

ORIGINAL COMMUNICATION

Safety of long-term consumption of plant sterol esters-enriched spread

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Objective: To evaluate both efficacy and safety in humans of long-term consumption of spreads containing plant sterol esters.

Design: Randomized double-blind placebo-controlled parallel trial.

Subjects: Hundred and eighty-five healthy volunteers (35–64 y).

Intervention: Volunteers daily consumed 20 g spread enriched with 1.6 g plant sterols as fatty acid esters or a control spread for 1 y. They continued their habitual diet and lifestyle. Outcome measures included efficacy markers such as total and LDL-cholesterol, a large range of safety parameters, and reporting of adverse events.

Results: Consumption of the plant sterol ester-enriched spread consistently lowered total and LDL cholesterol during the 1 y period on average by 4 and 6%, respectively ($0.01 < P < 0.05$). Plant sterols intake did on average not result in a lower carotenoid concentration (when expressed per LDL-cholesterol) after 52 weeks ($P > 0.05$). However, carotenoid concentrations changed over time. Plant sterols intake reduced lipid adjusted α - and β -carotene-concentrations by only 15–25% after 1 y, relative to control. Lipid-adjusted fat-soluble vitamin concentrations remained unchanged. Plant sterol concentrations in serum were increased from 2.76 to 5.31 ($\mu\text{mol}/\text{mmol}$ total cholesterol) for campesterol ($P < 0.0001$) and from 1.86 to 2.47 ($\mu\text{mol}/\text{mmol}$ total cholesterol) for β -sitosterol ($P < 0.0001$). The increase in total plant sterol concentration in red blood cells (5.29–9.62 $\mu\text{g}/\text{g}$) did not affect red blood cell deformability. Hormone levels in males (free and total testosterone) and females (luteinizing hormone, follicle stimulating hormone, β -estradiol and progesterone) as well as all clinical chemical and hematological parameters measured were unaffected. Adverse events reported were not different between subjects consuming control spread and subjects consuming plant sterol esters-enriched spread.

Conclusion: Consumption of a plant sterol esters-enriched spread is an effective way to consistently lower blood cholesterol concentrations and is safe to use over a long period of time.

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Descriptors: plant sterol esters; cholesterol lowering; safety; long-term study; efficacy; coronary heart disease; spread

Introduction

An increased blood cholesterol concentration is an established modifiable risk factor for coronary heart disease mortality (Stamler *et al*, 2000). Blood cholesterol concentrations may be modulated by changes in the intake of dietary fat and cholesterol (Tang *et al*, 1998). It has also been known since

the 1950s that plant sterols reduce blood cholesterol concentrations significantly when consumed at intake levels over 1 g per day (Pollak & Kritchevsky, 1981; Moghadasian & Frohlich, 1999).

Plant sterols, not synthesized by humans, occur naturally in vegetable oils and fats and have structural similarity to

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cholesterol. The exact mechanism for blood cholesterol lowering by plant sterols is not fully understood, but they appear to inhibit the absorption of dietary and biliary cholesterol from the gut. Spreads enriched with plant sterol esters have been shown to effectively reduce total and LDL-cholesterol in the short-term (2–8 weeks) without affecting HDL-cholesterol and triacylglycerol concentrations (Hendriks *et al*, 1999; Weststrate & Meijer, 1998).

Animal and human studies have shown that plant sterol esters are non-toxic (Baker *et al*, 1999; Hepburn *et al*, 1999; Waalkens-Berendsen *et al*, 1999; Weststrate *et al*, 1999; Ayesh *et al*, 1999; Sanders *et al*, 2000), indicating that these compounds are safe. Human studies, however, have focused on efficacy mainly with a general lack of long-term safety data. So far, short-term studies have reported no adverse events. Also, hypercholesterolemic patients have taken up to 25 g plant sterols per day for several months without reporting any side effects (Pollak & Kritchevsky, 1981).

Human trials have shown that in general α - and β -carotene and lycopene concentrations, but not concentrations of fat-soluble vitamins, are reduced by plant sterol consumption. These effects may be dependent on intake level (Hendriks *et al*, 1999; Hallikainen *et al*, 2000a). Long-term effects as well as functional consequences of these changes in α - and β -carotene concentrations in blood have not been previously studied in detail.

This study reports on the long-term effects of consuming a low-fat spread providing 1.6 g plant sterols daily for one year, on a large variety of outcome measures including efficacy markers such as total and LDL cholesterol, a large range of safety parameters and reporting of adverse events in healthy men and women.

Methods

Ethics

The protocol was approved by the independent Medical Ethics Committee of TNO. The procedures followed were in accordance with the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines for Good Clinical Practice and with the Helsinki Declaration of 1975, as revised in 1996.

Participants

Subjects were recruited from the pool of volunteers of TNO Nutrition and Food Research and through advertising. After having shown their interest in writing, respondents received study-specific written information and an oral briefing. All questions raised by candidate participants were answered. All subjects interested in participation filled in an informed consent form.

Eligibility was assessed by health questionnaire, physical examination and results of the pre-study laboratory tests. Volunteers (total cholesterol ≥ 8.0 mM) using medicine or

having a history of medical or surgical events, which might have significantly affected the study outcome (chronic gastrointestinal complaints, high blood pressure (according to WHO guidelines) or cardiovascular disease) were not eligible. Those with an unexplainable weight loss or gain of more than 7 kg in the 2 y prior to the pre-study screening and those using a slimming or other (medically) prescribed diet and those wishing to become pregnant or being pregnant and/or lactating were excluded. Also, subjects taking part in sport for more than 10 h per week, subjects not using spreads and subjects drinking more than 21 (women) or 28 (men) units of alcoholic beverage per week were excluded.

Study conduct

Study substance. Study substances included a control spread and a plant sterol esters-enriched spread. The control spread contained 34.6% (w/w) fat (edible vegetable oils: sunflower oil and rape seed oil) and 5.8 mg/kg carotenoids. Plant sterol esters-enriched spread contained 8.1% (w/w) free plant sterols, 34.8% (w/w) fat (fatty acids from the plant sterol esters and the edible vegetable oils) and 5.7 mg/kg carotenoids. Plant sterols consisted mainly of β -sitosterol (about 48% (w/w)), campesterol (about 27% (w/w)) and stigmasterol (about 19% (w/w)). Plant sterol esters enrichment in the spread corresponded to a daily intake of 1.6 g of free plant sterol equivalents per day. The test and control spreads were produced according to Good Manufacturing Practice.

Study design. Subjects were randomly allocated to the treatments using serum cholesterol concentration, gender and age as randomization parameters. Deblinding occurred for one person after a possibly related adverse event and for the full set of subjects after laboratory analyses were reported.

All participants consumed daily either 20 g of spread containing 1.6 g of plant sterols or the control spread for 1 y in a double-blind fashion. Spreads were dispensed every 4 or 5 weeks during the 1 y period. Four spare portions were added per 4 or 5 week period to compensate for possible loss or damage. At the start of the study subjects were instructed to consume the spreads according to their customary habits under free-living conditions, any time during the day at any frequency they wished. After 26 weeks, subjects were requested to consume 10 g spread with breakfast and 10 g with lunch or dinner, hypothesizing that cholesterol reduction may be further improved. Consumption moment of spreads was subsequently evaluated after 39 and 52 weeks. Subjects were instructed to use the low-fat products as spread and not for baking or frying. At each visit, the unconsumed portions were returned and registered. In case discrepancies occurred because half-empty containers were not returned, the worst possible compliance was assumed. Some subjects consumed reserve portions sporadically. Compliance was expressed as a percentage based on the ratio of spread consumed (spread dispensed minus spread returned) and spread dispensed (spread dispensed minus spare spread).

