Long-term immunomodulatory effects of a Mediterranean diet in adults at high-risk for cardiovascular disease in the PREDIMED randomized controlled trial.

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i) Supplemental Table 1 and Supplemental Table 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

ii) Abbreviations used: BP: blood pressure; CHD: coronary heart disease (CHD); CRF: cardiovascular risk factors; CRP: C-reactive protein; CVD: cardiovascular disease; EVOO: extra-virgin olive oil; FQQ: food frequency questionnaire; hs-CRP: ultra-sensitive C-reactive protein; ICAM-1: intercellular adhesion molecule-1; IL-6: Interleukin 6; LFD: low-fat diet; MCP-1: monocyte chemoattractant protein-1; MeDiet: Mediterranean diet; MMP-9: matrix metalloproteinase-9; MUFA: monounsaturated fat; LDL: low-density lipoprotein; oxLDL: oxidized low-density lipoprotein; HDL: high-density lipoprotein; PBMCs: peripheral blood mononuclear cells; PUFA: polyunsaturated fatty acid; PREDIMED: Prevention with Mediterranean Diet; ROO: refined olive oil; SFA: saturated fatty acids; TGF-β1: transforming growth factor beta 1; TNF-α: tumor necrosis factor alpha; VCAM-1: vascular cell adhesion molecule-1; VOO: virgin olive oil.

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ABSTRACT

Background: The Mediterranean Diet (MeDiet) has demonstrated short-term anti-inflammatory effects, but little is known about its long-term immunomodulatory properties.

Objective: To assess the long-term effects of the MeDiet on inflammatory parameters related to atherogenesis in adults at high risk of cardiovascular disease (CVD) compared to the effects of a low-fat diet (LFD).

Methods: We randomized 165 high-risk participants (half men, on average 66 year-old) without overt CVD to one of three diets: a MeDiet supplemented with extra-virgin olive oil (EVOO; 50 mL/day), a MeDiet supplemented with nuts (30 g/day), or a LFD. Follow-up data were collected at 3 and 5y. Repeated-measures ANOVA, adjusted for potential confounding variables, was used to evaluate changes in diet adherence, cardiovascular risk factors, and inflammatory parameters.

Results: The two MeDiet groups achieved a high degree of adherence to the intervention and the LFD group had reduced energy intake from fat by 13% by 5 y. Compared to baseline, at ∆3 and ∆5y, both MeDiets had significant reductions of ≥16% in plasma concentrations of high-sensitivity C-reactive, interleukin-6, tumor necrosis factor-α, and monocyte chemoattractant protein-1 ($P \leq 0.04$), whereas there were no significant changes in LFD group. The reductions in CD49d and CD40 expressions in T-lymphocytes and monocytes at Δ3y were ≥16% greater in both MeDiet groups compared with changes in LFD group ($P<0.001$). Compared to baseline, at Δ3y, the MeDiet groups had an increased HDL-cholesterol (≥8%) and decreased blood pressure (>4%), total- cholesterol (≥9%), LDL-cholesterol (≥8%) and triglyceride (≥15%)

concentrations, and total/HDL-cholesterol ratio (≥19%). At Δ5y, concentrations of glucose (13%) and glycated hemoglobin (8%) had increased in the LFD.

Conclusions: MeDiet's participants showed lower cellular and plasma concentrations of inflammatory parameters related to atherosclerosis at 3 and 5 years. This antiinflammatory role of the MeDiet could explain, in part, the long-term cardioprotective effect of the MeDiet against CVD.

Clinical trial registration: The trial is registered in the London-based Current Controlled Trials register with ISRCTN number 35739639.

Keywords: Mediterranean diet, adhesion molecules, cardiovascular disease, peripheral blood mononuclear cells, inflammation, long-term.

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Introduction

The Mediterranean diet (MeDiet) is recognized as one of the healthiest dietary patterns. Several epidemiological studies have shown that high adherence to the MeDiet is associated with a reduced risk of developing metabolic syndrome, hypertension, type 2 diabetes and some neurodegenerative diseases and cancers, as well as, a lower mortality and incidence of cardiovascular disease (CVD) (1,2,3). There is also consistent evidence demonstrating that the MeDiet improves classical cardiovascular risk factors (4,5). Accordingly, intervention studies such as the PREDIMED (PREvención con DIeta MEDiterránea) study (6,7) and the Lyon Diet Heart study (8) have demonstrated the beneficial effect of the MeDiet in the primary and secondary prevention of CVD, respectively.

Atherosclerosis is a complex degenerative process in which monocytes and T-cells play a key role. The cells migrate from the circulation to the subendothelial space where they differentiate into macrophages and later into foam cells after taking up oxidized low-density lipoprotein (oxLDL)(9,10,11). In parallel, the endothelium is activated due to the accumulation of modified LDL and upregulates the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin and other chemotactic agents, such as monocyte chemoattractant protein-1 (MCP-1)(12,13), which perpetuate the activation, recruitment and transmigration of monocytes, lymphocytes and other inflammatory cells across the endothelial layer into the subendothelial space, whereby initiating the formation of atheroma plaque (10,12).

Clinical and epidemiological studies have shown that adherence to the MeDiet is associated with antiatherogenic effects (14) such as reduced blood pressure (15,16), improved lipid profile (17,18), and diminished vascular inflammation (19,20), oxidative stress (21,22) and endothelial dysfunction (23,24).

Previous sub-studies of the PREDIMED trial revealed that a MeDiet supplemented with extra-virgin olive oil (EVOO) or nuts reduced systemic inflammatory biomarkers related to atherosclerosis [tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and C-reactive protein (CRP)] after 3 months (19) and 1 year (14,20) of intervention. In addition, at 3 and 12 months, monocyte expression of CD49d, an adhesion molecule crucial for leukocyte homing, and CD40, a proinflammatory ligand, decreased after both MeDiets (19,20).

Whether this anti-inflammatory effect of the MeDiet is maintained in the long-term remains to be elucidated. The aim of this study was to assess changes in the expression of adhesion molecules related to atheroma plaque formation and changes in the plasma concentrations of the main and more studied immunomodulatory biomarkers (hs-CRP, IL-6, TNF-α and MCP-1) related to atherosclerosis after 3 and 5 years of intervention in a sub-cohort of the PREDIMED study. These are secondary outcomes of our randomized controlled trial.

MATERIALS AND METHODS

Design

The PREDIMED study is a parallel-group, single-blind, multicenter, randomized, controlled 5-year clinical trial conducted in Spain to assess the effects of the MeDiet on the primary prevention of CVD (www.predimed.es) (5,6). The design, methodology and eligibility criteria for the PREDIMED study have been described elsewere (5,6).

Setting and participants

From October 2003 to November 2004 we screened 193 consecutive candidates to the PREDIMED study recruited in primary care centers associated with the Hospital Clínic of Barcelona, Spain. Twenty-nine of these candidates did not fulfill the inclusion criteria. Four participants withdrew before 5 years (1 from the MeDiet+EVOO group, 1 from the MeDiet+nuts group and 2 from the control group). Thus, 160 subjects completed the

study; 74 men (55 to 80 years of age) and 86 women (60 to 80 years of age) who were free of CVD at inclusion but had either type-2 diabetes mellitus or at least three of the following cardiovascular risk factors: current smoking, hypertension, high levels of LDL cholesterol, low levels of high-density lipoprotein (HDL) cholesterol, overweight/obesity, or family history of premature coronary heart disease (CHD). Further details of the inclusion and exclusion criteria can be found elsewhere (5,6).

