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Food and Drug Administration
Room 1061
5630 Fishers Lane
Rockville, MD 20852

RE: Food Labeling: Health Claims; Plant Sterol/
Stanol Esters and Coronary Heart Disease
Docket Nos. 00P-1275 and 00P-1276
66 Fed. Reg. 50824 (October 5, 2001)

This letter is submitted by the Altus Food Company ("Altus"), The Quaker Oats Company ("Quaker"), and Novartis Consumer Health, Inc. ("Novartis") in response to the Food and Drug Administration's ("FDA") request in the October 5, 2001 Federal Register, (66 FR 50824-6) for additional comments on its interim final rule permitting health claims stating that plant sterol/stanol esters may reduce the risk of coronary heart disease.

Altus is a joint venture of Quaker and Novartis . Altus is developing and marketing processed foods and beverages for the retail market and has recently introduced into test markets products that specifically support cardiovascular health. Altus strongly believes that food labeling should ensure that consumers are fully informed about the health-promoting benefits of certain food products but that consumers not be misled about those benefits or the nutritional content of their food.

Altus has brought to market products containing a mixture of free (not esterified) plant sterols and stanols. Altus and Novartis have previously provided the FDA with documentation of the safety and efficacy of the active ingredient in these products (GRAS Notice No. GRN 000039). The FDA has acknowledged in the preamble to the interim final rule (65 FR 54686) that free sterols and stanols are the active form of plant sterols and stanols. The FDA has also acknowledged that it had no questions about the safety of the ingredient, Reducol™ (Phytrol), for use in spreads. Novartis submitted additional information in November 2000 confirming the safety of the ingredient for use in cereals, bars and juice drinks. Altus recently completed clinical studies demonstrating the cholesterol reducing efficacy of these product forms.

In these comments Altus will briefly reiterate our previously submitted rationale for modifying the interim final rule to include free plant sterols and stanols, additional

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sources of plant sterols and stanols, and additional food formats containing these substances. In addition, we will submit new data that confirms the efficacy of these substances and food formats. We will also provide rationale that advisory statements are not justified scientifically and are therefore unwarranted and unneeded.

I. Eligibility of unesterified plant sterols and plant stanols for the health claim

Altus and Novartis have previously, and separately, commented, both on November 21, 2000, that free plant sterols and stanols should be included in the list of substances eligible to bear the claim. The FDA determined in its review of the relevant data that sterol and stanol esters are converted to free sterols and stanols before exerting their effect in the body (65 FR 54690), that the free form is the biologically "active moiety" and that the ester forms are actually secondary and created primarily to address manufacturing and marketing issues. Additionally, the FDA found that blood cholesterol and LDL-cholesterol levels were significantly reduced in studies in which the sterols and stanols were consumed in the free, unesterified form (65 FR 54704, especially Jones et al., 1999[1]). Novartis also submitted, in its November 21, 2000 comments, additional data demonstrating that free phytosterols could also be effective in reducing blood cholesterol levels when ingested in beverages, specifically a milk based drink.

The functionality of free plant sterols and stanols is additionally confirmed by a recently completed Altus clinical study in which subjects consumed one serving per day each of a low fat cereal, a low fat bar and a juice drink, all containing 0.6 g of free plant sterol and stanol[2]. The bar and cereal also contained at least 0.75 grams of oat beta-glucan per serving. The study included more than one hundred subjects in two groups who first received low fat dietary advice for six weeks and then, for an additional six weeks, one serving per day each of a bar, a cereal and juice drink. The test subjects received 1.8 grams per day of a mixture of free plant sterols and stanols and 2.7 grams per day of beta-glucan in oats. The test subjects LDL-cholesterol and total cholesterol were reduced 7% (-10.3 mg/dl) and 5% (-13.2 mg/dl), respectively, as compared to the placebo group. By comparison, Ripsin et al.[3] in a meta-analysis of twenty oat trials calculated an effect size of –3.4 to –10.5 mg/dl for total cholesterol for doses of oat soluble fiber of less than three grams. The effects in the Altus study test group were larger than can be attributed to beta-glucan alone, confirming that free plant sterols and stanols can be effective when incorporated into low fat foods and beverages.

Novartis has previously submitted data to the FDA on the cholesterol lowering efficacy of a milk-based beverage as well.[4] These studies confirm that phytosterols can be effective when incorporated into product forms other than spreads and salad dressings.

II. Daily intake levels necessary to reduce the risk of CHD

As we commented previously, we believe that the FDA has used an appropriate standard, i.e., the lowest daily amount that consistently and significantly lowers blood LDL-

cholesterol, to set the daily effective amount. This is consistent with the belief that even small changes in blood cholesterol levels will have a useful public health benefit. It is also consistent with the approach taken in developing the soluble fiber and soy protein health claims.

We believe that if all phytosterol cholesterol reduction studies are considered as a whole, plant sterols and plant stanols have approximately equal ability to reduce blood cholesterol levels. There are insufficient direct comparisons of sterols to stanols and free to esterified phytosterols to conclusively assign different efficacies to different forms. Further, we believe that differentiating the effectiveness of sterols and stanols will lead to unnecessary consumer confusion making it less likely that consumers will actually use beneficial products with these ingredients and will, therefore, fail to derive any benefit.

We do recommend that the effective amounts be expressed in grams of free sterols and stanols for all qualifying products. This reflects the actual substance measured analytically and the active component. It will reduce confusion and inconsistency in expressing the amount present in a food and the effective amount.

III. Eligibility of mixtures of plant sterols and plant stanols for the health claim

Both the Lipton/Unilever plant sterol ester and the McNeil/Raisio plant stanol ester ingredients are mixtures of sterols and stanols, albeit mixtures dominated by either sterols or stanols respectively. There is no scientific rationale for excluding intermediate mixtures from the health claim. The attached Altus study testing the Forbes-MediTech mixture of free plant sterols and stanols, as well as the previously submitted Novartis milk drink study and the Jones et al., 1999[1], study cited in the preamble to the interim final rule all confirm efficacy comparable to either the Unilever or Raisio ingredients. If the FDA accepts the argument that sterol and stanol efficacy are approximately equal there is no difficulty determining the effective amount of any mixture of sterols and stanols. If not, the effective dose can reasonably be specified as the sum of the % of the effective dose of sterols and the % of the effective dose of stanols present in the product. The sum would have to be equal to or greater than 100% for the product to qualify for the claim.

IV. Issues regarding safe use of plant sterol/stanol esters in foods and advisory label statements

The questions raised by the European Commission (EC), the Australia New Zealand Food Authority (ANZFA) and the American Heart Association are not material facts and do not warrant further action. The concerns involve the possibility of impaired vitamin status as a consequence of reduced fat soluble vitamin absorption and whether individuals who are heterozygous for sitosterolemia are at increased risk for cardiovascular disease. The actions of the EC and ANZFA are more cautious than warranted by the available scientific data.

There is no direct evidence that increased phytosterol consumption impairs vitamin A or any other fat soluble vitamin status. The effect of phytosterols on fat-soluble vitamins has been extensively studied. Published, well-controlled, randomized, double-blinded clinical trials have found no significant effects on the status of vitamins D, E, and K [5-12]. In these evaluations, carotenoids or dietary precursors of vitamin A have been reduced in some [5-9,12-15] but not all studies[5,6,10]. The Hendriks study[5] found that 1.6 g of plant sterols per day lowered plasma cholesterol concentrations without seriously affecting plasma carotenoid concentrations.

Novartis Consumer Health observed no negative effect on fat-soluble vitamin bioavailability, evidenced by unchanged plasma levels of fat-soluble vitamins after 4 weeks of consumption of a phytosterol enriched milk-based product at 1.8 g/day[4]. At the highest dose tested, i.e. 3.6 g/day, plasma α-carotene levels, but not β-carotene levels, were significantly lowered (i.e., 18%). In another clinical study, no effect on plasma vitamin A and E, and β-carotene levels were observed after treatment with a phytosterol enriched cereal product at a dose of 1.8 g/day[16].

Despite the reductions in β-carotene observed in some trials, plasma retinol levels remained unchanged, demonstrating that vitamin A status was not affected by the decreases seen in this vitamin A precursor[6-8,12,17,18]. Vitamin A is obtained from retinol, retinyl esters, and carotenoids in the diet. In addition, serum carotenoid concentrations, particularly in short-term studies, may not be accurate indicators of carotenoid availability in the body because they amount to only approximately one percent of the total tissue content of carotenoids[17].

In the various studies that showed a decrease in serum carotenoid levels after phytosterol treatment, the actual carotenoid concentrations remained within the ranges normally found in the population. Although both α-carotene and β-carotene have provitamin A activity, carotenoids are not essential nutrients and no recommended intake (or dietary reference intake) levels have been established[19]. No relevant health impairment at low (marginal) intake has been demonstrated. The recent evaluation of carotenoids by the Committee on the Scientific Evaluation of Daily Reference Intakes noted the following, "while there is evidence that β-carotene is an antioxidant *in vitro*, its importance to health is not known"[19]. Based upon the totality of scientific evidence that exists at this time, there is no persuasive reason to believe that modest reductions in serum levels of some carotenoids will affect health in the U.S. population.

Existing safety data provides no rationale for restricting the phytosterol intake of infants, children or pregnant or lactating women. Extensive safety studies conducted in animals and cited in the GRAS notifications submitted by Unilever, McNeil Consumer Healthcare, Novartis Consumer Health and Cargill found no effects of substantial intakes of a variety of mixtures and forms of phytosterols on reproduction or development. In all cases the FDA concluded that it had no questions about the use of various phytosterol preparations in a variety of food products. Animal studies demonstrate that these substances are poorly absorbed from the gastrointestinal tract, have low toxicity (NOAEL

= 6.6 g/kg/day), have no effect on reproduction, and are not genotoxic[20]. None of the human clinical studies[4-16,20] reported any adverse effects with phytosterol use, including one that evaluated intake for one year[7], and several[4,5,16] that reported intakes several times higher than that intended for Altus products. Furthermore, the products are designed and marketed to older adults with cardiovascular disease concerns. The sensory attributes, the packaging, and the advertising are all designed to appeal to older adults, making it less likely that they will be consumed by children or young women.

Commercially available plant stanol ester-containing spreads have been available in Finland since late 1995 with 260 million servings consumed since introduction[18]. Currently, approximately 140,000 adults in Finland are consuming the spread daily with no evidence of a safety issue[18]. Recently, the European Union (EU) approved plant sterol esters for use in margarine/spread under the EU Novel Foods Regulation and concluded they were safe for human use[21]. Switzerland, Brazil and Australia have also approved phytosterol use in vegetable spreads. Between 1954 and 1982, prior to the development of the cholesterol-reducing statin drugs, a phytosterol product was marketed in the United States and Canada as an anti-hypercholesterolemic agent at doses ranging between 9 and 30 g/day without any apparent adverse effects[17,22].

Among healthy individuals, the absorption rate of phytosterols is usually less than 5% of dietary levels, which is considerably lower than the absorption rate of cholesterol, which is over 40%[23]. Thus approximately 95% of dietary phytosterols enter the colon and are eliminated from the body. The exception is a rare, inherited condition known as phytosterolemia. Recent estimates suggest only approximately 50 cases have been identified world-wide[24]. This condition is inherited as a recessive trait and is characterized by the excessive absorption of phytosterols (20-30%)[25,26]. Phytosterolemia leads to elevated plasma concentrations of phytosterols, which can be as high as 1600 μmol/L, compared to an average of 30 μmol/L in healthy individuals[27]. These individuals are identified at a relatively early age and are treated with low phytosterol diets and binding agents such as cholestyramine. Since phytosterolemics are homozygotes, their parents are obligate heterozygotes. Individuals heterogygous for phytosterolemia are clinically and biochemically normal [28-31]. The normal response to phytosterol consumption by heterozygous subjects is reinforced by a recent report[32] that ingestion of approximately 3 grams per day by the heterozygous parents of a homozygous subject resulted only in increased campesterol and sitosterol blood levels comparable to those seen in normal subjects. There is no evidence that heterozygotes experience an intermediate response to phytosterol consumption and there is thus a low probability of their having an increased risk of cardiovascular disease.

It should be noted that the National Cholesterol Education Program (NCEP) of NHLBI[33] encourages intake of plant sterols and stanols and soluble fiber as part of a heart-healthy lifestyle, including a low fat, low cholesterol diet, weight management and exercise. NCEP guidelines also recommend the same therapeutic lifestyle changes for patients on drug therapy. Also, the AHA does state that plant sterols and stanols can be used by individuals needing to reduce blood cholesterol levels or for secondary prevention after an atherosclerotic event.

Requiring phytosterol containing products to carry a statement recommending medical supervision of their use is inappropriate. Based on extensive safety research the substances have been determined by experts to be generally regarded as safe for the food uses specified. As far as we are aware, there is no data supporting the need for medical supervision of foods which modestly reduce blood cholesterol levels including those that are low in fat or contain soluble fibers or soy protein. We are also unaware of any data which suggests that plant sterols and stanols interact in any negative manner with cholesterol reducing drugs. The NCEP Expert Panel suggested increased soluble fiber and phytosterol intake in conjunction with drug therapy for patients needing to reduce their cholesterol levels. Requiring labeling that recommends medical supervision on plant sterol and stanol containing foods is likely to inhibit the use of products which have been shown to be both safe and effective in reducing elevated cholesterol levels, which affect 100 million U.S. adults and is a leading risk factor for cardiovascular disease, a major U.S. health risk.

Summary

Failure to issue the final plant sterol/stanol rule in a timely manner and reopening the comment period continues to cause economic damage to Novartis, Quaker and Altus, as well as to other companies that are preparing to market sterol/stanol containing products. By further delaying the final rule, FDA is ensuring that a limited number of companies continue to retain the exclusive benefit of using the sterol/stanol health claim, while competitors must wait. This is inspite of the fact that the FDA has received information that confirms the safety and efficacy of alternate sterol/stanol mixtures, as well as alternate sources and alternate food formats.

In our understanding, the interim final rule procedure is intended to expedite the availability of health claims to leverage public health benefits. In this case, however, the process surrounding the interim final rule is having just the opposite effect by further delaying widespread use of the health claims as well as public health benefits. Health claims are not intended to be company specific; rather, they are intended to apply generally to all companies' products that meet the parameters of the health claim. This is in order to provide the general public with liberal access to the health benefits expressed in the health claim.

At the very outset, FDA's use of the interim health claim procedure prevented the industry from commenting on the health claim at a proposed rule stage, effectively foreclosing any chance to shape the health claim before it was approved for use by certain companies. In addition, both of the petitions which sought a health claim and the Interim Final Rule itself, are entirely product specific. As such, any competitor with a slightly varying product formulation is effectively precluded from taking advantage of the health claim. And finally, FDA's delay in issuing a final rule has further prolonged the exclusive use of the claim for certain companies while barring its use by competitors. These actions are not consistent with the intent of the Nutrition Labeling and Education Act ("NLEA") in providing for the development and use of health claims.

Altus, Quaker and Novartis recognize that FDA did attempt to approve the sterol/stanol health claim in the most expeditious manner possible. We urge FDA to make the changes outlined here as soon as possible.

As the FDA has previously acknowledged, the active form of plant sterols and stanols is the free, unesterified form, not the esters. Available research clearly demonstrates that free plant sterols and stanols can be successfully incorporated into a variety of food formats. Low fat, cholesterol-lowering foods containing plant sterols and stanols offer consumers an important alternative in helping reduce cardiovascular disease risk and should be included in the final plant sterol and stanol health claim rule.

Existing efficacy data for free and esterified plant sterols and stanols suggest that the cholesterol lowering potential is more similar than different among the different forms. There are insufficient direct comparisons to establish different efficacies. The amount of plant sterol and/or stanol required daily and per serving should be the same and stated in terms of free sterol content.

There is direct evidence that a variety of plant sterol and stanol mixtures reduce blood cholesterol levels in humans. As stated above, we believe that there is no sound basis for differentiating free or esterified plant sterols or stanols.

There is no scientific basis for requiring additional cautionary labeling statements on plant sterol and stanol containing foods which bear the health claim. There is no direct evidence of impairment of fat soluble vitamin status. There is extensive evidence in animal models that development and reproduction are not effected by relatively high intakes of plant sterols or stanols. There is no evidence that individuals who are heterozygous for sitosterolemia are at increased risk for cardiovascular disease as a consequence of plant sterol or stanol consumption and there is evidence that they respond as normal subjects do to ingestion of phytosterols. There has been no scientific rationale presented for recommending medical supervision for persons consuming products bearing cardiovascular risk reduction health claims.

As Altus and Novartis have stated in previous comments on this interim final health claim rule, we strongly urge the FDA not to use the health claim regulation to restrict the product forms permitted to use the claim. The existing GRAS and food additive review processes are much better suited to the regulation of food safety.

We respectfully request the opportunity to work with the FDA to allow the development and introduction of safe and efficacious products which will help reduce disease risk and improve the health of all Americans. The failure to issue the plant sterol and stanol health claim rule in a timely and scientifically grounded fashion will economically damage companies dependent on the agency for judicious review and approval of the claim. The FDA and the Congress need urgently to address whether the current approach to the management of the health claim review process is preventing the timely delivery of beneficial products to U.S. consumers.

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Cholesterol-lowering efficacy of a sitostanol-containing phytosterol mixture with a prudent diet in hyperlipidemic men[1–3]

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ABSTRACT

Background: Dietary plant sterols (phytosterols) have been shown to lower plasma lipid concentrations in animals and humans. However, the effect of phytosterol intake from tall oil on cholesterol and phytosterol metabolism has not been assessed in subjects fed precisely controlled diets.

Objective: Our objective was to examine the effects of sitostanol-containing phytosterols on plasma lipid and phytosterol concentrations and de novo cholesterol synthesis rate in the context of a controlled diet.

Design: Thirty-two hypercholesterolemic men were fed either a diet of prepared foods alone or a diet containing 1.7 g phytosterols/d for 30 d in a parallel study design.

Results: No overall effects of diet on total cholesterol concentrations were observed, although concentrations were lower with the phytosterol-enriched than with the control diet on day 30 ($P < 0.05$). LDL-cholesterol concentrations on day 30 had decreased by 8.9% ($P < 0.01$) and 24.4% ($P < 0.001$) with the control and phytosterol-enriched diets, respectively. HDL-cholesterol and triacylglycerol concentrations did not change significantly. Moreover, changes in circulating campesterol and β-sitosterol concentrations were not significantly different between phytosterol-fed and control subjects. In addition, there were no significant differences in fractional (0.091 ± 0.028 and 0.091 ± 0.026 pool/d, respectively) or absolute (0.61 ± 0.24 and 0.65 ± 0.23 g/d, respectively) synthesis rates of cholesterol observed between control and phytosterol-fed subjects.

Conclusion: Addition of blended phytosterols to a prudent North American diet improved plasma LDL-cholesterol concentrations by mechanisms that did not result in significant changes in endogenous cholesterol synthesis in hypercholesterolemic men. *Am J Clin Nutr* 1999;69:1144–50.

KEY WORDS Phytosterols, plant sterols, plasma cholesterol, low-density lipoprotein, LDL, high-density lipoprotein, HDL, sitostanol, humans, men, hyperlipidemia, tall oil

INTRODUCTION

Increasingly, dietary approaches to lowering heart disease risk are finding appeal over pharmacologic alternatives in the general population. One such approach has been to use naturally occurring plant sterols (phytosterols) as cholesterol-lowering adjuncts in foods (1–6). Sitostanol, the saturated derivative of the most common phytosterol, β-sitosterol, has successfully lowered circulating cholesterol concentrations in most human feeding trials. Decreases in total and LDL-cholesterol concentrations of 5–15% have been observed in studies lasting as long as 12 mo (7–16), although lack of efficacy has also been observed (17).

Although most studies using pure sitostanol or sitostanol ester have shown lowering of cholesterol in humans, results across studies show considerable variability that is likely due, in large part, to differences in study design and the method of administration of the phytosterol material (7–17). To date, no experiment has been conducted in which the sitostanol-containing phytosterols were administered over the 3 daily meals of a precisely controlled metabolic diet. How the addition of sitostanol-containing phytosterols alters plasma cholesterol concentrations in a dietary setting in which meal timing, composition, and quantity are rigorously maintained has not been established.

Both sitostanol and β-sitosterol are believed to reduce plasma cholesterol concentrations extrinsically by competitively blocking cholesterol absorption from the intestinal lumen (18, 19), displacing cholesterol from bile salt micelles (20), increasing bile salt excretion (21), or hindering the cholesterol esterification rate in the intestinal mucosa (22, 23). Additional intrinsic actions of phytosterols may include modification of hepatic acetyl-CoA carboxylase (24) and cholesterol 7-α hydroxylase enzyme activities (25) in animals and humans.

Whether the cholesterol-lowering ability of sitostanol-rich phytosterol mixtures influences cholesterogenesis has not been fully addressed. β-Sitosterol has been shown to lower plasma cholesterol concentrations while simultaneously stimulating (26, 27), inhibiting (28), or exerting (29) no effect on cholesterol synthesis in animals and humans. Previous reports of studies that examined the effect of phytosterols on cholesterogenesis in humans determined synthesis rates indirectly (14, 26, 30). However, none of those studies examined the effect of a precisely

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controlled, prudent North American diet with simultaneous administration of tall oil phytosterols on cholesterogenesis measured directly by the deuterium uptake method. Tall oil is the fat-soluble fraction of the hydrolysate obtained from trees during the pulping process.

Our objective was to examine whether tall oil phytosterol consumption alters lipoprotein-cholesterol concentrations and sterol metabolism in hyperlipidemic men when provided as a food supplement suspended in margarine. The hypothesis tested was that when sitostanol-containing phytosterols are provided to hyperlipidemic men over the 3 meals of a prudent, fixed-food North American diet, lipoprotein cholesterol profiles, plasma phytosterol concentrations, and de novo cholesterol synthesis rates will be different from when the diet is provided alone over 30 d.

SUBJECTS AND METHODS

Subjects

Thirty-two men (aged 25–60 y) with primary hypercholesterolemia were selected. Subjects were screened, after 12 h of fasting, for total cholesterol and triacylglycerol concentrations 1 and 2 wk before the start of the study. Criteria for acceptance were plasma total cholesterol concentrations between 6.5 and 10 mmol/L, total circulating triacylglycerol concentrations <3.5 mmol/L, and a body mass index (BMI; in kg/m^2) >18 or <37. Individuals who reported having diabetes, heart disease, or hypothyroidism or who had stopped using medication for hypercholesterolemia for <4 wk were excluded. Subjects were randomly assigned into the 2 treatment groups on the basis of plasma total cholesterol concentrations.

Protocol and diet

The study was a randomized, double-blind clinical trial. All subjects were provided for 30 d with a North American diet considered to be healthy in terms of macronutrient and fat content. The control diet group (n = 16) received a diet of prepared food alone; the phytosterol-enriched diet group (n = 16) received a diet with sitostanol-containing phytosterols [22 mg/kg body wt (1.5 g/70-kg man)] suspended in the margarine component of the diet. The diet was formulated to meet Canadian recommended nutrient intakes and to provide fat, fiber, and carbohydrate subcomponents consistent with recommendations of Health and Welfare Canada (31). Dietary protein, carbohydrate, and fat made up 15%, 50%, and 35% of ingested energy, respectively. The dietary fat was composed of 11%, 10%, and 14% of energy as saturated, polyunsaturated, and monounsaturated fats, with a blend of butter, corn oil, olive oil, and canola oil–based margarine.

Diets, fed in amounts determined to maintain body weight throughout the 30-d trial (32), were provided under supervision as 3 meals of equal energy each day. A 3-d rotating cycle was used, each cycle having similar macro- and micronutrient contents. Meals were prepared at the Clinical Nutrition Research Unit for consumption on site or, in certain cases, for takeout, as described previously (16). During meal preparation, foods were weighed precisely to the nearest 0.5 g. Subjects were instructed not to consume any foods or beverages other than those provided by the diet. Alcoholic and caffeinated beverage consumption was strictly prohibited over the course of the trial. Subjects were provided with decaffeinated, energy-free carbonated beverages to drink between meals.

Phytosterols were prepared from tall oil by solvent extraction and purification through repeated crystallization. Sitostanol made up ≈20% of the mixture by weight. The remaining phytosterols were mostly sitosterol and campesterol. The phytosterols were administered by suspending them in 30 g prewarmed margarine each day, providing a ratio of margarine to phytosterol of ≈20:1 (by wt). The 30 g margarine was divided equally among the 3 meals and mixed directly with food ingredients during preparation before cooking. When fluctuations in body weight occurred, adjustments to energy intakes were made during the initial 10-d period of the trial only. There were no changes to subjects' diets thereafter.

On day 29 of the trial, subjects were given orally 1.2 g D_2O (99.8% atom percent excess) per kg body weight at 0800. Deuterium uptake into cholesterol was measured over the following 24 h. Blood samples were collected just before and 24 h after deuterium dosing for red blood cell free cholesterol and water deuterium enrichment measurements.

Subjects underwent routine physical examinations and detailed blood chemistry analyses before and on day 30 of the study. A physician was on call continually throughout the trial for subjects to contact in case they experienced discomfort.

Analyses

Lipid and phytosterol analyses

Blood samples were collected from subjects before breakfast on days 0, 10, 20, 29, and 30 of the trial. Plasma was obtained after 20 min of centrifugation at 520 × g at 4°C. Plasma total, and LDL- and HDL-cholesterol and triacylglycerol concentrations were then determined. In addition, samples were collected from subjects on days 40 and 50, after the end of the diet.

Plasma total cholesterol, HDL-cholesterol, and triacylglycerol concentrations were measured in duplicate by using a VP autoanalyzer and commercial enzymatic kits (Abbott Laboratories, North Chicago, IL). HDL-cholesterol concentrations were measured in plasma after precipitation of apolipoprotein B lipoproteins with dextran sulfate and magnesium chloride (33). The concentration of LDL cholesterol was calculated according to the methods of Friedewald et al (34). CVs for replicate analyses of total cholesterol, HDL-cholesterol, and triacylglycerol concentrations were 2.74%, 6.53%, and 1.93%, respectively.

Plasma phytosterol concentrations were determined in duplicate by gas-liquid chromatography from the nonsaponifiable material of plasma lipid as reported previously (35). Briefly, 0.5-mL plasma samples were saponified with 0.5 mol methanolic KOH/L for 1 h at 100°C and the nonsaponifiable materials were extracted with petroleum ether. 5-α Cholestane was used as an internal standard. Samples were injected into a gas-liquid chromatograph equipped with a flame ionization detector (HP 5890 Series II; Hewlett Packard, Palo Alto, CA) and with a 30-m capillary column (SAC-5; Supelco, Bellefonte, PA). Detector and injector temperatures were 310° and 300°C, respectively. Duplicate samples were run isothermically at 285°C. Phytosterol peaks were identified by comparison with authenticated standards (Supelco).

