

## Serum sterol responses to increasing plant sterol intake from natural foods in the Mediterranean diet

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### Abstract

**Background** Phytosterols in natural foods are thought to inhibit cholesterol absorption. The Mediterranean diet is rich in phytosterol-containing plant foods.

**Aim of the study** To assess whether increasing phytosterol intake from natural foods was associated with a cholesterol-lowering effect in a substudy of a randomized trial of nutritional intervention with Mediterranean diets for primary cardiovascular prevention (PREDIMED study).

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**Methods** One hundred and six high cardiovascular risk subjects assigned to two Mediterranean diets supplemented with virgin olive oil (VOO) or nuts, which are phytosterol-rich foods, or advice on a low-fat diet. Outcomes were 1-year changes in nutrient intake and serum levels of lipids and non-cholesterol sterols.

**Results** Average phytosterol intake increased by 76, 158 and 15 mg/day in participants assigned VOO, nuts and low-fat diets, respectively. Compared to participants in the low-fat diet group, changes in outcome variables were observed only in those in the Mediterranean diet with nuts group, with increases in intake of fibre, polyunsaturated fatty acids and phytosterols ( $P < 0.020$ , all) and significant ( $P < 0.05$ ) reductions of LDL-cholesterol (0.27 mmol/l or 8.3%) and the LDL/HDL-cholesterol ratio (0.29 mmol/l or 11.5%). Variations in saturated fat, cholesterol or fibre intake were unrelated to LDL-cholesterol changes. In the whole group, changes in serum sitosterol-to-cholesterol, which reflect those of dietary phytosterol intake and absorption, correlated inversely to LDL-cholesterol changes ( $r = -0.256$ ;  $P = 0.008$ ). In multivariate analyses, baseline LDL-cholesterol, increases in serum sitosterol ratios and statin use were independently associated with LDL-cholesterol reductions.

**Conclusions** Small amounts of phytosterols in natural foods appear to be bioactive in cholesterol lowering.

**Keywords** Phytosterols · Mediterranean diet · Nuts · Olive oil · Cholesterol

### Introduction

Coronary heart disease (CHD) is the main cause of death and morbidity in industrialized countries. Mediterranean

countries have a low CHD mortality rate compared with Northern Europe or the US [33], which has been ascribed in part to dietary habits [20]. Recent findings from large cohort studies [15, 32] and controlled feeding trials, as recently reviewed [7], suggest that adherence to the Mediterranean dietary pattern is associated with reduced CHD mortality and beneficial effects on cardiovascular disease biomarkers, respectively.

The traditional Mediterranean diet is characterized by a high intake of vegetables, fruits and olive oil; a moderate intake of fish and alcohol, mostly wine; and a low intake of dairy products, meat and sweets [18, 35]. Ecological evidence suggested that this dietary pattern was associated with lower serum cholesterol than the so-called Western diet, with higher intakes of meat, dairy products and sweets [13]. This was confirmed by a feeding trial in which Mediterranean and Western diets were switched [8], and may be explained in part by a lower dietary content of saturated fatty acids (SFA) and a higher content of monounsaturated fatty acids (MUFA) [4] and fibre [2] in the traditional Mediterranean diet. Other factors in the diet apart from fat and fibre could influence serum cholesterol. These may include bioactive plant sterols.

Plant sterols or phytosterols are important components of a vegetable-based diet, being particularly abundant in cereals, nuts, seeds and oils derived from them [23]. They are structurally related to cholesterol, but have bulkier and more hydrophobic molecules, which confer them a higher affinity for intestinal micelles than cholesterol. Consequently, cholesterol is displaced from micelles and the amount available for absorption is limited [25]. Inhibition of cholesterol absorption by gram doses of phytosterols incorporated into various foods is a well-established non-pharmacological strategy for cholesterol lowering [29]. An important question is whether plant sterols in natural vegetable foods or oils are also bioactive and might help lower serum cholesterol. At least there is convincing evidence that they reduce cholesterol absorption [6, 26, 27]. Thus, feeding experiments in patients with an ileostomy showed that the phytosterol content of the diet was highly and inversely correlated to cholesterol absorption [6]. In addition, single meal studies with phytosterol-rich corn oil and wheat germ muffins showed a  $\approx 40\%$  reduction of cholesterol absorption that reverted to baseline when the same foods were given after being depleted of phytosterols [26, 27]. Recently, dietary phytosterol intake was inversely related to serum cholesterol in large population studies [1, 14], suggesting that they are indeed bioactive in the usual diet.

