Response of obligate heterozygotes for phytosterolemia to a low-fat diet and to a plant sterol ester dietary challenge

Peter O. Kwiterovich, Jr.,[1,*] Shirley C. Chen,[†] Donna G. Virgil,[*] Amy Schweitzer,[*] Dagmar R. Arnold,[*] and Lisa E. Kratz[*]

Lipid Research/Atherosclerosis Division,* Department of Pediatrics, Johns Hopkins University, 550 North Broadway, Baltimore, MD 21205; and Nutrition Science Group,† Unilever Bestfoods NA, 800 Sylvan Avenue, Englewood Cliffs, NJ 07632

Abstract **Twelve obligate heterozygotes from two kindreds were ascertained through phytosterolemic probands homozygous for molecular defects in the ATP binding cassette (ABC) half transporter, ABCG8. The response of these heterozygotes to a Step 1 diet low in fat, saturated fat, and cholesterol, and to 2.2 g daily of plant sterols (as esters) was determined in Protocol I (16 weeks) and Protocol II (28 weeks) during three consecutive feeding periods: Step 1/placebo spread; Step 1/plant sterol spread; and Step 1/placebo spread (washout). At baseline, half the heterozygotes had moderate dyslipidemia and one-third had mildly elevated campesterol and sitosterol levels. On the Step 1/placebo spread, mean LDL cholesterol decreased significantly, 11.2% in Protocol I (n = 12), and 16.0% in Protocol II (n = 7). Substitution with plant sterol spread produced a significant treatment effect on LDL levels in Protocols I and II. Conversely, the mean levels of campesterol and sitosterol increased 119% and 54%, respectively, during the use of plant sterol spread for 6 weeks in Protocol I, an effect mirrored for 12 weeks in Protocol II. During the placebo spread washouts, LDL levels increased, while those of plant sterols decreased to baseline levels in both protocols. In conclusion, phytosterolemic heterozygotes respond well to a Step 1 diet, and their response to a plant sterol ester challenge appears similar to that observed in normals.**—Kwiterovich, Jr., P. O., S. C. Chen, D. G. Virgil, A. Schweitzer, D. R. Arnold, and L. E. Kratz. **Response of obligate heterozygotes for phytosterolemia to a low-fat diet and to a plant sterol ester dietary challenge.** ***J. Lipid Res.*** **2003.** 44: **1143–1155.**

Supplementary key words lipoproteins • carotenoids • fat soluble vitamins • plant sterol ester enriched spread • margarine

Studies in humans with inherited disorders of cholesterol metabolism have provided unique and important insights into the mechanisms underlying both hypercholesterolemia and premature atherosclersosis (1). This has led to a more precise understanding of the effects of both dietary restriction of cholesterol and of pharmacological agents on reducing plasma cholesterol levels and preventing cardiovascular disease (1–4). For example, both dietary and drug treatments that lower the pool of cholesterol in the liver lead to an up-regulation of LDL receptors and to a decrease of plasma LDL cholesterol (1).

The effect of dietary cholesterol on plasma cholesterol levels is modest, due in part to the incomplete absorption of cholesterol by the intestine (5, 6). For example, Bosner et al. (7) found that the mean (1 SD) cholesterol absorption in humans was 56.2 (12.1)% with a range from 29.0% to 80.1%. This protective barrier in the normal human intestine is even more efficient for dietary plant sterols, such as sitosterol and campesterol, with estimates for sitosterol absorption ranging from 0.6% to 7.5%, and for campesterol from 5.5% to 16% (6). Ostlund and coworkers (8), using serum to establish unequivocal absorption into the systemic circulation and mass spectrometry for definitive identification of labeled tracers, recently reported lower estimates of absorption, namely, 0.5% for sitosterol and 1.9% for campesterol.

The plant sterols are structurally very similar to cholesterol, except that they always contain substitutions at the C24 position, and the mechanism that selectively prevents most of their intestinal absorption is not clear. The plant sterols, due to this structural similarity to cholesterol, prevent cholesterol absorption, most likely by precipitating cholesterol and competing for space in mixed micelles (9). An increased fecal excretion of cholesterol occurs, the hepatic concentration of cholesterol decreases, LDL receptors up-regulate, and the blood levels of total and LDL cholesterol subsequently fall (10–12). Plant sterols were first given in high doses of 6 g to 8 g per day to treat hypercholesterolemic patients. More recently, more modest doses of phytosterols, such as 2 g daily, have been incorporated into margarine and given to normal or moder-

Manuscript received 6 December 2002 and in revised form 6 March 2003.

Published, JLR Papers in Press, April 1, 2003.
DOI 10.1194/jlr.M200455-JLR200

[1] To whom correspondondence should be addressed.
e-mail: pkwitero@jhmi.edu

ately hypercholesterolemic subjects to lower their LDL cholesterol levels about 10% (10–19).

Recently, novel insights into the mechanisms affecting cholesterol and plant sterol absorption have been gained through the study of patients with the rare (<one in a million) autosomal recessive disorder, phytosterolemia (1). Patients with phytosterolemia have markedly elevated (>30-fold) plasma levels of plant sterols (20–23). This results from two metabolic abnormalities. First, patients with phytosterolemia hyperabsorb plant sterols, for example, between 15% and 60% of the average of 200 mg to 300 mg of plant sterols consumed daily (5, 20, 23). Second, unlike normal humans in whom almost all of any absorbed plant sterol is quickly secreted into the bile (5, 23), phytosterolemic homozygotes excrete only a fraction of the plant sterols into the bile (21, 23, 24). Phytosterolemic subjects also absorb a higher fraction of dietary cholesterol than normals, and they secrete less cholesterol into the bile (21, 23, 24). The cholesterol pool in the liver increases despite a low cholesterol synthetic rate, LDL receptors down-regulate, and LDL levels increase. The liver secretes the excess sterols into the blood on VLDL, which is converted into LDL, the major carrier of sterol in blood (23). About 10% to 25% of the sterol on LDL in phytosterolemia is plant sterol (20–23), and a similar proportion of these sterols is found in human tissues (25).

As a result of these two metabolic abnormalities, patients with phytosterolemia often have elevated levels of LDL sterol similar to those heterozygotes for familial hypercholesterolemia (FH); however, phytosterolemic subjects develop xanthomas (cholesterol deposits in skin and tendons) in the first decade of life, while FH heterozygotes do so in the third and fourth decades. Phytosterolemic homozygotes can also develop premature coronary artery disease at an earlier age than FH heterozygotes (21–23, 25). Phytosterolemic patients often develop aortic stenosis, an unusual finding in FH heterozygotes, and more typical of FH homozygotes (23). Unlike individuals with FH or other forms of hypercholesterolemia, phytosterolemic subjects respond dramatically to restriction in dietary cholesterol and plant sterols, and to bile acid sequestrants (21, 23, 26), often with the total and LDL sterol levels falling to within the normal range.

The molecular defects responsible for homozygous phytosterolemia are caused by two mutant alleles either in the gene that encodes the ATP binding cassette (ABC) half transporter, ABCG5, or in the gene encoding the half transporter ABCG8 (27, 28). These two genes are on chromosome 2p, where they are located in a head-to-head orientation. ABCG5 and ABCG8 are expressed exclusively in human liver and intestine, the sites of the two metabolic abnormalities in phytosterolemia. In mice, diets containing high cholesterol markedly increase the expression of ABCG5 and ABCG8 mRNA in liver and intestine (29). The current hypothesis, therefore, is that ABCG5 and ABCG8 have two normal functions: first, to limit the absorption of cholesterol and plant sterols, and second, to promote their excretion from the liver into the bile.

While it is clear that consuming plant sterols is contraindicated in phytosterolemic homozygotes, little information is available on the effect of increasing dietary plant sterols on the plasma total and LDL cholesterol, and plant sterol levels in the more common (<1 in 500) heterozygotes for phytosterolemia (30, 31). The intake of dietary plant sterols can be increased significantly using margarines containing either unsaturated plant sterol esters or saturated plant sitostanol esters, both of which lower total and LDL cholesterol levels in normal humans about 5–10% (10–12), and have similar effects on suppressing cholesterol absorption (12). Since unsaturated sterol esters have been shown to increase the average plasma sitosterol and campesterol levels in normals about 2-fold while saturated sitostanol esters decrease these levels to below the average (13), we selected unsaturated sterol esters as the dietary challenge.

Our objectives were to characterize the clinical and biochemical phenotype of obligate heterozygotes for phytosterolemia and their response to an unsaturated plant sterol ester-enriched spread, as judged by their change in LDL cholesterol levels and the extent of their increase in plant sterols levels.

METHODS

Study participants

Sixteen members of two families with homozygous phytosterolemic probands participated in the study. An Amish family was ascertained through the sudden death of an 11-year-old boy who had xanthomas and extensive atherosclerosis at autopsy. Of the 12 siblings of this Amish proband, five were found to be phytosterolemic homozygotes (22, 32). Of the 17 Amish obligate heterozygotes (two parents and 15 children of the homozygotes), 10 participated in this study. Two siblings and two spouses of the Amish homozygotes also participated as familial controls. Both parents of the two original phytosterolemic homozygotes described by Bhattacharyya and Connor (20) were also studied. The two families were not related to each other, and each had different mutations in the gene for the ABCG8 half transporter (27). The Amish phytosterolemics were homozygous for a missense mutation (Arg for Gly, G 574 A) in a residue that was conserved in mouse and human ABCG8 (27). The original phytosterolemic patient was homozygous for a nonsense mutation (1083 G–A) in exon 7 that introduced a premature terminal signal at codon 361, terminating ABCG8 (27). The subjects were healthy males (n = 9) and females (n = 7), aged 6 to 80 years. Informed consent was obtained for each subject. The Johns Hopkins Joint Committee on Clinical Investigation approved the study.

