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Long-Term Immunomodulatory Effects of a Mediterranean Diet in Adults at High Risk of Cardiovascular Disease in the PREvención con Dieta MEDiterránea (PREDIMED) Randomized Controlled Trial[1–3]

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Abstract

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

Objective: Our goal was to assess the long-term effects of the MedDiet on inflammatory markers related to atherogenesis in adults at high risk of cardiovascular disease (CVD) compared with the effects of a low-fat diet (LFD).

Methods: We randomly assigned 165 high-risk participants (one-half men; mean age: 66 y) without overt CVD to 1 of 3 diets: a MedDiet supplemented with extra-virgin olive oil, a MedDiet supplemented with nuts, or an LFD. Follow-up data were collected at 3 and 5 y. Repeated-measures ANOVA, adjusted for potential confounding variables, was used to evaluate changes in diet adherence, CVD risk factors, and inflammatory variables.

Results: The 2 MedDiet 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 with baseline, at 3 and 5 y, both MedDiet groups had significant reductions of ≥16% in plasma concentrations of high-sensitivity C-reactive protein, interleukin-6, tumor necrosis factor α, and monocyte chemoattractant protein 1 ($P \leq 0.04$), whereas there were no significant changes in the LFD group. The reductions in CD49d and CD40 expressions in T lymphocytes and monocytes at 3 y were ≥16% greater in both MedDiet groups than were the changes in the LFD group ($P < 0.001$) at 3 y. Compared with baseline, at 3 y, the MedDiet groups had increased HDL-cholesterol (≥8%) and decreased blood pressure (>4%) and total cholesterol, LDL-cholesterol, and triglyceride (≥8%) concentrations. At 5 y, concentrations of glucose (13%) and glycated hemoglobin (8%) had increased with the LFD.

Conclusions: The MedDiet participants had lower cellular and plasma concentrations of inflammatory markers related to atherosclerosis at 3 and 5 y. This anti-inflammatory role of the MedDiet could explain in part the long-term cardioprotective effect of the MedDiet against CVD. This trial was registered at controlled-trials.com as ISRCTN35739639. *J Nutr* 2016;146:1684–93.

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

Introduction

The Mediterranean diet (MedDiet)[12] is recognized as one of the healthiest dietary patterns. Several epidemiologic studies have shown that high adherence to the MedDiet 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 rate of mortality and incidence of cardiovascular disease (CVD) (1–3). There is also consistent

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evidence demonstrating that the MedDiet improves classic CVD 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 MedDiet on 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 LDL (9–11). In parallel, the endothelium is activated because of 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, 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, thereby initiating the formation of atheroma plaque (10, 12).

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

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

Whether this anti-inflammatory effect of the MedDiet is maintained in the long-term remains to be elucidated. The aim of this study (ISRCTN35739639) 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 [high-sensitivity C-reactive protein (hs-CRP), IL-6, TNF-α, and MCP-1] related to atherosclerosis after 3 and 5 y of intervention in a subcohort of the PREDIMED study. These are secondary outcomes of our randomized controlled trial.

Methods

Design. The PREDIMED study is a parallel-group, single-blind, multicenter, randomized, controlled 5-y clinical trial conducted in Spain to assess the effects of the MedDiet on the primary prevention of CVD (www.predimed.es) (5, 6). The design, methodology, and eligibility criteria for the PREDIMED study have been described elsewhere (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 y—1 from the group that consumed the Mediterranean diet supplemented with 50 mL extra-virgin olive oil/d (MedDiet+EVOO), 1 from the group that consumed the Mediterranean diet supplemented with 30 g mixed nuts/d (MedDiet+nuts), and 2 from the control low-fat diet (LFD) group. Thus, 160 subjects completed the study, including 74 men (55–80 y of age) and 86 women (60–80 y of age) who were free of CVD at inclusion but had either type 2 diabetes mellitus or ≥3 of the following CVD risk factors: current smoking, hypertension, high concentrations of LDL cholesterol, low concentrations of HDL cholesterol, overweight or obesity, or family history of premature coronary artery disease (CAD). 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 1 of 3 intervention groups: the MedDiet+EVOO, the MedDiet+nuts (walnuts, almonds, and hazelnuts), or the control LFD, as described elsewhere (5, 6).

Random assignment was performed centrally by means of a computer-generated random-number sequence. The baseline examinations included the administration of 14-item and 9-item questionnaires to assess adherence to the MedDiet and the LFD, respectively, a 137-item 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 anthropometric and BP measurements (Omron HEM-705CP), and obtained prespecified biological samples that were stored at −80° C until assay (4–6). These examinations were repeated at years 3 and 5 of follow-up.