The aim of the present study of a subsample of participants in the PREDIMED study [7], a large feeding trial of primary cardiovascular prevention, was to assess whether increasing phytosterol intake from natural foods in an

enhanced Mediterranean diet was associated with cholesterol lowering.

## Methods

### Study population

The PREDIMED study is a parallel group, multi-centre, randomized and controlled 5-year clinical trial. Eligibility of participants has been described elsewhere [7, 19]. Participants were randomized to three groups, two Mediterranean diets, supplemented with either virgin olive oil (VOO) or mixed nuts, or advice on a low-fat diet. At baseline and after intervention for 1 year, participants filled a 137-item validated food frequency questionnaire (FFQ) [17] and underwent anthropometric measurements and venipuncture.

This exploratory analysis includes 114 participants chosen at random from the lists of two recruiting centres (Hospital Clínic, Barcelona and Hospital Sant Joan, Reus) among those who provided FFQs and blood samples at baseline and 1 year and were not using plant sterol supplements. Recruitment took place between October 2004 and December 2005. All participants gave informed consent to a protocol approved by the local review boards.

### Diets

Trained dietitians delivered the dietary intervention, which was based on quarterly individual and group education meetings. The dietitians gave advice to follow either the recommended Mediterranean diets or the low-fat diet and provided written material with elaborate descriptions of target foods and seasonal shopping lists, meal plans and cooking recipes. The general procedures and dietary recommendations for the three groups have been described [7, 19]. The participants allocated to the Mediterranean diets also received free provisions of VOO (1 l/week) or mixed nuts (30 g/day, as 15 g walnuts, 7.5 g almonds and 7.5 g hazelnuts). Participants in the olive oil group were recommended to consume a minimum of 50 ml of the supplemental olive oil per day. The extra olive oil was provided to improve adherence, account for family needs and allow sufficient oil for deep frying.

Food intake during the preceding year was derived from the FFQs. The consumption of energy and nutrients was calculated from Spanish food composition tables. Intake of total phytosterols and their measured components in the diet at baseline and after intervention for 1 year was estimated from a database of Spanish foods [12]. The phytosterol content of the olive oil and nuts used in the study was analysed in a reference laboratory, as described [7].

The mean values of six measurements of total phytosterols in random samples of VOO, walnuts, almonds and hazelnuts were 156, 199, 224 and 175 mg/100 g, respectively. The average sitosterol content ranged from 79% in almonds to 96% in olive oil, while the average campesterol content was <6% in all foods.

#### Laboratory determinations

Coded samples of serum were shipped to a central laboratory and stored at  $-80^{\circ}\text{C}$  until assay. Cholesterol and triglycerides were measured by enzymatic procedures, and HDL-cholesterol was determined after precipitation with phosphotungstic acid and magnesium chloride. The concentration of LDL-cholesterol was calculated by the Friedewald equation.

Serum non-cholesterol sterols (campesterol, sitosterol and lathosterol) were determined by gas chromatography using a modification of the method of Heinemann et al. [10]. The ratios to cholesterol of the plant sterols, campesterol and sitosterol, are accepted as surrogate markers for the efficiency of cholesterol absorption, while the cholesterol precursor lathosterol is a reliable index of cholesterol synthesis [22]. Epicoprostanol (2  $\mu\text{g}$ ) was added to serum (0.1 ml) as internal standard. After alkaline hydrolysis, extraction, and derivatization to trimethylsilyl ethers, sterols were quantified on a 30-m nonpolar capillary column (TRB-Esterol; Teknokroma, Barcelona) with a gas chromatography apparatus (Autosystem<sup>TM</sup>, Perkin Elmer, Norwalk, CT, USA). Inter- and intra-assay coefficient variations were 5.0 and 3.2% for lathosterol, 1.9 and 1.6% for campesterol and 2.0 and 1.8% for sitosterol, respectively.

#### Statistical analyses

Mean values and SD were used to describe continuous variables. For analysis of laboratory variables, the average of two measurements was used as the final value. Values with a skewed distribution were transformed to their natural logarithm for analyses. Analysis of variance (ANOVA) or Chi-square tests, as appropriate, were used for comparisons of baseline variables among the three groups. We examined 1-year changes in energy, nutrient intake, serum lipids and non-cholesterol sterols by ANOVA with adjustment for age, sex and baseline body weight. Centre had no effect on outcomes and was excluded from further analyses. Pearson correlation coefficients were used to assess relationships between continuous variables. Multiple linear regression analysis was used to build a predictive model of LDL-cholesterol change. All statistical tests were two tailed and the significance level was set at  $P < 0.05$ . Analyses were performed using SPSS, version 12.0 (SPSS Inc., Chicago, IL, USA).