The subjects were free of active surgical or medical illnesses. Subjects were excluded from participating in the study if they had any of the following conditions: Types I, III, or V hyperlipoproteinemia; secondary hyperlipoproteinemia; body mass index >35.0 kg/m^2; were pregnant or lactating women; or used oral hypolipidemic therapy, systemic corticosteroids, androgens, or thyroid hormones (except stable-dose replacement therapy for ⩾2 months prior to enrollment).

Study protocols

The flow diagrams for the two study protocols are schematically summarized in **Fig. 1**. Protocol I was of 16 weeks duration and consisted of seven outpatient visits. Five subjects were also sampled 4 weeks after the conclusion of Protocol I (an eighth

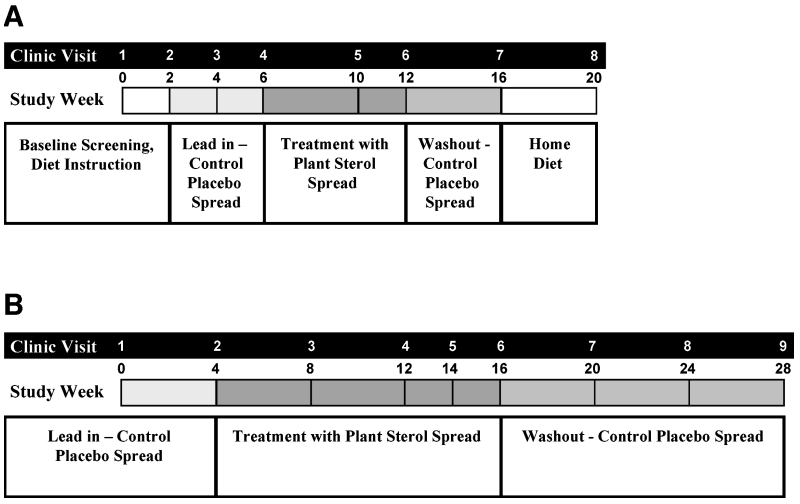

Fig. 1. Protocol I is schematically summarized in A and Protocol II is summarized in B.

visit). Protocol II was of 28 weeks duration and consisted of eight outpatient visits. Each study was single blind and placebo controlled.

Study design

In both Protocol I and Protocol II, each subject received an instruction at the first visit by a nutritionist on a low total-fat, saturated-fat, and cholesterol diet following the guidelines of the American Heart Association (33). The revised guidelines placed increased emphasis on foods and an overall eating pattern. The goal is a total fat intake of 30% of calories or less, a saturated fat intake of <10% of calories, and a daily cholesterol intake of <300 mg. While these guidelines collectively replace the "Step 1" designation used earlier, we simply refer to this diet here as the Step 1 diet. This Step 1 diet was followed throughout both Protocol I and Protocol II. In Protocol I, the first visit was followed by a 6 week baseline period; after 2 weeks of a stabilization period (Fig. 1A), a placebo spread [four servings (7 g per serving) of a 40% fat spread] was incorporated into the Step 1 diet. After 4 weeks on the placebo spread, each subject entered a 6 week treatment period, during which a plant sterol spread [four servings (0.55 g plant sterols per serving) in the form of sterol esters] was incorporated into the Step 1 diet. Protocol I concluded with a 4 week washout period during which time the subjects reverted back to the use of the placebo spread as part of their Step 1 diet.

In Protocol II, the subjects were given a placebo spread [two servings (14 g each) daily of a 40% fat spread] at their first visit to be incorporated into their Step 1 diet. After 4 weeks on placebo spread and diet, each subject entered a 12 week treatment period, during which time the plant sterol spread [two servings (14 g each) daily, each containing 1.1 g of plant sterols in the form of sterol esters] was incorporated into the Step 1 diet. Protocol II concluded with a 12 week washout period, during which time the subjects reverted back to the use of the placebo spread in their Step 1 diet.

For both protocols, evaluation included a physical examination and an electrocardiogram at baseline and final visit, vital signs at each visit, urinary pregnancy measurement at baseline, and safety laboratory evaluation (chemistry and hematology profiles and urinalysis) at visits one, three, four, and seven in Protocol I, and at visits one, five, and eight in Protocol II. Thyroid-stimulating hormone was measured in each subject at baseline. Plasma levels of total cholesterol, total triglycerides, and HDL cholesterol were determined at each visit in both protocols. The concentrations of the plasma plant sterols were measured at each visit in both protocols except at week 2, visit two in Protocol I. The plasma levels of apolipoprotein B (apoB), apoA-I, and lipoprotein [a] (Lp[a]) were assessed at each visit in both Protocol I and Protocol II. Retinol, tocopherols, and carotenoids were determined in plasma at visits three, four, five, six, and seven in Protocol I, and at each visit in Protocol II.

The test margarines were specially prepared spreads (Unilever Bestfoods, NA, Baltimore, MD). One was a plant sterol spread, the other was a placebo spread. The plant sterol spread was fortified with phytosterol (ester) concentrates derived from vegetable oil distillates. The sterols were a mixture of sitosterol, campesterol, brassicasterol, and stigmasterol (13). The vegetable oil sterols were esterified with fatty acids from sunflower oil to an esterification degree of 98%. The two spreads were provided in the form of 7 g individual packs for Protocol I. For Protocol II, the spreads were provided in tubs, along with a measuring spoon that provided a 14 g serving. The placebo and study spreads had identical fat levels (40%, or 2.8 g/serving) and fatty acid compositions. The chemical composition of the spreads has been described in detail (19). The plant sterol spread contained 0.55 g of phytosterol in each serving in Protocol I, and 1.1 g of phytosterol in each serving in Protocol II. Thus, the daily total intake of phytosterol was 2.2 g in both studies, but the package size was different.

Compliance with study spread consumption was evaluated by patient interview and by 3 day food records. Compliance was recorded as a percentage of scheduled intakes of study product consumed. Compliance was >87% in both protocols.

The intakes of dietary cholesterol and plant sterols, total fat, saturated fat, and unsaturated fat were estimated for each subject using a 3 day food record before, during, and after the sterol-containing test spread. The 3 day food records were then analyzed using Nutritionist Pro, First Data Bank, San Bruno, CA. The subjects were also asked to report the frequency, kind, and amount of supplements, including vitamins that they used at each visit.

Statistical methods

The effect of the three study diets (placebo spread, plant sterol ester spread, placebo spread) on the dependent variables (plasma levels of total cholesterol, total triglycerides, HDL cholesterol, LDL cholesterol, the ratio of LDL-HDL cholesterol, campesterol, sitosterol, Lp[a], apoB, apoA-I, the ratio of apoB-apoA-I, retinol, tocopherols, and carotenoids) were determined in Protocol I and Protocol II using a one-way mixed model

ANOVA for repeated measurements (SAS Proc Mixed). In this model, individual differences were accounted for using baseline (home diet) as a covariate (34), and by including a random subject effect in the model. The remaining time series (residual) correlation was modeled as one parameter autoregressive (34). We used the Tukey-Kramer multiple comparison test (35) to determine which of the study diets significantly affected the dependent variables. Additional posthoc comparisons of the same variables for subjects at baseline and at the end of the placebo spread run-in period were determined using paired Student's *t*-tests (35).

Laboratory methods

Total cholesterol, total triglycerides, and direct HDL cholesterol were measured in a Hitachi 717 Chemistry Analyzer in the Johns Hopkins Lipoprotein Analytical Laboratory with coefficients of variability (CVs) of <5%. The Lipid Standardization Program of the Centers for Disease Control, Atlanta, GA, certified the laboratory. LDL cholesterol was calculated by the Friedewald formula (36). The plasma levels of total apoB and apoA-I were measured in a Bering immunonephelometer with CVs of 5% (37). We measured Lp[a] by ELISA, as described, with a CV of 8% (38).

Lipoprotein phenotypes were defined as follows. First, an elevated plasma level of LDL cholesterol or triglycerides was defined as a value >90th percentile, and a low level of HDL cholesterol as a value <10th percentile, using the age- and sex-specific cut points from the Lipid Research Clinics Program (39). A Type IIa phenotype was defined as an elevated level of LDL cholesterol with normal triglycerides, and a Type IIb as elevated levels of LDL cholesterol and triglycerides. A Type IV phenotype was defined as an elevated triglyceride level with a normal LDL cholesterol level. HyperapoB phenotype was defined as an elevated level of apoB (>2.19 μmol/l in adults, >2.00 μmol/l in children) in the presence of a normal LDL cholesterol level. A normal phenotype was defined as the absence of any of these dyslipidemic phenotypes.

Campesterol and sitosterol were measured using selected ion monitoring gas liquid chromatography/mass spectrometry (GC/MS) as described (40), except to increase the plasma sample volume to 200 μl to permit more accurate measurement of the trace quantities of phytosterols in plasma of normal individuals. This method detects small amounts (<2.5 μmol/l) of plant sterols.

The plasma levels of lutein, α-cryptoxanthine, β-cryptoxanthine, lycopene, α-carotene, β-carotene, total carotenoids, retinol, γ-tocopherol, and α-tocopherol were determined using HPLC as described (41, 42).

RESULTS

The biochemical and clinical characteristics of the study population are summarized in **Table 1**. Twelve are obligate phytosterolemic heterozygotes (10 children of four Amish homozygotes and two parents of the original phytosterolemic homozygotes). Four additional Amish subjects were studied as familial controls: two adult siblings (II-11 and II-12) of the homozygous proband, and two spouses (II-13 and II-14) of two other homozygotes. Nine males and seven females were studied. At baseline, six of the 12 obligate phytosterolemic heterozygotes (Type IIb, n = 1; Type IV, n = 3; hyperapoB, n = 2), and both the Amish proband's siblings (Type IIa, n = 1; hyperapoB, n = 1) had a dyslipidemia (Table 1). Both the spouses were normal.

