**Title:**

Plasma Ceramides, Mediterranean Diet, and Incident Cardiovascular Disease in the PREDIMED Trial

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**Short Title:**

Ceramide, Mediterranean Diet and Cardiovascular Disease

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**Abstract**

**Background**

Although *in vitro* studies and investigations in animal models and small clinical populations have suggested that ceramides may represent an intermediate link between over-nutrition and certain pathological mechanisms underlying cardiovascular disease (CVD), no prospective studies have investigated the association between plasma ceramides and risk of CVD.

**Methods**

The study population consisted of 980 participants from the PREDIMED trial, including 230 incident cases of CVD and 787 randomly selected participants at baseline (including 37 overlapping cases), followed for up to 7.4 years. Participants were randomized to a Mediterranean diet (MedDiet) supplemented with extra-virgin olive oil, a MedDiet supplemented with nuts, or a control diet. Plasma ceramide concentrations were measured on a liquid chromatography tandem mass spectrometry metabolomics platform. The primary outcome was a composite of non-fatal acute myocardial infarction, non-fatal stroke, or cardiovascular death. Hazard Ratios (HRs) were estimated with weighted Cox regression models, using Barlow weights to account for the case-cohort design.

**Results**

The multivariable HRs [95% confidence interval (CI)] comparing the extreme quartiles of plasma concentrations of C16:0, C22:0, C24:0 and C24:1 ceramides were 2.39 (1.49-3.83, *P* trend <0.001), 1.91 (1.21-3.01, *P* trend =0.003), 1.97 (1.21-3.01, *P* trend =0.004), and 1.73 (1.09-2.74, *P* trend =0.01), respectively. The ceramide score, calculated as a weighted sum of concentrations of four ceramides, was associated with a 2.18 fold higher risk of CVD across extreme quartiles (HR =2.18, 95% CI, 1.36-3.49, *P* trend <0.001). The association between baseline ceramide score and incident CVD varied significantly by treatment groups (*P* interaction =0.01). Participants with a higher ceramide score and assigned to either of the two active intervention arms of the trial showed similar CVD risk to those with a lower ceramide score, whereas participants with a higher ceramide score and assigned to the control arm presented significantly higher CVD risk. Changes in ceramide concentration were not significantly different between MedDiet and control groups during the first year of follow-up.

**Conclusions**

Our study documented a novel positive association between baseline plasma ceramide concentrations and incident CVD. In addition, a Mediterranean dietary intervention may mitigate potential deleterious effects of elevated plasma ceramide concentrations on CVD.

**Key words**

Ceramide; Mediterranean diet; cardiovascular disease; coronary heart disease; stroke

**Introduction**

Ceramides are members of the sphingolipid family and essential precursors of complex sphingolipids. Since the early 1990s, studies in cultured cells and animal models have shown that the aberrant accumulation of ceramides may lead to the activation of several signaling and putative targets that may impair normal cellular function, including insulin action. [1](#_ENREF_1) Meanwhile, this evidence has also linked excess *de novo* ceramide biosynthesis to cellular stress stimuli, especially to the exposure to saturated free fatty acids. [1-3](#_ENREF_1) Ceramide and its metabolites have thus been proposed as an intermediate link between over-nutrition and certain underlying abnormalities driving cardio-metabolic disease risk, including insulin resistance and low-grade inflammation. [2-4](#_ENREF_2) However, existing evidence relating ceramides to health outcomes comes mostly from *in vitro* experiments and animal studies, and it is mainly based on intermediate outcomes of cardiovascular risk. No studies have prospectively investigated the association between ceramides and the incidence of hard cardiovascular disease (CVD) endpoints, e.g., coronary heart disease (CHD) and stroke, in a primary prevention setting. Very recently, Laaksonen et al. reported divergent associations of distinct plasma ceramides with CVD death and proposed the ratio of two ceramides as the strongest predictor of CVD death among patients with stable CHD using a case-control study design[5](#_ENREF_5).

Modification of overall dietary patterns, compared to individual dietary factors, has long been proposed as a more effective and actionable target for CVD prevention and intervention. [6](#_ENREF_6) Recently, the first randomized controlled trial targeting overall dietary patterns for the primary prevention of CVD, the PREvencion con DIeta MEDiterranea (PREDIMED) trial [7](#_ENREF_7), [8](#_ENREF_8), found that the Mediterranean diet (MedDiet) enriched with extra-virgin olive oil or nuts significantly reduced CVD events by approximately 30% compared to the control diet. [9](#_ENREF_9) Based on strong and consistent evidence on hard CVD endpoint from the PREDIMED trial [9](#_ENREF_9) and prospective cohort studies [10-13](#_ENREF_10), the 2015-2020 Dietary Guidelines for Americans [14](#_ENREF_14) and the American Heart Association (AHA) [15](#_ENREF_15) both recommend the MedDiet for CVD prevention. However, the biological mechanisms underlying cardio-protective effects of the MedDiet are not completely understood.

Advances in metabolite profiling technology (metabolomics), especially liquid chromatography tandem mass spectrometry (LC-MS) techniques, provide powerful tools to decipher the biological mechanisms of disease. Several structurally different ceramides are among the lipid metabolites profiled by current metabolomics platforms. Recent evidence from two short-term small intervention studies found that ceramide concentration could be transiently decreased by adopting a healthy dietary pattern [16](#_ENREF_16) and changes in primary dietary sources of fat. [17](#_ENREF_17) The MedDiet might also exert its effect through decreasing ceramide concentration. However, it is still largely unknown whether ceramide concentration responds to long-term dietary intervention in a large population. In the present study based on the PREDIMED trial, we hypothesized that 1) plasma ceramide concentrations at baseline were associated with incident clinical events of CVD, 2) the association between baseline plasma ceramide concentrations and incident CVD was modified by the MedDiet interventions, and 3) participants in MedDiet intervention groups showed more favorable changes in plasma ceramide concentration compared to those in the control group during the first year of follow-up.

**Methods**

Study design and population

This study was nested in the PREDIMED randomized trial, but adopted a case-cohort design [18](#_ENREF_18), [19](#_ENREF_19) by including all the available incident CVD cases diagnosed during follow-up and randomly sampling 10% of the enrolled participants at baseline in the PREDIMED trial. The case-cohort design preserves random intervention assignments and maintains the causal integrity of the randomized design of the trial. The PREDIMED trial ([www.predimed.es](http://www.predimed.es)) was conducted from 2003 through 2010 in 11 centers in Spain to assess the effects of the MedDiet on the primary prevention of CVD. At baseline, this trial enrolled 7,447 participants aged 55-80 years with high cardiovascular risk but initially free from diagnosed CVD, including CHD (angina, myocardial infarction, coronary revascularization procedures or existence of abnormal Q waves in the electrocardiogram), stroke (ischemic or hemorrhagic, including transient ischemic attacks), and symptomatic peripheral artery disease at baseline. Participants were randomly assigned to a MedDiet supplemented with extra-virgin olive oil (MedDiet+EVOO), a MedDiet supplemented with nuts (MedDiet+nuts), or a control diet consisting of advice to reduce the intake of all types of fat. During a mean follow-up time of 4.8 years (maximum follow-up: 7.4 years), 288 incident CVD events occurred. The protocol was approved by the Institutional Review Boards at all study locations and all participants provided written informed consent. Detailed information about the PREDIMED trial can be found elsewhere.[9](#_ENREF_9), [20](#_ENREF_20) The study population consisted of 980 participants with available EDTA plasma samples, including 230 incident cases of CVD and 787 randomly selected participants at baseline (sub-cohort). The sub-cohort included 37 overlapping cases of CVD. We excluded 2 participants with undetectable plasma ceramide concentrations.

Study samples and metabolomics profiling

All analyses used fasting (fasting for ≥8 hours) plasma EDTA samples collected at baseline and year 1. All samples were processed at each recruiting center no later than 2 hours after collection and stored in -80°C freezers. Samples from cases and sub-cohort participants were randomly distributed before being shipped to the Broad Institute in Boston, MA, for metabolomics assays. LC-MS techniques were used to quantitatively profile ceramides in plasma samples. Details of the LC-MS platform can be found elsewhere. [21-27](#_ENREF_21) Internal standard peak areas were monitored for quality control and to ensure system performance throughout analyses. Pooled plasma reference samples were also inserted every 20 samples as an additional quality control.