The same dietitian performed the interventions in the 3 study groups. All participants received quarterly individual and group educational sessions that included a face-to-face interview and a group session specific to each intervention group and included no more than 20 participants/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 American Heart Association guidelines (25). In the 2 MedDiet groups, participants were encouraged to increase their 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.

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[3] Supplemental Tables 1 and 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 http://jn.nutrition.org.

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[12] Abbreviations used: BP, blood pressure; CAD, coronary artery disease; CRP, C-reactive protein; CVD, cardiovascular disease; EVOO, extra-virgin olive oil; hs-CRP, high-sensitivity C-reactive protein; ICAM-1, intercellular adhesion molecule 1; LFD, low-fat diet; MCP-1, monocyte chemoattractant protein 1; MedDiet, Mediterranean diet; MedDiet+EVOO, Mediterranean diet supplemented with 50 mL extra-virgin olive oil/d; MedDiet+nuts, Mediterranean diet supplemented with 30 g mixed nuts/d; PBMC, peripheral blood mononuclear cell; PREDIMED, PREvención con DIeta MEDiterránea; VCAM-1, vascular cell adhesion molecule 1.

Participants in the 2 MedDiet groups were given supplementary foods at no cost. These foods included either EVOO (1 L/wk for the participants and their families) or mixed nuts (30 g/d: 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 3 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 update for Federalwide Assurance for the Protection of Human Subjects for International (Non-US) Institutions no. 00000738, approved the study protocol 16 July 2002.

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 y of intervention.

First, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll/Hypaque (Lymphoprep; Axis-Shield) density-gradient. The expression of adhesion molecules on the surface of PBMCs was analyzed via double direct immunofluorescence with the use of commercial monoclonal antibodies while following the manufacturer's instructions. The adhesion molecules analyzed included the following: 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), and 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) with the use of CellQuest software. The results are expressed as mean fluorescence intensity in arbitrary units.

Plasma was obtained after centrifugation of blood. Plasma and PBMCs were stored at −80°C until assay. Plasma concentrations of 4 inflammatory biomarkers related to different stages of the atherosclerotic process were measured. hs-CRP was determined by standard ELISAs (5). IL-6, TNF-α, and MCP-1 were determined with the use of the Bio-Plex Pro cytokine, adhesion molecule, and chemokine assays (Bio-Rad Laboratories), 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 were acquired with the use of the Luminex system. A high-speed digital processor efficiently managed the data output, which was further analyzed and presented as fluorescence intensity and target concentrations on the Luminex 200 system. Thereafter, the data were 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 as follows: blood glucose concentrations with the use of the glucose–oxidase method; serum insulin concentration by radioimmunoassay; cholesterol and TG concentrations by enzymatic procedures; HDL cholesterol concentration after precipitation with phosphotungstic acid and magnesium chloride; and apoA1 and B concentrations with the use of 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 EVOO intake and the α-linolenic acid (18:3n−3) plasma content by GC 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 to include all those patients without a previous diagnosis of the disease who fulfilled the diagnostic criteria of the American Diabetes Association 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) while assuming a maximum loss of 10% of participants. To detect a mean difference of 10 mean fluorescence units in the expression of monocyte CD49d with a conservative SD of 10, 20 subjects were needed to complete the study (α risk = 0.05; power = 0.9). Monocyte expression of CD49d was considered to be the primary outcome and was used to determine the sample size. Nonetheless, changes in all of the endpoints were of equal interest in this study.

We used descriptive statistics with means ± SDs 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 ln for analysis. We used descriptive statistics with means ± SDs for the baseline characteristics of the participants. Categorical variables are expressed as percentages. Differences in food and nutrient intake, adiposity, and CVD risk factors at baseline and at 3 and 5 y were assessed by a Student's *t* test. One-factor ANOVA was used, as appropriate, to determine differences in the baseline characteristics between the 3 study groups. Repeated-measures ANOVA was used to compare changes in food and nutrient intake, adiposity markers, and CVD risk factors, in which we tested the effects of interaction of 2 factors: time as a within-participants factor with 2 levels—at baseline and at 3 y, at baseline and at 5 y, and at 3 and 5 y—and the 3 intervention groups, adjusting for potential confounding variables, including age; sex; BMI; waist circumference; and antihypertensive drug, oral hypoglycemic agent, and lipid-lowering agent use. Changes in adhesion molecules and other inflammatory biomarkers were measured with the use of repeated-measures ANOVA in which we tested the effects of interaction of 2 factors: time as a within-participants factor with 3 levels—at baseline, at 3 y, and at 5 y—and the 3 intervention groups, adjusting for potential confounding variables, including age; sex; BMI; waist circumference; and aspirin, oral hypoglycemic agent, and statin use. To test the effects of individual factors, we calculated the differences between 3 y and baseline and 5 y 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 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 (95% CIs). The significance level was set at $P < 0.05$. All analyses were performed with the use of SPSS version 20.0.