Diets, physical activity and clinical measurements

All the participants were randomly assigned to one of three intervention groups: MeDiet supplemented with EVOO, MeDiet supplemented with mixed nuts (walnuts, almonds, and hazelnuts), or a control low-fat diet (LFD), as described elsewhere (5,6). Randomization was performed centrally by means of a computer-generated random-number sequence. The baseline examinations included the administration of a 14-item and 9-item questionnaire to assess adherence to the MeDiet and LFD, respectively, a 137-item food frequency questionnaire (FFQ), and the Minnesota leisure-time physical activity questionnaire (5,6). In addition, the study nurse administered a 47-item questionnaire about education, lifestyle, chronic illness and medication used, performed anthropometrical and blood pressure (BP) measurements (Omron HEM-705CP, Hoofddorp, the Netherlands), and obtained pre-specified biological samples that were stored at -80 ºC until assay (4-6). These examinations were repeated at years 3 and 5 of follow-up.

The same dietitian perfomed the interventions in the 3 study groups. All the participants received quarterly individual and group educational sessions, that included a face-to-face interview and a group session, that was specific for each intervention group and included no more than 20 participants per group. In the individual session, the dietitian gave personal recommendations directed to improve adherence to the MedDiet or LFD, depending on the intervention assigned. In the group sessions, participants were

provided with descriptions of seasonal foods, shopping lists, weekly meal plans and cooking recipes according to the intervention group assigned. Participants allocated to the LFD group were advised to reduce all types of fat and were given written recommendations according to the American Heart Association guidelines (25). In the 2 MeDiet groups, participants were encouraged to increase the intake of vegetables (≥2 servings/d), fresh fruit (≥3 servings/d), legumes, nuts, fish or seafood (≥3 servings/wk), and to use olive oil for cooking and dressings.

Participants in the two MeDiet groups were given supplementary foods at no cost. These foods included either EVOO (1 liter/week for the participants and their families) or mixed nuts (30 g/day: 15 g walnuts, 7.5 g hazelnuts, and 7.5 g almonds) according to the intervention group. The composition of the olive oil and nuts used in the study was measured by standard methods in a reference laboratory and is shown in **Table 1** (5). Energy restriction was not specifically advised nor was physical activity promoted in any of the three groups.

Ethics Statement

All participants provided signed informed consent. The Institutional Review Board of the Hospital Clinic (Barcelona, Spain), accredited by the US Department of Health and Human Services (DHHS) update for Federal wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738, approved the study protocol July 16, 2002. The trial was registered (ISRCTN35739639).

Laboratory measurements

The main outcome measurements were changes in circulating adhesion molecules involved in the first stages of atherosclerosis development at baseline and after 3 and 5 years of intervention.

First, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque (Lymphoprep™, Axis-Shield PoC AC) density-gradient. The expression of adhesion molecules on the surface of PBMCs was analyzed via double direct immunofluorescence using commercial monoclonal antibodies following the manufacturer's instructions. The adhesion molecules analyzed were: anti-CD14 and anti-CD2 monoclonal antibodies (Caltag) as markers of monocytes and T-lymphocytes, anti-CD11a and anti-CD11b (Bender Medsystems), anti-CD49d (Cytogmos), anti-CD40 (Caltag). Cell counts (5000 events for T-lymphocytes and 2000 for monocytes) and fluorescence analysis were performed in a FACSCalibur Flow Cytometer (Becton-Dickinson) using CellQuest software. The results are expressed as mean fluorescence intensity (MFI) in arbitrary units.

Plasma was obtained after centrifugation of blood. Plasma and PBMC were stored at -80 ºC until assay. Plasma concentrations of four inflammatory biomarkers related to different stages of the atherosclerotic process were measured. Ultra-sensitive (hs) CRP was determined by standard enzyme-linked immunosorbent assays (5). IL-6, TNF-α, and MCP-1 were determined using the Bio-Plex Pro™ cytokine, adhesion molecules and chemokine assays (Bio-Rad Laboratories Inc., Hercules, CA, USA), which are based on magnetic bead-based multiplex assays designed to measure multiple cytokines, adhesion molecules and chemokines in matrices of plasma. Data from reactions are acquired using the Luminex system. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity and target concentrations on the Luminex® 200™ System. Thereafter, the data are processed and analyzed with the Bio-plex Manager 6.1™. We performed all analyses in duplicate.

The analytes determined for each participant in frozen samples of whole serum or plasma as appropriate were: blood glucose levels using the glucose– oxidase method; serum insulin level by radioimmunoassay; cholesterol and triglyceride levels by

enzymatic procedures; HDL cholesterol levels after precipitation with phosphotungstic acid and magnesium chloride; and apolipoproteins A1 and B levels using turbidimetry. In a random sample of 90 participants (56%), we measured urinary tyrosol and hydroxytyrosol concentrations by gas chromatography–mass spectrometry as markers of adherence to extra virgin olive oil intake and the α-linolenic acid plasma content by gas chromatography as a measure of adherence to nut (walnut) intake (5,6).

Diagnostic criteria for new cases of diabetes

We considered new cases of type 2 diabetes mellitus as all those patients without a previous diagnosis of the disease who fulfilled the diagnostic criteria of the American Diabetes Association (ADA) for type-2 diabetes mellitus(26) (plasma glycemia ≥ 124 mg/dL and/or glycated hemoglobin ≥ 6.5%) during the follow-up period of the PREDIMED trial.

Statistical analyses

For a parallel design, the sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline, Brentford, United Kingdom) assuming a maximum loss of 10% of participants. To detect a mean difference of 10 MFI units in the expression ofmonocyte CD49d with a conservative standard deviation (SD) of 10, 20 subjects would be needed to complete the study (a risk = 0.05, power = 0.9). Monocyte expression of CD49d was considered the primary outcome and was used to determine the sample size. Nonetheless, changes in all the endpoints were of equal interest in this study.

We used descriptive statistics with the mean ± SD for the baseline characteristics of the participants. We transformed variables with a skewed distribution (CD49d for T-lymphocytes and monocytes and hs-CRP) to their natural logarithm for analysis. We used descriptive statistics with the mean ± SD for the baseline characteristics of the

participants. Categorical variables are expressed as percentages. Differences in food and nutrient intake, adiposity, and cardiovascular risk factors at baseline and at 3 and 5 years were assessed by the Student's t test. One-factor analysis of variance was used, as appropriate, to determine differences in the baseline characteristics among the 3 study groups. Repeated-measures ANOVA was used to compare changes in food and nutrient intake, adiposity parameters and cardiovascular risk factors, testing the effects of interaction of 2 factors: time as a within-participants factor with 2 levels (first, at baseline and at 3 years, second, at baseline and at 5 years, and third at 3 and 5 years) and the 3 intervention groups, adjusting for potential confounding variables as age, sex, body mass index (BMI), waist circumference, antihypertensive drugs, oral hypoglycemic agents and lipid-lowering agents. Changes in adhesion molecules and other inflammatory biomarkers were mesured using repeated-measures ANOVA testing the effects of interaction of 2 factors: time as a within-participants factor with 3 levels (at baseline, at 3 years, and at 5 years) and the 3 intervention groups, adjusting for potential confounding variables as age, sex, BMI, waist circumference, aspirin, oral hypoglycemic agents and statins. To test the effects of individual factors, we calculated the differences between 3 years and baseline and 5 years and baseline values for the adhesion molecules and inflammatory molecules and then applied an ANOVA test, with the intervention group as fixed factors. Significant interactions were assessed by the simple-effect analysis. All the multiple contrasts were adjusted by a Bonferroni post hoc test. Within- and between-group differences were expressed as estimated means and 95% CI. The significance level was set at $P< 0.05$. All analyses were performed using SPSS v. 20.0 (SPSS Inc, Chicago, IL).

