Inhibition of circulating immune cell activation: a molecular antiinflammatory effect of the Mediterranean diet[1–3]

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ABSTRACT

Background: Adherence to the Mediterranean diet (Med-Diet) is associated with a reduced risk of cardiovascular disease (CVD). However, the molecular mechanisms involved are not fully understood.

Objective: The objective was to compare the effects of 2 Med-Diets with those of a low-fat diet on immune cell activation and soluble inflammatory biomarkers related to atherogenesis in subjects at high risk of CVD.

Design: In a controlled study, we randomly assigned 112 older subjects with diabetes or ≥3 CVD risk factors to 3 dietary intervention groups: Med-Diet with supplemental virgin olive oil (VOO), Med-Diet with supplemental nuts, and low-fat diet. Changes from baseline in cellular and serum inflammatory biomarkers were assessed at 3 mo.

Results: One hundred six participants (43% women; average age: 68 y) completed the study. At 3 mo, monocyte expression of CD49d, an adhesion molecule crucial for leukocyte homing, and of CD40, a proinflammatory ligand, decreased ($P < 0.05$) after both Med-Diets but not after the low-fat diet. Serum interleukin-6 and soluble intercellular adhesion molecule-1, inflammatory mediators crucial in firm adhesion of leukocytes to endothelial surfaces, decreased ($P < 0.05$) in both Med-Diet groups. Soluble vascular cellular adhesion molecule-1 and C-reactive protein decreased only after the Med-Diet with VOO ($P < 0.05$), whereas interleukin-6, soluble vascular cellular adhesion molecule-1, and soluble intercellular adhesion molecule-1 increased ($P < 0.05$) after the low-fat diet.

Conclusions: Med-Diets supplemented with VOO or nuts downregulate cellular and circulating inflammatory biomarkers related to atherogenesis in subjects at high risk of CVD. The results support the recommendation of the Med-Diet as a useful tool against CVD. *Am J Clin Nutr* 2009;89:248–56.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death and disability in industrialized countries, particularly in older subjects, but incidence rates show marked geographic differences (1). Some areas of the world, such as the Mediterranean countries and Japan, have CVD rates lower than those of countries in Eastern and northern Europe and the United States (1, 2). A factor frequently invoked to explain this health advantage in southern Europe is customary adherence to the traditional Mediterranean diet (Med-Diet), which is characterized by an abundant intake of vegetable products, a moderate consumption of fish and wine, and a low intake of meat and dairy and industrial bakery products (3). The cardioprotective effect of the Med-Diet has been attributed to its ability to improve conventional risk factors, such as adiposity, blood pressure, serum lipids, and insulin resistance (4–8). However, alternative mechanisms, such as an antiinflammatory effect of the Med-Diet, have also been postulated (9).

Atherosclerosis has long been considered the result of lipid accumulation in the artery wall; however, compelling evidence now indicates that inflammation plays a key role at all stages of the disease (10). Early phases of atherosclerosis involve the recruitment of inflammatory cells from the circulation, their adhesion to the endothelial surface, and their final migration to the subendothelial space—a complex process mediated by inflammatory stimuli that involves cytokine production and up-regulation of adhesion molecules on endothelial cells and circulating peripheral blood mononuclear cells (PBMCs) (11). Ongoing inflammation is also crucial in the development of instability and rupture of atheromatous plaques and the subsequent appearance of ischemic events in advanced stages of the disease (10, 11).

The important role of inflammation in the pathogenesis of atherosclerosis has led to the belief that dietary preventive

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measures act in part by modifying related inflammatory pathways (9). Indeed, results from cross-sectional studies (12, 13) and feeding trials (7, 8) in Mediterranean populations suggest that the Med-Diet has antiinflammatory effects, as was also ascertained in a cross-sectional evaluation of the Nurses' Health Study (14). The effects of the Med-Diet on the expression of adhesion molecules in PBMCs, a crucial step for their firm adhesion to endothelial cells in the initial inflammatory events of atherogenesis (10, 11), has not been previously investigated. In a substudy of a larger clinical trial designed to compare the effects of 2 Med-Diets, one supplemented with virgin olive oil (VOO) and another supplemented with mixed nuts, with those of a low-fat diet on cardiovascular outcomes (PREDIMED Study) (8), we evaluated changes in serum inflammatory markers and adhesion molecule expression in circulating T lymphocytes and monocytes from subjects at high-risk of CVD at 3 mo.

SUBJECTS AND METHODS

Subjects and design

The PREDIMED (PREvención con DIeta MEDiterránea) Study is a parallel-group, multicenter, randomized, controlled clinical trial of 4-y duration aimed to assess the effects of the Med-Diet on the primary prevention of CVD (www.predimed.org; ISRCTN35739639) (8).

We selected 112 potential participants in primary care centers affiliated with the Hospital Clínic of Barcelona. Eligible participants were community-dwelling men aged 55–80 y and women aged 60–80 y who were free of CVD and met $\geq$1 of 2 criteria: type 2 diabetes or $\geq$3 of the following risk factors: smoking, hypertension (blood pressure $\geq$140/90 mm Hg or treatment with antihypertensive drugs), LDL-cholesterol concentration $\geq$160 mg/dL (or treatment with hypolipidemic drugs), HDL-cholesterol concentration $\leq$40 mg/dL, body mass index (in kg/m^2) $\geq$25, or family history of early-onset coronary heart disease. Exclusion criteria were a history of prior CVD, any severe chronic illness, drug or alcohol abuse, history of allergy or intolerance to olive oil or nuts, or low predicted likelihood of changing dietary habits according to the stages of change model. Of the eligible candidates who met entry requirements, 97% agreed to participate and provided informed consent to a protocol approved by our institutional review board.