To interpret the plant sterol levels in the study population at baseline, plasma sitosterol and campesterol levels (mg/dl) were determined in 61 unrelated control subjects. These controls included 44 normal Mennonites, who were unlikely to carry the mutant allele for phytosterolemia, but who share a similar diet and life style with the Amish. Seventeen other Caucasian controls who were not Mennonites were also studied. The mean (SD) sitosterol and campesterol levels (μmol/l), respectively, in the control subjects were: *1*) adult Mennonite (n = 21), 4.65

TABLE 1. Clinical and biochemical characteristics of the study population

Subjects	Sex	Age	TC	TG	HDL Cholesterol	LDL Cholesterol	ApoB	ApoA-I	Sitosterol	Campesterol	Lp[a]	Lipoprotein Phenotypes
					mmol/l				*μmol/l*		*mg/dl*	
Obligate heterozygotes												
I-1	M	75	5.58	1.58	1.03	3.82	2.26	51.9	8.0	13.2	0	HyperapoB
I-2	F	80	4.91	1.46	1.21	3.02	2.06	64.8	2.2	2.7	0	Normal
II-1	F	12	4.78	1.49	1.14	2.97	1.60	45.9	5.1	6.8	3	Type IV
II-2	M	10	4.03	0.69	1.27	2.45	1.28	45.6	6.4	10.3	4	Normal
II-3	M	9	4.16	0.75	1.58	2.25	1.13	54.8	7.1	9.6	0	Normal
II-4	F	6	4.70	0.85	1.24	3.07	1.68	47.8	15.3	22.8	0	Normal
II-5	F	30	4.96	0.92	1.06	3.49	1.88	45.2	7.3	5.5	0	Normal
II-6	M	36	4.44	1.29	1.11	2.74	1.64	50.0	7.2	10.7	0	Normal
II-7	M	18	6.18	1.31	1.21	4.37	2.42	59.6	8.9	10.9	7	Type IIb
II-8	F	19	6.07	1.31	1.11	4.37	2.5	53.3	14.4	21.5	8	HyperapoB
II-9	M	8	4.70	1.3	0.98	3.13	1.88	47.8	15.0	24.6	7	Type IV
II-10	M	12	4.65	1.38	1.14	2.89	1.71	49.3	21.7	30.6	15	Type IV
Proband's siblings												
II-11	M	46	7.73	1.50	1.16	5.87	3.32	53.0	11.7	17.3	16	Type IIa
II-12	M	52	6.46	2.76	1.03	4.16	2.62	42.6	8.6	11.5	7	HyperapoB
Spouse controls												
II-13	F	43	5.53	1.21	1.03	3.95	2.08	45.9	7.2	1.0	3	Normal
II-14	F	43	4.16	0.89	1.14	2.61	1.33	51.5	4.6	7.7	2	Normal

Apo, apolipoprotein; Lp[a], lipoprotein[a]; TC, total cholesterol; TG, triglycerides. The lipoprotein phenotypes were determined as described in Methods.

(2.43), and 6.71 (4.21); *2*) Mennonite, 5–15 years (n = 16), 6.17 (3.69), and 8.62 (4.34); *3*) Mennonite, 2–4 years (n = 7), 4.89 (1.83), and 6.68 (2.84); and *4*) adult Caucasian (n = 17), 5.20 (5.30), and 7.71 (4.11). The Mennonite controls were used for the Amish participants and the Caucasian controls for the parents of the original phytosterolemic homozygotes. Four of the 10 Amish obligate heterozygotes had both plasma sitosterol and campesterol levels that were two SD above the mean of the controls. One sibling (II-11) of the Amish homozygous proband also had levels of sitosterol and campesterol that exceeded these cutpoints. Both the Amish spouses and the other two obligate heterozygotes (I-1 and I-2) from the original family had plasma plant sterol levels that were within the normal ranges.

Individual responses of obligate heterozygotes to modified-fat and plant sterol ester challenge

We first examined the individual responses of the obligate heterozygotes to the Step 1 diet and to the plant sterol ester-enriched margarine to determine the patterns of responses to the dietary interventions.

Individual responses in LDL cholesterol levels. The change in LDL cholesterol levels in each obligate heterozygote during Protocol I is summarized in **Fig. 2A**. The pattern of decrease in LDL cholesterol levels from baseline to the end of the placebo spread period, with an additional fall during the plant sterol spread period, followed by an increase during the washout placebo spread period, was internally consistent, with each subject generally following this pattern. Five of the 14 subjects were also sampled 4 weeks after the conclusion of the 16 week period of Protocol I, and LDL cholesterol values remained stable (n = 3) or increased (n = 2) (Fig. 2A).

In the longer-term Protocol II, there was again a downward trend in LDL cholesterol in each subject from baseline to week 4 on the Step 1/placebo spread diet (Fig. 2B). After 12 weeks of the substitution of the plant sterol spread for the placebo spread, the subject (II-1) with highest LDL cholesterol at baseline had the greatest decrease in LDL cholesterol (Fig. 2B). The decrease in LDL cholesterol in the remaining six obligate heterozygotes was less accentuated, and one subject (I-2) had an increase in LDL cholesterol on the plant sterol ester spread (Fig. 2B). During the 12 week placebo spread washout period, however, the individual LDL cholesterol values increased, as expected, toward levels that were similar to those observed at baseline (Fig. 2B).

Individual responses in plant sterol levels. The response of the plasma plant sterols in the obligate heterozygotes to the challenge of 2.2 g per day of dietary plant sterols in the test margarine is summarized in **Fig. 3A–D**. In Protocol I, both sitosterol (Fig. 3A) and campesterol (Fig. 3C) increased during the plant sterol spread phase, reaching a maximum after 6 weeks of treatment, and declining thereafter on the placebo spread to values similar to those at baseline. However, at the end of Protocol I, three subjects (II-9, II-4, and II-10) had campesterol levels >25 μmol/l; the sitosterol levels in each of these three subjects were <24 μmol/l. Protocol II was therefore designed to assess for a longer period of time the effect of the plant sterol spread on the sitosterol and campesterol levels. In Protocol II, subjects II-9 and II-10 again had the largest increase in sitosterol and campesterol on the test margarine (Fig. 3B, D). Both these subjects also had sitosterol and campesterol levels at baseline that exceeded 2 SD above the mean of the age-appropriate controls (Table 1). The sitosterol levels did not exceed 24 μmol/l on the plant sterol spread, and fell to about 12 μmol/l on the placebo spread during the washout period, a value close to baseline (Fig. 3B). The campesterol levels exceeded 50 μmol/l in each of these two subjects on the plant sterol spread (Fig. 3D), but fell during the washout period on the placebo spread, and were about 25 μmol/l at 24 weeks and 28 weeks of Protocol II, close to the baseline values (Fig. 3D). Each of the rest of the subjects in Protocol II had an increase in sitosterol and campesterol on the plant sterol spread that then fell during the washout with the placebo spread to values similar to those observed at baseline.

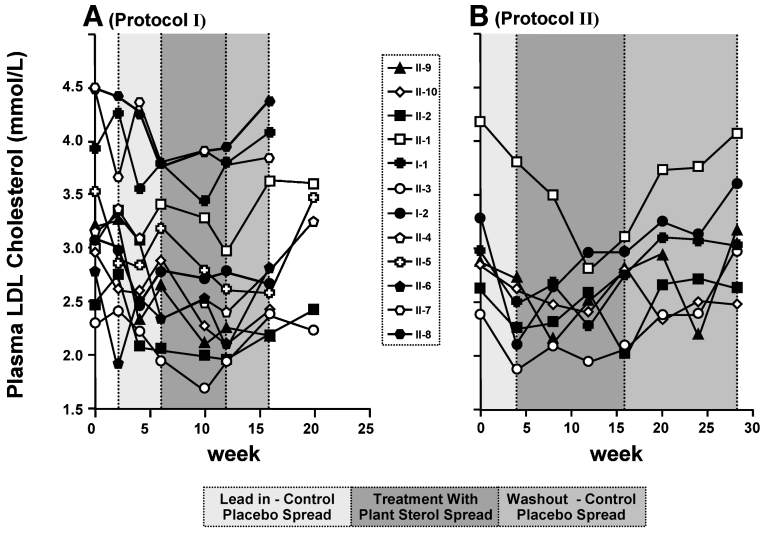

Fig. 2. Individual responses of LDL cholesterol to placebo spread and plant sterol ester spread in obligate phytosterolemic heterozygotes in Protocol I (A) and in Protocol II (B).

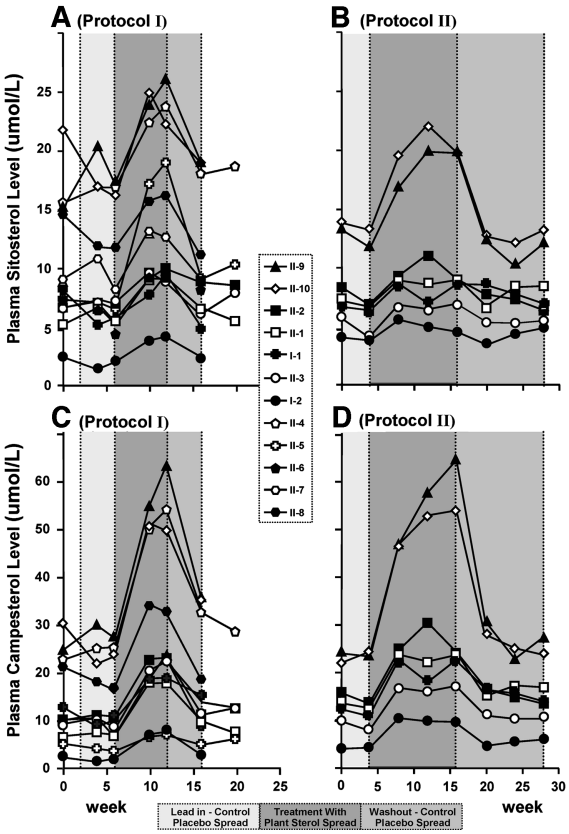

Fig. 3. Individual responses of plasma levels of sitosterol (top) and of campesterol (bottom) to placebo spread and plant sterol ester spread in obligate phytosterolemic heterozygotes in Protocol I (A, C) and Protocol II (B, D).