De novo cholesterol synthesis determination

Cholesterol biosynthesis was determined as the rate of incorporation of deuterium from body water into free sterol over 24 h. Labeled water equilibrates quickly with intra- and extracellular water body pools and permits direct determination of choles-

TABLE 1
Mean and percentage changes in subjects' body weights between days 0 and 30 in the control and phytosterol-enriched diet groups[1]

	Control	Phytosterol
Body weight (kg)		
Day 0	79.8 ± 9.6	87.8 ± 15.2
Day 30	78.9 ± 6.5	86.7 ± 15.1
Body weight change (%)	−1.1 ± 1.1	−1.2 ± 1.4

[1] $\bar{x}$ ± SD; n = 16 men per group. There was no significant difference in body weight between groups on days 0 or 30.

terol synthesis rates (36). Deuterium enrichment was measured in red blood cell free cholesterol and plasma water as reported previously (37–39).

The fractional synthesis rate (FSR) of cholesterol was determined as incorporation of precursor deuterium into plasma total cholesterol relative to the maximum theoretic enrichment by using the linear regression model described previously (37, 39). The absolute synthesis rate (ASR = FSR × M_1 pool) was calculated according to the model of Goodman et al (40) as follows:

$$M_1 \text{ pool} = 0.287 \text{ wt (kg)} + 0.0358 \text{ plasma total cholesterol (mmol/L)} - 2.40 \text{ TGGP} \quad (1)$$

where TGGP is a variable that is equal to 1, 2, or 3 depending on the serum triacylglycerol concentration: <2.267, 2.267–3.401, or >3.401 mmol/L, respectively.

Statistical methods

Plasma lipid concentration data were evaluated by using a two-factor repeated- measures analysis of variance (ANOVA) procedure with tests for time and diet effects, and time-by-diet interactions. When the time-by-diet interaction was $P < 0.10$, repeated measures one-way ANOVA procedures were used. Wilk's lambda test was used to analyze time effects, whereas Student-Neuman-Keuls' post hoc tests were used to identify significant effects of diet at particular times (41). For total cholesterol, consistent with our initial hypothesis, percentage changes between days 0 and 30 were compared by using ANOVA. When main time effects were significant, a quadratic model was fitted to individual data of each treatment to determine whether the pattern of the decline was different. Slopes of the different diets were tested by using unpaired Student's t tests as were the effects of tall oil–derived phytosterols on FSR and ASR values. The relation between plasma total cholesterol concentrations, phytosterols, ASR, and FSR were determined by using Pearson product-moment correlation coefficients. The accepted level of significance was $P < 0.05$.

RESULTS

Thirty-three subjects started the feeding trial; 32 subjects completed the entire study. All subjects tolerated the experimental diet without any reported adverse effects. In addition, results of blood chemistry and urine tests were normal at the start of the diet period and throughout the duration of the trial. Screening checkups conducted at each 10-d time point of the trial suggested no clinical irregularities. Overall, subjects maintained excellent health throughout the duration of the experiment, except for one subject in the phytosterol group who reported diarrhea considered to be associated with a bout of influenza over the final 4 study days.

The sitostanol-containing phytosterol mixture was found to be inert in that subjects reported no particular abnormal or atypical smell, taste, aftertaste, or mouth-feel of meals during either diet. Subjects were not able to identify which diet they were consuming. There were no reported abnormalities of stool consistency or color, with the exception noted above.

Mean body weight, age, and BMI did not differ between control and phytosterol-enriched diet groups (Table 1). There was no significant change in the mean body weight of subjects across the 30-d feeding period. Seven study subjects had their energy intake altered by 10–20% over the first week of each trial. Three subjects either lost weight or reported lack of satiation, and thus their energy intake was increased. Four subjects reported feeling overfull and thus their energy intakes were reduced. In instances in which energy intakes were adjusted, relatively steady weight was achieved over the remainder of the period.

Mean total cholesterol concentrations over the 50-d study are shown in Figure 1. Mean plasma lipid profiles during the 30-d feeding period are presented in Table 2. Total cholesterol concentrations measured over the 30-d feeding period showed substantial variation in pattern between subjects. For the control diet group, the tertile (n = 5) of subjects showing the greatest response to diet had a mean 23% decline from day 0 to day 30 ($P < 0.05$), whereas the tertile (n = 5) with the least response showed an average 1% increase in total cholesterol concentrations (NS; data not shown). The variability in cholesterol concentrations during the phytosterol-enriched diet was similar to that observed during the control diet. The tertile of subjects showing the greatest response to diet had a mean decline in cholesterol concentrations of 31% ($P < 0.05$), whereas the tertile with the least response showed an average decline in total cholesterol concentrations of 6% (NS; data not shown). Variance in response was not associated with the initial circulating lipid concentration, change in body weight, or number of meals consumed away from the Clinical Nutrition Research Unit.

There was a significant main effect of time on total cholesterol concentration (Table 2). For effects of diet, with use of the two-factor ANOVA model, there was no interaction between

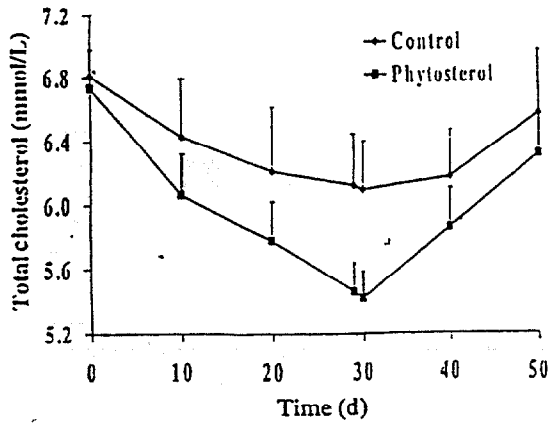

FIGURE 1. Effect of a phytosterol-enriched diet over time on mean (±SE) total cholesterol concentrations of hypercholesterolemic men.

TABLE 2
Plasma lipid concentrations in the control and phytosterol-enriched diet groups[1]

Plasma lipid and study day	Control	Phytosterol
	mmol/L	
Total cholesterol[2]		
Day 0	6.81 ± 1.30	6.73 ± 1.15
Day 10	6.43 ± 1.39	6.06 ± 1.05
Day 20	6.22 ± 1.60	5.78 ± 1.19
Day 30	6.10 ± 1.45	5.42 ± 0.92
LDL cholesterol[3]		
Day 0	5.00 ± 1.27	4.45 ± 1.37
Day 10	4.87 ± 1.30	3.94 ± 0.95[4,5]
Day 20	4.59 ± 1.42[6]	3.66 ± 0.84[4,5]
Day 30	4.56 ± 1.35[6]	3.37 ± 0.94[5,7]
HDL cholesterol		
Day 0	0.64 ± 0.18	0.75 ± 0.24
Day 10	0.64 ± 0.20	0.71 ± 0.26
Day 20	0.63 ± 0.20	0.71 ± 0.25
Day 30	0.60 ± 0.18	0.67 ± 0.18
Total triacylglycerols[8]		
Day 0	2.55 ± 1.20	3.33 ± 1.23
Day 10	2.00 ± 0.60	2.97 ± 1.09
Day 20	2.18 ± 0.90	3.08 ± 1.14
Day 30	2.02 ± 0.66	3.00 ± 1.60

[1] $\bar{x}$ ± SD; n = 16 men per group.
[2] Significant main effect of time, $P = 0.0001$.
[3] Significant main effects of diet, $P = 0.009$, and time, $P = 0.0001$.
[4,5] Significantly different from day 0 within study group: [4] $P < 0.01$, [5] $P < 0.05$. [7] $P < 0.001$.
[5] Significantly different from control, $P < 0.05$.
[8] Significant main effect of diet, $P = 0.013$.

time and dietary treatment for mean circulating total cholesterol concentrations, indicating no overall effect of diet. When total cholesterol concentrations were expressed as the difference between the mean of day 29 and 30 values compared with the concentration on day 0, a 10.4% decline was observed for the control diet. For the phytosterol-enriched diet, the decline was 19.5%. When a specific comparison was made between days 0 and 30 for total cholesterol concentrations, a significant difference was observed between diet groups ($P < 0.05$).

The individual mean total cholesterol data for days 0–30 with the control and phytosterol-enriched diets were fitted by using quadratic models. The quadratic term for total cholesterol with the control diet was significant ($r = 0.999$, $P = 0.012$), whereas that with the phytosterol-enriched diet was not ($P = 0.162$). The slope of the decline in total cholesterol during the phytosterol-enriched diet was linear ($r = 0.984$, $P = 0.001$).

The LDL-cholesterol concentrations over the 50-d study are shown in Figure 2. Despite dietary control, substantial variations in pattern between subjects were observed for LDL-cholesterol concentrations measured over the 30-d feeding period. With the control diet, the tertile ($n = 5$) of individuals showing the greatest response to diet had an average 24% decline ($P < 0.05$), whereas the tertile ($n = 5$) with the least response showed an average 4% drop in LDL-cholesterol concentrations (NS; data not shown). The variability was similar with the phytosterol-enriched diet. In the present study, the tertile of individuals showing the greatest response to diet had an average 37% decline ($P < 0.05$), whereas the tertile with the least response showed an average 8% drop in LDL-cholesterol concentrations (NS; data not shown).

Both the control and phytosterol-enriched diets caused a progressive decline in LDL-cholesterol concentrations over time, with a trend toward resumption of prediet concentrations over days 40 and 50 (Figure 2). Significant main effects of time and diet treatment were shown for LDL-cholesterol concentrations ($P < 0.05$; Table 2). A marginally significant ($P < 0.1$) interaction between time and dietary treatment was observed for LDL-cholesterol concentrations. LDL-cholesterol concentrations expressed as a percentage difference between the mean of days 29 and 30 compared with day 0 differed significantly ($P < 0.01$) between the control (8.9%) and phytosterol-enriched (24.4%) diets. On days 10, 20, and 30, mean plasma LDL-cholesterol concentrations were significantly lower ($P < 0.05$), by 8.7%, 9.0%, and 15.5%, respectively, in the group consuming the phytosterol-enriched diet compared with the control diet.

The quadratic terms of LDL-cholesterol curves for the control and phytosterol-enriched diets were not significant, but the slopes of the 2 dietary treatment lines were significantly different ($P = 0.041$, Student's unpaired t test). The decline in LDL-cholesterol concentrations with the phytosterol-enriched diet was steeper (−0.036 mmol/d, $r = 0.989$) than that with the control diet (−0.016 mmol/d, $r = 0.969$).

There was no significant difference at the start between the group mean HDL-cholesterol concentration in those consuming the phytosterol-enriched diet and those consuming the control diet, although the mean for the latter group was 12% lower than that of the phytosterol-enriched diet group (Table 2). Neither time nor diet showed significant effects on HDL-cholesterol concentrations in subjects over the duration of the trial.

For the plasma triacylglycerol concentration there was a significant main effect of diet but no time effect or time-by-diet interaction. The triacylglycerol concentration in the phytosterol-enriched diet group was initially higher than that in the control diet group and this difference was maintained throughout the experiment (Table 2).

Mean plasma campesterol and β-sitosterol concentrations in the control diet group did not vary across diet or time from those in the group given phytosterols (Table 3). Correction for variations in total cholesterol concentrations and expression of the values of β-sitosterol per mol cholesterol did not result in any significant diet or time effects or a time-by-diet interaction (Table 4). For the campesterol-cholesterol ratio, main effects of diet and time were significant, but there was no time-by-diet interaction (Table 4). The campesterol-cholesterol ratio in the phytosterol-enriched diet group was initially higher than that in the control group and this difference was maintained throughout the experiment.

The mean FSR did not differ significantly between the control (0.0911 ± 0.0280 pool/d; range: 0.0408–0.1420) and phytosterol-enriched (0.0914 ± 0.0250 pool/d; range: 0.0487–0.1320) diet groups. In addition, no significant difference was noted in the ASR between the control (0.613 ± 0.243 g/d; range: 0.200–1.10) and phytosterol-enriched (0.647 ± 0.234 g/d; range: 0.274–1.060) diet groups. Significant correlations between circulating phytosterol concentrations and various indexes such as total cholesterol, FSR, ASR, body weight, and BMI were observed. Plasma campesterol concentrations (day 30) correlated with total cholesterol concentrations in the control group ($r = 0.62$, $P = 0.011$), but not in the phytosterol-enriched diet

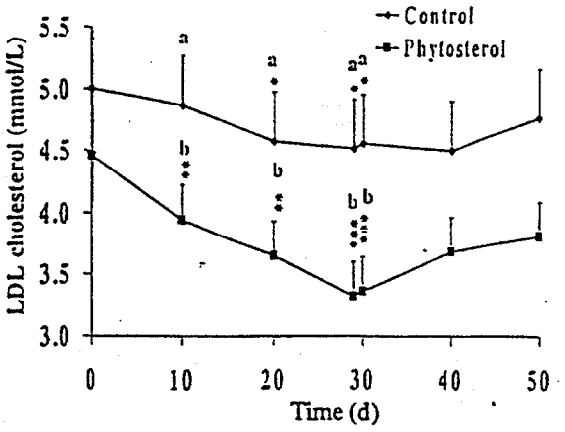

FIGURE 2. Effect of a phytosterol-enriched diet over time on mean (±SE) LDL-cholesterol concentrations of hypercholesterolemic men. *, **, ***Significantly different from day 0: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Between diets, time points with different letters are significantly different, $P < 0.05$.

group. Conversely, the correlation of percentage change in plasma campesterol concentrations with percentage change in total cholesterol concentrations was not significant across treatments. Circulating campesterol concentrations were negatively correlated with M_1 pool size on day 30 in the control ($r = -0.68$, $P = 0.004$) and phytosterol-enriched ($r = -0.48$, $P = 0.05$) diet groups. The FSR was negatively correlated with campesterol ($r = -0.4$, $P = 0.027$), β-sitosterol ($r = -0.39$, $P = 0.03$), the ratio of campesterol to cholesterol ($r = -0.37$, $P = 0.038$), and the ratio of β-sitosterol to cholesterol ($r = -0.34$, $P = 0.05$) concentrations. A negative correlation ($r = -0.36$, $P = 0.045$) was also found between the ASR and total cholesterol concentrations for pooled data of the 2 groups. Finally, BMI correlated with plasma total cholesterol ($r = 0.34$, $P = 0.04$), campesterol ($r = -0.41$, $P = 0.02$), and β-sitosterol ($r = -0.452$, $P = 0.009$) concentrations, and with the percentage change in campesterol on day 30 ($r = 0.425$, $P = 0.015$) in all subjects.

DISCUSSION

To our knowledge, this study is the first in which hypercholesterolemic subjects consumed precisely controlled diets enriched in phytosterols; previous studies used self-selected diets (5, 10, 11, 15, 17). Supplementation with 22 mg tall oil phytosterols•kg body $wt^{-1}•d^{-1}$ was effective in lowering circulating LDL-cholesterol concentrations in these subjects without changing either endogenous cholesterol synthesis or phytosterol concentrations. Although the reduction in LDL-cholesterol concentrations observed in this study agrees with that found by other investigators (9, 14), the observation of no change in cholesterogenesis does not agree with previous reports (26, 42).

The efficacy of phytosterols in lowering circulating lipid concentrations was shown previously for both unsaturated (1, 2, 5, 15) and saturated (8–16) phytosterols either esterified or nonesterified to fatty acids. Use of both pure unsaturated and saturated phytosterols has drawbacks, however. Unsaturated phytosterols must generally be consumed in amounts >4 g/d to be effective (1, 3, 4), although there are reports of efficacy with low doses of unsaturated phytosterols (5) or phytosterol esters (15). Similarly, sitostanol, although more effective than sitosterol in cholesterol-lowering ability, has limitations in that the material must be prepared chemically through hydrogenation from β-sitosterol, then modified to produce sitostanol ester.

In contrast, the present study showed the efficacy of a mixture of unsaturated and saturated phytosterols in lowering LDL cholesterol. After 30 d, our mixture decreased LDL-cholesterol concentrations by 15.5% over and above the action of diet alone. This degree of reduction was similar to that achieved with comparable doses of fully saturated stanol esters given over longer periods (10). When used in conjunction with a prudent North American diet, the extent of cholesterol lowering was approximately twice that attributable to phytosterols alone; LDL-cholesterol concentrations declined almost 25% in our hyperlipidemic subjects. Our mixture of phytosterols was obtained from tall oil without hydrogenation or chemical manipulation after extraction, and the tall oil starting material is available worldwide in abundant quantity.

Independent of the present observation of efficacy of this phytosterol mixture in reducing LDL-cholesterol concentrations is the observation that the full effect of this material is likely attained after more prolonged use. Data for LDL-cholesterol concentrations suggested a steeper decline over time in the group fed phytosterols than that in the control group. For total cholesterol concentrations, the curves differed in shape between the control and phytosterol-fed groups. Phytosterols may thus be acting on lipids in a manner that is mechanistically distinct from that of diet alone. The present data are consistent with those of Miettinen et al (10), who showed that only 80% of the eventual plateau in cholesterol lowering had been achieved at 60 d in mildly hypercholesterolemic subjects consuming self-regulated diets. The final plateau was obtained at about 6 mo (10). In both our study and that of Miettinen et al (10), discontinuation of phytosterols resulted in a rapid return of lipid concentrations to prestudy values (Figures 1 and 2).

Our results contrast with those of Denke (17), who provided 3 g unesterified sitostanol/d to hyperlipidemic subjects and found no significant effect on circulating lipid concentrations. The absence of action observed in the study by Denke may as likely have been a result of study design as of the biological inefficacy of free sitostanol. The present study differed from that of Denke in several ways. First, Denke's subjects were not consuming prepared diets fixed in composition, as in the present experiment. Second, sitostanol in the previous study was provided in capsules, not blended into the fat of each meal as in the present

TABLE 3
Plasma plant sterol concentrations in the control and phytosterol-enriched diet groups[1]

	Campesterol		β-Sitosterol	
Study day	Control	Phytosterol	Control	Phytosterol
		μmol/L		
0	21.2 ± 8.0	22.1 ± 9.7	7.5 ± 2.8	5.4 ± 2.4
10	22.8 ± 8.1	25.7 ± 9.3	7.2 ± 3.5	5.9 ± 2.0
20	21.5 ± 9.0	28.3 ± 17.9	6.4 ± 3.2	6.4 ± 2.9
30	26.4 ± 12.4	27.5 ± 11.7	6.1 ± 5.2	4.4 ± 1.8

[1] $\bar{x}$ ± SD; $n = 16$ men per group. There were no significant main effects of diet or time and no significant time-by-diet interactions.

TABLE 4
Plasma plant sterol concentrations according to cholesterol in the control and phytosterol-enriched diet groups[1]

	Campesterol[2]		β-Sitosterol	
Study day	Control	Phytosterol	Control	Phytosterol
	nmol/mol cholesterol			
0	3.03 ± 0.83	3.39 ± 1.65	1.07 ± 0.31	0.89 ± 0.40
10	3.58 ± 1.17	4.25 ± 1.38	1.09 ± 0.31	0.98 ± 0.31
20	3.44 ± 1.15	4.75 ± 2.29	1.00 ± 0.39*	1.14 ± 0.56
30	4.28 ± 1.68	5.18 ± 2.22	0.96 ± 0.62	0.85 ± 0.46

[1] $\bar{x}$ ± SD; n = 16 men per group.
[2] Significant main effects of diet (P = 0.049) and time (P = 0.0008).

study. Capsular phytosterols may not fully disperse or solubilize in the gut digesta before absorption, limiting their ability to reduce cholesterol absorption. Third, in the study by Denke, compliance was monitored by pill count, not by visual confirmation as in the present study. Thus, the previous study may have not completely confirmed compliance with phytosterol consumption. Also, distribution of the phytosterol intake across the 3 daily meals in the present study may have improved efficacy over more intermittent capsular administration.

The test diet alone produced a notable cholesterol-lowering effect without the addition of phytosterols. Although the diet's fat content was not exceptionally lower than that typically consumed by North Americans (43), several features may have been responsible for this improved lipid profile. First, the control diet was relatively high in mono- and polyunsaturated fats, which may have replaced the saturated fats typically found in subjects' habitual intakes. Second, this diet had a lower cholesterol content as a result of the unsaturated fat. Third, the diet was fed to avoid a positive energy balance, a metabolic state associated with increased circulating insulin concentrations and cholesterogenesis (44). In addition, lower circulating total cholesterol and LDL-cholesterol concentrations may have been due to elevated fiber intakes and no alcohol consumption. It is surprising that a distinct lipid-lowering effect was not observed in the control group of the longer-term sitostanol ester feeding study of Miettinen et al (10) because the rapeseed oil would have contributed unsaturated fats to the subjects' diets.

Plasma campesterol concentrations did not change during the study in either the control or phytosterol-enriched diet groups. Phytosterol concentrations were comparable with those reported previously in hypercholesterolemic subjects (10, 45, 46) and are consistent with results reporting that >8 times the normal intake (250–500 mg/d) of dietary β-sitosterol is required to substantially modify plasma sitosterol concentrations (21). Similarly, Lees et al (2) showed that daily administration of 3 g phytosterols, containing largely β-sitosterol, to hypercholesterolemic patients for 1 mo failed to increase plasma phytosterol concentrations.

Although plasma total and LDL-cholesterol concentrations declined with tall oil phytosterol feeding in this study, no compensatory increase in endogenous cholesterol synthesis was suggested by our data. A possible reason for our failure to detect differences in cholesterogenesis between groups was that cholesterol synthesis was compared on day 30 of the trial and not between days 0 and 30. Thus, the lack of response in the FSR and ASR of cholesterol between groups on day 30 may have been obscured by the effect of the control diet itself. Although the correlation between the ASR and plasma total cholesterol in all subjects suggests that endogenous cholesterol synthesis varies with plasma total cholesterol concentrations, this observed association provides insufficient evidence that cholesterol synthesis was different because of phytosterol supplementation.

In summary, the results of the present study show the efficacy of a widely available phytosterol mixture in lowering LDL cholesterol and altering the pattern of response in total cholesterol concentrations in hyperlipidemic men when provided in conjunction with a prudent diet. These changes in lipid profiles were similar in magnitude to those reported in a recent study in which subjects consumed ≈2 g wood- or vegetable-derived saturated stanol esters per day for 8 wk (47). We conclude that sitostanol-containing blends of unsaturated phytosterols have the potential to lower plasma lipid concentrations, which are a risk factor in the development of heart disease in susceptible populations.

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A Randomized, Double-Blind, Controlled Trial Examining the Lipid-Lowering Effects of Food Products Containing Free Tall Oil-Based Phytosterols and Oat β-Glucan

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ABSTRACT

This randomized, double-blind, controlled trial evaluated the influence of low-fat, low-saturated fat food products that contained free tall oil-based phytosterols (TOP) and oat β-glucan on serum lipid concentrations in adults with mild-to-moderate hypercholesterolemia. Following a 5-week National Cholesterol Education Program Step I diet lead-in period, 112 subjects incorporated one of two treatments into their diets for 6 weeks: food products (cereal, snack bar, and beverage) that provided 1.8 g TOP and 2.8 g β-glucan/day and contained ≤3.0 g total fat and ≤1.0 g saturated fat (TOP/β-glucan treatment) or similar control foods. The low-density lipoprotein cholesterol response from screening to the end-of-study was significantly larger in the TOP/β-glucan treatment than in the control (-7.6 vs. -0.6%; $p<0.001$). Likewise, total cholesterol decreased to a greater extent in the TOP/β-glucan treatment (-5.0 vs. -0.1% for control; $p<0.001$). High-density lipoprotein cholesterol and triglyceride responses did not differ between treatments. Results of this trial suggest that consumption of low-fat, TOP and β-glucan-containing foods is a useful adjunct in the dietary management of hypercholesterolemia.

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death in the United States (NHLBI 2000). Compelling evidence indicates that the risk of CHD can be decreased by reducing total and low-density lipoprotein (LDL) cholesterol concentrations (Downs 1998, Krauss 2000, Expert Panel 2001, Scandinavian Simvastatin Survival Group 1994). The National Cholesterol Education Program (NCEP) Step I diet, which emphasizes limiting saturated fatty acid and cholesterol intakes, has been shown to reduce LDL cholesterol by 3 to 10% (Davidson 1996, Bae 1991, Geil 1995, Kris-Etherton 1999, Ginsberg 1998, Walden 1997). Since a reduction by this amount is not always large enough to achieve desirable LDL cholesterol levels, an adjunct to diet therapy is often needed. The Adult Treatment Panel III of the NCEP recommends phytosterols and soluble fiber as adjunctive lipid-lowering therapies (Expert Panel 2001).

Regular consumption of plant sterols and/or stanols has been shown to significantly decrease total and LDL cholesterol concentrations (Weststrate 1998, Moghadasian 1999, Jones 1998, Hendricks 1999, Hallikainen 2000, Jones 1999, Jones 1998, Maki 2001, Davidson 2001). Plant sterols, also known as phytosterols, are structurally similar to cholesterol and compete with cholesterol for incorporation into micelles in the intestine. Usual levels of phytosterol consumption do not significantly affect cholesterol absorption. When consumed at high levels, however, plant sterols inhibit absorption of exogenous and endogenous cholesterol in the gastrointestinal tract (Ling 1995, Ikeda 1998).

Consumption of β-glucan has also been shown to reduce total and LDL cholesterol levels (Glore 1994, Davidson 1998, Davidson 1991, Ripsin 1992, Nicolosi 1999, Behall 1997). Beta-glucan is

the predominant soluble fiber in oat products. The mechanism by which β-glucan lowers cholesterol levels may be related to its viscosity, bile salt binding capacity, or fermentability (Davidson 1998, Marlett 1994). A meta-analysis of 19 clinical trials indicates that daily consumption of 3 g β-glucan can reduce total cholesterol levels by 5 to 6 mg/dL (Ripsin 1992).

There are no published studies to our knowledge that have examined the hypocholesterolemic effect of phytosterols and β-glucan when consumed together. The present study was conducted to evaluate the effect of a group of low-fat food products containing free tall oil-based phytosterols (TOP) and oat β-glucan on serum lipid levels in adults with mild-to-moderate hypercholesterolemia.

METHODS

This was a randomized, double-blind, controlled clinical trial conducted at the Chicago Center for Clinical Research, Chicago, IL. This study was performed according to Good Clinical Practice Guidelines, the Declaration of Helsinki (1996), and US 21 CFR Part 50 – Protection of Human Subjects, and Part 56 – Institutional Review Boards. An institutional review board (Schulman Associates IRB, Inc., Cincinnati, OH) approved the protocol prior to the initiation of the study. Study procedures were reviewed with subjects and each participant provided written informed consent before protocol-specific procedures were carried out.