Participants were randomly assigned into 3 diet groups: a recommended low-fat diet and 2 Med-Diets, supplemented with either VOO or mixed nuts. At baseline and 3 mo, participants completed a 137-item validated food-frequency questionnaire and a 14-item questionnaire assessing adherence to the Med-Diet, recorded medication use, had an evaluation of physical activity, and underwent various tests, including anthropometric and blood pressure measurements and collection of fasting blood and a spot urine sample, as previously described in detail (8). Main outcome measurements were 3-mo changes in adhesion molecules and CD40 expression by circulating PBMCs and plasma concentrations of inflammatory biomarkers.

Diets

The same dietitian delivered the intervention to the 3 groups. All participants received personalized dietary advice about the desired frequency of intake of specific foods during a 30-min session. We advised participants allocated to the low-fat diet to reduce intake of all types of fat and gave them a leaflet with written recommendations according to American Heart Association guidelines (15). For total fat intake, these recommendations were opposite those given to participants in the 2 Med-Diet groups, who received instructions to increase intake of vegetable fats and oils. In the Med-Diet groups, intervention was based on individual and group education, with a single group session scheduled 1 wk after recruitment with up to 20 participants per session. At this meeting, the dietitian gave advice to follow the recommended Med-Diet and provided written material with elaborate descriptions of target foods and seasonal shopping lists, meal plans, and cooking recipes. The recommendations for the Med-Diet groups included use of abundant olive oil for cooking and dressing; intake of $\geq$2 servings/d of vegetables and $\geq$3 servings/d of fresh fruit; intake of $\geq$3 servings/wk of legumes, nuts, and fish or seafood (1 serving of fatty fish); select white meats instead of red or processed meats; and cooking at least twice a week with garlic, onion, and tomato sauce simmered in olive oil. Advice was also given to eliminate or limit the consumption of cream, butter, margarine, cold meat, pâté, duck, sweetened beverages, pastries, industrial bakery products, fried snacks, and out-of-home precooked meals. All diets were ad libitum.

Participants allocated to the 2 Med-Diets also received free provisions of typical Mediterranean foods. Depending on group assignment, they were given a 3-mo allotment of either VOO (1 L/wk) or mixed nuts (30 g/d, as 15 g walnuts, 7.5 g almonds, and 7.5 g hazelnuts). The fatty acid composition of the olive oil and nuts used in the trial is shown in **Table 1**. We estimated energy and nutrient intakes from Spanish food-composition tables, as described (8).

Clinical and laboratory measurements

Anthropometric and blood pressure measurements were performed with standardized methods (8). Samples of serum, EDTA-plasma, and urine were coded and stored at −80°C until assay. A technician blinded to group allocation processed PBMCs on the same day of blood extraction. Cells were obtained from EDTA-venous blood over a 1.077 g/mL density solution made of 9.1% (wt:vol) sodium diatrizoate and 5.7% (wt:vol) polysaccharide and submitted to density-gradient centrifugation (Lymphoprep; Axis-Shield PoC AC, Oslo, Norway). Adhesion molecule expression on T lymphocytes and monocytes was determined by using a double-direct immunofluorescence test with fluorescein and phycoerythrin-coupled monoclonal antibodies. The negative control was immunoglobulin G mouse isotype conjugated with fluorescein and phycoerythrin. We used the following monoclonal antibodies, all conjugated to phycoerythrin or fluorescein as appropriate: anti-CD49d (Cytogmos, Barcelona, Spain), anti-CD11a (Bender Medsystems, Vienna, Austria), anti-CD11b (Bender), and anti-CD40 (Caltag, Burlingame, CA). Anti-CD14 (Caltag) and anti-CD2 (Caltag) monoclonal antibodies were used to identify monocytes and T lymphocytes, respectively. We treated PBMCs with saturating amounts of monoclonal antibodies for 1 h at 4°C and then washed them with phosphate-buffered saline containing 2% fetal calf serum. We gated lymphocytes and monocytes electronically by the forward and sidelight scatter pattern on a FACSCalibur Clinical Cytometer (Becton-Dickinson, San

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TABLE 1
Fatty acid composition of virgin olive oil and nuts used in the trial[1]

	Olive oil	Walnuts	Almonds	Hazelnuts
Total fat (%)	100	62.9 ± 0.3	50.2 ± 0.2	53.2 ± 0.3
Palmitic acid (% of total)	8.2 ± 0.2	6.3 ± 0.0	7.4 ± 0.1	7.4 ± 0.1
Stearic acid (% of total)	3.2 ± 0.1	2.6 ± 0.0	1.8 ± 0.0	1.9 ± 0.1
Oleic acid (% of total)	75.0 ± 0.8	14.0 ± 0.3	61.2 ± 0.4	72.1 ± 0.2
Linoleic acid (% of total)	6.8 ± 0.2	61.3 ± 0.4	26.7 ± 0.2	13.3 ± 0.2
α-Linolenic acid (% of total)	0.4 ± 0.0	14.3 ± 0.1	0.1 ± 0.0	0.8 ± 0.0

[1] Values are means ± SDs of 6 measurements of random samples from different lots. Adapted from reference 8.

Jose, CA) with the use of CellQuest software. We collected data from a minimum of 2000 events (cells) gated as monocytes and 5000 events gated as lymphocytes based on side scatter and forward scatter criteria. Results are expressed as mean fluorescence intensity (MFI) in arbitrary units (16).