Diet and lifestyle. Subjects used a self-selected diet with some dietary counseling, such as recipes with alternatives for spread use. Food intake over the preceding period was evaluated each 3 month period using a TNO food frequency questionnaire (modified version of the validated questionnaire (Grootenhuis *et al*, 1995)) assessing the continuity of intake of energy, macronutrients (protein, fats (saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids and cholesterol), carbohydrates and fiber). Questionnaires also surveyed smoking habits and consumption of spreads.

Laboratory analyses

Fasting venous blood was taken at baseline and after 13, 26, 39 and 52 weeks. Serum and plasma were stored at -80°C . Total and HDL-cholesterol and triacylglycerols were measured by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany; CVs < 3%); and LDL-cholesterol was calculated according to Friedewald *et al* (1972). Cholesterol was analyzed twice, first after each time point when blood samples were taken and subsequently in all samples at the same time after ending the trial. The results from both analyses were similar. The second cholesterol analysis has been chosen for this report in order to obtain maximum comparability between time points. Serum lipoprotein (a) was analyzed by rate nephelometry on a Hitachi 911 analyzer. Carotenoids (α -carotene, β -carotene, lycopene, lutein, β -cryptoxanthine, zeaxanthine; CVs 4–15%) and retinol (CV < 3%) and α -tocopherol (CV < 3%) were analyzed by high performance liquid chromatography (HPLC) followed by ultraviolet and fluorimetric detection, respectively. Vitamin K₁ (CV = 8%) was analyzed by HPLC followed by electrochemical reduction and fluorimetric detection. Vitamins D (25-OH-vitamin D), B₁₂ and folic acid (as overall safety parameters possibly related to coronary heart disease) were analyzed by radioimmunoassay.

Clinical chemical parameters (AST, ALT, ALP, GGT, LDH, total protein, albumin, total bilirubin, creatinin, urea, glucose, iron, sodium, potassium and chloride) and hematological parameters (hemoglobin, hematocrit, leukocytes, erythrocytes, MCV, MCH, MCHC, PTT, aPTT and platelets) were measured by routine clinical techniques (CVs < 4%). Adverse events were classified according to 'The ICD-10: International statistical classification of diseases and related health problems, 10th revision' (WHO, Geneva, 1996). Adverse events are termed in 'preferred terms' and were always given an alphanumeric code. For some adverse events no preferred term was available and therefore the reported term was used. Red blood cell morphology was evaluated after 52 weeks only in fixed and stained blood smears. Red blood cell deformability was analyzed in duplicate (Hardeman *et al*, 1994a,b) in 24 randomly selected subjects consuming control spread and 26 randomly selected subjects consuming plant sterol esters-enriched spread and

was expressed as the elongation index calculated at two shear stresses, namely 3 and 30 Pascal (CV 4–0.4%).

Urine was analyzed by dipstick at 0, 13, 26, 39 and 52 weeks in study. Evaluation included a routine semiquantitative assessment of leukocytes, erythrocytes, nitrite, protein, glucose, ketones and bilirubin in spot urine samples.

Plant sterols were analyzed in serum at 0, 26 and 52 weeks and in red blood cells at 52 weeks only. Serum plant sterol concentrations were analysed for all subjects, red blood cell plant sterol concentrations were analyzed in 23 randomly selected subjects consuming control spread and 23 randomly selected subjects consuming plant sterol esters spread. Blood samples and washed red blood cells were saponified and extracted and separated by capillary gas-liquid chromatography (Kempen *et al*, 1991) using epicoprostanol as an internal standard (CV = 10%)

Female hormones were analyzed in 54 peri- and postmenopausal women by an enzyme immunoassay using a AIA 600 analyzer (Eurogenetics); male hormones were analyzed in 84 males by immuno radiometric assays (PDC).

Statistical analysis

The statistical package used was SAS (SAS/STAT version 6.12, Statistical Analysis System Institute Inc., Cary, NC, USA). Data were expressed as mean \pm standard deviation (s.d.) for either treatment. Differences in study parameters were evaluated using two-sided analysis of variance (ANOVA) with gender and treatment as factors adjusting parameters for baseline values. Data on carotenoids as well as data on triacylglycerol, Lp(a) and vitamin K were not normally distributed and were natural log (ln) transformed before statistical analysis. Safety parameters, body weights and results of compliance, eating moment and food frequency questionnaires were presented descriptively. Individual baseline values on campesterol and β -sitosterol concentrations were subdivided in quintiles of the treatment group and differences in absolute and relative changes between the highest quintile and the remaining observations were analysed by ANOVA. The level of statistical significance was preset at 0.05 (two-sided).

Results

Recruitment and follow-up

Three-hundreds and fifty-three potentially eligible subjects were screened. Statin users were considered eligible in the initial recruitment phase, but were excluded (not eligible) later due to insufficient numbers to be analyzed as a sub-set. Two hundred and six eligible subjects were randomized to treatment in two phases: first a group of 196 subjects, followed by an additional 10 subjects. The 196 volunteers had a staggered start in seven groups of 19–31 persons each with about an equal number of men and women per group. The 10 volunteers randomized secondarily were excluded

from statistical analysis because they only participated in the study for the last 39 weeks.

All eligible subjects were considered healthy as assessed by health questionnaire, physical examination and results of the pre-study laboratory tests. Fasting blood cholesterol at pre-study screening was less than 8.0 mmol/l. All had normal Dutch eating habits and consumed spreads regularly. Women drank less than 22 and men less than 29 units alcoholic beverages per week, with each unit containing 10 g of alcohol.

Average age of the 185 volunteers was 48 y ranging from 35 to 64 y, body weight was on average 76 ± 12 kg ranging from 50 to 110 kg, body mass index was 24.9 ± 3.2 kg/m² ranging from 18.1 to 33.6 kg/m². Systolic and diastolic blood pressure as well as pulse rate and the blood chemistry parameters ALP, ALT, AST, GGT, cholesterol, triacylglycerols, bilirubin, urea, creatinin and glucose were normal. There were no differences in the above parameters between treatment groups (Table 1).

During the 52 week study, eight subjects dropped out: seven of these were consuming plant sterol esters; three of these dropped out for medical reasons and one subject consuming control spread; this volunteer dropped out for a medical reason. An additional three volunteers were lost to follow-up, because they decided not to participate on the first day of the study. Consequently, a complete set of data was obtained for 46 men and 50 women receiving control spread and 44 men and 45 women receiving spread enriched with plant sterol esters (Figure 1).

Adverse events

In total 876 adverse events were reported by 173 subjects of the total of 203 volunteers starting the clinical phase of the study.

Volunteers participating for 39 weeks and drop-outs. For those participating for 39 weeks only, no consistent pattern

of anomalies was observed. One subject consuming the plant sterol esters-enriched spread reported 53 migraine attacks. These migraine attacks were classified as unlikely to be related to the treatment. There appeared not to be a skewed distribution of adverse events in the four volunteers dropping out for medical reasons. The drop-out in the control group reported on various occasions hepatitis A, epididymitis, cystitis, pustules/rash and dropped out after diagnosis of a non-Hodgkin lymphoma. The three drop-outs in the plant sterol group reported: (1) headache, metrorrhagia and drop-out after a spleen infarction due to hepatitis; (2) common cold, fracture of the foot and drop-out due to surmenage/stress; (3) drop-out after spontaneous reoccurring postmenopausal bleeding.

Only the latter adverse event was classified as possibly related to treatment and was followed up. This 53-y-old woman showed considerable increases in oestradiol and decreases in follicle stimulating hormone suggesting renewing of the menstrual cycle. Therefore, all perimenopausal and postmenopausal women included in the study (all women of 50 y of age and older giving consent, Table 2) were evaluated on luteinizing hormone, follicle stimulating hormone, β -estradiol and progesterone. No consistent pattern of anomalies was observed: fluctuations in hormone concentrations were small and occurred in both groups of women. Consequently it was concluded that there was no indication that plant sterol esters-enriched spread consumption would significantly affect hormonal status possibly resulting in postmenopausal bleeding. In addition, serum total and free testosterone concentrations were evaluated in all men giving their consent. Also no effect of consumption of plant sterol esters-enriched spread on male hormones was observed (Table 2).

