Mediterranean diet reduces endothelial damage and improves the regenerative capacity of endothelium[1–3]

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ABSTRACT

Background: Endothelial dysfunction is a fundamental step in the atherosclerotic disease process. Activation or injury of the endothelium leads to a variety of inflammatory disorders, including the release of microparticles. Endothelial progenitor cells may contribute to the maintenance of the endothelium by replacing injured mature endothelial cells.

Objective: We studied the influence of dietary fat on the release of endothelial microparticles (EMPs) and endothelial progenitor cells (EPCs) in elderly subjects.

Design: Twenty healthy, elderly subjects (10 men and 10 women) consumed 3 diets following a randomized crossover design, each for 4 wk: a saturated fatty acid diet; a low-fat, high-carbohydrate diet; and a Mediterranean diet (MedDiet) enriched in monounsaturated fatty acids. We investigated total microparticles, EMPs from activated endothelial cells (activated EMPs), EMPs from apoptotic endothelial cells (apoptotic EMPs), EPCs, oxidative stress variables, and ischemic reactive hyperemia (IRH).

Results: The MedDiet led to lower total microparticle, activated EMP, and apoptotic EMP concentrations and higher EPC numbers than did the other diets ($P < 0.001$). We detected lower superoxide dismutase activity ($P < 0.001$), a higher plasma β-carotene concentration ($P < 0.001$), and lower urinary isoprostane and plasma nitrotyrosine concentrations after consumption of the MedDiet than after consumption of the other 2 diets ($P < 0.05$). Furthermore, the occurrence of IRH was higher after consumption of the MedDiet than after consumption of the other 2 diets ($P < 0.05$).

Conclusion: Consumption of the MedDiet induces a reduction in endothelial damage and dysfunction, which is associated with an improvement in the regenerative capacity of the endothelium, in comparison with 2 other diets. *Am J Clin Nutr* 2011;93:267–74.

INTRODUCTION

Endothelial dysfunction, a fundamental step in the atherosclerotic disease process, is a risk factor for the development of clinical events and may represent a marker of atherothrombotic burden. Atherosclerosis is promoted and perpetuated by an early and constant endothelial dysfunction (1). Maintenance of an intact monolayer endothelial cell barrier is crucial for normal vascular structure and function and exerts atheroprotective effects in vivo through the release of substances that promote anticoagulation, inhibit inflammation, and induce vasodilatation (2). Activation or injury of the endothelium leads to a variety of inflammatory disorders, including the release of microparticles (3). Microparticles of various cellular origins (platelets, leukocytes, granulocytes, and endothelial cells) are found in the plasma of healthy subjects, and their amount increases under pathologic conditions. Considerable interest has recently been aroused in a novel surrogate marker of endothelial injury, endothelial microparticles (EMPs) (4). Given their biochemical composition, nature, and biological effects, EMPs appear to be extensively involved in atherosclerosis (5).

Increases in plasma EMP concentrations, particularly those of endothelial origin, reflect cellular injury (eg, by cytokines, oxidative stress) (6) and now appear to be a surrogate marker of vascular dysfunction (7). Similarly, it has been observed that one of the mechanisms involved in the generation of EMPs may be an increase in oxidative stress. These EMPs are also playing a mayor biological role in inflammation, vascular injury, angiogenesis, and thrombosis. Elevated EMP concentrations are encountered in patients with a variety of diseases that involve the vascular system, such as acute coronary syndromes, peripheral arterial disease, diabetes mellitus, and metabolic syndrome. EMPs may

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be an initial step in the development of endothelial dysfunction in genetically predisposed subjects (8), and their number probably increases with age. Also, EMPs probably increase the risk of endothelial dysfunction in asymptomatic subjects (9).

Endothelial progenitor cells (EPCs) may contribute to the maintenance of the endothelium by replacing injured mature endothelial cells (10). The EPC number has been shown to be reduced in patients with cardiovascular disease (11), diabetes mellitus, and multiple coronary risk factors, which leads to speculation that atherosclerosis is caused by a consumptive loss of endothelial repairing capacity (12). Apoptotic bodies from endothelial cells, apoptotic EMPs, may also contribute to tissue repair mechanisms by stimulating the differentiation of progenitor cells (13). Lifestyle factors such as diet may influence this process. In Western countries, the high prevalence of cardiovascular disease is largely attributable to our contemporary lifestyle, which is often sedentary, and includes a diet high in saturated fat and sugars and low in n−3 (omega-3) fatty acids, fruit, vegetables, and fiber (14).

To date, no studies have analyzed the effects of dietary fat on EMP and EPC release. On the basis of the evidence discussed above, we explored the influence of dietary fat on the release of EMPs and EPCs in elderly subjects.