Enzyme-linked immunosorbent assays were performed per participant in thawed plasma with commercial kits (BLK, Barcelona, Spain) for soluble E-selectin, P-selectin, vascular cell adhesion molecule 1 (sVCAM-1), intercellular adhesion molecule 1 (sICAM-1), and the cytokine interleukin-6 (BLK and Elast Amplification System; PerkinElmer Life Sciences, Boston, MA).

Additional serum analytes determined were as follows: fasting glucose and immunoreactive insulin, with calculation of insulin resistance in nondiabetic participants by using the homeostasis model assessment (HOMA) method (insulin resistance = fasting insulin × fasting glucose/22.5, where insulin is in μU/mL and glucose is in mmol/L); total cholesterol, triglycerides, and HDL and LDL cholesterol; and high-sensitivity C-reactive protein (CRP), as described previously (8). In a random sample of 56 participants (53%), we measured urinary tyrosol and hydroxytyrosol concentrations by gas chromatography–mass spectrometry as markers of adherence to VOO intake (17). The plasma fatty acid composition was determined by gas chromatography, and the α-linolenic acid (ALA) content was used as a measure of adherence to nut (walnut) intake. For all laboratory methods, intra- and interassay CVs ranged from 1.8% to 8.9% and from 0.9% to 9.9%, respectively.

Statistical analyses

For a parallel design, statistical power calculations indicated that to detect mean differences of 10 MFI in monocyte CD49d expression with a conservative SD of 10 MFI, assuming a maximum loss of 10% participants, 21 subjects per group would need to complete the study (α risk = 0.05; power = 0.8) (ENE 2.0 statistical program; GlaxoSmithKline, Brentford, United Kingdom). CD49d expression was used to set sample size, but changes in all endpoints were of equal interest in this study.

We used descriptive statistics with means and SDs for the baseline characteristics of the participants. We transformed variables with a skewed distribution (inflammatory molecules) to their natural logarithm for analyses. One-factor analysis of variance or chi-square tests, as appropriate, were used to determine differences in baseline characteristics between the 3 study groups. Changes in all outcomes were assessed with repeated-measures analysis of variance for the 2 factors diet and time and their interactions. Significant interactions were analyzed by the simple effects test with multiple contrasts of Bonferroni. Within- and between-group differences are expressed as means and 95% CIs. All statistical tests were 2-tailed, and the significance level was set at $P < 0.05$. Analyses were performed by using SPSS (version 12.0; SPSS Inc, Chicago, IL).

RESULTS

Participant's characteristics and self-selected diets

We excluded 6 of 112 presumably eligible participants before randomization, 3 of whom declined to participate and 3 who did not meet inclusion criteria. The baseline characteristics of the 106 participants who entered the study are shown in **Table 2**. By study design, participants were mostly overweight subjects with a sizeable burden of CVD risk factors, which were well balanced among groups. No changes in medication use were made during the study period. One subject withdrew from the study because of intolerance to nuts. No other adverse effects were noted.

The self-selected dietary habits of the participants before the study began were similar between the 3 intervention groups and were close to those of the typical Med-Diet in several aspects: high mean intakes of olive oil, vegetables, fruit, cereals, and fish; moderate intakes of nuts, legumes, and alcoholic beverages; and low intakes of industrial bakery products and sweets (**Table 3**). Dietary habits deviated from the traditional Med-Diet, however, because of high mean intakes of meat and dairy products.

As shown in **Table 4**, the baseline diets were high in total fat and monounsaturated fatty acids (MUFAs), which was attributed in part to the customary use of olive oil. For the same reason (ie, low intake of vegetable fats other than olive oil or nuts), the total polyunsaturated fatty acid content of the diet was relatively low, whereas the proportion of marine n−3 fatty acids was high because of frequent fish intake.

Changes in food and nutrient intake

After 3 mo, intakes of VOO and nuts were higher in the respective Med-Diet groups than in the low-fat-diet group or the other Med-Diet group. VOO intake was lower in the low-fat-diet group than in either Med-Diet group (Table 3). Legume intake increased and cereal intake (mostly from white bread, which is customarily eaten by Mediterranean populations) decreased in all groups. No significant changes were observed in intake of other foods or in energy expended in physical activity. Objective measures of compliance confirmed food-frequency questionnaire data regarding intake of supplemental foods. At 3 mo, urinary tyrosol increased by 18 ng/mL (95% CI: 0.22, 36) from a baseline value of 28.9 ± 25.8 ng/mL ($P = 0.047$) and hydroxytyrosol increased by 99 ng/mL (95% CI: 5, 195) from a baseline value of 101.8 ± 110.0 ng/mL ($P = 0.037$) in the Med-Diet with VOO group. In this group, the mean plasma oleic

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TABLE 2
Participant's characteristics[1]

