PLASMA LIPIDOMIC PROFILES AND CARDIOVASCULAR EVENTS IN A RANDOMISED INTERVENTION TRIAL WITH MEDITERRANEAN DIET

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Short running head

Metabolomics and cardiovascular disease (PREDIMED)

Abbreviations

CVD- Cardiovascular disease

EVOO- extra-virgin olive oil

MedDiet- Mediterranean diet

PREDIMED- PREvención con DIeta MEDiterránea

Clinical trial registry number and website

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ABSTRACT

Background: Lipid metabolites may partially explain the inverse association between Mediterranean diet (MedDiet) and cardiovascular disease (CVD).

Objective: We evaluated the associations between 1) 202 lipid species and the risk of CVD (myocardial infarction, stroke or cardiovascular death); 2) a MedDiet intervention [supplemented with extra-virgin olive oil (EVOO) or nuts] and 1-year changes in these molecules; 3) 1-year changes in lipid species and subsequent CVD.

Design: Using a case-cohort design, we profiled 202 lipid species at baseline and after 1-year of intervention in the PREDIMED trial in 983 participants [230 cases and a random subcohort of 790 participants (37 overlapping cases)].

Results: Baseline concentrations of cholesterol esters were inversely associated with CVD. Shorter chain length and higher saturation of some lipids were directly associated with CVD. After adjustment for multiple testing, direct associations remained significant for 20 lipids and inverse associations remained significant for 6 lipids. When lipid families were weighted by the number of carbon atoms and double bonds, the strongest inverse association was found for cholesterol esters [adjusted HR=0.39 (95% CI, 0.22-0.68) between extreme quintiles, p(trend)=0.002]. Participants in the MedDiet group+EVOO experienced significant ($p < 0.05$) 1-year changes in 20 lipids and participants in the MedDiet group+nuts in 17 lipids, compared to the control group. Out of these, only changes in cholesterol ester CE(20:3) in the MedDiet+nuts group remained significant after correcting for multiple testing. None of the 1-year changes was significantly associated with CVD risk after correction for multiple comparisons.

Conclusions: Although the MedDiet interventions induced some significant 1-year changes in the lipidome, 1-year changes were not significantly associated with subsequent CVD risk.

Lipid metabolites with a longer acyl chain and a higher number of double bonds at baseline were significantly and inversely associated with the risk of CVD.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death worldwide, and the number of deaths worldwide attributable to CVD is expected to rise from 16.7 million in 2002 to 23.3 million in 2030 (1). Thus, preventive strategies to tackle this epidemic are of paramount importance.

For decades (2), the Mediterranean diet (MedDiet) has been linked to a reduced risk of CVD. The PREDIMED trial, a randomized trial designed to assess the effect of a MedDiet supplemented either with extra-virgin olive oil (EVOO) or mixed nuts compared to a control group (advised to follow a low-fat diet) found a 30% relative reduction versus control in the risk of CVD for both intervention groups (3).

Several mechanisms have been suggested to underlie the observed benefits of the MedDiet on CVD. MedDiets decrease inflammatory markers beyond other control diets according to a meta-analysis of randomized trials (4) and increase nitric oxide production (5). In addition, the MedDiet has been suggested to reduce LDL oxidation, decrease LDL atherogenicity and improve several characteristics of HDL particles (6,7,8). In addition, it exerts beneficial transcriptomic effects on inflammation and foam cell formation-related genes (4)(9).

However, the biological mechanisms underpinning the beneficial effects of the MedDiet for the primary prevention of CVD are not yet fully understood. Recent advances in metabolomics techniques allow for a high-throughput assessment of a large number of small-molecule metabolites involved in different biological pathways, offering a metabolic profile related to biological status (10,11).

The PREDIMED trial –a large randomized field trial (www.predimed.es)- showed a beneficial effect of a holistic dietary pattern on the primary prevention of CVD. This dietary pattern is characterized by a high consumption of healthy fats, which stem from EVOO and tree nuts to

a large degree. This high fat content may influence plasma lipid profiles. Therefore, the PREDIMED trial offers a unique opportunity to assess the association between the plasma lipidomic profile (or its changes during the nutritional intervention) and subsequent CVD. The aims of this study were to 1) describe the association of the baseline lipid metabolic profile of the PREDIMED participants with the risk of CVD, 2) assess the effect of the dietary intervention of the PREDIMED trial on lipid metabolite changes, and 3) depict the association between 1-year changes in the lipidomic profile and subsequent risk of CVD.