## Results

Of the initially chosen 114 participants, data from eight subjects were not included in the final analysis because five of them had a change in statin use during the study, which might interfere with sterol values, and three subjects provided FFQ data which were out of range for total energy intake (500–3,500 kcal/day for women and 800–4,000 kcal/day for men).

The clinical characteristics of the 106 participants are presented in Table 1. By study design, the sample was composed of older, mostly overweight subjects with a sizeable burden of cardiovascular risk factors, which were reasonably balanced among the three arms. Likewise, there were no among-group differences in serum non-cholesterol sterol-to-cholesterol ratios.

As shown in Table 2, baseline food, energy and nutrient intake were similar among groups. The self-selected participant's dietary habits reasonably conformed to the traditional Mediterranean food pattern in several aspects, as they recorded high intakes of olive oil, cereals, vegetables, fruits and fish; moderate intakes of legumes, nuts and alcoholic beverages and low intakes of industrial bakery products and sweets. They deviated from the traditional Med diet, however, because of high intakes of meat and dairy products. The baseline diet was high in fibre, due to high intakes of vegetables and fruits, and also in total fat and MUFA, which can be attributed in part to customary use of olive oil. For the same reason, the phytosterol content of the baseline diet was rather high.

The main dietary changes recorded at 1 year were high increases in VOO and nut consumption in the corresponding Mediterranean diet groups provided with these foods. As derived from the FFQs, olive oil intake only increased marginally by 11 g/day in the group supplemented with VOO because participants just exchanged their usual olive oil, mainly refined olive oil (15 g/day reduction), for the virgin variety supplied (24 g/day increase). Nut intake significantly increased by 29 g/day in participants in the Mediterranean diet with nuts. Participants in all groups increased consumption of fruits and vegetables by an average of 77 and 33 g/day, respectively, and marginally decreased meat consumption by an average 8 g/day, while maintaining a low level of alcohol intake of  $\approx 13$  g/day. Mean body weight changes at 1 year were  $-0.81$  kg in the VOO group,  $-0.41$  kg in the nuts group and  $0.34$  kg in the low-fat diet group ( $P = 0.223$ ).

Changes in energy and nutrient intake adjusted for age, sex and baseline body weight were usually minor (Table 3). At 1 year, participants in the Mediterranean diet with VOO increased intake of total phytosterols and sitosterol, but not campesterol, while the group assigned the Mediterranean diet with nuts increased intake of total

**Table 1** Baseline characteristics of participants

	Mediterranean diet with VOO ( <i>n</i> = 35)	Mediterranean diet with nuts ( <i>n</i> = 37)	Recommended low-fat diet ( <i>n</i> = 34)	<i>P</i> value <sup>a</sup>
Age (years)	66.1 ± 6.2	65.1 ± 6.3	67.6 ± 6.6	0.238
Men	18 (51.4)	24 (64.9)	20 (58.8)	0.512
Family history of CHD	8 (23.5)	7 (18.9)	11 (32.4)	0.415
Current smokers	7 (20.0)	14 (37.8)	11 (32.4)	0.243
BMI (kg/m <sup>2</sup> )	28.0 ± 3.1	28.3 ± 2.8	29.4 ± 3.9	0.171
Type 2 diabetes mellitus	16 (45.7)	19 (51.4)	17 (50.0)	0.884
Hypertension	31 (88.6)	30 (81.1)	30 (88.2)	0.587
Dyslipidemia	28 (80.0)	26 (70.3)	24 (70.6)	0.575
Statin treatment	11 (31.4)	14 (37.8)	9 (26.5)	0.588
Other lipid-lowering agents	4 (11.4)	2 (5.4)	2 (5.9)	0.567
Serum lipids (mmol/l)				
Total cholesterol	5.51 ± 0.83	5.24 ± 0.87	5.21 ± 0.79	0.243
LDL-cholesterol	3.54 ± 0.66	3.24 ± 0.79	3.15 ± 0.64	0.061
HDL-cholesterol	1.41 ± 0.33	1.34 ± 0.33	1.39 ± 0.31	0.691
Triglycerides <sup>b</sup>	1.08 ± 1.52	1.39 ± 1.51	1.35 ± 1.64	0.128
LDL/HDL ratio	2.62 ± 0.66	2.52 ± 0.72	2.30 ± 0.56	0.124
Triglycerides/HDL ratio <sup>b</sup>	1.91 ± 1.69	2.29 ± 1.75	2.40 ± 1.91	0.217
Serum non-cholesterol sterols-to-cholesterol (μmol/mmol)				
Lathosterol	1.38 ± 0.60	1.49 ± 0.64	1.45 ± 0.89	0.815
Campesterol	2.66 ± 1.46	2.68 ± 1.45	2.67 ± 1.58	0.997
Sitosterol	2.30 ± 1.26	2.27 ± 1.20	2.22 ± 1.20	0.961