RESULTS

Study population

Of the 165 participants included, equal numbers (n=55) were randomized into each of the three intervention groups. **Figure 1** shows the retention rates (≥ 96% for all) for the

3- and 5-year follow-ups. One participant was lost to follow-up in each of the 2 MeDiet groups and three in the control group.

All participants in this sub-study were selected at random and had similar characteristics to those of the whole PREDIMED cohort. **Table 2** shows the characteristics of the study subjects by intervention group. On average, the participants were 66 years old and nearly half were men. Most participants (85%) were overweight or obese, 64% had hypertension, 64% had dyslipidemia, and 77% were diabetic. The numbers of participants who changed medication increased in the 3 intervention groups throughout, but only aspirin use significantly increased in the 3 groups (P<0.001;all). However, the differences among groups in aspirin use did not attain statitistical significance (P=0.21).

Food, energy balance and dietary adherence

Adherence to the supplemental foods was good in the two MeDiet groups. Compared to baseline, urinary concentration of tyrosol and hydroxytyrosol increased in the MeDiet+EVOO group at 3 and 5 years of intervention (P<0.001;both), while the MeDiet+nuts group showed an increase in α-linolenic acid (P≤0.003) which was greater than in the other diet groups at both 3 and 5 years of intervention. A reduction in energy (P≤0.01;all), protein (P≤0.04;all), carbohydrate (P≤0.006;all) and cholesterol (P≤0.04;all) intake was observed in the 3 groups at 3 and 5 years compared to baseline **(Supplemental Table 1).** In both assessment periods total fat and MUFA intake significantly increased in the participants in the MeDiet+EVOO group while polyunsaturated fatty acid (PUFA) and saturated fatty acid (SFA) intake decreased. In the MeDiet+nuts group we observed an increase in total fat and PUFA and a decrease in SFA intake. Finally, the LFD group showed a significant decrease in the intake of fiber, total fat, SFA and PUFA; in fact, LFD group showed a reduction of 13% in energy from fat at 5 years.

As shown in **Supplemental Table 2,** participants in the MeDiet+EVOO group significantly increased EVOO consumption and decreased the refined olive oil (ROO) consumption, the consumption of pastries, cakes and sweets at 3 and 5 years. Nut consumption increased in the MeDiet+nuts group but decreased in the other two groups. At 3 and 5 years, the consumption of vegetables and legumes increased in the two MeDiet groups, whereas the consumption of cereals and meat and meat products decreased in the three groups. Fruit consumption increased in the two MeDiet groups at 3 years, but fish consumption increased after 5 years only in the MeDiet+nuts group. Physical activity was maintained in all the treatment groups throughout the intervention. Adherence to the MeDiet increased in all the groups, with among-group differences in favor of the two MeDiet arms.

Classical cardiovascular risk factors

As shown in **Table 3**, systolic and diastolic BP significantly decreased in the 2 MeDiet groups at 3 and 5 years. Compared to the LFD group, the MeDiet+EVOO and MeDiet+nuts groups showed a mean reduction of 6-7 and 10-11 mmHg in systolic BP and of 5 and 7-8 mmHg in diastolic BP, respectively, at 3 and 5 years. On the other hand, weight and the body mass index (BMI) decreased by ≥1% in the MeDiet+EVOO group at 3 and 5 years of intervention. Waist circumference reduced by ≥1.2% in the 3 intervention groups at 3 years, but only the MeDiet+nuts group showed a significant reduction at 5 years of intervention compared to baseline. Finally, at 3 and 5 years, the MeDiet+EVOO and MeDiet+nuts groups showed a reduction in triglyceride, total-cholesterol, and LDL-cholesterol cholesterol, a decrease in total- /HDL-cholesterol ratio and an increase in HDL-cholesterol concentrations. The LFD group showed a significant increase in glucose and glycated hemoglobin levels at 5 years.

Compared to the LFD group at both 3 and 5 years the MeDiet+EVOO group reducced BMI by 10% (P<0.001), while the MeDiet+nuts group reduced LDL-cholesterol by 31 %.

The number of new cases of diabetes (plasma glucose ≥124 mg/dL and glycated hemoglobin ≥ 6.5%) was greater in patients in the LFD group (7 cases) than the two MeDiet groups (one in each group) (P<0.001;both).

Adhesion molecules and CD40 expression in PBMC at 3 and 5 years

Table 4 shows that CD11a expression on lymphocyte and monocyte surfaces was down-regulated in the three intervention groups at the two time points. After 3 and 5 years, CD49d and CD40 expression in peripheral T-lymphocytes was down-regulated in both MeDiet groups while CD49d expression in T cells was increased in the LFD group. Participants in the control group also showed up-regulation of CD40 in T-lymphocytes at 5 years.

At 3 and 5 years, circulating monocytes showed a significant decrease in CD11b, CD49d and CD40 in the two MeDiet groups compared to baseline.

Comparisons among the 3 intervention groups showed a greater reduction of CD49d (≥ 16%) and CD40 (≥ 27%) expression in T- lymphocytes in the MeDiet+EVOO and MeDiet+nut groups than the LFD group after 3 and 5 years intervention.

In relation to monocytes, we observed a greater reduction in CD11b expression (≥40%) in the MeDiet+nut group after 5 years, while the expression of CD49d and CD40 (≥49%; both) was lower in both MeDiet groups, compared to the LFD group.

Plasma Inflammatory Biomarkers

At 3 and 5 years, participants in both MeDiets also showed significant reductions of ≥30% in plasma concentrations of hs-CRP (P≤0.02; both), ≥35% IL-6 (P≤0.005;both), ≥21% TNF-α (P≤0.04; both) and ≥16% MCP-1 (P≤0.009; both), whereas the changes

in LFD group were not significant (*P between* 0.3 and 0.7) (**Table 5**). Comparasions among groups showed significant reductions in the MeDiet+EVOO group for all inflammatory parameters evaluated ($P \leq 0.006$; all) compared with the LFD group, whereas those allocated in the MeDiet+nut group only showed significant reduction in MCP-1 and IL-6 ($P \leq 0.002$;both) compared with the LFD group.