SUBJECTS AND METHODS

Subjects and diets

The study included 20 free-living elderly subjects (age >65 y; 10 men and 10 women). Recruitment of the patients and dietary intervention took place between 1 January 2006 and 15 November 2007. Informed consent was obtained from all participants, all of whom underwent a comprehensive medical history, physical examination, and clinical chemistry analysis before enrollment. None of the subjects showed evidence of chronic illness (eg, hepatic, renal, thyroid, or cardiac dysfunction), and they were requested to maintain their regular physical activity and lifestyle and asked to record in a diary any event that could affect the outcome of the study (eg, stress, change in smoking habits, alcohol consumption, or intake of foods not included in the experimental design). Six participants had high blood pressure, 2 had hyperlipidemia, and 3 had diabetes mellitus. None of the participants had evidence of high alcohol consumption or family history of early-onset cardiovascular disease. None of the participants were active smokers. The study protocol was approved by The Human Investigation Review Committee at Reina Sofia University Hospital according to institutional and Good Clinical Practice guidelines.

Participants were randomly assigned to receive, in a crossover design, 3 diets each for a period of 4 wk: *1*) Mediterranean diet (MedDiet) enriched in monounsaturated fatty acids (MUFAs) with virgin olive oil, containing 15% of energy as protein, 47% as carbohydrate, and 38% as fat [24% MUFAs (provided by virgin olive oil), <10% saturated fatty acids (SFAs), and 4% polyunsaturated fatty acids (PUFAs), of which 0.4% was α-linolenic (ALA)]; *2*) SFA-rich diet, with 15% of energy as protein, 47% as carbohydrate, and 38% as fat (12% MUFAs, 22% SFAs, and 4% PUFAs, of which 0.4% was ALA); and *3*) low-fat, high-carbohydrate diet enriched in n−3 PUFAs (CHO-ALA diet), with 15% of energy as protein, 55% as carbohydrate, and <30% as fat (<10% SFAs, 12% MUFAs, and 8% PUFAs, of which 2% was ALA). The cholesterol intake was constant (<300 mg/d) throughout the 3 periods. The n−3 PUFA enrichment of the CHO-ALA diet was achieved with the use of natural food components rich in L of plant origin [based on walnuts (*Juglans regia* L.]. Carbohydrate intake from the CHO-ALA diet was based on the consumption of biscuits, jam, and bread. Eighty percent of the MUFA diet was provided by virgin olive oil, which was used for cooking, for salad dressing, and as a spread. Butter was used as the main source of SFAs during the SFA dietary period (*see* supplementary Table 1 under "Supplemental data" in the online issue). The composition of the experimental diets was calculated by using the US Department of Agriculture (15) food tables and Spanish food-composition tables for local foodstuffs (16).

Before the start of the intervention period, the volunteers completed a 3-d weighed food diary and an extensive food-frequency questionnaire (FFQ) (17), which allowed identification of foods to be modified. Fat foods were administered by dietitians in the intervention study. At the start of the intervention period, each patient was provided with a handbook for the diet to which they had been randomly assigned, which included 14 menus elaborated with regular solid foods. Advice was given on foods to choose and those to avoid when eating outside the home. At baseline, volunteers were provided with a supply of study foods to last for 2 wk and collected additional study foods every fortnight or when required. At these times, a 24-h recall of the previous day's food intake and a short food-use questionnaire based on the study foods were completed to monitor and motivate volunteers to adhere to the dietary advice. A points system was used to assess the number of food exchanges achieved in the 24-h recall and additional advice was given if either the 24-h recall or FFQ showed inadequate intake of food-exchange options. Volunteers were asked to complete 3-d weighed food diaries at baseline, week 2, and week 4. Weighed food intake over 2 weekdays and 1 weekend day was obtained by using scales provided by the investigators. A dietary analysis software program (Dietsource version 2.0; Novartis SA, Barcelona. Spain) was used in the nutritional evaluation of the menus. Biochemical laboratory personnel were unaware of the dietary period that each participant was following for each determination.

Biochemical determinations

Plasma samples

Blood was collected into tubes containing 1 g EDTA/L or 3.8% citrate and were stored in containers with ice and kept in the dark. Particular care was taken to avoid exposure to air, light, and ambient temperature. Plasma was separated from red blood cells by centrifugation at 1500 × *g* for 15 min at 4°C within 1 h of extraction.

Lipid analysis

Total cholesterol and triglycerides in plasma and lipoprotein fractions were assayed by means of enzymatic procedures. Apolipoprotein (apo) A-I and apo B were measured by inmunoturbidimetry. HDL cholesterol was measured by analyzing the supernatant fluid obtained after precipitation of the plasma aliquot with dextran sulfate-Mg^{2+}. LDL-cholesterol concentrations

were estimated by using the Friedewald formula based on the concentrations of cholesterol, triglycerides, and HDL cholesterol. Plasma glucose concentrations were measured by using the exoquinase method. Plasma insulin concentrations were measured by chemiluminescence.