METHODS

The present case-cohort study was conducted within the PREDIMED (PREvención con DIeta MEDiterránea) trial (3, 12). Briefly, the PREDIMED trial was a multicenter randomized controlled field trial designed to assess the effect of the MedDiet on the primary prevention of CVD. Participants were recruited during 2003-2009. Participants were 7447 men (aged 55 to 80 years) and women (aged 60 to 80 years) with no prior CVD but at high cardiovascular risk due to the presence of either type 2 diabetes or at least three of the following classical cardiovascular risk factors: current smoking, overweight/obesity, high LDL-cholesterol, low HDL-cholesterol, family history of early coronary heart disease or hypertension. Participants were randomly allocated in a 1:1:1 ratio to a MedDiet supplemented with EVOO (MedDiet+EVOO), to a MedDiet supplemented with nuts (MedDiet+nuts) or to a control group (advised to follow a low-fat diet and to reduce all types of fat). Supplemental Table 1 shows the dietary recommendations to the participants in the different allocation arms. Participants in the two MedDiet groups received individual and group dietary educational sessions by a trained dietitian at the baseline visit and quarterly thereafter. Participants in the control group also received dietary education at the baseline visit. During the first 3

years of the trial, participants in the control group received a leaflet explaining the low-fat diet yearly. The protocol was amended in October 2006. Thereafter, participants in the control group received a dietary intervention promoting a low-fat diet with the same intensity and frequency as the two MedDiet groups.

Compliance was assessed by monitoring yearly the adherence to the MedDiet with a 14-item MedDiet screener (13) and with measurement of biomarkers of key foods in the MedDiet in random samples of participants, concretely extra-virgin olive oil (hydroxytyrosol in urine) and walnuts (alpha-linolenic acid in plasma) (3).

The trial was stopped because of early benefit on the basis of end points documented through December 1, 2010, after a median intervention time and follow-up of 4.8 years.

For the present study, we followed a case-cohort design and included information from 794 randomly selected participants (approximately 10% of the original study sample) at baseline and all 233 incident cases of CVD with available plasma samples (55 of the total 288 incident CVD cases in the PREDIMED trial did not have available plasma samples). We excluded 5 participants because of unavailable lipid information and 2 participants in the initial quality check. Thus, we included 983 participants in our analysis: 230 incident CVD cases and 790 participants in the subcohort (including 37 overlapping cases). Furthermore, 907 participants (777 participants in the subcohort and 160 cases, including 30 overlapping cases) had available plasma samples after 1-year of intervention and were included in the lipidomic change analyses.

At baseline, participants completed an extensive questionnaire including information on sociodemographic characteristics and medical conditions, the Minnesota leisure time physical activity questionnaire (14) and a 14-item screener on their adherence to the traditional MedDiet (13).

The Institutional Review Boards of all the recruitment centers approved the overall PREDIMED trial design and the Institutional Review Boards of the University of Navarra, the Broad Institute of MIT and Harvard, and the Harvard TH Chan School of Public Health, approved the case-cohort subproject. All participants gave written informed consent. Participants underwent a blood withdrawal after an overnight fast by trained and certified nurses at baseline and after one year of intervention. Samples were processed by the study personnel according to the study protocol. EDTA plasma samples were coded and kept refrigerated until they were stored at –80ºC in freezers.

In June 2014, randomly ordered paired samples (baseline and 1-year of follow-up) were selected and shipped on dry ice to the Broad Institute for metabolomics analysis.

Metabolomic analyses of plasma.

Plasma polar and nonpolar lipids were profiled using a Nexera X2 U-HPLC system (Shimadzu Scientific Instruments; Marlborough, MA) coupled to an Exactive Plus orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA). Lipids were extracted from plasma (10 µL) using 190 µL of isopropanol containing 1,2-didodecanoyl-sn-glycero-3-phosphocholine as an internal standard (Avanti Polar Lipids; Alabaster, AL). After centrifugation (10 min, 9,000 x g, ambient temperature), supernatants (10 µL) were injected directly onto a 100 x 2.1 mm ACQUITY BEH C8 column (1.7 µm; Waters; Milford, MA). The column was eluted at a flow rate of 450 µL/min isocratically for 1 minute at 80% mobile phase A (95:5:0.1 vol/vol/vol 10 mM ammonium acetate/methanol/acetic acid), followed by a linear gradient to 80% mobile-phase B (99.9:0.1 vol/vol methanol/acetic acid) over 2 minutes, a linear gradient to 100% mobile phase B over 7 minutes, and then 3 minutes at 100% mobile-phase B. MS analyses were carried out using electrospray ionization in the

positive ion mode using full scan analysis over m/z 200-1100 at 70,000 resolution and 3 Hz data acquisition rate. Additional MS settings were: ion spray voltage, 3.0 kV; capillary temperature, 300°C; probe heater temperature, 300 °C; sheath gas, 50; auxiliary gas, 15; and S-lens RF level 60. Raw data were processed using Progenesis QI software (NonLinear Dynamics) for feature alignment, nontargeted signal detection, and signal integration. Targeted processing of a subset of lipids was conducted using TraceFinder software (version 3.2, Thermo Fisher Scientific; Waltham, MA. Lipids are denoted by headgroup and total acyl carbon content and total acyl double bond content. A full list of measured metabolites, HMDB id, and m/z values are available on Supplemental Table 2.

