

Olive oil containing olive oil fatty acid esters of plant sterols and dietary diacylglycerol reduces low-density lipoprotein cholesterol and decreases the tendency for peroxidation in hypercholesterolaemic subjects

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Plant sterols (PS) and MUFA are well-documented cholesterol lowering agents. We aimed to determine the effect of PS esterified to olive oil fatty acids (PS-OO) on blood lipid profile and lipid peroxidation in hypercholesterolaemic subjects. Twenty-one moderately overweight, hypercholesterolaemic subjects consumed three consecutive treatment diets, each lasting 28 d and separated by 4-week washout periods, using a randomized crossover design. Diets contained 30 % energy as fat, 70 % of which was provided by olive oil (OO), and differed only in the treatment oils: OO, PS esterified to sunflower oil fatty acids (PS-SO), and PS-OO. Both PS-SO and PS-OO treatments provided 1.7 g PS /d. PS-OO and PS-SO consumption resulted in a decrease ($P=0.0483$) in LDL-cholesterol (LDL-C) concentrations compared with the OO diet. Although total cholesterol and apo B-100 levels were not significantly affected, PS-SO and, to some extent, PS-OO reduced the total:HDL-cholesterol (HDL-C) ratio ($P=0.0142$) and the apo B-100:apo A-I ratio ($P=0.0168$) compared with the OO diet. There were no differences across diets in lipoprotein(a) (Lp(a)) and lipid peroxidation levels. However, following consumption of OO and PS-SO, Lp(a) concentrations increased ($P=0.0050$ and 0.0421 , respectively), while PS-OO treatment did not affect Lp(a) levels. Furthermore, there was a decrease ($P=0.0097$) in lipid peroxidation levels with PS-OO treatment during the supplementation phase. Our results suggest that supplementing an OO-rich diet with PS-OO favourably alters the plasma lipid profile and may decrease the susceptibility of LDL-C to lipid peroxidation in hypercholesterolaemic subjects.

Plant sterols: Olive oil fatty acids: Blood lipids: Oxidative stress: Hypercholesterolaemic subjects: Lipid peroxidation: Lipoprotein (a): LDL cholesterol: Apo B: Diacylglycerol

CHD is the leading cause of morbidity and mortality in Western countries. An elevated concentration of LDL-cholesterol (LDL-C) is a well-established independent risk factor for atherosclerosis¹. In addition, LDL oxidation has been implicated by several studies as one of the initial steps of atherogenesis, and therefore associated with higher risk of CHD^{2,3}. Elevated concentrations of oxidised LDL have recently been identified as a strong predictor for subsequent acute CHD events in healthy men⁴.

Plant sterols (PS) have long been reported to have significant cholesterol-lowering properties⁵. Studies conducted throughout the past decades suggested that an intake of 1.5–2 g PS/d is needed to achieve an optimal LDL-C lowering effect⁶. It is generally accepted that PS decrease circulating cholesterol concentrations by suppressing intestinal absorption of cholesterol due to the higher affinity of PS to micelles compared to cholesterol, resulting in less cholesterol being incorporated in chylomicrons^{7,8}. Several reports have demonstrated that the solubility of PS may play an important role in the process of PS incorporation into micelles⁹. Indeed, low intestinal

bioavailability of purified phytosterols was shown to be elevated by esterification to fatty acids, dissolving in dietary diacylglycerol (DAG) oil, or by emulsifying with lecithin micelles^{10–13}. To date, the most common process used to enhance the solubility of PS is by esterifying PS with *n*-6 PUFA, such as soyabean oil and sunflower oil (SO) fatty acids. PS can therefore be incorporated into fatty foods, such as margarines and spreads¹⁴. Emerging new approaches consist of esterifying PS to fatty acids associated with additional health benefits, such as fish oil fatty acids¹⁵.

Early epidemiological evidence showed a lower incidence of CHD in Mediterranean countries¹⁶ where olive oil (OO) is the primary source of fat¹⁷. The consumption of OO, which contains high levels of oleic acid (a MUFA), was inversely associated with IHD, presumably due to hypolipidaemic effects¹⁸. This notion has been further confirmed in several clinical intervention trials^{19–21}. MUFA supplementation was also shown to have protective effects against lipid peroxidation^{19,22}. Likewise, PS were also reported to have antioxidant properties²³.

Abbreviations: DAG, diacylglycerol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; Lp(a), lipoprotein (a); OO, Olive oil; SO, sunflower oil; PS, plant sterols; TBARS, thiobarbituric acid-reactive substance; TC, total cholesterol.

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The objective of this study was to assess whether a novel formulation of PS that had been esterified with OO fatty acids (PS-OO) would exert the effects of its components on the blood lipid profile and lipid peroxidation. We tested this hypothesis in mildly overweight, hypercholesterolaemic subjects who consumed an OO-rich diet that was further supplemented either with PS-OO or with PS esterified to SO fatty acids (PS-SO).