Group response of obligate heterozygotes for phytosterolemia to Step 1 diet and to a plant sterol ester challenge

Changes in nutrient intake. The group data for the obligate heterozygotes studied in Protocol I was next assessed, focusing on the plasma levels of LDL cholesterol and plant sterols. An important first step was to characterize the baseline (home) diet of these subjects, to document that they had modified their dietary intake as a result of their instruction on a Step 1 diet (see Methods), and that they continued to follow this diet throughout the three study periods (**Table 2**). The study group lowered their mean intakes of saturated fat and cholesterol significantly (Table 2). Total fat was also lowered from 31.1% of calories to 27.8% of calories, but this change was not significant. The modest increase in polyunsaturated fat was significant (Table 2). These changes were maintained throughout the study (Table 2), and there were no significant differences in these variables across the three experimental diet periods (by ANOVA, see Methods) (Table 2). During the three experimental periods, a significant difference was observed for energy intake, due mainly to decreased carbohydrate intake (Table 2). There was an ~30-fold increase in plant sterols during the plant sterol ester spread period (Table 2).

The group in Protocol II was very similar to that in Protocol I in regard to their marked increase in dietary plant sterols during the plant sterol ester spread period. The mean nutrient intakes for the seven obligate heterozygotes in Protocol II differed, however, from those of the 12 obligate heterozygotes in Protocol I in several ways. First, at the initial visit on the home diet, their mean intakes of total fat (29.1%), saturated fat (9.4%), and cholesterol (156 mg/day) were already at the Step 1 goals. There were no significant differences in Protocol II between these nutrients (or any others) when the home diet and the end of the placebo run-in period were compared (data not shown). Nevertheless, the group in Protocol II decreased their total fat and saturated fat to 26% and 7.1% of calories, a change that was maintained throughout the plant sterol ester spread period. In protocol II, during the placebo washout, the study group increased their protein, total-fat, saturated-fat, monounsaturated-fat, polyunsaturated-fat, and cholesterol intakes significantly, compared with the plant sterol ester spread study period, but they were still within the Step 1 goals.

TABLE 2. Nutrient intakes of obligate heterozygotes for sitosterolemia (n = 12) based on 3 day diet records (Protocol I)

Variables and Study Periods	Home Diet[a] (Baseline, Week 2)	Study Diets: Placebo Spread (Run-in, Weeks 4, 6)	Study Diets: Plant Sterol Ester Spread (Weeks 10, 12)	Study Diets: Placebo Spread (Washout, Week 16)	P^e
Energy, kcal	1,969 ± 130	1,908 ± 57[c]	1,668 ± 58[d]	1,840 ± 80[cd]	0.0156
Protein, g/day	79 ± 8	69 ± 3	62 ± 3	67 ± 4	0.2815
Carbohydrate, g/day	269 ± 17	280 ± 12[c]	247 ± 12[d]	292 ± 15[c]	0.0278
Fat, g/day	68 ± 9	59 ± 3	50 ± 3	52 ± 4	0.0834
Cholesterol, mg/day	267 ± 45[b]	175 ± 16	148 ± 17	145 ± 18	0.3751
Saturated fat, g/day	25 ± 4[b]	19 ± 1	16 ± 1	16 ± 2	0.1246
Monounsaturated fat, g/day	21 ± 3	20 ± 1	17 ± 1	18 ± 1	0.1087
Polyunsaturated fat, g/day	11 ± 1[b]	12 ± 1	11 ± 1	11 ± 1	0.2286
Plant sterols, mg/day	73 ± 9	70 ± 50[d]	2,063 ± 51[c]	55 ± 68[d]	0.0001

[a] Mean ± SE.
[b] Are significantly different from the corresponding values at the end of the run-in period (week 6), $P < 0.05$.
[c,d,cd] Least squares mean ± SE. For the three study diet periods, values in a row with different superscripts differ, $P < 0.05$.
[e] P values for treatment effect, as determined in Methods.

TABLE 3. Lipids and apolipoprotein concentrations of obligate heterozygotes for sitosterolemia (n = 12) at baseline, and in response to study diets (Protocol I)

Variables and Study Periods	Home Diet[a] (Baseline, Week 0)	Study Diets: Placebo Spread (Run-in, Weeks 4, 6)	Study Diets: Plant Sterol Ester Spread (Weeks 10, 12)	Study Diets: Placebo Spread (Washout, Week 16)	Overall P^e
Total cholesterol, mmol/l	4.96 ± 0.12[b]	4.49 ± 0.08[cd]	4.29 ± 0.08[d]	4.68 ± 0.10[c]	0.0050
Triacylglycerols, mmol/l	1.20 ± 0.09	1.06 ± 0.09	0.94 ± 0.09	1.24 ± 0.10	0.0648
HDL cholesterol, mmol/l	1.17 ± 0.05	1.14 ± 0.03	1.18 ± 0.03	1.17 ± 0.04	0.7235
LDL cholesterol, mmol/l	3.22 ± 0.20[b]	2.86 ± 0.08[cd]	2.69 ± 0.08[d]	2.95 ± 0.09[c]	0.0381
LDLC/HDLC	2.81 ± 0.22	2.62 ± 0.12	2.38 ± 0.12	2.61 ± 0.11	0.2567
Campesterol, μmol/l	14.09 ± 2.52	13.57 ± 1.41[d]	29.71 ± 1.41[c]	11.43 ± 1.40[d]	0.0163
Sitosterol, μmol/l	9.90 ± 1.60	9.20 ± 0.64[d]	14.17 ± 0.63[c]	10.00 ± 0.63[d]	0.0215
Lp[a], mg/dl	3.67 ± 1.36	5.04 ± 1.06	6.31 ± 1.06	7.20 ± 1.09	0.3371
ApoB, μmol/l	1.84 ± 0.12[b]	1.64 ± 0.04	1.57 ± 0.04	1.71 ± 0.04	0.0789
ApoA-I, μmol/l	51.48 ± 1.71[b]	47.42 ± 1.09	47.25 ± 1.09	50.59 ± 1.30	0.0813
ApoB/ApoA-I	0.035 ± 0.002	0.036 ± 0.001	0.034 ± 0.001	0.034 ± 0.002	0.9914

[a] Mean ± SE.
[b] Values are significantly different from the corresponding values at the end of the run-in period (week 6), $P < 0.05$.
[c,d,cd] Least squares mean ± SE. For the three study diet periods, values in a row with different superscripts differ, $P < 0.05$.
[e] P values for treatment effect, as determined in Methods.

Group response of plasma LDL cholesterol levels. There were significant falls in LDL cholesterol of 11.2% from baseline to 6 weeks in Protocol I and of 16.0% from baseline to 4 weeks in Protocol II (**Tables 3**, **4**). There was a significant treatment effect of the plant sterol-enriched margarine on LDL cholesterol levels in both Protocols I and II (Tables 3, 4). During the plant sterol spread period, LDL cholesterol in Protocol I decreased another 5.9% but did not fall further in Protocol II. During the washout periods of both Protocols I and II, the substitution of the placebo spread for the plant sterol spread produced significant increases in the mean LDL cholesterol levels of 9.7% and 15.8%, respectively (Tables 3, 4). These data taken together suggested that the plant sterol spread was responsible for the significant treatment effect on LDL cholesterol observed in both studies (Tables 3, 4).

Group response of plasma plant sterol levels. In both Protocol I and Protocol II, there was a significant treatment effect of the plant sterol ester spread on plasma levels of both sitosterol and campesterol (Tables 3, 4). The mean plasma campesterol levels increased about 2-fold in both Protocol I and Protocol II during the plant sterol ester spread period. The mean increase in the sitosterol level on the plant sterol ester spread was about half that seen with campesterol (Tables 3, 4). In the washout period, when the placebo spread was substituted for the plant sterol spread, both the mean campesterol and sitosterol levels decreased to those observed in the placebo run-in periods in Protocols I and II (Tables 3, 4). Despite differences in the duration of the plant sterol treatment and the placebo washout period in the two protocols, the qualitative pattern and the quantitative extent of response were very similar.