Endpoint ascertainment

The primary endpoint of the PREDIMED trial was a composite outcome of non-fatal acute myocardial infarction, non-fatal stroke, and cardiovascular death. Medical doctors in each recruitment center blinded with respect to the allocation group reviewed yearly all the participants' medical charts to assess any incident CVD outcome. Other sources of information such as consultation of the National Death Index were used to ascertain incident cases (3). Then, anonymized information was sent from the recruitment center to a blinded central Event Ascertainment Committee which adjudicated the events.

Statistical analyses

Baseline characteristics of the participants in the subcohort and of the participants who experienced a primary outcome (cases) were described as means and standard deviations (SD) for quantitative traits and as percentages for qualitative traits.

Baseline individual lipid values were normalized and scaled in multiples of 1 SD with Blom's

rank-based inverse normal transformation (15). Changes in the lipid values were calculated and the resulting difference was normalized and scaled in multiples of 1 SD with Blom's inverse normal transformation.

First, we calculated the correlation coefficients between all the individual lipid species. Second, in the subcohort, we estimated the effect of the intervention on the 1-year changes in the lipid values of the two intervention groups (MedDiet+EVOO and MedDiet+nuts) compared with the control group with multivariable linear regression models adjusted for the baseline lipid value. In order to correct for multiple testing, we used the procedure described by Benjamini and Yekutieli (16).

Third, we assessed the effect of the intervention (3 categories) on the changes after 1-year in lipid values with linear regression models using the change in the lipid values as the dependent variable and with no constant, in order to assess the lipid changes within each allocation arm among participants in the subcohort. These models were adjusted for the baseline lipid levels.

Fourth, we assessed if the two Mediterranean groups –separately for MedDiet+EVOO and MedDiet+nuts–had an effect on the lipidomic profile of the participants after 1 year of intervention by assessing whether changes in the average number of carbon atoms per acyl chain or the average number of double bonds per acyl chain in each lipid species were associated with each of the two Mediterranean diet intervention in comparison to the control group. As a preliminary step, we ran linear regression models with 1-year changes in each lipid signals as dependent variables and the allocation group as independent term (3 categories, control group as reference), and adjusted the model for baseline lipid levels. We obtained the beta coefficients for both intervention groups (vs. control) and their corresponding standard errors for each of the 202 assessed lipids. For lipids with more than

one acyl chain, we averaged the number of carbon atoms and double bonds per acyl chain. We then ran weighted linear regression models, in which we assessed if the average number of carbon atoms or the average number of double bonds of the acyl chain were independent predictors for the beta coefficients of the aforementioned regression models. As weights in this regression, we used the inverse of the standard error of the beta coefficient obtained in the aforementioned regression model. We presented the results graphically and separately for both intervention groups. Then, we ran the weighted linear regression models, in which we assessed if the average number of carbon atoms or the average number of double bonds of the acyl chain were independent predictors for the beta coefficients of the aforementioned regression models separately for those lipid families with information on at least 5 lipids. The obtained p values were penalized for multiple comparisons with the procedure described by Benjamini and Yekutieli (16) since we assessed 10 lipid families. Fifth, we calculated HRs and 95% CI for incident CVD per 1-SD in baseline individual lipid levels with weighted Cox regression models taking into account the case-cohort design with Barlow weights (17). We adjusted for age, sex, smoking (never, former, current), body mass index, family history of early coronary heart disease, leisure-time physical activity and educational level and stratified by allocation arm in all models. The HRs for individual lipids and their p values were grouped according to lipid species (family) and plotted in a two-dimensional graph defined by the number of carbon atoms (x axis) and the number of double bonds (y axis) in the acyl chain. Lipids with the same number of carbon atoms and double bonds were slightly pulled apart horizontally to visualize both results. Sixth, because the number of carbon atoms and double bonds appeared to be relevant to CVD, for each lipid species, we additionally calculated a weighted score for the baseline lipid value, weighted by its number of carbon atoms and by the number of double bonds in the

acyl chain. Since some lipids had no double bonds, we added a constant (constant=1) to the number of double bonds:

score= lipid level * # carbon atoms * (# double bonds + 1)

We calculated this score for lipid species (families) for which at least 5 molecules were measured, and assessed the association of the score for each lipid family with the risk of cardiovascular disease using weighted Cox models, as stated above and adjusted for age, sex, smoking (never, former, current), body mass index, family history of early coronary heart disease, leisure-time physical activity and educational level (17). As sensitivity analyses, we changed some assumptions: a) without adding 1 to the number of double bonds; b) using multiple imputation for missing values instead of the lowest lipid-specific detected value; c) adding the lipid metabolites without weighting.

