Effects of Supplemented Mediterranean Diets on Plasma-Phospholipid Fatty Acid Profiles and Risk of Cardiovascular Disease after 1 Year of Intervention in the PREDIMED Trial

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BACKGROUND: Plasma fatty acids (FAs) have been associated with cardiovascular disease (CVD) risk. Diet and endogenous metabolism influence the FA profile of the plasma phospholipid (PL) fraction. In the PREDIMED trial, we examined 1-year changes in the FA profile of plasma PL according to a nutritional intervention with Mediterranean diets, either supplemented with extra-virgin olive oil (MedDiet + EVOO) or mixed nuts (MedDiet + nuts), in a high cardiovascular risk population. We also analyzed if 1-year changes in PL FAs were associated with subsequent cardiovascular risk.

METHODS: We included 779 participants in our case-cohort study: 185 incident cases and 594 participants in the subcohort (including 31 overlapping cases). The end point was the incidence of CVD. We measured the FAs of plasma PL at baseline and after 1 year of intervention.

RESULTS: MedDiet + EVOO increased C17:0 and C20:3n9 in linear regression models [β coefficient$_{perSD}$: 0.215 (95% CI, 0.032–0.399) and 0.271 (0.107–0.434), respectively] and decreased 16:1n7 and C22:4n6 [β$_{perSD}$: −0.239 (95% CI, −0.416 to −0.061) and −0.287 (95% CI, −0.460 to −0.113), respectively] vs the control group. MedDiet + nuts increased C18:3n3 [β$_{perSD}$: 0.382 (95% CI, 0.225 – 0.539)], C18:2n6 [β$_{per\ SD}$: 0.250 (95% CI, 0.073 — 0.428)], C18:0 [β$_{perSD}$: 0.268 (95% CI, 0.085—0.452)], and C22:0 [β$_{per\ SD}$: 0.216 (95% CI, 0.031—0.402)]; and decreased the sum of six n6 FAs [β$_{per\ SD}$: −0.147 (95% CI, −0.268 to −0.027)] vs the control group. The 1-year increase in C18:2n6 was inversely associated with the subsequent CVD risk (HR$_{perSD}$: 0.64 (95% CI, 0.44–0.92)).

CONCLUSIONS: MedDiet interventions changed n6 FAs and C16:1n7c; other changes were specific for each group: MedDiet + EVOO increased C17:0 and C20:3n9, and MedDiet + Nuts C18:3n3, C18:2n6, C18:0, and C22:0 FAs.

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Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide (1) and effective prevention strategies are essential to control and reduce its burden. The PREDIMED study, a randomized trial of primary cardiovascular prevention using a nutritional intervention, revealed that the Mediterranean diet (MedDiet) supplemented with either extra-virgin olive oil (MedDiet + EVOO) or mixed nuts (MedDiet + nuts) reduced the risk of cardiovascular events by 30% after a median follow-up of 4.8 years in comparison to a low-fat diet (2). However, the mechanisms by which the MedDiet was able to reduce CVD risk still remain unclear.

Plasma phospholipids (PLs) were associated, depending on their content of double bonds, with CVD in the PREDIMED trial (3). These results suggested a potential role for fatty acids (FAs) esterified to plasma PLs in mediating CVD risk (3). The FA profile of circulating PLs reflects dietary FA intake over the previous few weeks as well as endogenous FA metabolism (4, 5). High concentrations of long-chain n3 FAs (6) and some n6 FAs, such as linoleic acid, have been associated with a lower risk of CVD and type 2 diabetes (7, 8). In this context, the plasma FA profi in PREDIMED participants may refl the effect of the dietary intervention, particularly the supplemental foods EVOO and nuts, and these FA profi changes may also be linked to the reduction in CVD risk conferred by the nutritional intervention through changes in the composition of PLs.

In this case-cohort study, nested within the PREDIMED trial, we aimed to (*a*) analyze 1-year changes in the plasma FA profi of the PL fraction according to the nutritional intervention in each arm of the trial, and (*b*) assess if 1-year FA changes in the PL fraction were associated with subsequent risk of CVD.

Materials and Methods

STUDY POPULATION AND DESIGN

The PREDIMED study http://www.predimed.es/ is a primary cardiovascular prevention randomized trial conducted in Spain with 7447 participants at high vascular risk. The methods and design have been reported elsewhere (2). Briefly, participants were randomly assigned to 1 of 3 nutritional interventions: (*a*) MedDiet + EVOO, (*b*) MedDiet + nuts, and (*c*) a control diet consisting of advice to reduce intake of all types of fat.

We designed an unstratified case-cohort (9) study nested within the PREDIMED trial aimed at assessing the metabolomic profiling of participants in the trial. We included 233 incident cases of CVD with a mean follow-up of 4.8 years (55 of the 288 incident cases of the PREDIMED trial had no available plasma samples) and a random sample of approximately 10% of PREDIMED participants at baseline (subcohort, which included 37 overlapping cases). Cases corresponded to the primary CVD end point of the trial that was a composite of myocardial infarction, stroke, or cardiovascular death (2). In a first stage, plasma lipidomics profiling was performed (3, 10). In a second stage, to complete the lipidomics data, FA profiling of the PL fraction was performed.

We excluded 18 participants in the initial quality check of the samples. For the FA profiling, there were 241 samples with insufficient volume for the analytic method—consequently 779 participants were included: 185 incident cases and 594 participants in the subcohort (including 31 overlapping cases). In addition, for 1-year analyses, cases that occurred before completing the first year of intervention were excluded (27 cases). In addition, for the 1-year measurements there were no samples for 79 subjects, and for 4 subjects there were no data on dietary covariates; consequently, for the present analyses, a total of 664 participants were included: 126 incident cases and 538 participants in the subcohort (including 25 overlapping cases). A flowchart with this information is shown in the online Data Supplement (Fig. S1).