TABLE 4. Lipids and apolipoprotein concentrations of obligate heterozygotes for sitosterolemia (n = 7) at baseline, and in response to study diets (Protocol II)

Variables and Study Periods	Home Diet[a] (Baseline, Week 0)	Study Diets: Placebo Spread (Run-in, Week 4)	Study Diets: Plant Sterol Ester Spread (Weeks 8, 12, 16)	Study Diets: Placebo Spread (Washout, Weeks 20, 24, 28)	Overall P^e
Total cholesterol, mmol/l	4.65 ± 0.26[b]	3.95 ± 0.14[d]	4.20 ± 0.09[d]	4.64 ± 0.09[c]	0.0001
Triacylglycerols, mmol/l	0.98 ± 0.16	0.70 ± 0.10[d]	0.97 ± 0.06[c]	1.06 ± 0.06[c]	0.0153
HDL cholesterol, mmol/l	1.19 ± 0.08	1.11 ± 0.04[d]	1.21 ± 0.02[c]	1.23 ± 0.02[c]	0.0280
LDL cholesterol, mmol/l	3.00 ± 0.22[b]	2.52 ± 0.11[d]	2.53 ± 0.06[d]	2.93 ± 0.06[c]	0.0001
LDLC/HDLC	2.59 ± 0.27[b]	2.33 ± 0.08	2.14 ± 0.06	2.543 ± 0.06	0.9999
Campesterol, μmol/l	14.14 ± 2.64	14.20 ± 2.21[d]	28.73 ± 1.79[c]	15.72 ± 1.79[d]	0.0001
Sitosterol, μmol/l	8.41 ± 1.36[b]	7.60 ± 0.61[d]	10.93 ± 0.49[c]	8.04 ± 0.49[d]	0.0001
Lp[a], mg/dl	4.43 ± 1.42	4.97 ± 1.60	7.02 ± 1.07	5.43 ± 1.09	0.2756
ApoB, μmol/l	1.57 ± 0.13	1.48 ± 0.05[d]	1.41 ± 0.04[d]	1.61 ± 0.04[c]	0.0010
ApoA-I, μmol/l	50.74 ± 2.52	46.25 ± 2.25[d]	51.15 ± 2.00[c]	51.92 ± 2.00[c]	0.0154
ApoB/ApoA-I	0.031 ± 0.003	0.032 ± 0.001	0.028 ± 0.001	0.032 ± 0.001	0.9999

[a] Mean ± SD.
[b] Values are significantly different from the corresponding values at the end of the run-in period (week 4), $P < 0.05$.
[c,d] Least squares mean ± SE. For the three study diet periods, values in a row with different superscripts differ, $P < 0.05$.
[e] P values for treatment effect, as determined in Methods.

Group response in other biochemical lipid variables. In Protocol I, the mean plasma levels of both total cholesterol and apoB decreased significantly after the Step 1/placebo spread run period (Table 3). There was a significant treatment effect for the plant sterol ester spread for total cholesterol, but that for the apoB did not reach statistical significance (Table 3). The plasma level of apoA-I decreased significantly after the Step 1/placebo spread run-in, but that of HDL cholesterol did not (Table 3). There was no significant treatment effect on either the HDL cholesterol or apoA-I levels in Protocol I (Table 3).

In Protocol II, the plasma levels of total cholesterol also decreased significantly after the Step 1/placebo spread, and a significant treatment effect was noted for total cholesterol. In contrast to Protocol I, apoB, triglycerides, HDL cholesterol, and apoA-I also manifested a significant treatment effect (Table 4).

There was no effect of either the Step 1 diet or the plant sterol ester spread on Lp[a] lipoprotein levels (Tables 3, 4).

Response of familial controls to the plant sterol ester challenge

The individual responses of the four familial controls to the plant sterol ester treatment were also assessed and compared with responses in unrelated subjects from the published literature (13, 19, 43–45) where a similar dose of plant sterol esters was used and both LDL cholesterol and plant sterols were measured (**Table 5**). The average fall in LDL cholesterol in the familial controls was 13.5%, similar to the high end of the range of unrelated subjects in the literature (13). The average plasma sitosterol and campesterol levels increased 31.2% and 80.9% in the familial controls, within the ranges previously reported in unrelated subjects (Table 5).

Fat-soluble vitamins and carotenoids. If plant sterols displace fat-soluble vitamins and carotenoids from bile acid micelles, their use might theoretically decrease the absorption of these fat-soluble nutrients and potentially promote deficiencies in such nutrients (13, 16, 17). We therefore also examined whether the use of plant sterol in a dose of 2.2 g/day significantly decreased carotenoids, retinol, and tocopherols. Ten variables were measured in Protocols I and II (lutein, α- and β-cryptoxanthine, lycopene, α- and β-carotene, total carotenoids, retinol, and α- and γ-tocopherols). The data are presented for Protocol II because these variables were measured at baseline and could be used as covariates in the statistical analysis. Protocol II was also longer than Protocol I, and therefore any effect of the plant sterol esters more pronounced. After correction for plasma total cholesterol levels, significant treatment effects of the plant sterol ester were found for plasma levels [mean (SEM) as μg/100 mg cholesterol] of β-cryptoxanthine, lycopene, and α-carotene (**Table 6**). There was no significant effect on any of the other variables examined (Table 6).

DISCUSSION

We report here the response of obligate heterozygotes from two well-characterized families with phytosterolemia to 2.2 g daily of plant sterol via the consumption of a plant sterol esters-enriched spread, as judged by the change in their plasma levels of LDL cholesterol and plant sterols. We used an ABA study design that provided two control periods (A), one before and one after the plant sterol ester challenge (B; ABA switchover). Each obligate heterozygote served as his or her control.

TABLE 5. Comparisons of mean plasma levels of LDL-cholesterol, sitosterol and campesterol responses to intake of plant sterol ester-containing margarine of subjects from published literature and individual responses of familial controls in the current study

Subjects	n	Design (Duration)	Daily Intake of Plant Sterol (Background Diet)	Percent Change in Plasma Levels After Treatment with Plant Sterol Esters		
				LDL Cholesterol	Sitosterol	Campesterol
Published studies (reference)						
Mildly hypercholesterolemic adults (13)	78	Incomplete Latin Square (3 weeks)	3.3 g (habitual diets)	−13.1	+38.8	+72.6
Normal to mildly hypercholesterolemic adults (19)	118, 46[b]	Two arm parallel (5 weeks)	2.2 g (Step 1)	−8.1	+33.3	+72.2
Hypercholesterolemic adults with or without statins (43)	58	Cross-over (8 weeks)	2.5 g (recommended diet for hypercholesterolemics)	−10.0	+16.7	+86.7
Hypercholesterolemic adults (44)	60	Cross-over (3 weeks)	1.8 g (habitual diets)	−6.2	+50.0	+79.8
Normal to mildly hypercholesterolemic adults (45)	185	Two arm parallel (52 weeks)	1.6 g (habitual diets)	−6.0	+49.1	+113.7
Current study (familial controls)[a]		ABA switchover[c]	2.2 g (Step 1)			
Adult sibling of homozygote	1	Protocol I		−12.5[d]	+27.0[d]	+75.9[d]
Adult sibling of homozygote	1	Protocol I		+10.0	+32.7	+53.3
Spouse of homozygote	1	Protocol II		−34.1	+30.2	+109.5
Spouse of homozygote	1	Protocol II		−17.2	+34.9	+84.9

[a] See Table 1 for subject descriptions and Fig. 1 for study design.
[b] n = 118 for the LDLC measurement; n = 46 for the plant sterol measurements.
[c] There were two control periods (A), one before and one after the plant sterol ester challenge (B).
[d] Comparison of values at the end of the lead-in period with control placebo spread and at the end of the treatment period with plant sterol ester spread.

TABLE 6. Plasma carotenoids and fat-soluble vitamin concentrations (μg/100 mg cholesterol)[a] of obligate heterozygotes for sitosterolemia (n = 7) at baseline, and in response to study diets (Protocol II)

Variables and Study Periods	Home Diet[b] (Baseline, Week 0)	Study Diets[e]			
		Placebo Spread (Run-in, Week 4)	Plant Sterol Ester Spread (Weeks 8, 12, 16)	Placebo Spread (Washout, Weeks 20, 24, 28)	Overall *P*[f]
Lutein	54 ± 7	80 ± 7	88 ± 6	94 ± 6	0.8724
α-Cryptoxanthine	16 ± 4	21 ± 4	25 ± 3	25 ± 3	0.4843
β-Cryptoxanthine	38 ± 7	60 ± 13[c]	47 ± 12[d]	57 ± 12[cd]	0.0413
Lycopene	111 ± 28	147 ± 23[cd]	121 ± 18[d]	190 ± 18[c]	0.0216
α-Carotene	21 ± 6	38 ± 4[c]	27 ± 3[d]	28 ± 3[d]	0.0260
β-Carotene	100 ± 22	146 ± 22	128 ± 19	158 ± 19	0.2080
Retinol	228 ± 30	423 ± 30	482 ± 25	494 ± 25	0.2066
α-Tocopherol	6,340 ± 650	10,120 ± 850	11,995 ± 682	12,457 ± 688	0.1111
γ-Tocopherol	1,076 ± 203	1,696 ± 170	1,878 ± 134	2,058 ± 135	0.2497

[a] Corrected for total cholesterol.
[b] Mean ± SE.
[c,d,cd] Values at baseline and at the end of the run-in period (week 4) are not different.
[e] Least squares mean ± SE. For the three study diet periods, values in a row with different superscripts differ, $P < 0.05$.
[f] *P* values for treatment effect, as determined in Methods.

The study population responded to a Step 1 diet with an average decrease in LDL cholesterol of 11.2% in Protocol I and of 16.0% in Protocol II, both highly statistically significant. In Protocol I, the ingestion of about 2.2 g of plant sterols daily produced a significant additional reduction of 5.9% in LDL cholesterol over that achieved by a Step 1 diet. Such a significant fall was not seen in Protocol II. Since the blood levels of the plant sterols increased significantly in Protocol II, and in a pattern similar to Protocol I (Fig. 3), lack of compliance to the ingestion of the test margarine does not explain the failure to find a decrease in LDL. The simplest explanation is that the observation was due to intraindividual heterogeneity, which might become of more importance, given the smaller sample size in Protocol II. For example, in one subject (I-2) in Protocol II, the LDL cholesterol levels increased notably during the plant sterol ester period (Fig. 2B). The mean dietary intakes of total fat, saturated fat, and cholesterol were lower at baseline and during the placebo run-in period in Protocol II than in Protocol I, but both groups followed a Step 1 diet throughout all the study periods. Moreover, in both Protocol I and Protocol II, there was a significant increase in LDL cholesterol in the placebo spread washout period, indicating that the plant sterol-enriched spread had an effect independent of the Step 1 diet. While sources of error, such as instrument effect, underestimate or overestimate of dietary intakes, and adherence effect (compliance bias) can influence the estimates of the intake of these nutrients (33), the changes in these the intakes of total fat, saturated fat, and cholesterol were consistent across our study population and both protocols.