Values are number (%) or mean ± SD

VOO virgin olive oil, CHD coronary heart disease

<sup>a</sup> *P* value by Chi-square test or ANOVA

<sup>b</sup> Data computed as logarithm and expressed as anti-logarithm

energy, fibre, energy from total fat, PUFA and total plant sterol and their components, with a reciprocal decrease in carbohydrate intake. Cholesterol intake decreased in the low-fat diet group. Compared to the low-fat diet group, there were no differences in nutrient changes in the Mediterranean diet group with VOO, while significant increases in intake of fibre, PUFA and phytosterols were observed in the Mediterranean diet with nuts group. Figure 1 shows the contribution of the main plant-derived food groups to phytosterol intake at 1 year in each intervention arm. In all diet groups, the main source was oils (essentially, olive oil), followed by cereals and then vegetables and fruits to the same extent, while the contribution of legumes and nuts was minor, except for the group supplemented with nuts, in which nuts were second to oils as the main contributors to phytosterol intake.

Table 4 shows the effects of diets on outcome variables adjusted for age, sex and baseline body weight. The Mediterranean diets with VOO or nuts were associated with significant reductions from baseline in LDL-cholesterol (−4.2 and −6.8%, respectively) and the LDL/HDL-

cholesterol ratio (−6.1 and −9.5%, respectively), while only participants in the nut-supplemented group showed an increase in HDL-cholesterol (5.2%). The treatment effect by comparison with the low-fat diet group was significant (*P* < 0.05) only for reduction in LDL-cholesterol (0.27 mmol/l or 8.3%) and the LDL/HDL-cholesterol ratio (0.29 mmol/l or 11.5%) in the Mediterranean diet with nuts group. In the group supplemented with nuts, the 1-year LDL-cholesterol responses were non-significantly higher in statin users compared with nonusers (−7.8% vs. −4.2%, respectively, *P* = 0.25). There were no effects of diets on concentrations of triglycerides, triglyceride/HDL ratios, lathosterol or campesterol, while sitosterol increased, albeit non-significantly, in the two Mediterranean diet groups.

Changes of serum lipids were significantly and negatively correlated (e.g., bigger reductions) to baseline levels, with *r* = −0.339 (*P* < 0.001) for total cholesterol, *r* = −0.408 (*P* < 0.001) for LDL-cholesterol and *r* = −0.322 (*P* = 0.001) for triglycerides. Changes in total and LDL-cholesterol were unrelated to those of dietary plant sterols, SFA, fibre or cholesterol intakes (all *r* values < 0.16;