Completers. Of the 185 volunteers participating for 52 weeks, subjects consuming control spread reported a total of 371 adverse events and subjects consuming plant sterol esters-enriched spread reported 414 adverse events. Of these

Table 1 Characteristics at baseline

Characteristics	All (n = 185)		Control spread (n = 96)		Plant sterol-enriched spread (n = 89)	
Male (%)	90	(49%)	46	(48%)	44	(49%)
Age (y)	48 ± 8	(35–64)	48 ± 8	(35–64)	48 ± 7	(35–64)
Body weight (kg)	76 ± 12	(50–110)	77 ± 11	(54–102)	75 ± 13	(50–110)
Body mass index (kg/m ²)	24.9 ± 3.2	(18.1–33.6)	25.1 ± 3.0	(20.1–33.2)	24.7 ± 3.3	(18.1–33.6)
Systolic blood pressure (mm Hg)	124 ± 14	(92–160)	123 ± 15	(92–158)	124 ± 14	(92–160)
Diastolic blood pressure (mm Hg)	76 ± 9	(52–100)	76 ± 10	(52–99)	77 ± 9	(56–100)
Pulse (beats per minute)	75 ± 13	(48–117)	75 ± 12	(48–106)	75 ± 13	(48–117)
ALP (U/l)	62 ± 19	(20–137)	60 ± 17	(20–105)	64 ± 21	(29–137)
ALT (U/l)	20 ± 8	(7–48)	20 ± 7	(8–48)	20 ± 8	(7–44)
AST (U/l)	20 ± 5	(8–35)	19 ± 4	(11–33)	20 ± 5	(8–35)
γ -GT (U/l)	23 ± 18	(5–126)	24 ± 19	(5–126)	23 ± 17	(6–106)
Cholesterol (mmol/l)	5.90 ± 0.98	(2.94–7.83)	5.88 ± 0.95	(3.08–7.80)	5.92 ± 1.03	(2.94–7.83)
Triacylglycerol (mmol/l)	1.25 ± 0.70	(0.37–3.63)	1.26 ± 0.70	(0.38–3.55)	1.24 ± 0.69	(0.37–3.63)
Glucose (mmol/l)	5.15 ± 0.44	(4.24–6.85)	5.18 ± 0.42	(4.36–6.46)	5.12 ± 0.47	(4.24–6.85)

Values are means ± s.d. (range) except for number males. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, asparagine aminotransferase; γ -GT, γ -glutamyltransferase.

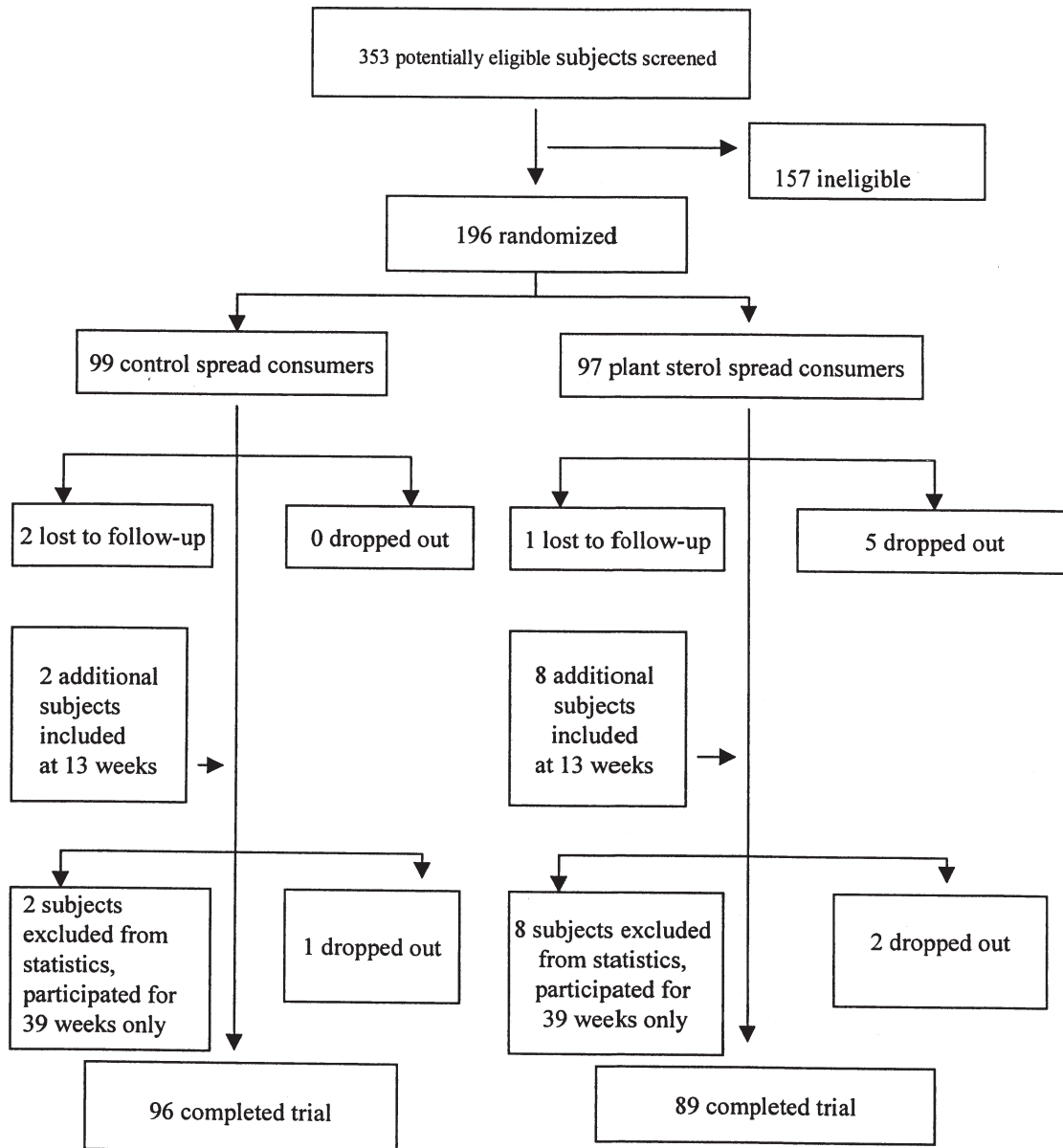


Figure 1 Recruitment and follow-up.

785 adverse events, two were serious, one was an unspecified skin cancer and another was a myocardial infarction. Both adverse events were classified as unlikely to be related to the treatment and occurred in the control group.

Twenty-one of the 785 adverse events were considered to be possibly related to treatment, the remainder being registered as unlikely or not to be related. Twelve adverse events, possibly related to treatment, occurred in the subjects consuming control spread and nine in subjects consuming the plant sterol esters-enriched spread. The subjects consuming control spread reported diarrhea, influenza-like symptoms, dyspepsia, itchy legs, abdominal pain (thrice), nausea,

flatulence, increased bowel action and thin and increased defecation frequency. The subjects consuming the plant sterol esters-enriched spread reported itchy belly, trunk or whole body (twice); cramps in the lower belly; abdominal pain; flatulence and increased defecation frequency. In conclusion, no differences between the two treatment groups in the adverse events were observed.

