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, dieticians 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$_{\text{max}}$) 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 $\geq 1.2$ mm thick. IMT and plaque$_{\text{max}}$ 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$_{\text{max}}$, 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 $\alpha$-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$_{\text{max}}$ 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