DISCUSSION

Adherence to the MeDiet down-regulates the expression of adhesion molecules on circulating T-lymphocyte (CD11a, CD49d and CD40) and monocyte (CD11a, CD11b, CD49d, CD40) surfaces as well as inflammatory biomarkers (TNF-α, IL-6, MCP-1, hs-CRP) in serum. These molecules play an essential role in the recruitment of monocytes from the bloodstream to the subendothelial space in the initial stages of atherogenesis and throughout its course. This anti-inflammatory effect of the MeDiet was maintained in the long-term and was also associated with an improvement in classical cardiovascular risk factors, including reduced blood pressure and waist circumference and a shift of the lipid profile towards less atherogenicity. A large body of scientific evidence supports the cardioprotective effect of the MeDiet (5,6,19,20,27). The best proof of the health effects of the MeDiet has been provided by the results of the PREDIMED study showing that a MeDiet supplemented with EVOO or nuts reduces the incidence of CVD events by 30% in subjects at high cardiovascular risk(6). In addition, the PREDIMED study has also investigated the mechanisms involved in this salutary effect. The results of the present study suggest that the MeDiet has a dual effect against CVD. First, it improves the classical cardiovascular risk factors(5,19,20) and, second, it has a significant anti-inflammatory effect(14,19,20) in the short- and long-term. Thus, the MeDiet reduces systolic and diastolic BP(5,17,18) and fasting glucose levels(17,27), improves insulin resistance(27,28), and decreases abdominal fat(28,29,3031). The lipid profile(5) also improved with a decrease in LDL cholesterol

and an increase in HDL cholesterol in both MeDiet groups. On the other hand, the MeDiet seems to exert its effects on classical risk factors at an early stage (3 months)(19). Experimental and clinical studies have shown that the MeDiet exerts its anti-inflammatory and immunomodulating effects through down-regulation of the expression of leukocyte adhesion molecules(19,20), decreasing pro-inflammatory interleukins (IL-1, IL-6), hs-CRP, TNF-α and its receptors, chemoattractant molecules (MCP-1), and soluble endothelial adhesion molecules (sVCAM-1, sICAM-1, sE- and sP-Selectin)(5,14,19,20). Moreover, the MeDiet also down-regulates the expression of molecules related to plaque instability, such as IL-18, MMP-9 or TGF-β1(20). The results of the present study confirm the long-term anti-inflammatory effects of the MeDiet.

An important question is whether it is the MeDiet pattern itself or specific food components that are responsible for these effects. Olive oil is one of the main components of the MeDiet. Besides MUFA, EVOO contains α-tocopherol and phenolic compounds with strong antioxidant and anti-inflammatory properties(32,33). *In vitro* and *ex vivo* studies with EVOO have shown down-regulation of the expression of systemic VCAM-1, ICAM-1, and E-selectin in circulating lymphocytes and monocytes(33) and decreases of plasma concentrations of IL-6, and CRP in patients with stable CHD(34). In addition, cross-sectional studies(35) have shown low concentrations of VCAM-1, ICAM-1, IL-6 and CRP in subjects with the highest consumption of EVOO.

In a study using a nutrigenomic approach, the 3-week intake of EVOO reduced the gene expression on PBMNCs of CD40L, its downstream products, and related genes involved in atherogenic and inflammatory processes in humans(36). These results are in accordance with the reduction of the expression of CD40 on T-lymphocytes and monocytes in a short- (3 and 12 months)(19,20) and long-term follow-up of 3 and 5 years.

On the other hand, nuts, another key component of the MeDiet, are rich in unsaturated fatty acids (α-linolenic acid in the case of walnuts), fiber, phytosterols, folic acid and vitamin E and polyphenols(37). Nut consumption has also been associated with decreased concentrations of IL-6, CRP and fibrinogen in cross-sectional studies(35,38), as well as lower plasma concentrations of sVCAM-1, sICAM-1 and sE-selectin in hypercholesterolemic patients in interventional studies(39). On the other hand, several studies have associated the immunomodulatory and anti-inflammatory effects of the MeDiet with the dietary pattern itself and not to specific foods(23,40,41,42) showing reductions in the concentrations of biomarkers of inflammation and endothelial dysfunction (CRP, IL6, ICAM-1 and VCAM-1) in subjects with higher adherence to the MeDiet. However, these former studies all evaluated the effects of the MeDiet at only 3 to 12 months after intervention.

After 3 and 5 years of intervention, the two MeDiet groups in the current study showed increased adherence to the MeDiet assessed by food questionnaires and to the supplemental foods assessed by changes in objective biomarkers such as plasma urinary tyrosol and hydroxytyrosol concentrations (as a measure of adherence to EVOO consumption recommendations) and the plasma α-linolenic acid proportion (as a measure of adherence to walnut consumption recommendations). Concomitantly, we observed a down-regulation of the expression of T-lymphocyte and monocyte adhesion molecules. Therefore, according to these results, the composition of the diet could lead to a modification in the expression of leukocyte adhesion molecules in participants assigned to the 2 MeDiet groups and could modify the expression of these adhesion molecules not only in the short- and medium-term but could also maintain or even increase these effects in the long-term, for up to at least 5 years of follow-up.

Our study has several strengths, one of which is its randomized design and reproduction of real life conditions, such as home-prepared foods, excellent completion rates, and good compliance, which were assessed with serum biomarkers and close

monitoring of the participants, the number of inflammatory leukocyte adhesion molecules evaluated, and, importantly, the long duration of the follow-up. Nonetheless,there are also limitations to our study. The results cannot be generalized to other populations because the participants were older subjects at high risk for CHD. Other limitation of the study could be that a great proportion of our patients had type 2 diabetes which may have a great effect on the development of atherogenesis (inflammation and inmune cell activation); therefore, these data should be replicated in another cohort with lower inicidence of type 2 diabetes.
On the other hand, the outcomes of the study were changes in classical cardiovascular risk factors and inflammatory molecules, while the effects on other variables related to arterial structure and function or oxidative stress were not studied.

CONCLUSION

The current study supports the recommendation of the MeDiet as a useful dietary strategy for CVD prevention. This healthy effect seems to be reached achieved through several mechanisms, including modulating inflammatory response and improving classical cardiovascular risk factors which are maintained in the long-term.

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The authors' responsibilities were as follows -RE, RC, MU-S, ES: study conception and design; RC, OC: laboratory and clinical data; RC, ES, MU-S, ER and RE: analysis and interpretation of the data; RC, ES, RML-R and RE: draft of the article; and RC, ES, MU-

S, ES, DC, OC, RML-R, JS-S, M-AM-G, ER and RE: critical revision and final approval. RC, MU-S, ES and RE wrote the paper. RE had primary responsibility for the final content. All the authors have read and approved the final manuscript. None of the authors declare a conflict of interest related to the study.

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TABLES

TABLE 1. Fatty acid, tocopherol, and sterol composition of the extra-virgin olive oil and nuts used in the trial[1].

Constituents	Extra Virgin Olive Oil	Walnuts	Almonds	Hazelnuts
Total fat, *%*	100	62.9 ± 0.3	50.2 ± 0.2	53.2 ± 0.3
Palmitic acid, *%*	8.2 ± 0.2	6.3 ± 0.0	7.4 ± 0.1	7.4 ± 0.1
Stearic acid, *%*	3.2 ± 0.1	2.6± 0.0	1.8± 0.0	1.9 ± 0.1
Oleic acid, *%*	75.0 ± 0.8	14.0 ± 0.3	61.2 ± 0.4	72.1± 0.2
Linoleic acid, *%*	6.8 ± 0.2	61.3 ± 0.4	26.7± 0.2	13.3± 0.2
α-Linolenic acid, *%*	0.4 ± 0.0	14.3 ± 0.1	0.1 ± 0.0	0.8 ± 0.0
α-Tocopherol, *mg/100 g*	14.7 ± 0.0	4.9 ± 0.1	48.4 ± 0.9	38.8 ± 1.5
β-Tocopherol, *mg/100 g*	4.3 ± 0.0	2.0 ± 0.1	5.4 ± 0.9	8.8 ± 1.5
γ-Tocopherol, *mg/100 g*	0.4 ± 0.0	50.2 ± 1.3	6.0 ± 0.2	20.7 ± 0.4
Total sterols, *mg/100 g*	156 ± 0	199 ± 8	224 ± 25	175 ± 9
β-Sitosterol, *%*	95.5 ± 0.1	84.0 ± 0.8	79.1 ± 0.5	82.8 ± 1.1
Campesterol, *%*	3.2 ± 0.0	5.3 ± 0.0	3.3 ± 0.0	5.2 ± 0.1
Δ-5-Avenasterol, *%*	<0.1	7.6 ± 0.9	6.3 ± 1.2	11.1 ± 0.2

[1] Values are mean ± SD of 6 measurements of random samples from different lots.