Finally, we modeled the risk of CVD per 1-SD in the 1-year change in individual lipid levels using weighted Cox regression models with the same adjustment as the previously described Cox regression models plus an additional adjustment for baseline lipid concentration (17). Again, HRs and their p values were grouped according to lipid species and plotted in a two-dimensional graph defined by the number of carbon atoms (x axis) and the number of double bonds (y axis) in the acyl chain. Lipids with the same number of carbon atoms and double bonds were slightly pulled apart horizontally to visualize both results. Then, in an additional analysis, we corrected the associations between the individual baseline lipids (or their 1-year changes) and CVD for multiple testing using the procedure described by Benjamini and Yekutieli (16).

All analyses were performed with STATA/SE 13.1 (College Station, TX: StataCorp LP).

RESULTS

For the present analysis, we included 983 participants (230 cases and 790 participants in the subcohort, of whom 37 overlapped) (Supplemental Figure 1). Their baseline characteristics are displayed in Table 1. As expected, the participants included in the subcohort showed similar baseline characteristics and a similar risk than the entire cohort of participants included in the PREDIMED trial. In addition, we have included the distribution of the subcohort participants' characteristics according to their allocation arm in Supplemental table 3.

Correlations between the 202 individual lipid metabolites were strongest within each lipid family but moderate to strong correlations (data not shown) were also observed for triacylglycerides with diacylglycerides, phosphatidylcholines, phosphatidylethanolamines, or phosphatidylcholine plasmalogens; phosphatidylcholines with phosphatidylserines or cholesterol esters; phosphatidylcholine plasmalogens with phosphatidylthanolamine plasmalogens and diacylglycerides; and lysophosphatylcholines with lysophosphatidylethanolamines or phosphatidylethanolamines.

1-Year Changes in Lipid Metabolites in the Intervention Groups

As shown in Table 2, participants in the MedDiet+EVOO group or in the MedDiet+nuts group showed statistically significant reductions versus the control group at 1-year in some lipids. Only changes in cholesterol ester CE(20:3) in the MedDiet+nuts group versus the control group, but no other association, remained statistically significant after correcting for multiple comparisons. The full list of 1-year changes in the lipid metabolites levels in both intervention groups compared to the control group is available on Supplemental Table 4. The full list of 1-year changes in the lipid concentrations in each of the three arms of the trial is available in Supplemental Table 5.

When we considered the lipidome as a whole, there was not a clear pattern in changes in lipid signals according to acyl chain length and number of double bonds. The change in the lipid signal was significantly higher with a higher average acyl chain carbon number in the MedDiet+EVOO group compared to the control group (Figure 1). This means that lipids with a longer mean acyl chain carbon number showed greater increases than lipids with shorter mean acyl chain carbon number or a higher number of double bonds in the MedDiet+EVOO than in the control group. In contrast, no significant changes in the lipid signal were observed in the MedDiet+nuts group compared to the control group (Figure 2) neither for the mean acyl chain length nor for the mean acyl chain saturation. When we repeated the analyses separately for 10 lipid families and after accounting for multiple comparisons, the only associations that remained significant were a greater increase in triacylglycerols with longer acyl-chains in the MedDiet+EVOO group vs. control and a greater increase in triacylglycerols with more double-bonds in the MedDiet+nuts group vs. the control group (Supplemental figures 2-21).