**Table 2** Key foods, energy and nutrients of self-selected prestudy diets in the three intervention groups

	Mediterranean diet with VOO ( <i>n</i> = 35)	Mediterranean diet with nuts ( <i>n</i> = 37)	Low-fat diet ( <i>n</i> = 34)	<i>P</i> value <sup>a</sup>
<b>Foods (g/day)</b>				
Olive oil	39 ± 18	36 ± 15	41 ± 20	0.485
Cereals	229 ± 130	197 ± 71	201 ± 94	0.378
Vegetables	332 ± 135	295 ± 102	307 ± 107	0.382
Fruits	217 ± 115	242 ± 120	242 ± 124	0.603
Legumes	16.7 ± 13.0	13.0 ± 6.0	14.5 ± 9.0	0.274
Nuts	11.4 ± 11.6	14.2 ± 16.6	15.0 ± 16.4	0.585
Fish or seafood	85 ± 53	81 ± 37	74 ± 24	0.470
Meat and meat products	93 ± 35	105 ± 36	115 ± 45	0.396
Industrial bakery products	27.5 ± 36	25.9 ± 48.7	27.0 ± 55.3	0.989
Dairy products	302 ± 190	254 ± 173	214 ± 130	0.074
Alcohol	12.5 ± 15.4	14.7 ± 17.0	14.9 ± 15.0	0.780
<b>Nutrients</b>				
Total energy (kcal/day)	2161 ± 630	2143 ± 525	2128 ± 626	0.725
Energy from protein (%)	16.8 ± 2.5	16.6 ± 2.7	16.6 ± 2.9	0.922
Energy from carbohydrate (%)	43.8 ± 6.0	41.5 ± 6.8	40.9 ± 7.2	0.201
Fibre (g/day)	29.7 ± 11.0	26.2 ± 7.8	26.4 ± 7.3	0.663
Energy from total fat (%)	36.6 ± 5.5	37.6 ± 6.3	38.6 ± 7.2	0.475
Saturated fatty acids (%)	9.8 ± 2.3	10.5 ± 2.2	10.2 ± 2.2	0.493
Monounsaturated fatty acids (%)	18.1 ± 3.5	17.7 ± 4.0	19.2 ± 4.3	0.329
Polyunsaturated fatty acids (%)	5.7 ± 1.8	6.4 ± 2.1	6.4 ± 2.1	0.305
Cholesterol (mg/day)	343 ± 108	365 ± 104	355 ± 101	0.717
Total plant sterols (mg/day)	351 ± 94	342 ± 90	373 ± 129	0.352
Sitosterol (mg/day)	222 ± 57	212 ± 51	230 ± 75	0.406
Campesterol (mg/day)	34.3 ± 12.4	31.5 ± 10.1	32.8 ± 9.9	0.263
Other sterols (mg/day) <sup>b</sup>	94 ± 34	99 ± 35	110 ± 57	0.309

Data as mean ± SD

<sup>a</sup> *P* value by ANOVA<sup>b</sup> Includes stigmasterol, stigmasterol and unspecified plant sterols

$P > 0.1$ ). However, changes of serum sitosterol ratios correlated negatively with those of serum total cholesterol ( $r = -0.285$ ;  $P = 0.003$ ) and LDL-cholesterol ( $r = -0.256$ , respectively;  $P = 0.008$ ).

Finally, we used a stepwise multiple regression analysis to build a predictive model of the lipid response, with LDL-cholesterol change as dependent variable and sex, age, diabetes status, statin use, baseline BMI and levels of LDL-cholesterol and non-cholesterol sterol ratios, and 1-year changes in weight, intake of SFA, fibre, dietary cholesterol and plant sterols, and non-cholesterol sterol ratios as independent variables. Baseline LDL-cholesterol [regression coefficient ( $B$ ) =  $-0.236$ ;  $P < 0.001$ ], change in serum sitosterol-to-cholesterol ratios ( $B = -0.158$ ;  $P = 0.011$ ) and statin use ( $B = -0.203$ ;  $P = 0.032$ ) were independently associated with LDL-cholesterol changes (adjusted  $R^2 = 0.22$ ).

## Discussion

In this study, asymptomatic older persons at high-cardiovascular risk who improved their diet after nutritional education and supplementation with VOO or mixed nuts during a 1-year period showed improved lipid profiles in comparison with those recommended a low-fat diet. The reductions in total cholesterol and LDL-cholesterol were unrelated to changes in dietary SFA, cholesterol or fibre intake, but they were associated with dietary enrichment with plant sterols, as indirectly estimated by increases in serum sitosterol ratios.

Observed changes in nutrient intake were not of great magnitude. Increased nut intake was mirrored by significant increases in healthy nutrients more than increased VOO intake, because participants in this group merely exchanged their usual refined oil for the virgin oil supplied.

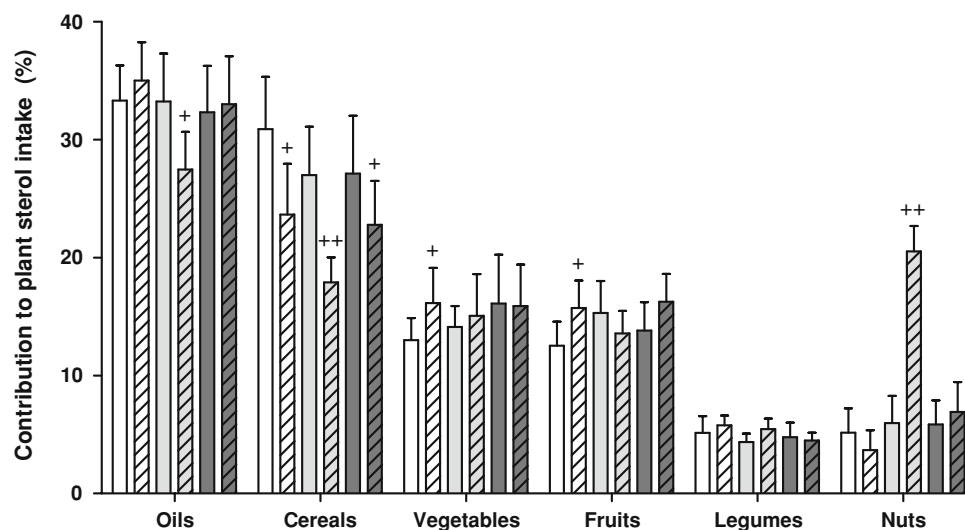
**Table 3** Changes in energy and nutrient intake