Ischemic reactive hyperemia determination

Laser-Doppler linear Periflux 5000 (Perimed SA, Stockholm, Sweden) was used to measure ischemic reactive hyperemia (IRH). The methods used were published previously elsewhere (18). Briefly, capillary flow in the second finger of the dominant arm of the patient was assessed for 1 min before (*t*0) and after (*t*d) ischemia was induced in the arm for 4 min by means of a sphygmomanometer, and ischemic reactive hyperemia was obtained

$$\mathrm{IRH} = [\mathrm{AUC}(t\mathrm{d}) - \mathrm{AUC}(t0)/\mathrm{AUC}(t0) \times 100\,\mathrm{AUC}(t0)] \quad (1)$$

where AUC is the area under the curve. This calculation was carried out at the same time of day (between 0800 and 1000).

α-Tocopherol and β-carotene measurements

Concentrations of α-tocopherol and β-carotene were measured in plasma samples according to the method described by Santos-González et al (19). The quantification of these substances was performed by reversed-phase HPLC. Separation was performed at 1 mL/min in a C_{18} column (5 μm particles, 5 × 0.45 cm) and with a mobile phase that consisted of a mixture of methanol and *n*-propanol (1:1) containing lithium perchlorate (2.12 g/L). Monitoring was carried out with a Coulochem II electrochemical detector (ESA, Chelmsford, MA) fitted with a model 5010 analytic cell with the electrodes set at potentials of −500 mV and +300 mV. α-Tocopherol or β-carotene was detected from the signal obtained at the second electrode. Eluted compounds were quantified by integration of peak areas and comparison with internal standards (α-tocopherol and β-carotene standards; Sigma Aldrich, Madrid, Spain).

Biomarkers of oxidative stress determination

The isoprostane content was measured in urine samples by using BIOXYTECH 8-Isoprostane Assay Immunoassay for 8-epi-Prostaglandin F2α (OXIS International, Portland, OR). This kit is a competitive enzyme-linked immunoassay. For measurement of oxidized LDL we used a commercial noncompetitive enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden). Oxidized apo B-100 in the sample reacts with antioxidized apo B-100 antibodies bound to microtitration wells and peroxidase-conjugated antioxidized LDL antibodies in the solution. The absorbance was evaluated in an enzyme immunoassay plate reader (DTX 880 Multimode Detector; Beckman Coulter Inc, Brea, CA) at a wavelength of 450 nm.

Nitric oxide (NO) is a free gas produced endogenously by a variety of mammalian cells. This molecule induces vasodilatation; it inhibits platelet aggregation and adhesion to the vascular endothelium. Total nitrite (nitrite and nitrate) was used as an indicator of NO production assayed following the Griess method (20). The reaction was monitored at 540 nm (ultraviolet-1603 spectrophotometer; Shimadzu, Columbia, MD).

Lipid peroxidation is a mechanism of cellular and molecular injury. Plasma concentrations of lipid peroxidation products (LPO) were measured by using the Bioxytech LPO-586 Kit (OXIS International Inc). The kit uses a chromatogenic reagent that reacts with malondialdehyde + 4-hydroxyalkenals. The absorbance was evaluated in a spectrophotometer (ultraviolet-1603; Shimadzu, Kyoto, Japan) at a wavelength of 586 nm.

Protein carbonyl (PC) was measured in plasma samples by using the method of Levine et al (21). The carbonyls were evaluated in a spectrophotometer (ultraviolet-1603) at 360 nm. Nitrotyrosine is a product of tyrosine nitration, and it may be used by an indicator of cell damage and inflammation caused by NO in cell. Nitrotyrosine was quantified by a "sandwich" enzyme-linked immunosorbent assay (Nitrotyrosine-EIA; Hycult Biotech, PB Uden, Netherlands). A standard curve was constructed by incubating in the wells serial dilutions of 4.5 mol nitrotyrosine standard/L in duplicate. The nitrotyrosine concentrations of the samples were estimated from the standard curve. The reaction was monitored at 450 nm (ultraviolet-1603 spectrophotometer).

Antioxidant enzyme activity

Catalase activity was measured spectrophotometrically in plasma samples according to Aebi (22). Total superoxide dismutase (SOD) activity was determined by using a colorimetric assay in plasma according to the laboratory method described by Nebot et al (23). Glutathione peroxidase activity was evaluated in plasma by using the Flohé and Gunzler method (24).

Determination of EMPs

A minimum 21-gauge needle was used to perform the venipuncture; the first 3 mL blood was discarded. Blood samples were processed according to the International Society of Thrombosis and Hemostasis recommendations for microparticles analysis. Platelet-free plasma (PFP) obtained after a first centrifugation at 1500 × *g* for 15 min followed by a 2-min centrifugation at 13,000 × *g* was stored frozen until used. For the quantification of EMPs, PFP was incubated by using fluorescein isothiocyanate-conjugated (FITC) Annexin V—a sensitive probe that binds to negatively charged phospholipid surfaces with a higher affinity for phosphatidylserine (Annexin V-FITC apoptosis detection kit I; Bender MedSystem, Vienna, Austria) and anti-CD31 monoclonal antibody (mouse anti-human CD31 phycoeritrine-labeled; BD Biosciences, Mississauga, Canada). The suspension was analyzed by flow cytometry (FC-500; Beckman Coulter Inc). $CD31^+$Annexin V^+ particles >0.1 μm and <1.0 μm were defined as apoptotic EMPs. To reduce the number of microparticles derived from nonendothelial cells, especially platelet-derived microparticles, which may occasionally show low expression of CD31, microparticles were selected as described previously (25). The number of circulating apoptotic EMPs is expressed as EMPs per 1 μL PFP.