Ascertainment of CVD outcomes

The primary outcome was a composite of non-fatal acute myocardial infarction (AMI), non-fatal stroke, or cardiovascular death. Information on outcomes was collected from continuous contact with participants and primary health care physicians, annual follow-up visits, yearly ad-hoc reviews of medical charts, and annual consultation of the National Death Index. Study physicians who were blinded collected information on primary outcomes to the intervention. Blinded to the intervention assignment, the Clinical End-Point Committee adjudicated the events according to the standard criteria. [28-33](#_ENREF_28)

Measurements of covariates

Medical conditions, family history of disease, and risk factors were collected through a questionnaire during the first screening visit. At baseline and during annual visits, participants completed a 14-item questionnaire in a personal interview with a registered dietitian to assess their adherence to the MedDiet. [34](#_ENREF_34) At baseline and annually, trained personnel measured participants’ body weight, height, waist circumference, and blood pressure according to the study protocol. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Participants’ triglyceride (TG), total cholesterol, low-density lipoprotein-cholesterol (LDL), high-density lipoprotein-cholesterol (HDL), and fasting blood glucose levels were measured using fasting plasma samples at baseline.

Statistical analysis

We transformed ceramide concentrations to the natural logarithm scale in order to render the distributions approximately Gaussian as well as to stabilize the variance. We categorized all the participants into quartiles of the ceramide concentration based on the distribution in the sub-cohort. Person-years of follow-up were calculated from baseline to the earliest CVD event, loss to follow-up, or the end of follow-up.

Weighted proportional hazards Cox regression models stratified on intervention group assignment (MedDiet+EVOO, MedDiet+nuts, and control) were applied to estimate hazard ratios (HRs) and their 95% confidence intervals (CIs) of CVD comparing participants in each quartile to the lowest quartile.We used the weighting scheme suggested by Barlow et al. [35](#_ENREF_35), [36](#_ENREF_36) to account for the over-representation of cases. To quantify a linear trend, we assigned the median value of ceramide concentration within each quartile and modeled this variable continuously. We also calculated HRs and 95% CIs of CVD associated with a 1-standard deviation (SD) increment in the transformed concentrations of ceramides. All multivariable models were simultaneously adjusted for age, sex, BMI, family history of premature CHD, smoking status, histories of hypertension, dyslipidemia, and diabetes. We calculated a baseline ceramide score as the weighted sum of concentrations of four different ceramides and modeled the ceramide score as a main exposure variable in the Cox model in the same fashion as individual ceramides. The weight for each ceramide was the regression coefficient for 1-SD increment in the ceramide concentration estimated from the multivariable Cox regression model. [21](#_ENREF_21) We also performed secondary analyses on the associations of ceramide concentrations with AMI and stroke separately. Because Laaksonen et al. [5](#_ENREF_5) suggested that ratios of ceramides could be stronger predictors of cardiovascular risk, we examined the similar ratios in relation to CVD as a secondary analysis. To test the robustness of our findings, in secondary analyses, the multivariable model adjusted for ratio of high-density lipoprotein cholesterol to low-density lipoprotein cholesterol (HDL/LDL) and for triglycerides (TG) as continuous variables instead of adjusting for dyslipidemia as a dichotomous variable, as well as further adjusted for other metabolites putatively associated with CVD that were targeted on the current metabolomics platform. These models additionally included other sphingolipid metabolites, including sphingomyelins and sphingosine. [37](#_ENREF_37), [38](#_ENREF_38) We also further adjusted for three branched-chain amino acids, i.e., valine, leucine and isoleucine, because they have been identified as strong predictors of insulin resistance and cardio-metabolic risk in our previous study [39](#_ENREF_39) and recent publications [21](#_ENREF_21), [40-43](#_ENREF_40). We further explored the association between the ceramide score and incident CVD risk in subgroups defined by several dichotomous risk factors at baseline, including sex (male, female), age (≤65 years, >65 years), BMI categories (≤30.0, >30.0 kg/m2), smoking status (never smoking, current/ever smoking), family history of premature CHD (yes, no), hypertension (yes, no), dyslipidemia (yes, no), and diabetes (yes, no). The interactions between these stratification variables and the ceramide score were tested by adding multiplicative terms into the multivariable Cox models; the likelihood ratio test was used for testing statistical significance of the interaction term.

We evaluated the added predictive ability of ceramides by comparing the c-statistics between one model including conventional risk factors of CVD, i.e., age, sex, systolic blood pressure, total and high-density lipoprotein cholesterol, current smoking and diabetes, and the other model including ceramide score in addition to the conventional risk factors, as well as estimating the net reclassification improvement (RNI) [44](#_ENREF_44) for the 7-year risk of CVD.

To examine whether the association between plasma ceramide concentration and incident CVD varied by intervention group, we first categorized participants into joint subgroups defined by intervention group assignment and whether their ceramide score was above/equal to or below the median value in the sub-cohort. Second, we constructed adjusted cumulative incidence curves for the joint subgroups by using Langholz et al.’s method for case-cohort design [45](#_ENREF_45) and included all the aforementioned covariates in the model. Third, we calculated the multivariable adjusted HRs for CVD from Cox regression models for each joint subgroup using participants with a low ceramide score (below the median) and in the intervention groups as a reference group. Lastly, we added a multiplicative term between intervention assignment and the ceramide score into the multivariable Cox models stratified on intervention assignment to test for interaction. To compare the temporal changes in ceramide concentrations between intervention and control groups, we employed linear mixed model to account for the within-individual repeated measurements and restricted this analysis to the random sub-cohort. All analyses were performed using SAS software, version 9.4 (SAS Institute, North Carolina), at a two-tailed *α* of 0.05.

**Results**

Baseline characteristics

The median follow-up of the analytic population was 4.5 years. The baseline characteristics of the sub-cohort were very similar to that of the full-cohort in the PREDIMED trial, [9](#_ENREF_9) except for a slightly higher proportion of participants with a family history of premature CHD (**Table 1**). At baseline, participants with a higher ceramide score had higher levels of total cholesterol, LDL, triglycerides, and diastolic blood pressure.

Plasma ceramide concentrations and CVD

The current metabolomics platform identified four different ceramides, including C16:0 (Number of carbon atoms: Number of double bonds), C22:0, C24:0, and C24:1 ceramides. Ceramide C24:0 had the highest relative concentration, while ceramide C16:0 had the lowest relative concentration (**Supplemental table 1**). We observed moderate and positive correlations in plasma concentration among the four ceramides, ranging from 0.49 to 0.63, except a high correlation of 0.90 between ceramides C22:0 and C24:0 (**Supplemental table 2**). All the ceramides were positively associated with incident CVD risk; the positive associations only differed in magnitude across different ceramide species and became slightly stronger after multivariable adjustment (**Table 2**). The multivariable HRs comparing the extreme quartiles of plasma concentrations of C16:0, C22:0, C24:0 and C24:1 ceramides were 2.39 (95% CI, 1.49-3.83, *P* for trend <0.001), 1.91 (95% CI, 1.21-3.01, *P* for trend =0.003), 1.97 (95% CI, 1.21-3.01, *P* for trend =0.004), and 1.73 (95% CI, 1.09-2.74, *P* for trend =0.01), respectively. The ceramide score was associated with a 2.18 fold higher risk of CVD across quartiles (HR =2.18, 95% CI, 1.36-3.49, *P* for trend <0.001). The HR associated with a 1-SD increment in the ceramide score was 1.41 (95% CI, 1.17, 1.68). The associations of ceramides and the ceramide score with CVD risk barely changed after further adjustment for sphingomyelins, sphingosine, and branched-chain amino acids and was slightly attenuated in model adjusting for HDL/LDL ratio and TG as continuous variables (**Supplemental Table 3**). Secondary analyses on stroke and AMI yielded similar associations between plasma ceramides and the specific CVD outcomes, compared to the main analysis of the composite CVD outcome (**Supplemental Table 4**). The associations between ceramide score and CVD risk were generally consistent across different risk strata subgroups (**Supplemental Table 5**). The addition of the ceramide score into the model with conventional risk factors of CVD improved the c-statistics from 0.70 (95% CI, 0.66-0.73) to 0.71 (95% CI, 0.67-0.74). Further, comparing the 7-year CVD risk predicted by the two models yielded an NRI of 0.22 (95% CI, 0.04-0.45, *P*=0.04). The HR associated with 1-SD increment in the ratio between ceramide C16:0 and C24:0 was 1.24 (95% CI, 1.05-1.46, *P* =0.01). However, other two ceramide ratios (C22:0/C24:0 and C24:1/C24:0) were not significantly associated with the incidence of CVD (**Supplemental table 6**).