TABLE 2. Baseline characteristics of the participants at high risk for cardiovascular disease included in the trial and classified according to the dietary intervention administered.

	MeDiet+EVOO	MeDiet+nuts	Low-fat diet	*P*[2]
Age, *years*	66.7 ± 6.0[1]	65.8 ± 5.6	66.3 ± 6.3	0.72
Men, *n (%)*	23 (43) [1]	31 (57)	20 (39)	0.20
Family history of early-onset CHD, *n*	15 (28)	9 (17)	11 (21)	1.00
Smoking status, *n (%)*				
Current smokers	9 (17)	11 (20)	9 (17)	0.15
BMI, *kg/m²*	29.4 ± 4.0	28.7 ± 3.1	29.1 ± 3.8	0.60
BMI ≥ 25 kg/m², *n (%)*	47 (87)	45 (83)	44 (85)	0.41
Waist circumference, *cm*	100 ± 10	101 ± 8	100 ± 10	0.83
Waist-to-height ratio	0.47 ± 0.06	0.47 ± 0.05	0.47 ± 0.06	0.97
Glucose, *mg/dL*	133 ± 53	136 ± 55	130 ± 42	0.86
Glycated hemoglobin, *mg/dL*	6.3 ± 2.1	6.0 ± 1.6	6.0 ± 1.3	0.61
Type 2 diabetes, *n (%)*	45 (83)	43 (80)	35 (67)	0.23
Years of diagnosis				
1-5y	18 (33)	21 (38)	12 (22)	0.21
> 5y	27 (50)	22 (41)	23 (44)	0.10
Hypertension, *n (%)*	38 (70)	29 (54)	35 (67)	0.10
Dyslipidemia, *n (%)*	32 (59)	34 (63)	36 (69)	0.40
Medications, *n (%)*				
ACE inhibitors	10 (19)	12 (22)	13 (25)	0.41
Diuretics	12 (22)	6 (11)	12 (23)	0.22
Other antihypertensive agents	10 (19)	8 (15)	9 (17)	0.84
Statins	17 (32)	14 (26)	10 (19)	0.56
Other-lipid-lowering agents	4 (7)	2 (4)	4 (8)	0.27
Insulin	3 (6)	7 (13)	3 (6)	0.51
Oral hypoglycemic drugs	29 (54)	24 (44)	27 (52)	0.87
Biguanides	11 (20)	14 (25)	17 (37)	0.44
Increase insulin secretion	14 (26)	13 (24)	16 (30)	0.43
Others	4 (7)	5 (9)	3 (6)	0.19
Aspirin or antiplatelet drugs	9 (17)	8 (15)	5 (10)	0.93
NSAIDS	5 (9)	9 (17)	6 (12)	0.52

[1]Values are means ± SDs, n=54 or 52 (LFD) unless noted otherwise.

[2]From Pearson's chi-square test for categorical variables and one-factor ANOVA for continuous variables. ACE, angiotensin converting enzyme; BMI, body mass index; CHD, coronary heart disease; EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts; NSAIDS, Non-steroidal anti-inflammatory drugs.

TABLE 3. Baseline values and changes in cardiovascular risk factors and adiposity after 3 and 5 years of follow-up with MeDiet+EVOO, MeDiet+Nuts, or LFD in subjects at high risk for cardiovascular disease.

		Intervention Group			
		MeDiet + EVOO	MeDiet + Nuts	Low-fat diet	Time x treatment [3]
Systolic blood pressure, *mmHg*	Baseline[1]	152 ± 15	148 ± 14	147 ± 16	
	3y[2]	-6.2 (-10.0, -2.3)*	-7.2 (-10.9, -3.6)*	-0.5 (-4.6, 3.5)	0.04
	5y[2]	-9.7 (-13.9, -5.5)*,[b]	-10.9 (-15.0, -6.9)*, [b]	-1.1 (-5.5, 3.3) [γ]	0.03
Diastolic blood pressure, *mmHg*	Baseline	85.1 ± 8.7	84.7 ± 9.1	81.0 ± 10.5	
	3y	-5.3 (-7.6, -3.0)*	-5.5 (-7.8, -3.3)*	0.1 (-2.4, 2.5)	0.002
	5y	-7.2 (-9.7, -4.6)*	-7.8 (-10.3, -5.3)*, [a, γ]	0.5 (-2.2, 3.3) [γ]	<0.001
Triglycerides, *mg/dL*	Baseline	135 ± 66	144 ± 74	137 ± 69	
	3y	-19.0 (-36.1, -1.8)*	-21.6 (-37.8, -5.4)*	-10.2 (-28.9, 8.6)	0.65
	5y	-22.2 (-42.1, -2.3)*	-24.4 (-43.2, -5.7)*	-13.7 (-35.4, 8.1)	0.75
Total-cholesterol, *mg/dL*	Baseline	228 ± 31	219 ± 36	213 ± 31	
	3y	-19.2 (-28.7, -9.8)*	-18.4 (-27.5, -9.4)*	-7.6 (-18.0, 2.8)	0.20
	5y	-31.1 (-41.2, -21.0)*, [b, γ]	-39.1 (-48.9, -29.4)*, [b, γ]	-22.7 (-33.9, -11.5)*, [γ]	0.10
HDL-Cholesterol, *mg/dL*	Baseline	51.4 ± 12.3	47.6 ± 9.4	51.7 ± 15.0	
	3y	7.5 (4.9, 10.0)*	6.5 (4.1, 8.9)*	3.9 (1.2, 6.7)*	0.16
	5y	4.4 (0.2, 8.5)*	7.4 (3.5, 11.3)*	2.8 (-1.7, 7.3)	0.30
LDL-Cholesterol, *mg/dL*	Baseline	144 ± 28	141 ± 34	130 ± 21	
	3y	-11.7 (-20.0, -3.6)*	-16.5 (-24.5, -8.5)*	-0.1 (-9.3, 9.2)	0.03
	5y	-23.8 (-33.8, -13.7)*, [b, γ]	-44.2 (-54.0, -34.4)*, [a, b, γ]	-7.7 (-19.0, 3.7) [γ]	<0.001
[Total-Cholesterol: HDL-Cholesterol] ratio	Baseline	4.7 ± 1.1	4.7 ± 1.1	4.2 ± 1.2	
	3y	-0.9 (-1.2, -0.6)*	-0.9 (-1.2, -0.6)*	-0.4 (-0.7, -0.2)*	0.02
	5y	-1.0 (-1.3, -0.6)*	-1.2 (-1.5, -0.8)*	-0.5 (-0.9, -0.1)*	0.12
Glucose, *mg/dL*	Baseline	133 ± 53	136 ± 55	130 ± 42	
	3y	0.8 (-11.6, 13.1)	2.1 (-9.5, 13.7)	1.4 (-12.0, 14.8)	0.99
	5y	-2.6 (-15.5, 10.2)	0.6 (-11.4, 12.7)	16.5 (2.7, 30.4)*, [γ]	0.11
Glycated hemoglobin, *mg/dL*	Baseline	6.3 ± 2.1	6.0 ± 1.6	6.0 ± 1.3	
	3y	0.2 (-0.2, 0.6)	0.3 (-0.1, 0.6)	0.3 (-0.1, 0.7)	0.92
	5y	0.1 (-0.3, 0.4)	0.2 (-0.2, 0.5)	0.5 (0.1, 0.9)*, [γ]	0.22
Weight, *Kg*	Baseline	76.3 ± 18.2	77.1 ± 14.5	75.7 ± 16.7	
	3y	-0.8 (-0.8, -0.7)*	-0.03 (-0.08, 0.02)	0.03 (-0.02, 0.09)	<0.001
	5y	-1.3 (-1.4, -1.2)*	-0.1 (-0.2, 0.1)	0.05 (-0.09, 0.2)	<0.001
BMI, *kg/m2*	Baseline	29.4 ± 4.0	28.7 ± 3.1	29.1 ± 3.8	
	3y	-0.3 (-0.3, -0.2)*	-0.02 (-0.03, 0.001)	0.01 (-0.01, 0.03)	<0.001
	5y	-0.5 (-0.6, -0.5)*	-0.02 (-0.07, 0.03)	0.02 (-0.03, 0.07)	<0.001
Waist circumference, *cm*	Baseline	100 ± 10	101 ± 8	101 ± 9	
	3y	-4.0 (-5.2, -2.8)*	-2.8 (-4.0, -1.6)*	-2.1 (-3.4, -0.8)*	0.08
	5y	-1.2 (-2.5, 0.2) [γ]	-1.6 (-2.9, -0.3)*	-1.5 (-3.0, 0.04)	0.90