Activated EMPs were analyzed by flow cytometry. PFPs were incubated by using monoclonal antibodies against CD144 (phycoeritrine) (mouse monoclonal antibody specific for vascular endothelial-cadherin of human origin; Santa Cruz Biotechnology Inc, Heidelberg, Germany) and CD62 (FITC) (mouse anti-human CD62E-selectin; Serotec, Oxford, United Kingdom). $CD144^+$ $CD62E^+$ particles were defined as activated EMPs, and the number of circulating activated EMPs is expressed as EMPs per 1 mL PFP.

Quantification of EPCs

To quantify EPCs, peripheral blood plasma in EDTA was incubated with anti-human monoclonal antibodies CD34

(mouse monoclonal antibody to the human CD34-antigen; Caltag Laboratories, Chatujak Bangkok, Thailand), CD133 (human monoclonal CD 133/1 antibody; Miltenyi-Biotec, Bergisch Gladbach, Germany), and vascular endothelial growth factor-receptor-2 (VEGFR2) (mouse monoclonal anti-human VEGFR2/KDR; R&D Systems, Minneapolis, MN). Red blood cells and platelets were lysed (Facs Lysing; BD Biosciences), and the remaining mononuclear cells were analyzed by flow cytometry (FC-500; Beckman Coulter Inc). Hematopoietic stem cells ($CD34^+$) were selected via a gating strategy according to the guidelines of the International Society of Hematopoietic and Graft Engineering (26). $VEGFR2^+CD34^+CD133^+$ cells were counted and defined as EPCs, as previously described (27). Each analysis included 50,000 events passing the gate. The number of circulating EPCs was expressed as a percentage of circulating total white blood cells.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc; SPSS Inc, Chicago, IL) was used for the statistical comparisons. The Kolmogorov-Smirnov test did not show a significant departure from normality in the distribution of variance values. To evaluate data variation, an analysis of variance (ANOVA) for repeated measures was used with a post hoc Bonferroni test. Sex was tested as a covariate in all tests, and the sex effect was tested in all ANOVAs performed. Pearson's linear correlation coefficient was calculated. Differences were considered to be significant when $P < 0.05$. All data presented in the text and tables are expressed as means ± SDs.

RESULTS

The baseline characteristics of the 20 healthy participants who completed the 3 dietary intervention periods showed, as expected, that height, waist circumference, triglycerides, and apo B were greater in men than in women (*see* supplementary Table 2 under "Supplemental data" in the online issue). We found no differences by sex in the baseline characteristics or in any of the analysis discussed below.

Analysis of the nutrient composition at the end of each dietary period showed good compliance during the different intervention stages (**Table 1**). The diets were isoenergetic, and nonsignificant differences were found in energy between the 3 diets after the intervention period. The percentage of energy from fat was significantly higher in the SFA diet and the MedDiet than in the CHO-ALA diet. Conversely, the percentage of energy from carbohydrate was significantly greater in the CHO-ALA diet than in the SFA and MedDiet diets. The percentage of energy from SFA was significantly greater in the SFA diet than in the other 2 diets. The percentage of energy from MUFAs was significantly greater in the MedDiet than in the other diets, whereas the percentage of energy from MUFAs was significantly greater in the SFA diet than in the CHO-ALA diet. Finally, the percentage of energy from PUFAs was significantly greater in the CHO-ALA diet than in the SFA diet and was significantly greater in the MedDiet than in the SFA diet. In addition, the percentage of energy from PUFAs was significantly greater in the CHO-ALA diet than in the MedDiet.

Plasma lipid and apo concentrations after ingestion of the 3 diets are shown in **Table 2**. The analysis showed lower concentrations of total cholesterol, LDL cholesterol, and apo B after consumption of the MedDiet ($P = 0.001$, $P = 0.008$, and $P = 0.012$, respectively) and the CHO-ALA diet ($P = 0.003$, $P = 0.011$, and $P = 0.009$, respectively) than after the SFA diet. No significant differences were observed in the other lipid variables between the 3 diets.