Interactions between plasma ceramide concentrations and the MedDiet interventions

**Figure 1** shows that the association between baseline ceramide score and the incidence of CVD clinical events varied significantly by intervention group assignment. Using the median ceramide score as the cut-point, participants with a higher ceramide score and randomized to either of the two active arms of the trial showed similar incidence of CVD to those with a lower ceramide score. However, the cumulative incidence curve for participants with a higher ceramide score and randomized to the control group diverged soon from those in other subgroups after the initiation of this trial. Compared to participants with a lower ceramide score and randomized to either of the two active intervention arms of the trial, the HRs were 2.76 (95% CI, 1.72-4.44) for participants with a higher ceramide score and randomized to the control group, 1.07 (95% CI, 0.64-1.78) for those with a lower ceramide score and randomized to the control group, and 1.26 (95% CI, 0.84-1.87) for those with a higher ceramide score and randomized to either of the two active intervention arms (*P* for interaction =0.01, **Supplemental table 7**). The association between the baseline ceramide score and incident CVD also varied significantly when the two intervention groups were examined separately (**Figure 1**). The interaction between the MedDiet+EVOO intervention and the ceramide score was more pronounced (*P* for interaction =0.009) than that between the MedDiet+nuts intervention and the ceramide score (*P* for interaction =0.05).

Changes in ceramide concentration

One-year changes in ceramide concentration were not significantly different between participants in either of the two intervention groups and those in the control group. (**Supplemental table 8**). We observed similar trends in ceramide concentrations when comparing each MedDiet group to the control group.

**Discussion**

In this prospective case-cohort study within the PREDIMED trial, we observed that plasma ceramide concentrations were strongly associated with elevated risk of the composite CVD outcome defined as non-fatal AMI, non-fatal stroke, or cardiovascular death, which was the primary end-point of the PREDIMED trial. The positive association was consistent for two major components of the composite CVD endpoint, namely AMI and stroke, and across different subgroups defined by baseline risk characteristics. In addition, the association between plasma ceramides and CVD risk varied significantly across intervention groups, suggesting that the MedDiet has the potential to mitigate the detrimental effect associated with elevated baseline plasma ceramide concentrations on CVD risk.

This study, to our knowledge, is the first prospective study in a clinical trial setting to investigate the association between plasma ceramide concentrations and hard CVD endpoints. Previously, *in vitro* and *in vivo* animal studies have provided substantial evidence relating ceramide accumulation to multiple mechanisms underlying pathogenesis of CVD. However, human data are still sparse and limited by their small sample size and cross-sectional study design. The role of ceramides in the development of insulin resistance has been intensively studied in the past two decades. Earlier studies using cultured cells and animal models suggested that endogenous ceramides antagonized insulin-stimulated glucose uptake and anabolism [46](#_ENREF_46), [47](#_ENREF_47) by blocking activation of Akt/PKB, a serine/threonine kinase that is obligate for insulin and growth-factor activation of anabolism and cell survival [48-53](#_ENREF_48). Human studies reported increased ceramide concentration in obese insulin-resistant participants [54](#_ENREF_54) and a negative correlation between muscle and plasma ceramide and insulin sensitivity [55-57](#_ENREF_55). Interestingly, our data did not support cross-sectional associations between ceramide concentrations and several baseline characteristics related to insulin resistance, e.g., BMI, prevalence of diabetes, and fasting glucose. It is possible that these associations were diluted among this study population given that all our participants were selected because they were high-risk subjects and most of them might had already developed insulin resistance at the time of the enrollment. Beyond insulin resistance, limited human studies have observed positive correlations between plasma ceramide concentrations and inflammatory makers, e.g., interleukin-6 [58](#_ENREF_58) and TNF-α, [59](#_ENREF_59) suggesting a relationship between excess ceramides and inflammation. Several lines of evidence in rodent models suggest that pharmacological inhibition of ceramide biosynthesis wards off atherogenesis. [60](#_ENREF_60), [61](#_ENREF_61) Ceramides and other sphingolipids may contribute to plaque erosion and therefore induce thrombosis [3](#_ENREF_3). Of note, these studies on plaque formation [60](#_ENREF_60), [61](#_ENREF_61) also found that inhibition of ceramide biosynthesis caused a reduction of circulating total cholesterol and LDL, which is consistent with our cross-sectional observations on ceramide concentrations and blood lipid profiles. Elevated ceramides were also implicated in cardiomyopathy. For example, Park, *et al.* observed that inhibition of a rate-limiting enzyme in ceramide biosynthesis (serine palmitoyltransferase [SPT]) improved systolic function and prolonged survival rates in a mouse model [62](#_ENREF_62). Laaksonen et al. observed an elevated risk of CVD death in CHD patients with higher plasma concentration of three ceramide species (C16:0, C18:0 and C24:1) but an insignificant lower risk of CVD death in those with higher concentration of ceramide C24:0 [5](#_ENREF_5). However, we found that all four ceramides were positively associated with the incidence of CVD. Further, the ratios of ceramide did not show stronger associations with CVD risk than individual ceramides or ceramide score. The evidence regarding whether distinct ceramide metabolites were divergently associated with insulin resistant were also inconsistent [63-65](#_ENREF_63). Further studies are still warranted to investigate the potential different biological effects of ceramides with different acyl-chain length.

We observed that the detrimental effect of higher ceramide concentrations on CVD risk was modified by the MedDiet intervention. The potential mechanisms for the MedDiet’s modulatory effects on the ceramide pathway are two-fold. First, consumption of key components of the MedDiet may directly influence ceramide biosynthesis. Studies using cultured myotubes and animal models found that exposure to saturated free fatty acids (FFAs), especially long-chain saturated FFAs, promoted ceramide formation [66](#_ENREF_66), [67](#_ENREF_67), while unsaturated FFAs prevented the excess ceramide accumulation stimulated by saturated FFAs [68](#_ENREF_68), and therefore postulated the rate-limiting SPT was specific to the composition of circulating FFAs [3](#_ENREF_3). The PREDIMED trial was effective in modifying intervention groups’ dietary patterns, [9](#_ENREF_9) which were characterized by a high intake of virgin olive oil, fruit, nuts, vegetables, and cereals; a moderate intake of fish and poultry; a low intake of dairy products, red meat, processed meats, and sweets; and wine in moderation, consumed with meals [69](#_ENREF_69). In conjunction with two supplemental foods, extra-virgin olive oil and nuts, the interventions might have changed circulating FFA composition through modifying dietary fat intake pattern, i.e., decreasing saturated fat intake and increasing monounsaturated and polyunsaturated fat intakes, and modulating *de novo* lipogenesis upon improvement in dietary carbohydrate quality. It is worth noting that our analysis did not find that the MedDiet was associated with favorable changes in ceramide concentrations during the first year of follow-up. However, we cannot rule out that MedDiet may directly mitigate aberrant ceramide accumulation in longer follow-up. Secondly, the MedDiet intervention could suppress deleterious effects following excess ceramide accumulation. Previous studies have suggested that the benefits of the Mediterranean dietary pattern on CVD could be mediated through several mechanisms, including the reduction of low-grade inflammation [70-74](#_ENREF_70), enhanced endothelial function [72](#_ENREF_72), [75](#_ENREF_75), [76](#_ENREF_76), lower oxidative stress [77](#_ENREF_77), [78](#_ENREF_78), and lower levels of oxidized low-density lipoprotein (LDL) [79](#_ENREF_79) and atherogenic lipoproteins [80](#_ENREF_80).

Our results should be interpreted in the context of several limitations. First, participants of this project were mostly European Caucasians, which might limit the generalizability of our findings to other populations. Secondly, participants were recruited based on their high CVD risk. Therefore, our findings might not be applicable in populations with low CVD risk. Finally, even though we carefully adjusted for many potential confounders, residual confounding cannot be ruled out.

Our study possesses several major strengths. First, this study was built on a large, successful randomized controlled trial of hard clinical CVD endpoints, which provided a unique and powerful setting to address our research questions, because of its well-characterized study population, high compliance to the interventions, and low rates of drop-out. Secondly, the case-cohort design preserved the randomized design of this intervention trial and maintained the causal integrity of a randomized exposure status.

In summary, our study documented for the first time a strong positive association between plasma ceramide concentrations and incident CVD risk by using a prospective design nested in a well-known randomized trial. In addition, the traditional MedDiet intervention showed the potential to mitigate deleterious effects on CVD risk related to elevated plasma ceramide concentrations. Further studies are warranted to replicate these results in other populations and investigate potential mechanisms.