	Mean changes from baseline at 12 months (95% CI)			Mediterranean diet with olive oil vs. low-fat diet		Mediterranean diet with nuts vs. low-fat diet	
	Mediterranean diet with VOO ( <i>n</i> = 35)	Mediterranean diet with nuts ( <i>n</i> = 37)	Low-fat diet ( <i>n</i> = 34)	Mean (95% CI) between group difference	<i>P</i> value	Mean (95% CI) between group difference	<i>P</i> value
Total energy (kcal/day)	−66 (−239 to 106)	181 (11 to 351)	−24 (−213 to 165)	−43 (−358 to 273)	1.00	204 (−109 to 519)	0.34
Energy from protein (%)	0.42 (−0.41 to 1.25)	−0.24 (−1.06 to 0.58)	−0.28 (−1.19 to 0.63)	0.70 (−0.82 to 2.22)	0.79	0.05 (−1.47 to 1.56)	1.00
Energy from carbohydrate (%)	−0.76 (−2.98 to 1.47)	−2.79 (−4.99 to −0.60)	1.15 (−1.29 to 3.59)	−1.91 (−5.97 to 2.16)	0.77	−3.94 (−7.98 to 0.11)	0.06
Fibre (g/day)	2.34 (−0.61 to 5.28)	6.22 (3.31 to 9.12)	−0.03 (−3.26 to 3.20)	2.37 (−3.01 to 7.74)	0.86	6.25 (0.89 to 11.60)	0.016
Energy from total fat (%)	1.29 (−1.09 to 3.66)	3.53 (1.19 to 5.87)	0.21 (−2.40 to 2.81)	1.08 (−3.26 to 5.42)	1.00	3.32 (−1.00 to 7.64)	0.19
SFA (%)	−0.64 (−1.39 to 0.11)	−0.67 (−1.41 to 0.07)	−0.25 (−1.07 to 0.57)	−0.39 (−1.76 to 0.98)	1.00	−0.42 (−1.78 to 0.94)	1.00
MUFA (%)	1.17 (−0.45 to 2.78)	1.49 (−0.10 to 3.09)	−0.01 (−1.78 to 1.76)	1.18 (−1.77 to 4.13)	0.99	1.51 (−1.43 to 4.44)	0.64
PUFA (%)	0.18 (−0.54 to 0.90)	2.70 (1.99 to 3.41)	0.25 (−0.54 to 1.04)	−0.07 (−1.38 to 1.25)	1.00	2.46 (1.14 to 3.77)	<0.001
Cholesterol (mg/day)	−26 (−61 to 9)	−25 (−59 to 10)	−60 (−98 to −21)	33 (−30 to 97)	0.61	35 (−28 to 98)	0.54
Total plant sterols (mg/day)	76 (26 to 126)	158 (110 to 205)	15 (−35 to 64)	62 (−25 to 148)	0.26	143 (58 to 228)	<0.001
Sitosterol (mg/day)	36 (8 to 63)	94 (68 to 121)	2 (−26 to 29)	34 (−14 to 82)	0.27	93 (46 to 139)	<0.001
Campesterol (mg/day)	0.82 (−3.02 to 4.66)	5.37 (1.74 to 9.00)	−1.56 (−5.36 to 2.23)	2.38 (−4.26 to 9.03)	1.00	6.93 (0.45 to 13.42)	0.032
Other sterols (mg/day) <sup>a</sup>	36 (19 to 54)	50 (33 to 66)	16 (−1 to 33)	20 (−10 to 50)	0.31	34 (4 to 63)	0.020

Changes are mean values (95% CI) and are calculated as 1-year value minus baseline value; adjusted for age, sex and baseline body weight