Risk of CVD by Baseline Lipid Metabolite by Acyl Chain Carbon Number and Double Bond Number

The associations between individual baseline lipid concentrations and the risk of CVD are shown graphically in Figure 3 according to the number of carbon atoms in the acyl chain and to the number of double bonds. All specific comparisons can be found in Supplemental table 6. A higher risk of CVD for those lipids with a lower number of carbon atoms in the acyl chain or with fewer double bonds was observed for phosphatidylcholines, phosphatidylethanolamine plasmalogens, phosphatidylinositols, diacylglycerols and triacylglycerols. On the other hand, a lower risk of CVD was found for those lipids with

increasing number of carbon atoms and more double bonds including phosphatidylcholines, phosphatidylcholine plasmalogens, cholesterol esters and triacylglycerols. Baseline levels of cholesterol esters with 20 or 22 carbon atoms in their acyl chain and 4, 5 or 6 double bonds showed inverse associations with CVD. On the contrary, higher baseline concentrations of hydroxy-phosphatidylcholines, lysophophatidylethanolamines and ceramides, and, to a lower extent, of phophatidylethanolamines with a medium-long acyl chain and phoshpatidylserine plasmalogens, were associated with a higher risk of CVD.

After adjustment for multiple testing, the associations remained statistically significant for the phosphatidylethanolamines PE(34:2), PE(36:2) and PE(36:1), the lysophophatidylethanolamines LPE(16:0), LPE(18:2), and LPE(18:0), the phosphatidylethanolamine plasmalogen PEP(36:3), the phosphatidylserine PS(38:4), the ceramides C(16:0) and C(22:0), the hydroxy-phosphatidylcholines [M+Na]+ OHPCMA(34:2), OHPCMA(36:4) and OHPC(36:4), and the diacylglycerols DAG(34:2), DAG(34:1), DAG(36:1), and DAG(36:0), and the triacylglycerols TAG(50:3), TAG(50:2) and TAG(52:3). All of them were directly associated with the risk of CVD; still after adjustment for multiple testing, an inverse significant association was observed for the phosphatidylcholine PC(40:10), the phosphatidylcholine plasmalogen PCP(36:5)a, the cholesterol esters CE(20:5), CE(20:4) and CE(22:5), and the triacylglycerol TAG(58:8).

Risk of CVD by Baseline Lipid Species Family, Weighted by Acyl Chain Carbon Number and Double Bond Number

To test our hypotheses that the length of the acyl chain and the number of double bonds were key elements to account for the association between lipid species and CVD, we weighted the metabolites by these two parameters. Results of the associations between the

baseline levels of the different lipid species (families of lipids)—with individual lipids weighted according to their number of double bonds and to the number of carbon atoms in each lipid species—and the future risk of CVD are displayed in Supplemental table 7. Higher (weighted) concentrations of lysophosphatidylethanolamines [HR (95% CI) for the 5th vs. the 1st quintile 2.43 (1.41,4.19), p for trend 0.003] and diacylglycerols [HR (95% CI) for the 5th v. the 1st quintile 1.71 (1.02,2.87), p for trend 0.016] were significantly associated with a higher risk of CVD. Contrarily, higher concentrations of cholesterol esters [HR (95% CI) for the 5th v. the 1st quintile 0.39 (0.22,0.68), p for trend 0.002] weighted by their number of double bonds and carbon atoms were significantly associated with a lower risk of CVD. When we changed the definition of the score using different assumptions, the only noticeable changes were that the linear trend for the association between baseline phosphatidylethanolamines and CVD became statistically significant [HR (95% CI) for the 5th v. the 1st quintile 1.62 (0.98,2.67), p for trend 0.030] and that the association of the cholesterol esters score was attenuated [HR (95% CI) for the 5th v. the 1st quintile 0.65 (0.37,1.13), p for trend 0.041], both with the unweighted approach.

Risk of CVD by Changes in Lipid Metabolite by Acyl Chain Carbon Number and Double Bond Number

In Figure 4 we show the associations between 1-year changes (per 1 SD) in individual lipid concentrations and the risk of CVD according to the number of carbon atoms in the acyl chain and to the number of double bonds. The specific estimates of the HRs for all comparisons can be found in Supplemental table 8. A higher risk of CVD for those lipids with fewer carbon atoms in the acyl chain and fewer double bonds were observed for phosphatydylcholines, phosphatidylcholine plasmalogens and cholesterol esters. But these

associations were of smaller magnitude and mainly absent for changes in these metabolite values as opposed to the associations observed with their baseline levels. In addition, after adjustment for multiple testing, none of these associations remained statistically significant. Therefore, we did not further assess associations of 1-y changes in lipids with CVD according to lipid family, nor did we build weighted scores for these changes.

DISCUSSION

To our knowledge, this is the first study assessing the association between lipid metabolomics data and subsequent risk of CVD in the context of a dietary intervention trial. These data suggest that: 1) the baseline lipid metabolic profile was associated with the risk of CVD in the PREDIMED trial; 2) the intervention, which assessed the effect of the MedDiet supplemented either with EVOO or nuts, induced some modest changes in the lipid profile of the participants, especially for MedDiet+nuts; however, 3) 1-year changes in the metabolic profile were not statistically associated with the subsequent risk of CVD.

