Changes in Ultrasound-Assessed Carotid Intima-Media Thickness and Plaque With a Mediterranean Diet

A Substudy of the PREDIMED Trial

Aleix Sala-Vila, Edwin-Saúl Romero-Mamani, Rosa Gilabert, Isabel Núñez, Rafael de la Torre, Dolores Corella, Valentina Ruiz-Gutiérrez, María-Carmen López-Sabater, Xavier Pintó, Javier Rekondo, Miguel-Ángel Martínez-González, Ramon Estruch, Emilio Ros

Objective—The Prevención con Dieta Mediterránea (PREDIMED) trial showed that a Mediterranean diet (MedDiet) supplemented with either extra virgin olive oil or 30 g/d of mixed nuts reduced incident cardiovascular events compared with a control (low fat) diet. The mechanisms of cardiovascular protection afforded by MedDiets remain to be uncovered. We assessed the effect of both supplemented MedDiets on internal carotid intima-media thickness (ICA-IMT) and plaque height, the ultrasound features that best predict future cardiovascular events, in subjects at high cardiovascular risk.

Approach and Results—In a PREDIMED subcohort (n=175), plaque height and carotid IMT of 3 prespecified segments (ICA, bifurcation, and common) were sonographically assessed at baseline and after intervention for a mean of 2.4 years. We evaluated 164 subjects with complete data. In a multivariate model, mean ICA-IMT progressed in the control diet group (mean [95% confidence interval], 0.052 mm [−0.014 to 0.118 mm]), whereas it regressed in the MedDiet+nuts group (−0.084 mm [−0.158 to −0.010 mm]; P=0.024 versus control). Similar results were observed for maximum ICA-IMT (control, 0.188 mm [0.077 to 0.299 mm]; MedDiet+nuts, −0.030 mm [−0.153 to 0.093 mm]; P=0.034) and maximum plaque height (control, 0.106 mm [0.001 to 0.210 mm]; MedDiet+nuts, −0.091 mm [−0.206 to 0.023 mm]; P=0.047).

Conclusions—Compared with a control diet, consumption of a MedDiet supplemented with nuts is associated with delayed progression of ICA-IMT and plaque. The results contribute mechanistic evidence for the reduction of cardiovascular events observed in the PREDIMED trial.


Key Words: carotid intima-media thickness ■ carotid stenosis ■ diet, Mediterranean ■ nuts ■ olive oil

Cardiovascular disease (CVD) mortality rates in Spain and other Mediterranean areas are low compared with those of Northern Europe1,2 and the United States.2 This North-to-South gradient in CVD risk could be explained in part by local dietary habits, such as adherence to the Mediterranean diet (MedDiet). This hypothesis is supported by observational studies,3,4 a secondary prevention trial (the Lyon Diet Heart Study),5 and the recent results of the randomized controlled clinical trial Prevención con Dieta Mediterránea (PREDIMED; www.predimed.org). In the PREDIMED study, conducted in 7447 participants at high cardiovascular risk but no CVD at enrollment, advice on a low-fat diet after intervention for a mean of 4.8 years.
The biological mechanisms by which EVOO and nuts, main components of the MedDiet intervention in PREDIMED, protect against CVD remain to be uncovered. Both foods have a favorable fatty acid profile. Their richness in bioactive phytochemicals with antioxidant and anti-inflammatory properties probably explains why their consumption decreases circulating inflammatory biomarkers related to atherogenesis. Thus, it is plausible that EVOO and nuts might reduce CVD via an antiatherogenic effect, thus delaying the development of atherosclerosis, the pathological basis of most CVD events.