Methods

Study population

Twenty-four volunteers (eleven males, thirteen postmenopausal females) were recruited from the Montreal area by an advertisement posted in local newspapers. The inclusion criteria were as follows: baseline LDL-C > 2.6 mmol/l (100 mg/dl), BMI ranging from 24 to 30 kg/m² and aged 30–65 years. Subjects were excluded if they had taken medications known to affect lipid metabolism, such as cholestyramine, colestipol, gemfibrozil, probucol, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, fish oil capsules and supplements containing PS, during the previous 3 months. Subjects who had been diagnosed with diabetes mellitus, kidney disease or liver disease, and those who smoked, consumed more than two glasses per day of alcoholic beverages and/or took two or more doses per week of laxatives or concentrated sources of fibre were also excluded. Subjects with thyroid disease (*n* 2) and with high blood pressure (*n* 4) were included in the study since they had been stable in response to thyroid and blood pressure treatments, and their medications were maintained throughout the trial. One subject on hormone replacement therapy was also included in the study and her regimen was maintained at the same dose throughout the study period.

Fasting blood samples were obtained for screening purposes. Before enrolment in the study, the candidates provided a medical history and underwent a complete physical examination conducted by the study physician. The study protocol was reviewed and approved by the Human Ethical Review Committee of the Faculty of Agriculture and Environmental Sciences for the School of Dietetics and Human Nutrition at McGill University (protocol number REB# 808-0403). All subjects received explanations about the protocol and written consent forms were obtained from each participant.

Experimental design, protocol and diets

The study was a semi-randomized, crossover, double blind, clinical intervention trial using a Latin square sequence. It consisted of three 28 d phases separated by a 4-week washout interval. During each dietary treatment phase, the subjects were provided with an OO-based, weight-maintaining, North-American diet. During the washout period, the subjects consumed their own habitual diets. All subjects received the control OO diet during the first phase, after which they were randomly assigned to the two other dietary treatments.

The basic diet contained approximately 15% energy as protein, 55% energy as carbohydrates, 30% energy as fat, of which approximately 70% was provided by OO, 80 mg cholesterol per 4186.8 kJ (1000 kcal); and 12 g fibre per 4186.8 kJ (1000 kcal). The OO of the basic diet

provided 0.02 g/d naturally occurring free PS. The basic diet composition is shown in Table 1. Table 2 lists the composition of the treatment oils. During the two randomized phases, an isoenergetic amount of OO was replaced by either (i) 21.4 g low-fat PS-SO margarine providing the equivalent of 1.7 g soyabean sterols (Take control[®], Unilever Bestfoods NA, Baltimore, MD) or (ii) 9.1 g PS-OO containing the equivalent of 1.7 g soyabean sterols enzymatically esterified to OO fatty acids, as well as 1.4 g DAG (Enzymotec Ltd, Migdal HaEmeq, Israel). No antioxidants were supplemented to the PS-OO matrix or other tested matrices.

The diets were provided to the subjects by the Mary Emily Clinical Nutrition Research Unit at McGill University. These diets consisted of three isoenergetic meals, prepared according to a 3-d cycle menu. All meals (breakfast, lunch and supper) were prepared in the metabolic kitchen of the clinic where the foods were weighed precisely to 0.5 g during meal preparation. Treatment oils were ingested at breakfast and under supervision to monitor compliance. The subjects were instructed to consume only foods and beverages provided by the clinic.

All three experimental diets were isoenergetic. Individual basal energy requirements were calculated by the Mifflin equation²⁴, and were then multiplied by a physical activity factor of 1.7 to meet the total energy requirements of mildly to moderately active healthy adults. Energy intake was adjusted during the first 2 weeks of the control phase to maintain a constant body weight. A comparable energy level was maintained during the two subsequent phases. Subjects were encouraged to keep a constant exercise level throughout the study to ensure that body weights remained unchanged.

Fasting blood samples were collected from the subjects on days 1, 2, 28 and 29 of each phase. On day 28 of each phase, postprandial plasma TAG concentrations were measured 4 h after breakfast. A complete blood count was done on day 29 for monitoring purposes.

Plasma lipids

Blood samples were collected in vacutainer tubes and centrifuged for 15 min at 1000 g at 4°C, within 30 min, to separate plasma from erythrocytes. Plasma and erythrocytes were immediately stored at –80°C until lipid analysis. Total cholesterol (TC), HDL-cholesterol (HDL-C), and TAG concentrations were measured by automated methods on the multianalyzer Dimension RxL Max utilizing enzymatic reagents Flex (Dade Behring Diagnostic, Marburg, Germany). LDL-C

Table 1. Average composition of the control olive oil-based diet over a period of 3 d (Values are means with their standard errors)

Diet component	Mean	SE
Proteins (% energy)	14.4	0.2
Carbohydrates (% energy)	54.0	1.0
Fat (% energy)	31.2	0.9
SFA (% energy)	6.8	0.6
MUFA (% energy)	18.6	0.6
PUFA (% energy)	3.5	0.1
Cholesterol (mg/1000 kcal)	80.3	1.0
Fibre (g/1000 kcal)	12.8	0.3