[1]Values are means ± SDs, n=54 or 52 (LFD) unless noted otherwise.

[2]Mean differences (95% CI). [*]*P*: Different from baseline, (*P*<0.05). [ɣ]*P*: Different from 3 and 5y of intervention (*P*<0.05).

[3]Time x treatment: comparison between measures obtained before and after intervention and among the 3 diet groups, *P*<0.05. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, *P*<0.05.

BMI, body mass index; EVOO, extra virgin olive oil; LFD, low-fat diet; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts.

TABLE 4. Baseline values and changes in adhesion molecule expression in circulating T- lymphocytes and monocytes after 3 and 5 years of follow-up with MeDiet+EVOO, MeDiet+Nuts, or LFD in subjects at high risk for cardiovascular disease.

		Intervention Group			Between-group changes *P* value for differences [3]		
		MedDiet + EVOO	MedDiet + Nuts	LFD	MeDiet+EVOO vs. LFD	MeDiet+EVOO vs. MeDiet+Nuts	MeDiet+Nuts vs. LFD
T-LYMPHOCYTES (MFI)							
CD11a	Baseline[1]	130 ± 33	126 ± 25	115 ± 32			
	3y[2]	-66.9 (-81.5, -52.3)*, a	-58.8 (-76.0, -41.7)*	-33.5 (-51.1, -16.0)*	0.03	0.31	0.92
	5y[2]	-71.8 (-88.5, -55.0)*	-55.6 (-75.4, - 35.8)*	-40.3 (-60.5, -20.1)*	0.03	0.01	1.00
CD49d	Baseline	46.2 ± 1.7	44.4 ± 1.7	35.7 ± 1.7			
	3y	-10.8 (-16.6, -6.1)*,a	-9.0 (-15.8, -3.9)*,a	18.5 (16.0, 20.0)*	<0.001	1.00	<0.001
	5y	-13.3 (-18.5, -9.1)*,a	-10.6 (-16.5, -6.1)*,a	15.3 (16.0, 14.0)*	<0.001	0.93	<0.001
CD40	Baseline	47.8±1.8	51.5±1.8	38.6±1.4			
	3y	-13.7 (-18.8, -9.4)*	-14.5 (-20.4, -15.1)*	0.4 (-3.5, 3.2)	0.01	1.00	0.02
	5y	-15.6 (-19.1, -12.7)*, a	-18.3 (-22.6, -14.8)*, a	17.4 (15.4, 19.5)*, γ	<0.001	1.00	<0.001
MONOCYTES (MFI)							
CD11a	Baseline	82.3±26.4	80.7±35.1	74.2±22.8			
	3y	-50.1 (-60.3, -39.9)*	-48.2 (-61.1, -35.4)*	-41.9 (-55.2, -28.6)*	0.34	1.00	1.00
	5y	-60.5 (-71.4, -49.6)*, a, γ	-54.4 (-68.2, -40.7)*, γ	-41.2 (-55.5, -27.0)*	0.03	0.33	1.00
CD11b	Baseline	45.5±16.0	43.6±13.1	42.4±15.2			
	3y	-10.0 (-17.4, -2.7)*	-7.5 (-15.1, 0.1)*	-4.3 (-12.9, 4.4)	0.85	1.00	1.00
	5y	-22.9 (-31.4, -14.4)*, γ, a	-17.3 (-26.0, -8.5)*, γ, a	-3.2 (-13.2, 6.9)	<0.001	0.82	0.01
CD49d	Baseline	35.8±1.7	40.8±1.6	33.6±1.4			
	3y	-18.9 (-22.7, -15.7)*, a	-24.3 (-29.9, -19.7)*, a	-4.5 (-7.7, -2.0)	<0.001	1.00	<0.001
	5y	-19.6 (-23.2, -16.6)*, a	-23.6 (-28.6, -19.4)*, a	0.3 (-1.2, 1.4)γ	<0.001	1.00	<0.001
CD40	Baseline	34.2±1.5	40.7±1.7	33.9±1.5			
	3y	-17.2 (-20.4, -14.4)*, a	-21.5 (-26.4, -17.5)*, a	-0.4 (-2.2, 0.9)	<0.001	1.00	<0.001
	5y	-18.5 (-21.9, -15.6)*, a	-22.7 (-27.7, -18.5)*, a	-2.2 (-4.1, -0.6)	<0.001	1.00	<0.001

[1]Values are means ± SDs, n=54 or 52 (LFD) unless noted otherwise.

[2]Mean differences (95% CI). [*]*P*: Different from baseline, (*P*<0.05). [ɣ]*P*: Different from 3 and 5y of intervention (*P*<0.05).

[3]*P* value: Significant differences (*P*<0.05) in changes between groups. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, *P*<0.05. EVOO, extra virgin olive oil; LFD, low-fat diet; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts; MFI, Mean fluorescence intensity.

TABLE 5. Baseline values and changes in inflammatory serum biomarkers after 3 and 5 years of follow-up with MeDiet+EVOO, MeDiet+Nuts, or LFD in subjects at high risk for cardiovascular disease.