We observed that baseline triacylglycerols, phosphatidylcholines, and lysophosphatidylethanolamines were differentially associated with CVD depending on the structure of the acyl chains such that the shorter the chain and the higher the saturation in the chain, the higher the risk of CVD. This pattern is consistent with other studies. Stegemann et al observed that triacylglycerols with a low carbon number and a lower number of double bonds were predictive of CVD (18). Analogously, this triacylglycerol pattern was previously associated with a larger BMI, waist circumference, and HOMA-IR in a cross-sectional study in the Framingham Offspring cohort (19).This same pattern was observed for triacylglycerol and phosphatidylcholine associations with the risk of diabetes in the Framingham Heart Study (20). The detrimental effect of saturation in triacylglycerols

may be attributable to the higher atherogenic potential of saturated fats.

For cholesterol esters, we observed that the longer and the more unsaturated the acyl chain, the lower the risk of CVD. This finding is consistent with a reported lower diabetes risk, although this latter association disappeared after multivariable adjustment (20).

The observation that the longer and the more unsaturated the acyl chain in phosphatidylcholines, the lower the associated CVD risk, is also consistent with the inverse association of carbon number and double bond content and risk of diabetes observed by Rhee et al (20). On the other hand, in the Bruneck Study, no clear association was observed for phosphatidylcholines and CVD (18). Phosphatidylcholines are the most abundant membrane lipids in mammals (21). If phosphatidylcholines with shorter chains and more highly saturated acyl chains are available, this could confer less fluidity to the cell membranes. Saturated fatty acid intake is known to be associated with adverse blood lipid profiles (raised LDL) and with the induction of insulin resistance (22). Therefore, it is likely that a lower number of double bonds in plasma lipids may be associated with a higher risk of CVD events. On the other hand, long-chain fatty acids with many double bonds (i.e. long-chain PUFA), mainly omega-3 PUFAs, are associated with reduced triglyceride levels, reduced myocardial oxygen demands and beneficial changes in endothelial function (23-25). These physiologic benefits, together with reduced heart rate (and lower heart rate variability) and decreased blood pressure observed in association with long-chain omega-3 fatty acids support the biologic plausibility for a protective effect of long-chain PUFA on cardiovascular clinical events. In addition, the intake of saturated fatty acids with a lower number of carbon atoms (16:0) are known to exert more detrimental effects on lipids and cardiovascular disease than those with a higher number of carbon atoms (18.0) (26). Long-chain PUFA are also precursors to bioactive lipid metabolites, including specialized pro-

resolving mediators and cytochrome P450–generated monoepoxides (27,28). Long-chain PUFAs are also assumed to have membrane stabilizing actions in the context of ischemia-induced ventricular fibrillation (29).

We also observed that higher baseline concentrations of diacylglycerols, ceramides, hydroxy-phosphatidylcholines, and medium-chained phosphatidylethanolamines were associated with an increased risk of CVD. A specific analysis on the association between ceramides and CVD has been previously reported (30). Diacylglycerols that showed stronger associations with the risk of CVD were consistent with the increasing saturation as those triacylglycerols that also showed a direct association with the risk of CVD. On the other hand, sphingolipids, such as ceramides, have been associated with insulin resistance and vascular dysfunction suggesting thus a potential mechanism to explain their association with CVD (31). Also, hydroxy-phosphatidylcholines can be formed as adducts under conditions of oxidative stress and/or inflammation and may be components of oxidized LDL, thus increasing the risk of CVD. Finally, Stegemann et al observed a higher risk of CVD for the ratio of phosphatidylethanolamines to phosphatidylcholines, which is consistent with our finding of higher risk with higher levels of phosphatidylethanolamines (18).

It could be thought that our results mainly support an inverse association of long-chain PUFA, primarily of the omega-3 type, with CVD, and this is already well known. However, the omic approach in conjunction with a randomized intervention trial does add novel findings because a) we were able to assess the role of lipids jointly classified according to these two features, namely, the length of the acyl chain and number of double bonds; b) we assessed these associations not only for baseline lipids, but also for their changes after one year of dietary intervention, using repeated measurements of the lipidome; c) we provide a systematic analysis of the effect of the dietary intervention on the whole lipidome. As far as

we know, these three approaches to assess the role of the lipidome on the risk of CVD have not been reported before.

In the present study, we have not found an association between induced changes in the lipidome by the intervention conducted in the PREDIMED trial and subsequent CVD. These results suggest that the effect of the intervention may be due to other factors, e. g. bioactive polyphenols with anti-inflammatory properties (32).