Table 2. Fatty acid and plant sterol composition of study formulations*

	OO	PS-SO	PS-OO
Plant sterol esters (g/100 g oil or margarine)	ND	12.1	28.6
Free plant sterol equivalents (g/100 g oil or margarine)			
β-sitosterol	0.2	3.7	8.4
Campesterol	> 0.1	1.9	4.6
Stigmasterol	> 0.1	1.4	3.9
Brassicasterol	ND	0.2	0.5
Others	0.1	0.5	1.0
Total	0.2	7.8	18.3
Fatty acids (% by weight total fatty acids)			
12:0	ND	0.2	ND
14:0	> 0.1	0.2	ND
16:0	12.9	8.3	12.8
16:1	1.0	0.1	0.9
17:0	ND	0.1	ND
18:0	2.9	6.2	3.0
18:1	69.8	41.8	70.6
18:2	11.5	36.4	10.7
18:3 <i>n</i> -3	0.8	5.5	0.8
20:0	0.5	0.5	0.5
20:1	0.3	0.3	0.2
22:0	0.2	0.4	0.2
24:0	0.1	0.2	0.1
Other fatty acids	0.2	ND	0.1

OO, control olive oil; PS-SO, plant sterols esterified with sunflower oil fatty acids; PS-OO, plant sterols esterified with olive oil fatty acids; ND, not detected.

* Typical values.

was calculated by the Friedewald equation²⁵ for individuals with TAG levels <4.5 mmol/l, while LDL-C was measured directly by the abovementioned enzymatic methods when TAG levels were >4.5 mmol/l. Apo A-I, apo B-100 and lipoprotein (a) (Lp(a)) levels were measured on the BN ProSpec Nephelometer (Dade, Behring Diagnostics, Marburg, Germany) utilizing the Dade Behring N Antisera assays to determine apo A-I, apo B-100, and Lp(a), respectively (Behring Diagnostics).

Plasma cholesterol precursor and plant sterols

Plasma PS concentrations were determined from the non-saponifiable material of plasma lipids as reported previously²⁶. Briefly, an internal standard, 5α-cholestane, was added to 0.5 ml plasma sample. Plasma samples were saponified with 0.5 M methanolic KOH for 1 h at 100°C and the non-saponifiable materials were extracted with petroleum diethyl ether. After extraction, the samples were derivatized with 1.3 ml TMS reagent (pyridine–hexamethyldisilazan–trimethylchlorosilane; 9:3:1, by volume); Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). The samples were then injected into GLC (HP 5890 Series II, Hewlett Packard, Palo Alto, CA, USA), equipped with a flame ionization detection and auto-injector system and with a 30 m capillary column. The column temperature was 285°C and the isothermal running conditions were maintained for 30 minutes. Detector and injector temperatures were set to 310°C and 300°C, respectively. Lathosterol, campesterol, stigmasterol and β-sitosterol peaks were identified by comparison with authenticated standards (Sigma-Aldrich Canada Ltd).

Plasma lipid peroxidation

Apo B-containing lipoproteins were precipitated with manganese chloride–heparin by ultracentrifugation²⁷. The LDL fraction was re-suspended in normal saline after centrifugation. The thiobarbituric acidreactive substance (TBARS) assay²⁸ was used to measure lipid peroxidation in the plasma LDL subfraction (OXItek, ZeptoMetrix Corporation, Buffalo, NY, USA).

Statistical analyses

Based on previous publications with a comparable design²⁹, twenty subjects would be required in order to detect a clinically significant difference (–0.48 mmol/l) in LDL-C levels, the primary outcome, using 0.84 mmol/l as a standard deviation, at the 0.05 level of significance and 80% power. A total enrolment of twenty-four patients was originally estimated as being required to enable a study dropout rate of 15%.

All data were expressed as means and their standard errors. Statistical significance was set at $P < 0.05$ for all analyses. Variables that were not normally distributed were log transformed before analysis. Differences in plasma variables were tested by repeated-measures ANOVA with the type of dietary matrix in each intervention arm as the within-subject factor and with endpoint values as the dependent variable. Baseline values were inserted into the model as covariates if their interaction with dietary matrices was found to be statistically significant. Subsequently, contrast analyses were used to identify differences between pairs of diets. When variables failed to demonstrate any treatment effect, the two-tailed paired Student's *t* test was used to compare baseline and endpoint values within each diet phase. Furthermore, a modified Cohen's effect size was calculated for endpoint values to evaluate changes from the baseline OO diet. Data were analyzed with the use of SAS software (version 8.0; SAS Institute Inc, Cary, NC, USA).

Results

Study subjects

Twenty-four subjects (eleven males, thirteen females) were recruited and twenty-one subjects (eleven males, ten females) completed the entire trial. The three females who dropped out during the first phase reported difficulties with the transportation to the clinic (*n* 1) or with daily clinic visits (*n* 1) and personal affairs (*n* 1). The baseline characteristics of study subjects who completed the trial are displayed in Table 3. No side effects were reported after consuming the treatment oils. Results from the complete blood count at the end of each phase were within the normal range for all subjects (data not shown). The mean baseline bodyweight for OO, PS-SO, and PS-OO (73.9 (SE 2.7) kg, 74.7 (SE 2.8) kg, and 74.4 (SE 2.8) kg, respectively) and the percentage changes in bodyweight values (–0.7 (SE 0.3) %, –0.4 (SE 0.2) %, and –0.7 (SE 0.2) %, respectively) did not differ between treatments. Baseline values (following washout periods) of all the characteristics presented in Table 3 were not statistically different between dietary phases (data not shown).

Table 3. Baseline characteristics of the study subjects (*n* 11 males, 10 females)

(Values are means with their standard errors)

	Mean	SE
Age (years)	54.19	1.62
Weight (kg)	73.69	2.72
BMI (kg/m ²)	25.93	0.62
Total cholesterol (mmol/l)	6.09	0.18
LDL-cholesterol (mmol/l)	3.91	0.12
HDL-cholesterol (mmol/l)	1.28	0.07
TAG (mmol/l)	1.77	0.25

Plasma lipid concentrations

Table 4 lists plasma lipid concentrations at the end of each treatment phase. Supplementation of an OO-based diet with either PS-SO or PS-OO resulted in reduced ($P=0.0218$ and 0.0185 , respectively) LDL-C levels compared with control OO, but there was only a mild tendency towards a reduction ($P=0.0839$) in TC levels. TC:HDL-C ratios following PS-SO treatment were lower ($P=0.0018$) compared with control OO, but they were not significantly different from those observed with PS-OO. Consumption of the PS-matrices did not influence plasma HDL-C or TAG (fasting and postprandial) concentrations (Table 4).

The effects of the dietary fats and PS matrices on apo concentrations are also presented in Table 4. Apo A-I concentrations were higher ($P=0.0052$ and $P<0.0001$) following PS-OO administration relative to control OO-diet and PS-SO, respectively, presumably due to differences in baseline values ($P<0.0001$). PS-containing diets had a strong tendency to induce ($P=0.0577$) lower (10–11%, change from baseline) apo B-100 concentrations. This was associated with a mild tendency towards decreased ($P=0.1030$) LDL-C:apo B ratios, especially following the PS-OO treatment (an effect size of 0.47). Apo B-100:apo A-I ratios were lower ($P=0.0052$) with

PS-SO (6%, relative to baseline) and, to a lesser degree ($P=0.2057$), with PS-OO (4%, relative to baseline) administrations compared with the control OO-diet. The effect of PS-SO consumption (an effect size of 0.35) on apo B-100:apo A-I ratios did not differ ($P=0.8698$) from the effect of PS-OO supplementation (an effect size of 0.31).

Plasma Lp(a) concentrations were not altered ($P=0.1182$) by the dietary treatments at the end of the feeding phases. When endpoint values were compared to baseline concentrations, however, consumption of PS-OO did not influence Lp(a) levels, while the control OO-based diet and PS-SO treatments resulted in an increase ($P=0.0050$ and $P=0.0421$, respectively) of Lp(a) concentrations (Fig. 1).

Plasma lipid peroxide concentrations

The dietary treatments did not impact ($P=0.1295$) endpoint plasma TBARS concentrations. However, when baselines and endpoints were compared (Fig. 2), the consumption of PS-OO was shown to have resulted in reduced (–13%; $P=0.0097$) TBARS levels, whereas the OO-based diet (–10%; $P=0.0993$) and the PS-SO (–3%; $P=0.1640$) treatments failed to produce a comparable effect.

Plasma neutral sterol concentrations

Consumption of PS-SO and PS-OO resulted in a statistically significant increase in plasma campesterol, stigmaterol, and β -sitosterol concentrations compared with the control OO-based diet (Table 5). Similar observations were obtained when plasma PS concentrations were normalized to cholesterol levels, although to a somewhat larger extent. The concentration of cholesterol precursor, lathosterol and its ratio to cholesterol were elevated ($P=0.0031$ and 0.0268 , $P=0.0564$ and 0.0047) by PS-SO and PS-OO treatments, respectively, compared with the control OO-diet.

Table 4. Fasting plasma lipid and apo concentrations in overweight, hyperlipidaemic subjects (*n* 21) consuming different oil supplements varying in fatty acid and plant sterol content in separate diets each lasting 4 weeks

(Values are means with their standard errors)

Plasma lipid	OO		PS-SO		PS-OO		<i>P</i> value*
	Mean	SE	Mean	SE	Mean	SE	
Cholesterol							
Total (mmol/l)	5.90	0.22	5.61	0.21	5.49	0.25	0.0839
LDL (mmol/l)	3.83 ^a	0.17	3.59 ^b	0.16	3.48 ^b	0.19	0.0483
HDL (mmol/l)	1.29	0.07	1.29	0.07	1.23	0.07	0.1904
Total:HDL	4.75 ^a	0.18	4.53 ^b	0.20	4.70 ^{ab}	0.22	0.0142
TAG							
Fasting (mmol/l)	1.86	0.27	1.62	0.19	1.75	0.25	0.1402
Postprandial (mmol/l)	2.63	0.21	2.56	0.23	2.70	0.28	0.9942
Apo							
ApoA-I (g/l)	1.30 ^a	0.06	1.30 ^a	0.06	1.31 ^b	0.05	0.0001†
ApoB-100 (g/l)	1.12	0.05	1.06	0.06	1.09	0.06	0.0577
ApoB-100:Apo A-I	0.89 ^a	0.04	0.84 ^b	0.05	0.84 ^{ab}	0.05	0.0168
LDL cholesterol:apoB-100	3.49	0.17	3.47	0.14	3.28	0.12	0.1030

OO, control olive oil; PS-SO, plant sterols esterified with sunflower oil fatty acids; PS-OO, plant sterols esterified with olive oil fatty acids.

* *P* values obtained by repeated-measures ANOVA, with †baseline concentrations included in the model as covariates. Values of plasma lipid concentrations were normalized using a log transformation.

^{a,b} Values not sharing a common superscript letter are significantly different at $P<0.05$.

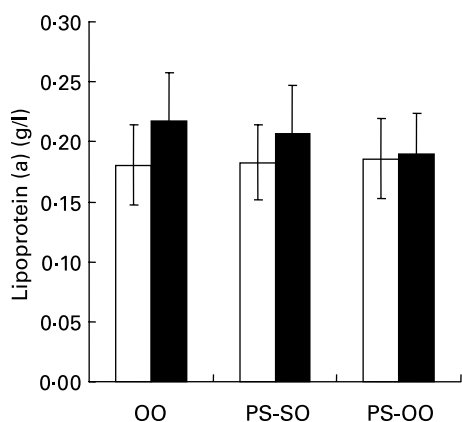


Fig. 1. Lipoprotein(a) (Lp(a)) levels in blood were measured at baseline (□) and after treatment (■). Values are means, with their standard errors shown by vertical bars, of Lp(a) concentrations of twenty-one patients. Statistical significance between the baseline and endpoint values was determined by the two-tailed paired-Student's *t* test for the OO ($P=0.005$) and the PS-SO ($P=0.0421$) treatments but not for PS-OO ($P=0.8123$). OO, control olive oil; PS-SO, plant sterols esterified with sunflower oil fatty acids; PS-OO, plant sterols esterified with olive oil fatty acids.

Discussion

Our results suggest that supplementation of an OO-based diet with OO fatty acids esterified to PS and mixed with dietary DAG reduces LDL-C levels and could lower LDL susceptibility to oxidation compared with an OO-based diet in hypercholesterolaemic mildly-overweight subjects. Consumption of both PS-OO and PS-SO diets favoured comparable beneficial reductions of CHD-related risk factors. However, in the context of an OO background diet, PS-OO feeding improved plasma antioxidant properties and protected against the increase in Lp(a) levels over the study period, while PS-SO supplementation did not show such an action.

In the current study, PS-OO and PS-SO supplementation to the base OO-diet tended to reduce TC concentrations by -8 and -6% , relative to baseline respectively. Importantly, we have shown that ingestion of 1.7 g/d PS esterified to OO or

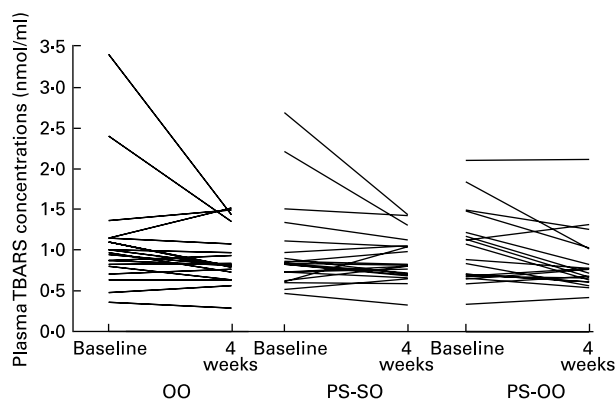


Fig. 2. Plasma thiobarbituric acid reactive substance (TBARS) levels in the LDL fraction were measured at baseline and endpoint (4 weeks) of each of three feeding phases (n 21 subjects). Statistical significance between the baseline and endpoint values was determined using the two-tailed paired-Student's *t* test only for the PS-OO treatment. ($P=0.0097$), but not for OO ($P=0.0993$) and the PS-SO ($P=0.1640$) treatments. OO: control olive oil; PS-SO: plant sterols esterified with sunflower oil fatty acids; PS-OO: plant sterols esterified with olive oil fatty acids.

SO fatty acids significantly decreased LDL-C concentrations by $6-9\%$ compared to the base OO-diet. These decreases are consistent with results from a recent meta-analysis⁶, in which an intake of $1.5-1.9$ g PS /d was associated with a $7.0-10.1\%$ reduction in LDL-C levels. Moreover, plasma levels of apo B-100 tended to lower with consumption of PS diets, but to a somewhat lesser extent than LDL-C concentrations. These observations suggest that the PS treatments may have affected the cholesterol content of the LDL particles more than their number. On the other hand, there was a significant reducing effect of PS-containing treatments on apo B-100:apo A-I ratio, suggesting that, in fact, these diets favour a beneficial suppression of apo B-100 levels compared with the control OO-diet. Taken together, our findings suggest that supplementation with PS provides superior protection against coronary artery disease risk factors than a healthy OO-based diet.

In this study, the PS concentrations in plasma increased following consumption of both PS-SO and PS-OO as compared with the base OO-diet. Plasma PS concentrations are naturally low since they are poorly absorbed. Nevertheless, high PS intake has been consistently shown to substantially increase circulating PS concentrations^{30,31}. In the current study, however, the degree of elevation in plasma PS concentrations and their ratios to cholesterol due to PS consumptions were 2–3-fold lower than what we had previously observed, when PS were administered equally across two to three daily meals³¹. Taken together with the somewhat limited LDL-C lowering effect, these findings suggest that a single morning dose of PS may result in a lower PS bioavailability and therefore lesser efficacy. The link between PS bioavailability and treatment efficacy warrants further investigation.

Compared with the baseline values, the serum lipid peroxides associated with LDL particles were significantly reduced following PS-OO treatment. Early publications suggested that OO consumption resulted in LDL enrichment with oleic acid and, consequently, in a greater resistance to oxidation³². Furthermore, a diet enriched in MUFA, rather than PUFA, was shown to inhibit LDL oxidation^{19,21,33}. The presence of high levels of PS in diet³⁴ or *in vitro*³⁵ has been associated with decreased lipid peroxidation. Interestingly, similar observations were recently noted following administration of MUFA-enriched PS mixed with DAG to atherosclerotic apo E deficient mice³⁶. Therefore, the antioxidant properties of MUFA-diets and PS could have contributed, at least in part, to the reduction in TBARS concentrations observed in our study on PS-OO.

In the current study, OO-diet and PS-SO supplementation resulted in increased Lp(a) levels over the study period, whereas no such deleterious effect was observed following PS-OO consumption. Elevated concentrations of Lp(a) have been suggested to be a risk factor for a variety of atherosclerotic and thrombotic disorders^{37,38}. The atherogenicity of Lp(a) may be mediated, at least in part, by associated proinflammatory oxidized phospholipids^{38,39}, but the link between Lp(a) and LDL oxidation in response to dietary modifications remains unclear. Although OO was shown in some studies to protect LDL from oxidation^{40,41}, there are several reports in which its consumption resulted in elevated Lp(a) concentrations^{42,43}. Likewise, PUFA-enriched diets have been shown to increase Lp(a) levels⁴⁴, presumably via interacting

Table 5. Plasma plant sterol and cholesterol precursor concentrations following the consumption of each dietary treatment by mildly-overweight hypercholesterolaemic subjects (*n* 21)

(Values are means with their standard errors)

Plasma sterol	OO		PS-SO		PS-OO		<i>P</i> value*
	Mean	SE	Mean	SE	Mean	SE	
Lathosterol (μmol/l)	6.03 ^a	0.75	6.42 ^b	0.53	7.21 ^{ab}	0.90	0.0085†
Campesterol (μmol/l)	9.26 ^a	0.83	18.11 ^b	1.75	18.68 ^b	2.07	<0.0001
Stigmasterol (μmol/l)	4.56 ^a	0.67	4.65 ^b	0.73	5.20 ^b	0.79	0.0282†
β-sitosterol (μmol/l)	5.48 ^a	0.61	7.62 ^b	0.94	7.73 ^b	0.96	0.0007
Lathosterol:cholesterol	1.06 ^a	0.14	1.22 ^b	0.14	1.43 ^b	0.19	0.0205†
Campesterol:cholesterol	1.61 ^a	0.15	3.36 ^b	0.36	3.71 ^b	0.47	<0.0001
Stigmasterol:cholesterol	0.84 ^a	0.14	0.93 ^{ab}	0.18	1.16 ^b	0.25	0.0121
β-sitosterol:cholesterol	0.93 ^a	0.08	1.38 ^b	0.15	1.50 ^b	0.18	<0.0001

OO, control olive oil; PS-SO, plant sterols esterified with sunflower oil fatty acids; PS-OO, plant sterols esterified with olive oil fatty acids.

* *P* values obtained by repeated-measures ANOVA, with †baseline concentrations included in the model as covariates.

Values of plasma lipids concentrations were normalized using a log transformation.

^{a,b} Values not sharing a common superscript letter are significantly different at *P*<0.05.

with several transcription factors, such as NF-κB and PPAR α ^{45,46}. In contrast, dietary DAG ingestion⁴⁷ was recently associated with a slight but significant reduction in Lp(a) concentrations, possibly as a result of decreased hepatic fat contents. In our current study, the PS-OO treatment, which contained equivalent amounts of OO fatty acids as the control OO-diet, in addition with DAG maintained low Lp(a) levels. Although the levels of DAG administered in the PS-OO dietary matrix were considerably lower than the dose of DAG that had been shown to be effective in human clinical studies^{42,48}, its presence could have contributed to maintaining Lp(a) levels. Taken together with the reduction in plasma TBARS concentrations, these results suggest that PS-OO may exert protective actions against oxidative stress.

In conclusion, our findings demonstrate that providing PS-containing matrices to hypercholesterolaemic, mildly overweight subjects fed with an OO-based diet results in optimized plasma lipid concentrations. Furthermore, consumption of PS-OO, but not PS-SO, may reduce the susceptibility of LDL to oxidative stress, which, in turn, could protect against increases in Lp(a) concentrations. Therefore, in the context of an OO-based diet, supplementation with PS-OO matrixed with DAG in OO may offer a greater level of protection against CHD than the traditional PS-SO formula to hypercholesterolaemic individuals.

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References

- Eisenberg D (1998) The importance of lowering cholesterol in patients with coronary heart disease. *Clin Cardiol* **21**, 81–84.
- Ross R (1999) Atherosclerosis-an inflammatory disease. *N Engl J Med* **340**, 115–126.
- Witztum JL & Steinberg D (1991) Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* **88**, 1785–1792.
- Meisinger C, Baumert J, Khuseynova N, Loewel H & Koenig W (2005) Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation* **112**, 651–657.
- Pollak OJ (1953) Reduction of blood cholesterol in man. *Circulation* **7**, 702–706.
- Katan MB, Grundy SM, Jones P, Law M, Miettinen T & Paoletti R (2003) Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clin Proc* **78**, 965–978.
- Ikeda I & Sugano M (1983) Some aspects of mechanism of inhibition of cholesterol absorption by beta-sitosterol. *Biochim Biophys Acta* **732**, 651–658.
- Heinemann T, Kullak-Ublick GA, Pietruck B & von Bergmann K (1991) Mechanisms of action of plant sterols on inhibition of cholesterol absorption. Comparison of sitosterol and sitostanol. *Eur J Clin Pharmacol* **40**, Suppl. 1, S59–S63.
- Ostlund RE Jr (2004) Phytosterols and cholesterol metabolism. *Curr Opin Lipidol* **15**, 37–41.
- Gremaud G, Dalan E, Piguat C, Baumgartner M, Ballabeni P, Decarli B, Leser ME, Berger A & Fay LB (2002) Effects of non-esterified stanols in a liquid emulsion on cholesterol absorption and synthesis in hypercholesterolemic men. *Eur J Nutr* **41**, 54–60.
- Meguro S, Higashi K, Hase T, Honda Y, Otsuka A, Tokimitsu I & Itakura H (2001) Solubilization of phytosterols in diacylglycerol versus triacylglycerol improves the serum cholesterol-lowering effect. *Eur J Clin Nutr* **55**, 513–517.
- Ostlund RE Jr, Spilburg CA & Stenson WF (1999) Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. *Am J Clin Nutr* **70**, 826–831.

13. Spilburg CA, Goldberg AC, McGill JB, Stenson WF, Racette SB, Bateman J, McPherson TB & Ostlund RE Jr (2003) Fat-free foods supplemented with soy stanol-lecithin powder reduce cholesterol absorption and LDL cholesterol. *J Am Diet Assoc* **103**, 577–581.
14. Weststrate JA & Meijer GW (1998) Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur J Clin Nutr* **52**, 334–343.
15. Demonty I, Chan YM, Pelled D & Jones PJ (2006) Fish-oil esters of plant sterols improve the lipid profile of dyslipidemic subjects more than do fish-oil or sunflower oil esters of plant sterols. *Am J Clin Nutr* **84**, 1534–1542.
16. Aravanis C, Corcondilas A, Dontas AS, Lekos D & Keys A (1970) Coronary heart disease in seven countries. IX. The Greek islands of Crete and Corfu. *Circulation* **41**, 188–100.
17. Stark AH & Madar Z (2002) Olive oil as a functional food: epidemiology and nutritional approaches. *Nutr Rev* **60**, 170–176.
18. Mattson FH & Grundy SM (1985) Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. *J Lipid Res* **26**, 194–202.
19. Kratz M, Cullen P, Kannenberg F, Kassner A, Fobker M, Abuja PM, Assmann G & Wahrburg U (2002) Effects of dietary fatty acids on the composition and oxidizability of low-density lipoprotein. *Eur J Clin Nutr* **56**, 72–81.
20. Kris-Etherton PM, Pearson TA, Wan Y, Hargrove RL, Moriarty K, Fishell V & Etherton TD (1999) High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. *Am J Clin Nutr* **70**, 1009–1015.
21. Puiggros C, Chacon P, Armadans LI, Clapes J & Planas M (2002) Effects of oleic-rich and omega-3-rich diets on serum lipid pattern and lipid oxidation in mildly hypercholesterolemic patients. *Clin Nutr* **21**, 79–87.
22. Reaven PD & Witztum JL (1996) Oxidized low density lipoproteins in atherogenesis: role of dietary modification. *Annu Rev Nutr* **16**, 51–71.
23. Berger A, Jones PJ & Abumweis SS (2004) Plant sterols: factors affecting their efficacy and safety as functional food ingredients. *Lipids Health Dis* **3**, 5.
24. Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA & Koh YO (1990) A new predictive equation for resting energy expenditure in healthy individuals. *Am J Clin Nutr* **51**, 241–247.
25. Friedewald WT, Levy RI & Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* **18**, 499–502.
26. Ntanos FY & Jones PJ (1998) Effects of variable dietary sitostanol concentrations on plasma lipid profile and phytosterol metabolism in hamsters. *Biochim Biophys Acta* **1390**, 237–244.
27. Gidez LI, Miller GJ, Burstein M, Slagle S & Eder HA (1982) Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure. *J Lipid Res* **23**, 1206–1223.
28. Lefevre G, Beljean-Leymarie M, Beyerle F, Bonnefont-Rousselot D, Cristol JP, Therond P & Torrealles J (1998) Evaluation of lipid peroxidation by measuring thiobarbituric acid reactive substances. *Ann Biol Clin (Paris)* **56**, 305–319.
29. Jones PJ, Ntanos FY, Raeini-Sarjaz M & Vanstone CA (1999) Cholesterol-lowering efficacy of a sitostanol-containing phytosterol mixture with a prudent diet in hyperlipidemic men. *Am J Clin Nutr* **69**, 1144–1150.
30. Jones PJ, Vanstone CA, Raeini-Sarjaz M & St-Onge MP (2003) Phytosterols in low- and nonfat beverages as part of a controlled diet fail to lower plasma lipid levels. *J Lipid Res* **44**, 1713–1719.
31. Vanstone CA, Raeini-Sarjaz M, Parsons WE & Jones PJ (2002) Unesterified plant sterols and stanols lower LDL-cholesterol concentrations equivalently in hypercholesterolemic persons. *Am J Clin Nutr* **76**, 1272–1278.
32. Aviram M & Eias K (1993) Dietary olive oil reduces low-density lipoprotein uptake by macrophages and decreases the susceptibility of the lipoprotein to undergo lipid peroxidation. *Ann Nutr Metab* **37**, 75–84.
33. Berry EM, Eisenberg S, Haratz D, Friedlander Y, Norman Y, Kaufmann NA & Stein Y (1991) Effects of diets rich in monounsaturated fatty acids on plasma lipoproteins—the Jerusalem Nutrition Study: high MUFAs vs high PUFAs. *Am J Clin Nutr* **53**, 899–907.
34. Homma Y, Ikeda I, Ishikawa T, Tateno M, Sugano M & Nakamura H (2003) Decrease in plasma low-density lipoprotein cholesterol, apolipoprotein B, cholesteryl ester transfer protein, and oxidized low-density lipoprotein by plant stanol ester-containing spread: a randomized, placebo-controlled trial. *Nutrition* **19**, 369–374.
35. van Rensburg SJ, Daniels WM, van Zyl JM & Taljaard JJ (2000) A comparative study of the effects of cholesterol, beta-sitosterol, beta-sitosterol glucoside, dehydroepiandrosterone sulphate and melatonin on *in vitro* lipid peroxidation. *Metab Brain Dis* **15**, 257–265.
36. Fuhrman B, Plat D, Herzog Y & Aviram M (2007) Consumption of a novel dietary formula containing plant sterols esterified to canola oil fatty acids and 1,3-diaclyglycerol in a canola oil matrix reduces oxidative stress in the atherosclerotic apolipoprotein E-deficient mice. *J Agric Food Chem* **55**, 2028–2033.
37. Boffa MB, Marcovina SM & Koschinsky ML (2004) Lipoprotein(a) as a risk factor for atherosclerosis and thrombosis: mechanistic insights from animal models. *Clin Biochem* **37**, 333–343.
38. Tsimikas S, Lau HK, Han KR, Shortal B, Miller ER, Segev A, Curtiss LK, Witztum JL & Strauss BH (2004) Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation* **109**, 3164–3170.
39. Tsimikas S, Bergmark C, Beyer RW, Patel R, Pattison J, Miller E, Juliano J & Witztum JL (2003) Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. *J Am Coll Cardiol* **41**, 360–370.
40. Aviram M (1996) Interaction of oxidized low density lipoprotein with macrophages in atherosclerosis, and the antiatherogenicity of antioxidants. *Eur J Clin Chem Clin Biochem* **34**, 599–608.
41. Fito M, Covas MI, Lamuela-Raventos RM, Vila J, Torrents L, de la Torre C & Marrugat J (2000) Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. *Lipids* **35**, 633–638.
42. Mensink RP & Katan MB (1992) Effect of dietary fatty acids on serum lipids and lipoproteins. A meta-analysis of 27 trials. *Arterioscler Thromb* **12**, 911–919.
43. Vessby B, Unsitupa M, Hermansen K, *et al.* (2001) Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study. *Diabetologia* **44**, 312–319.
44. Silaste ML, Rantala M, Alftan G, Aro A, Witztum JL, Kesaniemi YA & Horkko S (2004) Changes in dietary fat intake alter plasma levels of oxidized low-density lipoprotein and lipoprotein(a). *Arterioscler Thromb Vasc Biol* **24**, 498–503.
45. Maziere C, Conte MA, Degonville J, Ali D & Maziere JC (1999) Cellular enrichment with polyunsaturated fatty acids induces an

- oxidative stress and activates the transcription factors AP1 and NFkappaB. *Biochem Biophys Res Commun* **265**, 116–122.
46. Tai ES, Corella D, Demissie S, Cupples LA, Coltell O, Schaefer EJ, Tucker KL & Ordovas JM (2005) Polyunsaturated fatty acids interact with the PPARA-L162V polymorphism to affect plasma triglyceride and apolipoprotein C-III concentrations in the Framingham Heart Study. *J Nutr* **135**, 397–403.
 47. Teramoto T, Watanabe H, Ito K, Omata Y, Furukawa T, Shimoda K, Hoshino M, Nagao T & Naito S (2004) Significant effects of diacylglycerol on body fat and lipid metabolism in patients on hemodialysis. *Clin Nutr* **23**, 1122–1126.
 48. Rudkowska I, Roynette CE, Demonty I, Vanstone CA, Jew S & Jones PJ (2005) Diacylglycerol: efficacy and mechanism of action of an anti-obesity agent. *Obes Res* **13**, 1864–1876.