TABLE 1 FA, 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 means ± SDs of 6 measurements of random samples from different lots.

Results

Study population. Of the 165 participants included, equal numbers (n = 55) were randomly assigned to each of the 3

intervention groups. The retention rates (≥96% for all) for the 3- and 5-y follow-ups are shown in **Figure 1**. One participant was lost to follow-up in each of the 2 MedDiet groups and 3 were lost in the control group.

All participants in this substudy were selected at random and had characteristics similar to those of the whole PREDIMED cohort. The characteristics of the study subjects by intervention group are shown in **Table 2**. On average, the participants were 66 y old, and nearly one-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 between groups in aspirin use did not attain statistical significance ($P = 0.21$).

Food, energy balance, and dietary adherence. Adherence to the supplemental foods was good in the 2 MedDiet groups. Compared with baseline, urinary concentrations of tyrosol and hydroxytyrosol increased in the MedDiet+EVOO group at 3 and 5 y of intervention ($P < 0.001$; both), whereas the MedDiet+nuts group had an increase in α-linolenic acid ($P \leq 0.003$) that was greater than in the other diet groups at both 3 and 5 y of intervention. A reduction in energy ($P \leq 0.01$; all), protein ($P \leq 0.04$; all), carbohydrate ($P \leq 0.006$; all) and cholesterol ($P \leq 0.04$; all) intake was observed in the 3 groups at 3 and 5 y compared with baseline (**Supplemental Table 1**). In both assessment periods, total fat and MUFA intake significantly increased in the participants in the MedDiet+EVOO group, whereas PUFA and SFA intake decreased. In the MedDiet+nuts group, we observed an increase in total fat and PUFA intake and a decrease in SFA intake. Finally, the LFD group had a significant decrease in the intake of fiber, total fat, SFAs, and PUFAs; in fact, the LFD group had a reduction of 13% in intake of energy from fat at 5 y.

Participants in the MedDiet+EVOO group significantly increased EVOO consumption and decreased refined olive oil consumption, as well as consumption of pastries, cakes, and sweets, at 3 and 5 y (**Supplemental Table 2**). Nut consumption