<sup>a</sup> Includes stigmasterol, stigmasterol and unspecified plant sterols





**Fig. 1** Contribution of plant-based food groups to estimated daily intake per person of plant sterols in the three intervention groups of the PREDIMED participants at baseline and at 1 year intervention. Values are mean (95% IC).  $^+P < 0.05$ ,  $^{++}P < 0.001$  vs. baseline value. White bar indicates Med diet + VOO baseline, white bar with

striped lines indicates Med diet + VOO 1-year, light grey bar indicates Med diet + nuts baseline, light grey bar with striped line indicates Med diet + nuts 1-year, dark grey bar indicates Med diet + low-fat baseline and dark grey bar with striped line indicates Med diet + low-fat 1-year

Participants in the low-fat diet group showed little changes in nutrient intake because they followed a similar diet as that self-selected at the onset of the study. Noticeably, plant sterol intakes increased only in the VOO and nut groups, by 22 and 46%, respectively.

In spite of relatively small overall changes in macro-nutrient intake, beneficial changes in lipid profiles occurred only in the Mediterranean diet groups, particularly in the group given supplemental nuts, confirming the results of the 3-month assessment of a larger PREDIMED cohort [7]. The increase in total fat and unsaturated fatty acid consumption in the mixed nuts group might be relevant to the observed HDL-cholesterol increase, as reported after nut-enriched diets [9].

Feeding experiments in humans indicate that plant sterols in natural foods reduce intestinal cholesterol absorption [6, 26, 27] and two large population studies showed that phytosterol intake was inversely related to serum cholesterol [1, 14], suggesting that they are indeed bioactive in the usual diet. Our main research question was whether this bioactivity was operational to lower serum cholesterol in free-living persons who increased consumption of plant sterol-rich foods. The present results support an association between increasing dietary phytosterol intakes and LDL-cholesterol lowering. Plant sterol intake increased in the two Mediterranean diet groups, to a higher extent in the group with nuts. While both groups showed decreases of LDL-cholesterol from baseline, only the change after the nut-supplemented diet was significantly different from that observed in the low-fat diet. The cholesterol-lowering efficacy of nut intake in feeding trials

has often been higher than that predicted on the basis of fatty acid exchange [9], and the phytosterol content of nuts might be responsible in part for this effect [30]. Our findings are consistent with this hypothesis, as the small changes in intake of SFA or fibre, two important dietary determinants of serum cholesterol levels, were unrelated to those of serum LDL-cholesterol. While the latter were similarly unrelated to changes of dietary phytosterol intake, they correlated inversely with those of serum sitosterol ratios, a reliable index of intestinal sterol absorption [3, 22]. Inherent inaccuracies of the subjective FFQ technique to estimate the intake of nutrients, especially of those with high intra- and inter-individual variability such as plant sterols [25], in face of objective determinations of serum levels may explain these discrepancies. At any rate, daily plant sterol intake increased by an average of 158 mg in the Mediterranean diet with nuts group. Based on studies with phytosterol-enriched foods, the contribution to cholesterol lowering from these small doses in natural foods seems unexpected. However, a higher effect from naturally occurring plant sterols fits with the curvilinear relationship between phytosterol intake and serum cholesterol derived from clinical studies [29]. Recent studies have shown that treatment of hypercholesterolemic subjects with healthy diets enriched with high-fibre foods and pharmacological doses of phytosterols, together with nuts in an isoenergetic diet [11] or specific phytochemicals within the context of a weight-losing diet [16] can have marked beneficial effects on the lipid profile.

The effects of diet on serum non-cholesterol sterols deserve some consideration. Even though plant sterol