Artery vessel wall enlargement, an early feature of atherosclerosis, can be easily evaluated by ultrasound determination of carotid artery intima-media thickness (IMT), a noninvasive, well-standardized, and validated imaging technique. IMT has been usually measured in the common carotid artery (CCA) because this carotid segment is easily visualized and

Table 1. Baseline Clinical Characteristics, Lipid Profiles, Treatment Regimes, and Sonographic Variables of the Study Population by Group Allocation

<table>
<thead>
<tr>
<th></th>
<th>Control Diet (n=61)</th>
<th>MedDiet+EVOO (n=57)</th>
<th>MedDiet+Nuts (n=46)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>23 (38)</td>
<td>28 (49)</td>
<td>25 (54)</td>
<td>0.203</td>
</tr>
<tr>
<td>Age, y</td>
<td>66 (64–67)</td>
<td>67 (65–68)</td>
<td>66 (64–67)</td>
<td>0.481</td>
</tr>
<tr>
<td>Family history of early-onset CVD, n (%)</td>
<td>27 (44)</td>
<td>27 (47)</td>
<td>21 (46)</td>
<td>0.876</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.7 (28.8–30.6)</td>
<td>29.2 (28.2–30.2)</td>
<td>29.9 (28.9–30.9)</td>
<td>0.596</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>99 (97–101)</td>
<td>100 (97–102)</td>
<td>101 (99–103)</td>
<td>0.548</td>
</tr>
<tr>
<td>Leisure-time physical activity, MET-min/d</td>
<td>274 (214–334)</td>
<td>282 (224–341)</td>
<td>300 (227–373)</td>
<td>0.847</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>10 (16)</td>
<td>10 (18)</td>
<td>7 (15)</td>
<td>0.951</td>
</tr>
<tr>
<td>Former smoker, n (%)</td>
<td>19 (31)</td>
<td>13 (23)</td>
<td>18 (39)</td>
<td>0.200</td>
</tr>
<tr>
<td>Dyslipidemia, n (%)</td>
<td>50 (82)</td>
<td>42 (74)</td>
<td>33 (72)</td>
<td>0.402</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>206 (197–216)</td>
<td>216 (206–226)</td>
<td>212 (201–222)</td>
<td>0.386</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>51 (48–54)</td>
<td>53 (49–57)</td>
<td>50 (47–53)</td>
<td>0.433</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>133 (124–141)</td>
<td>133 (124–142)</td>
<td>134 (125–143)</td>
<td>0.974</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>128 (101–155)</td>
<td>132 (119–148)</td>
<td>139 (112–167)</td>
<td>0.419</td>
</tr>
<tr>
<td>Use of statins, n (%)</td>
<td>34 (56)</td>
<td>25 (44)</td>
<td>20 (43)</td>
<td>0.328</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>51 (84)</td>
<td>41 (72)</td>
<td>35 (76)</td>
<td>0.306</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>152 (148–157)</td>
<td>147 (142–152)</td>
<td>152 (146–158)</td>
<td>0.284</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>83 (81–85)</td>
<td>79 (77–82)</td>
<td>80 (78–83)</td>
<td>0.666</td>
</tr>
<tr>
<td>Use of antihypertensive drugs, n (%)</td>
<td>43 (71)</td>
<td>36 (63)</td>
<td>32 (70)</td>
<td>0.661</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>118 (107–130)</td>
<td>120 (109–131)</td>
<td>120 (109–131)</td>
<td>0.752</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>20 (33)</td>
<td>22 (39)</td>
<td>16 (35)</td>
<td>0.801</td>
</tr>
<tr>
<td>Use of oral antidiabetic agents, n (%)</td>
<td>15 (25)</td>
<td>12 (21)</td>
<td>7 (15)</td>
<td>0.495</td>
</tr>
<tr>
<td>Use of insulin, n (%)</td>
<td>4 (7)</td>
<td>3 (5)</td>
<td>2 (4)</td>
<td>0.880</td>
</tr>
</tbody>
</table>

Carotid ultrasound†

| CCA-IMTmean, mm | 0.79 (0.75–0.83) | 0.76 (0.72–0.80) | 0.81 (0.77–0.86) | 0.247 |
| CCA-IMTmax, mm  | 0.95 (0.89–1.02) | 0.97 (0.90–1.04) | 1.01 (0.93–1.09) | 0.543 |
| BIF-IMTmean, mm | 0.97 (0.91–1.04) | 0.92 (0.85–0.99) | 0.98 (0.90–1.06) | 0.431 |
| BIF-IMTmax, mm  | 1.38 (1.25–1.52) | 1.34 (1.19–1.48) | 1.44 (1.28–1.59) | 0.680 |
| ICA-IMTmean, mm‡ | 0.74 (0.66–0.82) | 0.73 (0.65–0.82) | 0.82 (0.73–0.92) | 0.297 |
| ICA-IMTmax, mm‡  | 1.01 (0.86–1.16) | 1.05 (0.89–1.20) | 1.12 (0.95–1.30) | 0.608 |
| Plaque, mm§       | 2.10 (1.88–2.32) | 1.89 (1.67–2.12) | 2.13 (1.88–2.39) | 0.308 |