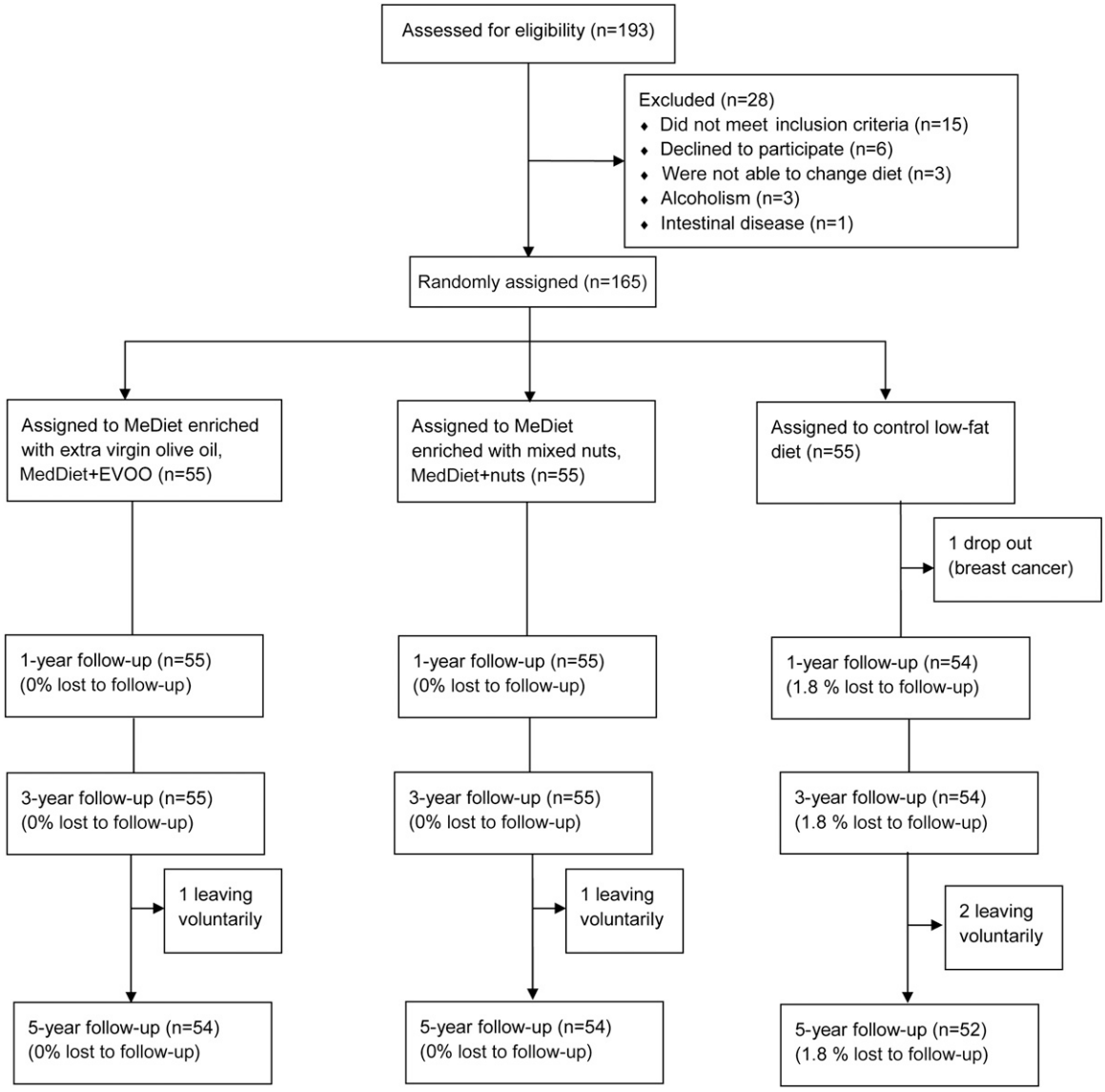

FIGURE 1 Flowchart of the study participants, with detailed information on the participants excluded. MedDiet, Mediterranean diet; MedDiet+EVOO, Mediterranean diet supplemented with 50 mL extra-virgin olive oil/d; MedDiet+nuts, Mediterranean diet supplemented with 30 g mixed nuts/d.

TABLE 2 Baseline characteristics of the participants at high risk of cardiovascular disease included in the trial and classified according to the dietary intervention administered[1]

	MedDiet+EVOO	MedDiet+nuts	LFD	P^2
Age, y	66.7 ± 6.0	65.8 ± 5.6	66.3 ± 6.3	0.72
Men	23 (43)	31 (57)	20 (39)	0.20
Family history of early-onset CAD	15 (28)	9 (17)	11 (21)	1.00
Current smokers	9 (17)	11 (20)	9 (17)	0.15
BMI, kg/m^2	29.4 ± 4.0	28.7 ± 3.1	29.1 ± 3.8	0.60
BMI ≥25 kg/m^2	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	45 (83)	43 (80)	35 (67)	0.23
Diagnosis				
1–5 y	18 (33)	21 (38)	12 (22)	0.21
>5 y	27 (50)	22 (41)	23 (44)	0.10
Hypertension	38 (70)	29 (54)	35 (67)	0.10
Dyslipidemia	32 (59)	34 (63)	36 (69)	0.40
Medications				
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
Drugs that 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 or *n* (%), *n* = 54 or 52 (LFD). ACE, angiotensin-converting enzyme; CAD, coronary artery disease; LFD, low-fat diet; MedDiet+EVOO, Mediterranean diet supplemented with 50 mL extra-virgin olive oil/d; MedDiet+nuts, Mediterranean diet supplemented with 30 g mixed nuts/d; NSAID, nonsteroidal anti-inflammatory drug.
[2] Pearson's chi-square test for categorical variables and 1-factor ANOVA for continuous variables.

increased in the MedDiet+nuts group but decreased in the other 2 groups. At 3 and 5 y, the consumption of vegetables and legumes increased in the 2 MedDiet groups, whereas the consumption of cereals and meat and meat products decreased in all 3 groups. Fruit consumption increased in the 2 MedDiet groups at 3 y, but fish consumption increased after 5 y only in the MedDiet+nuts group. Physical activity was maintained in all of the treatment groups throughout the intervention. Adherence to the MedDiet increased in all of the groups, with between-group differences in favor of the 2 MedDiet arms.