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**Figure legends:**

Figure 1. Adjusted cumulative incidence curves in joint subgroups defined by ceramide score and intervention group assignment. Cumulative incidence curves were adjusted for age (continuous) and sex (male, female), body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes (all yes, no)

Table 1. Baseline characteristics of study participants.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Sub-cohort \* | Cases |  | Quartiles of the ceramide score ‡ | | | | *P*-value § |
|  | (n=787) | (n=230) |  | Q1 (n=195) | Q2 (n=197) | Q3 (n=197) | Q4 (n=198) |
| Intervention group, % |  |  |  |  |  |  |  |  |
| Control | 234 (29.7) | 83 (36.1) |  | 67 (34.4) | 63 (32.0) | 57 (28.9) | 47 (23.7) | 0.29 |
| Mediterranean diet + EVOO | 291 (37.0) | 82 (35.7) |  | 62 (31.8) | 71 (36.0) | 78 (39.6) | 80 (40.4) |  |
| Mediterranean diet + Nuts | 262 (33.3) | 65 (28.3) |  | 66 (33.8) | 63 (32.0) | 62 (31.5) | 71 (35.9) |  |
| Men, % | 450 (57.2) | 91 (39.6) |  | 93 (47.7) | 101 (51.3) | 132 (67.0) | 124 (62.6) | <.001 |
| Family history of premature CHD, % | 196 (24.9) | 44 (19.1) |  | 56 (28.7) | 56 (28.4) | 43 (21.8) | 41 (20.7) | 0.13 |
| Smoking, % |  |  |  |  |  |  |  |  |
| Never | 491 (62.4) | 104 (45.2) |  | 119 (61.0) | 107 (54.3) | 135 (68.5) | 130 (65.7) | 0.08 |
| Current | 96 (12.2) | 46 (20.0) |  | 22 (11.3) | 27 (13.7) | 24 (12.2) | 23 (11.6) |  |
| Former | 200 (25.4) | 80 (34.8) |  | 54 (27.7) | 63 (32.0) | 38 (19.3) | 45 (22.7) |  |
| Baseline prevalent disease, % |  |  |  |  |  |  |  |  |
| Hypertension | 659 (83.7) | 190 (82.6) |  | 161 (82.6) | 167 (84.8) | 159 (80.7) | 172 (86.9) | 0.38 |
| Dyslipidemia | 579 (73.6) | 134 (58.3) |  | 134 (68.7) | 145 (73.6) | 146 (74.1) | 154 (77.8) | 0.24 |
| Diabetes | 372 (47.3) | 149 (64.8) |  | 105 (53.8) | 81 (41.1) | 93 (47.2) | 93 (47.0) | 0.09 |
| Age (years) | 67.2±5.9 | 69.5±6.5 |  | 67.3±5.8 | 67.5±6.0 | 67.1±6.0 | 66.9±6.0 | 0.80 |
| Body mass index (kg/m2) | 29.8±3.6 | 29.6±3.7 |  | 30.1±3.8 | 29.6±3.4 | 29.5±3.7 | 29.8±3.6 | 0.44 |
| Adherence to Mediterranean diet † | 8.8±1.9 | 8.4±1.8 |  | 8.9±2.0 | 9.0±1.7 | 8.5±2.0 | 8.8±1.8 | 0.07 |
| Fasting glucose (mg/dL) | 121.9±41.0 | 136.2±48.9 |  | 123.2±40.4 | 115.7±31.4 | 121.7±43.8 | 126.7±46.3 | 0.07 |
| Total cholesterol (mg/dl) | 210.3±37.1 | 212.1±35.7 |  | 186.6±32.4 | 201.7±30.7 | 215.9±30.7 | 236.2±35.4 | <.001 |
| HDL cholesterol (mg/dL) | 54.0±15.4 | 51.9±16.4 |  | 53.5±15.9 | 53.7±16.5 | 53.0±11.8 | 55.5±16.7 | 0.40 |
| LDL cholesterol (mg/dL) | 130.8±33.4 | 131.4±33.4 |  | 112.8±29.9 | 124.9±28.5 | 135.8±29.4 | 149.5±34.4 | <.001 |
| Triglyceride (mg/dL) | 135.0±79.3 | 151.5±83.4 |  | 114.6±81.9 | 122.7±56.9 | 133±55.0 | 168.6±102.2 | <.001 |
| Systolic blood pressure (mmHg) | 147.3±20.3 | 154.9±23.1 |  | 146.2±18.3 | 147.8±20.8 | 147.2±19.8 | 148.1±22.1 | 0.80 |
| Diastolic blood pressure (mmHg) | 82.0±10.5 | 83.0±11.6 |  | 80.3±10.4 | 82.0±10.2 | 81.5±10.5 | 84.2±10.7 | 0.003 |

Abbreviations: EVOO, extra-virgin olive oil; CHD, coronary heart disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein;

\* The sub-cohort also included 37 cases.

† Adherence to the Mediterranean diet was assessed by a 14-item dietary screener.

‡ Quartiles were calculated based on the distribution of the ceramide score (weighted sum of four ceramides) in the sub-cohort.

§ *P*-values indicate whether the baseline characteristics were different across quartiles of ceramide score in the sub-cohort. χ2 test was used for comparison of categorical variable and ANOVA was used for comparison of continuous variable.

Table 2. Associations of baseline plasma ceramide concentrations and the ceramide score with cardiovascular disease.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Quartiles of ceramide species concentration \* | | | | *P* trend |  | HR per 1 | *P* value |
|  | Q1 | Q2 | Q3 | Q4 |  | SD increment † |
| Ceramide (16:0) |  |  |  |  |  |  |  |  |
| Cases | 38 | 57 | 57 | 78 |  |  |  |  |
| MV1 ‡ | Ref. | 1.60 (1.00, 2.54) | 1.67 (1.04, 2.67) | 2.20 (1.40, 3.46) | <.001 |  | 1.43 (1.20, 1.70) | <.001 |
| MV2 § | Ref. | 1.72 (1.05, 2.81) | 1.87 (1.14, 3.07) | 2.39 (1.49, 3.83) | <.001 |  | 1.42 (1.19, 1.69) | <.001 |
| Ceramide (22:0) |  |  |  |  |  |  |  |  |
| Cases | 53 | 43 | 62 | 72 |  |  |  |  |
| MV1 | Ref. | 0.95 (0.60, 1.50) | 1.29 (0.83, 1.99) | 1.89 (1.22, 2.93) | 0.002 |  | 1.33 (1.12, 1.58) | 0.001 |
| MV2 | Ref. | 0.88 (0.54, 1.43) | 1.28 (0.81, 2.02) | 1.91 (1.21, 3.01) | 0.003 |  | 1.32 (1.10, 1.57) | 0.002 |
| Ceramide (24:0) |  |  |  |  |  |  |  |  |
| Cases | 48 | 56 | 59 | 67 |  |  |  |  |
| MV1 | Ref. | 1.26 (0.80, 1.97) | 1.40 (0.89, 2.18) | 1.88 (1.20, 2.95) | 0.006 |  | 1.29 (1.09, 1.53) | 0.003 |
| MV2 | Ref. | 1.20 (0.75, 1.94) | 1.51 (0.94, 2.42) | 1.97 (1.21, 3.20) | 0.004 |  | 1.32 (1.10, 1.57) | 0.002 |
| Ceramide (24:1) |  |  |  |  |  |  |  |  |
| Cases | 44 | 50 | 59 | 77 |  |  |  |  |
| MV1 | Ref. | 1.07 (0.67, 1.70) | 1.31 (0.82, 2.08) | 1.53 (0.98, 2.37) | 0.04 |  | 1.22 (1.04, 1.43) | 0.02 |
| MV2 | Ref. | 1.16 (0.72, 1.89) | 1.44 (0.88, 2.36) | 1.73 (1.09, 2.74) | 0.01 |  | 1.27 (1.08, 1.49) | 0.004 |
| Ceramide score |  |  |  |  |  |  |  |  |
| Cases | 45 | 51 | 59 | 75 |  |  |  |  |
| MV1 | Ref. | 1.14 (0.72, 1.81) | 1.53 (0.97, 2.41) | 2.04 (1.30, 3.18) | <.001 |  | 1.40 (1.17, 1.66) | <.001 |
| MV2 | Ref. | 1.25 (0.77, 2.03) | 1.68 (1.05, 2.69) | 2.18 (1.36, 3.49) | <.001 |  | 1.41 (1.17, 1.68) | <.001 |

Abbreviations: MV, multivariable model

\* Quartiles were calculated based on the distribution of the ceramide concentrations in the sub-cohort.