Analysis of the variables related to oxidative stress (Table 2) showed lower plasma SOD activity after consumption of the MedDiet than after consumption of the SFA ($P = 0.001$) and CHO-ALA ($P = 0.001$) diets. Similarly, we observed a higher

TABLE 1
Dietary targets for the percentage of nutrients from the different fatty acids and the final composition at the end of the intervention periods[1]

	SFA-enriched diet	CHO-ALA diet	Mediterranean diet
Target (% of energy)			
Fat	38	28	38
SFA	22	<10	<10
MUFA	12	12	24
PUFA	4	8	4
End of intervention			
No. of subjects	20	20	20
Energy (MJ/d)	8.2 ± 1.4[2]	8.3 ± 1.5	8.2 ± 1.3
Fat (% of energy)	40.3 ± 6.4[a]	28.6 ± 5.8[b]	39.1 ± 6.4[a]
SFA (% of energy)	20.7 ± 2.7[a]	8.6 ± 1.9[b]	8.7 ± 1.9[b]
MUFA (% of energy)	13.4 ± 2.3[a]	10.9 ± 1.9[b]	24.2 ± 2.9[c]
PUFA (% of energy)	3.9 ± 0.5[a]	7.6 ± 0.7[b]	4.1 ± 0.5[c]
CHO (% of energy)	43.6 ± 5.5[a]	53.7 ± 4.8[b]	44.6 ± 5.5[a]
Protein (% of energy)	16.0 ± 1.5	17.7 ± 1.5	16.3 ± 1.5
Total α-tocopherol (mg/d)	6.9 ± 2.3[a]	5.2 ± 2.4[a]	18.9 ± 2.7[b]
Total fiber (g)	27.4 ± 2.3	30.2 ± 7.3	27.9 ± 7.0
Total β-carotene (mg/d))	3.2 ± 2.4	3.1 ± 2.4	3.1 ± 2.2

[1] PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; CHO, carbohydrate; CHO-ALA diet, low-fat, high-carbohydrate diet enriched in n−3 PUFAs. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (one-factor ANOVA with a post hoc Bonferroni test).
[2] Mean ± SD (all such values).

TABLE 2
Plasma lipids, apolipoproteins, and oxidative stress variables after the 3 dietary intervention periods[1]

	SFA-enriched diet	CHO-ALA diet	Mediterranean diet	P^2
Total cholesterol (mmol/L)	5.2 ± 0.8[a]	4.8 ± 0.6[b]	4.6 ± 0.6[b]	0.001
Triglycerides (mmol/L)	1.2 ± 0.4	1.0 ± 0.4	1.1 ± 0.4	0.430
HDL cholesterol (mmol/L)	1.4 ± 0.3	1.4 ± 0.3	1.3 ± 0.3	0.370
LDL cholesterol (mmol/L)	3.2 ± 0.7[a]	2.9 ± 0.5[b]	2.8 ± 0.5[b]	0.010
Apolipoprotein A-I (g/L)	1.6 ± 0.27	1.5 ± 0.26	1.4 ± 0.27	0.260
Apolipoprotein B (g/L)	0.9 ± 0.2[a]	0.8 ± 0.1[b]	0.8 ± 0.1[b]	0.008
IRH (% of baseline)	59.6 ± 36.2[a]	88.1 ± 58.3[b]	88.0 ± 61.3[b]	0.010
Superoxide dismutase activity (U/L × 10^{-3})	2.9 ± 1.7[a]	4.2 ± 1.6[b]	1.7 ± 1.0[c]	0.001
β-Carotene (μmol/L × 10^{-3})	45.2 ± 23.6[b]	42.9 ± 25.2[b]	58.2 ± 38.6[a]	0.003
Catalase activity (U/dL × 10)	0.9 ± 0.9	0.8 ± 0.9	0.7 ± 0.7	0.760
Isoprostane (ng/L × 10^{-3})	6.4 ± 2.2[a]	6.1 ± 2.8[a,b]	5.1 ± 1.4[b]	0.050
Lipoperoxides (nmol/L)	0.8 ± 0.4[a]	0.6 ± 0.2[b]	0.6 ± 0.2[b]	0.040
α-Tocopherol (μmol/L)	28.8 ± 8.5	31.8 ± 10.5	31.5 ± 7.9	0.360
Oxidized LDL (U/L)	69.9 ± 18.95	61.9 ± 19.7	59.7 ± 15.5	0.436
Nitric oxide (μmol/L)	51.4 ± 29.7	69.7 ± 41.1	64.8 ± 34.1	0.174
Protein carbonyl activity (mmol/mL × 10^{-3})	5.2 ± 2.0	4.2 ± 2.0	4.2 ± 1.1	0.290
Nitrotyrosine (nmol/L)	8.4 ± 12.4[a]	6.9 ± 8.2[a,b]	4.9 ± 8.9[b]	0.050
Glutathione peroxidase activity (nmol · mL^{-1} · min^{-1})	55.9 ± 7.9	59.1 ± 16.9	52.1 ± 9.9	0.180

[1] All values are means ± SDs; n = 20 patients in each dietary intervention period. SFA, saturated fatty acid; IRH, ischemic reactive hyperemia; CHO-ALA diet, low-fat, high-carbohydrate diet enriched in n−3 polyunsaturated fatty acids. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (one-factor ANOVA with a post hoc Bonferroni test).
[2] Derived by repeated-measures ANOVA followed by Bonferroni's correction for multiple comparisons.

activity of this enzyme after consumption of the CHO-ALA diet than after consumption of the SFA diet ($P = 0.001$). On the other hand, consumption of the MedDiet resulted in higher concentration of β-carotene in plasma than did the CHO-ALA ($P = 0.004$) and SFA ($P = 0.023$) diets and lower urinary isoprostane and plasma nitrotyrosine concentrations than did the SFA diet ($P = 0.025$ and $P = 0.05$, respectively). Furthermore, we found lower plasma lipoperoxide concentrations after the MedDiet ($P = 0.05$) and after the CHO-ALA diet ($P = 0.028$) than after the SFA diet. No significant differences between the 3 diets were found in the other oxidative stress variables measured. In addition, we observed higher IRH after consumption of the MedDiet ($P = 0.03$) and CHO-ALA diet ($P = 0.05$) than after the SFA diet.

The concentrations of circulating microparticles and EPCs are shown in **Figure 1**. Total microparticle and apoptotic EMP concentrations (Figure 1, A and B) were lower after consumption of the MedDiet than after consumption of the SFA and CHO-ALA diets ($P = 0.001$). Consumption of the CHO-ALA diet also resulted in lower total microparticle and apoptotic EMP concentrations than did the consumption of the SFA diet ($P = 0.001$). Similar results were found from the analysis of the activated EMP concentrations (Figure 1C). Activated EMP concentrations were lower after consumption of the MedDiet than after consumption of the SFA diet ($P = 0.001$) and CHO-ALA diet ($P = 0.001$). Similarly, we observed a lower concentration of activated EMP concentrations after consumption of the CHO-ALA diet than after consumption of the SFA diet ($P = 0.05$). In addition, the number of EPCs (Figure 1D) was greater after consumption of the MedDiet than after consumption of the SFA or CHO-ALA diet ($P = 0.001$ in both cases). No significant differences in the EPC number were found between the SFA and the CHO-ALA diets.

Pearson's rank correlation coefficients of the circulating EPCs and plasma β-carotene concentrations are shown in **Figure 2**. The analysis showed a positive correlation between circulating EPC and plasma β-carotene concentrations. We found no significant correlation between circulating EPCs and the other studied variables.

DISCUSSION

Our results indicate that consumption of the MedDiet was associated with a lower concentration of total microparticles and activated and apoptotic EMPs and a higher number of EPCs than was consumption of the SFA or CHO-ALA diet. We similarly observed lower plasma microparticle and activated and apoptotic EMP concentrations after the CHO-ALA diet than after the SFA diet and lower plasma SOD activity and higher plasma β-carotene concentrations after the MedDiet than after the other 2 diets. We also found lower urinary isoprostane and plasma nitrotyrosine concentrations after the MedDiet than after the SFA diet. Furthermore, IRH was greater after the MedDiet and the CHO-ALA diet than after the SFA diet. These findings suggest a decrease in endothelial damage and endothelial dysfunction associated with improved regenerative capacity of the endothelium after the intake of the MedDiet, which is rich in virgin olive oil, as compared with the other 2 diets.

Circulating EMPs, a risk factor for several pathologic processes (3), are influenced by diet (28, 29). Del Turco et al (29) found that the administration of n−3 PUFAs (eicosapentaenoic acid and docosahexaenoic acid) resulted in a lower number of platelet- and monocyte-derived microparticles than did a low dose of olive oil, but that EMPs were not influenced by treatment. However, our results did not agree with theirs. Interestingly our analysis of plasma EMPs showed that consumption of the MedDiet led to lower plasma concentrations of these microparticles than did consumption of the other 2 diets. This discrepancy may have been due to the fact that Del Turco et al administered a low dose of olive oil (5.2 g/d) in the form of

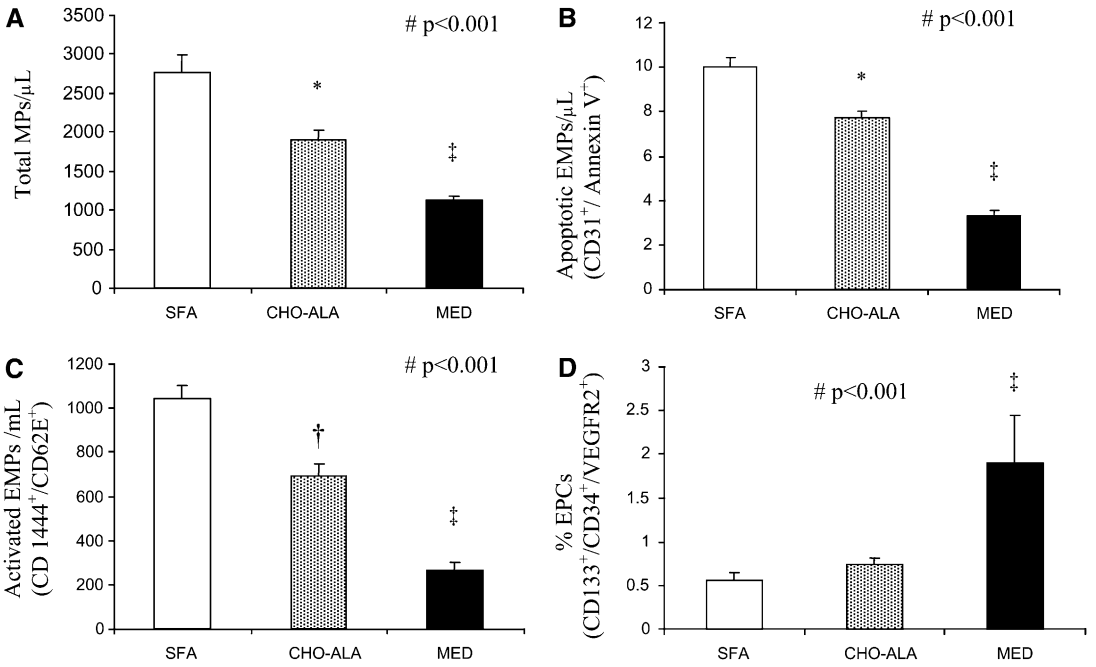

FIGURE 1. Mean (±SE) plasma concentrations of microparticles (MPs) (A), apoptotic endothelial MPs (EMPs) (B), and activated EMPs (C) and percentage of endothelial progenitor cells (EPCs) (D) after the 3 dietary intervention periods. SFA diet, saturated fat–enriched diet; MED, Mediterranean diet; CHO-ALA diet, low-fat, high-carbohydrate diet enriched in n−3 polyunsaturated fatty acids. n = 20 patients in each dietary intervention period. #ANOVA for repeated measured followed by Bonferroni's correction for multiple comparisons. *,†Significantly different from the SFA diet: *$P < 0.001$, †$P < 0.05$. ‡Significantly different from the SFA and CHO-ALA diets, $P < 0.001$.

capsules, whereas we administered an average of 51 g extra virgin olive oil daily in a natural form for cooking and dressing foods and salads and in the context of a Mediterranean diet.

Circulating EMP concentrations are thought to reflect a balance between cell stimulation, proliferation, and death. Proinflammatory, prothrombotic, proapoptotic, and oxidative substances have all been shown to stimulate EMP release (3). Potentially damaging oxidative stress can be generated by an excess of reactive oxygen species, which are kept in check by endogenous cellular antioxidant mechanisms. To ameliorate and cope with injury from oxidative damage and maintain redox homeostasis, aerobic organisms have developed efficient defense mechanisms against enzymatic and nonenzymatic antioxidants. SOD specializes in eliminating superoxide anion radicals derived from extracellular stimulants, including oxidative insults. Previous studies with Wistar rats showed that olive oil lowers antioxidant SOD activity (30). Our results agree with these findings, because we observed lower SOD activity after consumption of the olive oil–rich MedDiet than after consumption of the other 2 diets. These findings may indicate a reduced production of superoxide radicals as a result of the lower level of oxidative stress induced by the MedDiet. This metabolic situation generates a reduced induction of the antioxidant defense system, which is regulated by transcription factor Nrf2, which produces a lower concentration of oxidative enzymes such as SOD. Li et al (31) suggested that Nrf2 may play an important role in defending against oxidative stress, possibly via the activation of cellular antioxidant machinery. In addition, in vivo and in vitro data suggest that many dietary compounds can differentially regulate Nrf2-mediated antioxidant/antiinflammatory signaling pathways as the first line of defense or induce apoptosis once the cells have been damaged. Compliance with the traditional Mediterranean diet has been associated with a reduction in coronary heart disease, cancer, and overall mortality (32). This protective effect has been attributed, at least in part, to the richness of this diet in antioxidants (33). The Prevención con Dieta Mediterránea (PREDIMED) study suggested that a decrease in oxidative damage could be one of the protective mechanisms by which the Mediterranean diet exerts its protective effects on the development of coronary heart disease (33). We therefore speculate that lower circulating EMP

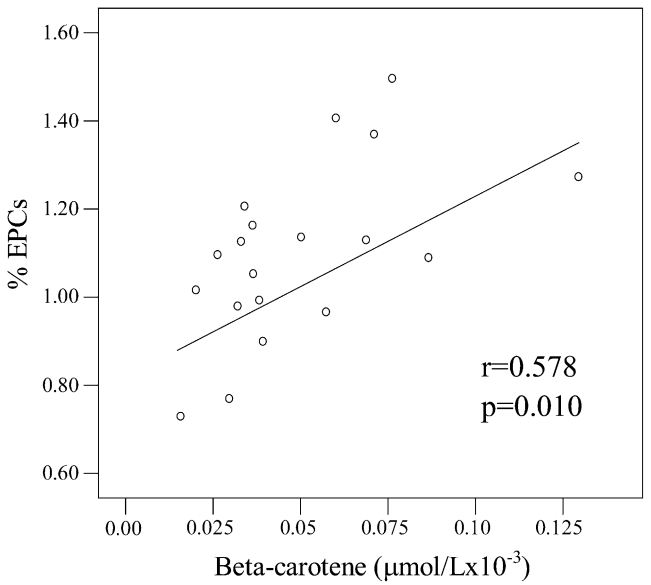

FIGURE 2. Pearson's correlation (r) between the percentage of endothelial progenitor cells (EPCs) and plasma β-carotene concentrations. n = 20 patients in each dietary intervention period.

concentrations are due in part to reduced oxidative damage, induced after consumption of the MedDiet and possibly as a result of the antioxidant effect of the micronutrients present in the MedDiet and virgin olive oil.

Cardiovascular disease risk factors reduce the availability of EPCs by affecting their mobilization and integration into injured vascular sites (34). Presumably, altering risk factors by modulating the concentration of oxidative stress, NO activity, or other physiologic processes could directly influence the mobilization or half-life of EPCs (35). A growing body of evidence suggests that aging (36) and cardiovascular disease risk factors affect the number and properties of EPCs. The demonstration of low plasma EPC concentrations is important, because it reflects impaired ability of EPCs to participate in the repair of injured endothelium (10, 11, 13). Endothelial damage ultimately represents a balance between the magnitude of injury and the capacity for repair. As a result of their reduced repair capacity, low EPC concentrations have been found to be associated with endothelial dysfunction (37). Our findings suggest that both the SFA and CHO-ALA diets could increase endothelium injury and impair its repair capacity. Furthermore, the MedDiet may improve the recovery and regenerative capacity of the endothelium and thus prevent the development of cardiovascular and other chronic diseases and their associated risk factors. In support of this hypothesis, we noted greater IRH and lower plasma nitrotyrosine concentrations after the MedDiet. It is possible that the decrease in EMP concentrations that we observed after the consumption of the MedDiet was associated with a higher concentration of circulating EPCs, which favors the balance between EPCs and EMPs and increases the regenerative capacity of the endothelium.

Many studies have consistently reported an association between lipid metabolism and the biology of human EPCs (37). In our study, we observed that the MedDiet and the CHO-ALA diet equally reduced plasma LDL-cholesterol concentrations; nevertheless, we did not observe significant differences in triglyceride concentrations between these 2 diets. Although marine n−3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) have been found to effectively lower triglyceride concentrations, ALA (an n−3 fatty acid of plant origin) has a much more limited effect in this direction (38), which may be the underlying cause of the lack of differences in triglyceride concentrations between the CHO-ALA diet and the MedDiet. However, only the MedDiet improved the regenerative capacity of the endothelium, as was indicated by the increase in the number of EPCs. Other mechanisms, such as reduced oxidative stress induced by ingesting the MedDiet, may therefore be involved in the explanation of this phenomenon (39). In agreement with this hypothesis, we observed lower urinary isoprostane concentrations after the MedDiet. This could have been due to the fact that the MedDiet has a greater antioxidant capacity, which improves EPC survival rates (40) and improves endothelial dysfunction. Another study that investigated the role of antioxidants on EPC numbers and function studied β-carotene, which has been shown to influence homing on EPCs (40). In our study, the MedDiet resulted in an increase in β-carotene concentrations that correlated with higher concentrations of circulating EPCs. Thus, higher plasma β-carotene concentrations might induce an increase in EPC numbers, which favors the regenerative capacity of the endothelium.

In conclusion, consumption of the MedDiet improved the regenerative capacity of the endothelium as a result of a better balance between damage and repair. The mechanisms involved in these processes may be associated with a reduced liberation of free radicals and less oxidative stress, due to the protective effect of the antioxidant components of the virgin olive oil–rich MedDiet.

The authors' responsibilities were as follows—JL-M: had full access to and takes responsibility for the integrity of the data in the study; JL-M and FP-J: conception and design; CM, EMY-S, AG-R, JD-L, FMG-M, FR, JL-M, RR, and JL-M: data acquisition; CM, JL-M, RR, JC, and FP-J: data analysis and interpretation; CM, JD-L, PP-M, JL-M, and FP-J: drafting of the manuscript; JD-L, PP-M, JL-M, and FP-J: critical revision of the article for important intellectual content; AG-R, JD-L, and JL-M: provision of study materials or patients; PP-M, JL-M: statistical expertise; JL-M: funding; RR, EMY-S, JC, AG-R, and JD-L: technical or logistic support; and JL-M and FP-J: study supervision. All decisions regarding the design, conduct, collection, analysis, or interpretation of the data and the decision to submit the manuscript for publication were made independently by the authors. None of the authors had any conflicts of interest that could have affected the performance of the work or the interpretation of the data.

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