Classic CVD risk factors. Systolic and diastolic BP significantly decreased in the 2 MedDiet groups at 3 and 5 y (**Table 3**). Compared with the LFD group, the MedDiet+EVOO and MedDiet+nuts groups had a mean reduction of 6–7 and 10–11 mm Hg in systolic BP and of 5 and 7–8 mm Hg in diastolic BP, respectively, at 3 and 5 y. On the other hand, weight and BMI decreased by ≥1% in the MedDiet+EVOO group at 3 and 5 y of intervention. Waist circumference was reduced by ≥1.2% in the 3 intervention groups at 3 y, but only the MedDiet+nuts group showed a significant reduction at 5 y of intervention compared with baseline. Finally, at 3 and 5 y, the MedDiet+EVOO and MedDiet+nuts groups had a reduction in TG, total cholesterol, and LDL cholesterol concentrations; a decrease in total-to-HDL cholesterol ratio; and an increase in HDL cholesterol concentrations. The LFD group had a significant increase in glucose and glycated hemoglobin concentrations at 5 y.

Compared with the LFD group at both 3 and 5 y, the MedDiet+EVOO group reduced their BMI by 10% ($P < 0.001$), whereas the MedDiet+nuts group reduced their LDL cholesterol concentrations 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 in the 2 MedDiet groups (one in each group) ($P < 0.001$; both).

Adhesion molecules and CD40 expression in PBMCs at 3 and 5 y. CD11a expression on lymphocyte and monocyte surfaces was downregulated in the 3 intervention groups at the 2 time points (**Table 4**). After 3 and 5 y, CD49d and CD40 expression in peripheral T lymphocytes was downregulated in both MedDiet groups, whereas CD49d expression in T cells was increased in the LFD group. Participants in the control group also had upregulation of CD40 in T lymphocytes at 5 y.

At 3 and 5 y, circulating monocytes were significantly decreased in CD11b, CD49d, and CD40 in the 2 MedDiet groups compared with baseline.

TABLE 3 Baseline values and changes in cardiovascular disease risk factors and adiposity after 3 and 5 y of follow-up with the MedDiet+EVOO, MedDiet+nuts, or LFD in subjects at high risk of cardiovascular disease[1]