† A logarithmic transformation was applied to the raw value.

‡ Model 1 stratified on intervention group and simultaneously adjusted for age (continuous) and sex (male, female).

§ Model 2 additionally adjusted for body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes (all yes, no).

|  |  |
| --- | --- |
| Figure 1A. Two Mediterranean Diet Groups Combined | |
| E:\Dropbox\predimed\output\gplot17.png  Control + Ceramide score ≥median  Intervention + Ceramide score ≥median  Control + Ceramide score <median  Intervention + Ceramide score <median  Cumulative incidence of cardiovascular disease  Years | Hazard ratio (95% CI)  Ceramide score ≥median vs. <median  Two MedDiet groups combined  Control group |

|  |  |
| --- | --- |
| Figure 1B. Mediterranean Diet + Extra-Virgin Olive Oil | |
| E:\Dropbox\predimed\output\gplot18.png  Control + Ceramide score ≥median  Intervention + Ceramide score ≥median  Control + Ceramide score <median  Intervention + Ceramide score <median  Cumulative incidence of cardiovascular disease  Years | MedDiet + EVOO  Hazard ratio (95% CI)  Ceramide score ≥median vs. <median  Control group |

|  |  |
| --- | --- |
| Figure 1C. Mediterranean Diet + Nuts | |
| E:\Dropbox\predimed\output\gplot19.png  Control + Ceramide score ≥median  Intervention + Ceramide score ≥median  Control + Ceramide score <median  Intervention + Ceramide score <median  Cumulative incidence of cardiovascular disease  Years | MedDiet + Nuts  Hazard ratio (95% CI)  Ceramide score ≥median vs. <median  Control group |

Figure 1. Adjusted cumulative incidence curves in joint subgroups defined by ceramide score and intervention group assignment. Cumulative incidence curves were adjusted for age (continuous) and sex (male, female), body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes (all yes, no)

Supplemental table 1. Distributions of plasma ceramides (m/z)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Arithmetic mean | Geometric mean | Median | Standard deviation | 5th percentile | 25th percentile | 75th percentile | 95th percentile |
| Ceramide (16:0) | 16778.2 | 15965.2 | 16225.0 | 5349.4 | 9475.0 | 13078.0 | 19758.0 | 26159.0 |
| Ceramide (22:0) | 98286.3 | 93423.7 | 93057.0 | 32055.0 | 55664.0 | 76914.0 | 116105.0 | 155088.0 |
| Ceramide (24:0) | 355086.6 | 341674.7 | 342510.0 | 99969.6 | 219385.0 | 286572.0 | 409883.0 | 539026.0 |
| Ceramide (24:1) | 130573.4 | 125085.2 | 126688.0 | 38908.4 | 76216.0 | 104476.0 | 153409.0 | 198174.0 |

Supplemental table 2. Correlations matrix among baseline plasma sphingolipid metabolites and branched-chain amino acids

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Ceramide (16:0) | Ceramide (22:0) | Ceramide (24:0) | Ceramide (24:1) | Sphingomyelin (24:0) | Sphingomyelin (22:0) | Sphingomyelin (16:0) | Sphingomyelin (18:0) | Sphingomyelin (18:1) | Sphingomyelin (24:1) | Sphingomyelin (16:1) | Sphingomyelin (18:2) | Sphingomyelin (14:0) | Sphingomyelin (22:1) | Sphingomyelin (20:0) | Sphingosine | Valine | Leucine | Isoleucine |
| Ceramide (16:0) | 1.00 | 0.60 | 0.58 | 0.63 | 0.45 | 0.50 | 0.62 | 0.53 | 0.48 | 0.45 | 0.53 | 0.41 | 0.44 | 0.48 | 0.51 | 0.02 | 0.03 | 0.02 | 0.03 |
| Ceramide (22:0) |  | 1.00 | 0.90 | 0.58 | 0.48 | 0.62 | 0.38 | 0.38 | 0.30 | 0.09 | 0.38 | 0.21 | 0.50 | 0.48 | 0.60 | 0.05 | 0.12 | 0.12 | 0.09 |
| Ceramide (24:0) |  |  | 1.00 | 0.49 | 0.67 | 0.72 | 0.49 | 0.38 | 0.34 | 0.23 | 0.49 | 0.28 | 0.52 | 0.58 | 0.63 | 0.05 | 0.08 | 0.08 | 0.05 |
| Ceramide (24:1) |  |  |  | 1.00 | 0.17 | 0.25 | 0.45 | 0.50 | 0.45 | 0.52 | 0.41 | 0.25 | 0.28 | 0.37 | 0.37 | 0.00 | 0.04 | 0.01 | 0.01 |
| Sphingomyelin (24:0) |  |  |  |  | 1.00 | 0.94 | 0.71 | 0.52 | 0.42 | 0.59 | 0.60 | 0.44 | 0.54 | 0.72 | 0.72 | 0.14 | 0.02 | 0.00 | -0.03 |
| Sphingomyelin (22:0) |  |  |  |  |  | 1.00 | 0.74 | 0.62 | 0.51 | 0.56 | 0.64 | 0.52 | 0.63 | 0.80 | 0.84 | 0.12 | 0.06 | 0.03 | -0.01 |
| Sphingomyelin (16:0) |  |  |  |  |  |  | 1.00 | 0.68 | 0.66 | 0.81 | 0.83 | 0.69 | 0.62 | 0.79 | 0.67 | 0.03 | -0.09 | -0.13 | -0.13 |
| Sphingomyelin (18:0) |  |  |  |  |  |  |  | 1.00 | 0.86 | 0.64 | 0.62 | 0.53 | 0.54 | 0.64 | 0.75 | 0.07 | -0.03 | -0.05 | -0.07 |
| Sphingomyelin (18:1) |  |  |  |  |  |  |  |  | 1.00 | 0.64 | 0.80 | 0.71 | 0.43 | 0.73 | 0.64 | -0.01 | -0.13 | -0.17 | -0.18 |
| Sphingomyelin (24:1) |  |  |  |  |  |  |  |  |  | 1.00 | 0.71 | 0.59 | 0.38 | 0.69 | 0.53 | 0.04 | -0.11 | -0.17 | -0.15 |
| Sphingomyelin (16:1) |  |  |  |  |  |  |  |  |  |  | 1.00 | 0.73 | 0.63 | 0.86 | 0.63 | 0.00 | -0.13 | -0.17 | -0.18 |
| Sphingomyelin (18:2) |  |  |  |  |  |  |  |  |  |  |  | 1.00 | 0.38 | 0.69 | 0.52 | -0.02 | -0.12 | -0.18 | -0.17 |
| Sphingomyelin (14:0) |  |  |  |  |  |  |  |  |  |  |  |  | 1.00 | 0.71 | 0.73 | 0.06 | 0.01 | 0.00 | -0.03 |
| Sphingomyelin (22:1) |  |  |  |  |  |  |  |  |  |  |  |  |  | 1.00 | 0.83 | 0.06 | -0.10 | -0.14 | -0.17 |
| Sphingomyelin (20:0) |  |  |  |  |  |  |  |  |  |  |  |  |  |  | 1.00 | 0.07 | 0.02 | 0.00 | -0.04 |
| Sphingosine |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | 1.00 | 0.03 | 0.03 | 0.03 |
| Valine |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | 1.00 | 0.93 | 0.91 |
| Leucine |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | 1.00 | 0.95 |
| Isoleucine |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | 1.00 |

Values are age- and sex-adjusted partial Spearman correlation coefficients in the sub-cohort.