Our study has several strengths. First, this study was built on a successful dietary intervention trial that demonstrated beneficial effects of MedDiet interventions for the primary prevention of CVD. Second, we conducted repeated measurements of lipid metabolites at baseline and 1 year of intervention, enabling us to examine the effects of the interventions on changes in lipids.

We also acknowledge some limitations of this study. First, although we observed changes in the lipidome between intervention arms, we did not see appreciable changes within each intervention arm after one year of intervention, even though the lipid composition of the intervention diets differed. Several studies have observed short-term changes in the metabolome induced by a dietary intervention but these changes were nevertheless toned down after 6 months of follow-up (33,34). Second, the PREDIMED participants were mostly European whites and roughly half of them had diabetes, which may limit the generalizability of our results. Third, we observed mainly non-significant changes in metabolite levels induced by the intervention and no associations between changes in the metabolite levels and future risk of CVD. It is possible that 1-year changes in metabolites were too small to detect subtle mediational effects. It is also possible that mechanisms other than lipidomic changes may account for the observed benefit on clinical hard events. Fourth, some of our analyses are based on observational data. In spite of having adjusted for the main potential

confounders, residual confounding may still have played a role in the observed associations. Fifth, the specific benefit of the MedDiet might have been confounded because during the first period of the trial the comparator group received less attention than during the second period. However, the diet for the control group was precisely defined from the very beginning of the trial and we did not change the definition or the targets of the control group, we only modified the intensity and frequency of contacts with the dietitians.

In conclusion, the MedDiet interventions induced some significant 1-year changes in the lipidome, but one-year changes were not significantly associated with subsequent CVD risk. Our study suggests that some lipid classes may be related to the risk of CVD. The effect may be different in the different lipid species although for several lipid species we have observed that those lipids with a shorter and more saturated acyl chain were more strongly associated with the risk of CVD. On the contrary, lipid metabolites with a longer acyl chain and a higher number of double bonds were inversely associated with the risk of CVD.

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Table 1. Baseline participants' characteristics in the random subcohort and cases

Characteristic	Entire PREDIMED cohort	Subcohort[1]	Cases
n	7447	790	230
Sex, % women	57.5	57.1	39.6
Age, years	67 (6)	67 (6)	70 (6)
Allocation arm, %			
MedDiet+EVOO	34.2	37.1	35.7
MedDiet+nuts	33.0	33.2	28.3
Family history of early CHD, %	22.4	24.9	19.1
Smoking status, %			
Former smoker	24.7	25.4	35.1
Current smoker	14.1	12.3	20.0
Dyslipidemia, %	72.3	73.5	58.3
Type 2 diabetes, %	48.5	47.1	64.8
Hypertension, %	82.8	83.7	82.6
Waist circumference, cm	100 (11)	100 (10)	102 (11)
Body mass index, kg/m^2	30.0 (3.8)	29.8 (3.6)	29.6 (3.7)
Leisure time physical activity, METs-min/d	230 (239)	258 (258)	237 (238)
Education, %			
Secondary school or higher	22.2	23.5	19.6
Total energy intake, kcal/d	2275 (606)	2334 (614)	2366 (686)
Baseline adherence to the MedDiet[2]	9 (2)	9 (2)	8 (2)

Data are mean (SD) unless otherwise stated

[1]: The randomly selected subcohort included 37 cases.

[2]: Based on the 14-item screener

Abbreviations: MedDiet: Mediterranean diet; CHD: coronary heart disease

Table 2. Significant lipid changes after 1 year of intervention in the two intervention groups of the PREDIMED trial compared to the control group among participants in the subcohort