	Intervention group			Time × treatment[2]
	MedDiet+EVOO	MedDiet+nuts	LFD	
Systolic blood pressure, mm Hg				
Baseline[1]	152 ± 15	148 ± 14	147 ± 16	
Δ 3 y	−6.2 (−10.0, −2.3)*	−7.2 (−10.9, −3.6)*	−0.5 (−4.6, 3.5)	0.04
Δ 5 y	−9.7 (−13.9, −5.5)*	−10.9 (−15.0, −6.9)[b]*	−1.1 (−5.5, 3.3)†	0.03
Diastolic blood pressure, mm Hg				
Baseline	85.1 ± 8.7	84.7 ± 9.1	81.0 ± 10.5	
Δ 3 y	−5.3 (−7.6, −3.0)*	−5.5 (−7.8, −3.3)*	0.1 (−2.4, 2.5)	0.002
Δ 5 y	−7.2 (−9.7, −4.6)*	−7.8 (−10.3, −5.3)[a]*†	0.5 (−2.2, 3.3)†	<0.001
TGs, mg/dL				
Baseline	135 ± 66	144 ± 74	137 ± 69	
Δ 3 y	−19.0 (−36.1, −1.8)*	−21.6 (−37.8, −5.4)*	−10.2 (−28.9, 8.6)	0.65
Δ 5 y	−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	
Δ 3 y	−19.2 (−28.7, −9.8)*	−18.4 (−27.5, −9.4)*	−7.6 (−18.0, 2.8)	0.20
Δ 5 y	−31.1 (−41.2, −21.0)*†	−39.1 (−48.9, −29.4)[b]*†	−22.7 (−33.9, −11.5)*†	0.10
HDL cholesterol, mg/dL				
Baseline	51.4 ± 12.3	47.6 ± 9.4	51.7 ± 15.0	
Δ 3 y	7.5 (4.9, 10.0)*	6.5 (4.1, 8.9)*	3.9 (1.2, 6.7)*	0.16
Δ 5 y	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	
Δ 3 y	−11.7 (−20.0, −3.6)*	−16.5 (−24.5, −8.5)*	−0.1 (−9.3, 9.2)	0.03
Δ 5 y	−23.8 (−33.8, −13.7)*†	−44.2 (−54.0, −34.4)[a,b]*	−7.7 (−19.0, 3.7)†	<0.001
Total:HDL cholesterol ratio				
Baseline	4.7 ± 1.1	4.7 ± 1.1	4.2 ± 1.2	
Δ 3 y	−0.9 (−1.2, −0.6)*	−0.9 (−1.2, −0.6)*	−0.4 (−0.7, −0.2)*	0.02
Δ 5 y	−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	
Δ 3 y	0.8 (−11.6, 13.1)	2.1 (−9.5, 13.7)	1.4 (−12.0, 14.8)	0.99
Δ 5 y	−2.6 (−15.5, 10.2)	0.6 (−11.4, 12.7)	16.5 (2.7, 30.4)*†	0.11
Glycated hemoglobin, mg/dL				
Baseline	6.3 ± 2.1	6.0 ± 1.6	6.0 ± 1.3	
Δ 3 y	0.2 (−0.2, 0.6)	0.3 (−0.1, 0.6)	0.3 (−0.1, 0.7)	0.92
Δ 5 y	0.1 (−0.3, 0.4)	0.2 (−0.2, 0.5)	0.5 (0.1, 0.9)*†	0.22
Weight, kg				
Baseline	76.3 ± 18.2	77.1 ± 14.5	75.7 ± 16.7	
Δ 3 y	−0.8 (−0.8, −0.7)*	−0.03 (−0.08, 0.02)	0.03 (−0.02, 0.09)	<0.001
Δ 5 y	−1.3 (−1.4, −1.2)*	−0.1 (−0.2, 0.1)	0.05 (−0.09, 0.2)	<0.001
BMI, kg/m^2				
Baseline	29.4 ± 4.0	28.7 ± 3.1	29.1 ± 3.8	
Δ 3 y	−0.3 (−0.3, −0.2)*	−0.02 (−0.03, 0.001)	0.01 (−0.01, 0.03)	<0.001
Δ 5 y	−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	
Δ 3 y	−4.0 (−5.2, −2.8)*	−2.8 (−4.0, −1.6)*	−2.1 (−3.4, −0.8)*	0.08
Δ 5 y	−1.2 (−2.5, 0.2)†	−1.6 (−2.9, −0.3)*	−1.5 (−3.0, 0.04)	0.90

[1] Values are means ± SDs or mean differences (95% CIs), n = 54 or 52 (LFD). [a]Different from LFD, $P < 0.05$; [b]different from MedDiet +EVOO, $P < 0.05$. *Different from baseline, $P < 0.05$; †different from Δ 3 y, $P < 0.05$. LFD, low-fat diet; MedDiet+EVOO, Mediterranean diet supplemented with 50 mL extra-virgin olive oil/d; MedDiet+nuts, Mediterranean diet supplemented with 30 g mixed nuts/d.
[2] Comparison between measures obtained before and after intervention and between the 3 diet groups, $P < 0.05$.

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

In relation to monocytes, we observed a greater reduction in CD11b expression (≥40%) in the MedDiet+nut group after 5 y, whereas the expression of CD49d and CD40 (≥49%; both) was lower in the 2 MedDiet groups than that in the LFD group.

TABLE 4 Baseline values and changes in adhesion molecule expression in circulating T lymphocytes and monocytes after 3 and 5 y of follow-up with the MedDiet+EVOO, MedDiet+nuts, or LFD in subjects at high risk of cardiovascular disease[1]

