.11)	0.06	2.45 (1.24, 4.83)	<0.01
Model 1						
Per s.d.*	1.84 (1.30, 2.59)	<0.01	1.46 (1.00, 2.13)	0.05	2.68 (1.35, 5.33)	<0.01
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	2.49 (1.27, 4.90)		2.93 (1.28, 6.71)		1.89 (0.56, 6.38)	
Q3	2.91 (1.47, 5.74)		2.58 (1.12, 5.97)		3.52 (1.04, 11.87)	
Q4	2.52 (1.26, 5.02)		1.88 (0.78, 4.51)		3.36 (1.05, 10.77)	
p for trend		0.01		0.35		0.02
Model 2						
Per s.d.	1.82 (1.27, 2.61)	<0.01	1.41 (0.96, 2.07)	0.07	3.38 (1.49, 7.66)	<0.01
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	2.46 (1.23, 4.91)		3.10 (1.31, 7.32)		1.60 (0.46, 5.54)	
Q3	2.86 (1.42, 5.74)		2.73 (1.13, 6.59)		3.40 (0.99, 11.70)	
Q4	2.53 (1.24, 5.18)		1.83 (0.71, 4.54)		4.37 (1.24, 15.38)	
p for trend		0.01		0.43		0.01
	Medium-chain acylcarnitines (C8-C14)					
Crude per s.d.	1.88 (1.04, 3.42)	0.03	1.55 (0.71, 3.37)	0.26	3.28 (1.26, 8.55)	0.01
Model 1						
Per s.d.	1.76 (0.95, 3.28)	0.07	1.34 (0.63, 2.85)	0.44	3.45 (1.14, 10.38)	0.02
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	1.09 (0.59, 2.00)		1.41 (0.62, 3.19)		0.85 (0.33, 2.22)	
Q3	0.98 (0.52, 1.84)		1.27 (0.57, 2.80)		0.58 (0.18, 1.84)	
Q4	1.64 (0.93, 2.91)		1.42 (0.66, 3.06)		2.37 (1.01, 5.67)	
p for trend		0.09		0.44		0.07
Model 2						

Per s.d.*	1.59 (0.85, 2.98)	0.14	1.23 (0.56, 2.72)	0.60	2.92 (1.01, 8.62)	0.05
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	1.16 (0.62, 2.17)		1.26 (0.55, 2.89)		1.05 (0.34, 3.18)	
Q3	1.01 (0.52, 1.95)		1.21 (0.52, 2.75)		0.70 (0.20, 2.44)	
Q4	1.55 (0.86, 2.79)		1.30 (0.59, 2.87)		2.37 (0.95, 5.87)	
p for trend		0.16		0.56		0.09
Long-chain carnitines (C16-C26)						
Crude per s.d.	1.66 (1.06, 2.59)	0.03	1.32 (0.77, 2.26)	0.31	2.69 (1.12, 6.42)	0.03
Model 1						
Per s.d.	1.41 (0.85, 2.32)	0.17	1.15 (0.65, 2.05)	0.62	2.09 (0.76, 5.82)	0.15
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	0.90 (0.48, 1.69)		1.00 (0.45, 2.19)		0.77 (0.27, 2.20)	
Q3	0.93 (0.49, 1.74)		1.43 (0.65, 3.12)		0.42 (0.13, 1.34)	
Q4	1.15 (0.62, 2.16)		1.09 (0.48, 2.49)		1.14 (0.40, 3.23)	
p for trend		0.60		0.67		0.73
Model 2						
Per s.d.	1.37 (0.82, 2.29)	0.22	1.15 (0.64, 2.08)	0.63	2.09 (0.70, 6.18)	0.18
Across quartiles						
Q1	1 (ref.)		1 (ref.)		1 (ref.)	
Q2	0.95 (0.49, 1.82)		1.00 (0.44, 2.26)		0.84 (0.27, 2.57)	
Q3	0.96 (0.51, 1.81)		1.53 (0.69, 3.39)		0.46 (0.14, 1.49)	
Q4	1.18 (0.62, 2.24)		1.11 (0.48, 2.56)		1.13 (0.43, 4.15)	
p for trend		0.59		0.62		0.68

*An inverse normal transformation was applied to raw values of acylcarnitines and a weighted sum of these values was computed to calculate the scores (**short-chain acylcarnitines**: C2carnitine, C3DCCH3carnitine, C3carnitine, C4carnitine, C4OHcarnitine, C5carnitine, C5:1carnitine, C5:DCcarnitine, C6carnitine, C7carnitine; **medium-chain acylcarnitines**: C8carnitine, C9carnitine, C10carnitine, C10:2carnitine, C12carnitine, C12:1carnitine, C14carnitine, C14:1carnitine, C14:2carnitine*; ***long-chain acylcarnitines:** C16carnitine, C18carnitine, C18:1carnitine, C18:1OHcarnitine, C18:2carnitine, C20carnitine, C20:4carnitine, C26carnitine). We excluded from the analysis the non-stroke CVD cases. Model 1: Adjusted for age, sex, BMI, family history of premature heart disease, smoking, and stratified by intervention group (only in overall analyses). Model 2: additionally adjusted for physical activity (metabolic equivalent task minutes/day), hypertension, dyslipidemia and diabetes.*

FIGURE LEGENDS:

Figure 1. Multivariate adjusted HRs (95% CI) of incident CVD and quartiles of baseline acylcarnitine scores stratified by intervention group (Mediterranean interventions versus control group).

Multivariate adjusted HRs (95% CI) of incident CVD and quartiles of baseline acylcarnitine scores stratified by intervention group (Mediterranean interventions versus control group). An inverse normal transformation was applied to raw baseline values and a weighted sum of these values was computed to calculate the scores. **A: short-chain acylcarnitines**: C2carnitine, C3DCCH3carnitine, C3carnitine, C4carnitine, C4OHcarnitine, C5carnitine, C5:1carnitine, C5:DCcarnitine, C6carnitine, C7carnitine; **B: medium-chain** acylcarnitines: C8carnitine, C9carnitine, C10carnitine, C10:2carnitine, C12carnitine, C12:1carnitine, C14carnitine, C14:1carnitine, C14:2carnitine; **C: long-chain acylcarnitines:** C16carnitine, C18carnitine, C18:1carnitine, C18:1OHcarnitine, C18:2carnitine, C20carnitine, C20:4carnitine, C26carnitine. N=980 for the three carnitine scores. Adjusted for age, sex, BMI, physical activity (mets min/d), family history of premature heart disease, and smoking. *P for interaction* between each MedDiet intervention group (EVOO and Nuts) as (binary, yes/no) and the acylcarnitine scores (continuous), with 2 product terms (EVOO*acylcarnitine score and Nuts*acylcarnitine score) and 2 degrees of freedom. *P for interaction* = 0.04, 0.09, and 0.48 for short-, medium- and long-chain acylcarnitine scores, respectively.