Compliance

Consumption of spreads was evaluated for each 13 week period and did not differ between subjects consuming control and

Table 2 Female and male hormone concentrations

Week number	Control spread					Plant sterol-enriched spread				
	0	13	26	39	52	0	13	26	39	52
<i>Female hormones</i> (n = 26 women)						<i>Female hormones</i> (n = 28 women)				
Luteinizing hormone (U/l)	19 ± 15	17 ± 12	14 ± 9	16 ± 11	13 ± 10	19 ± 15	19 ± 15	14 ± 11	20 ± 13	15 ± 11
Follicle stimulating hormone (U/l)	69 ± 50	67 ± 52	53 ± 39	61 ± 52	44 ± 37	68 ± 55	74 ± 63	55 ± 43	75 ± 56	55 ± 47
β-Oestradiol (ng/l)	39 ± 33	45 ± 48	30 ± 27	36 ± 37	34 ± 32	70 ± 102	48 ± 53	41 ± 37	35 ± 30	43 ± 54
Progesterone (μg/l)	0.9 ± 2.4	0.3 ± 0.7	0.6 ± 2.4	1.0 ± 2.7	0.6 ± 1.6	0.4 ± 0.9	1.2 ± 3.9	1.1 ± 3.5	0.2 ± 0.2	0.5 ± 1.1
<i>Male hormones</i> (n = 43 men)						<i>Male hormones</i> (n = 41 men)				
Total testosterone (μg/l)	4.9 ± 1.1				5.0 ± 1.6	4.8 ± 1.8				4.6 ± 1.5
Free testosterone (ng/l)	17.4 ± 4.5				15.5 ± 3.7	17.4 ± 4.5				15.5 ± 3.7

Values are means ± s.d.

plant sterol esters-enriched spread. Individual consumption varied between 1789 ± 82 and 1807 ± 35 g per 13 week period and did not differ between the two groups at 13 ($P=0.85$), 26 ($P=0.32$), 39 ($P=0.48$) and 52 weeks ($P=0.96$).

Overall yearly percentage of compliance was high, namely 98.9 ± 1.8% for controls and 98.9 ± 2.1% for plant sterol esters users ($P=0.99$). Individual percentage of compliance ranged between 90.4 and 100.8% for subjects consuming control spread and ranged between 87.9 and 105.6% for subjects consuming plant sterol esters-enriched spread.

Study outcomes

Diet and lifestyle. Nutrient intake was representative for the adult Dutch population. Nutrient intake and smoking

habits did not vary significantly during the study and did not differ between subjects consuming control and plant sterol esters-enriched spread at all time points evaluated (Table 3).

Blood lipids. Compared with the control product, consumption of the plant sterol esters-enriched spread lowered total cholesterol concentrations on average by 4%. Similarly, consumption of plant sterol esters in the spread lowered LDL-cholesterol on average by 6% (Figure 2). The reductions in total and LDL-cholesterol were sustained throughout the whole year and were similar for both men and women. HDL-cholesterol, like triacylglycerols and lipoprotein (a) concentrations were not affected (data not shown).

Table 3 Nutrient intake and smoking

Week number	Control spread (n = 96)				Plant sterol-enriched spread (n = 89)			
	13	26	39	52	13	26 ^a	39	52
Energy (kJ × 10 ⁻³)	9.9 ± 2.5	9.4 ± 2.4	9.2 ± 2.3	9.0 ± 2.4	9.4 ± 2.2	9.2 ± 2.4	8.8 ± 2.2	8.8 ± 2.2
Protein (g)	97 ± 26	92 ± 27	89 ± 24	89 ± 24	93 ± 19	89 ± 24	88 ± 21	88 ± 24
Fat								
Total (g)	93 ± 31	89 ± 29	86 ± 28	83 ± 31	86 ± 25	85 ± 28	81 ± 25	82 ± 28
Saturated fatty acids (g)	37 ± 13*	35 ± 12	34 ± 11	33 ± 13	33 ± 10	33 ± 11	32 ± 9	32 ± 11
Monounsaturated fatty acids (g)	32 ± 12	31 ± 11	30 ± 11	29 ± 11	30 ± 9	29 ± 9	28 ± 8	28 ± 9
Polyunsaturated fatty acid (g)	17 ± 7	16 ± 7	16 ± 7	15 ± 7	16 ± 7	16 ± 8	15 ± 9	16 ± 8
Cholesterol (mg)	264 ± 86	242 ± 75	243 ± 77	245 ± 88	250 ± 72	237 ± 71	237 ± 69	236 ± 76
Carbohydrates (g)	265 ± 79	252 ± 70	246 ± 65	243 ± 71	256 ± 73	247 ± 71	236 ± 67	236 ± 65
Fiber (g)	27 ± 8	27 ± 8	26 ± 7	25 ± 7	27 ± 8	28 ± 8	27 ± 8	26 ± 8
Non-cigarette smokers (n)	71	72	74	74	59	59	60	58
Smoking 0–15 cigarettes (n)	19	18	16	17	19	19	19	22
Smoking > 16 cigarettes (n)	6	6	6	5	11	10	10	9
Non-cigar smokers (n)	83	85	82	81	75	75	72	69
Smoking 0–2 cigars (n)	13	11	14	15	14	13	17	20

Values are means ± s.d. ^an = 88.

* $P=0.045$ for differences between groups.

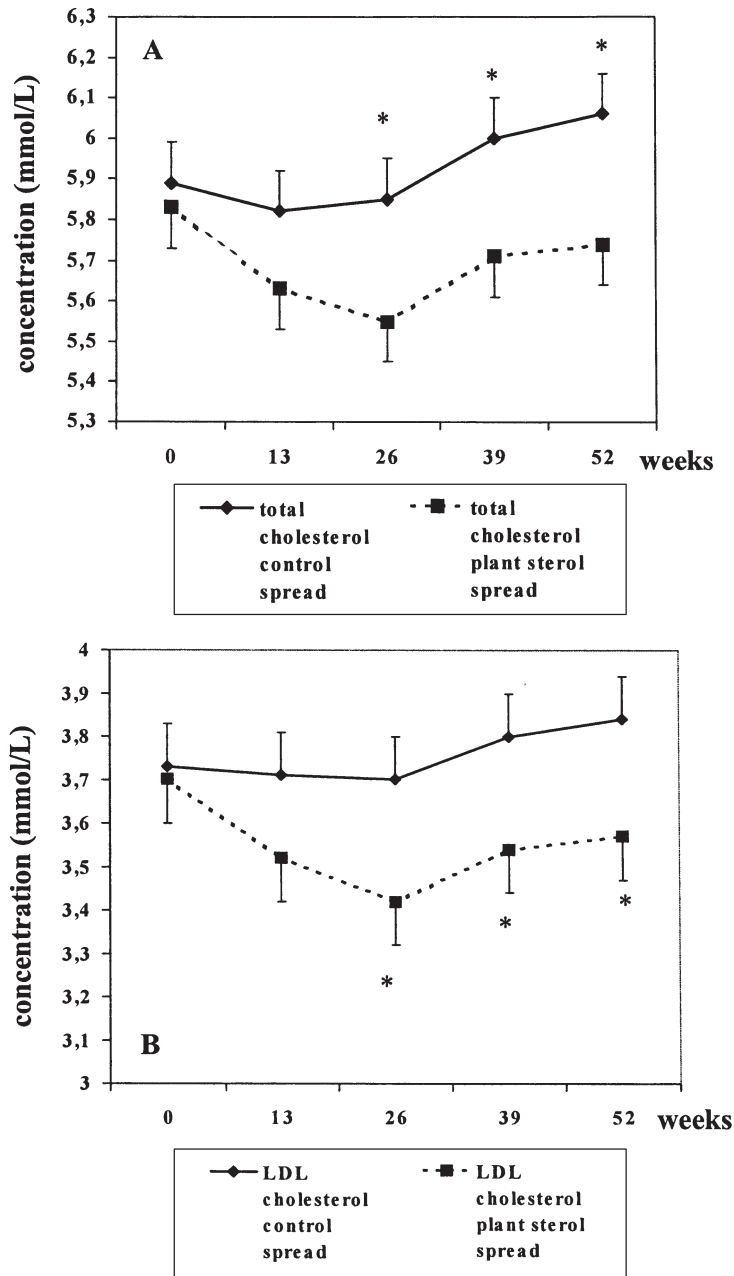


Figure 2 Total cholesterol (A) and LDL cholesterol (B) concentration in subjects control consuming spread (n=gb) and plant sterol esters-enriched spread (n=dg).