The Research Ethics Committees of all recruitment centers approved the overall PREDIMED trial design according to the ethical guidelines of the Declaration of Helsinki. The Research Ethics Committee of the University of Navarra approved the case-cohort subproject. All participants provided their written informed consent (Clinical trial registry number: Controlled-Trials.com number, ISRCTN35739639).

Laboratory measurements. Analyses of FA spectra of plasma PL were performed at the Department of Molecular Epidemiology, German Institute of Human Nutrition Potsdam-Rehbruecke, with a modified method using extraction with tert-butyl methyl ether/methanol, solid-phase separation, hydrolysis and methylation with trimethyl sulfonium hydroxide (TMSH), and subsequent analysis by gas chromatography (11, 12). A detailed description of the method can be found in the Supplemental Material.

The FA composition of plasma PL was expressed as the percentage of each FA area relative to total area of all detected FA. The inter-assay coefficients of variation (CV%, $n = 10$) for each plasma FA using flame ionization detection were smaller than 6.4% for all FA. The information about the CV% for specific FAs can be found in Table S1 (Supplementary Material).

The omega system was used for the FA nomenclature.

OUTCOME ASCERTAINMENT

In each recruitment center physicians blinded to the intervention reviewed all the participants' medical charts each year to investigate any incident cardiovascular outcomes. Four sources of information to ascertain incident cases (also blinded with respect to the intervention) were used: repeated contacts with participants, contacts with family physicians, a yearly review of medical records, and consultation of the National Death Index. Then, anonymized information was sent to a blinded central Event Ascertainment Committee who finally adjudicated the events. A detailed description of the adjudication of the composite event is included in the online Supplementary Methods.

COVARIATE ASSESSMENT

At baseline and at yearly follow-up visits, a questionnaire was administered about lifestyle variables, educational achievement, history of illnesses, medication use, and family history of disease. Physical activity was assessed with the validated Spanish version of the Minnesota Leisure-Time Physical Activity questionnaire (13). A 137-item validated semiquantitative food-frequency questionnaire was used by trained dietitians to ascertain dietary habits (14). We used Spanish food composition tables to estimate energy and nutrient intakes (15). Anthropometric and blood pressure measurements were directly measured and registered by trained nurses.

Dyslipidemia was defined as: LDL-cholesterol ≥160 mg/dL (4.1 mmol/L) or HDL-cholesterol ≤40 mg/dL (1.03 mmol/L) for men and ≤50 mg/dL (1.29 mmol/L) in women, including for participants receiving lipid-lowering therapy.

STATISTICAL ANALYSIS

Baseline characteristics of the participants according to their primary outcome status were described as means and standard deviations (SD) for quantitative traits and as percentages for qualitative traits.

Two multivariable linear regression models were designed to analyze the effect of the intervention on each individual 1-year FA change: a first model to compare the MedDiet + EVOO vs control groups and a second model to compare the MedDiet + nuts vs control groups. Linear regression models were adjusted for age, sex, body mass index (BMI), smoking, family history of early coronary heart disease, leisure-time physical activity, center of recruitment, years of education, and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups (2). Additionally, each model was adjusted for each corresponding baseline FA. We also repeated these analyses without adjusting for the baseline FAs, which is included in the online Tables S2 and S3. *P*-values were adjusted for multiple comparisons using the false discovery rate described by Simes (16).

Both FA changes and baseline FA were introduced in the models as continuous variables (per 1 SD): baseline individual FA values (%) were normalized and scaled in multiples of 1 SD with the Blom inverse normal transformation (17); changes in FA (1-year value minus the baseline value) were calculated and the resulting difference was also normalized and scaled with the same method.

We analyzed the FA changes (Blom inverse normally transformed values) within each MedDiet group with an analysis of covariance (ANCOVA) adjusted for the same potential confounders (age, sex, BMI, smoking, family history of early coronary heart disease, leisure-time physical activity, center of recruitment, years of education, and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups).

In those FA that signifi increased or decreased in the MedDiet groups as compared to the control group after 1 year of intervention, the 1-year FA change was introduced in Cox regression models to analyze its association with the risk of CVD. Robust standard errors accounting for intracluster correlations were used. To analyze the combined effect of n6 FA on CVD risk, a new variable, n6 score, was calculated as the sum of the following fatty acids: C18:3n6, C20:2n6, C20:3n6, C22:4n6, and C22:5n6 (excluding linoleic and arachidonic acids, which did not consistently decrease in both MedDiet groups), and was assessed using the same regression models. Cox regression models with Barlow weights (9) were used to calculate the hazard ratios (HR) and their 95% CI for the risk of CVD for each 1 SD of FA change. These models were also adjusted for age, sex, BMI, smoking, family history of early coronary heart disease, leisure-time physical activity, and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups (2) and they were stratifi by center, sex, and educational level using the Stata command *strata*. Again, each model was additionally adjusted for the respective baseline FA (as a continuous variable, for 1 SD).

Statistical significance was set a priori at <0.05 after false discovery rate (FDR) and STATA 16.0 was used for the analyses.

Results

The baseline characteristics of participants and the incidence of CVD according to the intervention group are shown in Table 1.