Supplemental Table 3. Associations of baseline plasma ceramide concentrations and the ceramide score with cardiovascular disease.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Quartiles of ceramide species concentration *1* | | | | *P* trend |  | HR per 1 | *P* value |
|  | Q1 | Q2 | Q3 | Q4 |  | SD increment *2* |
| Ceramide (16:0) |  |  |  |  |  |  |  |  |
| Model 1 *3* | Ref. | 1.72 (1.05, 2.81) | 1.87 (1.14, 3.07) | 2.39 (1.49, 3.83) | <.001 |  | 1.42 (1.19, 1.69) | <.001 |
| Model 2 *4* | Ref. | 1.68 (1.03, 2.75) | 1.74 (1.06, 2.84) | 2.10 (1.30, 3.40) | 0.003 |  | 1.36 (1.13, 1.63) | <.001 |
| Model 3 *5* | Ref. | 1.93 (1.14, 3.27) | 2.02 (1.18, 3.46) | 2.64 (1.49, 4.68) | 0.001 |  | 1.58 (1.26, 1.98) | <.001 |
| Model 4 *6* | Ref. | 1.68 (1.03, 2.73) | 1.85 (1.13, 3.04) | 2.32 (1.45, 3.71) | <.001 |  | 1.41 (1.18, 1.68) | <.001 |
| Model 5 *7* | Ref. | 1.89 (1.12, 3.19) | 2.00 (1.17, 3.43) | 2.54 (1.43, 4.53) | 0.002 |  | 1.55 (1.24, 1.96) | <.001 |
| Ceramide (22:0) |  |  |  |  |  |  |  |  |
| Model 1 | Ref. | 0.88 (0.54, 1.43) | 1.28 (0.81, 2.02) | 1.91 (1.21, 3.01) | 0.003 |  | 1.32 (1.10, 1.57) | 0.002 |
| Model 2 | Ref. | 0.85 (0.52, 1.39) | 1.17 (0.73, 1.88) | 1.55 (0.95, 2.53) | 0.04 |  | 1.20 (1.00, 1.45) | 0.05 |
| Model 3 | Ref. | 0.87 (0.53, 1.42) | 1.30 (0.81, 2.09) | 1.89 (1.17, 3.06) | 0.005 |  | 1.30 (1.08, 1.57) | 0.006 |
| Model 4 | Ref. | 0.85 (0.52, 1.38) | 1.26 (0.80, 1.99) | 1.84 (1.17, 2.90) | 0.003 |  | 1.30 (1.09, 1.56) | 0.004 |
| Model 5 | Ref. | 0.84 (0.51, 1.37) | 1.28 (0.80, 2.06) | 1.83 (1.13, 2.95) | 0.007 |  | 1.28 (1.06, 1.55) | 0.01 |
| Ceramide (24:0) |  |  |  |  |  |  |  |  |
| Model 1 | Ref. | 1.20 (0.75, 1.94) | 1.51 (0.94, 2.42) | 1.97 (1.21, 3.20) | 0.004 |  | 1.32 (1.10, 1.57) | 0.002 |
| Model 2 | Ref. | 1.19 (0.74, 1.93) | 1.43 (0.88, 2.30) | 1.63 (0.98, 2.69) | 0.04 |  | 1.20 (1.00, 1.43) | 0.06 |
| Model 3 | Ref. | 1.21 (0.75, 1.97) | 1.47 (0.89, 2.44) | 1.91 (1.13, 3.25) | 0.01 |  | 1.31 (1.06, 1.61) | 0.01 |
| Model 4 | Ref. | 1.19 (0.74, 1.91) | 1.51 (0.94, 2.42) | 1.94 (1.19, 3.15) | 0.004 |  | 1.30 (1.08, 1.56) | 0.005 |
| Model 5 | Ref. | 1.19 (0.74, 1.94) | 1.48 (0.89, 2.45) | 1.88 (1.11, 3.19) | 0.02 |  | 1.29 (1.05, 1.58) | 0.02 |
| Ceramide (24:1) |  |  |  |  |  |  |  |  |
| Model 1 | Ref. | 1.16 (0.72, 1.89) | 1.44 (0.88, 2.36) | 1.73 (1.09, 2.74) | 0.01 |  | 1.27 (1.08, 1.49) | 0.004 |
| Model 2 | Ref. | 1.19 (0.73, 1.94) | 1.37 (0.83, 2.26) | 1.52 (0.95, 2.43) | 0.07 |  | 1.17 (1.00, 1.38) | 0.05 |
| Model 3 | Ref. | 1.18 (0.72, 1.93) | 1.44 (0.87, 2.40) | 1.73 (1.06, 2.82) | 0.02 |  | 1.26 (1.06, 1.50) | 0.009 |
| Model 4 | Ref. | 1.12 (0.69, 1.83) | 1.40 (0.85, 2.29) | 1.67 (1.05, 2.66) | 0.02 |  | 1.26 (1.07, 1.48) | 0.006 |
| Model 5 | Ref. | 1.14 (0.69, 1.87) | 1.40 (0.84, 2.33) | 1.67 (1.02, 2.73) | 0.03 |  | 1.24 (1.04, 1.48) | 0.01 |
| Ceramide score |  |  |  |  |  |  |  |  |
| Model 1 | Ref. | 1.25 (0.77, 2.03) | 1.68 (1.05, 2.69) | 2.18 (1.36, 3.49) | <.001 |  | 1.41 (1.17, 1.68) | <.001 |
| Model 2 | Ref. | 1.22 (0.75, 1.98) | 1.57 (0.97, 2.54) | 1.85 (1.14, 3.01) | 0.008 |  | 1.31 (1.09, 1.59) | 0.005 |
| Model 3 | Ref. | 1.34 (0.81, 2.23) | 1.81 (1.10, 2.98) | 2.46 (1.40, 4.35) | 0.001 |  | 1.51 (1.20, 1.90) | <.001 |
| Model 4 | Ref. | 1.25 (0.77, 2.03) | 1.67 (1.04, 2.67) | 2.13 (1.33, 3.42) | <.001 |  | 1.39 (1.16, 1.67) | <.001 |
| Model 5 | Ref. | 1.34 (0.80, 2.23) | 1.80 (1.09, 2.96) | 2.40 (1.36, 4.25) | 0.002 |  | 1.49 (1.18, 1.87) | <.001 |

Abbreviations: BCAA, branched-chain amino acid

*1* Quartiles were calculated based on the distribution of the ceramide concentrations in the sub-cohort.

*2* A logarithmic transformation was applied to the raw value.

*3* Multivariable model in the main analysis stratified on intervention group and simultaneously adjusted for age (continuous) and sex (male, female), body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes (all yes, no).

*4* Multivariable model stratified on intervention group and simultaneously adjusted for age (continuous) and sex (male, female), body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), ratio of baseline serum high-density lipoprotein cholesterol and low-density lipoprotein cholesterol (continuous), baseline serum triglyceride (continuous) histories of hypertension and diabetes (both yes, no).

*5* Model 1 further adjusted for sphingomyelin score and sphingosine (both continuous). Sphingomyelin score was calculated as the weighted sum of concentrations of eleven different sphingomyelins. The weight for each sphingomyelin was the regression coefficient for 1-SD increment in this sphingomyelin concentration estimated from the multivariable Cox regression model.

*6* Model 1 further adjusted for branched-chain amino acid (BCAA) score (continuous). BCAA score was calculated as the weighted sum of concentrations of three different BCAAs. The weight for each BCAA was the regression coefficient for 1-SD increment in this BCAA concentration estimated from the multivariable Cox regression model.

*7* Model 1 further adjusted for sphingomyelin score, sphingosine and BCAA score (all continuous).