**Table 4** Changes in serum lipids and non-cholesterol sterols

	Mean changes from baseline at 12 months (95% CI)		Mediterranean diet with olive oil vs. low-fat diet		Mediterranean diet with nuts vs. low-fat diet		
	Mediterranean diet with VOO ( <i>n</i> = 35)	Mediterranean diet with nuts ( <i>n</i> = 37)	Low-fat diet ( <i>n</i> = 34)	Mean (95% CI) between group difference	<i>P</i> value	Mean (95% CI) between group difference	<i>P</i> value
Lipids (mmol/l)							
Total cholesterol	−0.15 (−0.31, 0.00)	−0.11 (−0.26, 0.04)	0.04 (−0.12, 0.20)	−0.19 (−0.47 to 0.08)	0.26	−0.15 (−0.42 to 0.12)	0.51
LDL-cholesterol	−0.15 (−0.29, −0.00)	−0.22 (−0.36, −0.08)	0.05 (−0.10, 0.20)	−0.20 (−0.46 to 0.06)	0.20	−0.27 (−0.53 to −0.01)	0.036
HDL-cholesterol	0.02 (−0.03, 0.07)	0.07 (0.02, 0.11)	0.00 (−0.05, 0.05)	0.02 (−0.07 to 0.11)	1.00	0.06 (−0.02 to 0.15)	0.24
Triglycerides <sup>a</sup>	−1.06 (−1.18, 1.04)	1.07 (−1.03, 1.18)	−1.05 (−1.06, 1.06)	−1.01 (−1.21 to 1.18)	1.00	1.12 (−1.07 to 1.34)	0.37
LDL/HDL ratio	−0.16 (−0.29, −0.02)	−0.24 (−0.37, −0.11)	0.05 (−0.10, 0.19)	0.20 (−0.44 to 0.04)	0.13	−0.29 (−0.52 to −0.05)	0.014
Triglyceride/HDL ratio <sup>a</sup>	−1.08 (−1.21, 1.04)	1.03 (−1.08, 1.15)	−1.05 (−1.19, 1.06)	−1.27 (−1.67, 1.03)	0.09	1.02 (−1.28, 1.32)	0.91
Non-cholesterol sterols/cholesterol (μM/mM)							
Lathosterol	0.05 (−0.15, 0.25)	−0.04 (−0.23, 0.16)	0.07 (−0.14, 0.27)	−0.02 (−0.37 to 0.34)	1.00	0.10 (−0.45 to 0.25)	1.00
Campesterol	0.04 (−0.31, 0.39)	−0.05 (−0.39, 0.29)	−0.06 (−0.41, 0.30)	0.10 (−0.52 to 0.71)	1.00	0.01 (−0.60 to 0.62)	1.00
Sitosterol	0.22 (−0.02, 0.47)	0.22 (−0.02, 0.45)	0.06 (−0.19, 0.31)	0.16 (−0.27 to 0.59)	1.00	0.15 (−0.27 to 0.58)	1.00

Changes are mean values (95% CI) and are calculated as 1-year value minus baseline value; adjusted for age, sex and baseline body weight

<sup>a</sup> Data computed as logarithm and expressed as anti-logarithm

absorption is poor, it is enhanced by increased amounts in the intestinal lumen, resulting in increased serum phytosterol concentrations, as documented in feeding trials of natural phytosterol-rich foods [24, 31, 34]. The fact that in our study only sitosterol levels increased in comparison with campesterol can be explained by the relative abundance of sitosterol in Mediterranean foods competing with campesterol for absorption [3].

Inverse correlations were observed between changes in serum sitosterol and those of total and LDL-cholesterol. In addition, the LDL-cholesterol response was independently associated with serum sitosterol increases at multivariate analysis. For these reasons, the presumed atherogenicity of increased serum phytosterols [3, 28] must be questioned when dealing with populations consuming diets rich in vegetables, nuts and plant-derived oils, such as the Mediterranean diet.

The fact that participants undergoing statin treatment showed a trend towards more pronounced LDL-cholesterol reductions than untreated ones also supports a phytosterol effect, because decreased cholesterol synthesis by statins is compensated by increased intestinal absorption of sterols [21], and in this situation dietary plant sterols should be more effective to lower blood cholesterol. Indeed, as recently shown by de Jong et al. [5], there is a synergistic cholesterol-lowering effect of plant sterol supplement consumption and statins, with effectiveness maintained on the long term.

Our study has limitations, such as the small number of participants in each group, which might reduce statistical power and the assessment of nutrient intake based on subjective self-reports. The nutritional education on the low-fat diet was less intense than the intervention in the two Mediterranean diet groups is an added limitation. In fact, fat intake at 1 year was similar to baseline in the group assigned the low-fat diet. This was partly because of the study design, but also because participants belonged to a Mediterranean culture, where people have a long-lasting preference for using olive oil. Therefore, the differences in outcomes between the Mediterranean diet groups and the low-fat diet group might be attributed mainly to the supplemental foods provided. Strengths of the study are that it reproduces real-life conditions with home-prepared foods and that plant sterol intake was determined from composition tables constructed with Spanish foods [12].

In conclusion, the results of this study suggest that part of the beneficial effects of the enhanced Mediterranean diets on the lipid profile is due to increased consumption of plant sterols, which might be bioactive even in small amounts in their natural food matrices. More work is warranted to confirm that intake of natural phytosterol-rich foods has a cholesterol-lowering effect in free-living populations.



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