Carotenoids and vitamins. Carotenoid concentrations were evaluated at 0, 26 and 52 weeks (Table 4). Average concentrations of lutein, β -cryptoxanthine and zeaxanthine were not different between both groups after 52 weeks of spread consumption. Only the average α - and β -carotene and lycopene concentrations were 15–20% lower in subjects consuming plant sterol esters-enriched spread for 26 and 52 weeks. These differences were not observed after correction for LDL cholesterol concentrations, except for α -carotene

after 52 weeks. However, carotenoid concentrations changed over time. Plant sterol esters-enriched spread consumption reduced all plasma carotenoid concentrations (except for lycopene) more when compared to control spread consumption, both after 26 and 52 weeks. Reductions ranged between about 10 and 35% for zeaxanthine, lutein and α -carotene (corrected for LDL-cholesterol concentrations) and between about 10 and 20% for β -carotene (corrected for LDL-cholesterol concentrations). The decreases were essentially

Table 4 Carotenoid concentrations

Week number	Control spread (n = 96)					Plant sterol-enriched spread (n = 89)				
	0	26 ^a	52	$\Delta 26-0$	$\Delta 52-0$	0	26 ^b	52	$\Delta 26-0$	$\Delta 52-0$
β -Carotene (nmol/l)	399 ± 221	368 ± 195	384 ± 225	-29 ± 139	-15 ± 179	410 ± 217	322 ± 176	310 ± 190**	-91 ± 24***	-100 ± 142***
β -Carotene/LDL (nmol/mmol)	111 ± 67	104 ± 55	107 ± 66	-7 ± 44	-4 ± 48	118 ± 68	101 ± 62	90 ± 50	-17 ± 33**	-28 ± 39***
α -Carotene (nmol/l)	74 ± 56	93 ± 57	93 ± 55	19 ± 40	19 ± 37	91 ± 108	81 ± 64*	78 ± 109***	-10 ± 63***	-13 ± 71***
α -Carotene/LDL (nmol/mmol)	21 ± 19	26 ± 16	26 ± 16	5 ± 13	4 ± 12	26 ± 29	25 ± 20	22 ± 22**	-0 ± 16***	-4 ± 19***
Lycopene (nmol/l)	370 ± 158	330 ± 157	364 ± 180	-38 ± 139	-6 ± 162	338 ± 174	283 ± 160*	309 ± 164*	-57 ± 147	-29 ± 147
Lycopene/LDL (nmol/mmol)	103 ± 47	96 ± 50	102 ± 60	-6 ± 43	-1 ± 51	96 ± 50	88 ± 54	91 ± 52	-7 ± 42	-4 ± 45
Lutein (nmol/l)	193 ± 88	195 ± 78	202 ± 80	1 ± 56	9 ± 53	218 ± 84*	183 ± 67	194 ± 70	-36 ± 61***	-25 ± 68***
Lutein/LDL (nmol/mmol)	54 ± 26	56 ± 26	56 ± 27	2 ± 18	2 ± 18	62 ± 26*	57 ± 25	57 ± 25	-5 ± 18**	-5 ± 19**
β -Cryptoxanthine (nmol/l)	182 ± 106	213 ± 118	197 ± 101	31 ± 92	16 ± 73	198 ± 126	194 ± 133	177 ± 96	-5 ± 92***	-20 ± 77***
β -Cryptoxanthine/LDL (nmol/mmol)	49 ± 26	63 ± 42	55 ± 31	14 ± 31	6 ± 18	57 ± 40	60 ± 43	53 ± 30	2 ± 32**	-5 ± 24*
Zeaxanthine (nmol/l)	53 ± 23	49 ± 19	55 ± 22	-4 ± 16	2 ± 16	57 ± 23	44 ± 17	50 ± 17	-13 ± 15***	-6 ± 17**
Zeaxanthine/LDL (nmol/mmol)	15 ± 6	14 ± 7	15 ± 7	-0 ± 5	0 ± 5	16 ± 8	14 ± 7	15 ± 7	-3 ± 5**	-1 ± 6

Values are means ± s.d. ^an = 95; ^bn = 88.

*0.01 < P < 0.05; **0.001 < P < 0.01; ***P < 0.001 for differences between groups.

the same after 26 and 52 weeks. The only exception was α -carotene corrected for LDL-cholesterol concentration, for which a significant treatment × time period interaction was observed ($P = 0.02$).

Similarly, the fat-soluble vitamins were evaluated at 0, 26 and 52 weeks (Table 5). Retinol (vitamin A) and vitamin K₁ did not change. The decreases over time in 25-OH-vitamin D (vitamin D) concentrations were larger in subjects consuming plant sterol esters-enriched spread as compared to those consuming control spread. α -Tocopherol (vitamin E) concentrations were decreased with plant sterol esters consumption. This decrease, however, was due to the decrease in LDL-cholesterol; vitamin E concentration corrected for LDL-cholesterol concentration did not change.

Osteocalcin carboxylation degree (as an indirect measure for the status of vitamin K), vitamin B₁₂ and folic acid and MDA were analyzed at 26 and 52 weeks only and were not different after 6 months or 1 y of consumption of plant sterol esters-enriched spread as compared to control spread (Table 5).

Clinical chemistry. Clinical chemical parameters analyzed included AST, ALT, ALP, GGT, LDH, total protein, albumin, total bilirubin, creatinin, urea, glucose, iron, sodium, potassium and chloride (Table 6). ALP was slightly higher in the group consuming the plant sterol ester-enriched product at 52 weeks only. LDH was lower at the start of the study and at all time points thereafter in subjects consuming plant sterol esters-enriched spread. No treatment effect was observed on any of these parameters. A lower LDH concentration

therefore reflects a specific biological characteristic of this population rather than an effect of plant sterol esters consumption. The statistical power of the study for the detection of an overall significant difference at a probability level of $P = 0.05$ was high. Differences of 10% or less would have been detected in the clinical chemical parameters except for liver enzymes. For the latter, significant differences of 10–30% would have been detected.

Hematology. There was no effect on any of the hematological parameters tested, namely hemoglobin, hematocrit, leukocytes, erythrocytes, platelets, MCV, MCH, MCHC, PTT and aPTT. Also, urinalysis, including leukocytes, erythrocytes, nitrite, protein, glucose, ketones and bilirubin was not consistently different between the two groups (data not shown).

Histological evaluation of red blood cell morphology showed that one person consuming plant sterol esters-enriched spread showed a sporadic light Burr cell morphology. Thus it was concluded that red blood cell morphology did not show any relevant deviations.

Plant sterols. The concentration of the main plant sterols in spreads, campesterol and β -sitosterol were increased from 4.62 to 7.78 $\mu\text{mol}/\text{mmol/l}$ total cholesterol in serum and from 5.30 to 9.62 $\mu\text{g}/\text{day}$ in red blood cells of subjects consuming plant sterol esters-enriched spread after 52 weeks compared with control (Table 7).

Elongation index reflecting red blood cell deformability was not affected both at 3 Pa (subjects consuming control

Table 5 Vitamins and related parameters

Week number	Control spread (n = 96)				Plant sterol-enriched spread (n = 89)					
	0	26 ^a	52	Δ26-0 ^a	Δ52-0	0	26 ^a	52	Δ26-0 ^a	Δ52-0
Retinol (μg/ml)	0.62 ± 0.13	0.61 ± 0.12	0.60 ± 0.13	- 0.01 ± 0.08	- 0.02 ± 0.08	0.61 ± 0.12	0.61 ± 0.14	0.60 ± 0.14	- 0.01 ± 0.10	- 0.01 ± 0.10
α-Tocopherol (g/ml)	12.5 ± 2.5	13.0 ± 2.9	13.0 ± 2.7	0.6 ± 1.9	0.5 ± 1.6	12.3 ± 2.4	11.9 ± 2.3**	12.0 ± 2.3**	- 0.4 ± 1.6***	- 0.2 ± 2.1***
α-Tocopherol/LDL (μmol/mmol)	8.0 ± 1.5	8.4 ± 1.9	8.1 ± 1.6	0.5 ± 1.5	0.1 ± 1.2	8.0 ± 1.7	8.4 ± 2.1	8.1 ± 1.9	0.4 ± 1.4	0.1 ± 1.7
25-OH-Vitamin D (nmol/l)	80 ± 26	73 ± 27	83 ± 29	- 7 ± 18	3 ± 18	82 ± 21	68 ± 23	79 ± 24 ^d	- 14 ± 15**	- 3 ± 17*
Vitamin K ₁ (pg/l)	505 ± 543	496 ± 509 ^c	83.3 ± 5.0	- 1 ± 375 ^c		598 ± 550 ^b	504 ± 464 ^c	85.6 ± 4.6	- 84 ± 622 ^c	
Osteocalcin carboxylation degree (%)			85.3 ± 4.1				83.6 ± 5.3			
Vitamin B ₁₂ (pmol/l)		295 ± 105	292 ± 105				285 ± 95	286 ± 108		
Folic acid (nmol/l)		13.1 ± 5.5	14.5 ± 6.1				13.0 ± 6.2	15.0 ± 6.6		
Malondialdehyde (mmol/l)		0.32 ± 0.15	0.44 ± 0.19				0.33 ± 0.25	0.48 ± 0.32 ^e		

Values are means ± s.d. ^an = 95 for control spread and n = 88 for plant sterol enriched spread. ^bn = 89. ^cn = 93 for control spread and n = 86 for plant sterol spread. ^dn = 88. ^en = 47. *0.01 < P < 0.05 and **0.001 < P < 0.01 for differences between groups.

Table 6 Clinical chemical parameters

Week number	Control spread (n = 96)				Plant sterol-enriched spread (n = 89)				
	0	13	26	52	0	13	26	39	52
AST (U/l)	21 ± 5	21 ± 6	22 ± 6	22 ± 10	20 ± 5	21 ± 6	22 ± 6	21 ± 5	22 ± 6
ALT (U/l)	17 ± 7	18 ± 8	19 ± 8	21 ± 24	16 ± 7	18 ± 8	18 ± 7	17 ± 8	19 ± 10
ALP (U/l)	60 ± 17	59 ± 17	64 ± 18	59 ± 17	65 ± 21	65 ± 21	69 ± 21	66 ± 23	65 ± 21*
GGT (U/l)	23.5 ± 17.6	24.6 ± 18.7	25.0 ± 17.6	25.7 ± 18.3	22.6 ± 17.3	23.7 ± 18.2	24.3 ± 18.3	25.9 ± 24.3	26.3 ± 23.1
LDH (U/l)	321 ± 47	338 ± 55	314 ± 47	303 ± 50	300 ± 42**	323 ± 56	299 ± 46*	284 ± 44*	285 ± 40**
Total protein (g/l)	73 ± 4	73 ± 4	75 ± 4	72 ± 6	74 ± 3	73 ± 4	75 ± 4	73 ± 4	73 ± 5
Albumin (g/l)	46 ± 2	47 ± 2	47 ± 2	46 ± 4	46 ± 2	47 ± 2	47 ± 3	47 ± 2	46 ± 3
Total bilirubin (μmol/l)	9.8 ± 5.2	10.2 ± 5.4	11.5 ± 5.5	9.7 ± 5.1	9.1 ± 3.5	9.6 ± 4.1	10.3 ± 3.8	9.7 ± 3.8	9.1 ± 3.3
Creatinin (μmol/l)	79 ± 12	78 ± 12	78 ± 12	75 ± 13	77 ± 11	77 ± 11	77 ± 11	76 ± 12	73 ± 11
Urea (mmol/l)	5.2 ± 1.4	5.3 ± 1.3	5.9 ± 1.5	5.5 ± 1.5	5.2 ± 1.2	5.3 ± 1.2	5.9 ± 1.4	5.6 ± 1.2	5.3 ± 1.3
Glucose (mmol/l)	5.35 ± 0.47	5.18 ± 0.56	5.34 ± 0.46	5.23 ± 0.55	5.40 ± 0.56	5.12 ± 0.64	5.31 ± 0.65	5.39 ± 0.48	5.27 ± 0.54
Iron (μmol/l)	19.9 ± 6.6	19.1 ± 5.5	20.4 ± 6.7	18.2 ± 6.2	20.0 ± 6.6	20.1 ± 6.1	20.7 ± 6.7	19.8 ± 6.2	19.0 ± 6.5
Sodium (mmol/l)	140 ± 2	143 ± 2	144 ± 1	138 ± 6	140 ± 2	143 ± 2	143 ± 2	140 ± 2	138 ± 3
Potassium (mmol/l)	4.3 ± 0.3	4.4 ± 0.3	4.2 ± 0.2	4.1 ± 0.2	4.4 ± 0.3	4.4 ± 0.3	4.2 ± 0.3	4.3 ± 0.3	4.1 ± 0.3
Chloride (mmol/l)	103 ± 2	104 ± 3	107 ± 2	104 ± 4	103 ± 2	103 ± 2	106 ± 2	98 ± 2	104 ± 2

Values are means ± s.d. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, asparagine aminotransferase; γ-GT, γ-glutamyltransferase; LDH, lactate dehydrogenase. *0.01 < P < 0.05 and **0.001 < P < 0.01 for differences between groups.

Table 7 Serum and red blood cell plant sterol concentrations after 52 weeks

	Control spread	Plant sterol-enriched spread
Serum ($\mu\text{mol}/\text{mmol}$ total cholesterol)	(n = 96)	(n = 88)
Campesterol	2.76 \pm 1.18	5.31 \pm 2.01***
β -Sitosterol	1.86 \pm 0.83	2.47 \pm 0.93***
Red blood cells ($\mu\text{g}/\text{g}$ red blood cells)	(n = 23)	(n = 23)
Campesterol	2.89 \pm 1.64	6.21 \pm 2.37**
β -Sitosterol	2.40 \pm 1.10	3.41 \pm 0.97**

Values are means \pm s.d.

0.001 < P < 0.01 and *P < 0.001 for differences between groups.

spread, 0.352 \pm 0.05; subjects consuming plant sterol esters-enriched spread, 0.341 \pm 0.05; P = 0.08) and at 30 Pa (subjects consuming control spread, 0.596 \pm 0.03; subjects consuming plant sterol esters-enriched spread, 0.591 \pm 0.03; P = 0.25).

Discussion

The long-term efficacy and safety of a plant sterol esters-enriched spread commercially available in the European Union, the United States and Australia was extensively studied. A sustained cholesterol-lowering effect of daily consumption of about 20 g spread enriched with 1.6 g plant sterols was observed in this double-blind, placebo-controlled trial during 1 y in apparently healthy normocholesterolemic and mildly hypercholesterolemic male and female volunteers. The cholesterol-lowering effect of the plant sterol esters-enriched spread was consistent throughout the study, namely, total cholesterol was reduced on average by about 4% and LDL-cholesterol on average by about 6%. The reductions in total and LDL-cholesterol observed are significant and may, on a population basis, substantially contribute to the prevention of coronary heart disease (Law *et al*, 1994).

This study showed also that carotenoid concentrations were decreased, whereas fat-soluble vitamins were not seriously affected. None of the additional safety parameters measured, including clinical chemical parameters and hematological parameters, but also including female and male reproductive hormones, osteocalcin carboxylation degree, vitamin B₁₂, folic acid and MDA concentrations, were affected.

The reductions in serum cholesterol concentrations following plant sterol consumption reported here are slightly lower than those reported for short-term trials (eg Hendriks *et al*, 1999; Weststrate & Meijer, 1998) and in one other one-year study (Miettinen *et al*, 1995). Efficacy may depend on many different factors like intake level (Hendriks *et al*, 1999; Hallikainen *et al*, 2000a), background diet (Hallikainen *et al*, 2000b; Hallikainen & Uusitupa, 1999; Denke, 1995), composition of the sterol mixture (Jones *et al*, 2000; Sierksma *et al*, 1999) and specific characteristics of the subjects studied

(Hallikainen *et al*, 1999; Tammi *et al*, 2000; Vuorio *et al*, 2000), like genetic background, compliance and eating moment. Miettinen *et al* (1995) reported a slightly higher efficacy, most likely due to the larger intake of plant stanols as compared with the plant sterol intake in the trial reported here (2.6 g stanols vs 1.6 g sterols per day). Also, the plant stanol trial was conducted in North Carelia, Finland, with a population known for its high cholesterol concentrations and inherited lipid metabolism disorders. Interestingly, studies conducted in the USA using 3 g plant stanols per day (Nguyen *et al*, 1999; Blair *et al*, 2000) showed a lower efficacy than the data reported by Miettinen *et al* (1995).

After 13 weeks in-study, the majority of the volunteers consumed all spread with breakfast. Since the proposed mechanism of cholesterol lowering is the inhibition of cholesterol absorption from the intestine, it may be expected that a constant presence of plant sterols in the intestines may be needed for an optimal effect. Therefore, consumption of a plant sterol esters-enriched spread with at least two meals per day was considered relevant. We wanted to explore whether cholesterol reduction could be enhanced by increasing the number of daily eating occasions. Consequently, a request was issued half way through the study, to eat 10 g of spread with both breakfast and lunch. Although the request resulted in a clear increase in the proportion of volunteers consuming spread with two meals (from about 40 to about 70%), cholesterol lowering efficacy was not significantly affected. This is in accordance to what has been demonstrated recently (Plat *et al*, 2000), namely that frequency of plant stanol esters-enriched spread intake did not affect blood cholesterol lowering efficacy.

A direct comparison on the efficacy of plant sterols and stanols has been performed several times so far. Four double-blind placebo-controlled studies directly compared the efficacy of plant sterols and stanols (Weststrate & Meijer, 1998; Hallikainen *et al*, 2000b; Jones *et al*, 2000; Normen *et al*, 2000). These short-term human trials, either dietary controlled or in free-living conditions, showed a similar (Weststrate & Meijer, 1998; Hallikainen *et al*, 2000b; Normen *et al*, 2000) or a better (Jones *et al*, 2000) blood cholesterol lowering efficacy for plant sterol esters-enriched spreads compared with spreads enriched with plant stanol esters.

The major objective of this study was to examine the clinical and safety parameters during 1 y of consumption of plant sterol esters in healthy volunteers. Consumption of plant sterol esters-enriched spread appeared to have no adverse side effects, defined as reported adverse events or undesirable changes in clinical chemical parameters, hematological parameters and urinalysis. The absence of side effects is in agreement with the observations in earlier, shorter-term, clinical and safety studies (Hendriks *et al*, 1999; Weststrate & Meijer, 1998; Baker *et al*, 1999; Hepburn *et al*, 1999; Waalkens-Berendsen *et al*, 1999; Weststrate *et al*, 1999; Ayesha *et al*, 1999; Sanders *et al*, 2000; Miettinen *et al*, 1995; Hallikainen *et al*, 1999, 2000a,b; Hallikainen & Uusitupa,

Table 8 Quintiles of serum campesterol and β -sitosterol concentrations at baseline and their percental change (in brackets) after 52 weeks

	Plant sterol-enriched spread ^a				
	Q1	Q2	Q3	Q4	Q5
Campesterol	7.97 (124)	12.54 (92)	16.48 (102)	20.12 (45)	30.97 (51)
β -Sitosterol	5.49 (80)	8.26 (21)	10.56 (25)	12.3 (28)	19.37 (10)

Values are means ($\mu\text{mol/l}$) and (percental change). ^a $n=88$.

1999; Denke, 1995, Jones *et al*, 2000; Sierksma *et al*, 1999; Tammi *et al*, 2000; Vuorio *et al*, 2000; Nguyen *et al*, 1999; Blair *et al*, 2000).

Plant sterols are slightly absorbed (< 5%) and could subsequently be exchanged with cholesterol in an equilibrium fashion in various sterol pools, including cell membranes. Red blood cells are easily accessible cells with an average life span of about 4 months. We therefore analyzed the sterol levels and profiles of red blood cell membranes sampled at the end of the study. In subjects consuming the plant sterol ester-enriched margarine, plant sterol concentrations in red blood cells were higher than in their control counterparts but had similar relative increase to that observed in serum. This close relationship between the increase in plant sterol concentrations between serum and red blood suggests that plant sterols did not accumulate in cell membranes. Also, red blood cell cholesterol concentrations (which are up to 1000-fold higher) were not affected, suggesting that plant sterols do not actively replace cholesterol in the cell membrane. Even with the possibility of exchange of plant sterols for cholesterol in the RBC membrane, this did not contribute to any changes in membrane rigidity. No effect on RBC deformability index was seen in healthy volunteers after 1 y use of plant sterol ester-enriched spread.

Interestingly, the absolute concentrations in serum campesterol and β -sitosterol concentrations did not differ for those in the highest quintile of serum plant sterol concentrations as compared to the remaining observations (Table 8). Subjects with the highest baseline concentration of serum plant sterol, however, seemed to have a smaller relative increase in these concentrations as compared to the subjects with the lowest baseline concentration after one year consumption of the plant sterol-enriched spread. This finding may suggest that serum plant sterol concentrations reach an upper limit after which no further increase is observed.

In a previous study, three doses of plant sterol esters ranging from 0.8 to 3.2 g/day were effective in substantially reducing total and LDL-cholesterol but only the middle dose (about 1.6 g plant sterols per day) did not affect total serum lipids-corrected carotenoid concentrations. In this 1 y study, carotenoid concentrations changed over time and reductions in time were larger for subjects consuming spread enriched with 1.6 g plant sterol-esters as compared to those consuming control spread. Decreases were small, in the range of 15–20%, and remained constant over time. Plasma concentrations of

other carotenoids were also affected. These somewhat lowered carotenoid concentrations may reflect a new steady-state. Differences in carotenoid level may also, in part, reflect regression to normal values, because baseline values for plant sterol esters-enriched spread consumers were higher as compared with control spread consumers.

Also the levels of the fat-soluble vitamins retinol (vitamin A) and vitamin K₁ did not change. Average 25-OH-vitamin D concentrations were not different at all time points but the small decreases over time were larger in subjects consuming plant sterol esters-enriched spread as compared with those in subjects consuming control spread. Vitamin E concentrations were slightly decreased after plant sterol consumption when expressed per volume of serum or per total lipid but not when expressed per LDL concentration. Since vitamin E is present in high concentrations in LDL, vitamin E concentration per LDL concentration may physiologically be the most relevant parameter. Also, mean vitamin E concentrations for both treatments were well within the normal range, 7.7–18.5 $\mu\text{g/ml}$. These data suggest that carotenoids and fat-soluble vitamins are not affected by long-term consumption of plant sterol esters-enriched spread to an extent that seems biologically significant.

This study therefore indicates that daily consumption of 1.6 g of plant sterol in the long-term, consistently lowers blood cholesterol levels and does not appear to have any adverse health effects.

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References

Ayesh R, Weststrate JA, Drewitt PN & Hepburn PA (1999): Safety evaluation of phytosterol esters. Part 5. Faecal short-chain fatty acid and microflora content, faecal bacterial enzyme activity and serum female sex hormones in healthy normolipidaemic volunteers consuming a controlled diet either with or without a phytosterol ester-enriched margarine. *Food Chem. Toxicol.* 37, 1127–1138.

- Baker VA, Hepburn PA, Kennedy SJ, Jones PA, Lea LJ, Sumpter JP & Ashby J (1999): Safety evaluation of phytosterol esters: Part 1. Assessment of oestrogenicity using a combination of *in vivo* and *in vitro* assays. *Food Chem. Toxicol.* **37**, 13–22.
- Blair SN, Capuzzi DM, Gottlieb SO, Nguyen T, Morgan JM & Cater NB (2000): Incremental reduction of serum total cholesterol and low density lipoprotein cholesterol with the addition of plant stanol ester containing spread to statin therapy. *Am. J. Cardiol.* **86**, 46–52.
- Denke MA (1995): Lack of efficacy of low-dose sitostanol therapy as an adjunct to a cholesterol-lowering diet in men with a moderate hypercholesterolemia. *Am. J. Clin. Nutr.* **61**, 392–396.
- Friedewald WT, Levy RI & Frederickson DS (1972): Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18**, 499–502.
- Grootenhuys PA, Westenbrink S, Sie CMTL, Neeling JND de, Kok FJ & Bouter LM (1995): A semiquantitative food frequency questionnaire for use in epidemiologic research among the elderly: Validation by comparison with dietary history. *J. Clin. Epidemiol.* **48**, 859–868.
- Hallikainen MA & Uusitupa MI (1999): Effects of 2 low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects. *Am. J. Clin. Nutr.* **69**, 403–410.
- Hallikainen MA, Sarkkinen ES & Uusitupa MI (1999): Effects of low-fat stanol ester enriched margarines on concentrations of serum carotenoids in subjects with elevated serum cholesterol concentrations. *Eur. J. Clin. Nutr.* **53**, 966–969.
- Hallikainen MA, Sarkkinen ES & Uusitupa MI (2000a): Plant stanol esters affect serum cholesterol concentrations of hypercholesterolemic men and women in a dose-dependent manner. *J. Nutr.* **130**, 767–776.
- Hallikainen MA, Sarkkinen ES, Gylling H, Erkkila AT & Uusitupa MI (2000b): Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. *Eur. J. Clin. Nutr.* **54**, 15–25.
- Hardeman MR, Goedhart PT, Dobbe JGG & Lettinga KP (1994a): Laser-assisted optical rotational cell analyser (LORCA); I. A new instrument for measurement of various structural hemorheological parameters. *Clin. Hemorheol.* **14**, 605–618.
- Hardeman MR, Goedhart PT & Schut NH (1994b): Laser-assisted optical rotational cell analyser (LORCA); II. Red cell deformability: elongation index versus cell transit time. *Clin. Hemorheol.* **14**, 619–630.
- Hendriks HFJ, Weststrate JA, van Vliet T & Meijer GW (1999): 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.* **53**, 319–327.
- Hepburn PA, Homer SA & Smith M (1999): Safety evaluation of phytosterol esters: Part 2. Subchronic 90-day oral toxicity study on phytosterol esters: A novel functional food. *Food Chem. Toxicol.* **37**, 521–532.
- Jones PJ, Raeni-Sarjaz M, Ntanos FY, Vanstone CA, Feng JY & Parsons WE (2000): Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. *J. Lipid Res.* **41**, 697–705.
- Kempen HJM, de Knijff P, Boomsma DI, van der Voort HA, Gevers Leuven JA & Havekes L (1991): Plasma levels of lathosterol and phytosterol in relation to age, sex, anthropometric parameters, plasma lipids, and apolipoprotein E phenotype in 160 Dutch families. *Metabolism* **6**, 604–611.
- Law MR, Wald MJ & Thompson SG (1994): By how much and how quickly does a reduction in serum cholesterol concentrations lower risk of ischaemic heart disease? *Br. Med. J.* **308**, 367–373.
- Miettinen TA, Puska P, Gylling H, Vanhanen H & Vartiainen E (1995): Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *New Engl. J. Med.* **333**, 1308–1312.
- Moghadasian MH & Frohlich JJ (1999): Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: clinical and experimental evidence. *Am. J. Med.* **107**, 588–594.
- Nguyen TT, Dale LC, von Bergmann K & Croghan IT (1999): Cholesterol-lowering effect of stanol ester in a US population of mildly hypercholesterolemic men and women: a randomized controlled trial. *Mayo. Clin. Proc.* **74**, 1198–1206.
- Normen L, Dutta P, Lia A & Andersson H (2000): Soy sterol esters and beta-sitostanol ester as inhibitors of cholesterol absorption in human small bowel. *Am. J. Clin. Nutr.* **71**, 908–913.
- Plat J, van Onselen EN, van Heugten MM & Mensink RP (2000): Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters. *Eur. J. Clin. Nutr.* **54**, 671–677.
- Pollak OJ & Kritchevsky D (1981): *Monographs in Atherosclerosis*. New York: Basel.
- Sanders DJ, Minter HJ, Howes D & Hepburn PA (2000): The safety evaluation of phytosterol esters. Part 6. The comparative absorption and tissue distribution of phytosterols in the rat. *Food Chem. Toxicol.* **38**, 485–491.
- Sierksma A, Weststrate JA & Meijer GW (1999): Spreads enriched with plant sterols, either esterified 4,4-dimethylsterols or free 4-desmethylsterols, and plasma total- and LDL-cholesterol concentrations. *Br. J. Nutr.* **82**, 273–282.
- Stamler J, Daviglus ML, Garside DB, Dyer AR, Greenland P & Neaton JD (2000): Relationship of baseline serum cholesterol levels in 3 large cohorts of younger men to long-term coronary, cardiovascular, and all-cause mortality and to longevity. *JAMA* **284**, 311–318.
- Tammi A, Ronnema T, Gylling H, Rask-Nissila L, Viikari J, Tuominen J, Pulkki K & Simell O (2000): Plant stanol ester margarine lowers serum total and low-density lipoprotein cholesterol concentrations of healthy children: the STRIP project. *J. Pediatr.* **136**, 503–510.
- Tang JL, Armitage JM, Lancaster T, Silagy CA, Fowler GH & Neil HAW (1998): Systematic review of dietary intervention trials to lower blood total cholesterol in free-living subjects. *Br. Med. J.* **316**, 1213–1220.
- Vuorio AF, Gylling H, Turtola H, Kontula K, Ketonen P & Miettinen TA (2000): Stanol ester margarine alone and with simvastatin lowers serum cholesterol in families with familial hypercholesterolemia caused by the FH-North Karelia mutation. *Arterioscler. Thromb. Vase. Biol.* **20**, 500–506.
- Waalkens-Berendsen DH, Wolterbeek APM, Wijnands MVW, Richold M & Hepburn PA (1999): Safety evaluation of phytosterol esters. Part 3. Two-generation reproduction study of phytosterol esters in rats: a novel functional food. *Food Chem. Toxicol.* **37**, 683–696.
- 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.
- Weststrate JA, Ayesh R, Bauer-Plank C & Drewitt PN (1999): Safety evaluation of phytosterol esters. Part 4. Faecal concentrations of bile acids and neutral sterols in healthy normolipidaemic volunteers consuming a controlled diet either with or without a phytosterol ester-enriched margarine. *Food Chem. Toxicol.* **37**, 1063–1071.