Supplemental Table 4. Associations of baseline plasma ceramide concentrations and the ceramide score with stroke and myocardial infarction.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Quartiles of ceramide species concentration *1* | | | | *P* trend |  | HR per 1 | P value |
|  | Q1 | Q2 | Q3 | Q4 |  | SD increment *2* |
| Stroke | | | | | | | | |
| Ceramide (16:0) |  |  |  |  |  |  |  |  |
| Cases | 18 | 29 | 24 | 44 |  |  |  |  |
| MV1 *3* | Ref. | 1.62 (0.86, 3.05) | 1.36 (0.71, 2.63) | 2.49 (1.38, 4.49) | 0.005 |  | 1.47 (1.18, 1.84) | <.001 |
| MV2 *4* | Ref. | 1.82 (0.94, 3.53) | 1.48 (0.75, 2.93) | 2.72 (1.47, 5.03) | 0.003 |  | 1.48 (1.18, 1.85) | <.001 |
| Ceramide (22:0) |  |  |  |  |  |  |  |  |
| Cases | 25 | 21 | 35 | 34 |  |  |  |  |
| MV1 | Ref. | 1.00 (0.53, 1.90) | 1.45 (0.82, 2.59) | 1.87 (1.04, 3.34) | 0.02 |  | 1.33 (1.08, 1.63) | 0.007 |
| MV2 | Ref. | 0.96 (0.50, 1.86) | 1.52 (0.83, 2.79) | 1.82 (0.99, 3.34) | 0.03 |  | 1.31 (1.05, 1.63) | 0.02 |
| Ceramide (24:0) |  |  |  |  |  |  |  |  |
| Cases | 22 | 33 | 28 | 32 |  |  |  |  |
| MV1 | Ref. | 1.63 (0.90, 2.98) | 1.43 (0.78, 2.62) | 1.95 (1.07, 3.57) | 0.04 |  | 1.28 (1.04, 1.58) | 0.02 |
| MV2 | Ref. | 1.57 (0.84, 2.92) | 1.62 (0.85, 3.08) | 1.97 (1.02, 3.78) | 0.04 |  | 1.29 (1.03, 1.63) | 0.03 |
| Ceramide (24:1) |  |  |  |  |  |  |  |  |
| Cases | 21 | 24 | 30 | 40 |  |  |  |  |
| MV1 | Ref. | 1.07 (0.57, 2.03) | 1.25 (0.67, 2.33) | 1.67 (0.93, 3.00) | 0.07 |  | 1.27 (1.04, 1.55) | 0.02 |
| MV2 | Ref. | 1.16 (0.60, 2.23) | 1.32 (0.69, 2.53) | 1.86 (1.01, 3.42) | 0.04 |  | 1.31 (1.06, 1.60) | 0.01 |
| Ceramide score |  |  |  |  |  |  |  |  |
| Cases | 20 | 26 | 32 | 37 |  |  |  |  |
| MV1 | Ref. | 1.25 (0.67, 2.35) | 1.77 (0.95, 3.28) | 2.15 (1.18, 3.91) | 0.006 |  | 1.42 (1.15, 1.75) | 0.001 |
| MV2 | Ref. | 1.38 (0.73, 2.63) | 1.88 (0.99, 3.57) | 2.27 (1.22, 4.24) | 0.006 |  | 1.42 (1.14, 1.78) | 0.002 |
| Acute myocardial infarction | | | | | | | | |
| Ceramide (16:0) |  |  |  |  |  |  |  |  |
| Cases | 14 | 14 | 26 | 20 |  |  |  |  |
| MV1 *3* | Ref. | 1.12 (0.51, 2.47) | 2.17 (1.06, 4.43) | 1.66 (0.80, 3.47) | 0.06 |  | 1.35 (1.02, 1.78) | 0.04 |
| MV2 *4* | Ref. | 1.11 (0.49, 2.54) | 2.26 (1.09, 4.65) | 1.74 (0.83, 3.67) | 0.05 |  | 1.34 (1.03, 1.76) | 0.03 |
| Ceramide (22:0) |  |  |  |  |  |  |  |  |
| Cases | 20 | 12 | 17 | 25 |  |  |  |  |
| MV1 | Ref. | 0.66 (0.30, 1.44) | 0.93 (0.46, 1.86) | 1.76 (0.90, 3.44) | 0.10 |  | 1.33 (0.97, 1.83) | 0.07 |
| MV2 | Ref. | 0.57 (0.26, 1.28) | 0.88 (0.43, 1.79) | 1.83 (0.91, 3.65) | 0.09 |  | 1.35 (0.97, 1.87) | 0.08 |
| Ceramide (24:0) |  |  |  |  |  |  |  |  |
| Cases | 18 | 12 | 19 | 25 |  |  |  |  |
| MV1 | Ref. | 0.74 (0.34, 1.61) | 1.23 (0.61, 2.48) | 1.85 (0.94, 3.64) | 0.05 |  | 1.31 (0.97, 1.77) | 0.08 |
| MV2 | Ref. | 0.69 (0.30, 1.55) | 1.28 (0.61, 2.67) | 2.08 (1.00, 4.33) | 0.03 |  | 1.36 (0.99, 1.88) | 0.06 |
| Ceramide (24:1) |  |  |  |  |  |  |  |  |
| Cases | 15 | 18 | 21 | 20 |  |  |  |  |
| MV1 | Ref. | 1.18 (0.57, 2.48) | 1.48 (0.71, 3.08) | 1.24 (0.60, 2.56) | 0.45 |  | 1.12 (0.87, 1.45) | 0.37 |
| MV2 | Ref. | 1.30 (0.60, 2.80) | 1.65 (0.75, 3.63) | 1.41 (0.68, 2.94) | 0.28 |  | 1.18 (0.92, 1.51) | 0.19 |
| Ceramide score |  |  |  |  |  |  |  |  |
| Cases | 15 | 19 | 17 | 23 |  |  |  |  |
| MV1 | Ref. | 1.30 (0.63, 2.70) | 1.40 (0.66, 2.99) | 1.93 (0.95, 3.93) | 0.07 |  | 1.35 (0.99, 1.83) | 0.06 |
| MV2 | Ref. | 1.34 (0.63, 2.85) | 1.52 (0.70, 3.28) | 2.09 (1.00, 4.36) | 0.05 |  | 1.38 (1.01, 1.88) | 0.04 |

Abbreviations: MV, multivariable model

*1* Quartiles were calculated based on the distribution of the ceramide concentrations in the sub-cohort.

*2* A logarithmic transformation was applied to the raw value.

*3* Model 1 stratified on intervention group and simultaneously adjusted for age (continuous) and sex (male, female).

*4* Model 2 additionally adjusted for body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes (all yes, no).

Supplemental table 5. Hazard ratio of cardiovascular disease associated with 1-standard deviation increment in ceramide score by risk strata subgroups.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Hazard ratio (95% CI) | *P*-value | *P* interaction |
| Sex |  |  |  |
| Male | 1.32 (1.01, 1.72) | 0.04 | 0.33 |
| Female | 1.58 (1.22, 2.04) | <0.001 |  |
| Age |  |  |  |
| ≤65 years | 1.72 (1.18, 2.53) | 0.005 | 0.29 |
| >65 years | 1.38 (1.12, 1.69) | 0.002 |  |
| Body mass index |  |  |  |
| ≤30 kg/m2 | 1.47 (1.16, 1.87) | 0.002 | 0.96 |
| >30 kg/m2 | 1.54 (1.13, 2.09) | 0.006 |  |
| Smoking status |  |  |  |
| Current/ever | 1.44 (1.14, 1.83) | 0.002 | 0.99 |
| Never | 1.46 (1.10, 1.93) | 0.010 |  |
| Family history of CHD |  |  |  |
| Yes | 1.47 (0.86, 2.53) | 0.16 | 0.70 |
| No | 1.44 (1.19, 1.74) | <0.001 |  |
| Diabetes |  |  |  |
| Yes | 1.48 (1.18, 1.85) | <0.001 | 0.97 |
| No | 1.38 (0.99, 1.91) | 0.06 |  |
| Hypertension |  |  |  |
| Yes | 1.32 (1.07, 1.61) | 0.009 | 0.08 |
| No | 1.88 (1.22, 2.92) | 0.005 |  |
| Dyslipidemia |  |  |  |
| Yes | 1.33 (1.08, 1.63) | 0.008 | 0.21 |
| No | 1.77 (1.19, 2.61) | 0.005 |  |

Hazard ratios were calculated from Cox models stratified on intervention group and simultaneously adjusted for age (continuous) and sex (male, female), body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes (all yes, no) except the stratification variable. P for interaction were derived from Cox models adjusted as above, including an interaction term between the stratification variable and the ceramide score.

Supplemental table 6. Associations of baseline ceramide ratios and the ceramide score with cardiovascular disease.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Quartiles of ceramide ratio \* | | | | *P* trend |  | HR per 1 | *P* value |
|  | Q1 | Q2 | Q3 | Q4 |  | SD increment † |
| Ceramide (16:0)/Ceramide (24:0) |  |  |  |  |  |  |  |  |
| Cases | 44 | 46 | 63 | 77 |  |  |  |  |
| MV1 ‡ | Ref. | 1.02 (0.64, 1.64) | 1.35 (0.86, 2.12) | 1.56 (1.01, 2.41) | 0.02 |  | 1.23 (1.06, 1.43) | 0.008 |
| MV2 § | Ref. | 1.13 (0.69, 1.87) | 1.48 (0.92, 2.39) | 1.61 (1.01, 2.56) | 0.03 |  | 1.24 (1.05, 1.46) | 0.01 |
| Ceramide (22:0)/Ceramide (24:0) |  |  |  |  |  |  |  |  |
| Cases | 50 | 54 | 56 | 70 |  |  |  |  |
| MV1 | Ref. | 1.18 (0.76, 1.83) | 1.24 (0.80, 1.92) | 1.60 (1.04, 2.46) | 0.03 |  | 1.21 (1.03, 1.42) | 0.02 |
| MV2 | Ref. | 1.26 (0.79, 2.00) | 1.14 (0.72, 1.80) | 1.49 (0.95, 2.33) | 0.12 |  | 1.16 (0.99, 1.37) | 0.07 |
| Ceramide (24:1)/Ceramide (24:0) |  |  |  |  |  |  |  |  |
| Cases | 48 | 55 | 66 | 61 |  |  |  |  |
| MV1 | Ref. | 1.06 (0.67, 1.67) | 1.13 (0.72, 1.76) | 0.85 (0.54, 1.34) | 0.51 |  | 0.98 (0.85, 1.13) | 0.82 |
| MV2 | Ref. | 1.13 (0.70, 1.82) | 1.20 (0.76, 1.91) | 0.95 (0.59, 1.54) | 0.89 |  | 1.02 (0.88, 1.19) | 0.76 |

Abbreviations: MV, multivariable model

\* Quartiles were calculated based on the distribution of the ceramide ratios in the sub-cohort.