Data are expressed as mean (95% confidence interval) except for quantitative variables, expressed as n (%). BIF-IMT indicates intima-media thickness of bifurcation; BMI, body mass index; CCA-IMT, intima-media thickness of common carotid artery; CVD, cardiovascular disease; EVOO, extra virgin olive oil; HDL, high-density lipoprotein; ICA-IMT, intima-media thickness of internal carotid artery; LDL, low-density lipoprotein; MedDiet, Mediterranean diet; and MET-min, minutes at a given metabolic equivalent level (units of energy expenditure in physical activity, 1 MET-min is roughly equivalent to 1 kcal).

*χ² test and ANOVA, as appropriate.
†Adjusted for sex, age, ever smoking, BMI, energy intake, use of statins, use of antidiabetic drugs, and use of antihypertensive drugs.
‡Measured in 155 participants (n=58, n=55, and n=42, respectively).
§Measured in subjects with focal intrusions into the lumen ≥1.2-mm thick (n=46, n=46, and n=34, respectively).
CCA-IMT is a more reproducible measure than IMT of other carotid segments, such as the bifurcation or internal carotid artery (ICA). CCA-IMT has been widely used as a surrogate marker of future coronary events, but there is increasing evidence that maximum ICA-IMT and particularly carotid plaque are more representative of atherosclerosis and better predict CVD than CCA-IMT.

In a prior PREDIMED substudy, participants with a high carotid atherosclerotic burden allocated to the MedDiets supplemented with either EVOO or nuts showed regression of mean CCA-IMT at 1 year compared with those assigned to the control diet. However, changes in IMT of other carotid segments or plaque were not investigated in that study. We hypothesized that, in subjects at high vascular risk participating in the PREDIMED study, MedDiets supplemented with EVOO or nuts for >2 years would favorably influence the carotid lesions that best predict CVD risk, namely maximum ICA-IMT and plaque height.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

All 200 participants successfully underwent baseline ultrasound measurements of carotid IMT and plaque, but 25 participants refused to undergo a second procedure. No significant differences were observed between the refusers and compliers in terms of baseline clinical characteristics, lipid profiles, treatment regimes, and sonographic variables (data not shown). Complete clinical and ultrasound information was available in 164 participants after intervention for a mean of 2.4 years (range, 1.6–3.1 years).

Participants in the 3 groups were well balanced for baseline clinical characteristics (Table 1). As shown in Table 2, the main nutrient changes during follow-up were increases in polyunsaturated fatty acids in the MedDiet with nuts group (mean [95% confidence interval], 1.7 g/d [0.6–2.8...
In this randomized clinical trial conducted in subjects at high cardiovascular risk but no CVD at enrollment, we found that, in spite of supplemental EVOO, monounsaturated fatty acid intake did not increase in the corresponding MedDiet group because participants replaced customarily used common olive oil by the extra virgin variety supplied. Subjects in the 3 groups reported similar baseline adherence to the MedDiet, but the 14-item score improved only in the 2 MedDiet groups. No between-group differences were observed in consumption of fruits, vegetables, cereals, meats, dairy products, desserts, or alcohol were observed (data not shown).