		Intervention Group			Between-group changes *P* value for differences [3]		
		MedDiet + EVOO	MedDiet + Nuts	LFD	MeDiet+EVOO vs. LFD	MeDiet+EVOO vs. MeDiet+Nuts	MeDiet+Nuts vs. LFD
MCP-1, *pg/mL*	Baseline[1]	4.3 ± 2.3	4.6 ± 2.2	3.8 ± 1.2			
	3y [2]	-1.4 (-1.9, -0.9)*, a	-0.7 (-1.3, -0.1)*	-0.3 (-1.0, 0.4)	0.001	0.04	0.50
	5y [2]	-1.2 (-1.9, -0.6)*	-1.4 (-2.1, -0.7)*, γ	-0.1 (-0.9, 0.7)	0.003	1.00	0.002
IL-6, *pg/mL*	Baseline	1.3 ± 1.2	1.4 ± 1.3	1.0 ± 0.8			
	3y	-0.5 (-0.9, -0.2)*	-0.4 (-0.8, -0.1)*	0.1 (-0.3, 0.5)	0.006	1.00	0.08
	5y	-0.6 (-0.9, -0.3)*	-0.6 (-0.9, -0.2)*	0.02 (-0.3, 0.4)	0.003	1.00	0.001
TNF-α, *pg/mL*	Baseline	3.6 ± 2.8	3.6 ± 4.2	2.3 ± 1.8			
	3y	-1.6 (-2.5, -0.7)*	-1.0 (-1.9, -0.04)*	0.3 (-0.8, 1.5)	<0.001	0.91	0.02
	5y	-1.9 (-2.7, -1.1)*	-1.2 (-2.0, -0.3)*	-0.4 (-1.4, 0.6)	0.006	0.82	0.10
hs-CRP, *mg/mL*	Baseline	3.7 ± 1.7	3.5 ± 1.8	3.4 ± 1.7			
	3y	-1.8 (-2.4, -1.4) *, b	-1.3 (-1.8, -1.0) *, b	1.4 (0.9, 1.7)	<0.001	0.16	0.003
	5y	-2.0 (-2.7, -1.4)*, b	-1.5 (-2.0, -1.1)*	1.1 (0.7, 1.7)	0.001	0.31	0.08

[1]Values are means ± SDs, n=54 or 52 (LFD) unless noted otherwise.

[2]Mean differences (95% CI). **P*: Different from baseline, (*P*<0.05). *ᵞP*: Different from 3 and 5y of intervention (*P*<0.05).

[3]*P* value: Significant differences (*P*<0.05) in changes between groups. [a]MeDiet+EVOO vs. MeDiet+nuts and [b]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet are significantly different, *P*<0.05. EVOO, extra virgin olive oil; LFD, low-fat diet; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts.

LEGENDS

FIGURE 1. Flowchart of the study participants. The diagram includes detailed information on the participants excluded. Abbreviations: EVOO, extra virgin olive oil and MeDiet, Mediterranean diet.

Supplemental TABLE 1. Baseline values and changes in energy and nutrient intake after 3 and 5 years of follow-up with MeDiet+EVOO, MeDiet+Nuts, or LFD in subjects at high risk for cardiovascular disease.

		Intervention Group			
		MedDiet + EVOO	MedDiet + Nuts	LFD	Time x treatment [3]
Energy, *kcal/d*	Baseline[1]	2681 ± 634	2640± 562	2432 ± 746	
	3y[2]	-268 (-420, -116)*	-207 (-352, -63)*, [a]	-235 (-399, -72)*	0.85
	5y [2]	-633 (-794, -472)*, ɣ	-383 (-533, -233)*, [a], ɣ	-415 (-585, -244)*, ɣ	0.06
Protein, *g/d*	Baseline	113 ± 29.1	105 ± 22.1	103 ± 21.3	
	3y	-11.8 (-18.4, -5.2)*	-6.5 (-12.9, -0.1)*,[a]	-14.6 (-21.7, -7.5)*	0.23
	5y	-20.5 (-27.2, 13.8)*, ɣ	-11.1 (-17.3, -5.0)*, [a], ɣ	-19.3 (-26.3, -12.2)*, ɣ	0.09
Carbohydrate, *g/d*	Baseline	306 ± 115	277 ± 83.5	257 ± 257	
	3y	-60.2 (-85.5, -34.9)*	-40.5 (-64.4, -16.5)*	-38.6 (-66.0, -11.3)*	0.43
	5y	-108 (-136, -81)*, ɣ	-71.6 (-97.7, -45.5)*, ɣ	-64.0 (-93.8, -34.2)*, ɣ	0.06
Fiber, *g/d*	Baseline	32.0 ± 10.4	30.5 ± 8.5	27.8 ± 6.6	
	3y	0.5 (-2.3, 3.3) [a]	1.8 (-0.9, 4.4) [a]	-2.3 (-5.4, 0.7)	0.13
	5y	0.7 (-2.1, 3.6) [a]	1.9 (-0.7, 4.6) [a]	-4.6 (-7.6, -1.5)*, ɣ	0.002
Total fat, *g/d*	Baseline	107 ± 27	105 ± 25	101 ± 29	
	3y	11.2 (4.1, 18.3)*, [a]	13.6 (6.9, 20.3)*,[a]	-2.1 (-9.8, 5.6)	0.007
	5y	7.3 (0.4, 14.2)*, [a]	7.2 (0.5, 13.8)*,[a]	-13.2 (-22.6, -3.8)*, ɣ	0.001
SFA, *g/d*	Baseline	31.2 ± 10.4	29.3 ± 7.9	27.3 ± 9.5	
	3y	-5.6 (-7.9, -3.3)*	-2.3 (-4.5, -0.06)*	-1.7 (-4.1, 0.8)	0.04
	5y	-7.6 (-10.0, -5.3)*, ɣ	-4.3 (-6.8, -1.7)*, ɣ	-4.7 (-7.9, -1.5)*, ɣ	0.12
MUFA, *g/d*	Baseline	49.4 ± 12.0	52.7 ± 12.3	49.2 ± 14.6	
	3y	8.5 (4.5, 12.6)*, [a, b]	0.1 (-3.7, 3.9)	-0.7 (-5.1, 3.7)	0.003
	5y	7.9 (4.3, 11.5)*, [a, b]	1.0 (-2.5, 4.5)	-1.7 (-6.6, 3.3)	0.001
PUFA, *g/d*	Baseline	18.8 ± 7.0	18.6 ± 6.5	17.0 ± 6.8	
	3y	-3.1 (-5.5, -0.7)*, [b]	3.2 (1.0, 5.4)*, [a]	-1.9 (-4.4, 0.6)	<0.001
	5y	-5.3 (-7.5, -3.1)*, [b], ɣ	2.7 (0.6, 4.8)*, [a]	-4.0 (-7.0, -1.0)*	<0.001
Linoleic acid, *g/d*	Baseline	14.9 ± 5.1	16.1 ± 7.1	13.8 ± 6.3	
	3y	-0.8 (-3.0, 1.4) [b]	1.6 (-0.4, 3.5) [a]	-1.2 (-3.5, 1.1)	0.13
	5y	-1.6 (-3.6, 0.5) [a, b]	-0.4 (-2.4, 1.5) [a], ɣ	-3.4 (-5.7, -1.2)*, ɣ	0.14
α-linolenic acid, *g/d*	Baseline	1.8 ± 0.8	1.8 ± 0.8	1.7 ± 0.8	
	3y	-0.5 (-0.7, -0.2)*, [b]	0.4 (0.1, 0.6)*, [a]	-0.6 (-0.8, -0.3)*	<0.001
	5y	-0.7 (-0.9, -0.4)*, [b], ɣ	0.3 (0.1, 0.6)*, [a]	-0.6 (-0.9, -0.3)*	<0.001
Marine n-3 fatty acids, *g/d*	Baseline	1.0 ± 0.6	0.9 ± 0.5	0.8 ± 0.4	
	3y	0.01 (-0.10, 0.20) [a]	0.09 (-0.05, 0.20) [a]	-0.07 (-0.20, 0.09)	0.33
	5y	0.01 (-0.10, 0.20)	0.20 (0.03, 0.40)*	-0.02 (-0.30, 0.20)	0.17
Cholesterol, *mg/d*	Baseline	423 ± 120	418 ± 112	396 ± 111	
	3y	-57.6 (-85.3, -29.8)*	-28.1 (-55.3, -0.9)*	-44.7 (-74.6, -14.8)*	0.33
	5y	-83.7 (-117, -50.5)*	-43.8 (-79.0, -8.7)*	-69.5 (-11.3, -26.1)*	0.26

[1]Values are means ± SDs, n=54 or 52 (LFD) unless noted otherwise.