	Intervention group			Between-group changes, *P*-difference[2]		
	MedDiet+EVOO	MedDiet+nuts	LFD	MedDiet+EVOO vs. LFD	MedDiet+EVOO vs. MedDiet+nuts	MedDiet+nuts vs. LFD
T lymphocytes (MFI)						
CD11a						
Baseline	130 ± 33	126 ± 25	115 ± 32			
Δ 3 y	−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
Δ 5 y	−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			
Δ 3 y	−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
Δ 5 y	−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			
Δ 3 y	−13.7 (−18.8, −9.4)*	−14.5 (−20.4, −15.1)*	0.4 (−3.5, 3.2)	0.01	1.00	0.02
Δ 5 y	−15.6 (−19.1, −12.7)[a]*	−18.3 (−22.6, −14.8)[a]*	17.4 (15.4, 19.5)*[†]	<0.001	1.00	<0.001
Monocytes (MFI)						
CD11a						
Baseline	82.3 ± 26.4	80.7 ± 35.1	74.2 ± 22.8			
Δ 3 y	−50.1 (−60.3, −39.9)*	−48.2 (−61.1, −35.4)*	−41.9 (−55.2, −28.6)*	0.34	1.00	1.00
Δ 5 y	−60.5 (−71.4, −49.6)[a]*[†]	−54.4 (−68.2, −40.7)*[†]	−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			
Δ 3 y	−10.0 (−17.4, −2.7)*	−7.5 (−15.1, 0.1)*	−4.3 (−12.9, 4.4)	0.85	1.00	1.00
Δ 5 y	−22.9 (−31.4, −14.4)[a]*[†]	−17.3 (−26.0, −8.5)[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			
Δ 3 y	−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
Δ 5 y	−19.6 (−23.2, −16.6)[a]*	−23.6 (−28.6, −19.4)[a]*	0.3 (−1.2, 1.4)[†]	<0.001	1.00	<0.001
CD40						
Baseline	34.2 ± 1.5	40.7 ± 1.7	33.9 ± 1.5			
Δ 3 y	−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
Δ 5 y	−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 or mean differences (95% CIs), n = 54 or 52 (LFD). [a]Different from LFD, $P < 0.05$. *Different from baseline, $P < 0.05$; [†]different from 3 y of intervention, $P < 0.05$. LFD, low-fat diet; MedDiet+EVOO, Mediterranean diet supplemented with 50 mL extra-virgin olive oil/d; MedDiet+nuts, Mediterranean diet supplemented with 30 g mixed nuts/d; MFI, mean fluorescence intensity.
[2] Significant differences in changes between groups, $P < 0.05$.

Plasma inflammatory biomarkers. At 3 and 5 y, participants in both MedDiet groups also had significant reductions in plasma concentrations of hs-CRP (≥30%; $P \leq 0.02$ for both), IL-6 (≥35%; $P \leq 0.005$ for both), TNF-α (≥21%; $P \leq 0.04$ for both), and MCP-1 (≥16%; $P \leq 0.009$ for both), whereas the changes in the LFD group were not significant (P = 0.3–0.7) (**Table 5**). Comparisons between groups showed significant reductions in the MedDiet+EVOO group for all inflammatory markers evaluated ($P \leq 0.006$; all) compared with the LFD group, whereas those allocated to the MedDiet+nut group only had a significant reduction in MCP-1 and IL-6 ($P \leq 0.002$; both) compared with the LFD group.

Discussion

Adherence to the MedDiet downregulates the expression of adhesion molecules on circulating T lymphocyte (CD11a, CD49d, and CD40) and monocyte (CD11a, CD11b, CD49d, and CD40) surfaces, as well as inflammatory biomarkers (TNF-α, IL-6, MCP-1, and 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 MedDiet was maintained in the long-term and also was associated with an improvement in classic CVD risk factors, including reduced BP and waist circumference and a shift in the lipid profile toward less atherogenicity. A large body of scientific evidence supports the cardioprotective effect of the MedDiet (5, 6, 19, 20, 27). The best proof of the health effects of the MedDiet has been provided by the results of the PREDIMED study, which showed that a MedDiet supplemented with EVOO or nuts reduces the incidence of CVD events by 30% in subjects at high risk of CVD (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 MedDiet has a dual effect against CVD. First, it improves the classic CVD risk factors (5, 19, 20), and, second, it has a significant anti-inflammatory effect (14, 19, 20) in the short- and long-terms. Thus, the MedDiet reduces systolic and diastolic BP (5, 17, 18)

TABLE 5 Baseline values and changes in inflammatory serum biomarkers after 3 and 5 y of follow-up with the MedDiet+EVOO, MedDiet+Nuts, or LFD in subjects at high risk of cardiovascular disease[1]

	Intervention group			Between-group changes, *P*-difference[2]		
	MedDiet+EVOO	MedDiet+nuts	LFD	MedDiet+EVOO vs. LFD	MedDiet+EVOO vs. MedDiet+nuts	MedDiet+nuts vs. LFD
MCP-1, pg/mL						
Baseline	4.3 ± 2.3	4.6 ± 2.2	3.8 ± 1.2			
Δ 3 y	−1.4 (−1.9, −0.9)*	−0.7 (−1.3, −0.1)[b]*	−0.3 (−1.0, 0.4)	0.001	0.04	0.50
Δ 5 y	−1.2 (−1.9, −0.6)*	−1.4 (−2.1, −0.7)*†	−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			
Δ 3 y	−0.5 (−0.9, −0.2)*	−0.4 (−0.8, −0.1)*	0.1 (−0.3, 0.5)	0.006	1.00	0.08
Δ 5 y	−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			
Δ 3 y	−1.6 (−2.5, −0.7)*	−1.0 (−1.9, −0.04)*	0.3 (−0.8, 1.5)	<0.001	0.91	0.02
Δ 5 y	−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, g/L						
Baseline	3.7 ± 1.7	3.5 ± 1.8	3.4 ± 1.7			
Δ 3 y	−1.8 (−2.4, −1.4)[a]*	−1.3 (−1.8, −1.0)[a]*	1.4 (0.9, 1.7)	<0.001	0.16	0.003
Δ 5 y	−2.0 (−2.7, −1.4)[a]*	−1.5 (−2.0, −1.1)*	1.1 (0.7, 1.7)	0.001	0.31	0.08