	Med-Diet with VOO ($n = 35$)	Med-Diet with nuts ($n = 35$)	Low-fat diet ($n = 36$)	P^2
Age (y)	66 ± 11[3]	66 ± 7	69 ± 6	0.22
Men [*n* (%)]	16 (45.7)	24 (68.6)	20 (55.6)	0.15
Family history of CHD [*n* (%)]	9 (25.7)	10 (28.6)	6 (16.7)	0.13
Current smokers [*n* (%)]	8 (22.9)	12 (34.3)	9 (25.0)	0.61
BMI (kg/m^2)	28.0 ± 2.9	27.8 ± 3.2	28.4 ± 4.0	0.81
BMI ≥ 25 kg/m^2 [*n* (%)]	29 (82.9)	29 (82.9)	29 (80.6)	0.96
Type 2 diabetes [*n* (%)]	21 (60.0)	24 (68.6)	25 (69.4)	0.65
Hypertension [*n* (%)]	22 (62.9)	17 (48.6)	27 (75.0)	0.11
Dyslipidemia [*n* (%)]	24 (68.6)	23 (65.7)	23 (63.9)	0.92
Medications [*n* (%)]				
ACE inhibitors	11 (31.4)	11 (31.4)	14 (38.9)	0.75
Diuretics	10 (28.6)	7 (20.0)	12 (33.3)	0.44
Other antihypertensive agents	10 (28.6)	13 (37.1)	14 (38.9)	0.62
Statins [*n* (%)]	13 (37.1)	13 (37.1)	8 (22.2)	0.30
Other lipid-lowering agents [*n* (%)]	4 (11.4)	1 (2.9)	2 (5.6)	0.33
Insulin [*n* (%)]	2 (5.7)	5 (14.3)	3 (8.3)	0.45
Oral hypoglycemic drugs [*n* (%)]	12 (34.3)	15 (42.9)	20 (55.6)	0.19
Aspirin or antiplatelet drugs [*n* (%)]	4 (11.4)	4 (11.4)	5 (13.9)	0.94

[1] Med-Diet, Mediterranean diet; VOO, virgin olive oil; CHD, coronary heart disease; ACE, angiotensin-converting enzyme.
[2] From Pearson's chi-square test for categorical variables and one-factor ANOVA for continuous variables.
[3] Mean ± SD (all such values).

acid content increased by 2.3 mol% (95% CI: 0.37, 4.24; $P = 0.023$) from a baseline value of 25.3 ± 3.0 mol%. Increases in urinary phenolics were observed in 18 of 19 participants, which indicated 95% compliance. In participants allocated to the Med-Diet with nuts group, the baseline plasma ALA content was 0.27 ± 0.07 mol%, which increased 0.09 mol% (95% CI: 0.03, 0.14; $P = 0.005$). Plasma ALA increased in 17 of 18 participants (95% compliance). In the low-fat-diet group, the baseline plasma oleic acid content of 27.4 ± 4.6 mol% decreased by 2.07 mol% (95% CI: −3.58, −0.56; $P = 0.014$). Increments in urinary phenolics and the plasma oleic acid content observed in the Med-Diet with VOO, as well as the increased plasma ALA concentration in participants assigned to the Med-Diet with nuts group, were all significantly different compared with the changes observed in the low-fat-diet group ($P < 0.05$ for all).

The 14-point Med-Diet score was higher in the 2 Med-Diet groups than in the low-fat-diet group (Table 3). Baseline values and 3-mo changes in energy and nutrient intakes are shown in Table 4. No between-group differences were observed at baseline. At 3 mo, MUFA intake was higher in the Med-Diet with VOO group than in the low-fat-diet group. Participants assigned to consume the Med-Diet with nuts had higher total fat and polyunsaturated fatty acid intakes from supplemental nuts than did those assigned to the low-fat diet and Med-Diet with VOO groups, respectively. No major nutrient changes were observed in the low-fat-diet group.

Adhesion molecule and proinflammatory ligand CD40 expression on PBMCs

Immune cell adhesion molecule and CD40 expression were similar at baseline in the 3 intervention groups (**Table 5**). Although T lymphocyte CD49d expression decreased from baseline in the 2 Med-Diet groups at 3 mo, no differences in changes between groups were observed (**Figure 1**A). After the Med-Diet with VOO, monocyte CD49d and CD40 expression were downregulated by 19% and 8%, respectively. In participants allocated to the Med-Diet with nuts group, monocyte CD49d and CD40 expression were also decreased by 22% and 7%, respectively. The changes in monocyte CD49d expression after the Med-Diet with VOO were significantly different from those observed in the low-fat-diet group, whereas the changes in CD40 expression observed after the Med-Diet with nuts significantly differed from those in the low-fat-diet group (Figure 1B). Monocyte CD11b expression decreased from baseline in the 2 Med-Diet groups, but the changes did not differ from those observed in the low-fat-diet group.

Some participants were under stable treatment with CVD risk–reducing agents with known antiinflammatory properties, such as angiotensin-converting enzyme (ACE) inhibitors and statins (18, 19). Thus, 36 participants used ACE inhibitors: 34 used statins and 55 used both agents. Adhesion molecule and CD40 responses in both T lymphocytes and monocytes were similar regardless of drug treatment (data not shown).

Circulating inflammatory biomarkers

Baseline plasma concentrations of inflammatory biomarkers were similar between groups (Table 5). After the intervention period, sICAM-1 and interleukin-6 decreased in both Med-Diet groups and increased in the low-fat-diet group, whereas sVCAM-1 and CRP decreased only in the Med-Diet group given VOO (**Figure 2**). The level of sVCAM-1 also increased in the low-fat-diet group. Interestingly, the changes in plasma sVCAM-1 in the Med-Diet with VOO group correlated weakly but significantly with those of the urinary concentration of tyrosol—a measure of VOO intake ($r = -0.255$, $P = 0.019$).

Changes in inflammatory biomarkers were independent of treatment with ACE inhibitors or statins, except for the sVCAM-1 concentration, which decreased only in participants allocated to the Med-Diet with nuts group who were not using ACE inhibitors ($P = 0.004$). Platelet and leukocyte count were also monitored, but only a nonsignificant decrease in white cell count was observed in the Med-Diet plus VOO group.