[2]Mean differences (95% CI). **P*: Different from baseline, (*P*<0.05). ɣ*P*: Different from 3 and 5y of intervention (*P*<0.05).

[3]Time x treatment: comparison between measures obtained before and after intervention and among the 3 diet groups, *P*<0.05. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, *P*<0.05. EVOO, extra virgin olive oil; LFD, low-fat diet; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; Refined OO, refined olive oil; SFA, Saturated fatty acids.

Supplemental TABLE 2. Baseline values and changes in consumption of key food items, 14-point Mediterranean diet score and physical activity after 3 and 5 years of follow-up with MeDiet+EVOO, MeDiet+Nuts, or LFD in subjects at high risk for cardiovascular disease.

		Intervention Group			
		MeDiet + EVOO	MeDiet + Nuts	LFD	Time x treatment [3]
EVOO, *g/d*	Baseline[1]	12.4 ± 17.6	11.6 ± 13.9	12.5 ± 16.8	
	3y [2]	39.8 (35.0, 44.7)*,[a, b]	3.7 (-1.1, 8.4)	4.4 (-0.8, 9.7)	<0.001
	5y [2]	39.4 (34.6, 44.3)*, [a, b]	4.7 (-0.2, 9.6)	2.7 (-2.7, 8.1)[γ]	<0.001
Refined OO, *g/d*	Baseline	21.6 ± 16.1	19.5 ± 15.2	23.4 ± 17.8	
	3y	-21.1 (-26.6, -15.6)*,[a,b]	2.8 (-2.5, 8.2)	0.6 (-5.4, 6.5)	<0.001
	5y	-18.3 (-23.8, -12.9)*,[a, b]	0.6 (-4.8, 5.9)	-1.8 (-7.7, 4.1)	<0.001
Total nuts, *g/d*	Baseline	17.1 ± 17.7	21.1 ± 21.2	15.6 ± 15.9	
	3y	-10.0 (-15.6, -4.3)*,[b]	8.3 (2.9, 13.6)*,[a]	-10.1 (-16.2, -4.0)*	<0.001
	5y	-13.0 (-18.9, -7.2)*,[b, γ]	6.4 (1.0, 11.7)*,[a]	-11.1 (-19.0, -3.3)*	<0.001
Vegetables, *g/d*	Baseline	400 ± 163	377 ± 190	363 ± 140	
	3y	118 (64, 172)*,[a]	90 (38, 141)*	16 (-42, 75)	0.04
	5y	73 (11, 134)*,[a, γ]	34 (-22, 91)*,[a, γ]	-42 (-104, 21)[γ]	0.04
Legumes, *g/d*	Baseline	20.9 ± 14.7	18.9 ± 8.0	19.0 ± 9.0	
	3y	7.5 (3.3, 11.6)*,[a]	8.1 (4.1, 12.0)*	2.8 (-1.7, 7.2)	0.17
	5y	7.3 (3.9, 10.8)*,[a]	9.1 (5.3, 13.0)*,[a]	0.2 (-4.4, 4.8)	0.01
Fruits, *g/d*	Baseline	421 ± 183	458 ± 202	409 ± 217	
	3y	146 (81, 212)*	62 (0, 124)*	78 (7, 149)*	0.16
	5y	-0.3 (-74.1, 73.4) [γ]	-10.8 (-80.5, 58.8) [γ]	-7.3 (-86.8, 72.2) [γ]	0.98
Cereals, *g/d*	Baseline	313 ± 122	281 ± 99	267 ± 114	
	3y	-86 (-118, -54)*	-55 (-84, -26)*	-52 (-85, -18)*	0.25
	5y	-122 (-158, -86)*, [γ]	-96 (-135, -57)*, [γ]	-109 (-156, -63)*, [γ]	0.64
Fish or seafood, *g/d*	Baseline	117 ± 60	119 ± 49	102 ± 34	
	3y	3.9 (-9.9, 17.7)	6.5 (-6.5, 19.5)	0.7 (-14.1, 15.5)	0.85
	5y	6.9 (-9.4, 23.2)	16.4 (1.0, 31.8)*,[a]	4.2 (-13.4, 21.8)	0.54
Meat or meat products, *g/d*	Baseline	154 ± 68	152 ± 65	153 ± 54	
	3y	-17.0 (-33.2, -0.7)*	-17.0 (-32.4, -1.4)*	-31.5 (-49.0, -14.0)*	0.39
	5y	-19.8 (-36.3, -3.3)*	-22.1 (-40.1, -4.1)*	-36.7 (-58.1, -15.4)*	0.44
Pastries, cakes or sweets, *g/d*	Baseline	16.7 ± 16.5	15.0 ± 16.2	16.9 ± 21.5	
	3y	-6.1 (-11.3, -1.0)*	-1.0 (-5.8, 3.8)	-2.9 (-8.5, 2.6)	0.35
	5y	-10.3 (-15.7, -4.8)*, [γ]	-2.2 (-7.5, 3.0)	-1.9 (-7.7, 3.8)	0.06
Dairy products, *g/d*	Baseline	419 ± 197	366 ± 250	408 ± 225	
	3y	-16 (-89, 58)	-55 (-123, 14)	-8 (-87, 71)	0.62
	5y	-34 (-101, 33)	-37 (-99, 25)	-37 (-109, 35)	0.99
Alcohol, *g/d*	Baseline	11.1 ± 14.2	15.0 ± 26.3	11.5 ± 15.6	
	3y	-0.7 (-5.7, 4.2)	-1.2 (-5.8, 3.5)	-0.5 (-5.8, 4.9)	0.98
	5y	-1.9 (-5.0, 1.2)	-1.1 (-4.5, 2.3)	-3.4 (-7.4, 0.7)	0.70
Wine, *mL/d*	Baseline	68 ± 98	62 ± 90	67 ± 112	
	3y	7.3 (-12.3, 26.9)	2.9 (-15.9, 21.6)	11.8 (-9.4, 33.0)	0.82
	5y	5.5 (-16.7, 27.8)	5.2 (-19.2, 29.6)	-4.6 (-34.0, 24.8)[γ]	0.84
Physical Activity, *kcal/d*	Baseline	285 ± 220	260 ± 207	238 ± 211	
	3y	12.8 (-49.4, 75)	1.9 (-57.0, 60.6)	6.3 (-58.6, 71.1)	0.97
	5y	35.7 (-28.3, 99.7)	2.5 (-56.7, 61.7)	-1.3 (-66.7, 64.0)	0.67
MeDiet Score	Baseline	8.4 ± 1.6	8.2 ± 1.6	8.0 ± 1.6	
	3y	1.8 (1.5, 2.0)*,[a]	1.4 (1.2, 1.7)*,[a]	0.4 (0.1, 0.7)*	<0.001
	5y	1.8 (1.4, 2.1)*,[a]	1.7 (1.4, 2.1)*,[a]	0.4 (0.1, 0.8)*	<0.001

[1]Values are means ± SDs, n=54 or 52 (LFD) unless noted otherwise.

[2]Mean differences (95% CI). [*]*P*: Different from baseline, (*P*<0.05). [ɣ]*P*: Different from 3 and 5y of intervention (*P*<0.05).

[3]Time x treatment: comparison between measures obtained before and after intervention and among the 3 diet groups, *P*<0.05. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, *P*<0.05**.**

LFD, low-fat diet; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts.