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[1–3]

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ABSTRACT

Background: Fish-oil fatty acid esters of plant sterols (FO-PS) were shown to have hypotriglyceridemic and hypocholesterolemic properties in animal models.

Objective: The objective of the study was to evaluate the hypolipidemic effects of FO-PS supplementation in healthy hypercholesterolemic persons fed an olive oil (OO)–based diet.

Design: Twenty-one moderately overweight, hyperlipidemic subjects participated in a semi-randomized, single-blind, 4-period crossover study including 4 experimental isoenergetic diets of 4 wk each and 4-wk intervening washout periods. Diets contained 30% of energy as fat, of which 70% was from extra-virgin OO, and differed only in the supplement oil: OO, fish oil, FO-PS, or sunflower oil esters of plant sterols (SU-PS). Both fish oil and FO-PS provided 5.4 g total eicosapentaenoic and docosahexaenoic acids/d. FO-PS, SU-PS, and OO provided the equivalent of 1.7, 1.7, and 0.02 g free plant sterols/d, respectively.

Results: Fish oil and FO-PS resulted in fasting and postprandial plasma triacylglycerol concentrations that were markedly lower than those observed with OO and SU-PS ($P = 0.0001$), but to a different extent. LDL cholesterol was significantly lower after supplementation with FO-PS and SU-PS than at the end of the control OO diet ($P = 0.0031$ and 0.0407, respectively). HDL cholesterol was not affected. FO-PS and SU-PS resulted in a lower ratio of total to HDL cholesterol and lower apolipoprotein (apo) B concentrations than did OO and fish oil. The ratio of apoB to apoA was significantly lower after SU-PS consumption than after consumption of OO ($P = 0.0126$) and fish oil ($P = 0.0292$). FO-PS and SU-PS resulted in similar ratios of apoB to apoA. HDL_2 and the ratio of HDL_2 to HDL_3 were significantly higher at the end of the FO-PS treatment than at the end of the OO ($P = 0.0006$), fish oil ($P = 0.0036$), and SU-PS ($P = 0.0016$) treatments.

Conclusion: Supplementation of an OO-based diet with FO-PS may reduce cardiovascular disease risk more than does supplementation with fish oil or SU-PS. *Am J Clin Nutr* 2006;84:1534–42.

KEY WORDS Apolipoproteins, cardiovascular risk factors, fish oil, LDL cholesterol, HDL cholesterol, n−3 polyunsaturated fatty acids, olive oil, plant sterols, plasma lipids, triacylglycerols

INTRODUCTION

Coronary heart disease (CHD) remains one of the leading causes of mortality and morbidity in developed countries (1, 2). Lowering LDL-cholesterol concentrations has been identified by the National Cholesterol Education Program (NCEP) for the detection, evaluation, and treatment of high blood cholesterol in adults as the primary target of therapy for CHD risk (3). However, other risk factors may significantly affect CHD risk, and a growing body of evidence indicates that elevated plasma triacylglycerol concentrations may be considered an independent risk factor (4-6). A recent meta-analysis showed that an increase of 1 mmol triacylglycerol/L was associated with 14% and 37% higher risks of cardiovascular disease in men and women, respectively (7). In addition, high triacylglycerol concentrations are often associated with other atherogenic factors, such as abdominal obesity, low HDL-cholesterol concentrations, small LDL particles, high blood pressure, and insulin resistance (8, 9). This combination of metabolic abnormalities, which is known as the metabolic syndrome, increases CHD risk at any given concentration of LDL cholesterol (3).

During the past 10 y, much effort has gone toward the development of nonpharmacologic approaches to decrease CHD risk. Plant sterols, also called phytosterols, are plant components that are structurally similar to cholesterol (10) and that are thought to interfere with the intestinal absorption of cholesterol by displacing cholesterol from micelles (11, 12). In fact, consumption of 2 g plant sterols or stanols/d is among the recommendations of the NCEP in lowering LDL-cholesterol concentrations (13). However, plant sterols were not shown to have any beneficial effect on circulating triacylglycerol concentrations (11, 14). Because long-chain n−3 polyunsaturated fatty acids (n−3 LC-PUFAs) from fish oil have a potent hypotriglyceridemic effect (15, 16), plant sterols have been recently esterified to fish-oil fatty acids to obtain a final product that would simultaneously reduce plasma

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LDL-cholesterol and triacylglycerol concentrations. Preliminary studies in animal models suggest that the fish-oil plant sterol esters (FO-PS) may reduce both plasma cholesterol and triacylglycerol concentrations (17-19). However, the efficiency of these novel plant sterol esters has not been shown in humans.

In the current study, we tested the effect of a novel fish-oil supplement containing plant sterols esterified to fish-oil fatty acids via an enzymatic process (ie, FO-PS) on the lipid profile of overweight, hyperlipidemic subjects. Our working hypothesis was that, when supplemented to an olive oil (OO)-based diet, this novel FO-PS supplement would cause an additional improvement in both plasma cholesterol and triacylglycerol concentrations. The effects of the FO-PS esters were compared with those of a nonesterified triacylglycerol fish oil and with sunflower oil of plant sterols (SU-PS).

TABLE 1
Average composition of the control olive oil–based diet over a period of 3 d[1]

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

[1] All values are $\bar{x}$ ± SE. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA; polyunsaturated fatty acids.

SUBJECTS AND METHODS

Subjects

Twenty-four hypercholesterolemic men and postmenopausal women aged 30–65 y were recruited by newspaper advertisement in Montreal, Canada, and the surrounding areas. On the basis of previous research by our group (20), a difference of 0.56 mmol/L in endpoint LDL-cholesterol concentrations could be expected between the phytosterol-containing diets and the control diet. With the use of the SD obtained in that study (ie, 0.84), a sample size of 20 was sufficient to detect a difference of 0.48 mmol/L with 95% CIs and 80% power. Twenty-four subjects were enrolled to compensate for eventual dropouts. Subjects were screened for LDL cholesterol after 12 h of fasting. Criteria for inclusion in the study were plasma LDL-cholesterol concentrations >2.56 mmol/L and body mass index (BMI; in kg/m^2) between 24 and 30. Persons who reported having taken lipid-lowering drugs, high-dose dietary supplements, or fish-oil capsules during the previous 3 mo were excluded. Before enrollment, subjects provided a complete medical history and underwent a routine physical examination. Detailed blood chemistry analyses were also performed to rule out any abnormality. Volunteers with diabetes mellitus, kidney disease, or liver disease were excluded. Smokers and volunteers drinking more than the equivalent of 2 glasses (or 284 mL) wine/d were also excluded. One woman taking thyroid hormone therapy was included in the study because she had been shown to be stable in the past few months, and no change was planned in her medication. Women taking hormone replacement therapy were asked to maintain their current regimen for the duration of the study. A physician was on call continually throughout the trial for subjects to contact in case they experienced discomfort.

All subjects provided written informed consent by completing forms that had been approved by the Ethics Review Board (protocol no. REB 808-0403). The experimental protocol was approved by the Faculty of Medicine Ethics Review Board at McGill University (Montreal).

Protocol and diets

Subjects underwent a semirandomized, crossover, single-blind clinical trial at the Mary Emily Clinical Nutrition Research Unit of McGill University. The study consisted of 4 phases of 29 d each, during which subjects were provided with an OO-based, weight-maintaining, North American diet. The composition of the control OO diet is shown in **Table 1**. In accordance with the Canadian Nutrient Recommended Intakes, the diet provided ≈55% of energy from carbohydrates, 30% from fat, and 15% from protein. Cholesterol and fiber contents were 80 mg/1000 kcal and 13 g/1000 kcal, respectively. The diet was identical during the 4 dietary phases, except for the treatment oil. The plant sterol and fatty acid compositions of the different treatments are shown in **Table 2**. All subjects received the control OO diet during the first 1-mo phase. They were then randomly assigned to the 3 remaining dietary treatments with the use of a Latin-square design. During the control OO phase, 70% of energy was provided as extra-virgin OO. During the other 3 phases, a small proportion of OO was replaced by the treatment oils: *1*) 7.6 g fish oil providing a total of 1.7 g eicosapentaenoic acid (EPA) and 3.7 g docosahexaenoic acid (DHA), *2*) 9.6 g fish oil containing FO-PS and providing the equivalent of 1.7 g free plant sterols as well as a total of 1.7 g eicosapentaenoic acid (EPA) and 3.7 g docosahexaenoic acid (DHA), or *3*) 21.4 g low-fat SU-PS margarine (Take Control; Unilever Bestfoods NA, Baltimore, MD) providing the equivalent of 1.7 g free plant sterols. The control OO provided the equivalent of 0.02 g naturally occurring free plant sterols/d. The fish oil was manufactured by Ocean Nutrition Canada Ltd (Halifax, Canada) and contained 51% DHA and 23% EPA (% of total fatty acids). The FO-PS was synthesized by Enzymotec Ltd (Migdal HaEmeq, Israel) by using the same batch of fish oil. Tocopherol mixtures (0.2% by wt) were added to the base stock fish oil used as a control fish-oil treatment and as a source of fatty acids for the esterification of plant sterols. No antioxidant beyond the original formulation was introduced to the tested plant sterol esters of fish oil.

The nutrient content of the diet was adjusted by using FOOD PROCESSOR software (version 7.81; Food Processor, Salem, OR). Three isocaloric meals, of similar macronutrient and micronutrient composition, were prepared daily for each subject in the metabolic kitchen of the research unit. Food ingredients were weighted to the nearest 0.5 g. Breakfast, during which treatment oils were ingested, was consumed under supervision at the clinic. The single morning dose of fish oil and FO-PS was stirred into orange juice to neutralize fish-oil organoleptic properties. The control OO was provided in orange juice as well, and the SU-PS margarine was served on French toast, English muffins, or omelets. In both cases, there was no delay between the consumption of the plant sterol esters and the remainder of the meal. Lunch and dinner were packed for consumption at work or home. A 3-d rotating menu was offered to provide a variety of foods. Volunteers were instructed to eat and drink only the food prepared at the

TABLE 2
Fatty acid and plant sterol composition of study formulations[1]

	Control olive oil	Fish oil	Fish-oil esters of plant sterols	Sunflower oil esters of plant sterols
Plant sterol esters (% by wt of oil or margarine)	ND	ND	22.1	12.1
Free plant sterol equivalents (% by wt of oil or margarine)				
β-Sitosterol	0.17	ND	8.1	3.7
Campesterol	0.009	ND	4.9	1.9
Stigmasterol	0.0001	ND	3.3	1.4
Brassicasterol	ND	ND	0.8	0.2
Others	0.06	ND	1.1	0.5
Total	0.23	ND	18.2	7.8
Fatty acids (% by wt of total fatty acids)				
12:0	ND	ND	ND	0.19
14:0	0.01	0.30	0.45	0.15
15:0	ND	0.03	0.05	ND
16:0	12.90	0.64	1.08	8.25
16:1	0.96	0.40	0.68	0.11
17:0	ND	0.07	0.16	0.08
18:0	2.91	0.18	0.53	6.22
18:1	69.76	3.84	7.52	41.80
18:2	11.52	0.61	1.21	36.38
18:3n−3	0.79	0.66	1.34	5.49
18:4	ND	1.40	3.94	ND
20:0	0.51	0.04	0.11	0.46
20:1	0.25	2.65	3.10	0.29
20:2	ND	0.47	0.60	ND
20:4n−6	ND	1.10	0.68	ND
20:4n−3	ND	1.63	2.55	ND
20:5n−3	ND	23.28	14.75	ND
22:0	0.15	0.03	ND	0.44
22:1	ND	0.69	0.68	ND
22:5n−3	ND	5.00	4.60	ND
22:6n−3	ND	51.05	49.04	ND
24:0	0.08	ND	ND	0.17
24:1	ND	0.30	0.24	ND
Other fatty acids	0.16	5.62	6.68	ND

[1] Values are typical values. ND, not detected.

clinic, but they were allowed water. Consumption of alcoholic and caffeinated beverages was strictly prohibited during the treatment phases. Subjects were provided with decaffeinated, energy-free beverages to drink between meals. Each treatment phase was followed by a wash-out period of 3 to 4 wk during which subjects consumed their habitual diet and did not visit the clinic. The subjects were regularly asked to maintain their usual level of physical activity throughout the study and to report any symptom, disease onset, medication consumption, or change in their habits.

Basal energy requirements were calculated individually for each subject by using the Mifflin equation (21). Basal energy requirements were then multiplied by 1.7 to supply the additional energy needs for mild-to-moderate activity. Body weight was monitored daily during treatment phases. If subjects gained or lost weight during the first week of the first phase, energy intake was adjusted to maintain constant body weight. The same energy intakes were provided in the next 3 phases.

Fasting blood samples were collected on days 1, 2, 28, and 29 of each treatment phase for measurement of plasma lipid concentrations. On day 28 of each phase, postprandial plasma triacylglycerol concentrations were measured 4 h after breakfast. On day 29 of each phase, an additional blood sample was taken for a complete blood count analysis to ensure that no subject had developed anemia.

Laboratory analyses

Plasma lipids, apolipoproteins and lipoprotein(a)

Blood samples were collected in Vacutainer tubes (Becton Dickinson, Mississauga, Canada) containing polymer gel and silica activator. After 30 min, tubes were centrifuged at 1000 × *g* for 15 min at 4 °C to isolate plasma. Serum samples were stored at −80 °C until lipids were measured. Serum total cholesterol, HDL-cholesterol, and triacylglycerol concentrations were measured by an enzymatic method using the corresponding Flex reagents on a multianalyzer (Dimension RxL Max; Dade Behring Diagnostics, Marburg, Germany). LDL-cholesterol concentrations were calculated with the equation of Friedewald et al (22) in samples containing <4.5 mmol triacylglycerol/L. When plasma triacylglycerols were >4.5 mmol/L, LDL-cholesterol concentrations were measured directly by using the Flex reagents on the Dimension RxL Max multianalyzer. Subclasses 2 and 3 of HDL cholesterol (HDL_2 and HDL_3) were obtained by dual precipitation as described previously (23, 24). In brief, plasma apolipoprotein (apo) B–containing lipoproteins were precipitated with manganese chloride-heparin (1.12 mol $MnCl_2$/L, 20 000 USP units heparin/mL; 50:6 by vol) by ultracentrifugation at 1500 × *g* for 1 h at 4 °C. Part of the supernatant fluid was used to analyze total HDL cholesterol, and the remainder

was mixed with dextran sulfate (14.3 mg/mL) to precipitate HDL_2 cholesterol. After ultracentrifugation at 1500 × *g* for 30 min at 4 °C, HDL_3 cholesterol was measured in the supernatant fluid. Total HDL and HDL_3 cholesterol were measured by using a cholesterol enzymatic kit (Roche Diagnostics, Laval, Canada). Correction factors of 1.1 and 1.21 were used for total HDL and HDL_3 cholesterol, respectively, to take into account the dilution by the reagents. HDL_2 cholesterol was calculated from the difference between total HDL and HDL_3 cholesterol. Apo A-I and apo B were measured by using the N Antisera kit for apo A-I and apo B assays, respectively, on the BN ProSpec Nephelometer (Dade Behring Diagnostics). To reduce day-to-day variation, endpoint lipid concentrations were obtained from the averages of values obtained on days 28 and 29. Lipoprotein(a) [Lp(a)] concentrations were measured in plasma samples collected on day 28 of each phase by using the N Latex Lp(a) assay (Dade Behring Diagnostics) on the BN ProSpec Nephelometer.

Plasma fatty acid profile

Plasma samples from days 1, 2, 28, and 29 were analyzed for fatty acid composition by using gas-liquid chromatography. Total lipids were extracted by a modified Folch extraction (25), and fatty acids were methylated according to the procedure of Morrison and Smith (26). Briefly, an internal standard (heptadecanoic acid, 1 mg/mL) and methanol were added to the serum samples. Total lipids were extracted by using a ratio of chloroform to methanol (4:1, by vol) in the presence of deionized, distilled water. The aqueous phase was separated by centrifugation, and the organic supernatant phase was transferred into another tube. The aqueous phase was then re-extracted by using a ratio of hexane to chloroform (4:1, by vol). The supernatant fluid was added to the first extraction and dried under nitrogen. The methylating reagent (boron fluoride–methanol:hexane:methanol (7:6:7, by vol) was added to the samples, which were heated at 100 °C for 55 min. After the samples were cooled at room temperature, hexane and deionized distilled water were added to the samples. The tubes were centrifuged at 2500 rpm for 5 min at 4 °C (Sorvall model RT 6000B; DuPont Co, Wilmington, DE), and the top layer was transferred to a disposable culture glass tube, dried under nitrogen, redissolved in a smaller volume of hexane containing 50 ppm butylated hydroxytoluene (BHT), and transferred to amber vials.

Fatty acid profiles were determined by using a gas chromatograph (Clarus 500 GC; Perkin-Elmer, Shelton, CT) equipped with a 100-m × 0.2-mm SP-2560 fused silica capillary column (Supelco, Bellefonte, PA) and a flame ionization detector. The column temperature was held at 100 °C for 1 min, increased to 210 °C at a rate of 8 °C/min, and held at 210 °C for the remainder of the run. The injector temperature was set at 250 °C and the detector temperature at 275 °C. Helium was used as the carrier gas at a flow rate of 45 mL/min. Fatty acid methyl esters were identified on the basis of the retention time of known standards (Sigma-Aldrich Canada Ltd, Oakville, Canada). Results were expressed as a percentage of total identified fatty acids by weight.

Plasma plant sterol concentrations

Plasma concentrations of campesterol and β-sitosterol were determined by using gas-liquid chromatography as reported previously (27). An internal standard of 5α-cholestane (Sigma-Aldrich Canada Ltd) was added to each plasma sample. Samples were saponified with 0.5 mol methanolic KOH/L. Nonsaponified materials were then extracted 2 times with petroleum ether and dried under nitrogen flux. The extracts were derivatized by using TMS reagent (pyridine:hexamethyldisilazan:trimethylchlorosilane 9:3:1 by vol) (Sigma-Aldrich Canada Ltd; 28). After evaporation under nitrogen, the samples were dissolved in hexane and injected into a gas-liquid chromatograph (HP 5890 Series II; Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and an auto-injector system. Separation was achieved on a 30-m SAC-5 capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 μm (Supelco). The carrier gas (helium) flow rate was 7.5 psi. Samples were injected at 300 °C. The oven temperature remained at 160 °C for 1 min after injection, was increased to 245 °C at a rate of 15 °C/min, and then was kept constant for 5 min, after which it rose to 285 °C at a rate of 15 °C/min. Then, the temperature was kept constant at 285 °C for 20 min. The detector was set at 310 °C. Plant sterols (ie, campesterol and β-sitosterol) were identified by using authentic standards (Sigma-Aldrich Canada Ltd).

Plasma retinol and α-tocopherol concentrations

Plasma concentrations of the fat-soluble vitamins, ie, retinol and α-tocopherol, were measured by using light-protected, reverse-phase HPLC as previously described (29). Briefly, an internal standard solution (10 mg/dL) of retinol acetate (Sigma-Aldrich, St Louis, MO) was added to 250-μL plasma samples. Fat-soluble compounds were extracted with 200 μL methanol and 1000 μL hexane and then were mixed by vortex for 1 min. The organic phase was separated by centrifugation for 10 min at 13 000 × *g* at room temperature. The hexane layer (750 μL) was then transferred and dried under nitrogen. The extracts were dissolved in 250 μL methanol, mixed by vortex, and injected into an HPLC column (JASCO, Dunmow, United Kingdom) equipped with an ultraviolet detector and an auto-injector system. Separation was achieved on a 150 × 4.6–mm octadecylsilane 3-μm particle column (Supercosil LC-18DB; Sigma-Aldrich), with methanol as a mobile phase, at a flow rate of 1.5 mL/min. Fat-soluble vitamins were identified by using authentic standards (Sigma-Aldrich) and multiwavelength detection (325 and 292 nm for retinol and α-tocopherol, respectively) and were quantified by using standard curves.

LDL lipid peroxidation

LDL particles were precipitated according to the method of Gidez et al (23). Briefly, a manganese chloride–heparin solution was added to plasma samples. After centrifugation, the supernatant was discarded and the LDL fraction was resuspended in normal saline. Lipid peroxidation in plasma LDL subfractions was measured by using the thiobarbituric acid–reactive substance (TBARS) assay (Oxitek; ZeptoMetrix Corporation, Buffalo, NY) as described in the manufacturer's manual.

Statistical analyses

Results are expressed as means ± SEMs, and $P < 0.05$ was considered significant. Variables that were not normally distributed were log transformed before analysis. Differences in plasma variables were evaluated by using, first, repeated-measures analysis of variance (ANOVA) for carryover effect and, second, repeated-measures ANOVA with the type of dietary matrix in

TABLE 3
Baseline characteristics of the subjects who completed all dietary treatments

Characteristic	Value
Male (*n*)	11
Female (*n*)	10
Age (y)	54.2 ± 1.6[1]
Weight (kg)	73.7 ± 2.7
BMI (kg/m^2)	25.9 ± 0.6
Cholesterol (mmol/L)	
Total	6.09 ± 0.18
LDL	3.91 ± 0.12
HDL	1.28 ± 0.07
Triacylglycerols (mmol/L)	1.77 ± 0.25

[1] $\bar{x}$ ± SE (all such values).

each intervention arm as the within-subject factor and with endpoint values as the between-subject factors. Baseline values, sex, age, BMI, or all of those variables were inserted into the model as covariates if their interactions with dietary matrixes were found to be statistically significant. Subsequently, contrast analyses were used to identify differences between pairs of diets. All statistical analyses were conducted by using SAS software (version 8.2; SAS Institute, Cary, NC).

RESULTS

Subjects

Twenty-four subjects who fit the study criteria were included in the study. Three subjects withdrew from the study before completion: 2 dropped out for personal reasons, including lack of time and difficulty in reaching the research clinic, and 1 abandoned the study because of gastrointestinal discomfort associated with fish-oil consumption. The remaining 21 subjects completed all 4 treatment phases. The characteristics of the 21 study subjects at the time of selection are shown in **Table 3**.

Most subjects maintained good health throughout the study, and no major adverse events were reported. Approximately one-half of the patients reported burping after ingestion of the fish oil ($n = 9$) and the FO-PS ($n = 10$). Some patients complained about a fishy aftertaste ($n = 5$ and 2 for the fish oil and the FO-PS, respectively). Nausea ($n = 1$ for each of the fish-oil treatments) and gastrointestinal discomfort ($n = 4$ and 3 for the fish-oil and FO-PS treatments, respectively) were reported by a few subjects. In most cases, the symptoms were present for 2–4 h after consumption of the supplement, and their intensity decreased from day to day. Chewing sugarless gum helped decrease the intensity of the side effects. One subject had diarrhea for 2–3 d at the beginning of the 2 fish-oil treatments, and another patient had diarrhea for 4 d during the fish-oil phase. In the latter case, it is suspected that the disorder was of infectious origin. Except for a light abdominal discomfort reported by 3 patients at the beginning of the control OO diet, no gastrointestinal symptoms were reported during the control OO or the SU-PS phase.

At endpoints, body weights were 73.3 ± 2.6, 74.4 ± 2.8, 72.4 ± 2.8, and 74.3 ± 2.8 kg for the control OO, control fish-oil, FO-PS, and SU-PS groups, respectively. Because body weights at the end of each treatment phase were significantly correlated with body weights at the beginning of the phases ($P = 0.0001$), initial BMI values were used as covariates in the model.

Biochemical endpoints

Plasma lipid concentrations at the end of each treatment phase are shown in **Table 4**. Supplementation of a control OO-based diet with FO-PS and SU-PS resulted in LDL-cholesterol concentrations that were significantly ($P = 0.0031$ and 0.041, respectively) lower than those observed with the control diet. A strong tendency toward a reduction in total cholesterol concentrations ($P = 0.067$) was also observed. These observations were associated with significantly lower total:HDL cholesterol after supplementation with FO-PS and SU-PS than after the control OO diet and fish-oil supplementation. Apo B concentrations were 5–6% lower after SU-PS and FO-PS supplementation than after the control OO and fish-oil diet. In addition, SU-PS resulted in apoB:apo A that were significantly lower than those observed with control OO ($P = 0.013$) or fish oil ($P = 0.029$). ApoB:apoA at the end of the FO-PS phase did not differ significantly from the ratios observed after consumption of SU-PS and were significantly lower than those obtained with fish oil ($P = 0.0049$).

The effect of the diets on HDL-cholesterol subfractions is also shown in Table 4. HDL_2 concentrations were higher at the end of the FO-PS phase than at the end of the other 3 diets. In addition, fish oil and FO-PS resulted in lower HDL_3 subfraction concentrations than did OO and SU-PS. These modifications resulted in ratios of HDL_2 to HDL_3 (HDL_2:HDL_3) that were 62%, 7%, and 46% higher after supplementation with FO-PS than after consumption of control OO ($P = 0.0006$), fish oil ($P = 0.0036$), and SU-PS ($P = 0.0016$), respectively.

Significant differences were observed between the effects of the dietary treatments on fasting and postprandial plasma triacylglycerol concentrations. Indeed, supplementation of the control OO diet with fish oil and FO-PS resulted in fasting triacylglycerol concentrations that were 40% ($P = 0.0004$) and 46% ($P = 0.0002$) lower, respectively, than those observed with OO alone. Moreover, FO-PS and fish oil resulted in plasma triacylglycerol concentrations that were 39% ($P < 0.0001$) and 32% ($P = 0.0001$) lower, respectively, than those observed with SU-PS. It is interesting that fasting plasma triacylglycerol concentrations were significantly ($P = 0.03$) lower after consumption of FO-PS than after fish-oil supplementation. In the postprandial state, plasma triacylglycerol concentrations were 40% and 30% lower at the end of the FO-PS and fish-oil phases, respectively, than at the end of the control OO and SU-PS phases. Similar to the differences in fasting triglycerol concentrations, postprandial triacylglycerol concentrations after the consumption of FO-PS were significantly different from those observed with fish oil ($P < 0.0001$).

Plasma Lp(a) and TBARS concentrations were not significantly altered by the dietary treatments (Table 4). Consumption of SU-PS resulted in significantly ($P = 0.0013$ and 0.021) higher plasma campesterol concentrations than did the control OO and fish-oil diets, respectively (Table 4). FO-PS also resulted in high campesterol concentrations, which did not differ from those observed after supplementation with SU-PS. However, plasma β-sitosterol concentrations were not significantly altered by the different diets. Plasma concentrations of retinol and α-tocopherol were not significantly affected by plant sterol ester supplementation. Indeed, retinol concentrations were 5.9% lower after SU-PS supplementation (median: 64.2 μg/dL; range: 48.2–96 μg/dL) than after consumption of the control OO diet

TABLE 4
Fasting plasma lipid, apolipoprotein, plant sterol, and thiobarbituric acid–reactive substance concentrations in overweight, hyperlipidemic subjects consuming different oil supplements varying in fatty acid and plant sterol content for 4 wk[1]

Plasma lipid	Control olive oil	Fish oil	Fish-oil esters of plant sterols	Sunflower oil esters of plant sterols	P^2
Cholesterol					
Total (mmol/L)	5.90 ± 0.22[3]	5.69 ± 0.23	5.48 ± 0.23	5.61 ± 0.21	0.0668[4]
LDL (mmol/L)	3.83 ± 0.17[a]	3.95 ± 0.18[b]	3.73 ± 0.17[b]	3.59 ± 0.16[b]	0.0321[4,5]
HDL (mmol/L)	1.29 ± 0.07	1.25 ± 0.08	1.30 ± 0.07	1.29 ± 0.07	0.2217
Total:HDL	4.75 ± 0.18[a]	4.81 ± 0.24[a]	4.38 ± 0.21[b]	4.53 ± 0.20[b]	0.0040
HDL_2 (mmol/L)	0.35 ± 0.05[a]	0.44 ± 0.06[a]	0.48 ± 0.06[b]	0.37 ± 0.05[a]	0.0032[6]
HDL_3 (mmol/L)	0.94 ± 0.04[a]	0.80 ± 0.04[b]	0.84 ± 0.04[b]	0.90 ± 0.04[a]	0.0069
HDL_2:HDL_3	0.37 ± 0.05[a]	0.56 ± 0.07[a,b]	0.60 ± 0.07[c]	0.41 ± 0.05[a]	0.0045[6]
Triacylglycerols					
Fasting (mmol/L)	1.86 ± 0.27[a]	1.10 ± 0.13[b]	0.99 ± 0.12[c]	1.62 ± 0.19[a]	0.0012
Postprandial (mmol/L)	2.63 ± 0.21[a]	1.80 ± 0.27[b]	1.53 ± 0.15[c]	2.56 ± 0.23[a]	0.0002
Apolipoproteins					
Apo A-I (g/L)	1.30 ± 0.06	1.25 ± 0.05	1.31 ± 0.04	1.30 ± 0.06	0.0929
Apo B (g/L)	1.12 ± 0.05[a]	1.09 ± 0.05[a]	1.05 ± 0.06[b]	1.06 ± 0.06[b]	0.0100
Apo B:Apo A-I	0.89 ± 0.04[a,b]	0.90 ± 0.05[a]	0.82 ± 0.05[b,c]	0.84 ± 0.05[c]	0.0031
LDL cholesterol:apo B	3.49 ± 0.17	3.65 ± 0.10	3.60 ± 0.05	3.47 ± 0.14	0.2410[4]
Lp(a) (g/L)	0.22 ± 0.04	0.22 ± 0.04	0.22 ± 0.04	0.21 ± 0.04	0.7723[4]
TBARS (nmol/mL)	1.04 ± 0.15	1.05 ± 0.07	1.05 ± 0.10	0.87 ± 0.06	0.1143
Plant sterols					
Campesterol (μmol/L)	9.26 ± 0.83[a]	12.06 ± 1.29[a]	20.25 ± 2.04[a,b]	18.12 ± 1.75[b]	0.0016[6]
β-Sitosterol (μmol/L)	5.48 ± 0.61	6.93 ± 0.79	8.31 ± 0.89	7.62 ± 0.94	0.2294[6]

[1] $n = 21$. Apo, apolipoprotein; Lp(a), lipoprotein(a). Means in a row with different superscript letters are significantly different, $P < 0.05$ (repeated-measures ANOVA and contrasts.)

[2] Obtained by using repeated-measures ANOVA, with baseline concentrations, sex, or BMI included in the model as covariates. For fasting and postprandial triacylglycerols, P value was obtained by an ANOVA on log-transformed values. Values of plasma plant sterol concentrations were normalized by using a log transformation as well.

[3] $\bar{x}$ ± SE (all such values).

[4–6] Included in the model as covariate: [4] sex, [5] BMI, [6] baseline concentrations.

(median: 67.2 μg/dL; range: 44.1–85.2 μg/dL), but this difference was not significant ($P = 0.43$). FO-PS (median: 72.1 μg/dL; range: 46.2–87.4 μg/dL) resulted in retinol concentrations that did not differ from those observed with control OO. SU-PS and FO-PS resulted in α-tocopherol concentrations (median: 1660 μg/dL; range: 990–3650 μg/dL for SU-PS; median: 1550 μg/dL; range: 900–3170 μg/dL for FO-PS) that were 10% and 4% higher, respectively, than the concentrations measured at the end of the control OO diet (median: 1490 μg/dL; range: 700–3510 μg/dL); however, this effect was not significant ($P = 0.77$).

The effect of the different treatments on the plasma fatty acid profile is shown in **Table 5**. EPA and DHA concentrations were markedly higher after fish-oil and FO-PS supplementation than after the control OO diet ($P < 0.0001$ and < 0.0001; $P < 0.0001$ and $= 0.0036$, respectively) and supplementation with SU-PS ($P < 0.0001$ and $= 0.0034$; $P = 0.0022$ and 0.0466, respectively). Fish-oil supplementation resulted in oleic acid concentrations that were lower than those observed with control OO ($P = 0.0334$) and SU-PS ($P = 0.0019$). Both fish-oil diets and the SU-PS resulted in lower arachidonic acid concentrations than did the control OO diet ($P = 0.0003$, 0.0007, and 0.0303 for fish oil, FO-PS, and SU-PS, respectively); the concentrations with the fish-oil treatments were lower than those with the SU-PS ($P = 0.0034$ and 0.0215 for fish oil and FO-PS, respectively). Lower linoleic acid concentrations were observed with supplementation of both fish-oil matrixes than with control OO ($P = 0.0054$ and 0.0365 for fish oil and FO-PS, respectively) and SU-PS ($P = 0.0001$ and 0.0031 for fish oil and FO-PS, respectively). Supplementation of FO-PS resulted in lower and consumption of SU-PS resulted in higher α-linolenic acid concentrations than were observed after consumption of the control OO diet ($P = 0.00177$ and 0.0054, respectively).

DISCUSSION

The current results show that, in overweight, hyperlipidemic subjects consuming an OO-based diet, FO-PS supplementation has a potent hypotriglyceridemic effect that may be even more pronounced than the one observed with regular fish-oil supplementation, and it results in better lipoprotein and apolipoprotein profiles than are seen with fish oil. Moreover, the triacylglycerol-lowering properties of FO-PS, in addition to their increasing effect on HDL_2 cholesterol subfractions, make these novel plant sterol esters more beneficial than is SU-PS.

The hypotriglyceridemic effect of fish oil is well documented (15, 16). The 40% reduction in fasting plasma triacylglycerols observed in the current study in patients ingesting 7.6 g fish oil/d is proportional to the 14% decrease in triacylglycerol associated with the average daily consumption of 2.7 g fish oil reported in clinical trials in normotriglyceridemic patients (30). Postprandial triacylglycerol concentrations, which may be a better indicator of CHD than are fasting measurements (31), were also substantially lowered by FO-PS and fish-oil supplementation. The high-dose FO-PS supplement used in this experiment was necessary to provide the equivalent of 1.7 g free plant sterols/d. The lower triacylglycerol concentrations observed with FO-PS than with fish oil suggest a contribution of the plant sterol ester

TABLE 5
Plasma fatty acid profile in overweight, hyperlipidemic subjects consuming different oil supplements varying in fatty acid and plant sterol content for 4 wk[1]

	Control olive oil	Fish oil	Fish-oil esters of plant sterols	Sunflower oil esters of plant sterols	P^2
Plasma fatty acids (% of total identified fatty acids)					
Saturated					
Myristic (14:0)	0.87 ± 0.06[3]	0.79 ± 0.07	0.77 ± 0.08	0.85 ± 0.06	0.3823
Palmitic (16:0)	21.77 ± 0.83	24.66 ± 1.52	25.20 ± 1.94	22.00 ± 0.95	0.3562
Stearic (18:0)	7.02 ± 0.38	9.09 ± 0.56	9.28 ± 0.84	7.23 ± 0.33	0.0959
Monounsaturated					
Oleic (18:1n−9)	30.83 ± 1.16[a]	21.70 ± 0.70[b]	22.00 ± 1.00[a,b]	27.28 ± 0.97[a]	0.0228[4,5]
Polyunsaturated					
Linoleic (18:2n−6)	27.17 ± 0.97[a]	23.99 ± 0.92[c]	23.92 ± 1.38[c]	29.16 ± 1.00[b]	0.0032
α-Linolenic (18:3n−3)	0.51 ± 0.03[a]	0.45 ± 0.03[a,c]	0.42 ± 0.02[c]	0.57 ± 0.03[b]	0.0019
Arachidonic (20:4n−6)	8.21 ± 0.45[a]	5.74 ± 0.39[c]	5.91 ± 0.35[c]	7.65 ± 0.48[b]	0.0009
Eicosapentaenoic (20:5n−3)	0.65 ± 0.05[a]	5.93 ± 0.37[b]	5.63 ± 0.33[b]	1.02 ± 0.31[a]	0.0001[4]
Docosatetraenoic (22:4n−6)	1.07 ± 0.21	1.34 ± 0.19	1.13 ± 0.24	1.51 ± 0.25	0.2975
Docosahexaenoic (22:6n−3)	1.90 ± 0.15[a]	6.31 ± 0.37[b]	5.72 ± 0.32[b]	2.73 ± 0.23[a]	0.0010[4]

[1] $n = 21$. Means in a row with different superscript letters are significantly different, $P < 0.05$ (repeated-measures ANOVA and contrasts).
[2] Obtained by using repeated-measures ANOVA, with baseline concentrations or sex included in the model as covariate.
[3] $\bar{x}$ ± SE (all such values).
[4,5] Included in the model as covariate: [4] baseline concentration, [5] sex.

component to the triacylglycerol-lowering effect. Such a hypothesis is supported by data from other studies showing a hypotriglyceridemic effect of ascorbic acid esters of plant sterols in hamsters (32) and of vegetable oil fatty acid esters of plant stanols in hypertriglyceridemic subjects (33). However, the effect on triacylglycerols of SU-PS supplementation did not differ significantly from that of the control OO diet ($P = 0.104$). Data on the possible hypotriglyceridemic effect of plant sterols are still scarce, and confirmation of such an effect would require further investigations. On the other hand, the current data show that the novel FO-PS are efficient in lowering plasma triacylglycerol concentrations in the context of an OO-based diet, which, on its own, has been reported to induce a 10% triacylglycerol reduction compared with an average American diet (34).

Although n−3 LC-PUFAs from fish are associated with a lower risk of CHD because of their numerous beneficial effects on plasma triacylglycerols (35, 36), inflammation, coagulation, endothelial function (36) and heart rate (36, 37), their effect on plasma cholesterol is usually minor. Indeed, total and LDL-cholesterol concentrations are usually not affected by n−3 LC-PUFA supplementation (38–40). Because plant sterols have a well-established effect of lowering LDL-cholesterol concentrations (14, 41), we expected that plant sterols esterified to fish-oil fatty acids would cause an overall decrease in plasma total and LDL-cholesterol concentrations in hypercholesterolemic subjects. In the current study, LDL-cholesterol concentrations after supplementation with SU-PS and FO-PS were significantly lower than those at the end of the control OO diet phase. However, the reduction in LDL cholesterol was less pronounced (−3% and −6% with FO-PS and SU-PS, respectively) than was expected with the equivalent of 1.7 g free plant sterols [ie, −8.5% according to the meta-analysis of Katan et al (14)].

One explanation for the lower efficacy of plant sterols in the current study than in other trials may be that the plant sterol esters were given in a single dose. Most studies of the hypocholesterolemic effect of plant sterols have used 2–3 doses of plant sterols/d. Our one-dose design was based on the results of Plat et al (42), who observed no difference in LDL-cholesterol reduction between normocholesterolemic and hypercholesterolemic subjects consuming 2.5 g plant sterols/d at lunch or divided over the 3 daily meals (−9.9% and −10.2%, respectively). In another study in which 2.7 g plant sterols was given to hypercholesterolemic subjects in a single dose at lunchtime, LDL cholesterol was 14.6% lower than at baseline (43). It may be hypothesized that the time of administration affects the magnitude of the hypocholesterolemic effect of plant sterols. In the current study, plant sterol esters were given at breakfast. Results of a recent study in mildly hypercholesterolemic subjects showed that the LDL cholesterol–lowering effect of a single dose of 2.8–3.2 g plant sterols/d provided in a yogurt drink >30 min before breakfast was significantly less pronounced than the effect of the same plant sterol drink ingested with lunch (44). The latter results raise questions as whether plant sterol efficacy is affected by postprandial state, diurnal positioning of dose, or both. In the current study, all treatments were ingested at meal time, and no delay occurred between the consumption of the plant sterol esters and the meal. Therefore, differences between treatments could not be attributed to differences in intake occasion. The plant sterol dose itself does not appear to explain the absence of a significant effect on LDL cholesterol in the current study. Although the dose used in our experiment (1.7 g/d) was lower than the doses used in the previous once-a-day studies (1.7 g/d compared with 2.5–3.2 g/d, respectively; 42-44), a previous study showed that increasing the dosage from 1.6 to 3.2 g free sterol equivalent/d did not further increase LDL cholesterol–lowering efficacy (45).

Plasma HDL-cholesterol concentrations usually are inversely correlated with triacylglycerol concentrations (46). However, despite their potent hypotriglyceridemic properties, n−3 LC-PUFAs from fish oil have been reported to have no effect (16) or, when supplemented at high doses, to decrease total HDL-cholesterol concentrations (47). On the other hand, EPA and DHA may alter HDL-cholesterol subclasses. Increases in the HDL_2 subfraction have been reported with supplementation of 4 g DHA/d in hyperlipidemic men and patients with type 2

diabetes (48, 49). The effect of EPA on HDL_2 subclasses is less clear; a lowering effect on HDL_3 concentrations [with no effect on HDL_2 (48)] and higher HDL_2 measurements (49) have each been observed. When supplemented simultaneously, 1.48 g DHA/d and 1.88 g EPA/d were shown to increase HDL_2 concentrations in subjects with familial combined hyperlipidemia, a disorder characterized by low HDL_2 concentrations (50). HDL-cholesterol concentrations usually are not significantly affected by plant sterols (10, 14, 51), but a slight increase has been reported in a few studies (52, 53). In the current trial, FO-PS resulted in higher HDL_2-cholesterol concentrations and HDL_2:HDL_3 than did the 3 other diets. This effect may be due to the EPA and DHA components of FO-PS. However, despite a similar fatty acid composition, the fish oil did not significantly improve HDL_2 concentrations or HDL_2:HDL_3 relative to the control diet, which suggests that the esterification to plant sterols may beneficially affect the HDL subclass–modifying properties of n−3 LC-PUFAs from fish oil. HDL_2-cholesterol concentrations have been shown to be inversely associated with the risk of established CHD, and this association is stronger than the association with total HDL cholesterol (54). It is therefore possible that the higher HDL_2 concentrations observed when FO-PS are supplemented to a monounsaturated fatty acid–rich diet may result in a greater decrease in the risk of myocardial infarction than does supplementation with fish oil.

In our study, apoB:apoA was lower at the end of the SU-PS treatment than after the control OO diet and after supplementation with fish oil. Although apoB:apoA after FO-PS supplementation did not differ significantly from the ratio observed after SU-PS supplementation, the difference between FO-PS and control OO was not significant. However, apoB:apoA at the end of the FO-PS phase was significantly lower than that at the end of the fish-oil phase. In addition, FO-PS resulted in lower total:HDL cholesterol, another risk factor for cardiovascular disease, than did control OO and fish oil. The importance of this variable was shown in the Quebec Cardiovascular Study (55), which concluded that the variation in total:HDL cholesterol may be associated with more substantial alterations in metabolic indexes that are predictive of ischemic heart disease risk and related to the insulin resistance syndrome than is the variation in LDL cholesterol:HDL cholesterol. Whether such a beneficial effect in total:HDL cholesterol could be obtained by concomitant ingestion of fish oil and plant sterols remains to be elucidated.

It is assumed that the active form of plant sterols is the free form. Therefore, the rate of hydrolysis of plant sterol esters of different fatty acids in the intestinal tract could be a factor influencing their efficacy. In the current study, the similar increases in plasma campesterol and sitosterol concentrations observed after the ingestion of FO-PS and SU-PS suggest that the bioavailability of plant sterols is unaltered when they are esterified to fish-oil fatty acids. Reciprocally, n−3 LC-PUFAs from fish oil may be as bioavailable when they are esterified to plant sterols as when they are delivered in the customary fish-oil form. Indeed, the proportions of EPA and DHA were increased similarly at the end of the fish-oil and FO-PS supplementation phases, and both fish-oil treatments had a strong hypotriglyceridemic effect.

In summary, supplementation of an OO-based diet with FO-PS resulted in a marked decrease in plasma triacylglycerol concentrations in overweight, hyperlipidemic subjects. Similarly to the traditional vegetable oil fatty acid esters of plant sterols used in the current study, the novel FO-PS significantly reduced LDL-cholesterol concentrations. However, the LDL cholesterol–lowering effect was less pronounced than expected. This may be due to the time and frequency of plant sterol administration in the current experiment. Nevertheless, for hyperlipidemic persons whose triacylglycerol needs to be lower, FO-PS may provide additional effects by resulting in lower total:HDL cholesterol, lower apo B concentrations and apoB:apoA, and higher HDL_2:HDL_3 than does fish oil. FO-PS may also present some advantages over SU-PS; in addition to a potent triacylglycerol-lowering effect, FO-PS resulted in higher HDL_2 concentrations and HDL_2:HDL_3. These beneficial effects may result in further reductions in cardiovascular disease risk.

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PJHJ and DP designed the trial; ID coordinated the study with the participation of Y-MC; ID was responsible for patient recruitment and management, conducted the TBARS and plasma fatty acid laboratory analyses, performed statistical analyses, interpreted the data, and wrote the draft of the manuscript; Y-MC was responsible for the plant sterol and HDL subfraction measurements; ID and Y-MC were responsible for interpretation of the data; and all authors participated in critical review of the manuscript and approved the final version. DP is the Director of Clinical Studies at Enzymotec Ltd. None of the other authors had any personal or financial conflict of interest.

REFERENCES

1. Jemal A, Ward E, Hao Y, Thun M. Trends in the leading causes of death in the United States, 1970–2002. JAMA 2005;294:1255–9.
2. Banegas JR, Rodriguez-Artalejo F, Graciani A, Villar F, Herruzo R. Mortality attributable to cardiovascular risk factors in Spain. Eur J Clin Nutr 2003;57(suppl):S18–21.
3. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive summary of the third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA 2001;285:2486–97.
4. Jeppesen J, Hein HO, Suadicani P, Gyntelberg F. Triglyceride concentration and ischemic heart disease: an eight-year follow-up in the Copenhagen Male Study. Circulation 1998;97:1029–36.
5. Assman G, Schulte H, von Eckardstein A. Hypertriglyceridemia and elevated lipoprotein(a) are risk factors for major coronary events in middle-aged men. Am J Cardiol 1996;77:1179–84.
6. Cullen P. Evidence that triglycerides are an independent coronary heart disease risk factor. Am J Cardiol 2000;86:943–9.
7. Austin MA, Hokanson JE, Edwards KL. Hypertriglyceridemia as a cardiovascular risk factor. Am J Cardiol 1998;81:7B–12B.
8. Eckel H, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet 2005;365:1415–28.
9. Shepherd J, Betteridge J, Van Gaal L on behalf of a European Consensus Panel. Nicotinic acid in the management of dyslipidemia associated with diabetes and metabolic syndrome: a position paper developed by a European Consensus Panel. Curr Med Res Opin 2005;21:665–82.
10. Moreau RA, Whitaker BD, Hicks KB. Phytosterols, phytostanols, and

their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress Lipid Res 2002;41:457–500.
11. Ostlund RE. Phytosterols in human nutrition. Annu Rev Nutr 2002;22:533–49.
12. De Jong A, Plat J, Mensink RP. Metabolic effects of plant sterols and stanols. J Nutr Biochem 2003;14:362–9.
13. Stone NJ, Van Horn L. Therapeutic lifestyle change and Adult Treatment Panel III: evidence then and now. Curr Atheroscler Rep 2002;4:433–43.
14. Katan MB, Grundy SM, Jones P, Law M, Miettinen T, Paoletti R. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin Proc 2003;78:965–78.
15. Kris-Etherton PM, Harris WS, Appel LJ. AHA Scientific Statement: Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular diseases. Circulation 2002;106:2747–57.
16. Harris WS. n−3 Fatty acids and serum lipoproteins: human studies. Am J Clin Nutr 1997;65(suppl):145S–54S.
17. Ewart SH, Cole LK, Kralovec J, et al. Fish oil containing phytosterol esters alters blood lipid profiles and left ventricule generation of thromboxane A_2 in adult guinea pigs. J Nutr 2002;132:1149–52.
18. Russell JC, Ewart SH, Kelly SE, Kralovec J, Wright JLC, Dolphin PJ. Improvement of vascular dysfunction and blood lipids of insulin-resistant rats by a marine oil-based phytosterol compound. Lipids 2002;37:147–52.
19. Demonty I, Ebine N, Jia X, Jones PJ. Fish oil fatty acid esters of phytosterols alter plasma lipids but not red blood cell fragility in hamsters. Lipids 2005;40:695–702.
20. Jones PJ, Ntanios FY, Raeini-Sarjaz M, Vanstone CA. Cholesterol-lowering efficacy of a sitostanol-containing phytosterol mixture with a prudent diet in hyperlipidemic men. Am J Clin Nutr 1999;69:1144–50.
21. Mifflin MD, St-Jeor ST, Hill LA, Scott BJ, Daugherty SA, Koh YO. A new predictive equation for resting energy expenditure in healthy individuals. Am J Clin Nutr 1990;51:241–7.
22. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499–502.
23. Gidez LI, Miller GJ, Burstein M, Slagle S, Eder HA. Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure. J Lipid Res 1982;23:1206–23.
24. Bachorik PS, Albers JJ. Precipitation methods for quantification of lipoproteins. Methods Enzymol 1986;129:78–100.
25. Folch J, Lees M, Sloan Stanley GH. A simple method for the extraction and precipitation of total lipids from animal tissues. J Biol Chem 1957;226:497–509.
26. Morrison WR, Smith LM. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J Lipid Res 1964;5:600–8.
27. Ntanios FY, Jones PJH. Effects of variable dietary sitostanol concentrations on plasma lipid profile and phytosterol metabolism in hamsters. Biochim Biophys Acta 1998;1390:237–44.
28. Lütjohann D, Meese CO, Crouse JR III, von Bergmann K. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. J Lipid Res 1993;34:1039–46.
29. Sowell AL, Huff DL, Yeager PR, Caudill SP, Gunter EW. Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. Clin Chem 1994;40:411–6.
30. Normén L, Shaw CA, Fink CS, Awad AB. Combination of phytosterols and omega-3 fatty acids: a potential strategy to promote cardiovascular health. Curr Med Chem Cardiovasc Hematol Agents 2004;2:1–12.
31. Patsch JR, Miesenbock G, Hopferwieser T, et al. Relation of triglyceride metabolism and coronary artery disease. Studies in the postprandial state. Arterioscler Thromb 1992;12:1336–45.
32. Ebine N, Demonty I, Jia X, Jones PJ. Plant stanol ascorbate esters reduce body weight gain through decreased energy absorption in hamsters. Int J Obes 2006;30:751–7.
33. Plat J, Brufau M, Dasselaar M, Mensink RP. A stanol yoghurt drink alone or combined with a low-dose OTC statin lowers non-HDL-C, but also lowers TGs & elevates HDLC in metabolic syndrome patients. Atherosclerosis 2006;7(suppl):452 (abstr).
34. Kris-Etherton PM, Pearson TA, Wan Y, et al. High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. Am J Clin Nutr 1999;70:1009–15.
35. Castro IA, Barroso LP, Sinnecker P. Functional foods for coronary heart disease risk reduction: a meta-analysis using a multivariate approach. Am J Clin Nutr 2005;82:32–40.
36. Holub DJ, Holub BJ. Omega-3 fatty acids from fish oils and cardiovascular disease. Mol Cell Biochem 2004;263:217–25.
37. Mozzafarian D, Geelen A, Brouwer IA, Geleijnse JM, Zock PL, Katan MB. Effect of fish oil on heart rate in humans. A meta-analysis of randomized controlled trials. Circulation 2005;112:1945–52.
38. GISSI-Prevenzione Investigators (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico). Dietary supplementation with n−3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Lancet 1999;354:447–55.
39. Cairns JA, Gill J, Morton B, et al. Fish oils and low-molecular-weight heparin for the reduction of restenosis after percutaneous transluminal coronary angioplasty. The EMPAR Study. Circulation 1996;94:1553–60.
40. Sirtori CR, Crepaldi G, Manzato E, et al. One-year treatment with ethyl esters of n−3 fatty acids in patients with hypertriglyceridemia and glucose intolerance: reduced triglyceridemia, total cholesterol and increased HDL-C without glycemic alterations. Atherosclerosis 1998;137:419–27.
41. Berger A, Jones PJH, Abumweis SS. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. Lipid Health Dis 2004;3:5.
42. Plat J, Van Onselen ENM, Van heugten MMA, Mensink RP. Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant sterols. Eur J Clin Nutr 2000;54:671–7.
43. Matvienko OA, Lewis DS, Swanson M, et al. A single daily dose of soybean phytosterols in ground beef decreases serum total cholesterol and LDL cholesterol in young, mildly hypercholesterolemic men. Am J Clin Nutr 2002;76:57–64.
44. Doornbos AME, Meynen EM, Duchateau GSMJE, van der Knapp HCM, Trautwein EA. Intake occasion affects the serum cholesterol lowering of a plant sterol-enriched single-dose yoghurt drink in mildly hypercholesterolaemic subjects. Eur J Clin Nutr 2006;60:325–33.
45. Hendriks HF, Weststrate JA, van Vliet T, Meijer GW. Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. Eur J Clin Nutr 1999;53:319–27.
46. Austin MA. Plasma triglycerides as a risk factor for coronary heart disease: the epidemiologic evidence and beyond. Am J Epidemiol 1989;129:249–59.
47. Nestel PJ. Effects of n−3 fatty acids on lipid metabolism. Annu Rev Nutr 1990;10:149–67.
48. Mori TA, Burke V, Puddey IB, et al. Purified eicosapentaenoic and docosaheaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. Am J Clin Nutr 2000;71:1085–94.
49. Woodman RJ, Mori TA, Burke V, Puddey IB, Watts GF, Beilin LJ. Effects of purified eicosapentaenoic and docosahexaenoic acids on glycemic control, blood pressure, and serum lipids in type 2 diabetic patients with treated hypertension. Am J Clin Nutr 2002;76:1007–15.
50. Calabresi L, Villa B, Canavesi M, et al. An omega-3 polyunsaturated fatty acid concentrate increases plasma high-density lipoprotein 2 cholesterol and paraoxonase levels in patients with familial combined hyperlipidemia. Metabolism 2004;53:153–8.
51. Kris-Etherton PM, Hecker KD, Bonanome A, et al. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am J Med 2002;113:71S–88S.
52. Gylling H, Miettinen TA. Cholesterol reduction by different plant stanol mixtures and with variable fat intake. Metabolism 1999;48:575–80.
53. Gylling H, Miettinen TA. Serum cholesterol and cholesterol and lipoprotein metabolism in hypercholesterolaemic NIDDM patients before and during sitostanol ester-margarine treatment. Diabetologia 1994;37:773–80.
54. Ballantyne FC, Clark RS, Simpson HS, Ballantyne D. High density and low density lipoprotein subfractions in survivors of myocardial infarction and in control subjects. Metabolism 1982;31:433–7.
55. Lemieux I, Lamarche B, Couillard C, et al. Total cholesterol/HDL cholesterol ratio vs LDL cholesterol/HDL cholesterol ratio as indices of ischemic heart disease risk in men: the Quebec Cardiovascular Study. Arch Intern Med 2001;161:2685–92.