Table 1. Baseline characteristics of the study participants by intervention group.[a]			
	Control diet (n = 240)	MedDiet + EVOO (n = 285)	MedDiet + nuts (n = 250)
Age*	68.3 (6.6)	67.7 (6)	67.4 (6)
Sex, % women	51.2	56.5	47.6
BMI, kg/m^{2}*	30.2 (3.9)	29.9 (3.6)	29.1 (3.5)
LTPA, METS-min/day*	237 (244)	250 (241)	260 (264)
Type 2 diabetes, %	58.7	56.1	59.6
Dyslipidemia, %	65.4	64.9	71.2
Family history of CHD, %	22.9	18.9	22.4
Hypertension, %	85.8	84.2	77.2
Smoking			
Non smokers, %	63.3	57.5	54.8
Current smokers, %	9.6	13.7	15.6
Former smokers, %	27.1	28.8	29.6
Education, %			
Primary or less	78.8	77.6	76.2
Secondary	19.4	18.0	19.3
University graduate	1.8	4.3	4.5
PREDIMED center[b], %			
North Spain centers (2)	18.5	16.6	17.2
East Spain centers (4)	43.2	48.4	48.8
South Spain centers (2)	23.8	18.8	19.3
Islands (2)	14.5	16.2	14.7
Energy intake, kcal*	2318 (677)	2308 (582)	2358 (643)
Adherence to MedDiet[c]*	8.53 (1.82)	8.78 (1.98)	8.87 (1.84)
CVD incident cases, %	24.2	18.9	16.4

*Standard deviation in parentheses.
[a]Abbreviations: LTPA, leisure-time physical activity; METS, metabolic equivalent of tasks; CHD, coronary heart disease.
[b]Parentheses indicate number of centers in each geographical region.
[c]14-item questionnaire.

FA PROFILE CHANGES ACCORDING TO THE INTERVENTION GROUP

Table 2 shows FA changes in the MedDiet + EVOO group as compared to the control group and adjusted mean changes within each group. Multivariable models showed a statistically signifi reduction in the following fatty acids for the MedDiet supplemented with EVOO compared to the control group (Table 2): C16:1n7c (palmitoleic acid), C20:3n6 (dihomo-γ-linolenic acid (DGLA)), C20:4n6 (arachidonic acid), and C22:4n6 (adrenic acid). On the other hand, MedDiet + EVOO was associated with signifi increases in C17:0 (margaric acid) and C20:3n9 (eicosatrienoic acid) compared to the control group. After FDR correction, only the increase in eicosatrienoic acid (P = 0.014) and the reduction in adrenic acid (P = 0.014) remained statistically signifi

We observed that 1-year intervention with MedDiet supplemented with nuts signifi reduced C16:1n7c (palmitoleic acid), C18:1n9c (oleic acid), C20:3n6 (DGLA), C22:4n6 (adrenic acid), and C22:5n6 (docosapentanoic acid) compared to the control group (Table 3). In contrast, the intervention with MedDiet + nuts signifi increased C18:0 (stearic acid), C22:0 (behenic acid), C18:3n3 (α-linolenic acid (ALA)), and C18:2n6c (linoleic acid (LA)). After FDR correction, the increase in C18:0 (P = 0.022, stearic acid), C18:3n3 ($P <$ 0.001, ALA), and C18:2n6c (P = 0.027, LA), and the decrease in C20:3n6 (P = 0.007, DGLA), C22:4n6 (P = 0.007, adrenic acid), and C22:5n6 (P = 0.007, docosapentanoic acid) remained statistically signifi

Table 2. One-year changes[a] of FAs in PLs in the MedDiet + EVOO vs control diet groups.