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TABLE 3
Consumption of key food items, physical activity, and 14-point Mediterranean diet score at baseline and 3 mo[1]

Variable	Med-Diet with VOO (n = 35)	Med-Diet with nuts (n = 35)	Low-fat diet (n = 36)	P[2]		
				Time[3]	Group[4]	Interaction[5]
VOO (g/d)						
Baseline[6]	14.0 ± 21.5[7]	8.1 ± 11.3	8.3 ± 14.4	0.035	<0.001	0.006
Final	31.4 ± 18.1[a,8]	11.3 ± 13.6[b]	5.2 ± 7.8[b,8]			
Refined OO (g/d)						
Baseline	23.5 ± 18.2	20.2 ± 15.3	25.6 ± 14.8	0.004	0.82	0.53
Final	12.0 ± 19.4	16.2 ± 20.9	14.3 ± 10.0			
Total nuts (g/d)						
Baseline	14.3 ± 16.6	18.7 ± 20.7	13.9 ± 14.3	<0.001	<0.001	0.001
Final	10.2 ± 15.5[a]	49.2 ± 21.4[b,8]	11.0 ± 10.1[a]			
Vegetables (g/d)						
Baseline	342 ± 141	320 ± 114	307 ± 169	0.368	0.063	0.309
Final	360 ± 216	323 ± 105	344 ± 243			
Legumes (g/d)						
Baseline	21.6 ± 17.5	17.9 ± 10.0	20.1 ± 10.9	0.006	0.834	0.526
Final	46.7 ± 67.8	25.8 ± 11.3	36.6 ± 36.4			
Fruit (g/d)						
Baseline	253 ± 120	253 ± 97	208 ± 95	0.062	0.626	0.31
Final	252 ± 118	253 ± 86	263 ± 160			
Cereals (g/d)						
Baseline	274 ± 119	300 ± 108	258 ± 109	<0.001	0.54	0.44
Final	241 ± 119	259 ± 107	233 ± 101			
Fish or seafood (g/d)						
Baseline	93 ± 56	87 ± 42	70 ± 28	0.141	0.619	0.107
Final	99 ± 62	83 ± 25	80 ± 53			
Meat or meat products (g/d)						
Baseline	109 ± 50	113 ± 37	106 ± 48	0.123	0.499	0.52
Final	92 ± 35	97 ± 45	102 ± 60			
Pastries, cakes, or sweets (g/d)						
Baseline	22 ± 8	21 ± 8	20 ± 7	0.359	0.156	0.345
Final	19 ± 8	19 ± 8	17 ± 7			
Dairy products (g/d)						
Baseline	262 ± 128	241 ± 115	220 ± 108	0.095	0.081	0.445
Final	257 ± 125	225 ± 118	223 ± 131			
Alcohol (g/d)						
Baseline	14.1 ± 15.9	12.1 ± 34.2	13.1 ± 14.5	0.62	0.906	0.481
Final	12.2 ± 16.7	13.7 ± 10.9	11.0 ± 14.1			
Wine (mL/d)						
Baseline	69.9 ± 102.9	50.7 ± 79.4	64.1 ± 103.4	0.414	0.807	0.626
Final	71.0 ± 120.7	64.3 ± 90.7	64.2 ± 103.6			
Physical activity (kcal/d)						
Baseline	337.8 ± 245.2	292.1 ± 246.4	268.8 ± 264.3	0.546	0.202	0.59
Final	328.1 ± 243.3	256.5 ± 200.3	306.2 ± 242.2			
Med-Diet score						
Baseline	7.7 ± 2.0	7.8 ± 1.4	8.0 ± 1.8	<0.001	<0.001	<0.001
Final	10.2 ± 1.7[a,8]	10.1 ± 1.8[a,8]	7.4 ± 1.6[b,8]			

[1] Med-Diet, Mediterranean diet; VOO, virgin olive oil; OO, olive oil. Means within a row with different superscript letters are significantly different, $P < 0.05$ (simple-effect analysis by Bonferroni's multiple contrast).
[2] Data analyzed by repeated-measures 2-factor ANOVA.
[3] Comparison between before and after intervention.
[4] Comparison between the 3 diet groups.
[5] Comparison between measures obtained before and after intervention and between the 3 diet groups.
[6] No significant differences between groups at baseline.
[7] Mean ± SD (all such values).
[8] Significantly different from baseline, $P < 0.04$ (simple-effect analysis by Bonferroni's multiple contrast).

Cardiovascular disease risk factors

Changes in CVD risk factors were similar to those reported for a larger PREDIMED cohort (8). Adiposity measures were essentially unchanged from baseline at 3 mo. Systolic blood pressure decreased by 5.64 mm Hg (95% CI: −10.49, −0.80; $P = 0.023$) and −8.81 mm Hg (95% CI: −13.00, −4.63; $P < 0.001$) in the Med-Diet with VOO and nuts groups, respectively, whereas diastolic blood pressure decreased −2.02 mm Hg (95% CI: −3.89, −0.15; $P = 0.036$) only in the Med-Diet with nuts group. Fasting glucose (−8.02 mg/dL; 95% CI: −14.40, −1.64;

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TABLE 4
Baseline energy and nutrient intake and 3-mo changes[1]