Subjects

Potential participants (21 to 75 years of age) were recruited from the Chicago metropolitan area and pre-screened by telephone. Eligibility was further assessed at screening visits (weeks -6, -5, and -1). Subjects had to have LDL cholesterol between 130 and 200 mg/dL, triglycerides ≤350 mg/dL, and a body mass index (BMI) ≤38.0 kg/m^2. Participants also had to be in apparent good health, as indicated by a physical examination, an electrocardiogram, and serum chemistry, hematology, and urinalysis panels. Women of childbearing potential were required to have a negative urine pregnancy test and to use an approved method of contraception throughout the study.

Subjects were excluded if they had Type I, III, IV, or V secondary hyperlipoproteinemia. Use of hypolipidemic medication (including niacin or its analogues at doses of >400 mg/day) within four weeks of the week -6 visit was also exclusionary, as was the use of hypolipidemic supplements (including plant sterol, omega-3 fatty acid, and dietary fiber supplements) within

one week of the week -6 visit. Other exclusionary medications included drugs for regulating hemostasis (except for a stable dose of aspirin), hypoglycemic medications, systemic corticosteroids, androgens, phenytoin, erythromycin, and thyroid hormone (except stable-dose replacement therapy for ≥2 months prior to enrollment).

Poorly controlled hypertension (systolic blood pressure ≥160 mm Hg and/or diastolic blood pressure ≥100 mm Hg) was an exclusion criterion for this study, although subjects with adequately controlled hypertension were allowed to participate provided that their dose of anti-hypertensive therapy had remained constant for 2 months prior to the week -6 visit. The following were also exclusionary: diabetes mellitus or a fasting glucose ≥126 mg/dL at the week -5 visit; history of cancer within the previous 5 years (with the exception of non-melanoma skin cancer or basal cell carcinoma); current or recent history (within 6 months) of significant atherosclerotic, hepatic, gastrointestinal, pulmonary, endocrine, or renal disease; and a recent history (within the past 12 months) or a strong potential for substance abuse.

Clinic Visits

Subjects visited the clinic at weeks -6, -5, and -1 (screening), at week 0 (baseline), and at weeks 2, 5, and 6 (treatment) for assessments of vital signs, height (week -6 only) and weight, and a serum lipid profile [total, LDL, and high-density lipoprotein (HDL) cholesterol, and triglycerides]. Serum chemistry, hematology, and urinalysis panels were completed at week -5, and a urine pregnancy test (for women of childbearing potential) was performed at baseline (week 0). An electrocardiogram and a physical examination were performed at week -1. At week -6, subjects were instructed to follow their usual diets. At week -5, they were instructed on

the NCEP Step I diet. Compliance with this diet was reinforced at each subsequent visit. The Eating Pattern Assessment Tool (EPAT) (Peters 1994) was completed at weeks -6, 0, and 6, to aid in the assessment of dietary fat and saturated fat intakes during dietary counseling. Three-day diet records were dispensed at weeks -6, -1 and 5, and collected and analyzed at weeks -5, 0, and 6, respectively. Diet records were analyzed using the University of Minnesota Nutrition Data System for Research (NDS-R), version 4.03_31 software (2000).

Subjects were randomized at baseline (week 0) to one of two double-blind treatments: food products that provided 1.8 g TOP and 2.8 g β-glucan per day and contained ≤3.0 g total fat and ≤1.0 g saturated fat (TOP/β-glucan treatment) or similar food products that provided <1.0 g β-glucan and no TOP (control). Assessments of concomitant medication use and adverse events were performed at each treatment visit (weeks 2, 5, and 6).

Study Products

Study products included a group of low-fat food products that contained TOP and β-glucan, or a control group of low-fat food products that provided a low dose of β-glucan and did not contain TOP (Table 1). Food products for each treatment included a cereal, a snack bar, and a beverage. Subjects were instructed to consume one of each of these products daily, for a total of three doses of study product per day.

Each product in the TOP/β-glucan treatment was formulated to be identical in taste and appearance to the same type of product (cereal, snack bar, or beverage) in the control. Study products were also similar in nutrient composition, with the primary differences being the

phytosterol and β-glucan contents. The group of TOP/β-glucan products provided 1.8 g TOP and 2.8 g β-glucan per day. The group of control products provided <1.0 g β-glucan per day and no TOP. The source of β-glucan in the treatment cereal was whole grain rolled oats. The control cereal was primarily corn flakes with some whole grain rolled wheat and crisp rice. The β-glucan source in the treatment snack bar was whole grain rolled oats and oat bran concentrate. The control snack bar contained primarily crisp rice.

The free TOP used in the TOP/β-glucan food products was supplied by Reducol™ (Novartis Consumer Health SA, Nyon, Switzerland, produced by Forbes Meditech, Vancouver, Canada). Reducol™ has a composition of 44% sitosterol, 25% sitostanol, 12% campesterol, and 6% campestanol.

Laboratory Measurements

Medical Research Laboratories (Highland Heights, KY) performed laboratory measurements including urinalyses and serum chemistry, hematology, and lipid profiles. Serum chemistry analysis was conducted on the Hitachi 747 (Roche Diagnostics, Indianapolis, IN) and serum hematology testing utilized the Coulter STKS (Coulter Corporation, Miami, FL). Urinalyses were completed using the Clinitek Atlas (Bayer Diagnostics, Tarrytown, NY).

Serum lipids were analyzed according to the Centers for Disease Control and Prevention standardized fasting serum lipid profile (Myers 1989). Cholesterol and triglycerides were measured enzymatically using the Hitachi 747 (Roche Diagnostics, Indianapolis, IN). Heparin and manganese chloride were used to isolate HDL cholesterol. Low-density lipoprotein

cholesterol was calculated using the Friedewald equation (LDL cholesterol = total cholesterol – HDL cholesterol – triglycerides/5) (Friedewald 1972). Since this equation is not valid when the triglyceride concentration is above 400 mg/dL, LDL cholesterol was not calculated under these circumstances.

Statistical Analyses

Statistical analyses were conducted using the SAS version 8.0 statistical analysis package (SAS Institute, Cary, NC). Analyses are presented for an intent-to-treat sample. This sample includes all subjects who completed at least one post-randomization assessment of lipids after receiving at least one dose of study product. The last observations were carried forward to impute missing values.

Baseline comparability of treatments for demographic, anthropometric, and lipid values was assessed by analysis of variance (ANOVA), chi-square tests, or other techniques as appropriate. Parametric and non-parametric analyses were used as required to compare treatments for responses. Analysis of variance models were generated to compare responses between groups for the following variables: LDL, total, and HDL cholesterol; triglycerides; and the ratio of LDL cholesterol to HDL cholesterol. For these analyses, screening, baseline, and end-of-study values were defined as averages of values collected at weeks -5 and -6, weeks -1 and 0, and weeks 5 and 6, respectively. Differences between treatments in dietary intake were also assessed by ANOVA. Possible differences in the incidence of adverse events were measured with Fisher's exact (2-tail) test. All tests for significance were performed at α=0.05, two-tailed.

Post hoc analyses were performed to assess the possible impact of the following factors on lipid responses to treatment: median baseline LDL cholesterol level (≤154.5 vs. >154.5 mg/dL), median screening LDL cholesterol concentration (≤155.8 vs. >155.8 mg/dL), the percentage of time that a fat source was consumed with the study product, and the median percentage of time that a fat source was consumed with the study product (≤35.4 vs. >35.4%).

RESULTS

Subjects and Demographics

Two-hundred and sixty-eight people were screened to identify the 112 subjects randomized. Seven (6.3%) of the randomized subjects dropped out of the study prior to completing the intervention period. Reasons reported for discontinuation of subjects in the TOP/β-glucan treatment included non-compliance with the protocol (n=1) and withdrawal of consent (n=1). Subjects who dropped out of the control did so due to non-compliance with the protocol (n=1), withdrawal of consent (n=2), and adverse events [rash on arm and groin, abdominal pain in lower left quadrant (n=1); angioedema, mouth swelling (n=1)] judged by the Investigator to be possibly or probably related to the study product.

Demographic and baseline characteristics of subjects are shown in Table 2. Subjects in the TOP/β-glucan treatment were significantly older than those in the control (59.3 ± 1.4 vs. 55.2 ± 1.1 years, respectively; p=0.028). There were no significant differences between treatments in sex, race, height, weight, BMI, or blood pressure. The approximate mean height and weight of subjects were 169 cm and 80 kg, respectively. Subjects had a mean BMI of ~28 kg/m^2. Fifty-one percent of subjects in the TOP/β-glucan treatment were female as were 62% in the control. The majority of subjects (~75%) were Non-Hispanic White.

Mean compliance with study product consumption for all three food products exceeded 98% for subjects in the TOP/β-glucan treatment and averaged 95% in the control. There were no significant differences between treatments in mean overall compliance with study product intake or for consumption of the cereal, snack bar, or beverage independently.

Dietary Analyses

Results of three-day diet record analyses at screening, baseline, and the end-of-study are presented in Table 3. Study product intake was included in these analyses. Reported intakes of energy, carbohydrate, fat, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol were not significantly different between treatments at screening, baseline, or the end-of-study. The percent of energy from protein at screening was greater in the TOP/β-glucan treatment than in the control (17.9 ± 0.5 vs. 16.4 ± 0.6%; p=0.026). Compared to the control, dietary fiber intake in the TOP/β-glucan treatment was greater at the end-of-study (16.8 ± 1.0 vs. 21.6 ± 1.1 g; p=0.003). Likewise, reported consumption of soluble fiber at the end-of-study was greater in the TOP/β-glucan treatment (7.4 ± 0.4 g) than in the control (5.1 ± 0.3 g; p<0.001).

Lipids

There were no significant differences between treatments at screening or baseline in total, LDL, or HDL cholesterol concentrations; triglyceride levels; or the LDL/HDL cholesterol ratio. Mean LDL cholesterol levels at screening, baseline, and the end-of-study are presented in Figure 1. Subjects in both treatments entered the study with a mean LDL cholesterol level of ~160 mg/dL. Mean LDL cholesterol concentration at the end-of-study was 6.3% lower in the TOP/β-glucan treatment than in the control (147.8 ± 2.2 vs. 158.0 ± 3.0 mg/dL; p=0.007).

Lipid concentrations at screening, percent changes from screening to baseline, and percent changes from screening to the end-of-study are presented in Table 4. There were no significant

differences between treatments in the percent change from screening to baseline for any of the lipid parameters. The LDL cholesterol response from screening to the end-of-study was significantly larger for subjects in the TOP/β-glucan treatment (-7.6 ± 1.1%) than for those in the control (-0.6 ± 1.3%; $p<0.001$). Likewise, the percent change from screening to the end-of-study for total cholesterol was significantly greater for subjects in the TOP/β-glucan treatment (-5.0 ± 1.0%) than for those in the control (-0.1 ± 1.0%; $p<0.001$). There was also a significant difference between treatments in the percent change from screening to the end-of-study for the LDL/HDL cholesterol ratio (-4.15 ± 1.23 vs. 4.26 ± 1.76 for TOP/β-glucan and control, respectively; $p<0.001$). There were no significant differences between treatments in the percent changes from screening to the end-of-study for HDL cholesterol or triglycerides.

The LDL cholesterol response from baseline to the end-of-study was significantly larger in the TOP/β-glucan treatment (-3.7 ± 1.2%) than in the control (0.40 ± 1.5%; $p=0.013$). Likewise, the percent change from baseline for total cholesterol was greater in the TOP/β-glucan treatment (-2.3 ± 1.0 vs. 0.8 ± 1.1% for control; $p=0.043$). In addition, the LDL/HDL cholesterol ratio decreased from baseline by 1.8 ± 1.3% in the TOP/β-glucan treatment and increased by 4.8 ± 1.7% in the control ($p=0.003$). There were no significant differences between treatments in the percent changes from baseline to the end-of-study for HDL cholesterol or triglycerides.

Result of *post hoc* analyses did not provide evidence for significant interactions between treatment response and the following: median baseline LDL cholesterol level, median screening LDL cholesterol level, the percentage of time that a fat source was consumed with the study

product, and the median percentage of time that a fat source was consumed with the study product. For this reason, these results are not reported.

Safety

With the exception of an increased incidence of rhinitis in the TOP/β-glucan treatment (17.5% vs. 7.3% for control; p=0.026), there were no other statistically or clinically important differences between treatments in the incidence of adverse events overall or for any body system. All reported cases of rhinitis in the TOP/β-glucan treatment were considered mild in severity and judged by the Investigator to be unrelated to the study product.

DISCUSSION

Results of this randomized, double-blind, controlled trial demonstrate that subjects with mild-to-moderate hypercholesterolemia can reduce their LDL and total cholesterol levels by incorporating a group of TOP and β-glucan-containing foods into a low-fat diet. In this study, all subjects followed a NCEP Step I diet. Fasting lipid levels of those who consumed a group of low-fat, low-saturated fat food products that provided 1.8 g TOP and 2.8 g β-glucan per day were compared to those of subjects who consumed similar food products that provided <1.0 g β-glucan per day and no TOP. Mean LDL and total cholesterol levels at the start of the study were essentially identical for each treatment. At the end of the study, there was a 6.3% difference between treatments in LDL cholesterol concentrations and a 5.4% difference in total cholesterol levels, both in favor of the TOP/β-glucan treatment.

A lipid-lowering effect of the TOP and β-glucan-containing food products was found despite the low dietary cholesterol intakes of subjects at the end of this study (~213 vs. ~264 mg at screening for both treatments). A diet low in cholesterol reduces the amount of cholesterol available in the gastrointestinal tract (Lichtenstein 1990). Since both phytosterols and β-glucan inhibit cholesterol absorption in the intestine (Ling 1995, Ikeda 1998, Davidson 1998), it is likely that the reduction in the amount of cholesterol available resulted in a decreased effect of the TOP and β-glucan-containing food products. Even so, significant differences between treatments in LDL and total cholesterol levels were still demonstrated.

There is substantial evidence suggesting that sterol/stanol products and β-glucan-containing products are effective lipid-lowering agents (Weststrate 1998, Moghadasian 1999, Jones 1998,

Hendricks 1999, Hallikainen 2000, Jones 1999, Jones 1998, Maki 2001, Davidson 2001, Glore 1994, Davidson 1998, Davidson 1991, Ripsin 1992, Nicolosi 1999, Behall 1997). For this reason, we expected that our TOP and β-glucan-containing food products would reduce LDL and total cholesterol levels. Whether or not TOP and β-glucan had additive cholesterol-lowering effects in the present study is not known. The purpose of this study was not to determine the extent to which the individual components of treatment would affect serum lipid concentrations; rather, the purpose was to determine the lipid-altering effects of a specific group of food products that contain both TOP and β-glucan. Further research would be needed to determine the degree to which each treatment component affected LDL and total cholesterol levels.

Roughly one-half of adults in the United States have elevated total cholesterol levels (Sempos 1993). Based on the NCEP ATP II guidelines (population-based data are not available for the ATP III guidelines), each 5% reduction in LDL cholesterol in the population would decrease the number of people who qualify for drug therapy by 5 to 7 million (Sempos 1993, Expert Panel 1993). Therefore, the 6.3% LDL cholesterol reduction produced by the TOP and β-glucan-containing food products in this study has important public health implications.

The NCEP ATP III guidelines encourage the use of phytosterols and soluble fiber as therapeutic options to enhance LDL cholesterol-lowering (Expert Panel 2001). Therefore, it makes sense to identify specific food products that can be used as adjuncts to a low-fat diet. In the current study, the addition of the TOP and β-glucan-containing foods to the NCEP Step I diet doubled the lipid-lowering effect that resulted from the implementation of the low-fat diet alone. During the diet lead-in phase, LDL cholesterol levels in the TOP/β-glucan treatment decreased by 4.0%, from

160.5 mg/dL at screening to 154.1 mg/dL at baseline. When the TOP and β-glucan-containing products were added to the low-fat diet, LDL cholesterol was reduced by an additional 4.0% (from 154.1 to 147.8 mg/dL).

As the food industry develops products for adjunctive cholesterol-lowering therapies, it is important that they keep the needs of consumers in focus. People are unlikely to consider a product for long-term use unless it tastes good and is practical to consume. The group of TOP and β-glucan-containing foods used in the present study included a cereal, a snack bar, and a beverage. Compliance with consumption of these products was at least 95%, suggesting that the taste of these products was acceptable and that they were easy to incorporate into a low-fat diet. Therefore, these products can be recommended to the public with the expectation that people will consider them for long-term use.

In conclusion, the results of the present study provide evidence that consumption of a group of low-fat, TOP and β-glucan-containing foods is a useful adjunct in the dietary management of hypercholesterolemia.

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Table 1. Nutrient Compositions of the TOP/β-Glucan and Control Food Products[1]

	TOP/β-Glucan Food Products				Control Food Products			
Dietary Component	**Snack Bar**	**Cereal**	**Beverage**	**Total**	**Snack Bar**	**Cereal**	**Beverage**	**Total**
Energy, kcal	150.0	200.0	170.0	520.0	150.0	200.0	170.0	520.0
CHO, g	29.0	42.0	43.0	114.0	32.0	47.0	43.0	122.0
Protein, g	3.0	5.0	0.0	8.0	2.0	3.0	0.0	5.0
Total Fat, g	3.0	3.0	0.0	6.0	2.5	1.0	0.0	3.5
SFA, g	0.5	1.0	0.0	1.5	0.5	1.0	0.0	1.5
PUFA, g	0.5	0.5	0.0	1.0	0.0	0.0	0.0	0.0
MUFA, g	2.0	0.5	0.0	2.5	1.5	0.0	0.0	1.5
Cholesterol, mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TOP[2], g	0.6	0.6	0.6	1.8	0.0	0.0	0.0	0.0
β-glucan, g	1.3	1.5	0.0	2.8	0.3	0.5	0.0	0.8
Dietary Fiber, g	3.0	4.0	0.0	7.0	0.0	2.0	0.0	2.0
Sodium, mg	105.0	260.0	10.0	375.0	180.0	410.0	10.0	600.0

[1]Nutrient information is given for one serving. One serving is equal to a 40 g snack bar, a 10 fluid oz beverage, or a 53 g serving of cereal. Subjects in each treatment consumed one serving of each of the three products per day.

[2]Free TOP supplied by Reducol™ (44% sitosterol, 25% sitostanol, 12% campesterol, and 6% campestanol).

Abbreviations: TOP = tall oil-based phytosterols, CHO = carbohydrate, SFA = saturated fatty acids, PUFA = polyunsaturated fatty acids, MUFA = monounsaturated fatty acids.

Table 2. Demographic and Baseline Characteristics of Subjects

Parameter	TOP/β-Glucan Treatment	Control
Sex, n (%)		
Male	28 (49)	21 (38)
Female	29 (51)	34 (62)
Race, n (%)		
Non-Hispanic White	45 (79)	40 (73)
Other	12 (21)	15 (27)
Age, years, Mean ± SEM	59.3 ± 1.4*	55.2 ± 1.1
Height, cm, Mean ± SEM	169.7 ± 1.5	169.4 ± 1.2
Weight, kg, Mean ± SEM	79.4 ± 1.9	80.9 ± 2.0
BMI, kg/m^2, Mean ± SEM	27.5 ± 0.6	28.1 ± 0.6
Systolic BP, mm Hg, Mean ± SEM	122.8 ± 2.1	120.2 ± 1.9
Diastolic BP, mm Hg, Mean ± SEM	76.7 ± 1.2	76.0 ± 1.0

*p=0.028 vs. control

Abbreviations: TOP = tall oil-based phytosterols, SEM = standard error of the mean, BMI = body mass index, BP = blood pressure.

Table 3. Diet Record Analyses at Screening, Baseline, and End-of-Study, According to Treatment Assignment[1]

	TOP/β-Glucan Treatment			Control		
	Mean ± SEM			Mean ± SEM		
Dietary Variable	**Screening (Week -5)**	**Baseline (Week 0)**	**End-of-Study (Week 6)**	**Screening (Week -5)**	**Baseline (Week 0)**	**End-of-Study (Week 6)**
Energy, kcal	1913.7 ± 112.1	1805.7 ± 63.7	1959.3 ± 74.6	1873.5 ± 93.6	1756.6 ± 91.6	2015.5 ± 86.0
Total CHO, % energy	50.5 ± 1.4	51.9 ± 1.4	60.0 ± 1.3	51.1 ± 1.3	51.6 ± 1.3	59.5 ± 1.0
Protein, % energy	17.9 ± 0.5*	17.3 ± 0.5	15.2 ± 0.4	16.4 ± 0.6	16.9 ± 0.6	14.8 ± 0.6
Total Fat, % energy	31.6 ± 1.2	30.5 ± 1.1	25.8 ± 1.0	32.5 ± 1.1	31.8 ± 1.0	27.0 ± 0.9
SFA, % energy	9.8 ± 0.5	9.2 ± 0.4	8.3 ± 0.4	10.8 ± 0.5	10.3 ± 0.6	9.2 ± 0.4
PUFA, % energy	6.5 ± 0.3	6.6 ± 0.3	5.5 ± 0.3	6.4 ± 0.3	6.7 ± 0.3	5.7 ± 0.3
MUFA, % energy	12.5 ± 0.5	12.0 ± 0.5	9.7 ± 0.4	12.6 ± 0.5	12.2 ± 0.5	9.9 ± 0.4
Cholesterol, mg	262.0 ± 21.5	230.6 ± 18.6	216.0 ± 18.7	265.3 ± 24.0	234.2 ± 19.1	210.0 ± 21.7
Dietary Fiber, g	20.2 ± 1.2	19.2 ± 1.1	21.6 ± 1.1*	18.0 ± 1.3	18.5 ± 1.4	16.8 ± 1.0
Soluble Fiber, g	6.7 ± 0.4	6.7 ± 0.4	7.4 ± 0.4*	6.2 ± 0.4	6.4 ± 0.4	5.1 ± 0.3

[1]Reported study product intake was included in the analysis.

*$p<0.05$ vs. control at this visit (screening, baseline, or end-of-study)

Abbreviations: TOP = tall oil-based phytosterols, SFA = saturated fatty acids, PUFA = polyunsaturated fatty acids, MUFA = monounsaturated fatty acids, CHO = carbohydrate.

Table 4. Lipid Concentrations at Screening, Percent Changes (Δ) from Screening to Baseline, and Percent Changes from Screening to End-of-Study, According to Treatment Assignment[1,2]

Lipid Parameters & Week of Study	TOP/β-Glucan Treatment (n = 57)	Control (n = 55)
LDL Cholesterol (mg/dL)		
Screening	160.5 ± 2.3	159.6 ± 2.9
% Δ from screening to baseline	-3.7 ± 1.0	-0.3 ± 1.5
% Δ from screening to end-of-study	-7.6 ± 1.1*	-0.6 ± 1.3
Total Cholesterol (mg/dL)		
Screening	242.0 ± 3.2	243.2 ± 3.5
% Δ from screening to baseline	-2.6 ± 0.8	-0.5 ± 1.1
% Δ from screening to end-of-study	-5.0 ± 1.0*	-0.1 ± 1.0
HDL Cholesterol (mg/dL)		
Screening	54.0 ± 1.7	56.0 ± 1.9
% Δ from screening to baseline	-1.4 ± 1.0	0.0 ± 1.0
% Δ from screening to end-of-study	-3.2 ± 1.2	-3.9 ± 1.3
Triglycerides (mg/dL)		
Screening	133.5 (53.0, 240.5)	125.5 (46.5, 258.5)
% Δ from screening to baseline	2.3 (-36.4, 51.6)	-1.1 (-45.7, 61.6)
% Δ from screening to end-of-study	6.1 (-37.8, 74.5)	11.1 (-31.2, 116.9)
LDL-C/HDL-C		
Screening	3.14 ± 0.10	3.03 ± 0.11
% Δ from screening to baseline	-1.94 ± 1.26	0.15 ± 1.61
% Δ from screening to end-of-study	-4.15 ± 1.23*	4.26 ± 1.76

[1]Mean ± SEM for all lipid parameters except triglycerides. Triglycerides are median (minimum, maximum).

[2]Screening = average of values at weeks -6 and -5, baseline = average of values at weeks -1 and 0, end-of-study = average of values at weeks 5 and 6.

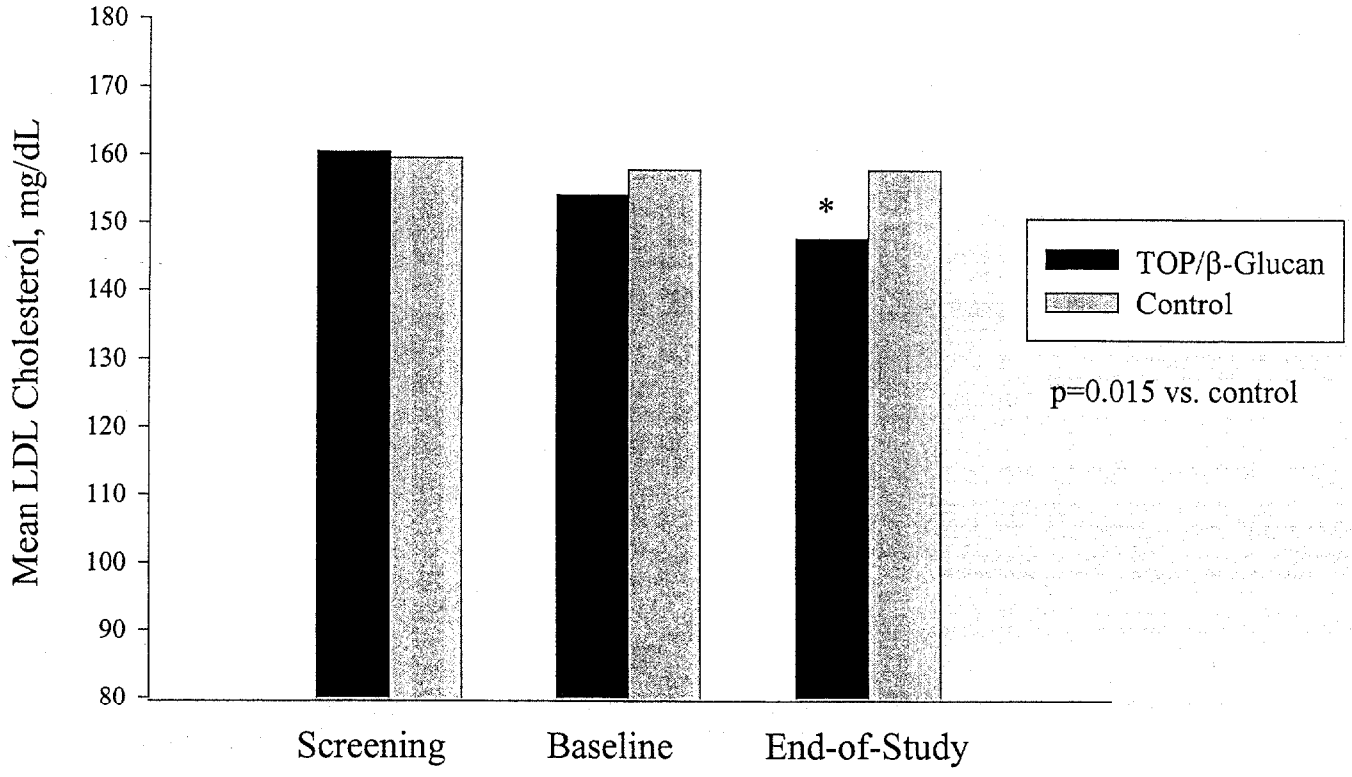
*$p<0.001$ vs. control

Figure Legend

Figure 1. LDL cholesterol levels at screening, baseline, and end-of-study, according to treatment assignment. Screening is the average of values at weeks -6 and -5, baseline is the average of values weeks -1 and 0, and end-of-study is the average of values at weeks 5 and 6.

Abbreviations: LDL = low-density lipoprotein, TOP = tall oil-based phytosterols.

Figure 1.


Mean LDL Cholesterol, mg/dL
180
170
160
150
140
130
120
110
100
90
80
*
Screening
Baseline
End-of-Study
TOP/β-Glucan
Control
p=0.015 vs. control

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Review

Oat Products and Lipid Lowering

A Meta-analysis

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Objectives.—To test the a priori hypothesis that consumption of oats will lower the blood total cholesterol level and to assess modifiers and confounders of this association.

Data Sources.—A computerized literature (MEDLINE) search and the Quaker Oats Co identified published and unpublished trials as of March 1991. Raw data were requested for all trials.

Study Selection.—Trials were included in summary effect size estimates if they were randomized and controlled, if a formal assessment of diet and body weight changes occurred, and, if raw data were not received, if there was enough information in the published report to perform calculations.

Data Synthesis.—Twenty trials were identified. Using the methods of DerSimonian and Laird, a summary effect size for change in blood total cholesterol level of −0.13 mmol/L (−5.9 mg/dL) (95% confidence interval [CI], −0.19 to −0.017 mmol/L [−8.4 to −3.3 mg/dL]) was calculated for the 10 trials meeting the inclusion criteria. The summary effect size for trials using wheat control groups was −0.11 mmol/L (−4.4 mg/dL) (95% CI, −0.21 to −0.01 mmol/L [−8.3 to −0.38 mg/dL]). Calculation of Keys scores demonstrated that substituting carbohydrates for dietary fats and cholesterol did not account for the majority of blood cholesterol reduction. Larger reductions were seen in trials in which subjects had initially higher blood cholesterol levels (≥5.9 mmol/L [≥229 mg/dL]), particularly when a dose of 3 g or more of soluble fiber was employed.

Conclusion.—This analysis supports the hypothesis that incorporating oat products into the diet causes a modest reduction in blood cholesterol level.

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IN 1963, DeGroot and colleagues[1] were the first to report that the addition of an oat product to the diet of humans resulted in lowered blood cholesterol levels. In that trial, 21 male volunteers substituted for the usual bread in their diet bread containing 140 g of oatmeal. At the end of 3 weeks, the blood total cholesterol level was reduced 11%. Since that time a substantial amount of research has accumulated; both metabolic ward studies and trials of free-living subjects have been conducted in an attempt to determine whether oats do have an effect on blood lipid levels.

Anderson and colleagues[2-5] have published the results of several uncontrolled metabolic ward trials and have demonstrated total cholesterol reductions from 13% to 26%. They have also published a controlled metabolic ward study comparing oat bran with wheat bran and demonstrated a net total cholesterol reduction of 9% for the oat bran group.[6] Although many metabolic ward studies have shown rather impressive lipid reductions,[1,3-6] trials of free-living subjects have reported considerably more variability in lipid response.[7-23] A few have demonstrated virtually no benefit,[12,18] while others have shown reductions greater than 10%.[11]

At least some of the variability can be accounted for by differences in study subjects and protocols. Various oat preparations have been used, including cereals, muffins, breads, and entrees. Some trials have employed oat bran as the intervention while others have used oatmeal, and doses have differed from trial to trial. Some have enrolled an all-male cohort of subjects and others have used various combinations of younger and older men and women. The initial serum cholesterol level of subjects also has varied from trial to trial, with some including normocholesterolemic subjects and others enrolling only those with hypercholesterolemia. With all of the differences between trials, it is difficult, by qualitative inspection alone, to sift through the information and understand the factors that might account for the variability of the serum lipid response to oats.

In a recent oat bran trial completed by Keenan et al,[10] post hoc analysis of the data revealed that subjects' lipid responses appeared to vary by age and gender. Young women had virtually no response to oat bran, while older women showed a marked drop in cholesterol level (−0.37 mmol/L [−14.5 mg/dL]); younger and older men had intermediate responses. It is known that lipid levels differ according to gender and menopausal status, and there is evidence that suggests that bowel transit times differ according to gender and menopausal status as well.[24] In addition, at least three oat product trials have demonstrated a relation-

ship between the initial blood cholesterol levels of subjects and subsequent reduction in cholesterol level attributed to the oat product.[8-10] Therefore, it is plausible to suggest that age and gender as well as initial cholesterol level could be playing a role in the variability of lipid response. This meta-analysis formally summarizes the oat product literature of clinical trials of free-living subjects, with attention to whether blood cholesterol response varies by age, gender, dose and/or initial blood cholesterol level.

METHODS

Design of the Meta-analysis

A computerized literature (MEDLINE) search was conducted to identify all published oat product trials as of March 1991. In addition, a list of all known investigators of the lipid-oats association (regardless of funding source) was supplied by the Quaker Oats Co (Barrington, Ill). From this list unpublished trials were solicited and, when possible, included to maximize the database and reduce the possibility of publication bias. Letters were sent to all investigators, describing the proposal and inviting collaboration in the effort. Collaborators were requested to provide certain aspects of their raw data (lipid values, some dietary variables, body weight, age, and gender) as well as thorough descriptions of their study protocols. The response was very positive; of the 20 trials initially identified (12 published, five abstracts, three unpublished), raw data were received for 14 trials, for a response rate of 70%. For one of the three unpublished trials, no information could be obtained and the investigator declined to collaborate, so 19 trials were reviewed for this meta-analysis. The following are the a priori hypotheses:

- Oat products act as a lipid-lowering agent in human subjects, reducing the blood total cholesterol level and its low-density lipoprotein (LDL) component.
- A dose-response relationship exists between the amount of oat product consumed and the degree to which blood lipid levels are reduced.
- Subjects with high initial total cholesterol levels will demonstrate greater reductions in total cholesterol levels than will those with lower initial total cholesterol levels.
- The variables age and gender modify the response of total cholesterol levels—older women will demonstrate a greater reduction than all other age-gender subgroups.

It was necessary to receive the raw data from each trial to test the last hypothesis, since most published reports did not provide an age-gender breakdown of results. In oat product trials that measured changes in lipoprotein fractions, LDL was the fraction of total cholesterol shown to be most affected by oat products; changes in high-density lipoprotein and triglyceride levels were minimal or absent.[7-12,23,25] Although LDL would be the preferred lipid variable for use in determining intervention effects, not all the trials measured LDL levels; therefore, the total cholesterol level was used as the lipid variable of primary interest.

All 19 trials were reviewed and summarized. However, to be included in the primary calculation of the summary effect size, trials needed to meet the following a priori criteria:

- Trials had to have been controlled and randomized. Without a control group, there is no way to estimate any change in blood lipid levels that is occurring independent of the effect of the intervention.
- If a comparison product was used, it had to have been one with very low or no soluble fiber (eg, wheat bran).
- If a trial tested the intervention against a special background diet (eg, a low-fat, low-cholesterol diet), there had to have been a sufficient lead-in period (a minimum of 4 weeks for change to a low-fat, low-cholesterol diet[26]) so that the effects of the special diet on the change in blood lipid levels during the oat product intervention could reasonably be assumed to be negligible.
- All trials had to have made a formal assessment of dietary behavior and body weight changes in treated and control subjects.
- If investigators did not submit their raw data for analysis, the published report had to have an adequate description of design and the necessary information to calculate the appropriate effect size and associated SE.
- Crossover trials were analyzed in the primary analysis as parallel design trials when the raw data were available, using the information from just the first phase. This was done to avoid any problems with order effects (ie, group-by-phase interaction).

Because these criteria are somewhat subjective, the summary effect size was recalculated in several ways that allowed for the inclusion of trials without a lead-in diet phase and for including the results (by pooling) from both phases of crossover trials. The results were then compared.

To determine whether using the results of one treatment group to create multiple effect sizes by comparison with multiple control groups (from the same trial) had artificially decreased the amount of heterogeneity of the assembled effect sizes, the summary effect size was calculated using the information from multiple comparisons and then again after eliminating duplicate use of the same treatment group from Keenan et al (excluding the American Heart Association step 1 [AHA-1] diet-only comparison),[10] Beling (unpublished results, 1991, excluding the comparison with the no-diet group), and O'Brien et al (excluding the special diet only comparison),[16] and the results were compared.

When sufficient dietary data were included in the published report, Keys scores were calculated to determine whether the reduction in total cholesterol level could be attributed to dietary changes other than the inclusion of oats in the diet. Keys scores are calculated using the changes in saturated fatty acids, polyunsaturated fatty acids, dietary cholesterol, and energy intake to determine whether the diet has changed from preintervention to postintervention and to determine the degree to which any dietary change could have affected the blood total cholesterol level.[26,27] For the subset of trials for which Keys scores could be calculated, an adjusted individual effect size was computed for each trial by subtracting expected (estimated by Keys scores) from observed reductions in the total cholesterol level. A new summary effect size was then calculated by substituting the adjusted individual effect sizes into the DerSimonian and Laird[28] calculations described below.

The preliminary cholesterol level is the mean total cholesterol level in the oat-treated group just prior to the intervention phase. For trials in which a lead-in diet period preceded the intervention phase, the preliminary total cholesterol level is the value at the end of the lead-in period.

Soluble fiber (in grams) is used as the variable to represent dose when evaluating the existence of a dose-response relationship between oats and lipid reduction.

To assess the presence of age-gender interaction, only those trials that had enrolled subjects of both older and younger ages as well as men and women are included. The dichotomous age variable (<50 vs ≥ 50 years) is constructed as in the oat product trial that reported the age-gender interaction.[10] As such, it is a surrogate variable for menopausal status in women. Individual effect sizes are created for each age-gender subgroup within a trial (ie, each trial yielded four effect sizes), and linear regression methods were used to determine whether age and gender or their interaction could predict effect size. As a second look at the age-gender hypothesis, individual regression models were run on each trial for which raw data were available.

Statistical Methods

To calculate the net mean change in total cholesterol level (individual study ef-

fect size) during the intervention phase of each trial, the mean change in total cholesterol level for the control subjects was subtracted from the mean change for the treated subjects. A negative effect size indicates a reduction during the intervention phase. The variance was calculated as follows:

$$V(ES)=s^2[(1/n_{treated})+(1/n_{control})],$$

where V is the variance of the effect size (ES); s, the pooled SD of the change in total cholesterol level for the treated and control groups; and n, the sample size.

DerSimonian and Laird[28,29] proposed a method for calculating the summary effect size of trials assembled for a meta-analysis, and their technique was used here. Briefly, the assumption underlying their technique is that the estimates of treatment effect (individual effect sizes) are normally distributed. The null hypothesis of a lack of heterogeneity (assessed by the use of a χ^2 distribution) tests whether the variability between the effect sizes exceeds the variability within each effect size. This method allows for the creation of a correction factor (to be incorporated into the SE) that reflects the degree of heterogeneity between the effect sizes. As heterogeneity increases, the corrected SE increases and, consequently, confidence intervals (CIs) constructed about the final summary effect size widen. Correction for heterogeneity is made whether or not the χ^2 value for heterogeneity is statistically significant.

Linear regression analyses were performed using the general linear models (GLM) procedure of the SAS program.[30] The dependent variable is the individual effect size in millimoles per liter, and all models are weighted using the inverse of the variance of each effect size. This method is analogous to that of DerSimonian and Laird,[28] except that it is possible for the variance correction factor to be negative using linear regression methods.

RESULTS

Qualitative Review

Trials Excluded From the Summary Effect Size Calculations.—Table 1 describes trials that were not included in the calculation of the summary effect size. In all, seven trials were excluded completely. Four of these (F.W.T., unpublished results, 1991)[14,17,19] did not employ control subjects with respect to the oat product. In the trial of Thye, controls were used, but this trial tested a combination of exercise and oat products against exercise and no oat products, so the controls were deemed inadequate for the meta-analysis. In the trial of Hegsted et al,[17] rice bran and oat bran were tested independently; no other control was used.

Table 1.—Trials Not Included in Summary Effect Size Estimates

Source, y	No. of Subjects	Intervention*	Results†	Reason for Exclusion
O'Kell and Duston,[13] 1988	45	Dry, uncooked oats: ½ cup for men, ⅔ cup for women	No significant difference between "on oats" and "off oats" periods	No final mean values given; final No. of subjects not given; no discussion of statistical methods; no diet or body weight data provided
Hegsted et al,[17]‡ 1989	11	OB, 100 g; RB, 100 g	OB and RB each lowered cholesterol level by 7%	No normal diet control; only comparisons were between OB and RB
Storch et al,[18] 1984‡	12	OB, 53 g; WB, 53 g	Authors claim a 12% reduction in total cholesterol level; after correction for control group's change, net decrease was 7%	Abstract only; insufficient data to calculate effect size
Welch et al,[19] 1990‡	12	OB, 90 g	Mean change, −0.40 mmol/L (−16 mg/dL); significantly different from baseline	Uncontrolled
Stewart and Welch,[14] 1990‡	15	OM, 78 g	Mean change, −0.10 mmol/L (−4 mg/dL); not significantly different from baseline	Uncontrolled
Thye, 1991§‡	12	OB, 100 g	−0.27±0.51 mmol/L (−10±20 mg/dL)	No control group for oat products; the exercise intervention was controlled
	10	OM, 100 g	−0.66±0.46 mmol/L (−26±18 mg/dL)	
Reynolds et al,[20] 1989	43	84 g of cereal containing 24 g of OB; 84 g of corn flakes	Net change, −0.26 mmol/L (−10 mg/dL)	Abstract only; insufficient data to calculate effect size

*OB indicates oat bran; RB, rice bran; WB, wheat bran; and OM, oatmeal.
†These results are changes demonstrated by treated subjects; they do not take into account changes in control subjects, since these trials are either uncontrolled or have not published enough information to calculate a net effect size.
‡Raw data were received.
§Unpublished results.

Three other trials[13,18,20] were excluded because there was inadequate information available in the published report to calculate an individual effect size. The trials of Storch et al[18] and Reynolds et al[20] otherwise met the inclusion criteria. The trial of O'Kell and Duston[13] did not indicate that any formal diet or body weight assessment was performed, so it is questionable whether this trial would have been used even if sufficient information had been available.

Two additional trials, included at the bottom of Table 2 (Demark-Wahnefried et al[15] and O'Brien et al[16]), did not meet the more rigorous criteria but met the broadened criteria. Consequently, they were excluded from the first summary effect size calculation, when the a priori criteria were rigorously enforced, and included in a second calculation, when the broadened criteria were used.

Trials Included in the Summary Effect Size Calculations.—Table 2 describes the trials included in the calculation of the summary effect size. A total of 10 trials (S.B., unpublished results, 1991)[7-12,21-23] met the a priori inclusion criteria: they generated 19 individual effect sizes because some single trials included multiple treatment groups. When the trials of Demark-Wahnefried et al[15] and O'Brien et al[16] were included, 22 individual effect sizes were available for the summary effect size calculation. Subjects ranged in age from 20 to 73 years old. Eight trials utilized a parallel design (S.B., unpublished results, 1991),[7-9,11,15,21,23] three used a 2×2 oat bran and wheat bran crossover design,[10,12,23] and one used a 3×3 design[22] that tested oat bran, rice bran, and wheat bran. Two trials measured changes in apolipoprotein A-I and B levels in addition to measuring changes in total cholesterol and LDL levels.[22,23] The duration of treatment phases ranged from 18 days to 12 weeks, and 10 to 137 subjects were enrolled in each treatment or control group. The majority of the trials assessed diet by use of a 3- or 4-day written food record.

Quantitative Analysis

Calculation of the Summary Effect Size.—The first calculations yielded a summary effect size of −0.15 mmol/L (−5.9 mg/dL) (SE, 0.03 mmol/L [1.3 mg/dL]) with a 95% CI of −0.22 to −0.09 mmol/L (−8.4 to −3.3 mg/dL). The heterogeneity of the assembled effect sizes was not statistically significant (χ^2=26.5, P=.10).

When the broadened inclusion criteria were employed, all trials in Table 2 were included, and the results from the second phases of the crossover trials of Keenan et al[10] and Turnbull and Leeds[23,25] were also included (the diet-only comparison of the trial of Keenan et al[10] was excluded, so this was analyzed strictly as a crossover trial). The summary effect size (for the 22 individual effect sizes) was −0.13 mmol/L (−5.1 mg/dL) (SE, 0.03 mmol/L

Table 2.—Trials Included in the

Source, y	Study Design	Younger Men/Older Men/ Younger Women/ Older Women, %†	Background Diet	Intervention‡ (No. of Subjects)
Van Horn et al,[7] 1986**	Parallel, 6-wk phase	33/17/35/15	AHA-1, 6-wk lead-in	AHA-1 + OB, 57 g (69)[a]
				AHA-1 + OM, 57 g (69)[b]
				AHA-1 diet only (70)
Van Horn et al,[8] 1988**	Parallel, 6-wk phase	33/4/43/20	AHA-1, 4-wk lead-in	AHA-1 + OM, 57 g (113)[c]; AHA-1 diet only (123)
Van Horn et al,[9] 1991**	Parallel, 8-wk phase	33/17/33/17	Usual	OM, 57 g (42)[d]; control (38)
Davidson et al,[11] 1991**	Parallel, 6-wk phase, 6-wk follow-up	27/32/9/32	AHA-1, 8-wk lead-in	AHA-1 + OB, 28 g (23)[e]
		19/52/10/19	AHA-1, 8-wk lead-in	AHA-1 + OB, 57 g (20)[f]
		14/41/9/36	AHA-1, 8-wk lead-in	AHA-1 + OB, 84 g (21)[g]
		30/10/15/45	AHA-1, 8-wk lead-in	AHA-1 + OM, 28 g (20)[h]
		19/48/5/29	AHA-1, 8-wk lead-in	AHA-1 + OM, 57 g (21)[i]
		25/20/25/30	AHA-1, 8-wk lead-in	AHA-1 + OM, 84 g (20)[j]
				AHA-1 + WB, 28 g (15)
Gold and Davidson,[21] 1988**	Parallel, 4-wk phase	50/0/50/0	Usual	OB, 34 g (19)[k]
				Half OB (17 g), half WB (17 g) (28)[l]
				WB, 34 g (25)
Keenan et al,[10] 1991**††	Crossover with concurrent diet controls, 6-wk phases	35/32/12/21	AHA-1, 6-wk lead-in	AHA-1 + OB, 57 g; AHA-1 + WB, 57 g (75 [total for crossover])[m]; AHA-1 diet only (67)[n]
Kestin et al,[22] 1990**††	3×3 crossover testing OB, RB, and WB; three 4-wk phases	67/33/0/0	Low fiber, 3-wk lead-in	OB, 95 g (8 [parallel])[o]; WB, 35 g (6 [parallel]); RB, 60 g (24 [total 3×3 design])
Turnbull and Leeds,[23,25] 1989 and 1987**††	Crossover; 4-wk phases	13/38/25/25	Low fat, 4-wk lead-in	Low fat + OM, 150 g[p]; low fat + Wheatabix, 150 g (17 [total])
Swain et al,[12] 1990‡‡	Crossover; 6-wk phases, 2-wk washout	20/0/80/0	Usual, 1-wk control period	OB, 100 g[q]; WB, 100 g (20 [total])
Beling, 1991**§§	Parallel, 4-wk phase	43/29/13/16	AHA-1, 4-wk lead-in	AHA-1 + OB, 40 g (119); AHA-1 diet only (137)[r]; control (no diet, no OB) (91)[s]
Demark-Wahnefried et al,[15] 1990**‖‖¶¶	Parallel, 12-wk phase	36/18/20/26	LFLC *or* usual, no lead-in	LFLC only (15); LFLC+OB, 50 g (18)[t]; usual diet + OB, 50 g (15); usual diet + processed OB, 42.5 g (20)
O'Brien et al,[16] 1985‖‖	Parallel, 18-day phase	##	High complex carbohydrate, high fiber, 3-day lead-in	Special diet + OB, 50 g (15)[u]; special diet + WB, 50 g (15)[v]; special diet only (15)

*AHA-1 indicates American Heart Association step 1 diet; OB, oat bran; OM, oatmeal; WB, wheat bran; RB, rice bran; and LFLC, low fat, low cholesterol.
†Younger men and women are those less than 50 years of age; older men and women are those 50 years of age or older.
‡The superscript lowercase letters indicate correspondences with Fig 1.
§Soluble fiber values are *estimates* in many trials. The values from the Nutrition Coding Center of the University of Minnesota are 2 g of soluble fiber in 28 g of OB and 1 g of soluble fiber in 28 g of OM.
‖These values are the means of the cholesterol levels of oat-treated subjects after the lead-in diet phase and before the intervention phase.
¶These values are pooled SDs of the oat and control groups.
#*Confidence intervals were constructed using the methods of DerSimonian and Laird.*[26]

[1.12 mg/ dL]) with a 95% CI of −0.19 to −0.07 mmol/L (−7.3 to −2.9 mg/dL). The heterogeneity of these effect size estimates was not statistically significant (χ^2=25.8, P=.20).

Keys Scores.—Table 3 provides Keys scores generated for the trials that had included diet data in published reports.[7-12,15] Using each arm of these trials as the unit of observation, the correlation between the observed change in total cholesterol level and the expected change as determined by Keys scores was .63 (P=.001).

The vast majority of the oat-treated groups demonstrated greater-than-predicted reductions in mean total cholesterol level. In the trial of Swain et al,[12] Keys scores for both the oat- and wheat-treated groups were almost identical; the observed reduction in total cholesterol level for the oat period was slightly less (0.05 mmol/L [1.9 mg/dL]) than predicted by Keys scores. In the trial of Davidson et al,[11] the 57-g oatmeal group demonstrated just a slight reduction in total cholesterol level beyond that predicted; this is an exception to the general trend demonstrated by the other five oat-treated groups in this trial that the reduction in total cholesterol level was far greater than predicted by the Keys scores.

For this subset[7-12,15] of 13 individual effect sizes for which Keys scores were calculated, the summary effect size before any adjustment for expected changes in total cholesterol level was −0.17 mmol/L (−6.5 mg/dL), with a 95% CI of −0.25 to −0.09 mmol/L (−3.5 to −9.7 mg/dL) and an SE of 0.04 mmol/L (1.6 mg/dL). The summary effect size after adjustment for expected changes in total cholesterol level (estimated by Keys scores) was −0.18 mmol/L (−6.8 mg/dL), with a 95% CI of −0.31 to −0.05 (−12 to −2 mg/dL) and an SE of 0.06 mmol/L (2.5 mg/dL). The χ^2 values for heterogeneity between the individual effect sizes were 10.8 and 46 before and after adjustment, respectively; the latter value indicates statistically significant between-effect size heterogeneity (P<.005).

Predictor Variables

The preliminary total cholesterol level for each trial was highly predictive of the subsequent reduction in total cholesterol level. R^2=0.46; the reduction in effect size per unit of preliminary total cholesterol was −0.14 (SE, 0.037, P=.001).

Neither age nor gender nor their interaction term demonstrated an ability to predict subsequent response to oats. The mean of the effect sizes for the four subgroups were: young men, −0.25±0.29 mmol/L (−9.8±11.2 mg/dL); older men,

Final Effect Size Estimate*

Estimated Soluble Fiber in Intervention, g§	Preintervention Mean Serum Cholesterol Level,‖ mmol/L (mg/dL)	Effect Size,¶ mmol/L (mg/dL)	95% Confidence Interval,# mmol/L (mg/dL)
4.1	5.1 (196)	−0.11±0.45 (−4.2±17.4)	−0.26 to 0.04 (−10.0 to 1.6)
2.2	5.0 (195)	−0.14±0.43 (−5.3±16.7)	−0.28 to 0.01 (−10.9 to 0.30)
2.2	5.0 (193)	−0.08±0.48 (−3.2±18.4)	−0.20 to 0.04 (−7.9 to 1.5)
2.2	6.6 (254)	−0.32±0.50 (−12.3±19.5)	−0.55 to −0.09 (−21.2 to −3.5)
2.0	7.0 (269)	−0.25±0.74 (−9.8±28.7)	−0.79 to 0.28 (−30.5 to 10.9)
4.1	6.9 (266)	−0.70±0.68 (−27.0±26.2)	−1.2 to −0.22 (−45.6 to −8.5)
6.1	6.9 (265)	−0.51±0.59 (−19.6±22.8)	−0.87 to −0.14 (−33.6 to −5.6)
1.1	6.8 (264)	−0.29±0.65 (−11.3±25.0)	−0.73 to 0.15 (−28.3 to 5.7)
2.2	6.9 (265)	−0.23±0.70 (−8.7±27.1)	−0.73 to 0.28 (−28.1 to 10.7)
3.2	7.1 (275)	−0.56±0.69 (−21.7±26.8)	−1.1 to −0.06 (−41 to −2.4)
2.5	4.6 (179)	−0.25±0.40 (−9.6±15.6)	−0.50 to 0.00 (−19.4 to 0.24)
1.2	4.7 (183)	+0.01±0.47 (0.57±18.12)	−0.24 to 0.28 (−9.5 to 10.8)
3.1	5.9 (229)	OB vs WB: −0.11±0.54 (−4.2±20.9); OB vs diet only: −0.32±0.66 (−12.3±25.7)	OB vs WB: −0.4 to 0.14 (−13.9 to 5.5); OB vs diet only: −0.59 to −0.04 (−23.0 to −1.5)
5.8	5.8 (223)	−0.27±0.51 (−10.4±19.7)	−0.86 to 0.32 (−33.1 to 12.2)
6.0	6.3 (243)	−0.29±0.61 (−11.3±23.6)	−0.92 to 0.34 (−35.7 to 13.2)
5.8	4.8 (186)	−0.03±0.41 (−1±15.9)	−0.21 to 0.18 (−8.0 to 7.0)
2.9	5.5 (212)	OB vs AHA-1: −0.09±0.59 (−3.3±22.7); OB vs no diet, no OB: 0.00±0.52 (+0.13±20.2)	OB vs AHA-1: −0.23 to 0.06 (−8.9 to 2.3); OB vs no diet, no OB: −0.14 to 0.15 (−5.4 to 5.7)
3.6	7.2 (278)	LFLC vs LFLC+OB, 50 g: +0.21±0.79 (+8.3±30.4); *uncontrolled changes:* LFLC: −1.2 (−46), LFLC+OB, 50 g: −1.1 (−41), usual diet+OB, 50 g: −0.92 (−35.6), usual diet + processed OB, 42.5 g: −0.73 (−28)	LFLC vs LFLC+OB, 50 g: −0.36 to 0.79 (−13.8 to 30.4)
7.6	7.1 (276)	OB vs WB: −0.28±1.1 (−10.7±41.7); OB vs diet only: −0.12±0.82 (−4.5±31.6)	OB vs WB: −1.1 to 0.53 (−41.9 to 20.5); OB vs diet only: −0.73 to 0.49 (−28.1 to 19.1)

**Raw data were received.
††These studies were analyzed as parallel-design trials; statistics in this table were calculated using parallel-design methods.
‡‡Raw data were not available; this study was analyzed as a crossover trial in all calculations. The authors stated that no group-by-phase interaction existed.
§§Unpublished results, 1991.
‖‖Data from these studies are included in the second of two summary effect sizes mentioned in the text (see the "Results" section for details).
¶¶Of the three treatment groups, the effect size could be calculated only for LFLC vs LFLC + OB, 50 g; the other two treatment groups did not have comparable control groups.
##One third of the subjects were women; no age breakdown was provided.

−0.31±0.35 mmol/L (−12.0±13.7 mg/dL); young women, −0.21±0.44 mmol/L (−8.2±17.2 mg/dL); and older women, −0.28±0.28 mmol/L (−10.8±11.0 mg/dL).

Davidson et al[11] recently performed a trial that tested a dose-response hypothesis. Because the raw data for the trial of Davidson et al[11] were available, a linear regression model was built with dose (grams of soluble fiber) and preliminary total cholesterol level as independent variables and change in total cholesterol level as the dependent variable. R^2=0.26; the reduction in effect size per unit of soluble fiber was −3.3 (SE, 1.07; P=.002), and the reduction in effect size per unit of preliminary total cholesterol was −0.36 (SE, 0.059; P<.0001).

To assess a dose-response relationship between individual effect sizes and the dose of soluble fiber for trials in the meta-analysis, linear regression methods were used to describe the interactive association of dose and preliminary total cholesterol level with effect size. With a dichotomous dose variable (<3 g vs ≥3g) and a continuous range of preliminary total cholesterol levels, the interaction term was statistically significant (P=<.05), and R^2=0.61. Table 4 shows mean effect sizes when the individual effect sizes are split into preliminary total cholesterol/dose subgroups. Trials that had the largest reduction in total cholesterol level were those whose subjects had the highest preliminary total cholesterol level and tested the higher doses. The association of the dose of the oat product and subsequent total cholesterol reduction appears to be blunted when initial cholesterol levels are low. There was considerable variability of the individual effect sizes even within the four subgroups suggested by the interaction.

COMMENT

Meta-analyses: Goals and Limitations

Meta-analyses are typically conducted to qualitatively describe the available research, compute a pooled estimate that reflects the available evidence, explain contradictory results between independent trials, and perform subgroup analyses that would not be possible within independent trials.[31,32] Two major criticisms have been leveled at meta-analyses. First, "file drawer" bias occurs when pooled estimates are derived solely from published reports and consequently may be a skewed representation of the entire body of research.[33,34] Second, assembled trials may be quite heterogeneous, in terms of both study design (ie, phase duration, subject selection, blood-drawing protocols) and the relative qual-

Table 3.—Energy Values, Keys Scores, and Body Weight Changes*

Source, y	Diet Assessment Tool	Intervention†	Energy Intake, kJ/d		Change in Total Cholesterol Level, mmol/L (mg/dL)		Change in Body Weight, kg
			Initial	Final	Predicted‡	Observed	
Van Horn et al,[7] 1986	3-Day food record	OB, 57 g	6896	6565	+0.13 (+5.1)	−0.14 (−5.4)	−0.27
		OM, 57 g	7384	7274	−0.07 (−2.7)	−0.17 (−6.5)	−0.27
		AHA-1 diet only	7514	7064	+0.08 (+2.9)	−0.03 (−1.2)	−0.41
Van Horn et al,[8] 1988	3-Day food record	OM, 57 g	6510	7039	−0.06 (−2.3)	−0.16 (−6.0)	+0.09
		AHA-1 diet only	6300	6422	0.00 (+0.11)	−0.07 (−2.8)	−0.27
Van Horn et al,[9] 1991	3-Day food record	OM, 57 g	8022	8341	−0.06 (−2.4)	−0.40 (−15.5)	−0.29
		AHA-1 diet only	8429	8488	+0.08 (+3.1)	−0.09 (−3.5)	+0.29
Keenan et al,[10] 1991	Baseline: FFQ; each phase: 4-day food record	OB-WB§	7602	7400	+0.01 (+0.27)	−0.15 (−5.8)	0
		WB-OB§	7804	7388	−0.01 (−0.42)	−0.11 (−4.3)	−0.4
		AHA-1 diet only	7972	7619	0.00 (+0.09)	+0.35 (+13.5)	−1.3
Swain et al,[12] 1990	Baseline: FFQ; each phase: 4-day food record	OB, 87 g	8673	10202	−0.41 (−15.8)	−0.36 (−13.9)	+0.3
		WB, 87 g	8673	9723	−0.39 (15.0)	−0.34 (−13.1)	+0.2
Davidson et al,[11] 1991	4-Day food record	OM, 28 g	5968	6140	−0.06 (−2.2)	−0.28 (−10.8)	−0.36
		OB, 28 g	6346	6644	+0.04 (+1.6)	−0.23 (−9.0)	−0.55
		OM, 57 g	5842	6560	−0.19 (−7.2)	−0.22 (−8.6)	−0.55
		OB, 57 g	7144	7321	−0.17 (−6.6)	−0.67 (−26.0)	−0.59
		OM, 84 g	6334	7253	−0.16 (−6.3)	−0.54 (−21.0)	+0.18
		OB, 84 g	6821	7493	−0.16 (−6.3)	−0.52 (−20.0)	−0.41
		WB, 28 g	5909	5788	0.00 (+0.01)	+0.02 (+0.77)	−1.0
Demark-Wahnefried et al,[15] 1990	Daily food record	LFLC diet + OB, 50 g	9400	6833	−0.28 (−14.6)	−1.1 (−41.4)	−4.1
		OB, 50 g	8984	8102	−0.38 (−4.2)	−0.92 (−35.6)	−1.6
		OB, 42.5 g	9059	7753	−0.11 (−5.6)	−0.72 (−28.1)	0
		LFLC diet only	9253	6699	−0.14.(−10.8)	−1.2 (−46.0)	−3.0

*OB indicates oat bran; OM, oatmeal; AHA-1, American Heart Association step 1 diet; WB, wheat bran; LFLC, low fat, low cholesterol; and FFQ, food frequency questionnaire.
†See Table 2 for a more complete description of interventions.
‡Predicted changes were calculated using Keys scores: $\Delta C = 1.35\,(2\Delta S - P) + 1.5\Delta Z$, where C is the total blood cholesterol level; S, the percentage of kilocalories consisting of saturated fatty acids; P, the percentage of kilocalories consisting of polyunsaturated fatty acids; and $Z = \sqrt{\text{Dietary Cholesterol (milligrams)}/1000 \text{ kilocalories}}$.
§OB-WB indicates subjects who consumed OB in the first phase and WB in the second phase; WB-OB, subjects who consumed WB in the first phase and OB in the second phase.

ity of each trial.

In our meta-analysis, "file drawer" bias was minimized by actively soliciting research from all known investigators, regardless of whether the results had been published; the inclusion criteria set the minimum standard of quality. Additional variance caused by heterogeneity between the individual effect sizes was estimated by the methods of DerSimonian and Laird.[28] To determine whether heterogeneity had been artificially decreased by creating more than one individual effect size using one treatment group and multiple control group comparisons (ie, duplicating the use of a single treatment group result), the summary effect size was recalculated in the two ways done formerly (once with the a priori inclusion criteria enforced and once with broadened criteria), but removing any effect sizes that were generated as a result of the multiple use of treatment groups. Table 5 shows the results of the calculations and recalculations. It is clear by comparison that inclusion or exclusion of the duplicate information does not affect the summary effect size in any important way.

It could be argued that trials using wheat bran as a comparison are better controlled than trials incorporating oats isocalorically into the diet of treated subjects without providing a comparison product for controls. Table 5 displays the summary effect size calculations when trials are stratified according to this difference in design. Clearly, the stratified analysis supports the same conclusion as the unstratified one. For the stratified analysis, only the 28-g oat bran and 28-g oatmeal individual effect sizes were used from the trial of Davidson et al[11] (these treatments were comparable by weight to the control treatment of 28 g of wheat cereal), which reduced the magnitude of the summary effect size because the four excluded treatment groups had high initial cholesterol levels (average of 6.9 mmol/L [268 mg/dL]).

There is heterogeneity with respect to other aspects of study design, which is the case in virtually every meta-analysis. For example, the intervention phase durations of these trials ranged from 18 days to 3 months; investigators used a variety of recruitment methods to obtain their subjects, and the number of subjects in each trial differed widely. When this information is pooled, definitions necessarily broaden. Interpretations are valid, however, provided conclusions are drawn with the broadened definitions in mind: short-term intervention trials employing a dose of approximately 3 g of soluble fiber and enrolling primarily healthy middle-class men and women with the motivation and the resources to make dietary changes.

Summary Effect Size

Two potential confounders are changes in total cholesterol level attributable to regression toward the mean[35] and dietary changes known to affect lipid levels. Since uncontrolled trials were excluded from the summary effect size and the individual effect sizes were computed by adjusting for any change in total cholesterol level occurring in the control groups, regression toward the mean with respect to the summary effect size was not a confounder in this analysis. Regression toward the mean did not importantly confound with respect to initial cholesterol level as a predictor variable because, with two exceptions (S.B., unpublished data, 1991),[9] trials either did not recruit exclusively hypercholesterolemic subjects,[7,8,12,21] or, if they did, the initial cholesterol value was the mean of multiple measures.[10,11,22,25]

It has been suggested[12] that the reported lipid-lowering effect of oat prod-

Table 4.—Effect Sizes for Change in Total Cholesterol Level by Dose and Initial Cholesterol Level

Intervention Dose	Effect Size, mmol/L (mg/dL)* Initial Cholesterol Level <5.9 mmol/L (<229 mg/dL)	Initial Cholesterol Level ≥5.9 mmol/L (≥229 mg/dL)
<3.0 g of soluble fiber from oats	−0.09±0.10 (−3.4±3.8)†	−0.27±0.04 (−10.5±1.6)‡
≥3 g of soluble fiber from oats	−0.13±0.12 (−5.2±4.8)§	−0.41±0.21 (−16.0±8.3)‖

*Values are mean±SD.
†There were six effect sizes.
‡There were four effect sizes.
§There were three effect sizes.
‖There were six effect sizes.

ucts is really the result of a substitution of carbohydrates for dietary fat and cholesterol. Keys and colleagues[26] studied men under metabolic ward conditions and developed the equation we used in which a change in blood total cholesterol level could be predicted by knowing the dietary changes in saturated fatty acids, polyunsaturated fatty acids, and cholesterol. Keys et al[26] and others[27] have tested the use of Keys scores in free-living subjects using diet records as their source of data, and they found that the predictive ability is still very good. Use of Keys scores is intended to measure group changes when components of a diet are being changed isocalorically; they are not intended for extrapolation to the individual subject, and both Keys et al[26] and more recent investigators[36] have demonstrated the variability of an individual's serum cholesterol response to dietary changes. Keys scores (Table 3) did not predict well in the trial of Demark-Wahnefried et al,[15] in which subjects reduced their energy intake and consequently lost weight in their attempts to adhere to the low-fat, low-cholesterol diet. In the trial of Swain et al,[12] energy consumption increased 1470 kJ/d during the treatment period, so the use of Keys scores may not have been entirely appropriate. The usefulness of Keys scores in this meta-analysis is limited to acting as a standard from which to judge whether reductions in total cholesterol level could be primarily attributed to the substitution of oats for dietary fats and cholesterol. The summary effect size calculated for a subset of trials after adjustments for changes in total cholesterol level due to substitution differed very little from the unadjusted summary effect size of this same subset of trials and very little from the unadjusted value of the entire sample of trials.

The trials of O'Brien et al[16] and Demark-Wahnefried et al[15] were originally excluded because there was not a sufficient lead-in period to ensure that the effects of the special diets (high-fiber, high–complex carbohydrate diet in the trial of O'Brien et al[16] and low-fat, low-cholesterol diet in the trial of Demark-Wahnefried et al[15]) on the change in blood lipid levels was negligible. However, any effect of a special diet should cancel out in randomized controlled trials when adjustments are made for the control group's change in total cholesterol level, so the two trials in question could be considered with the others. The summary effect size was calculated with and without the two trials in question, and it is clear that their inclusion did not increase the heterogeneity of the assembled individual effect sizes (χ^2 values were 25.8 and 26.5 for inclusion and exclusion, respectively).

The Figure illustrates why there appears to be such confusion about whether or not oats truly lower blood cholesterol levels. Apparently, many investigators overestimated the expected effect size when planning the sample sizes of trials, perhaps because outcomes were expected to mirror the results of early metabolic ward trials, in which total cholesterol changes as large as −0.75 mmol/L (−25 mg/dL) were reported[3,4,6]; consequently, power was lacking to detect a difference of 0.13 to 0.15 mmol/L (5 to 6 mg/dL).

By no means, however, has all of the variability in response between the trials been explained. The unpublished trial by Beling (1991) was the largest single trial that showed no significant reduction of total cholesterol level attributable to the oat product. The controls and treated subjects demonstrated significant reductions in total cholesterol levels during the lead-in diet period, but reductions beyond that point were slight. All of the groups demonstrated weight loss, from −1 to −2.7 kg. This trial and the trial of Demark-Wahnefried et al[15] were similar, in that both demonstrated weight loss, and the treated subjects in both failed to show significant reductions in total cholesterol levels compared with controls. This leads to speculation that the lipid reduction due to weight loss overshadows any contribution to reduction by oats and suggests that the effect of oats on blood lipid levels is best seen in weight-stable individuals. However, this ecologic observation has just two trials in evidence; more information is needed to draw any firm conclusions about a potential interaction of weight loss and oat product consumption on lipid reduction.

Age-Gender Interaction

As described previously, Keenan et al[10] reported a significant age-gender interaction and found that older women had the most marked total cholesterol reduction of the age-gender subgroups, but this observation was not supported in the meta-analysis. A major limitation of this retrospective subgroup analysis is that stratification did not occur for age and gender in any of these trials, so the randomization scheme could have been broken. Additionally, statistical power may have been insufficient to detect differences between subgroups since most trials had very few subjects in at least one of the subgroups. It is possible that a response in total cholesterol level modified by the interaction of age and gender could still be found in a trial specifically designed to test this hypothesis.

Initial Cholesterol Level

The finding that initial cholesterol level played an important predictive role in the outcome of the intervention was previously reported in three individual clinical trials.[8-10] The negative results of the highly publicized trial of Swain et al,[12] in which the mean preliminary total cholesterol level of the 20 subjects was just 4.8 mmol/L (186 mg/dL), are likely attributable to the low initial total cholesterol level of those subjects. In addition, the 9% reduction in total cholesterol level for the metabolic ward trial reported by Anderson et al[6] can be explained in part by the high initial cholesterol level of subjects (6.9 mmol/L [266 mg/dL]) and by the higher dose of oats employed (13.4 g of soluble fiber). The remaining difference may be attributable to differences in the ability to measure diet variables between free-living subjects whose diet is self-reported and subjects on metabolic wards whose diet is precisely measured and controlled.

Dose-Response

The determination of a dose-response relationship between oats and lipid lowering is difficult because the mechanism of action has continued to be elusive. The potential mechanisms have been thoroughly discussed,[5,37,38] and research continues in this area. For this analysis, grams of soluble fiber was chosen to represent the dose of the oat product because it is the best representation of β-glucan, the primary soluble fiber in oats. This measure, however, has limitations: The amount of soluble fiber obtained by measuring a quantity of oats will vary according to the solubilizing technique.[39] For the trials that did not directly measure the soluble fiber in their product, an estimate from the database

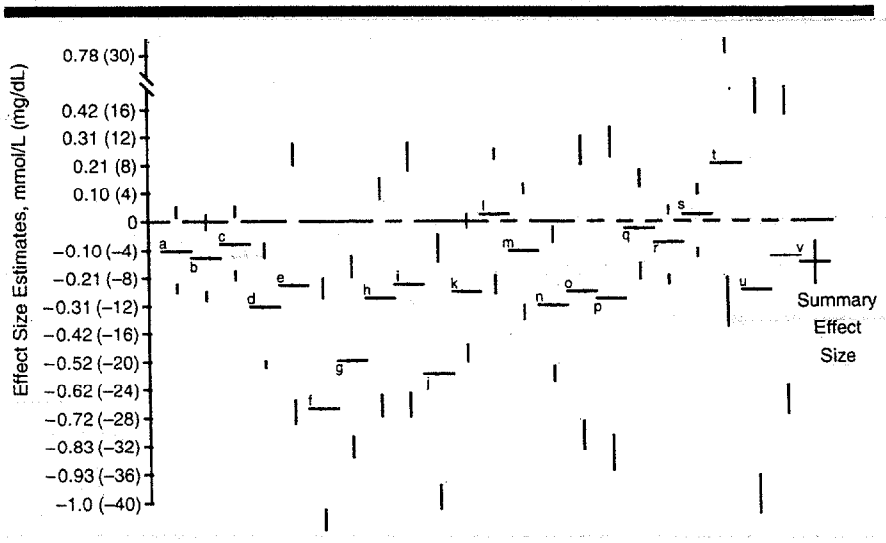
Table 5.—Description of Summary Effect Sizes Using Varying Inclusion Criteria

Inclusion Criteria*	Source	No. of Effect Sizes	χ^2	P†	Summary Effect Size, mmol/L (mg/dL)	95% Confidence Interval, mmol/L (mg/dL)
Trials using a priori criteria	Van Horn et al,[7-9] Keenan et al,[10] Davidson et al,[11] Swain et al,[12] Gold and Davidson,[21] Kestin et al,[22] Turnbull and Leeds,[23] Beling‡	19	26.5	.10	−0.15 (−5.9)	−0.22 to −0.09 (−8.4 to −3.3)
Trials using broadened criteria	Van Horn et al,[7-9] Keenan et al,[10] Davidson et al,[11] Swain et al,[12] Demark-Wahnefried et al,[15] O'Brien et al,[16] Gold and Davidson,[21] Kestin et al,[22] Turnbull and Leeds,[23] Beling‡	22	25.8	.20	−0.13 (−5.1)	−0.19 to −0.07 (−7.3 to −2.9)
Trials using a priori criteria but excluding the multiple use of treatment groups†	Van Horn et al,[7-9] Keenan et al,[10] Davidson et al,[11] Swain et al,[12] Gold and Davidson,[21] Kestin et al,[22] Turnbull and Leeds,[23] Beling‡§	17	21.3	15	−0.16 (−6.1)	−0.22 to −0.09 (−8.7 to −3.5)
Trials using broadened criteria but excluding the multiple use of treatment groups†	Van Horn et al,[7-9] Keenan et al,[10]§ Davidson et al,[11] Swain et al,[12] Demark-Wahnefried et al,[15] O'Brien et al,[16]§ Gold and Davidson,[21] Kestin et al,[22] Turnbull and Leeds,[23] Beling‡§	19	22.7	.20	−0.14 (−5.6)	−0.20 to −0.09 (−7.9 to −3.3)
Trials using a priori criteria and stratifying by type of control subjects						
Control groups with wheat bran as a comparison product	Keenan et al,[10] Davidson et al,[11] Swain et al,[12] Gold and Davidson,[21] Kestin et al,[22] Turnbull and Leeds[23]	12	16.3	.20	−0.22 (−8.6)	−0.35 to −0.10 (−13.4 to −3.8)
Control groups with no comparison product	Van Horn et al,[7-9] Keenan et al,[10] Beling‡	7	8.5	.20	−0.11 (−4.4)	−0.18 to −0.04 (−7.1 to −1.7)

*See the text for descriptions of a priori and broadened criteria.
†Tests the null hypothesis that within–effect size variance is equal to between–effect size variance.
‡Unpublished results, 1991.
§One effect size only.

of the Nutrition Coding Center of the University of Minnesota, Minneapolis, was used, but this estimate could be somewhat imprecise because the amount of β-glucan may vary according to the variety of the oat as well as its growing environment.[40] This last point may help to explain at least some of the unexplained variability in total cholesterol response between trials. It is also possible that soluble fiber is an incomplete measure of dose; other components of oats as well as the way in which the oat product is prepared may play a role in the mechanism of action.[39]

There is the strong suggestion of a dose-response relationship between the amount of soluble fiber and the degree of lipid reduction, and there is significant evidence of an interaction between dose and initial cholesterol level with respect to total cholesterol reduction. Trials enrolling subjects with higher initial cholesterol levels (≥5.8 mmol/L [≥229 mg/dL]) and employing a dose of 3 or more g of soluble fiber demonstrated fivefold greater reductions in total cholesterol levels than trials whose subjects had lower initial cholesterol levels and that employed lower doses of fiber. This interaction is important when attempting to apply the results of this analysis to an individual. Three grams of soluble fiber is the equivalent of one large bowl of ready-to-eat oat bran cereal or three packets of instant oatmeal (oat bran has approximately twice the amount of soluble fiber as oatmeal). Thus, although individuals with high initial cholesterol levels would likely benefit from a single serving of oats, individuals whose blood cholesterol levels are already low may demonstrate little change in total cholesterol level by introducing a single serving of oats into their diet.



Effect size estimates. The summary effect size was calculated using a priori inclusion criteria. The lowercase letters near each effect size bar correspond to the effect sizes in Table 2. Shaded bars indicate 90% confidence intervals; solid line extensions, 95% confidence intervals.

Epidemiologic Aspects

The large number of subjects analyzed here does not automatically mean these findings can be extrapolated to the general population. These results are generalizable to people who resemble the study subjects—motivated adults who are able to make dietary changes.

In addition to its lipid-lowering benefits, research efforts are ongoing to determine whether dietary fiber can protect against certain forms of intestinal cancer.[37] It is feasible to project that the consumption of fiber is or will be on the rise as people look for healthy ways to alter their risk factor profiles. The potential side effects or disadvantages of fiber consumption then begin to take on more importance. There is evidence to suggest that components of dietary fiber, such as phytates and oxalates, can bind minerals in the gut (ie, zinc, calcium, iron, magnesium, phosphorus, and copper), decreasing their bioavailabil-

ity. The balance of the current research suggests that an increase in mineral excretion with fiber intake is not significant[37]; however, these trials are mostly short-term (usually 2 to 4 weeks) and are conducted with healthy adults. Special populations at risk of mineral deficiencies may need to be studied separately to determine whether major increase in fiber will increase this risk.

Ideally, a large-scale, long-term clinical trial (ie, 6 months or longer) should be conducted to verify the results of this review.

CONCLUSION

After careful consideration of the available evidence and investigation of potential confounders, this analysis provides strong support for the hypothesis that approximately 3 g per day of soluble fiber from oat products can lower the total cholesterol level 0.13 to 0.16 mmol/L (5 to 6 mg/dL) and that the reduction is greater in those with initially higher blood cholesterol levels. It is especially advantageous from a public health perspective that this modest reduction can occur by incorporating into the diet a food product with considerable nutritional value.

Modest reductions in blood cholesterol levels can have a dramatic impact when realized by large numbers of people. It has been estimated that a 1% reduction in the serum cholesterol level could reduce heart disease mortality in the United States by 2%.[41,42] Keeping in mind that oats and the dietary changes that accompany their incorporation may not be realistic for all people, if even the segment of the population at increased risk for cardiovascular disease were able to reduce its total cholesterol level 2% to 3%, this would have a very beneficial impact on rates of heart disease.

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Effect of a milk drink enriched with increasing doses of free tall oil phytosterols on plasma lipid levels of mildly hypercholesteroleamic subjects

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ABSTRACT

Objective:

Design:

Subjects:

Interventions:

Results:

Conclusions:

Descriptors:

INTRODUCTION

Cardiovascular disease (CVD) is a significant cause of disability and death around the world with an enormous cost to the health care system. World-wide more than 10 million deaths per year are related to CVD and in industrialised countries nearly 50% of total deaths are due to CVD. The causal relation between serum cholesterol levels and CVD was shown in several studies (Kannel et al, 1979; Pooling Research Project Group, 1978; Wilson et al, 1987) and the lowering of total and LDL cholesterol was identified as a target for intervention. A recent analysis of the primary prevention WOSCOPS (West of Scotland Coronary Prevention Study, West of Scotland Coronary Prevention Study Group, Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS) , Circulation, 1998: 97:1440-1445.) shows a clear relation of a reduction in LDL and the related change in CVD risk. A reduction of LDL cholesterol >10% reduces the risk of CVD by about 12%, whereas a LDL reduction by 10-19% causes a risk reduction of up to 41%.

Reduced energy, fat and cholesterol intake reduce total and LDL cholesterol and the risk of CVD, but a significant portion of the population is unable to comply to such dramatic dietary modifications. There is a clear need for a low cost, low risk interventions that can treat that portion of the population that has not responded well to dietary and other non-pharmaceutical interventions, but because of the risk of serious adverse events and cost, the use of more drastic treatments is not justifiable.

It has been known for nearly 50 years that the ingestion of phytosterols has a positive effect on cholesterol metabolism in humans (Pollak, 1953; Farquhar et al, 1956). In the past 10 years, new interest has focused on the consequences of plant sterols ingestion on blood cholesterol level (reviewed by Jones et al; 1997). The magnitude of reported effects varied widely displaying differences in study design, amount and quality of phytosterol intake and starting cholesterol levels of patients. Total serum cholesterol levels were reduced by 0.5 - 26%, serum low density lipoprotein cholesterol (LDL cholesterol) levels by 2 - 33% whereas levels of serum high density lipoprotein cholesterol (HDL cholesterol) remained unchanged.

Most of the recent studies used preparations wherein the sterols or stanols were esterified with fatty acids (Miettinen, Gylling, Hendriks, Weststrate) to increase fat solubility. However, for physiological actions esterification of sterols to fatty acids is not necessary and Jones and co-workers (1999) showed that free sterols have a pronounced cholesterol lowering effect at low doses. Esterified sterols are rapidly hydrolysed during lipolysis of dietary fats and act in the digestive tract as free sterols (Mattson et al, 1977; Mattson et al, 1982; Swell et al, 1954).The fat soluble esterified sterols or stanols were mostly administered in foods with fat contents of 40-80% of total weight (margarine or mayonnaise). These studies generally resulted in quite impressive lowering of LDL and total cholesterol, reduction in LDL cholesterol of up to 14%. However, from a nutritional standpoint, it would be much more desirable to investigate the action of low fat phytosterol containing foods on cholesterol metabolism. Esterified sterols and stanols were shown to decrease fat soluble (pro)vitamins levels in a dose dependant manner (Hendriks et al. 1999). This negative action may not be seen if free sterols are used. Sierska et al, (1999) studied the effects of a low dose of free sterols on alpha- and beta-carotene and lycopene and showed no negative influence on plasma levels of the carotenoids.

The main objective of this study was to investigate the dose-dependency of the total and LDL-cholesterol lowering effects of 3 doses of free tall oil sterols delivered in a non fat based food, namely a milk drink. In addition, the effects of the free sterols on lipid soluble (pro)vitmains were studied.

SUBJECTS AND METHODS

Subjects

Volunteers were recruited from the population living in Chicago, USA. To be included in the study, subjects had to have a fasting plasma LDL cholesterol level > 125 mg/dL; to be between 30 -80 years of age; not currently taking any lipid lowering therapy, and to have a alcohol consumption < 10 drink per week.
Subjects were excluded on the base of having total cholesterol > 309 mg/dL at Visit 2 or Visit 3, triglycerides ≥ 354 mg/dL, fasting blood glucose > 125 mg/dL, a history of kidney, liver, and/or haematological disease or plasma urea > 18 mmol/L, serum creatinin > 177 µmol/L, total bilirubin > 34 µmol/L, SGOT or SGPT > 2 times upper limit of normal, uncontrolled hypertension (treated or untreated) with systolic blood pressure ≥ 160 mm Hg or diastolic blood pressure ≥ 95 mm Hg, Body Mass Index (BMI) ≥ 35, less than 20% or greater than 40% of calories consumed by fat, lipid-lowering drug received within 6 weeks prior to Visit 0, history or signs of heart disease - unstable angina or intermediate coronary syndrome or clinically significant ventricular arrhythmias at study entry or myocardial infarction within the previous 3 months, history of drug or alcohol abuse; current alcohol intake of more than an average of 10 drinks per week, familial sitosterolemia, history of ileal bypass surgery or any GI disorder that could affect cholesterol or fat absorption. Only subjects who had less than a 15% variation in LDL cholesterol between Visits 1 and 2, and an average fasting LDL > 125 mg/dL at Visits 1 and 2 were eligible for randomisation

A total of 270 volunteers were screened of which 132 were included in the study. The study protocol was approved by the local Ethics Committee and all subjects gave written consent. The study was conducted according to Good Clinical Practice.

Study design

This study followed a randomised, placebo-controlled, parallel arm design in which 3 different doses of tall oil sterols (0.9, 1.8 or 3.6 g/day) or placebo was given for a period of four weeks. The overall study plan is shown in table 1.

Table 1: Study plan

	Phase I: Screening			**Phase II: Active Medication**		
Week	Screen	-2	-1	0	3.5	4
Visit	0	1	2	3	4	5
Medical History	X					
Diet Questionnaire	X					X
Physical Examination	X	X	X	X	X	X
Adverse Event Report		X	X	X	X	X
Standard safety[1]	X			X		X
Lipids	X	X	X	X	X	X
Vitamins				X		X

[1]Standard safety included: Hematology (hemoglobin, hematocrit, WBC and differential RBC, platelet count), Blood Chemistry (glucose, BUN, uric acid, serum creatinine, total bilirubin, alkaline phosphatase, LDH, SGOT, SGPT), and Urinalysis (specific gravity, pH, protein, glucose, WBC and RBC as indicated by dipstix)

At visit 0 subjects underwent a selection procedure including a diet questionnaire, a complete a medical history interview with a physician and a brief physical examination. A fasting blood sample for safety evaluations as well as for lipid determination was taken. If subjects complied with inclusion/exclusion criteria they returned to the clinic for visit 1 and 2 (as per table 1). At visit 3 only subjects who had a difference in LDL cholesterol between visits 1 and 2 of less than 15% and an average fasting LDL > 125 mg/dL between visits 1 and 2 were randomised to either placebo or one of the 3 active groups. Randomised subjects underwent another clinical and physical examination and were provided with study products. Subjects returned to the clinic after 3.5 and 4 weeks for the final clinical and physical examinations.

All clinical visits were performed in the morning and subjects were instructed not to eat 12 hours prior to the clinic visits.

Study products

The tall oil sterol (TOS) enriched milk drinks were prepared by Wander AG, Neuenegg, Switzerland. Composition of active and placebo drinks is shown in table 2. The milk drinks were fortified with different doses of unesterified TOS (Reducol™, Forbes Medi-Tech, Vancouver, Canada) with 44% sitosterol, 25% sitostanol, 12% campesterol, and 6% campestanol. Subjects consumed 3 drinks of 90 mL per day with their main meals, ingesting 0 g, 0.9 g, 1.8 g or 3.6 g TOS per day.

Table 2: Composition of milk drinks

Variables	Placebo Drink	TOS Drink		
		0.9 g/d	1.8 g/d	3.6g/d
Water (g/100g)	79.5	79.8	80.0	80.0
Protein (g/100g)	2.0	1.9	2.0	1.9
Fat (g/100g)	3.6	3.6	3.6	3.6
Tall oil Sterols (g/100g)	0	0.31	0.63	1.26
Carbohydrates (g/100g)	14.3	13.8	13.4	12.6
Minerals (g/100g)	0.6	0.6	0.6	0.6
Specific weight (g/mL)	1.06	1.06	1.06	1.06

Analysis

Actual methods needed!

Blood samples for serum and plasma determination were centrifuged (3000 rpm x 10 min, +4°C) within 1 hour after drawing. Plasma and serum was separated and stored at -20°C until analysis.

Blood lipids
Total and HDL cholesterol and tryglicerides were determined with a Hitachi 747 analyser (Hitachi Inc., Tokio, Japan) using enzymatic methods (Roche UK Ltd, Lewes, England). LDL cholesterol was calculated by using the Friedewald equation (Friedewald, Levy, Fredrickson, 1987).

(Pro)Vitamins

Vitamin A and E and alpha- and beta-carotene was determined using a HPLC method as describe by Thurnham et al () and Zaman et al (). Proteins in the serum were precipitated with methanol and the vitamin/lipid complexes were extracted into hexane. The dried extract was redissolved in THF and methanol and quantification of the vitamins was achieved by reverse phase HPLC.

Statistical analysis

Statistical analysis was performed by using SAS® statistical software. The primary efficacy measurement was the change in plasma LDL cholesterol during the treatment period. The average of the lipid values obtained at Visits 1, 2 and 3 was used as the baseline value. The average of the lipid values obtained at Visits 4 and 5 was used as the post-treatment value. Three outcome variables were analysed: the absolute LDL cholesterol level post-treatment; the change in the LDL cholesterol from baseline; and the percentage change from baseline. The three outcome variables were analysed using analysis of covariance, with treatment group included as the only main effect. Since the age of the subjects was found to differ significantly between treatment groups, age was included as a covariate in the analysis. The baseline value was also included as a covariate in the analyses of the absolute value post-treatment and the change from baseline. Dunnett's test was used to determine the significance of any difference between each treated group and the placebo group. The significance of the difference from 0 of the change and relative change from baseline within each group was analysed either by the paired t-test or by Wilcoxon's matched pairs signed ranks test, as appropriate.
Sub-study measurements comprised levels of vitamins A and E, and alpha- and beta-carotene at Visit 3 and Visit 5. The values of these parameters at Visits 3 and 5, the change from Visit 3 to Visit 5, and the relative change from Visit 3 to Visit 5 were analysed by analysis of covariance. Age was included as a covariate in all analyses; the value at Visit 3 was included as a covariate in the analyses of the absolute value post-treatment and the change from Visit 3 to Visit 5. The carotene values were non-normally distributed, and were therefore ranked prior to analysis. The significance of the difference from 0 of the change and relative change from Visit 3 within each group was analysed either by the paired t-test or by Wilcoxon's matched pairs signed ranks test, as appropriate. In view of the non-normality of the distributions of the carotene levels, the mean difference between the value in each treated group and the value in the placebo group was not computed. Dunnett's test was used to determine the significance of any difference between each treated group and the placebo group with respect to values at the two visits and the changes and relative changes between visits.

For all safety analyses the intent-to-treat (ITT) population, defined as all subjects who were randomised, took any study treatment and had at least one efficacy data point after baseline, was used . The efficacy analyses were done on the per-protocol population (PP), as defined by inclusion/exclusion criteria, at least 80% compliance (as measured by number of study treatment doses taken divided by the number of doses that should have been taken between Visits 3 and 5), no more than 3 days off treatment prior to Visit 5, Visit 5 no more than 6 weeks after Visit 3.

RESULTS

General
One hundred and thirty-two subjects were enrolled (33 per group) and 123 subjects (93%) completed the trial. Three subjects discontinued study treatment due to an adverse event: one subject, a 65 year-old male who was randomised to TOS at a dose of 0.9 g/day,

discontinued because of severe pain in his right kneecap, which started 7 days after he started study treatment and was recorded as definitely not related to treatment. One subject, a 76 year-old female who was given TOS a dose of 1.8 g/day, discontinued because of moderate constipation which started after she had been on study treatment for 19 days, and was recorded as possibly related to treatment. One subject, a 50 year-old female who was given TOS at a dose of 0.9 g/day, was discontinued for elevated ALT and AST. The adverse event was rated as severe, but probably not related to study treatment; the event's start date was actually recorded as one day prior to the subject starting to take study treatment. Her AST was 37 IU/L at Visit 0, 47 IU/L at Visit 3 and 57 IU/L at Visit 5; her ALT was 59 IU/L at Visit 0, 74 IU/L at Visit 3 and 90 IU/L at Visit 5. The laboratory's upper limit of normal for females for both parameters is 34 IU/L. One subject given 0.9 g/day TOS was discontinued after Visit 4 when it was discovered that he had never stopped taking daily vitamin A and niacin, which constituted a protocol deviation. One subject given 3.6 g/day TOS received his initial supply of study treatment, but then did not receive the follow-up supply by mail. 4 dropped out due to personal reasons, e.g. 1 subject was not able to drink the 3 required portions per day. There was no difference between treatment groups with respect to the proportion of subjects who completed the trial.

Seventy-eight (59%) of the 132 subjects randomised were male; 98 (74%) were white, 21 (16%) black, four (3%) Asian and nine (7%) some other race. There was no significant difference between treatment groups with respect to either of these two variables.

The subjects baseline characteristics are summarised in table 3. The age of the subjects randomised to the 4 treatments group differed significantly ($p < 0.05$); specifically, subjects given 1.8 g/day TOS were eight years older, on average, than those given placebo ($p < 0.05$). Baseline total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides levels, and blood pressure did not differ significantly between groups.

Table 3: Baseline characteristics of subjects in placebo and treatment groups

Variables	Placebo Drink	TOS Drink 0.9 g/d	TOS Drink 1.8 g/d	TOS Drink 3.6g/d
Age (y)	52.2 (48.1,56.2)[#]	57.4 (54.2,60.6)	60.5 (55.7,65.2)	54.4 (50.5,58.3)
Weight (kg)	81.4 (76.1,86.8)	81.0 (75.6,86.4)	74.8 (69.5,80.2)	80.9 (75.1,86.8)
BMI (kg/m^2)	27.7 (26.5,29.0)	27.2 (25.9,28.6)	26.9 (25.2,28.6)	27.4 (26.1,28.6)
Lipids (mg/dL)				
Total Cholesterol	245.5 (235.3,255.7)	237.8 (227.7,247.8)	242.3 (232.1,252.5)	237.6 (227.5,247.6)
LDL Cholesterol	166.9 (158.5,175.4)	159.7 (151.4,168.1)	161.0 (152.5,169.5)	159.9 (151.6,168.3)
HDL Cholesterol	48.8 (44.7,52.9)	51.4 (47.3,55.4)	50.6 (46.5,54.7)	49.5 (45.4,53.5)
Triglycerides	151.1 (127.6,174.6)	135.2 (112.0,158.3)	153.5 (130.0,177.1)	141.3 (118.1,164.5)
Blood pressure (mm Hg)				
SBP	111.0 (106.2,115.7)	117.8 (113.3,122.3)	114.7 (109.1,120.3)	114.4 (109.3,119.5)
DBP	72.6 (70.1,75.1)	74.7 (71.9,77.5)	70.9 (68.0,73.7)	75.0 (72.1,78.0)

[#]Mean (95% confidence interval)

Body weight and diet

There were slight but significant increases in weight among all subjects given placebo ($p < 0.005$), 0.9 g/day TOS ($p < 0.005$) and 1.8 g/day TOS ($p < 0.05$). The maximum increase in weight was 3.9 kg for male subjects and 2.9 kg for female subjects, both in the placebo group. There was no significant difference between groups with respect to the changes experienced during the study (Table 4).

Dietary nutrient intake was monitored by a dietary questionnaire and an EPAT score was calculated. There was a slight decrease in all four groups, which reached statistical significance in the group given 1.8 g/day TOS ($p < 0.05$). None of the other changes reached

statistical significance, and there was no significant difference between treatment groups (Table 4).

More infos re EPAT requested from Prorocare

Table 4 : Change from baseline in weight and diet (EPAT scores)

Treatment group	Mean	95% C.I.	Minimum	Median	Maximum
Weight (kg)					
Placebo	0.8	(0.3,1.4)	-3.8	1.0	3.9
0.9 g/day	0.5	(0.2,0.8)	-1.1	0.4	2.9
1.8 g/day	0.4	(0.0,0.8)	-2.5	0.6	2.4
3.6 g/day	0.5	(-0.1,1.0)	-4.2	0.5	3.0
EPAT scores					
Placebo	-0.5	(-2.0,1.0)	-11	0.0	6
0.9 g/day	-1.0	(-2.3,0.3)	-10	-1.0	7
1.8 g/day	-1.5	(-2.8,-0.2)	-11	-1.5	7
3.6 g/day	-2.0	(-4.0,0.1)	-18	-1.0	8

Blood lipids

Table 5 shows lipid values post-treatment, the relative change from baseline, and the relative difference from placebo. For total cholesterol, there were highly significant differences between treatment groups with respect to all three parameters ($p < 0.001$). The groups given 1.8 g/day TOS and 3.6 g/day TOS experienced significant decreases in total cholesterol levels during treatment. At the end of the treatment period, these two groups had total cholesterol levels which were significantly lower than those in the placebo group, and decreases in total cholesterol which were significantly different from the small increase in the placebo group.

A significant difference between treatment groups in the absolute value post-treatment ($p < 0.001$) was seen for LDL cholesterol. The values in the three groups treated with TOS were all significantly lower than the value in the group given placebo. All three treated groups had significant absolute and relative decreases in LDL cholesterol. The change and relative change in LDL cholesterol post-treatment differed between the four groups (both $p < 0.001$), and all three treated groups experienced changes which were significantly different from the change in the placebo group.

For HDL cholesterol, there was no overall difference between treatment groups in absolute HDL cholesterol levels post-treatment. However, the groups given TOS in dosages of 0.9 g/day and 1.8 g/day had values which were significantly higher than the value in the placebo group. The group given 0.9 g/day TOS experienced significant absolute and relative increases in HDL cholesterol during treatment, but there was no difference between groups with respect to either the change or the relative change in HDL cholesterol post-treatment.

LDL/HDL ratios were significant different between treatment groups at the end of the treatment period ($p < 0.001$), and all three treated groups had significantly lower ratios than the placebo group post-treatment. Similarly, there were highly significant differences between groups with respect to the change and relative change in the LDL/HDL ratio from baseline (both $p < 0.001$), and all three treated groups had significant absolute and relative decreases which differed from the increase in the placebo group.

The group given 0.9 g/day TOS had significantly lower triglycerides levels post-treatment than the placebo group. There was no other significant difference between groups, and none of the changes observed in triglycerides levels reached statistical significance at the 5% level.

Table 5: Total, LDL, and HDL cholesterol concentration , LDL/HDL ratio, and triglycerides concentrations after a 4 week consumption of milk drinks with or without TOS fortification.

Variables	Placebo Drink	TOS Drink		
		0.9 g/d	1.8 g/d	3.6g/d
	(n=24)	(n=23)	(n=28)	(n=23)
Total Cholesterol				
Absolute (mg/dL)	242 (235,249)[#]	232 (224,239)	229 (222,235)*	220 (212,227)****
Change from baseline (mg/dL)	3.4 (-3.9,10.8)	-6.9 (-14.4,0.5)	-9.9 (-16.7,-3.1)***	-18.9 (-26.3,-11.5)****
Change from baseline (%)	1.3 (-1.8,4.3)	-2.8 (-5.8,0.3)	-4.0 (-6.7,-1.2)***	-7.8 (-10.8,-4.7)****
Difference to Placebo (%)	-	-4.3 (-9.4, 0.8)	-5.5 (-10.4,-0.6)*	-9.1 (-14.3,-4.0)****
LDL Cholesterol				
Absolute (mg/dL)	164 (158,171)	152 (145,159)*	150 (144,156)***	142 (136,149)****
Change from baseline (mg/dL)	3.4 (-3.2,10.1)	-9.2 (-15.9,-2.5)**	-11.2 (-17.3,-5.1)****	-18.7 (-25.4,-12.0)****
Change from baseline (%)	1.9 (-2.1,5.9)	-5.4 (-9.5,-1.4)**	-6.6 (-10.3,-3.0)****	-11.3 (-15.3,-7.3)****
Difference to Placebo (%)	-	-7.4 (-14.2,-0.6)*	-8.6 (-15.1,-2.1)**	-13.2 (-20.0,-6.5)****
HDL Cholesterol				
Absolute (mg/dL)	49 (47,51)	53 (51,55)****	51 (50,53)*	50 (48,52)
Change from baseline (mg/dL)	-0.5 (-2.5,1.5)	2.8 (0.8,4.9)*	1.4 (-0.4,3.3)	0.2 (-1.8,2.2)
Change from baseline (%)	-0.5 (-4.7,3.7)	5.8 (1.5,10.0)*	2.6 (-1.2,6.5)	0.5 (-3.8,4.8)
Difference to Placebo (%)	-	5.0 (-2.2,12.2)	1.9 (-4.9,8.8)	0.5 (-6.7,7.7)
LDL/HDL ratio				
Absolute	3.5 (3.4,3.7)	3.1 (2.9,3.2)****	3.1 (3.0,3.3)****	3.0 (2.8,3.1)****
Change from baseline	0.1 (-0.0,0.3)	-0.4 (-0.5,-0.2)****	-0.3 (-0.4,-0.1)****	-0.4 (-0.6,-0.3)****
Change from baseline (%)	3.5 (-1.0,8.0)	-10.2 (-14.8,-5.6)****	-8.6 (-12.7, -4.4)****	-11.5 (-16.0, -6.9)****
Difference to Placebo (%)	-	-12.7 (-20.4,-5.0)****	-11.1 (-18.4,-3.7)***	-14.6 (-22.3,-6.8)****
Triglycerides				
Absolute (mg/dL)	142 (129,155)	133 (120,147)**	138 (126,150)	137 (123,150)
Change from baseline (mg/dL)	3.6 (-9.6,16.9)	-4.9 (-18.4,8.5)	-0.5 (-12.7,11.6)	-1.6 (-15.0,11.7)
Change from baseline (%)	1.6 (-9.2,12.3)	-0.1 (-11.0,10.8)	2.3 (-7.6,12.2)	3.8 (-7.1, 14.6)
Difference to Placebo (%)	-	-1.3 (-19.6,17.0)	1.1 (-16.4,18.6)	2.4 (-16.0,20.7)

[#]Mean (95% confidence interval)
Asterisks indicate a significant difference to placebo or to baseline: *P<0.05, **P<0.01, ***P<0.005, ***P<0.001

(Pro)Vitamins

Table 6 shows vitamin A and E and alpha- and beta-carotene values at Visits 3 and 5, together with the absolute and relative change from Visit 3 to Visit 5. At Visit 3, there was no overall difference between treatment groups in vitamin A levels, but subjects given 1.8 g/day TOS had significantly lower levels of vitamin A than the placebo group ($p < 0.05$). Subjects given the placebo had a 4.4% increase in vitamin A levels between Visits 3 and 5, which was significant at the 5% level. At Visit 5, there was still no overall difference between treatment groups, but the placebo group had significantly higher levels of vitamin A than all three treated groups. There was no significant difference between treatment groups with respect either to the change or to the relative change in vitamin A between Visits 3 and 5. Vitamin E concentrations were not affected by TOS consumption. No significant difference between groups were seen at visit 3 or 5, and none of the changes or relative changes was significantly different from 0.

No significant difference between treatment groups either at Visit 3 or at Visit 5 was seen in alpha-carotene levels. Subjects randomised to 3.6 g/day TOS experienced a significant decrease in alpha-carotene levels between Visits 3 and 5 ($p < 0.01$), and this decrease was significantly different ($p < 0.05$) from the small increase observed in the placebo group. None of the relative changes in alpha-carotene was significantly different from 0, and there was no difference between treatment groups on this parameter. Beta-carotene levels showed no significant difference between groups, and none of the changes or relative changes was significantly different from 0.

Table 6: Vitamin A and E, and alpha- and beta-carotene before and after a 4 week consumption of milk drinks with or without TOS fortification.

Variables	Placebo Drink	TOS Drink		
		0.9 g/d	1.8 g/d	3.6g/d
	(n=31)	(n=32)	(n=33)	(n=33)
Vitamin A				
Visit 3 (umol/L)	2.32 (2.15,2.49)[#]	2.14 (2.01,2.27)	2.06 (1.91,2.21)*	2.15 (2.02,2.29)
Visit 5 (umol/L)	2.39 (2.24,2.53)	2.14 (1.97,2.31)***	2.09 (1.93,2.25)****	2.20 (2.05,2.34)**
Change V5 to V3 (umol/L)	0.07 (-0.02,0.15)	-0.02 (-0.14,0.11)	0.01 (-0.09,0.12)	0.01 (-0.10,0.12)
Change V5 to V3 (%)	4.4. (0.1,8.7)	-0.6 (-6.6,5.3)	0.8 (-3.9,5.5)	1.5 (-3.8,6.8)
Vitamin E				
Visit 3 (umol/L)	37.6 (33.3,42.0)	38.5 (33.3,43,8)	37.7 (33.8,41.7)	35.4 (31.9,39.0)
Visit 5 (umol/L)	38.7 (34.3,43.0)	36.2 (32.3,40.1)	38.0 (34.8,41.3)	35.2 (31.9,38.6)
Change V5 to V3 (umol/L)	1.0 (-1.7,3.7)	-2.5 (-7.6,2.7)	0.2 (-2.6,2.9)	-0.1 (-2.6,2.3)
Change V5 to V3 (%)	5.0 (-1.2,11.3)	-1.5 (-10.5,7.6)	4.0 (-5.5,13.5)	1.1 (-5.5,7.6)
Alpha-Carotene				
Visit 3 (umol/L)	0.31 (0.19,0.43)	0.29 (0.27,0.52)	0.40 (0.27,0.52)	0.45 (0.29,0.60)
Visit 5 (umol/L)	0.39 (0.24,0.53)	0.29 (0.21,0.37)	0.43 (0.29,0.57)	0.30 80.21,0.38)
Change V5 to V3 (umol/L)	0.07 (-0.06,0.20)	0.00 (-0.08, 0.08)	0.03 (-0.09, 0.16)	-0.15 (-0.28,-0.02)**
Change V5 to V3 (%)	75.8 (8.2,143.4)	16.4 (-10.6,43.4)	71.0 (2.4,139.6)	-9.7 (-34.0,14.7)
Beta-Carotene				
Visit 3 (umol/L)	0.85 (0.58,1.13)	1.02 (0.75,1.29)	1.23 (0.90,1.56)	1.09 (0.72,1.47)
Visit 5 (umol/L)	0.92 (0.56,1.28)	1.00 (0.70,1.31)	1.12 (0.86,1.38)	0.93 (0.67,1.20)
Change V5 to V3 (umol/L)	0.08 (-0.30,0.45)	-0.01 (-0.32,0.30)	-0.11 (-0.41,0.18)	-0.16 (-0.40, 0.08)
Change V5 to V3 (%)	80.9 (-14.6,176.5)	27.6 (-3.7,58.9)	39.5 (-17.0,96.1)	53.6 (-43.3,150.5)

[#]Mean (95% confidence interval)
Asterisks indicate a significant difference to placebo or to baseline: *P<0.05, **P<0.01, ***P<0.005, ***P<0.001

DISCUSSION

In the present double blind, placebo controlled study in mildly hypercholesterolemic subjects a milk drink enriched with different levels of TOS reduced both LDL and total cholesterol in a dose dependant manner. The daily intake of a low dose of 0.9 g TOS already yielded in significant decrease in LDL-cholesterol and in a highly significant decrease of LDL/HDL ratio. The consumption of the non fat based milk drink containing TOS resulted in decreases in total cholesterol of 4.3 – 9.1%, in 7.4 – 13.2% in LDL cholesterol, and in 12.7 – 14.6% in the LDL/HDL ratio. HDL was either not changed or significantly increased (+5.8% in 9.9/d TOS group).

Several short and long term studies (e.g. Vanhanen et al, 1993; Miettinen et al, 1995, Weststrate & Meijer, 1999, Hendriks et al, 1999) have shown that a daily intake of sterols or stanols can significantly lower total and LDL cholesterol by up to 10-15% with no change in HDL cholesterol. Sterol/stanol doses varied from 0.7 g per day up to 3.4 g per day and generally larger effects were seen with larger doses. However, studies investigating the dose dependancy for lipid lowering by sterols/stanols are rare. Miettinen et al (1995) compared the effects of 2 doses in a long term study. However the design of the study was not such to clearly differentiate the effects of the different doses. Subjects consumed for the first 6 months 2.6 g/d, for the remaining 6 months of this 12 months study half of the subjects stayed at 2.6 g/d where as the other half reduced the dose to 1.8 g/d. At the end of the study the difference between the 2 doses was a little as 0.2 mmol/L (7.7 mg/dL) suggesting that the concentration dependency of cholesterol lowering may already be flattened out at doses bigger then 2 g.

Check Hallikainen et al, 2000, J. Nutr 130(4):767-776 → Dose response study!!!!

Hendriks et al (1999) studied the dose-response effects of a soy bean derived esterified sterol mixture in a carefully designed study. The 3 doses used (0.83 g, 1.61 g, 3.24 g) did not show a strong tendency of increased effects of higher doses. While the lowest dose reduced total cholesterol by 4.9% the highest dose reduced it by 6.8%. A similar tendency was seen for LDL cholesterol (6.7% vs 9.9%) as well as for LDL/HDL ratios (6.5% vs 7.8%). The 4 fold increase in dose resulted in a further LDL lowering of 48% (relative) or 0.1 mmol/L (3.8 mg/dL). In our study dose dependency was somewhat stronger. While the lowest dose of 0.9 g/d TOS resulted in a similar lowering in both total (4.3%) and LDL cholesterol (7.4%), the highest dose (3.6 g/d) resulted in a bigger reduction (TC: 9.1%, LDL 13.2%). The 4 fold increase reduced LDL by a further 0.25 mmol/L (9.5 mg/dL) or a relative 78%. The 2 studies differed in many things but the probably 2 most relevant were the population and the sterol form. Hendriks et al (1999) studied the effects in normocholesterolaemic subjects with an initial cholesterol level of approx. 5.2 mmol/L (200 mg/dL). In our study subjects were mildly hypercholesterolemic with a starting total cholesterol level of about 6.25 mmol/L (240 mg/dL). Clearly, there is a correlation between the degree of hypercholesterolemia and the degree of cholesterol reduction. The effect is more marked in subjects with elevated cholesterol levels (Pollak & Kritchevsky, 1981).
Hendriks and co-workers used in their study sterol-esters whereas we used the free and unesterified from. Beside differences in composition and source of the mixtures (soy versus tall oil) this difference may be important in regard of effect. Esterified phytosterols and stanols are converted *in vivo* to, and are physiologically equivalent to, proportional amounts of free phytosterols and stanols. Evidence indicates that the active form of the sterol esters is the free sterol. Pancreatic cholesterol esterase hydrolyses both cholesterol esters and phytosterol esters to their free forms [Swell et al, 1954]. Cholesterol is not absorbed in the esterified form but must first be cleaved before it can be absorbed into the body. Hellman et al [1953] fed labeled cholesterol to rats and observed that the labeled sterol appeared in the lymph in the free fraction before it appeared in the ester fraction. Although the above experiment has not been performed with phytosterol esters, it can be inferred that only phytosterols in the free form are absorbed. Direct comparisons between free phytosterols and esterified phytosterols in the rat found that the esterified forms were less effective as inhibitors of cholesterol absorption [Pollak & Kritchevsky, 1981]. Similarly, Mattson et al [1982] reported lower efficacy of sitosterol oleate than free sitosterol in inhibiting absorption of cholesterol in the human and stigmasterol oleate was less effective than free stigmasterol in decreasing cholesterol absorption in the human [Matson et al, 1977]. These results indicate that cleavage of the sterol esters controls the availability of phytosterols for interaction with the cholesterol absorption mechanism. It can also be inferred that it is primarily the free phytosterols which interact with the cholesterol absorption mechanism.

To our knowledge the present trial is the first study showing that a non fat based food is an excellent vehicle for delivering the benefits of sterols. In the past most studies used

margarine or mayonnaise as delivery vehicle for the sterols/stanols. The fat content of these products were in the range of about 80% and only more recent studies (Hallikainen & Uusitupa, 1999) used fat reduced products (40% fat , w/w). Heinemann et al (1986), Becker et al (1992, 1993) and Denke at al (1995) used pastils or capsules for sterol/stanol delivery. In capsules sterols were suspended in oil. While Heinemann's and Becker's studies were rather small (below 10 patients) they showed significant cholesterol lowering effects. Denke and co-workers however failed to show any cholesterol lowering effect. This was probably due to timing of administration. For best effects phytosterols should be ingested with meals in order to block absorption of endo- and exogenous cholesterol form the intestinal lumen (Pollak & Kritchevsky, 1981). This can be easily achieved if phytosterols are delivered via a food.

While the positive action of plant phytosterols is wildly accepted and regarded as a useful dietary alternative to drug treatment for mildly hypercholesterolemic subjects, there is some concern about the possible negative effect of sterols/stanols on fat soluble vitamins and provitmains. The reduction of cholesterol by plant sterols is thought mainly due to decreased cholesterol absorption, therefore sterols may also interfere with the absorption of fat soluble vitamins. In a long term study using sitostanol ester enriched spreads Gylling et al (1996) showed that alpha-tocopherol and carotene concentrations were significantly decreased after consumption of 2 or 3 g. Lipid standardised alpha-tocpherol levels did however not change. Weststrate & Meijer (1998) recorded a significant decrease of approx. 20% of (alpha&beta)-carotene (lipid standardised or not) after consumption of 3 g esterified plant sterols. In a recent study Hendriks et al (1999) discovered the same effects as Weststrate but additionally showed that the lowering effects were dose-dependant. The highest dose of 3.24 g of esterified plant sterols decreased plasma lipid soluble vitamins and carotenoids by 8 to 20%, effects were somewhat reduced a lower plant sterol doses of 1.61 g and 0.83 g per day. The plasma reductions were however only significant for carotenoids. Hallikainen & Uusitupa (1999) showed a significant decrease in the concentration of beta-carotene and alpha-tocopherol after 8 week consumption of 2.2 g esterified sterols. However, there were no significant changes when the values were related related to the serum total cholesterol concentration. In the study of Hallikainen and co-workers (1999) beta-carotene concentrations decreased significantly after consumption of both approx.2.2 g vegetable or tall oil derived stanol esters. Gylling et al (1999) investigated whether sitostanol ester margarine affects serum levels of (pro)vitmains. Vitamin D, retinol and the ratio of alpha-tocopherol to cholesterol were unchanged by sitostanol esters. Beta- and alpha-carotene were significantly reduced in the sitostanol group from baseline and in relation to controls by up to 25%.
In contrast to the above studies with esterified plant sterols Sierksam et al (1999), investigating the cholesterol lowering effects of free soy sterols, could not detect any negative effect on (alpha&beta)-carotene concentrations. This may have been an effect of the low daily intake of 0.8 g per day. In our study, the consumption of free sterols had no negative effect on vitamin A, vitamin E and beta-carotene concentrations. There was no significant difference between treatment groups with respect to either change or relative change. Alpha-carotene levels did not show any significant difference between treatment groups, however subjects consuming 3.6g/d TOS experienced a significant decrease in alpha-carotene levels during the 4 week treatment phase. It is unclear why free sterols may have no lesser negative effect on carotenoid concentrations and further studies will be needed to verify and understand the reported finding.

CONCLUSIONS

This present study shows that the daily consumption of 3 milk drinks delivering 0.9 g, 1.8 g, or 3.6 g of TOS significantly lowers total and LDL cholesterol in mildly hypercholesterolemic subjects. Total cholesterol was lowered by 6.9 – 18.9 mg/dL, LDL cholesterol was reduced by 9.2 – 18.7 mg/dL. LDL/HDL ratios were also significantly decreased by 0.28-0.43 in the 3

treatment groups This effects were seen without any negative effects on serum concentrations of vitamin A and E and beta-carotene. Alpha-carotene did not differ significantly between the active groups and placebo, however was significantly reduced during the 4 week treatment phase in the group consuming 3.6g/D TOS.
A milk drink can serve as a useful delivery vehicle for phytosterols and positively influence lipid status of mildly hypercholesterolemic subjects.

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Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects

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Objective: To investigate the dose-response relationship between cholesterol lowering and three different, relatively low intake levels of plant sterols (0.83, 1.61, 3.24 g/d) from spreads. To investigate the effects on lipid-soluble (pro)vitamins.
Design: A randomized double-blind placebo controlled balanced incomplete Latin square design using five spreads and four periods. The five study spreads included butter, a commercially available spread and three experimental spreads fortified with three different concentrations of plant sterols.
Subjects: One hundred apparently healthy normocholesterolaemic and mildly hypercholesterolaemic volunteers participated.
Interventions: Each subject consumed four spreads, each for a period of 3.5 week.
Results: Compared to the control spread, total cholesterol decreased by 0.26 (CI: 0.15–0.36), 0.31 (CI: 0.20–0.41) and 0.35 (CI: 0.25–0.46) mmol/L, for daily consumption of 0.83, 1.61 and 3.24 g plant sterols, respectively. For LDL-cholesterol these decreases were 0.20 (CI: 0.10–0.31), 0.26 (CI: 0.15–0.36) and 0.30 (CI: 0.20–0.41). Decreases in the LDL/HDL ratio were 0.13 (CI: 0.04–0.22), 0.16 (CI: 0.07–0.24) and 0.16 (CI: 0.07–0.24) units, respectively. Differences in cholesterol reductions between the plant sterol doses consumed were not statistically significant. Plasma vitamin K1 and 25-OH-vitamin D and lipid standardized plasma lycopene and alpha-tocopherol were not affected by consumption of plant sterol enriched spreads, but lipid standardized plasma (alpha + beta)-carotene concentrations were decreased by about 11 and 19% by daily consumption of 0.83 and 3.24 g plant sterols in spread, respectively.
Conclusions: The three relatively low dosages of plant sterols had a significant cholesterol lowering effect ranging from 4.9–6.8%, 6.7–9.9% and 6.5–7.9%, for total, LDL-cholesterol and the LDL/HDL cholesterol ratio, respectively, without substantially affecting lipid soluble (pro)vitamins. No significant differences in cholesterol lowering effect between the three dosages of plant sterols could be detected. This study would support that consumption of about 1.6 g of plant sterols per day will benefically affect plasma cholesterol concentrations without seriously affecting plasma carotenoid concentrations.
Sponsorship: Unilever Research Vlaardingen, NL
Descriptors: cholesterol-lowering diet; plant sterols; dosage; spreads; lipid soluble (pro)vitamins

Introduction

One of the major modifiable risk factors for coronary heart disease (CHD) mortality is plasma cholesterol concentration. Several large scale intervention trials have shown that cholesterol lowering drug therapy in hypercholesterolaemic patients beneficially affects CHD mortality risk (Gould *et al*, 1998). Mildly hypercholesterolaemic subjects usually have dietary guidelines prescribed as a first treatment to lower their blood cholesterol. Normocholesterolaemic subjects may apply such a diet as a part of a healthy life-style. However, the percentage reduction in blood total cholesterol attributable to dietary advice is modestly effective in free-living subjects; that is a reduction of total cholesterol varying between 3 and 6% (Tang *et al*, 1998). Dietary factors contributing significantly to a reduction of total cholesterol by dietary advice include high concentrations of linoleic acid and low concentrations of saturated fatty acids (Keys *et al*, 1965; Mensink & Katan, 1992: Katan *et al*, 1994).

The cholesterol lowering properties of plant sterols have been known since the 1950s (Pollak, 1952). High dosages of plant sterols have been applied to lower plasma cholesterol concentrations in hypercholesterolaemic subjects (Ling & Jones, 1995). No obvious side effects have been observed in humans, except in individuals with phytosterolemia (Lütjohann & Bergmann, 1997). The mechanisms of hypocholesterolaemic action may include inhibition of cholesterol absorption (Pollak & Kritchevsky, 1981). Recently, it was suggested that substitution of sitostanol-ester enriched spread for a portion of normal dietary spread is suitable as a strategy to reduce serum cholesterol in the

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population. A one-year intervention study (Miettinen *et al,* 1995) showed that consumption of sitostanol-ester enriched spread by subjects with mild hypercholesterolaemia was effective in lowering serum total cholesterol and LDL cholesterol by 10 and 14%, respectively. Sitostanol-ester, which is derived from hydrogenation of sitosterol from pine tree woodpulp and subsequently esterified with a free fatty acid, is not the only effective cholesterol lowering plant sterol. Sterols from commonly used edible oils (soybean, rapeseed and sunflower oils), that is the 4-desmethylsterols sitosterol, campesterol and stigmasterol, have a similar cholesterol lowering effect (Weststrate & Meijer, 1998).

However, plant sterols, including stanols, may also lower plasma concentrations of other lipophilic compounds as reported for carotene, lycopene and alpha-tocopherol (Weststrate & Meijer, 1998; Gylling *et al,* 1996). Therefore, an optimal plant sterol or stanol intake level has to be selected, reducing cholesterol concentrations optimally, but having minimal effect on other lipophilic compounds. Relatively few studies, however, have evaluated the dose-effect relation of dietary plant sterols or stanols. Usually one intake level has been applied in efficacy studies using mildly hypercholesterolaemic subjects. Sitostanol and sitostanol ester dosages in the various studies ranged from 0.7 g/d (Miettinen & Vanhanen, 1994) to 3.4 g/d (Pollak, 1952). Miettinen *et al,* 1995 compared 1.8 g/d and 2.6 g/d of sitostanol ester in the second part of his study showing a slightly stronger cholesterol lowering effect of the high dose on both total and LDL cholesterol. Sitosterol has been consumed in dosages ranging from 0.7 g/d (Miettinen & Vanhanen, 1994) to 6 g/d (Weisweiler *et al,* 1984) in hypercholesterolaemic subjects and soy bean oil sterols in dosages from 0.7 g/d (Pelletier *et al,* 1995) to 3.0 g/d (Mensink & Katan, 1992) in normocholesterolaemic and mildly hypercholesterolaemic volunteers.

The main objective of this present study was to investigate the dose-dependency of the cholesterol-lowering effects of three relatively low dosages, namely 0.83, 1.61 and 3.24 g/d, of plant sterols derived from commonly used edible oils in spreads. These three spreads, and a control spread of similar fatty acid composition but not enriched in plant sterols and butter were used by normocholesterolaemic and mildly hypercholesterolaemic subjects consuming their habitual Dutch diet. In addition, the effects on lipid-soluble (pro)vitamins including (alpha + beta)-carotene, lycopene, alpha-tocopherol, vitamin K1 and 25-OH-vitamin D were evaluated.

Subjects and methods

The study was conducted according to Good Clinical Practice at the TNO Nutrition and Food Research Institute, Zeist, Netherlands. The study protocol was approved by the TNO Medical Ethical Committee.

Subjects

Subjects were recruited from a pool of volunteers containing no TNO employees. Respondents received a verbal briefing and received the same information in writing. They signed for informed consent and filled in a questionnaire on life style, disease history and dietary habits. Each of the respondents was physically examined and blood was collected after an overnight fast, for routine blood chemistry.

A total of 147 subjects was screened of which 100 were included in the study. All volunteers included were apparently healthy. Health status was assessed by disease history questionnaire, physical examination and routine blood chemistry. Eligible subjects did not suffer from chronic gastrointestinal complaints and/or cardiovascular disease, high blood pressure (according to WHO guidelines), high blood cholesterol (fasting serum total cholesterol < 7.5 mmol/L). They also did not use prescribed medication (except oral contraceptives) and were not on a diet for medical reasons. Eligible subjects reported intensive exercise for not more than 10 h/week, consumption of a habitual Dutch diet including the use of spreads (assessed by dietary questionnaire) and consumption of alcoholic beverages for less than 22 units/week when female and for less than 29 units/week when male.

Experimental design

The study had a double-blind, placebo-controlled, balanced incomplete Latin square design using five spreads and four periods. This design controls for between subject variation and for variation over time. In addition this design results in a balanced randomization of treatment orders over the subjects, so that any systematic effect of the order in which treatments were given will not create bias in the comparison of the treatment means. Subjects received in four consecutive periods of 24 or 25 d (3.5 weeks) 25 g spread per day. The included subjects were randomly allocated to the spreads. One hundred volunteers were included and consequently data were obtained for each spread from eighty subjects. The five spreads included butter and a control spread, that is a commercially available spread Flora (Van den Bergh Foods, Crawley, UK) and three test spreads, which were spreads fortified with three concentrations of plant sterols, namely 3.37% (w/w), 6.47% (w/w) and 13.06% (w/w) derived from commonly used edible oils. These three test spreads should provide 0.85 g/d, 1.62 g/d and 3.26 g/d of plant sterols, respectively, assuming 100% compliance of the subjects to the treatments. Flora has a very similar fat composition as the spreads containing plant sterols, and was used as the control spread for the quantitative evaluation of the cholesterol lowering effects of plant sterols. Butter was used as alternative, commonly used spread, with a completely different fat composition. The spreads were intended to replace an equivalent amount of the spread(s) habitually used by the volunteers. To ensure all the spread was consumed, the volunteers were instructed not to use it for cooking or frying. Half a portion was to be used with lunch and the other half with dinner, for example by mixing it with the meal on the subjects' plate or by spreading it on bread or toast eaten at dinner. Volunteers and TNO personnel were blinded. Unilever packed all test spreads in tubs of 25 g and delivered them in boxes with a blind code. The tubs were subsequently labelled with the subject number and study period.

Spreads were dispensed to the subjects twice per spread period. The volunteers received a leaflet containing instructions on storage conditions and consumption of the spreads, containing also space for the specification of deviations from the instructions. Each subject was provided with one portion for each study day and four spare portions per study period in case of damage or loss. Portions not consumed (including spare portions) were returned to TNO halfway and at the end of the period. Compliance was calculated based on subjects specification of deviations and on the number of portions returned. In case of conflicting

information, the worst case was assumed. During the study, subjects maintained their daily routines and used a self-selected diet.

Fasting blood was sampled at the end of each period (after 3.5 weeks). Analyses were carried out after ending the clinical part of the study when all blood samples were collected. Body weight was measured half way and at the end of each period. Also, health status and medicine use were registered by questionnaire half way and at the end of each period. Adverse events were reported by the volunteers by filling in a questionnaire halfway and at the end of each period. The medical investigator consulted the volunteers when additional information was needed. Dietary intake was assessed with a food frequency questionnaire at the end of each period.

Spreads

The plant sterol enriched spreads were prepared by Van den Bergh Foods, Purfleet, UK. The spreads were fortified with plant sterol (-ester) concentrates derived from vegetable (predominantly soybean) oil distillates (Henkel Corporation, LaGrange, USA). The vegetable oil sterols were esterified with fatty acids from sunflower-seed oil to an esterification degree of 82% (Unilever Research Laboratory, Vlaardingen, The Netherlands). The sterol concentrates were refined and used in spread production together with other edible oils and fats (sunflower-seed oil, rapeseed oil and hard stock) to produce spreads as close as possible in fatty acid composition as the non-fortified control. Total fat content, fatty acid composition, free sterols, carotenoids and alpha-tocopherol content of spreads were measured as described previously (Weststrate & Meijer, 1998).

The butter and spread composition is given in Table 1. As expected, the plant sterol enriched spreads have a fatty acid composition very similar to that of the control spread (Flora), while the fatty acid composition of butter is different. Butter contains more saturated fatty acids as compared to Flora (59.9 and 15.3 g/100 g, respectively). As a consequence, unsaturated fatty acids contribute much less to butter as compared to Flora; oleic acid contents are 15.1 and 20.1 g/100 g, respectively and the sum of linoleic and linolenic acid content are 1.5 and 33.0 g/100 g, respectively. In addition, butter has a higher content of trans fatty acids as compared to Flora (2.9 and 0.4 g/100 g, respectively). The experimental spreads have a trans fatty acid content of 0.8 g/100 g.

Blood lipids and routine blood chemistry

Blood was collected after an overnight fast from the antecubital vein using Vacutainer® tubes. For serum collection, blood was collected in tubes containing clot activator. Blood was centrifuged within one hour after collection and serum stored at −18°C. Alkaline phosphatase, ALP; alanine transaminase, ALT; aspartate transminase, AST; gamma-glutamate transaminase, gamma-GT; total cholesterol; HDL-cholesterol, after precipitation with polyethylene glycol: and triglycerides were determined using commercial test kits (Boehringer, Mannheim, Germany) on a Hitachi 911 automatic analyser (Hitachi Instrument Division, Ibaraki-ken, Japan). Serum LDL-cholesterol concentration was calculated using the formula by Friedewald *et al*, 1972.

Plasma lipid-soluble (pro)vitamins

Blood was collected in ice-chilled tubes containing lithium heparin and put in a cool dark box immediately after collection. Blood was centrifuged within half an hour after collection, and plasma stored at −70°C until analyses. Lycopene, (alpha + beta)-carotene, and alpha-tocopherol were determined by Unilever Research (Vlaardingen, Netherlands) in all samples except for those obtained after butter consumption. Plasma was extracted by addition of ethanol and n-heptane containing ethyl-beta-apo-8′-carotenoate and alpha-tocopheryl-acetate as internal standards, followed by a second extraction step with *n*-heptane/diethyl ether (1:1 (v/v)). A normal phase Nucleosil 5N(CH_3)$_2$ column (Machery & Nagel, Dueren, Germany) was used with a flow of 0.8 ml/min using

Table 1 Composition of spreads

Component	*Butter*	*Flora*[a]	3.37% (w/w) *plant sterols*	6.47% (w/w) *plant sterols*	13.06% (w/w) *plant sterols*
Total fat as fatty acids (g/100 g)	83.6	69.4	70	70.6	69.8
Major fatty acids (g/100 g)					
Total Saturated fatty acids	59.9	15.3	15.3	15.7	15.7
Lauric (C12:0)	3.9	1.7	1.6	1.7	1.7
Myristic (C14:0)	10.7	0.7	0.7	0.7	0.7
Palmitic (C16:0)	28.4	9.3	9.3	9.5	9.6
Stearic (C18:0)	7.8	2.5	2.7	2.7	2.6
Oleic (C18:1 cis)	15.1	20.1	19.6	20	20.3
Linoleic (C18:2, 9c, 12c)	1.1	31.5	32.2	32.2	31
Linolenic (C18:3 9c, 12c, 15c)	0.4	1.5	1.4	1.3	1.4
Total trans	2.9	0.4	0.8	0.8	0.8
Total sterols (mg/100 g)	221	298	3370	6466	13057
Major sterols (mg/100 g)					
Cholesterol	213	2.9	14.2	27.6	111
Brassicasterol		13.9	56.4	101	204
Campesterol	2.2	63.3	825	1597	3290
Campestanol		4	25.4	82.7	122
Stigmasterol		12.6	602	1198	2494
Beta-sitosterol	1.9	159	1629	3044	6233
Sitostanol		–	56	120	162
Delta-5-Avenasterol		9.1	46	96.5	137
Delta-7-Stigmastenol		16.1	27.8	35.9	60.2
Alpha-tocopherol (mg/100 g)	1.8	31.7	32.7	30.1	26.3
(Alpha + beta)-Carotene (mg/100 g)	0.6	0.5	0.7	0.8	0.8

[a]Control: not enriched in sterols.

n-heptane as the mobile phase. The carotenoids were detected at 450 nm, alpha-tocopherol was detected at 294 nm (Weststrate & Van't Hof, 1995).

Plasma carotenoid and alpha-tocopherol concentrations were standardised for plasma lipid (total cholesterol + total triacylglycerol) concentrations, because the sterol enriched spreads affected the concentrations of blood lipoproteins, which are the plasma carriers of carotenoids and alpha-tocopherol.

Vitamin K1 and 25-OH-vitamin D were analysed at the TNO Institute in all samples except for those obtained after butter consumption. For vitamin K1 analyses plasma was extracted by addition of methanol and hexane and 2',3'-dihydrophylloquinone was added as internal standard. Vitamin K1 was analysed by HPLC, using an Inertsil 5 ODS-3 reversed-phase column (Chrompack) with post column electrochemical reduction and fluorimetric detection (Haard *et al*, 1986). For vitamin D analyses plasma was extracted with a mixture of dichloromethane, methanol and water, and 25-OH-vitamin D was isolated by chromatography on a small silica column. The 25-OH-vitamin D content was analysed using a competitive protein binding assay (Berg *et al*, 1991).

Dietary intake

Dietary intake, excluding the spreads, was assessed at the end of each of the four periods using a modified version of the validated TNO food frequency questionnaire (Grootenhuis *et al*, 1995). The questionnaire was applied to assess continuity in the intake of macronutrients, main fats, total energy, alcohol and fibre. The questionnaire was modified to obtain more detailed information on the intake of specific brands of spreads and dietary fats in the habitual diet and consequently to better evaluate fat composition (like SAFA, MUFA, PUFA) of the habitual diet during each period. Subjects completed the questionnaire during their visit to the Institute at the last day of each period. The dietitian then checked the questionnaires for completeness, and, where necessary, asked subjects to check questions at home and return answers by mail. For calculation of the nutrient intake the 'Netherlands Food Composition Table (NEVO)' was used. Specific fat composition data were expanded upon a large number of extra brands of spreads and dietary fats, as provided by Unilever.

Statistical analyses

Differences in variables measured between spreads were evaluated by a two-sided ANOVA, using the following factors: subject, spread, period and residual effects (including carry-over). Two analyses were performed: residual effects corrected for direct effects and direct effects corrected for residual effects. Residual effects were observed for total and LDL-cholesterol and consequently the direct effects were corrected for these residual effects. Residual effects observed were small, and correction for them did not affect the results. In this present study residual effects may well be a consequence of the design and may not have a biological basis.

Since the lipid-soluble (pro)vitamins were not analysed after butter consumption, for those parameters the above mentioned design could not be used. Correction for residual effects was not possible because data for the previous period were incomplete, that is, absent when butter was consumed in the previous period. In addition, the number of spreads (for which data were available) was not the same for all subjects; 3 for 60 subjects and 4 for 20 subjects. Therefore, analysis was carried out with a simplified model not including residual effects and for $n = 60$ subjects.

Data are expressed as the mean ± s.d. Presented changes and their 95% confidence interval (CI) are calculated using the least square means. Data which were not distributed normally were ln transformed before statistical analysis (only (alpha + beta)-carotene and vitamin K1). The percental change and the 95% confidence intervals calculated from the transformed data were transformed again to the original scale in order to facilitate interpretation. The least significant difference between spreads is expressed as a percentage (last column of Table 5). The null hypothesis was rejected at the 0.05 level of probability. The statistical analyses were performed using Genstat 5 Release 3.1 (Lawes Agricultural Trust, Rothamsted Experimental Station).

The study was unblinded after reporting of the adverse events, evaluation and statistical analysis of food intake data and all biochemical data except vitamin D and K.