The moderate dyslipidemia in the obligate heterozygotes, observed at baseline here and elsewhere (30, 31), might reflect some moderate down-regulation of the LDL receptor in these subjects, which might accompany increased sterol absorption. Further metabolic studies will be required to answer this issue. While the response of LDL cholesterol to the dietary changes employed here are of relatively low magnitude, on a population basis even a mean decrease of only 5% to 10% may have a significant effect on the overall incidence of coronary artery disease (CAD) (2–4).

Conversely, the mean plasma levels of campesterol and sitosterol increased 119% and 54%, respectively, during the use of the plant sterol-enriched spread in Protocol I, an effect that was mirrored in Protocol II. While such changes were highly statistically significant, they were of similar magnitude to those seen in normal and mildly hypercholesterolemic unrelated subjects in other studies of plant sterol esters (13, 19, 43–45) and in our familial controls (Table 5). It must also be noted that the levels of plant sterols remained a minute fraction of the total blood sterols (Tables 3 and 4). The greater increase in the plasma level of campesterol than sitosterol in the obligate heterozygotes given the plant sterol-enriched spread may be related to the fact that humans have a greater intestinal absorption of campesterol than of sitosterol, a differential related to the decreased uptake of plant sterols by intestinal cells with an increasing number of carbon atoms at C 24 (6, 8, 46). In that regard, campesterol has one carbon, while sitosterol has two carbons at C 24. Once the effect of the plant sterol-enriched spread reached a plateau, no further increase was observed in either Protocol I or Protocol II, suggesting that the obligate heterozygotes eliminated the plant sterols sufficiently to prevent their accumulation. This tenet was supported by the fact that the mean plasma levels of both campesterol and sitosterol returned to baseline levels during the placebo spread washout period. In normal humans, Ostlund et al. (8) reported that the mean (SD) half-life of plasma sitosterol was 2.94 (0.15) days and that of plasma campesterol 4.06 (0.33) days. Despite the relatively long half-life of these plant sterols, one would predict that these levels would return to baseline by the time we sampled our obligate heterozygotes on the placebo spread in the washout period, unless there was a continued secretion of plant sterols ab-

normally accumulated in liver. These observations are consistent with the metabolic findings of Salen and coworkers (30), who reported that the somewhat increased plant sterol absorption in heterozygotes for phytosterolemia was offset by rapid elimination from the liver to prevent accumulation of plant sterols.

Protocol II examined the effect of the plant sterol spread on the plant sterol levels for a longer period of time, and also followed the participants for a greater time on the placebo spread to ensure that the increase in the plant sterols observed in Protocol I actually reached a plateau and then fell to the baseline levels and remained there. Very similar qualitative and quantitative patterns of increase in the mean plant campesterol and sitosterol levels, followed by a prompt decrease and stabilization, were observed in Protocol II as in Protocol I (Tables 3, 4).

The mean plasma sitosterol and campesterol levels (mg/dl) determined by GC/MS (see Methods) in our control groups were similar to those reported by others (5, 31, 47, 48). For example, the range of total plasma plant sterols in human serum under normal conditions was found to be between 7.5 to 42.5 μmol/l. Stalenhoef et al. (31) reported mean plasma levels of 12.5 μmol/l for campesterol and 8.2 μmol/l for sitosterol in 33 healthy subjects; the campesterol levels varied 6.6-fold (range 3.5 to 22.9 μmol/l) and the sitosterol levels varied 7.9-fold (range 1.9 to 15.2 μmol/l). The mean baseline levels of sitosterol of 9.9 μmol/l and of campesterol of 14.2 μmol/l in our obligate heterozygotes were about 2-fold higher than those found in our age- and population-specific controls. Four of the 10 Amish obligate heterozygotes had both sitosterol and campesterol levels that were two SD or higher above the mean for our controls. However, these values ranged from 14.5 to 29.6 μmol/l, and were clearly not elevated to a range intermediate between normals and phytosterolemic homozygotes (>450 μmol/l). Thus, while theoretically heterozygotes might have a 50% deficiency in the intestinal absorption and hepatic elimination of plant sterols leading to a more significant increase in their plasma plant sterols, this does not appear to be the case, and the function of the normal ABCG8 allele appears to compensate for the mutant allele, thereby leading to the recessive phenotype (20–32).

The implication for the development of CAD when plant sterol levels are in the range of 12.0 to 48.2 μmol/l is not known. In a cross-sectional study, Glueck and coworkers (49) reported such modestly elevated levels in probands and relatives from families with premature CAD. However, these observations may be confounded by dietary intake of sterols and age factors. Berge et al. (50) have recently found that the plasma levels of campesterol and sitosterol are heritable, and that two common DNA sequence variations (D19H and T400K) in the ABCG8 gene are associated with lower concentrations of these plant sterols. Thus, the ABCG8 gene appears to influence the plant sterol levels in the general population. In fact, a higher ratio of plasma plant sterols to cholesterol levels appears to be correlated positively with the fractional absorption of cholesterol but negatively with cholesterol synthesis (51). In the Scandinavian Simvastatin Survival Study, those patients with CAD who had a lower ratio of plasma plant sterols to cholesterol, and consequently a greater biosynthesis of cholesterol, were found to have a greater reduction in plasma cholesterol levels with treatment, and reduced recurrences of CAD events (52).

Reduced cholesterol solubilization in bile acid micelles has been proposed as an important mechanism in the inhibition of absorption of cholesterol by phytosterols (9). Since campesterol and sitosterol are more hydrophobic than cholesterol, they have a higher affinity than cholesterol for micelles, thus restricting the solubility of cholesterol. This should affect both dietary and biliary cholesterol absorption, and our observation that the plant sterols appear effective even in the face of a reduced-cholesterol diet supports this mechanism. One study (53) found that the addition of 3 g/day of sitostanol to a low-cholesterol diet was not efficacious in lowering plasma cholesterol in moderate hypercholesterolemic men, an observation that might be explained by the likelihood that the stanols used in this study were not in solution.

The absorption of cholesterol and plant sterols by intestinal cells was previously postulated to be nonspecific, but now a high-affinity receptor-mediated mechanism has been proposed (12). If this hypothesis is correct, then plant sterols might also inhibit the high-affinity receptor-mediated uptake of cholesterol, as well as displace cholesterol from micelles. Either of these two mechanisms might lead to decreased cholesterol absorption and esterification in the intestine, less subsequent excretion into the blood stream on chylomicrons, decreased hepatic uptake of cholesterol from chylomicron remnants, induction of LDL receptors, and reduction in LDL cholesterol levels. The decrease in LDL cholesterol is most likely not as effective as it could be, due to the compensatory increase in hepatic cholesterol biosynthesis (54, 55).

The identification of a high-affinity receptor that normally mediates the absorption of cholesterol and plant sterols has been elusive. If ABCG5 and ABCG8 co-coordinately functioned as such a receptor, one might expect mutations in the genes for these half ABC transporters to produce decreased intestinal absorption of sterols. The opposite occurs in the phytosterolemic homozygotes, and therefore it has been postulated that ABCG5 and ABCG8 normally mediate the egress of cholesterol and plant sterol from inside the intestinal cell back out into the intestinal lumen. In the intestine, cholesterol feeding up-regulates the genes for ABGG5 and ABCG8, an effect that is regulated by the oxysterol receptors, liver X-activated receptor (LXR)α, and LXRβ, transcription factors that regulate the expression of genes involved in cholesterol efflux, storage, catabolism, and elimination (29). In enterocytes, LXR agonists also markedly up-regulate the gene for ABCA1, a full-sized ABC transporter that mediates the efflux of cholesterol from cells (29). However, mutations in ABCA1 cause Tangier disease (56), and it is unlikely that ABCA1 is the primary receptor that regulates sterol absorption. Scavenger receptor class B type I (SR-BI) is another cell surface receptor postulated to play a role in cholesterol absorption (57). Ezetimibe, a drug that

inhibits the absorption of cholesterol in humans (58), binds with high affinity to SR-BI, and overexpression of SR-BI in Chinese hamster ovary cells resulted in increased cholesterol uptake, which was blocked by ezetimibe (57). However, SR-BI knockout mice have normal cholesterol absorption, indicating that SR-BI may have a role in cholesterol absorption, but is not essential for it to occur (57).

We used the amount of test margarine that one is likely to encounter in individuals attempting to lower their total and LDL cholesterol an additional amount over that obtained with a low total-fat, saturated-fat, and cholesterol diet. We did not examine, therefore, the dose-response relationship between the amount of dietary plant sterol and the LDL cholesterol level. Others (16) found no evidence for a statistically significant dose-response relationship between cholesterol lowering and three different, relatively low, intake levels of plant sterols (0.83, 1.61, and 3.24 g/day). The absence of a clear dose dependency may be due to the compensatory increase in cholesterol synthesis that occurs after ingestion of higher dosages of plant sterols (54, 55).

In a double-blind, randomly-crossed-over controlled feeding study of mildly hypercholesterolemic adults fed 2.2 g plant sterols daily, Judd et al. (34) found that baseline levels were a significant predictor of the final concentrations for all blood lipids and lipoproteins. We also observed in both Protocol I and Protocol II that those obligate heterozygotes for phytosterolemia with the highest baseline LDL cholesterol levels had the greatest fall in LDL cholesterol during the treatment period. Those with the lowest LDL levels also had a response, however, and the response does not appear to be simply a function of the baseline LDL levels. Furthermore, in examining a treatment effect of plant sterol esters, differences in baseline LDL levels were taken into account (see Methods). Our pediatric subjects, <18 years of age, responded similarly to the young and older adults in the study. Tammi et al. (59) found that doubling dietary plant sterol intake in 13-month-old children almost doubled the serum plant sterol concentrations. Becker et al. (60) previously reported that children with heterozygous FH responded to either sitosterol or sitostanol with a significant reduction of LDL cholesterol.

In agreement with at least a half dozen other reports (11, 17, 48), we found no significant treatment effect of the plant sterol esters on the levels of HDL cholesterol or apoA-I, its major apolipoprotein, in Protocol I. The treatment effect on the levels of HDL cholesterol and apoA-I in Protocol II may have been due to the increase in HDL and apoA-I in the placebo washout period when the dietary fat content of the diet increased. The reduction in LDL cholesterol that we observed was accompanied by a significant fall in the apoB levels, in agreement with a number of previous reports (11). This observation further suggests that treatment with a plant sterol-enriched margarine decreases the number of atherogenic LDL particles, and is not simply decreasing the core cholesteryl ester content.

In agreement with a number of other studies (12–15, 34), we found no evidence that the use of a plant sterol ester-enriched food decreased significantly the plasma levels of total carotenoids, retinol, and tocopherols. Even though we observed significant reductions of β-cryptoxanthine, lycopene, and α-carotene, the levels were within the broad limits of the normal population ranges. Thus, in regard to plasma carotenoids and fat-soluble vitamins, there appears to be no problem based on these relatively short-term studies in a special population of subjects. A concern has also been raised that a plant sterol-enriched margarine may decrease the availability of α- and β-carotene, even after correcting for changes (decreases) in LDL-C levels (12). Speculatively, this may decrease the availability of such antioxidants and promote the oxidation (and thus atherogenicity) of LDL, but this study provides no information in that regard except that the levels of β-carotene did decrease significantly on the plant sterol spread.

A larger cohort of older obligate heterozygotes for phytosterolemia will be required to determine if such carriers are at high risk for CAD. Future longer-term studies will also be required in large free-living populations to determine if small increases in plasma plant sterols might be associated with the development of atherosclerosis. We believe that this is unlikely, given the good prognosis of populations that consume diets low in animal fat and enriched with plant products. Normal humans, and apparently obligate heterozygotes for phytosterolemia, have a remarkable capacity to excrete plant sterols from the liver into bile, thus preventing their accumulation in the body.

The authors thank Dr. Beverly Clevidence for performing the analyses of the plasma carotenoids, retinol, and tocopherols, and Dr. Matthew Kramer for help with the statistical analyses. Both Dr. Clevidence and Dr. Kramer are with the United States Department of Agriculture. We thank Dr. Gert Meijer for his helpful discussions about the study. Dr. Richard Kelley oversaw the analyses of the plant sterol levels and provided critical review of the manuscript. We also thank Dr. D. Holmes Morton, Christine L. Hendrickson, Donna Robinson, and Lee Ann Drum of the Clinic for Special Children in Strasburg, PA; Dr. Brian Henry and Jo Ingraham of the Due West Family Medical Clinic, Due West, SC; and Dr. Alvin Graber and Valerie Haviland of Nappanee Medical Clinic, Nappanee, IN for their generous support in the conduct of both protocols. The authors thank Sharon Blackburn for providing the graphic arts. This study was partly supported by an unrestricted research grant from Unilever Bestfoods.

REFERENCES

1. Goldstein, J. L., and M. S. Brown. 2001. The cholesterol quartet. *Science.* **292:** 1310–1315.
2. Kwiterovich, P. O., Jr. 1997. The effect of dietary fat, antioxidants and pro-oxidants on blood lipids and lipoproteins and atherosclerosis. *J. Am. Diet. Assoc.* **97(Suppl.):** S31–S41.
3. Kwiterovich, P. O., Jr. 1998. State-of-the-art update and review: clinical trials of lipid-lowering agents. *Am. J. Cardiol.* **82:** 3U–17U.
4. Law, M. R., N. J. Wald, and S. G. Thompson. 1994. By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? *BMJ.* **308:** 367–372.

5. Salen, G., E. H. Ahrens, Jr., and S. M. Grundy. 1970. The metabolism of B-sitosterol in man. *J. Clin. Invest.* **49:** 952–967.
6. Ostlund, R. E., Jr. 2002. Phytosterols in human nutrition. *Annu. Rev. Nutr.* **22:** 533–549.
7. Bosner, M. S., L. G. Lange, W. F. Stenson, and R. E. Ostlund, Jr. 1999. Percent cholesterol absorption in normal men and women quantified with dual stable isotopic tracers and negative ion mass spectrometry. *J. Lipid Res.* **40:** 302–308.
8. Ostlund, R. E., Jr., J. B. McGill, C-M. Zeng, D. F. Covey, J. Stearns, W. F. Stenson, and C. A. Spilburg. 2002. Gastrointestinal absorption and plasma kinetics of Δ5-phytosterols and phytostanols in humans. *Am. J. Physiol. Endocrinol. Metab.* **282:** E911–E916.
9. Armstrong, M. J., and M. C. Carey. 1987. Thermodynamic and molecular determinants of sterol solubilities in bile salt micelles. *J. Lipid Res.* **10:** 1144–1155.
10. Ling, W. H., and P. J. Jones. 1995. Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sci.* **57:** 195–206.
11. Jones, P. J. H., D.E. MacDougall, F. Ntanios, C. A. Vanstone. 1997. Dietary phytosterols as cholesterol-lowering agents in humans. *Can. J. Physiol. Pharm.* **75:** 217–227.
12. Lu, K., M-H. Lee, and S. B. Patel. 2001. Dietary cholesterol absorption: more than just bile. *Trends Endocrinol. Metab.* **12:** 314–320.
13. Weststrate, J. A., and G. W. Meijer. 1998. Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolemic and mildly hypercholesterolemic subjects. *Eur. J. Clin. Nutr.* **52:** 334–343.
14. Jones, P. J. H., and F. Ntanios. 1998. Comparable efficacy of hydrogenated versus nonhydrogenated plant sterol esters on circulating cholesterol levels in humans. *Nutr. Rev.* **56:** 245–252.
15. Jones, P. J. H., M. Raeini-Sarjaz, F. Ntanios, C. Vanstone, J. Y. Feng, and W. Parsons. 2000. Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. *J. Lipid Res.* **41:** 697–705.
16. Hendricks, H. F. J., J. A. Westrate, T. van Vliet, G. W. Meijer. 1999. Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolemic and mildly hypercholesterolemic subjects. *Eur. J. Clin. Nutr.* **53:** 319–327.
17. Miettinen, T. A., and H. K. Gylling. 1999. Regulation of cholesterol metabolism by dietary plant sterols. *Curr. Opin. Lipidol.* **10:** 9–14.
18. Jones, P. J., and M. Raeini-Sarjaz. 2001. Plant sterols and their derivatives: the current spread of results. *Nutr. Rev.* **59:** 21–24.
19. Maki, K. C., M. H. Davidson, D. M. Umporowicz, E. J. Schaefer, M. R. Dickline, K. A. Ingram, S. Chen, J. R. McNamara, B. W. Gebhart, J. D. Ribaya-Mercado, G. Perrone, S. J. Robins, and W. C. Franke. 2001. Lipid responses to plant-sterol-enriched reduced-fat spreads incorporated into a National Cholesterol Education Program Step 1 diet. *Am. J. Clin. Nutr.* **74:** 33–43.
20. Bhattacharyya, A. K., and W. E. Connor. 1974. Beta-sitosterolemia and xanthomatosis. A newly described lipid storage disease in two sisters. *J. Clin. Invest.* **53:** 1033–1043.
21. Miettinen, T. A. 1980. Phytosterolemia, xanthomatosis, and premature atherosclerotic arterial disease: a case with high plant sterol absorption, impaired sterol elimination and low cholesterol synthesis. *Eur. J. Clin. Invest.* **10:** 27–35.
22. Kwiterovich, P. O., Jr., P. S. Bachorik, H. H. Smith, V. A. McKusick, W. E. Connor, B. Teng, and A. D. Sniderman. 1981. Hyperapobetalipoproteinemia in two families with xanthomas and phytosterolemia. *Lancet.* **1:** 466–469.
23. Bjorkhem, I., and M. Boberg. 1995. Inborn errors in bile acid biosynthesis and storage of sterols other than cholesterol. *In* The Metabolic Bases of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, editors. McGraw-Hill, New York. 2073–2102.
24. Lutjohann, D., I. Bjorkham, V. F. Beil, and K. von Bergmann. 1995. Sterol absorption and sterol balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitostanol treatment. *J. Lipid Res.* **36:** 1763–1773.
25. Salen, G., I. Horak, M. Rothkopt, J. L. Cohen, J. Speck, G. S. Tint, V. Shore, B. Dayal, T. Chen, and S. Shefer. 1985. Lethal atherosclerosis associated with abnormal plasma and tissue sterol composition in sitosterolemia with xanthomatosis. *J. Lipid Res.* **26:** 1126–1133.
26. Salen, G., P. O. Kwiterovich, Jr., S. Shefer, G. S. Tint, I. Horak, V. Shore, B. Dayal, and E. Horak. 1985. Increased plasma cholestanol and 5 alpha-saturated plant sterol derivatives in subjects with sitosterolemia and xanthomatosis. *J. Lipid Res.* **26:** 203–209.
27. Berge, K. E., H. Tian, G. A. Graf, L. Yu, N. V. Grishin, J. Schultz, P. Kwiterovich, B. Shan, R. Barnes, and H. H. Hobbs. 2000. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science.* **290:** 1771–1775.
28. Lu, K., M-H. Lee, S. Hazard, A. Brooks-Wilson, H. Hidaka, H. Kojima, L. Ose, A. F. H. Stalenhoef, T. Mietinnen, I. Bjorkem, E. Bruckert, A. Pandya, H. B. Brewer, Jr., G. Salen, M. Dean, A. Srivastava, and S. B. Patel. 2001. Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving Sterolin-1 and Sterolin-2, encoded by ABCG5 and ABCG8, respectively. *Am. J. Hum. Genet.* **69:** 278–290.
29. Repa, J. J., K. E. Berge, C. Pomajzl, J. A. Richardson, H. H. Hobbs, and D. J. Mangelsdorf. 2002. Regulation of ATP-binding cassette sterol transporters, ABCG5 and ABCG8, by the oxysterol receptors, LXR alpha and beta. *J. Biol. Chem.* **277:** 19793–19800.
30. Salen, G., G. S. Tint, S. Shefer, V. Shore, and L. Nguyen. 1992. Increased sitosterol absorption is offset by rapid elimination to prevent accumulation in heterozygotes with sitosterolemia. *Arterioscl. Thromb.* **12:** 563–568.
31. Stalenhoef, A. F. H., M. Hectors, and N. M. DeMacker. 2001. Effect of plant sterol-enriched margarine on plasma lipids and sterols in subjects heterozygous for phytosterolemia. *J. Int. Med. Res.* **249:** 163–166.
32. Beaty, T. H., P. O. Kwiterovich, Jr., M. J. Khoury, S. White, P. S. Bachorik, H. H. Smith, B. Teng, and A. D. Sniderman. 1986. Genetic analysis of plasma sitosterol, apoprotein B and lipoproteins in a large Amish pedigree with sitosterolemia. *Am. J. Hum. Genet.* **38:** 492–504.
33. Krauss, R. M., B. Howard, L. J. Appel, S. R. Daniels, R. J. Deckelbaum, J. W. Erdman, P. Kris-Etherton, I. Goldberg, T. A. Kotchen, A. H. Lichtenstein, W. E. Mitch, R. Mullis, K. Robinson, J. Wylie-Rosett, S. St. Jeor, J. Suttie, D. Tribble, and T. L. Bazzarre. 2001. Revision 2000: a statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *J. Nutr.* **131:** 132–146.
34. Judd, J. T., D. J. Baer, S. C. Chen, B. A. Clevidence, R. A. Muesing, M. Kramer, and G. W. Meijer. 2002. Plant sterol esters lower plasma lipids and most carotenoids in mildly hypercholesterolemic adults. *Lipids.* **37:** 33–42.
35. SAS/STAT User's Guide. Version 8. 1999. SAS Institute Inc., Cary, NC. 1–3884.
36. Friedewald, W. T., R. I. Levy, and D. S. Fredrickson. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18:** 499–502.
37. Bachorik, P. S., K. L. Lovejoy, M. D. Carroll, and C. L. Johnson. 1997. Apolipoprotein B and AI distributions in the United States, 1988–1991: results of the National Health and Nutrition Examination Survey III (NHANES III). *Clin. Chem.* **43:** 2364–2378.
38. Weiss, S. R., P. S. Bachorik, L. C. Becker, T. F. Moy, and D. M. Becker. 1998. Lipoprotein (a) and coronary heart disease risk factors in a racially mixed population: the Johns Hopkins Sibling Study. *Ethn. Dis.* **8:** 60–72.
39. The Lipid Research Clinics Population Studies Data Book. 1980. The Prevalence Study. Vol. I. US Department of Health and Human Services, National Institutes of Health, NIH Publication No. 80–1527. 1–136.
40. Kelley, R. I. 1995. Diagnosis of Smith-Lemli-Opitz Syndrome by gas chromatography/mass spectrometry of 7-dehydrocholesterol in plasma, amniotic fluid and cultured skin fibroblasts. *Clin. Chim. Acta.* **236:** 45–58.
41. Khachik, F., G. R. Beecher, M. B. Goli, W. R. Lusby, and J. C. Smith. 1992. Separation and identification of carotenoids and their oxidation products in the extracts of human plasma. *Anal. Chem.* **64:** 2111–2122.
42. Bieri, J. G., E. D. Brown, and J. C. Smith. 1985. Determination of individual carotenoids in human plasma by high performance liquid chromatography. *J. Liq. Chromatogr.* **8:** 473–484.
43. Neil, H. A., G. W. Meijer, and L. S. Roe. 2001. Randomized controlled trial of use by hypercholesterolaemic patients of a vegetable oil sterol-enriched fat spread. *Atherosclerosis.* **15:** 329–337.
44. Mussner, M. J., K. G. Parhoefer, K. von Bergmann, P. Schwandt, U. Broedl, and C. Otto. 2002. Effects of a phytosterol enriched margarine on hemorheology and plasma lipoproteins in mild to moderate hypercholesterolemia. *Metabolism.* **51:** 189–194.
45. Hendriks, H. F. J., E. J. Brink, G. W. Meijer, H. M. G. Princen, F. Y. Ntanios. 2003. Safety of long-term consumption of plant sterol esters-enriched spread. *Eur. J. Clin. Nutr.* **57:** 681–692.