	MedDiet+EVOO (n=293) vs. control (n=235)		MedDiet+nuts (n=262) vs. control (n=235)	
	Change (SD)		Change (SD)	
Lipid	Mean (95%CI)	p value[1]	Mean (95%CI)	p value[1]
Lysophosphatidyletha-nolamines				
LPE (22:6)	0.15 (0.00,0.31)	0.048	-	-
Phosphatidylcholines				
PC (36:4)b	-0.21 (-0.37,-0.04)	0.014	-0.19 (-0.36,-0.02)	0.027
PC (38:4)	-0.18 (-0.35,-0.02)	0.027	-0.18 (-0.35,-0.01)	0.037
PC (38:3)	-	-	-0.23 (-0.39,-0.07)	0.004
PC (38:2)	-	-	-0.17 (-0.33,-0.02)	0.031
PC (40:6)	-	-	-0.28 (-0.44,-0.12)	0.001
Phosphatidylcholine plasmalogens				
PCP (34:3)	-	-	0.18 (0.02,0.34)	0.032
PCP (34:2)	0.20 (0.03,0.36)	0.021	-	-
Phosphatidylethano-lamines				
PE (34:0)	-	-	-0.17 (-0.33,-0.01)	0.033
PE (38:5)	-0.18 (-0.34,-0.02)	0.027	-	-
PE (38:4)	-0.18 (-0.34,-0.03)	0.022	-	-
Phosphatidyletha-nolamine plasmalogens				
PEP (36:5)	-0.23 (-0.38,-0.08)	0.003	-	-
PEP (36:1)	0.18 (0.02,0.34)	0.031	-	-
PEP (38:5)	-0.18 (-0.34,-0.03)	0.018	-	-
Phosphatidylserines				
PS (40:6)	-	-	-0.23 (-0.39,-0.07)	0.004
Phosphatidylserine plasmalogens				
PSP (36:3)	-	-	-0.18 (-0.35,-0.01)	0.041
Ceramides				
Ceramide (24:1)	0.25 (0.09,0.41)	0.002	-	-
Sphingomyelines				
SM (18:1)	0.18 (0.01,0.34)	0.038	-	-
SM (18:0)	0.18 (0.02,0.34)	0.032	-	-
SM (24:1)	0.24 (0.08,0.40)	0.003	-	-
Cholesterol esters				
CE (14:0)	-	-	-0.19 (-0.35,-0.03)	0.023
CE (16:1)	-0.22 (-0.38,-0.05)	0.010	-0.27 (-0.44,-0.10)	0.002
CE (20:3)	-	-	-0.37 (-0.53,-0.21)	<0.001[2]
Diacylglycerols				

DAG (32:0)	-0.18 (-0.34,-0.02)	0.028		
DAG (36:4)			0.23 (0.07,0.39)	0.006
Triacylglycerols				
TAG (42:0)	-0.16 (-0.31,-0.01)	0.040	-	-
TAG (44:0)	-0.17 (-0.33,-0.01)	0.033	-	-
TAG (46:0)	-0.18 (-0.34,-0.03)	0.024	-	-
TAG (48:0)	-0.18 (-0.34,-0.02)	0.025	-	-
TAG (50:0)	-0.16 (-0.32,-0.01)	0.043	-	-
TAG (52:6)	-	-	0.16 (0.01,0.32)	0.042
TAG (52:5)	-	-	0.20 (0.05,0.36)	0.011
TAG (52:4)	-	-	0.19 (0.03,0.35)	0.020
TAG (54:5)	-	-	0.19 (0.03,0.35)	0.019

[1]: p values are unadjusted for multiple comparisons

[2] : this difference remained statistically significant after adjustment for multiple comparisons

Abbreviations: LPC: lysophosphatydil choline; PC: phosphatidylcholine; PCP: phosphatidylcholine plasmalogen; LPE: lysophosphophatidyl ethanolamine; PE: phosphatidylethanolamines; PEP: phosphatidylethanolamine plasmalogen; PS: phosphatidylserine; PSP: phosphatidylserine plasmalogen; SM: sphingomyelin; CE: cholesterol ester; DAG: diacylglycerol; TAG: triacylglycerol

Results from multivariable linear regression models with 1-year changes in the lipid values as dependent variable and the allocation groups as independent terms (3 categories, control group as reference), and adjusted for baseline lipid value.

The data are LC-MS peak areas that have been transformed by Blom's inverse normal transformation.

Figure 1. One-year changes in each lipid signal according to lipid carbon number and double bond content for participants in the Mediterranean diet group supplemented with extra-virgin olive oil (n=293) versus the control group (n=235).

Results from weighted linear regression models weighted by the inverse of the variance.

Figure 2. One-year changes in each lipid signal according to lipid carbon number and double bond content for participants in the Mediterranean diet group supplemented with nuts (n=262) versus the control group (n=235).

Results from weighted linear regression models weighted by the inverse of the variance.

Figure 3. Hazard ratios for one standard deviation higher baseline lipid concentration and the composite CVD endpoint.

Lipid species were inverse normally transformed and HRs were calculated from weighted Cox models adjusted for age, sex, smoking habit, BMI, family history of early CHD, leisure time physical activity and education, and stratified by allocation arm in the trial (17).

Figure 4. Hazard ratios for one standard deviation of 1-year change in lipid concentration and the composite CVD endpoint.

Lipid species were inverse normally transformed and HRs were calculated from weighted Cox models adjusted for age, sex, smoking habit, BMI, family history of early CHD, leisure time physical activity, education and baseline lipid concentration, and stratified by allocation arm in the trial (17).