Variables	Med-Diet with VOO ($n = 35$)	Med-Diet with nuts ($n = 35$)	Low-fat diet ($n = 36$)	P[2]		
				Time[3]	Group[4]	Interaction[5]
Energy (kcal/d)						
Baseline[6]	2153 ± 640[7]	2164 ± 506	1869 ± 571	0.503	0.154	0.392
Final	1979 ± 651	2106 ± 689	1807 ± 761			
Protein (% of energy)						
Baseline	17.2 ± 2.7	16.4 ± 2.8	18.5 ± 2.8	0.003	0.149	0.03
Final	17.8 ± 3.2[a,b]	16.3 ± 2.7[a]	19.3 ± 4.0[b,8]			
Carbohydrate (% of energy)						
Baseline	43.8 ± 6.5	43.1 ± 7.4	44.7 ± 7.1	0.007	0.757	0.88
Final	40.9 ± 7.4	41.6 ± 7.8	42.4 ± 7.9			
Total fat (% of energy)						
Baseline	34.6 ± 5.3	33.2 ± 5.6	34.4 ± 5.7	0.003	0.520	0.001
Final	35.6 ± 6.7[a,b]	37.7 ± 6.4[a,8]	34.2 ± 5.7[b]			
SFA (%)						
Baseline	9.8 ± 2.3	9.0 ± 1.9	9.2 ± 2.5	0.744	0.168	0.457
Final	9.6 ± 2.5	9.0 ± 2.3	9.4 ± 2.7			
MUFA (%)						
Baseline	16.4 ± 3.2	15.6 ± 2.8	16.4 ± 3.4	0.795	0.021	0.031
Final	17.6 ± 4.1[a,8]	16.7 ± 3.7[a,b]	15.5 ± 3.1[b]			
PUFA (%)						
Baseline	5.8 ± 2.2	5.6 ± 1.9	6.2 ± 2.2	0.028	0.001	<0.001
Final	5.6 ± 2.1[a]	8.7 ± 2.1[b,8]	6.6 ± 2.8[a,b]			
α-Linolenic acid (g/d)						
Baseline	1.6 ± 1.1	1.5 ± 0.9	1.3 ± 0.7	<0.001	<0.001	<0.001
Final	1.4 ± 0.9[a]	2.6 ± 0.7[b,8]	1.4 ± 1.1[a]			
Marine n−3 fatty acids (g/d)						
Baseline	0.95 ± 0.5	0.82 ± 0.5	0.8 ± 0.4	0.043	0.443	0.044
Final	0.96 ± 0.6[a]	0.84 ± 0.5[a]	1.2 ± 1.7[b]			
Cholesterol (mg/d)						
Baseline	450 ± 117	437 ± 115	392 ± 122	0.939	0.530	0.272
Final	414 ± 97	422 ± 111	376 ± 86			

[1] Med-Diet, Mediterranean diet; VOO, virgin olive oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Means within a row with different superscript letters are significantly different, $P < 0.05$ (simple-effect analysis by Bonferroni's multiple contrast).
[2] Data analyzed by repeated-measures 2-factor ANOVA.
[3] Comparison between before and after intervention.
[4] Comparison between the 3 diet groups.
[5] Comparison between measures obtained before and after intervention and between the 3 diet groups.
[6] No significant differences between groups at baseline.
[7] Mean ± SD (all such values).
[8] Significantly different from baseline, $P < 0.04$ (simple-effect analysis by Bonferroni's multiple contrast).

$P = 0.02$) and the HOMA index decreased (−0.86; 95% CI: −2.14, −0.03; $P = 0.008$) in the Med-Diet with VOO group. In addition, changes in serum glucose correlated with urinary concentration of tyrosol in participants from this group (r: −0.314; $P = 0.036$). HDL cholesterol increased by 5.18 mg/dL (95% CI: 2.68, 7.67; $P < 0.001$) and 2.45 mg/dL (95% CI: 0.35, 4.60; $P = 0.024$) in the Med-Diet with VOO and nuts groups, respectively, whereas the cholesterol/HDL ratio decreased by 0.62 (95% CI: −0.92, −0.33; $P = 0.002$) and −0.37 (95% CI: −0.68, 0.07; $P = 0.023$), respectively, in both Med-Diet groups. No changes were observed in total cholesterol, LDL cholesterol, or triglycerides for any group.

DISCUSSION

In this 3-mo dietary interventional trial, older subjects at high risk of CVD who increased adherence to Med-Diets supplemented with VOO or mixed nuts showed reduced immune cell activation and decreased concentrations of plasma inflammatory biomarkers related to atherogenesis, and the changes were largely independent of ongoing treatment with CVD risk–reducing agents with antiinflammatory properties. This overall antiinflammatory effect of the Med-Diets combines with a reduced potency of CVD risk factors and thus adds to the increasing evidence for their cardioprotective role (6, 9, 20, 21). These results contrast with those of participants advised to consume a low-fat diet, in whom essentially no changes in immune cell activation or conventional risk factors were observed, but increases in plasma inflammatory markers occurred.

The beneficial effect of the Med-Diet against CVD has been attributed to improved control of classic CVD risk factors (4–6, 20, 21). However, the knowledge that atherosclerosis is an inflammatory disease (10, 11) has stimulated research on the antiinflammatory effects of the Med-Diet as another potential

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TABLE 5
Baseline peripheral blood mononuclear cell expression of cell surface inflammatory mediators (adhesion molecules and CD40) and concentrations of circulating inflammatory molecules[1]