46. Heinemann, T., G. Axtmann, and K. von Bergmann. 1993. Comparison of the intestinal absorption of cholesterol with different plant sterols in man. *Eur. J. Clin. Invest.* **23:** 827–831.
47. Salen, G., V. Shore, G. S. Tint, T. Forte, S. Shefer, I. Horak, E. Horak, B. Dayal, L. Nguyen, A. K. Batta, F. T. Lindgren, and P. O. Kwiterovich. 1989. Increased sitosterol absorption, decreased removal and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. *J. Lipid Res.* **30:** 1319–1330.
48. Miettinen, T. A., P. Puska, H. Gylling, H. VanHanen, and E. Vartiainen. 1995. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *N. Engl. J. Med.* **333:** 1308–1312.
49. Glueck, C. T., J. Speirs, P. Streicher, E. Illig, and J. Vandegrift. 1991. Relationships of serum plant sterols (phytosterols) and cholesterol in 595 hypercholesterolemic subjects, and familial aggregation of phytosterols, cholesterol, and premature coronary heart disease in hyperphytosterolemic probands and their first-degree relatives. *Metabolism.* **40:** 842–848.
50. Berge, K. E., K. von Bergmann, D. Lutjohann, R. Guerra, S. M. Grundy, H. H. Hobbs, and J. C. Cohen. 2002. Heritability of plasma noncholesterol sterols and relationship to DNA sequence polymorphism in ABCG5 and ABCG8. *J. Lipid Res.* **43:** 486–494.
51. Miettinen, T. A., R. S. Tilvis, and Y. A. Kesaniemi. 1990. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am. J. Epidemiol.* **131:** 20–31.
52. Miettinen, T. A., H. Gylling, T. Strandberg, and S. Sarna. 1998. Baseline serum cholestanol as predictor of recurrent coronary events in subgroup of Scandinavian Simvastatin Survival Study: Finnish 4S investigators. *BMJ.* **316:** 1127–1130.
53. Denke, M. A. 1995. Lack of efficacy of low-dose sitostanol therapy as an adjunct to a cholesterol-lowering diet in men with moderate hypercholesterolemia. *Am. J. Clin. Nutr.* **61:** 392–396.
54. Vanhanen, H. T., S. Blomqvist, C. Ehnholm, M. Hyvonen, M. Jauhiainen, I. Torstila, and T. A. Miettinen. 1993. Serum cholesterol, cholesterol precursors, and plant sterols in hypercholesterolemic subjects with different apoE phenotypes during dietary sitostanol ester treatment. *J. Lipid Res.* **34:** 1535–1544.
55. Miettinen, T. A., and H. T. Vanhanen. 1994. Dietary sitostanol related to absorption, synthesis, and serum level of cholesterol in different apolipoprotein subtypes. *Atherosclerosis.* **105:** 217–226.
56. Remaley, A. T., S. Rust, M. Rosier, C. Knapper, L. Naudin, C. Broccardo, K. M. Peterson, C. Koch, I. Arnould, C. Prades, N. Duverger, H. Funke, G. Assman, M. Dinger, M. Dean, G. Chimini, S. Santamarina-Fojo, D. S. Fredrickson, P. Denelfe, and H. B. Brewer, Jr. 1999. Human ATP-binding cassette transporter 1 (ABC1): genomic organization and identification of the genetic defect in the original Tangier disease kindred. *Proc. Natl. Acad. Sci. USA.* **96:** 12685–12690.
57. Altmann, S. W., H. R. Davis, X. Yao, M. Laverty, D. S. Compton, L. Zhu, J. H. Crona, M. A. Caplen, L. M. Hoos, G. Tetzloff, T. Priestley, D. A. Burnett, C. D. Strader, and M. P. Graziano. 2002. The identification of intestinal scavenger receptor class B, type I (SR-B1) by expression cloning and its role in cholesterol absorption. *Biochim. Biophys. Acta.* **1580:** 77–93.
58. Sudhop, T., D. Lutjohann, A. Kodal, M. Igel, D. L. Tribble, S. Shah, I. Perevozskaya, and K. von Bergmann. 2002. Inhibition of intestinal cholesterol absorption by ezetimibe in humans. *Circulation.* **106:** 1943–1948.
59. Tammi, A., T. Ronnemaa, L. Valsta, R. Seppanen, L. Rask-Nissila, T. A. Miettinen, H. Gylling, J. Vilkari, M. Anttolainen, and O. Simell. 2001. Dietary plant sterols alter the serum plant sterol concentration but not the cholesterol precursor sterol concentrations in young children (The STRIP Study). *J. Nutr.* **131:** 1942–1945.
60. Becker, M., D. Staab, and K. von Bergmann. 1993. Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. *J. Pediatr.* **122:** 292–296.