† A logarithmic transformation was applied to the raw value.

‡ Model 1 stratified on intervention group and simultaneously adjusted for age (continuous) and sex (male, female).

§ Model 2 additionally adjusted for body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes (all yes, no).

Supplemental table 7. Joint associations of plasma ceramide level and intervention group assignment with risk of cardiovascular disease.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Intervention | Control | *P* for interaction |
| Two Mediterranean diet groups combined | | | |
| Ceramide score |  |  |  |
| <median | Ref. | 1.07 (0.64, 1.78) | 0.01 |
| ≥median | 1.26 (0.84, 1.87) | 2.76 (1.72, 4.44) |  |
| Ceramide (C16:0) |  |  |  |
| <median | Ref. | 1.23 (0.77, 1.98) | 0.08 |
| ≥median | 1.35 (0.92, 1.99) | 2.71 (1.67, 4.41) |  |
| Ceramide (C22:0) |  |  |  |
| <median | Ref. | 1.12 (0.69, 1.82) | 0.02 |
| ≥median | 1.27 (0.86, 1.88) | 2.74 (1.72, 4.38) |  |
| Ceramide (C24:0) |  |  |  |
| <median | Ref. | 1.29 (0.80, 2.10) | 0.17 |
| ≥median | 1.33 (0.90, 1.97) | 2.67 (1.66, 4.31) |  |
| Ceramide (C24:1) |  |  |  |
| <median | Ref. | 1.38 (0.84, 2.26) | 0.34 |
| ≥median | 1.39 (0.95, 2.03) | 2.42 (1.52, 3.85) |  |
| Mediterranean diet + Extra-virgin olive oil | | | |
| Ceramide score |  |  |  |
| <median | Ref. | 0.95 (0.53, 1.72) | 0.009 |
| ≥median | 1.16 (0.68, 1.98) | 2.57 (1.49, 4.45) |  |
| Ceramide (C16:0) |  |  |  |
| <median | Ref. | 1.11 (0.63, 1.97) | 0.37 |
| ≥median | 1.08 (0.62, 1.90) | 2.37 (1.34, 4.19) |  |
| Ceramide (C22:0) |  |  |  |
| <median | Ref. | 1.16 (0.65, 2.08) | 0.02 |
| ≥median | 1.36 (0.75, 2.44) | 2.83 (1.61, 4.98) |  |
| Ceramide (C24:0) |  |  |  |
| <median | Ref. | 1.53 (0.86, 2.72) | 0.06 |
| ≥median | 1.71 (0.96, 3.06) | 3.01 (1.67, 5.41) |  |
| Ceramide (C24:1) |  |  |  |
| <median | Ref. | 1.48 (0.82, 2.67) | 0.24 |
| ≥median | 1.44 (0.82, 2.54) | 2.41 (1.39, 4.20) |  |
| Mediterranean diet + Nuts | | | |
| Ceramide score |  |  |  |
| <median | Ref. | 1.09 (0.60, 1.98) | 0.05 |
| ≥median | 1.32 (0.73, 2.39) | 2.79 (1.57, 4.94) |  |
| Ceramide (C16:0) |  |  |  |
| <median | Ref. | 1.11 (0.63, 1.97) | 0.04 |
| ≥median | 1.08 (0.62, 1.90) | 2.37 (1.34, 4.19) |  |
| Ceramide (C22:0) |  |  |  |
| <median | Ref. | 1.16 (0.65, 2.08) | 0.09 |
| ≥median | 1.36 (0.75, 2.44) | 2.83 (1.61, 4.98) |  |
| Ceramide (C24:0) |  |  |  |
| <median | Ref. | 1.53 (0.86, 2.72) | 0.70 |
| ≥median | 1.71 (0.96, 3.06) | 3.01 (1.67, 5.41) |  |
| Ceramide (C24:1) |  |  |  |
| <median | Ref. | 1.48 (0.82, 2.67) | 0.76 |
| ≥median | 1.44 (0.82, 2.54) | 2.41 (1.39, 4.20) |  |

Hazard ratios were calculated from Cox models simultaneously adjusted for age (continuous) and sex (male, female), body mass index (kg/m2, continuous), family history of premature coronary heart disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes (all yes, no) except the variable of stratification. *P* for interaction were derived from Cox models stratified on intervention assignment and adjusted as above, including an interaction term between the intervention assignment and the ceramide score.

Supplemental table 8. Adjusted mean concentrations of ceramide (m/z) in the control and Mediterranean diet + extra-virgin olive oil groups at baseline and year 1 in the sub-cohort.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Baseline | Year 1 | *P* interaction |
| Ceramide (16:0) |  |  |  |
| Control | 15398.1 (14768.2-16054.9) | 14870.3 (14257.7-15509.2) | 0.40 |
| Two MedDiets combined | 16202.1 (15767.5-16648.7) | 15954.3 (15525.6-16394.9) |  |
| Control | 15398.3 (14760.8-16063.4) | 14870.1 (14250.1-15517.1) | 0.47 |
| MedDiet + EVOO | 16326.3 (15718.4-16957.7) | 16078.7 (15480.0-16700.5) |  |
| Control | 15398.0 (14763.0-16060.2) | 14870.4 (14253.0-15514.6) | 0.47 |
| MedDiet + Nuts | 16065.3 (15438.1-16718.1) | 15816.8 (15196.9-16461.9) |  |
| Ceramide (22:0) |  |  |  |
| Control | 89756.8 (86121.4-93545.8) | 88416.3 (84811.2-92174.7) | 0.90 |
| Two MedDiets combined | 95004.7 (92481.6-97596.7) | 93310.0 (90827.7-95860.2) |  |
| Control | 89758.1 (86088.3-93584.4) | 88419.3 (84779.0-92216.0) | 0.92 |
| MedDiet + EVOO | 96016.0 (92485.6-99681.2) | 94346.2 (90877.2-97947.6) |  |
| Control | 89756.5 (86079.6-93590.5) | 88415.5 (84769.6-92218.3) | 0.89 |
| MedDiet + Nuts | 93893.9 (90251.8-97683.0) | 92168.0 (88580.3-95901.1) |  |
| Ceramide (24:0) |  |  |  |
| Control | 330485.5 (318694.0-342713.3) | 325684.2 (313987.8-337816.2) | 0.80 |
| Two MedDiets combined | 346430.9 (338333.0-354722.7) | 343079.5 (335046.6-351304.9) |  |
| Control | 330491.2 (318628.5-342795.5) | 325702.5 (313932.5-337913.8) | 0.99 |
| MedDiet + EVOO | 349566.1 (338284.7-361223.7) | 344403.3 (333288.5-355888.7) |  |
| Control | 330482.1 (318566.9-342842.9) | 325673.1 (313856.0-337935.1) | 0.63 |
| MedDiet + Nuts | 342981.7 (331273.7-355103.4) | 341627.0 (329925.6-353743.5) |  |
| Ceramide (24:1) |  |  |  |
| Control | 121845.5 (117293.7-126574.0) | 119324.8 (114840.6-123984.2) | 0.16 |
| Two MedDiets combined | 126439.0 (123343.2-129612.6) | 127189.2 (124070.3-130386.5) |  |
| Control | 121847.4 (117279.2-126593.5) | 119320.1 (114819.2-123997.4) | 0.06 |
| MedDiet + EVOO | 128359.7 (124032.0-132838.3) | 131019.6 (126602.2-135591.1) |  |
| Control | 121843.2 (117200.1-126670.3) | 119330.5 (114757.0-124086.3) | 0.64 |
| MedDiet + Nuts | 124339.5 (119852.5-128994.5) | 123038.8 (118585.2-127659.7) |  |

Abbreviations: MedDiet, Mediterranean diet; EVOO, extra-virgin olive oil

Adjusted mean values were estimated from linear mixed model with compound symmetry variance structure specified. *P* for interaction indicates statistical significance of the interaction between times of blood collection (baseline and year 1) and intervention group assignment.