	One-year adjusted mean changes (95%CI)				
	Control	MedDiet + EVOO	β (95% CI)[b]	*P*-value	FDR-corrected *P*-values[c]
C14:0	0.29 (−0.10 to 0.154)	−0.02 (−0.14 to 0.08)	−0.068 (−0.238 to 0.102)	0.433	
C16:0	0.08 (−0.05 to 0.21)	0.01 (−0.11 to 0.12)	−0.080 (−0.252 to 0.091)	0.360	
C18:0	−0.14 (−0.27 to −0.01)	−0.01 (−0.13 to 0.10)	0.121 (−0.056 to 0.298)	0.179	
C20:0	−0.12 (−0.26 to −0.01)	0.04 (−0.08 to 0.16)	0.165 (−0.016 to 0.346)	0.074	
C22:0	−0.13 (−0.26 to −0.01)	0.01 (−0.11 to 0.12)	0.122 (−0.057 to 0.300)	0.183	
C15:0	−0.01 (−0.15 to 0.12)	0.06 (−0.05 to 0.18)	0.072 (−0.104 to 0.248)	0.420	
C17:0	−0.13 (−0.26 to −0.01)	0.09 (−0.02 to 0.21)	0.215 (0.032 to 0.399)	0.022	
C16:1n7c	0.17 (0.03 to 0.30)	−0.06 (−0.18 to 0.05)	−0.239 (−0.416 to −0.061)	0.009	0.081
C18:1n7c	0.05 (−0.08 to 0.18)	−0.03 (−0.14 to 0.09)	−0.063 (−0.241 to 0.115)	0.489	
C18:1n9c	0.06 (−0.07 to 0.18)	0.13 (0.02 to 0.24)	0.080 (−0.087 to 0.247)	0.350	
C20:1n9	−0.05 (−0.19 to 0.08)	0.08 (−0.03 to 0.20)	0.134 (−0.043 to 0.311)	0.139	
C16:1n7t	0.06 (−0.07 to 0.18)	−0.11 (−0.22 to −0.01)	−0.167 (−0.340 to 0.006)	0.059	
C18:1n7t	−0.01 (−0.14 to 0.11)	−0.02 (−0.13 to 0.08)	−0.008 (−0.171 to 0.155)	0.925	
C18:1n9t	0.03 (−0.09 to 0.15)	0.02 (−0.08 to 0.13)	−0.003 (−0.167 to 0.161)	0.972	
C18:3n3	−0.17 (−0.29 to −0.06)	−0.03 (−0.13 to 0.07)	0.152 (−0.000 to 0.304)	0.051	
C20:5n3	−0.06 (−0.18 to 0.07)	0.03 (−0.08 to 0.14)	0.081 (−0.085 to 0.247)	0.340	
C22:5n3	−0.01 (−0.13 to 0.08)	−0.05 (−0.17 to 0.06)	−0.051 (−0.225 to 0.122)	0.561	
C22:6n3	0.01 (−0.12 to 0.13)	0.03 (−0.08 to 0.14)	0.029 (−0.140 to 0.198)	0.735	
C18:2n6c	−0.13 (−0.26 to −0.01)	−0.01 (−0.12 to 0.10)	0.121 (−0.050 to 0.292)	0.166	
C18:2n6t	−0.14 (−0.27 to 0.01)	0.01 (−0.10 to 0.13)	0.143 (−0.035 to 0.322)	0.116	
C18:3n6	0.03 (−0.09 to 0.16)	−0.04 (−0.15 to 0.07)	−0.073 (−0.238 to 0.093)	0.390	
C20:2n6	0.08 (−0.04 to 0.21)	−0.03 (−0.14 to 0.09)	−0.101 (−0.274 to 0.072)	0.253	
C20:3n6	0.17 (0.05 to 0.30)	−0.03 (−0.14 to 0.08)	−0.193 (−0.364 to −0.022)	0.027	
C20:4n6	0.09 (−0.04 to 0.23)	−0.12 (−0.24 to −0.01)	−0.211 (−0.395 to −0.027)	0.025	
C22:4n6	0.21 (0.08 to 0.34)	−0.08 (−0.20 to 0.03)	−0.287 (−0.460 to −0.113)	0.001	0.014
C22:5n6	0.15 (0.02 to 0.28)	0.01 (−0.11 to 0.12)	−0.156 (−0.332 to 0.020)	0.082	
C20:3n9	−0.12 (−0.24 to 0.01)	0.18 (0.07 to 0.29)	0.271 (0.107 to 0.434)	0.001	0.014

[a]Changes in FA (1-year value minus the baseline value) were calculated and the resulting difference was normalized and scaled in multiples of 1 SD with the Blom inverse normal transformation. FA original values were expressed as the percentage of each FA area relative to total area of all detected FA.
[b] β regression coefficients were obtained from linear regression models adjusted for age, sex, BMI, smoking, family history of early coronary heart disease, leisure-time physical activity, center of recruitment, years of education, and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups. Additionally adjusted for the respective baseline FA.
[c]Corrected *P*-values ≤0.100 are shown.

The results presented in Tables 2 and 3 were repeated without adjusting for the respective baseline FA and there were only negligible changes (Tables S2 and S3).

Figures 1 and 2 present 1-year changes by intervention group. Figure 1 shows the FA that significantly increased in one or both MedDiet groups compared to the control diet group. The FAs C17:0 (margaric acid), C18:0 (stearic acid), C22:0 (behenic acid), C18:3n3 (ALA), C18:2n6 (LA), and C20:3n9 (eicosatrienoic acid) were significantly reduced in plasma PL after 1 year of follow-up only among participants allocated to the control group. In contrast, significant increases were observed in the MedDiet + EVOO group for C17:0 (Fig. 1A, margaric acid) and C20:3n9 (Fig. 1F, eicosatrienoic acid), and in the MedDiet + nuts group for C18:0 (Fig. 1B, stearic acid), C18:3n3 (Fig. 1D, ALA), and C18:2n6 (Fig. 1E, LA).

Table 3. One-year changes[a] in plasma phospholipid fatty acids (FA) in the MedDiet + nuts vs control diet groups.