	Med-Diet+VOO	*n*	Med-Diet+nuts	*n*	Low-fat diet	*n*	*P*[2]
Cell surface inflammatory mediators (MFI)							
T lymphocytes							
CD11a	121 ± 34[3]	30	128 ± 30	26	121 ± 25	23	0.64
CD49d	19.7 ± 6.3	23	19.2 ± 3.8	22	19.6 ± 6.7	21	0.96
CD40	6.0 ± 2.1	28	5.9 ± 1.9	22	5.9 ± 2.2	22	0.98
Monocytes							
CD11a	128 ± 60	28	108 ± 47	25	117 ± 60	26	0.44
CD11b	51.5 ± 20.0	29	43.9 ± 12.4	24	46.6 ± 17.8	25	0.26
CD49d	28.9 ± 10.1	22	22.5 ± 8.5	24	25.5 ± 11.1	23	0.10
CD40	22.7 ± 12.1	29	16.4 ± 9.0	25	20.0 ± 9.9	26	0.10
Circulating inflammatory mediators							
sE-selectin (ng/mL)	35.0 ± 24.0	28	36.7 ± 17.7	33	40.6 ± 20.8	30	0.57
sP-selectin (ng/mL)	57.4 ± 51.6	20	69.6 ± 51.4	31	66.1 ± 55.8	26	0.72
sVCAM-1 (ng/mL)	1033 ± 311	33	962 ± 363	28	1023 ± 298	32	0.66
sICAM-1 (ng/mL)	290 ± 104	33	270 ± 113	28	239 ± 103	32	0.16
Interleukin-6 (pg/mL)	6.8 ± 4.6	33	6.8 ± 6.0	24	5.9 ± 5.3	32	0.73
CRP (mg/L)	4.0 ± 4.9	31	2.2 ± 1.9	27	2.8 ± 2.7	27	0.15

[1] Med-Diet, Mediterranean diet; VOO, virgin olive oil; MFI, mean fluorescence intensity (arbitrary units); sE-selectin, soluble E-selectin; sP-selectin, soluble P-selectin; sVCAM-1, soluble vascular cell adhesion molecule 1; sICAM-1, soluble intercellular adhesion molecule 1; CRP, high-sensitivity C-reactive protein.
[2] Differences between groups by one-way ANOVA.
[3] Mean ± SD (all such values).

mechanism to explain its association with improved cardiovascular outcomes (7, 8, 12, 13).

In our study, the supplemental foods provided to the 2 Med-Diet groups accounted for most nutrient changes and thus might be responsible in part for the observed antiinflammatory effects. One of them was VOO, a paradigmatic Med-Diet food that is rich in MUFA and antioxidant phenolics. Experimental and clinical studies have shown that olive oil down-regulates endothelial VCAM-1, ICAM-1, and E-selectin expression (22) and decreases plasma concentrations of sICAM-1, sVCAM-1, sE-selectin, interleukin-6, and CRP in high-risk patients (23–25). Another supplemental food was mixed nuts, a classic Mediterranean food rich in antioxidants, and walnuts, a good source of the plant-derived n−3 fatty acid ALA, as the major component. Frequent intake of nuts has been associated with decreased concentrations of interleukin-6, CRP, and fibrinogen in a population free of CVD (26), whereas a Med-Diet supplemented with walnuts decreased concentrations of sVCAM-1 in healthy and hypercholesterolemic subjects (23, 27, 28). An ALA-rich Med-Diet was shown to be very efficient for secondary prevention of coronary events and death in the Lyon Diet Heart Study (20).

The recruitment and adhesion of PBMCs to the endothelium is an early event in fatty streak formation in which adhesion molecules and cytokines play a key role, followed by activation of resident macrophages and lymphocytes to secrete additional cytokines that in turn activate other cell types and lead to a self-perpetuating inflammatory process in the vascular wall that is instrumental in more advanced stages of atherosclerosis, such as plaque growth, vulnerability, and rupture and leads to thrombosis and acute ischemic events (10, 11). Inhibition of both cell-mediated and humoral inflammatory pathways, as shown in our study, provides a molecular mechanism for an antiatherosclerotic effect of the Med-Diet diet. Interestingly, the changes were opposite those observed after the recommended low-fat diet that, given the small changes in nutrients from baseline, was basically a control diet, similar to the Med-Diets except for the VOO and nut supplements. Noticeably, the antiinflammatory effects of the Med-Diets were essentially similar in subjects under stable treatment with risk-reducing agents that have demonstrable antiinflammatory properties, namely ACE inhibitors (18) and statins (19), and in those not treated with these drugs. Thus, the antiinflammatory effect of the Med-Diet appears to be complementary to that of pharmacologic treatment. Also, the benefit of dietary change occurred in older individuals with a sizeable burden of risk factors for CVD. This suggests that the potential antiatherosclerotic effect of healthy foods is not limited to early disease.

Although not explored in our study, a probable mechanistic explanation for the observed effects lies in reduced oxidative stress after high intakes of natural antioxidants present in the supplemental foods. Constitutive adhesion molecule and cytokine production with subsequent PBMC recruitment is low or absent in normal vessels, but is significantly induced in atherosclerosis by immune challenges, such as intimal retention of oxidatively modified LDL (10). The redox-sensitive nuclear transcription factor κB, the main signaling pathway for expression of genes encoding proinflammatory cytokines, is inhibited by phenolic antioxidants similar to those present in VOO and nuts (29). A recent PREDIMED substudy indicated that the 2 Med-Diets were associated with a reduced oxidative status, as shown by lower serum concentrations of oxidized LDL compared with the low-fat diet (30).