[1] Values are means ± SDs or mean differences (95% CIs), $n = 54$ or 52 (LFD). [a]Different from LFD, $P < 0.05$; [b]different from MedDiet+EVOO, $P < 0.05$. *Different from baseline, $P < 0.05$; †different from 3 y of intervention, $P < 0.05$. hs-CRP, high-sensitivity C-reactive protein; LFD, low-fat diet; MCP-1, monocyte chemoattractant protein 1; MedDiet +EVOO, Mediterranean diet supplemented with 50 mL extra-virgin olive oil/d; MedDiet+nuts, Mediterranean diet supplemented with 30 g mixed nuts/d.
[2] Significant differences in changes between groups, $P < 0.05$.

and fasting glucose concentrations (17, 27), improves insulin resistance (27, 28), and decreases abdominal fat (28, 29, 30, 31). Lipid profiles (5) also improved with a decrease in LDL cholesterol and an increase in HDL cholesterol in the 2 MedDiet groups. Furthermore, the MedDiet seems to exert its effects on classic risk factors at an early stage (3 mo) (19). Experimental and clinical studies have shown that the MedDiet exerts its anti-inflammatory and immunomodulating effects by downregulating the expression of leukocyte adhesion molecules (19, 20) and decreasing proinflammatory interleukins (IL-1, and IL-6), hs-CRP, TNF-α and its receptors, chemoattractant molecules (MCP-1), and soluble endothelial adhesion molecules (soluble VCAM-1, ICAM-1, and E- and P-selectin) (5, 14, 19, 20). Moreover, the MedDiet also downregulates the expression of molecules related to plaque instability, such as IL-18, matrix metalloproteinase 9, and TGF-β1 (20). The results of the present study confirm the long-term anti-inflammatory effects of the MedDiet.

An important question is whether it is the MedDiet pattern itself or specific food components that are responsible for these effects. Olive oil is one of the main components of the MedDiet. Besides MUFAs, 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 downregulation of the expression of systemic VCAM-1, ICAM-1, and E-selectin in circulating lymphocytes and monocytes (33) and decreases in plasma concentrations of IL-6 and CRP in patients with stable CAD (34). In addition, cross-sectional studies (35) have shown low concentrations of VCAM-1, ICAM-1, IL-6, and CRP in subjects who consume the highest amount of EVOO.

In a study that used a nutrigenomic approach, the 3-wk 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-mo) (19, 20) and long-term follow-up of 3 and 5 y.

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

After 3 and 5 y of intervention, the 2 MedDiet groups in the current study showed increased adherence to the MedDiet as assessed by food questionnaires and to the supplemental foods as 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 downregulation 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 MedDiet groups and not only could modify the expression of these adhesion molecules in the short- and medium-term but could also maintain or even increase these effects in the long-term, for ≥5 y of follow-up.

Our study has several strengths, including its randomized design, 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 these participants were older subjects at high risk of CAD. Other limitation of the study could be that a great proportion of our patients had type 2 diabetes, which may have had 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.

However, the outcomes of the study included changes in classic CVD risk factors and inflammatory molecules, whereas the effects on other variables related to arterial structure and function or oxidative stress were not studied.

In sum, the current study supports the recommendation of the MedDiet as a useful dietary strategy for CVD prevention. This healthy effect seems to be achieved through several mechanisms, including the modulation of inflammatory response and improvement in classic CVD risk factors that are maintained in the long-term.

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RC, ES, MU-S, and RE conceived of and designed the study and wrote the paper; RC and OC conducted the research; RC, ES, MU-S, ER, and RE analyzed and interpreted the data; RC, ES, R-ML-R, and RE drafted the article; and RE had primary responsibility for the final content. All authors critically revised the manuscript and read and approved the final manuscript.

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