	One-year adjusted mean changes (95%CI)				
	Control	MedDiet + nuts	β (95%CI)[b]	*P* value	FDR-corrected *P* values[c]
C14:0	0.29 (−0.10 to 0.154)	−0.01(−0.12 to 0.11)	−0.052 (−0.226 to 0.123)	0.562	
C16:0	0.08 (−0.05 to 0.21)	−0.05 (−0.17 to 0.07)	−0.133 (−0.311 to 0.046)	0.145	
C18:0	−0.14 (−0.27 to −0.01)	0.12 (−0.01 to 0.24)	0.268 (0.085 to 0.452)	0.004	0.022
C20:0	−0.12 (−0.26 to −0.01)	0.03 (−0.10 to 0.16)	0.155 (−0.033 to 0.343)	0.107	
C22:0	−0.13 (−0.26 to −0.01)	0.08 (−0.05 to 0.21)	0.216 (0.031 to 0.402)	0.022	0.066
C15:0	−0.01 (−0.15 to 0.12)	−0.06 (−0.19 to 0.06)	−0.046 (−0.227 to 0.136)	0.624	
C17:0	−0.13 (−0.26 to −0.01)	−0.01(−0.12 to 0.11)	0.116 (−0.074 to 0.306)	0.23	
C16:1n7c	0.17 (0.03 to 0.30)	−0.06 (−0.18 to 0.07)	−0.229 (−0.413 to −0.044)	0.015	0.057
C18:1n7c	0.05 (−0.08 to 0.18)	0.01 (−0.12 to 0.13)	−0.042 (−0.227 to 0.142)	0.654	
C18:1n9c	0.06 (−0.07 to 0.18)	−0.15 (−0.27 to −0.04)	−0.212 (−0.384 to −0.039)	0.017	0.057
C20:1n9	−0.05 (−0.19 to 0.08)	−0.05 (−0.17 to 0.08)	0.002 (−0.182 to 0.186)	0.98	
C16:1n7t	0.06 (−0.07 to 0.18)	0.09 (−0.03 to 0.21)	0.034 (−0.145 to 0.214)	0.71	
C18:1n7t	−0.01 (−0.14 to 0.11)	0.05 (−0.07 to 0.16)	0.067 (−0.102 to 0.236)	0.438	
C18:1n9t	0.03 (−0.09 to 0.15)	−0.04 (−0.16 to 0.07)	−0.077 (−0.247 to 0.093)	0.376	
C18:3n3	−0.14 (−0.29 to −0.06)	0.20 (0.09 to 0.31)	0.382 (0.225 to 0.539)	<0.001	<0.001
C20:5n3	−0.06 (−0.18 to 0.07)	0.03 (−0.10 to 0.15)	0.062 (−0.110 to 0.234)	0.479	
C22:5n3	−0.01 (−0.13 to 0.08)	0.04 (−0.08 to 0.17)	0.050 (−0.129 to 0.230)	0.581	
C22:6n3	0.01 (−0.12 to 0.13)	−0.06 (−0.18 to 0.05)	−0.072 (−0.247 to 0.104)	0.424	
C18:2n6c	−0.13 (−0.26 to −0.01)	0.12 (−0.01 to 0.24)	0.250 (0.073 to 0.428)	0.006	0.027
C18:2n6t	−0.14 (−0.27 to 0.01)	0.07 (−0.05 to 0.20)	0.213 (0.028 to 0.398)	0.025	0.068
C18:3n6	0.03 (−0.09 to 0.16)	0.01 (−0.10 to 0.13)	−0.018 (−0.190 to 0.154)	0.839	
C20:2n6	0.08 (−0.04 to 0.21)	−0.09 (−0.18 to 0.05)	−0.163 (−0.343 to 0.016)	0.075	
C20:3n6	0.17 (0.05 to 0.30)	−0.14 (−0.26 to −0.01)	−0.305 (−0.482 to −0.127)	0.001	0.007
C20:4n6	0.09 (−0.04 to 0.23)	0.03 (−0.10 to 0.16)	−0.055 (−0.246 to 0.136)	0.574	
C22:4n6	0.21 (0.08 to 0.34)	−0.10 (−0.22 to 0.03)	−0.310 (−0.490 to −0.130)	0.001	0.007
C22:5n6	0.15 (0.02 to 0.28)	−0.13 (−0.26 to −0.01)	−0.299 (−0.481 to −0.117)	0.001	0.007
C20:3n9	−0.12 (−0.24 to 0.01)	−0.09 (−0.20 to 0.03)	−0.007 (−0.177 to 0.164)	0.939	

[a] Changes in FA (1-year value minus the baseline value) were calculated and the resulting difference was normalized and scaled in multiples of 1 SD with the Blom inverse normal transformation. FA original values were expressed as the percentage of each FA area relative to total area of all detected FA.
[b] β regression coefficients were obtained from linear regression models adjusted for age, sex, BMI, smoking, family history of early coronary heart disease, leisure-time physical activity, center of recruitment, years of education, and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups. Additionally adjusted for the respective baseline FA.
[c] Corrected *P*-values ≤0.100 are shown.

In Fig. 2, we depict the FA that signifi decreased in one or both MedDiet groups as compared to the control diet group. A signifi 1-year reduction was observed in C20:4n6 (arachidonic acid) only for the MedDiet + EVOO group and in n6 FAs only for the MedDiet + nuts group (Fig. 2). Thus, we summed 1-year changes in these FAs and calculated a new variable: n6 change score (excluding linoleic and arachidonic acids, which did not decrease or even increased with the intervention). We observed that the MedDiet + nuts group experienced a very strong reduction in the n6 score (Fig. 2). We also observed that the MedDiet + EVOO group presented a smaller but still signifi reduction compared to the control group ($P = 0.005$).

When we compared 1-year vs baseline measurements of each FA within each MedDiet group and

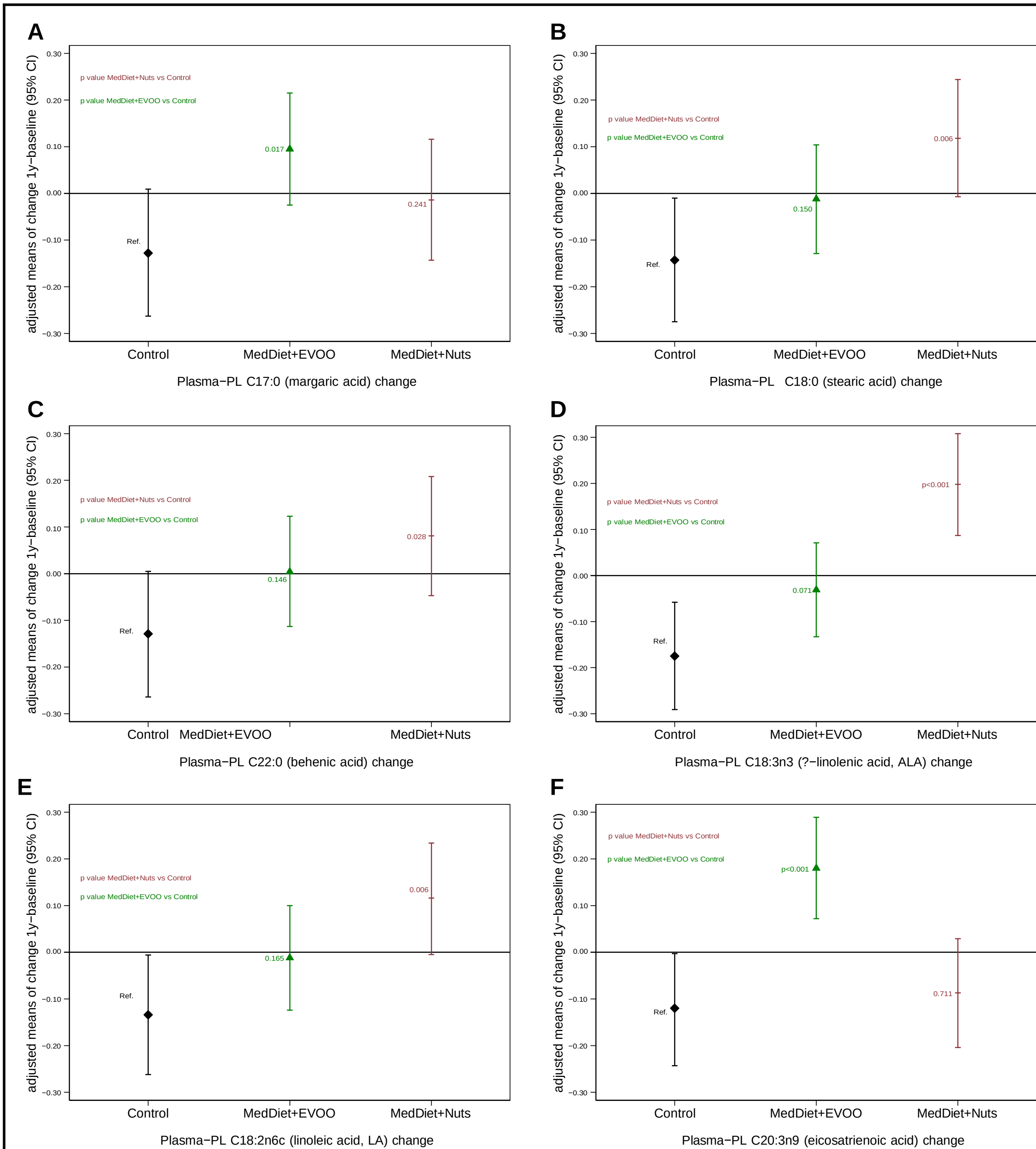

Fig. 1. Adjusted# mean 1-year changes, according to the intervention group, of plasma phospholipids FAs that significantly increased in one of both MedDiet groups. (A), C17:0 (margaric acid); (B), C18:0 (stearic acid); (C), C22:0 (behenic acid); (D), C18:3n3 (α-linolenic acid, ALA); (E), C18:2n6c (linoleic acid, LA); (F), C20:3n9 (eicosatrienoic acid).
#Age, sex, BMI, smoking, family history of early coronary heart disease, leisure-time physical activity, center of recruitment, years of education, and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups.

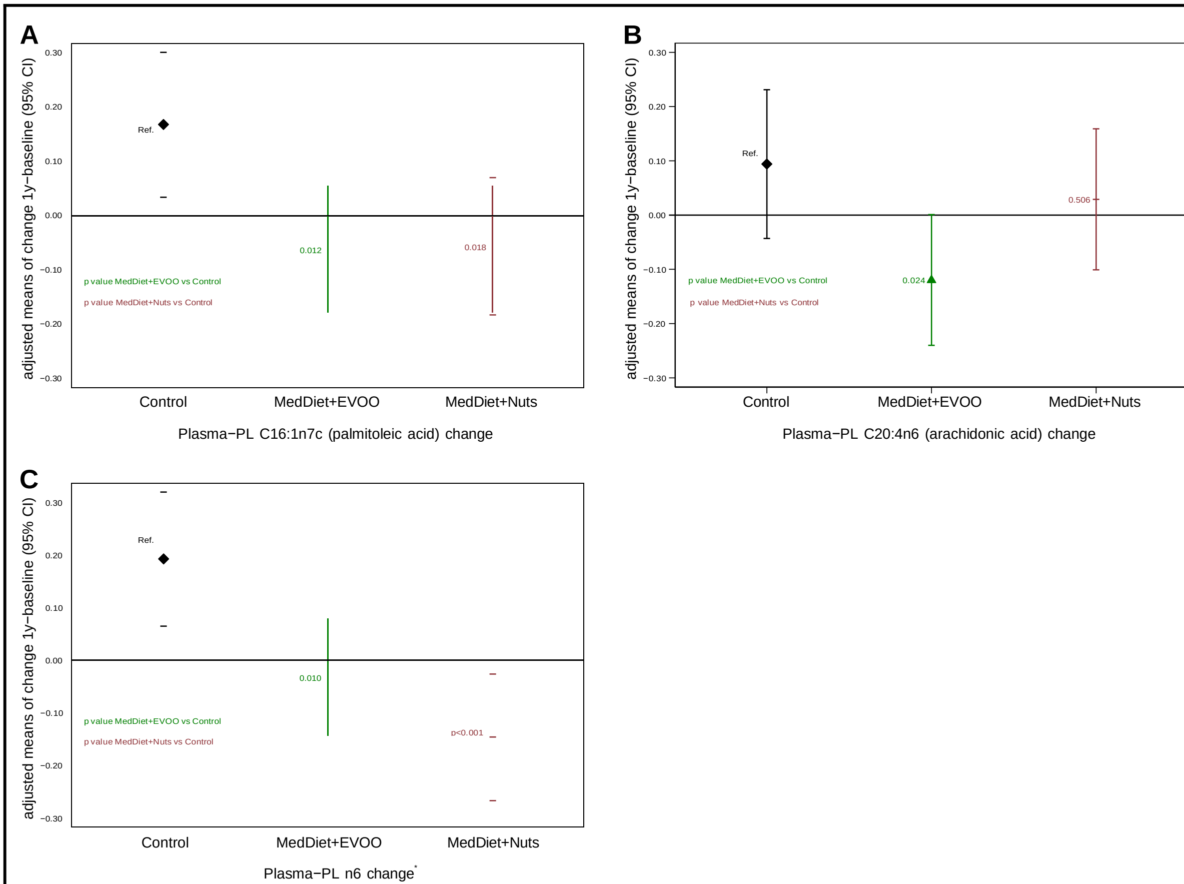

Fig. 2. Adjusted# mean 1-year changes, according to the intervention group, of plasma phospholipids FAs that significantly decreased in one of both MedDiet groups. (A), C16:1n7c (palmitoleic acid); (B), C20:4n6 (arachidonic acid); (C), n6 change score*.
#Age, sex, BMI, smoking, family history of early coronary heart disease, leisure-time physical activity, center of recruitment, years of education, and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups.
***Sum of 1-year changes in C18:3n6 (γ-linolenic acid), C20:2n6 (eicosadienoic acid), C20:3n6 (dihomo-γ-linolenic acid), C22:4n6 (adrenic acid), and C22:5n6 (docosapentanoic acid).**

adjusted for the same confounders, we observed statistically significant increases in C17:0 (margaric acid) and C20:3n9 (eicosatrienoic acid) in the MedDiet + EVOO group ($P = 0.002$ and $P = 0.021$, respectively). Regarding the MedDiet + nuts group, the increase in C18:3n3 (ALA) and the decrease in the n6 score reached statistical significance ($P = 0.011$ and $P = 0.023$, respectively; data not shown).

Finally, we analyzed if the statistically significant 1-year changes in FAs identified by comparing MedDiet groups to the control diet group were associated with subsequent CVD risk. We observed a strong inverse association between 1-year increase in linoleic acid (C18:2n6c, LA) and risk of CVD, $HR_{per\ SD}$: 0.64 (95% CI, 0.44–0.92) as shown in Table 4. Changes in C18:0 (stearic acid) and C18:3n3 (ALA) pointed into the same direction, but they failed to reach statistical significance [$HR_{per\ SD}$: 0.76 (95% CI, 0.55–1.06) and 0.80 (0.54–1.18), respectively].

Discussion

In this case-cohort study nested within the PREDIMED trial, we found that the intervention with MedDiet supplemented either with EVOO or nuts significantly changed the plasma PL FA profile of participants over the first year of intervention. Changes in FA profile were

Table 4. Hazard ratios and 95% CI for the association between 1-year FA changes (per 1 SD) and CVD.

	HR (95% CI)[a]	*P*-value
C17:0 change	1.00 (0.72–1.38)	0.982
C18:0 change	0.76 (0.55–1.06)	0.110
C22:0 change	0.93 (0.68–1.28)	0.667
C16:1n7c change	1.12 (0.87–1.43)	0.375
C18:3n3 change	0.80 (0.54–1.18)	0.259
C18:2n6c change	0.64 (0.44–0.92)	0.015
C20:4n6 change	1.03 (0.76–1.38)	0.867
n6 change score	0.96 (0.68–1.36)	0.835
C20:3n9 change	1.03 (0.76–1.38)	0.861

[a]Cox regression models adjusted for age, sex, BMI, smoking, family history of early coronary heart disease, leisure-time physical activity, center of recruitment, years of education, and propensity scores that used 30 baseline variables to estimate the probability of assignment to each of the intervention groups.

different for each MedDiet group, although we observed as a common feature a significant decrease in the n6 FAs score (based on 1-year change results for n6 FAs; we did not include linoleic and arachidonic acids in the score) compared to the control group.

Plasma PLs FA profiles do not only directly reflect dietary FA intake (18), but they also reflect endogenous metabolism including digestion (interaction with the gut microbiota), enterohepatic circulation, absorption, nutrient interactions, tissue FA use, and other factors (4, 11, 19). Thus, the observed changes are likely to reflect both dietary FA intake itself and the effects of the metabolic MedDiet intervention. Specifically, for the MedDiet + EVOO, an important increase in C17:0 FA was found. This saturated FA has been proposed as a biomarker of dairy intake in combination with the C15:0 FA (20). Since we did not observe a significant increase in C15:0 (pentadecanoic acid), we hypothesize that the increase in C17:0 (margaric acid) may be due to the endogenous metabolism of this FA. In this regard, diets rich in vegetables and fruits, naturally rich in fiber, have been reported to be associated with a higher content in odd-chain FAs in the PL fraction (21, 22), probably as the result of fiber fermentation by gut microbiota (23). In fact, PREDIMED participants allocated to the MedDiet groups were encouraged to increase the consumption of fresh vegetables and fruits, which is consistent with these results.

A sizable increase in C20:3n9 FA (eicosatrienoic acid) was observed in the MedDiet + EVOO group. This FA tends to be higher in subjects with a deficiency of the dietary essential FAs C18:2n6 (LA) and C18:3n3 (ALA) (24). The population of PREDIMED was not characterized by malnutrition and we found that neither LA nor ALA decreased in the EVOO group after 1 year of intervention. This supports the contention that the intake of both LA and ALA was sufficient in our participants. However, we found that the downstream metabolites of LA significantly decreased after 1 year of intervention with MedDiet + EVOO. Consequently, we hypothesize that perhaps the high consumption of oleic acid, due to the supplementation with EVOO, may have activated an alternative pathway to n6 and n3 catabolism. It is possible that the desaturation pathway of oleic acid was activated and enzymes with shared roles in other pathways may not have been available for the n6 and n3 pathways. But, it is also unclear whether the endogenous formation of C20:3n9 (eicosatrienoic acid) may be increased by the high intake of oleic acid in this situation.

For the MedDiet + nuts group, the change in FA profile was characterized by a strong increase in linoleic acid and a reduction in other n6 FAs, while arachidonic acid did not change. Furthermore, not only α-linolenic acid (C18:3n3, ALA), but also the saturated FAs C18:0 (stearic acid) and C22:0 (behenic acid) increased. The increase in C18:2n6 (LA) and C18:3n3 (ALA) of plasma PLs probably reflects the increased intake of nuts, particularly walnuts, which are particularly rich in these two FAs, during the intervention in the MedDiet + nuts group. Thus, C18:2n6 (LA) and C18:3n3 (ALA) of plasma PLs can be considered as biomarkers of adherence to the intervention as it was previously found for plasma FA (25).

Importantly, we found that the increase over 1 year in C18:2n6 (LA) was associated with a lower subsequent risk of CVD. This applied also to the increase in C18:3n3 (ALA), although results were not statistically signifi Therefore, it appears that the benefi effects of the MedDiet supplemented with nuts may be in part exerted through increasing C18:2n6 (LA) and C18:3n3 (ALA) intake, refl by their increased content in PLs. Supporting these results, a benefi effect of consuming C18:2n6 (LA) on cardiovascular risk has been previously reported (26, 27). In this context, old trials assessing the effect of replacing saturated fats with polyunsaturated fatty acids (PUFA) (mainly LA) reported a reduction in the risk of atherosclerotic CVD (28–31). Moreover, several prospective cohort studies have found an association between replacing saturated fat by PUFA (especially LA) and a lower risk of cardiovascular disease (26, 32). High concentrations of C18:2n6 (LA) in plasma PL have been also favorably associated with cardiovascular risk factors (33).

We also observed an increase in C18:0 (stearic acid) and C22:0 (behenic acid) FA in the MedDiet + nuts group. Circulating levels of C18:0 may reflect de novo lipogenesis rather than dietary intake (21, 34), however, we did not observe an increase in C14:0 (myristic acid) or C16:0 (palmitic acid) levels. Thus, the hypothesis of increased de novo lipogenesis seems unlikely. In the case of the C22:0 (behenic acid), it is not so clear if it is more influenced by diet or by endogenous metabolism. In the literature, the association between C22:0 and lower risk of CVD and CVD-related diseases seems to be more consistent than for C18:0, given that associations in both directions have been found for the latter (35–37). In the present study, we observed that a 1-year increase in C18:0 in plasma PLs appeared to be inversely associated with CVD, although it did not reach statistical significance. Recently, both C18:0 and C22:0 FAs were inversely associated with mortality (CVD and non-CVD death) (34) and with atrial fibrillation (38) in a large prospective cohort of the US.

We observed a decrease in palmitoleic acid (C16:1n7c) for both MedDiet groups. This FA is synthesized during de novo lipogenesis in response to high carbohydrate/low fat diets, which lead to higher levels in PLs. Hence, it is consistent to find lower levels of palmitoleic acid after the intervention with MedDiet, which is especially rich in unsaturated fats (39).

Finally, we observed changes in FAs that cannot be directly linked to changes in their dietary intake, such as C20:3n9 (eicosatrienoic acid) or C16:1n7c (palmitoleic acid). This supports the hypothesis that the MedDiet may exert its beneficial effects against CVD by modifying the endogenous metabolism of FAs linked to PL composition.

This study has several strengths. Repeated measures of FAs in the context of a randomized controlled trial gave us the possibility to analyze the effects of a controlled dietary intervention on the FA profi Furthermore, the case-cohort design nested in this large long-term intervention trial allowed us to investigate the long-term effects of FA changes on CVD risk while controlling for potential confounding factors, and to generalize these fi to all PREDIMED participants. One limitation of the present study is that these results may not be generalized to other populations because our study subjects lived in a Mediterranean area and were at high risk of CVD. Another limitation is that although the analyses on CVD risk were adjusted for several potential confounders, the possibility of residual confounding cannot be discarded. In addition, the FA profi of PLs was only determined at 2 time points (baseline and 1 year) and it would be interesting to observe changes for a longer period. Finally, changes due to endogenous metabolism cannot be demonstrated in this study.

In conclusion, an intervention with MedDiet, either supplemented with EVOO or nuts, resulted in relevant changes in the FA profi of PLs in the PREDIMED trial. Both MedDiet interventions signifi changed the content of several n6 FAs and palmitoleic acid, while other changes were specifi to each MedDiet group. MedDiet + EVOO signifi increased C17:0 (margaric acid) and C20:3n9 (eicosatrienoic acid), and MedDiet + nuts signifi increased C18:3n3 (ALA), C18:2n6 (LA), C18:0 (stearic acid), and C22:0 (behenic acid) FAs. Moreover, the 1-year increase in C18:2n6 (LA) was inversely associated with the subsequent risk of CVD.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: FA, fatty acid; CVD, cardiovascular disease; PL, plasma phospholipid; MedDiet, Mediterranean diet; EVOO, extra-virgin olive oil; BMI, body mass index; LA, linoleic acid; ALA, α-linolenic acid.

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