No between-group differences were observed in changes of weight, waist circumference, systolic and diastolic blood pressure, fasting glucose, or the lipid profile. Similarly, no differences were seen regarding the incidence of diabetes mellitus or metabolic syndrome. The Figure depicts changes from baseline in main carotid outcomes (ICA-IMTmean [upper], ICA-IMTmax [middle], and plaque max [lower]) in the 3 treatment arms. Whereas a significant progression was observed for ICA-IMTmax (0.188 mm [0.077 to 0.299 mm]) and plaque max (0.106 mm [0.001 to 0.210 mm]) in participants in the control diet group, those allocated to MedDiet with nuts showed significant regression (ICA-IMTmean, −0.084 mm [−0.158 to −0.010 mm]) or arrested progression (ICA-IMTmax and plaque max, −0.030 mm [−0.153 to 0.093 mm] and −0.091 mm [−0.206 to 0.023 mm], respectively). No significant changes were observed in subjects on the MedDiet with EVOO group. Changes in secondary carotid atherosclerosis outcomes were similar between the 3 treatment groups (Table 3).

When comparing baseline and the time point closest to the second sonographic measurement (Table 4), changes in objective biomarkers were mean increases from baseline to end of the trial of 0.10% for plasma α-linolenic acid in the MedDiet with nuts group and of 337 μg/L in urinary hydroxytyrosol in the MedDiet with EVOO group (P=0.003 versus control diet group for both), respectively, indicating good compliance with the supplemental foods. Attesting to the validity of the food frequency questionnaire, the Pearson correlation coefficient between calculated dietary α-linolenic acid and plasma α-linolenic acid was 0.263 (P=0.024). Likewise, the plasma change of α-linolenic acid was significantly correlated to changes in total carotid plaque max (millimeters). As seen, allocation into the MedDiet+nuts group versus the control diet group (B=−0.198 [95% confidence interval, −0.343 to −0.054], use of antihypertensive drugs at baseline (0.132 [0.001 to 0.263]), and baseline plaque max (−0.137 [−0.217 to −0.057]) were the only variables significantly associated with changes in plaque max (R²=0.122).

**Discussion**

Discussion
compared with a control group based on advice to follow a low-fat diet, intervention with a MedDiet supplemented with 30 g/d of mixed nuts for a mean of 2.4 years induced regression of ICA-IMT mean and delayed the progression of both ICA-IMT max and plaque max, the ultrasound features that best predict future CVD events. Although ICA-IMT max and plaque max progressed in the control group, there were no changes in the MedDiet supplemented with EVOO group.

A consistent body of evidence, mostly from prospective studies, points to the MedDiet as the dietary pattern most beneficial for cardiovascular health. The richness of the plant-based MedDiet in potentially beneficial foods, such as fruit, vegetables, legumes, nuts, cereals, and olive oil, is believed to explain its cardioprotective effects. Our results reinforce the notion that a MedDiet enriched with tree nuts could be a sound strategy to delay the progression of atherosclerosis, the harbinger of future cardiovascular events.

A recent report from the PREDIMED study has shown that MedDiets supplemented with either EVOO or nuts reduce incident CVD events compared with advice on a low-fat diet. Of note, the MedDiet with nuts was also associated with a significant 49% reduction in risk of stroke. Our findings of delayed IMT progression and, more importantly, delayed plaque progression might explain in part the reduction of CVD events, particularly stroke, observed in the arm supplemented with nuts in the PREDIMED trial. This adds evidence to that collected in earlier reports of the PREDIMED trial on the salutary effects of the enhanced MedDiets on traditional cardiovascular risk factors; novel risk factors, such as markers of oxidation, inflammation, or endothelial dysfunction; circulating immune cell activation; and the incidence of high cardiovascular risk conditions, namely the metabolic syndrome and diabetes mellitus.

Mean baseline IMT values at all carotid segments were close to those reported for Southern European subjects of similar age and cardiovascular risk in an ecological study conducted in 5 European countries, which concurs with reportedly low IMT values in Mediterranean areas compared with Northern Europe, a geographical gradient of carotid IMT similar to that observed for CVD risk. In this regard, our findings of reduced IMT progression with enhanced MedDiets suggest that nutritional factors might play a role in delaying the progression of atherosclerosis in Southern Europe. Few clinical trials have examined the effects of the MedDiet on IMT progression. In the Dietary Intervention Randomized Controlled Trial-Carotid study, carotid IMT was determined before and after 2 years of nutrition intervention with 3 weight-loss diets, including 1 MedDiet arm. Participants were younger, mostly male, and had a lower baseline IMT. No significant IMT changes were observed, but regression of carotid vessel wall volume was documented, suggesting an antiatherosclerotic effect.

Because of physiological and mechanical reasons (blood flow velocity and shear stress), the various carotid segments are differently prone to atherosclerosis. In this sense, CCA-IMT is taken as a measure of early atherosclerosis when enlarged, whereas ICA-IMT increases are more representative of advanced atherosclerosis. Therefore, the beneficial effect on ICA-IMT rather than CCA-IMT suggests that if bioactive components of the MedDiet with nuts (such as polyunsaturated fatty acids, arginine, and beneficial minerals such as calcium, potassium, and magnesium) prevent cardiovascular events, they must do so in part by delaying the progression of atherosclerotic disease in critical arterial beds rather than...
by preventing its onset. We found that baseline plaque height had the strongest association with the change in plaque\textsubscript{max} (nearly 0.14 mm regression for each millimeter of plaque height at baseline). Thus, it seems plausible that participants with a high atherosclerotic burden might be those who benefit most from the intervention, a notion put forth in a previous PREDIMED report.\textsuperscript{23} Although reduction in height after the MedDiet with nuts suggests that plaques have been stabilized (ie, rendered less vulnerable to rupture), characterization by more precise vascular imaging techniques, such as multiple contrast magnetic resonance,\textsuperscript{24} might provide firmer evidence.

Our study has limitations, such as the relatively small number of study subjects, with low statistical power to detect significant between-diet changes in cardiovascular risk factors. The small size of the sample was circumvented by the inclusion of confounding variables in the multivariate analyses, although cardiovascular risk factors and medication use were balanced between the 3 groups both at baseline and during follow-up. The main reason for a lack of a prespecified sample size is that, as far as we know, there are no published data on progression of the main outcomes (ICA-IMT\textsubscript{max} and plaque\textsubscript{max}) in white populations that we could use to make assumptions for power calculations. The majority of clinical trials assessing changes of IMT progression with different interventions have used CCA-IMT. There are few published data on the progression of total plaque area,\textsuperscript{25,26} but we did not measure this variable. Ours was an exploratory study with a sample large enough as to find evidence of changes in the progression of all main outcome variables between the study groups. Also, the generalization of our findings to younger and healthier individuals from other geographical locations is uncertain, even though the MedDiet has also shown cardiovascular benefit in adult US populations.\textsuperscript{3} There are also strengths to our study, such as the evaluation of dietary adherence by objective biomarkers and the sonographic characterization of all carotid segments, important given the questionable predictive value of reduced cardiovascular events of IMT regression merely assessed at the CCA.\textsuperscript{27,28}

In conclusion, in this randomized clinical trial conducted in subjects at high cardiovascular risk, we found that, compared with a control diet, intervention with a MedDiet supplemented with 30 g/d of nuts for a mean of 2.4 years was associated with regression or delayed progression of the surrogate markers of future CVD (ICA-IMT and plaque). These findings add mechanistic evidence for the reduction of cardiovascular events observed in the arm supplemented with nuts in the PREDIMED trial.\textsuperscript{9} Our results also suggest that a MedDiet rich in polyunsaturated fats and bioactive molecules from plant sources delays atherosclerosis progression, which likely reduces plaque vulnerability and proneness to rupture, thereby preventing ischemic events.

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All authors have made a substantial contribution to the study, as follows: (1) conception and design (R. Estruch, E. Ros, A. Sala-Vila), and analysis and interpretation of data (all authors); (2) drafting of the manuscript (E. Ros, A. Sala-Vila) and revising it critically for important intellectual content (all other authors); and (3) final approval of the manuscript (all authors). We thank Emili Corbella for expert assistance with statistical analyses.

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Disclosures

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In the Prevención con Dieta Mediterránea (PREDIMED) trial in subjects at high cardiovascular risk, Mediterranean diets enriched with either virgin olive oil or nuts reduced incident cardiovascular disease by 30% compared with a control diet. Data are scarce on the effects of the Mediterranean diets on carotid atherosclerosis progression. The novel results of this PREDIMED study in 175 participants are that supplementation of the Mediterranean diet with 30 g/d of mixed nuts for a mean of 2.4 years induced regression and delayed progression of internal carotid artery intima-media thickness and plaque, respectively, the ultrasound features that best predict future cardiovascular events. These findings contribute mechanistic evidence to the cardiovascular protection of the Mediterranean diet observed in the PREDIMED trial. Bioactive components of this diet might prevent cardiovascular disease in part by delaying the progression of atherosclerosis.
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MATERIAL AND METHODS

Subjects
The present clinical trial was conducted in a sub-cohort of the PREDIMED study (ISRCTN35739639). The protocol has been reported in detail elsewhere (1). For this analysis we randomly selected 200 participants recruited in the Barcelona-North site between February 2008 and July 2009. Participants were men aged between 55 and 80 years and women aged between 60 and 80 years at high cardiovascular risk but no cardiovascular disease at enrolment. Criteria for eligibility were the presence of either type-2 diabetes or at least 3 cardiovascular risk factors: current smoking, hypertension, dyslipidemia, overweight or obesity, and family history of early-onset coronary heart disease. Main exclusion criteria were a prior history of cardiovascular disease, any severe chronic illness, substance abuse, and history of allergy or intolerance to olive oil or nuts (supplemental foods given in two arms of the study) (1).

We obtained data about medical history, medication use, lifestyle, anthropometric and blood pressure measurements, and laboratory determinations in fasting venous blood and urine samples. All measurements were taken at baseline and yearly thereafter using the same procedures. Carotid ultrasonography was performed only at baseline and at the two year visit.

The study protocol was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the ethics committee of the institution. Written informed consent was obtained from all subjects at the first visit.

Assessment of risk factors
Participants were considered as diabetic, hyperlipidemic or hypertensive if they had a previous diagnosis of these conditions and/or they were treated with antidiabetic, cholesterol-lowering, or antihypertensive agents, respectively. Smoking status was categorized into never, current or past smoking according to self-reports. Physical activity was determined with the Minnesota Leisure-Time Physical Activity questionnaire. Height, weight, and waist circumference were measured with standard methods. Trained personnel measured systolic and diastolic blood pressure in triplicate with a validated semi-automatic oscillometer (Omron HEM-705CP; Hoofddorp, The Netherlands).

Diets
The dietary habits of participants were assessed using a validated 137-item food frequency questionnaire (2) completed by a trained dietician in face-to-face interviews. Participants were asked about the frequency of consumption of each food item during the past year, specifying usual portion sizes. Nine possibilities of frequency were offered, from never to >6 times per day. Nutrient intakes were computed using Spanish food composition tables and were adjusted for energy intake by the residual method (3). After the screening visit, suitable candidates were randomly assigned to one of three interventions: MedDiet with extra-virgin olive oil (EVOO), MedDiet with nuts or control diet. The two groups allocated MedDiets received intensive education to follow the MedDiet and supplemental foods at no cost. EVOO (1 L/week) was provided to one group and 30 g/day of mixed nuts (15 g walnuts, 7.5 g hazelnuts and 7.5 g almonds) to the other group. In the control group, participants received also intensive education to follow a low-fat diet and small non-food gifts.

At baseline and quarterly, diabeticians run individual and group sessions separately for each group. In each session, a dietary screener of adherence to the MedDiet was used to track changes of dietary habits. The score was determined by 12 questions on food consumption frequency and 2 questions on food intake habits considered characteristic of the MedDiet (each question scored 0 or 1) (4).

Carotid ultrasonography
B-mode ultrasound imaging of the carotid arteries was performed with an ultrasound apparatus (Sequoia Acuson; Siemens, Erlangen, Germany) equipped with a multi-frequency transducer (5-8 MHz) and ECG synchronization. A standardized imaging protocol was used for intima-media thickness (IMT) measurements, as described in detail elsewhere (5). Main outcomes were maximum plaque height (plaque_{max}) and mean and maximum IMT in the internal carotid artery. Secondary outcomes were mean and maximum IMT at the bifurcation and common carotid artery. IMT was defined as the average of multiple distance readings between the far wall lumen–intima and media-adventicia interfaces taken bilaterally at common carotid artery 1 cm prebifurcation, bifurcation, and internal carotid artery 1 cm after the flow divider. Plaques were sought by using B-mode and colour Doppler examinations in both longitudinal and transverse planes to take into consideration circumferential asymmetry and were defined as focal intrusions into the lumen ≥1.2 mm thick. IMT and plaque_{max} were measured offline by using edge-finding software in the predefined segments of the arterial wall. All procedures were performed by 2 certified sonographers (RG and IN) who were blinded to clinical information. Inter-observer variability was examined in 15 subjects. The maximum CV of paired readings of IMT at any site was 5.3% and that of plaque_{max}, 9.3%.

Laboratory determinations
Fasting blood and spot urine samples were collected at baseline and at 1 and 3 years of follow-up or at the study termination, whichever came first. Serum lipids and glucose concentrations were determined by standard enzymatic methods in the hospital clinical laboratory. To determine adherence to supplemental foods, we measured at once both baseline and changes of urinary hydroxytyrosol (the main phenolic compound in EVOO) and plasma proportions of α-linolenic acid (as a measure of adherence to nut [walnut] consumption) at the closest time-point to the second ultrasound measurement in a random sub-samples of participants (32% and 44%, respectively), as described (6).

Statistical analyses
When appropriate, the ANOVA or chi-square tests were used to assess whether the 25 participants that refused to undergo a second ultrasound measurement were comparable to the study participants in terms of baseline clinical characteristics, lipid profiles, treatment regimes and sonographic variables.

Because many subjects had been treated with hypolipidemic drugs, and high-dose statins may induce the regression of IMT and plaque, we adjusted for statin treatment when we assessed the associations of other covariates with IMT and plaque. To this end, for each subject we standardized the dose received of statin drugs to simvastatin.

Baseline imbalances in cardiovascular risk factors between treatment groups were assessed by chi-square and ANOVA, as appropriate.

Between-group differences in baseline carotid variables were assessed by ANCOVA with adjustment for sex, age, ever smoking, BMI, energy intake, use of statins, use of antidiabetic drugs, and use of antihypertensive drugs. The effect of intervention on changes of carotid outcomes was assessed by ANCOVA adjusting for the variables listed above plus follow-up time and in-trial changes in statin dose standardized to simvastatin.

Between-group differences in food and nutrient consumption were also assessed by ANCOVA with adjustment for sex, age, ever smoking, BMI, energy intake, use of statins, use of antidiabetic drugs, and use of antihypertensive drugs at baseline. When appropriate, multiple comparisons for ANCOVA were assessed by Bonferroni post-hoc tests.

To investigate whether change in plaque_{max} was predicted by baseline plaque height, we performed a multivariate linear regression adjusting for sex, age, ever smoking, BMI, energy intake, use of statins, use of antidiabetic drugs, and use of antihypertensive drugs at baseline, in-trial changes in statin dose standardized to simvastatin, allocation into the MedDiet + EVOO group (vs. control group) and allocation into the MedDiet + NUTS group (vs. control group).
Between-group differences in biomarkers of adherence to dietary intervention were assessed by ANOVA with Bonferroni post-hoc test.

In those subjects free of diabetes mellitus at baseline (n = 106), we calculated the odds ratios (OR) and 95% confidence intervals (CI) of the newly-reported incidence by a logistic regression model including sex, age, baseline energy intake, BMI at baseline, allocation into MedDiet + EVOO group (vs. control group), and allocation into MedDiet + NUTS group (vs. control group). A similar model was constructed for metabolic syndrome (n = 70 participants free of disease at baseline), although not including BMI at baseline because its co-linearity with waist circumference.

Finally, Pearson’s correlation coefficients were used to calculate univariate associations between continuous variables.

In all cases statistical significance was set at the p<0.05 level. Analyses were performed using SPSS software, release 16.0 (SPSS Inc., Chicago, IL).

References