Our study had limitations. One limitation related to our attempt to ensure the participants' compliance by providing dietary instructions. In the Med-Diet groups, adherence to the supplemental foods provided was good, as determined by objective

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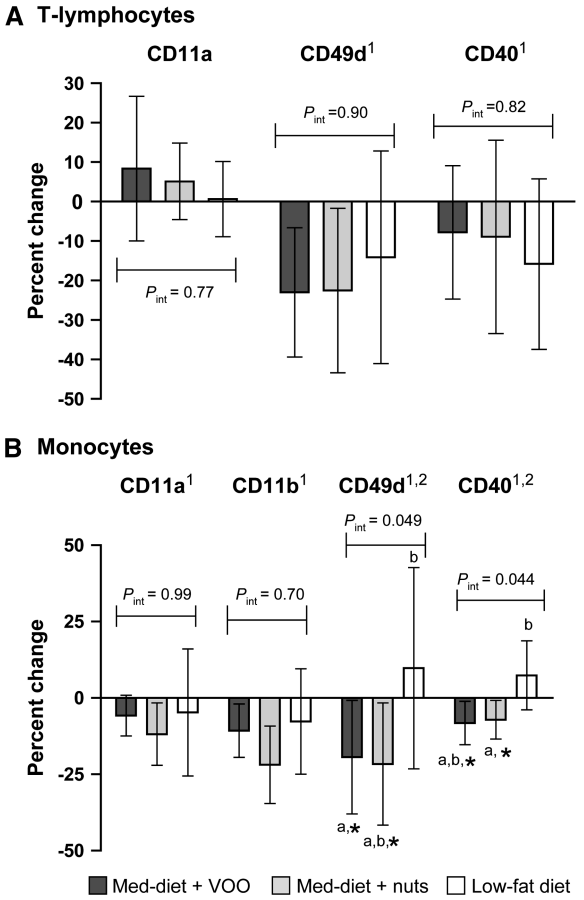

FIGURE 1. Mean percentage changes in adhesion molecules and proinflammatory ligand CD40 expression on T lymphocytes (A) and monocytes (B). Error bars are 95% CIs. Data were analyzed by repeated-measures 2-factor ANOVA. In all cases, $n \geq 21$ (*see* Table 5). P_{int}, comparison between measures obtained before and after intervention and between the 3 diet groups. $^{1}P_{time} < 0.05$ for comparison between before and after intervention. $^{2}P_{group} < 0.05$ for comparison between the 3 diet groups. $^{*}P < 0.05$ for difference from baseline based on a simple-effect analysis by Bonferroni's multiple contrast. ^{a,b}Bars with different lowercase letters are significantly different, $P < 0.05$ (simple-effect analysis by Bonferroni's multiple contrast). Med-diet, Mediterranean diet; VOO, virgin olive oil.

measurements, but participants allocated the low-fat diet did not receive a personalized intervention and barely changed their dietary habits. In fact, there were no significant changes in total fat intake in the low-fat-diet group, probably because participants belonged to a Mediterranean culture, where people prefer using olive oil. However, because the recommended low-fat diet was not the usual diet, participants in this group also changed food habits in a healthy way. Thus, as noted, an important part of the observed differences in outcomes might be attributed to supplemental VOO and nuts. Also, a 3-mo period provides no information about the sustainability of the antiinflammatory effects or their influence on incident CVD events. Our study also had strengths, such as its design (controlled clinical trial), excellent completion rates, good compliance with supplemental foods, and the number of inflammatory biomarkers evaluated.

In conclusion, Med-Diets supplemented with VOO or nuts reduce the potency of CVD risk factors and down-regulate cellular and humoral inflammatory pathways related to atherosclerosis. That these beneficial effects are observed in older subjects at high risk of CVD suggests that it is never too late to change dietary habits to improve health status. The results support recommendations to consume a Med-Diet as a useful tool to prevent CVD.

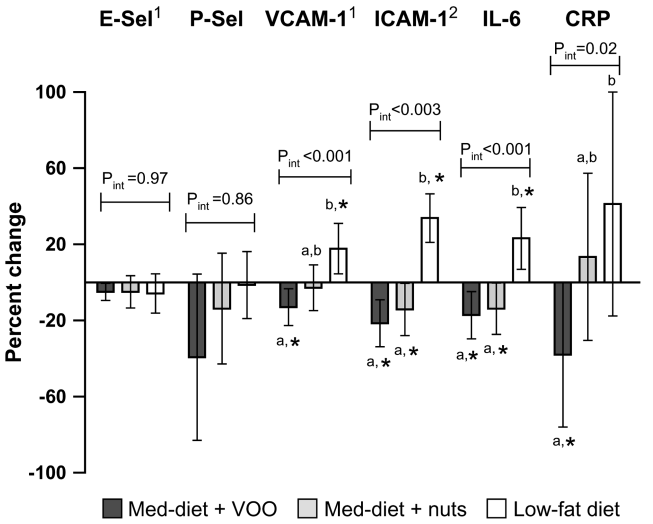

FIGURE 2. Mean percentage changes in plasma concentration of soluble adhesion molecules and other circulating inflammatory mediators. Error bars are 95% CIs. Data were analyzed by repeated-measures 2-factor ANOVA. Error bars are 95% CIs. In all cases, $n \geq 20$ (*see* Table 5). P_{int}, comparison between measures obtained before and after intervention and between the 3 diet groups. $^{1}P_{time} < 0.05$ for comparison between before and after intervention. $^{2}P_{group} < 0.05$ for comparison between the 3 diet groups. $^{*}P < 0.05$ for difference from baseline based on a simple-effect analysis by Bonferroni's multiple contrast. ^{a,b}Bars with different lowercase letters are significantly different, $P < 0.05$ (simple-effect analysis by Bonferroni's multiple contrast). Med-diet, Mediterranean diet; VOO, virgin olive oil; CRP, C-reactive protein; IL-6, interleukin-6; ICAM-1, soluble intercellular adhesion molecule 1; VCAM-1, soluble vascular cell adhesion molecule 1; P-Sel, soluble P-selectin; E-Sel, soluble E-selectin.

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