Results

General

The study started with 100 volunteers, 42 males and 58 females, with a mean age of 37 ± 10 y ranging from 19–58 y, and a mean body mass index of 22.8 ± 2.5 kg/m^2 ranging from 17.7–28.6 kg/m^2. Baseline fasting total-, LDL- and HDL-cholesterol concentrations were 5.10 ± 0.97 mmol/L (range: 2.71–7.42), 2.97 ± 0.83 mmol/L (range: 1.12–5.22), and 1.65 ± 0.38 mmol/L (range: 0.75–2.63), respectively. All participants completed the study.

Compliance was generally very good, only about 1% of the portions of the spreads was not consumed, distributed equally over the five spreads. Average spread consumption was 24.7 g/d for the 3.37% (w/w) plant sterol spread, 24.9 g/d for the 6.47% (w/w) plant sterol spread and 24.8 g/d for the other three spreads. Consequently, the average daily plant sterol consumption was 0.83 g, 1.61 g and 3.24 g, respectively.

Body weights were 72.3 ± 11.0, 71.4 ± 10.9, 72.1 ± 11.2, 72.2 ± 11.2 and 72.0 ± 10.4 kg after butter and Flora consumption and after daily consumption of 0.83, 1.61 and 3.24 g plant sterols, respectively. Body weights differed only slightly after daily consumption of 1.61 and 3.24 g plant sterols, the difference being 0.3 kg ($P < 0.01$). This small difference is not expected to have affected the outcome of this study.

Nutrient intake, excluding spread intake, during the four periods is shown in Table 2. Total fat intake and the contribution of saturated, monounsaturated and polyunsaturated fatty acids to fat intake, dietary cholesterol and energy intake did not change during consumption of spreads, nor did any other calculated dietary intake parameter.

No side effects occurred after the consumption of any of the spreads applied in these concentrations and under these conditions. Side effect testing included the liver enzymes ALP, ALT, AST and gamma-GT (Table 3) and adverse events reporting.

Blood lipids

Total, LDL-, and the LDL/HDL cholesterol ratio were all decreased by plant sterol consumption, while triacylglycerol concentration was not affected (Table 4).

Table 2 Intake of energy, protein, fats, carbohydrates, dietary fibre and alcohol as assessed by a food frequency questionnaire at the end of each treatment period, covering that treatment period. Intakes are expressed as grammes and as percent energy (en%) during consumption of butter, Flora and 0.83, 1.61 and 3.24 g plant sterols. Values do not include intake of spreads[a]

Nutrients	*Butter* ($n=79$)	*Flora* ($n=79$)	0.83 g *plant sterols* ($n=79$)	1.61 g *plant sterols* ($n=79$)	3.24 g *plant sterols* ($n=80$)
Energy (kJ)	9536±2715	9432±2381	9270±3196	9304±2752	9762±3243
Protein (g)					
total	93.1±26.7	91.9±24.6	91.6±35.5	91.2±29.0	94.0±31.6
vegetable	33.8±13.3	33.0±9.8	32.8±12.2	32.8±11.6	34.8±14.5
Fat (g)					
total	87.5±33.0	87.1±28.6	83.1±32.8	85.1±31.8	90.5±35.3
SAFA[b]	34.9±13.5	34.5±11.7	33.6±13.3	33.9±12.9	35.7±14.4
MUFA[b]	30.2±11.6	29.9±10.2	28.2±10.8	29.2±11.1	31.6±12.8
PUFA[b]	15.7±7.8	16.0±6.6	14.6±6.9	15.2±7.4	16.4±8.1
cholesterol (mg)	254.0±94.9	244.8±83.8	249.5±166.9	248.7±100.2	257.1±100.3
Carbohydrates (g)					
total	261.4±79.8	256.4±71.0	259.2±94.3	254.5±77.3	267.4±96.2
mono/-disaccharides	124.5±43.6	124.5±46.2	124.6±54.8	124.2±44.1	128.4±51.3
polysaccharides	136.8±47.4	131.8±39.1	134.6±52.3	130.3±45.3	139.1±54.2
Dietary fibre (g)	26.2±9.2	25.7±6.9	25.7±8.6	25.8±8.2	26.3±9.5
Alcohol (g)	9±11	10±13	8±9	10±12	9±11
Protein (en%)					
total	16.9±2.9	16.8±2.7	17.0±2.8	16.8±2.7	16.6±2.2
vegetable	6.0±1.4	6.0±1.1	6.1±1.2	6.0±1.1	6.0±1.1
Fat (en%)					
total	33.5±6.3	33.9±5.6	32.9±5.2	33.3±5.5	33.9±5.5
SAFA	13.5±3.3	13.5±2.9	13.4±2.5	13.3±2.7	13.5±2.86
MUFA	11.6±2.3	11.6±2.1	11.2±2.0	11.4±2.0	11.8±2.2
PUFA	5.9±1.8	6.2±1.6	5.7±1.7	5.9±1.7	6.0±1.7
cholesterol/energy (mg/MJ)	26.9±7.2	26.0±6.2	26.4±7.4	26.7±6.6	26.7±6.5
Carbohydrates (en%)					
total	46.8±7.2	46.3±6.3	47.6±6.3	26.9±6.5	46.7±6.7
mono-/disaccharides	22.3±5.6	22.4±6.0	22.8±6.2	23.0±5.8	22.5±5.8
polysaccharides	24.5±5.0	23.9±4.5	24.8±4.9	23.9±4.7	24.2±4.0
Alcohol (en%)	2.8±3.2	3.1±3.8	2.6±2.6	3.1±4.0	2.8±3.7
Dietary fibre/Energy (g/MJ)	2.8±0.8	2.8±0.8	2.9±0.9	2.9±1.0	2.8±0.7

[a]Butter contained 20.9 g fat and spreads contained 17.5 g fat, and contributed for about 8 and 7% of energy intake, not included in this table.
[b]SAFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids.

Table 3 ALP, ALT, AST and ;-GT concentrations after consumption of butter, Flora and 0.83, 1.61 and 3.24 g plant sterols[a,b]

	Butter ($n=80$)	*Flora* ($n=80$)	0.83 g *plant sterols* ($n=80$)	1.61 g *plant sterol* ($n=80$)	3.24 g *plant sterol* ($n=80$)
ALP (U/L)	60±14	59±14	58±15	58±15	59±15
ALT (U/L)	15±6	15±6	15±7	15±6	15±8
AST (U/L)	19±5	19±5	19±5	20±5	20±6
;-GT (U/L)	18.2±9.3	17.6±8.7	17.8±9.4	18.2±9.6	18.1±9.3

[a]ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate transaminase; Gamma-GT = gamma-glutamate transaminase.
[b]No statistical significant differences between treatments were present.

Total and LDL-cholesterol concentrations decreased after the consumption of plant sterol enriched spread as compared to Flora ($P<0.001$) and butter ($P<0.001$). Decreases in total cholesterol as compared to Flora were 0.26 (CI: 0.15–0.36), 0.31 (CI: 0.20–0.41) and 0.35 (CI: 0.25–0.46) mmol/L, for daily consumption of 0.83, 1.61 and 3.24 g plant sterols, respectively. For LDL-cholesterol these decreases were 0.20 (CI: 0.10–0.31), 0.26 (CI: 0.15–0.36) and 0.30 (CI: 0.20–0.41) mmol/L, respectively. Differences in cholesterol reductions between the plant sterol doses consumed were not significant. Total and LDL-cholesterol concentrations decreased after Flora consumption as compared to butter consumption.

HDL-cholesterol was not affected after consumption of the plant sterol enriched spreads as compared to Flora consumption. However, HDL-cholesterol was decreased after consumption of 1.61 and 3.24 g plant sterols as compared to butter consumption ($P<0.01$). Decreases were 0.047 (CI: 0.013–0.081) and 0.051 (CI: 0.017–0.085) mmol/L, respectively.

Analysis of variance for the LDL/HDL cholesterol ratio shows a pattern very similar to that of total cholesterol and LDL-cholesterol. The LDL/HDL ratio decreased after the consumption of plant sterol enriched spreads as compared to Flora and as compared to butter. Decreases as compared to Flora were 0.13 (CI: 0.04–0.22), 0.16 (CI: 0.07–0.24) and 0.16 (CI: 0.07–0.24) units, after 0.83, 1.61 and 3.24 g

plant sterols, respectively. The LDL/HDL cholesterol ratio for Flora and butter were the same.

Plasma lipid-soluble (pro)vitamins

Plasma concentrations of vitamin K1 and 25-OH-vitamin D were not affected by consumption of the spreads enriched in plant sterols. The compounds (alpha + beta)-carotene, lycopene and alpha-tocopherol were decreased after consumption of plant sterol enriched spreads as compared to Flora consumption. Plasma carotenoid and alpha-tocopherol data were also lipid standardized to correct for the decreases in plasma lipids (Table 5).

(Alpha + beta)-carotene concentrations were decreased with 12%, 11% and 19% after consumption of 0.83, 1.61 and 3.24 g plant sterols, respectively, as compared to consumption of Flora. The decrease in (alpha + beta)-carotene concentrations was larger after consumption of 3.24 g plant sterols as compared to consumption of 0.83 and 1.61 g plant sterols ($P < 0.05$). (Alpha + beta)-carotene concentrations per total plasma lipids were also decreased, namely by about 8 and 15% after consumption of 0.83 and 3.24 g plant sterols, respectively.

Lycopene concentrations decreased by about 11–15% after consumption of spreads enriched with plant sterols, respectively. No differences between the dose levels of plant sterols were observed. However, plasma lycopene concentrations per total lipid in plasma was not affected by consumption of any of the spreads enriched in plant sterols (Table 5).

Alpha-tocopherol concentrations were decreased by about 6 and 8% after consumption of 1.61 and 3.24 g plant sterols, respectively. The highest plant sterol concentration decreased alpha-tocopherol concentrations further as compared to the lowest concentration. However, alpha-tocopherol concentrations per total lipid in plasma were not affected by consumption of any of the spreads enriched in plant sterols.

Discussion

In this double-blind, placebo-controlled trial in normocholesterolaemic and mildly hypercholesterolaemic adult volunteers no statistical significant dose-dependency was observed in the cholesterol lowering effects of three different dosages of plant sterols. Intake of total plant sterols as low as 0.83 g/d, already resulted in significant decreases of blood cholesterol. Daily intake with lunch and dinner of plant sterol enriched spreads decreased total cholesterol, LDL cholesterol and the LDL/HDL ratio, but did not affect plasma triacylglycerol and HDL cholesterol concentrations. Decreases in total cholesterol were 4.9–6.8%, decreases in LDL cholesterol 6.7–9.9% and decreases in LDL/HDL ratio were 6.5–7.9% as compared to control spread consumption. The reductions in total and LDL-cholesterol observed are large and may, on a population basis, substantially contribute to the prevention of coronary heart disease (Law *et al*, 1994).

Theoretically, factors other than spread consumption may have affected the outcome of the study. However, the study was double-blinded and compliance appeared to be extremely good. Also, intake of nutrients including dietary fats and total energy, did not differ significantly during consumption of the spreads as assessed by food frequency questionnaire. Moreover, consumption of plant sterol enriched spreads appeared to have no adverse side

Table 4 Total, LDL and HDL cholesterol, LDL/HDL cholesterol ratio, triacylglycerol concentrations after consumption of Flora, 0.83, 1.61 and 3.24 g plant sterols and Butter

	Flora ($n = 80$)	0.83 g *plant sterols* ($n = 80$)		1.61 g *plant sterols* ($n = 80$)		3.24 g *plant sterols* ($n = 80$)		*Butter* ($n = 80$)	
Lipid parameter	*Mean* ± *s.d.*	*Mean* ± *s.d.*	*Decrease* (%) *CI*[a]	*Mean* ± *s.d.*	*Decrease* (%) *CI*[a]	*Mean* ± *s.d.*	*Decrease* (%) *CI*[a]	*Mean* ± *s.d.*	*Decrease* (%) *CI*[a]
Total cholesterol (mmol/l.)	5.16 ± 0.94	4.94 ± 0.91[b,c,3]	0.26 (4.9%) 0.15 – 0.36	4.84 ± 0.92[b,c,3]	0.31 (5.9%) 0.20 – 0.41	4.81 ± 0.93[b,c,3]	0.35 (6.8%) 0.25 – 0.46	5.27 ± 0.94[c,2]	– 0.14 (– 2.6%) – 0.24 – – 0.03
LDL cholesterol (mmol/l.)	3.05 ± 0.85	2.86 ± 0.78[b,c,3]	0.20 (6.7%) 0.10 – 0.31	2.77 ± 0.76[b,c,3]	0.26 (8.5%) 0.15 – 0.36	2.75 ± 0.79[b,c,3]	0.30 (9.9%) 0.20 – 0.41	3.15 ± 0.83[c,2]	– 0.12 (– 4.0%) – 0.22 – – 0.02
HDL cholesterol (mmol/l.)	1.64 ± 0.40	1.65 ± 0.41	0.01 (0.3%) – 0.03 – 0.04	1.63 ± 0.41[b,3]	0.02 (1.3%) – 0.01 – 0.06	1.61 ± 0.40[b,3]	0.02 (1.5%) – 0.01 – 0.06	1.65 ± 0.39	– 0.03 (– 1.6%) – 0.06 – – 0.01
LDL cholesterol/HDL cholesterol	2.01 ± 0.91	1.87 ± 0.82[b,c,3]	0.13 (6.5%) 0.04 – 0.22	1.84 ± 0.80[b,c,3]	0.16 (7.9%) 0.07 – 0.24	1.86 ± 0.89[b,c,3]	0.16 (7.8%) 0.07 – 0.24	2.02 ± 0.82	– 0.04 (– 2.1%) – 0.13 – 0.04
Triacylglycerols (mmol/l.)	1.13 ± 0.62	1.03 ± 0.48	0.09 (8.4%) – 0.00 – 0.19	1.05 ± 0.47	0.07 (6.1%) – 0.03 – 0.17	1.08 ± 0.61	0.05 (4.8%) – 0.05 – 0.15	1.10 ± 0.43	– 0.01 (– 1.0%) – 0.11 – 0.09

[a]Decrease of least square means as compared to Flora consumption, absolute value (%) and confidence interval of the decrease. Superscript characters indicate a significant difference between study substances: [b]as compared to butter, [c]as compared to Flora. Superscript figures indicate the level of significance: [2]$P < 0.05$; [3]$P < 0.01$; [4]$P < 0.001$.

effects, defined as several liver enzyme concentrations and adverse events reporting.

In this present study a mixture of plant sterols obtained from commonly used edible oils (predominantly soybean) was applied consisting mainly of sitosterol also containing appreciable amounts of campesterol and stigmasterol. This mixture resulted in reductions of total and LDL cholesterol of 4.9–6.8% and 6.7–9.9%, respectively. These reductions are slightly lower as those reported in literature on both soybean derived plant sterols and wood pulp derived sitostanol esters. Pelletier *et al,* 1995 applied soybean plant sterols and reported slightly higher reductions in total and LDL-cholesterol (10 and 15%, respectively). In most other studies the plant sterols applied were sitostanol ester (Miettinen *et al,* 1995; Vanhanen *et al,* 1993; Vanhanen *et al,* 1994). The study by Miettinen *et al* (1995), treating 153 subjects with a mild hypercholesterolaemia, resulted in a 10% reduction of total cholesterol and a 14% reduction of LDL cholesterol after 12 months in the study. After three months of sitostanol consumption, however, the reduction of total cholesterol was approximately 6.5%, which is similar to the reduction reported in this present study. Vanhanen *et al,* reported reductions of LDL cholesterol of 10–15% as compared to control in two studies with hypercholesterolaemic subjects (Vanhanen *et al.* 1993. 1994) and Heinemann *et al,* 1986 report a cholesterol reduction of 15% in severe hypercholesterolaemic subjects as well. Weststrate & Meijer (1998) compared spreads enriched in plant sterols derived from commonly used edible oils and enriched in sitostanol ester. Reductions of plasma total and LDL cholesterol in their study were 8 and 13%, respectively, independent of the plant sterols applied. These reductions correspond to the reductions in total and LDL cholesterol reported in this study.

In this present study the effects of the three dosages of plant sterols did not show a clear difference in efficacy. However, 95% confidence intervals (as compared to control) suggest increasing cholesterol reductions with increasing plant sterol content (Table 4). Dose-effect relationship was further investigated by analysing the significance of a trend in the three dose levels of plant sterols, using their orthogonal polynomials. This trend may either be linear or quadratic, because only three dose levels were included. The analysis, however, did not show a linear ($P = 0.076$ for total cholesterol) nor a quadratic ($P = 0.972$ for total cholesterol) trend in any of the serum lipids analysed. The statistical power of the study as executed indicated that an overall significant difference between plant sterol concentrations, at a probability of $P = 0.05$ and expressed as a percentage of the grand mean, was 2.2 and 3.7% for total and LDL cholesterol, respectively. This corresponds to a difference of 0.11 mmol/L for both parameters. The overall effect on total cholesterol varied between 0.15 and 0.46 mmol/L with the average decrease for the three concentrations of plant sterols being about 0.05 mmol/L higher with increasing dose, namely 0.26, 0.31 and 0.35 mmol/L for 0.83, 1.61 and 3.24 g plant sterol consumption. Therefore, the statistical power of the study may not have been sufficient to detect the small differences in effect between the three plant sterol concentrations.

Information on the dose dependency for blood lipid lowering by plant sterols is scarce. Only one experiment has directly compared the efficacy of different doses of plant sterols. In Miettinen's long-term experiment (Miettinen *et al.* 1995) with a dose of 2.6 g sitostanol ester per day

Table 5 Plasma lipid-soluble (pro)vitamin concentrations after consumption of Flora, and 0.83, 1.61 and 3.24 g plant sterols ($n = 60$)

	Flora	0.83 g *plant sterols*		1.61 g *plant sterols*		3.24 g *plant sterols*		
Lipid parameter	*Mean ± s.d.*	*Mean ± s.d.*	*% change*[4]	*Mean ± s.d.*	*% change*[4]	*Mean ± s.d.*	*% change*[4]	*Least significant difference (%)*
(Alpha + beta)-carotene (μmol/L)	0.57 ± 0.47[a]	0.50 ± 0.47[b2]	− 11.7	0.52 ± 0.51[b2]	− 11.0	0.40 ± 0.28[c3]	− 19.2	7.8
Lycopene (μmol/L)	0.40 ± 0.22[a]	0.38 ± 0.21[b1]	− 11.0	0.36 ± 0.22[b1]	− 12.8	0.37 ± 0.19[b2]	− 15.4	10.6
Alpha-tocopherol (μmol/L)	28.1 ± 5.3[a]	26.9 ± 5.1[ab]	− 3.10	25.8 ± 5.2[bc2]	− 5.9	25.6 ± 5.3[c3]	− 7.8	3.6
(Alpha + beta)-carotene/(TC + TG)[5] (μmol/mmol)	0.10 ± 0.09[a]	0.09 ± 0.09[b1]	− 8.2	0.10 ± 0.11[ab]	− 5.5	0.07 ± 0.05[c3]	− 14.9	7.6
Lycopene/(TC + TG) (μmol/mmol)	0.07 ± 0.03	0.06 ± 0.03	− 7.1	0.06 ± 0.04	− 7.9	0.06 ± 0.03	− 10.2	10.6
Alpha-tocopherol/(TC + TG)(μmol/mmol)	4.59 ± 0.72	4.63 ± 0.83	− 2.1	4.51 ± 0.65	− 0.3	4.47 ± 0.61	− 0.6	3
Vitamin K1 (pg/mL)	228 ± 167	198 ± 148	− 5.4	206 ± 136	− 7.4	168 ± 112	− 18.6	23
25-OH-vitamin D (nmol/L)	68 ± 25	71 ± 26	4.1	73 ± 27[6]	7	70 ± 26[7]	2.8	18.5

[a] [b] [c] Values with different superscript characters within a row are significantly different ($P < 0.05$).

[1] [2] [3] Superscript figures indicate the level of significance as compared to Flora: [1], $P < 0.05$; [2], $P < 0.01$; [3], $P < 0.001$.

[4] % change of least square means as compared to Flora consumption.

[5] TC = total cholesterol; TG = triacylglycerol.

[6] $n = 59$.

[7] $n = 58$.

for 12 months, half of the subjects received a dose of 1.8 g per day during the last six months. At the end of the study the difference in cholesterol reduction between the two dosages was only 0.2 mmol/L. This suggests that concentration dependency of the cholesterol lowering effect may be shallow in this concentration range. Miettinen *et al*, 1995 suggest that the practical interpretation is that these two doses produce a similar cholesterol-lowering effect.

Dose-dependency may not only be shallow, some indications for a threshold dosage are reported. Miettinen *et al*, 1995 reported small reductions of cholesterol (2.4% as compared to an increase of 1.9% in control group) after consumption of 0.7 g plant sterols per day. VanHanen *et al* reported a non significant reduction of cholesterol (7%) after intake of 0.8 g plant sterols per day. These experiments, however, were performed with sitostanol ester derived from wood pulp, but not with plant sterols derived from commonly used edible oils. The experiment presented here showed a substantial cholesterol lowering effect of 0.83 g plant sterols. Also Pelletier *et al*, 1995 reported a significant cholesterol reduction using a low dose, namely 0.74 g, soybean plant sterols. These data suggest that, at least for these plant sterols, a low dose is effective.

One hypothesis put forward to explain the absence of a dose-dependency is that a compensatory increase in cholesterol synthesis occurs after consumption of higher dosages of plant sterols. VanHanen *et al* (1993) evaluated concentrations of precursors of cholesterol and estimated that consumption of 2 g sitostanol per day (but not 0.8 g/d) by hypercholesterolaemic subjects increased cholesterol synthesis by 2 mg per day per kg body weight. Combining this with reported inhibitions of 30% (Vanhanen *et al*, 1994) to 60% (Gylling & Miettinen, 1994) in the absorption of cholesterol originating from both the diet as well as from the enterohepatic circulation, leads to the conclusion that increased cholesterol synthesis may at least in part compensate for a reduction in cholesterol absorption.

Since the reduction of cholesterol by plant sterols is thought to be mainly due to inhibition of cholesterol absorption, plant sterols might also interfere with the absorption of other fat-soluble compounds like the fat-soluble vitamins. To the best of our knowledge, the effects of plant sterol intake on the status of vitamins D and K has not been reported on. In this present study no effects of any of the spreads enriched in plant sterols were observed. In a long-term study using a sitostanol-ester enriched spread (Gylling *et al*, 1996) alpha-tocopherol and carotene concentrations were decreased after consumption of 2.6 g sitostanol ester per day. Lipid standardized alpha-tocopherol concentration did, however, not change. In a previous study with spread enriched in plant sterols derived from commonly used edible oils applying an intake of 3 g plant sterols per day (Weststrate & Meijer, 1998), plasma (alpha + beta)-carotene and lycopene concentrations were reduced. Expressed per plasma lipid concentration, however, lycopene concentration was not affected but (alpha + beta)-carotene concentration was decreased. In this present study similar results were obtained. We observed a decrease in plasma (alpha + beta)-carotene, lycopene and alpha-tocopherol concentration after consumption of plant sterol enriched spreads. Correction for the reductions in the total plasma lipids, however, showed only plasma (alpha + beta)-carotene concentration to be reduced by about 10% after consumption of two of the three spreads tested, that is the spreads enriched in the low and the high dose of plant sterols. Carotenoids may have positive effects on health (Poppel, 1993; Manson *et al*, 1993). Minimization or compensation for the carotenoid lowering effects of plant sterol enriched spreads should be considered.

All three doses of plant sterols were effective in substantially reducing total and LDL cholesterol. There may or may not be a dose dependency. Our study shows that both the lowest and the highest dose, but not the middle dose affect plasma carotenoid concentrations to a limited extent. Therefore, this study would support that consumption of about 1.6 g of plant sterols per day will beneficially affect plasma cholesterol concentrations without seriously affecting plasma carotenoid concentrations.

Conclusions

This present study shows that daily intake of spreads enriched with 0.83–3.24 g plant sterols derived from commonly used edible oils decreases total cholesterol by 0.15–0.46 mmol/L as compared to consumption of control spread. LDL-cholesterol is decreased by 0.10–0.41 mmol/L as compared to control. LDL/HDL ratio decreased by 0.04–0.24 as compared to control. The test spreads do not affect HDL cholesterol as compared to control spread, but the spreads with the highest plant sterol contents do slightly reduce HDL cholesterol as compared to butter. Lipid standardised plasma alpha-tocopherol and lycopene were not affected by the plant sterol enriched spreads. However, lipid standardized plasma (alpha + beta)-carotene was reduced by daily consumption of 0.83 and 3.24 g plant sterols.

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Effects of 2 low-fat stanol ester–containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects[1–3]

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ABSTRACT

Background: Full-fat sitostanol ester–containing margarine reduces serum total and LDL cholesterol, but the effect of plant stanol ester–containing margarine as part of a low-fat, low-cholesterol diet has not been studied.

Objective: We investigated the cholesterol-lowering effects of 2 novel, low-fat stanol ester–containing margarines as part of a low-fat diet recommended for hypercholesterolemic subjects.

Design: In a parallel, double-blind study, 55 hypercholesterolemic subjects were randomly assigned after a 4-wk high-fat diet (baseline) to 3 low-fat margarine groups: wood stanol ester–containing margarine (WSEM), vegetable oil stanol ester–containing margarine (VOSEM), and control margarine (no stanol esters). The groups consumed the margarines for 8 wk as part of a diet resembling that of the National Cholesterol Education Program's Step II diet. The daily mean total stanol intake was 2.31 and 2.16 g in the WSEM and VOSEM groups, respectively.

Results: During the experimental period, the reduction in serum total cholesterol was 10.6% ($P < 0.001$) and 8.1% ($P < 0.05$) greater and in LDL cholesterol was 13.7% ($P < 0.01$) and 8.6% ($P = 0.072$) greater in the WSEM and VOSEM groups, respectively, than in the control group. Serum campesterol concentrations decreased 34.5% and 41.3% ($P < 0.001$) in the WSEM and VOSEM groups, respectively. Serum HDL cholesterol, sitostanol, campestanol, β-carotene, and fat-soluble vitamin concentrations did not change significantly from baseline.

Conclusions: We conclude that the low-fat, plant stanol ester–containing margarines are effective cholesterol-lowering products in hypercholesterolemic subjects when used as part of a low-fat, low-cholesterol diet. They offer an additional, clinically significant reduction in serum cholesterol concentrations to that obtained with a low-fat diet alone. *Am J Clin Nutr* 1999;69:403–10.

KEY WORDS Cholesterol, low-fat diet, plant stanol esters, sitostanol, campestanol, campesterol, apolipoproteins, hypercholesterolemia, margarine, humans

INTRODUCTION

An increased concentration of LDL cholesterol is the main risk factor for atherosclerotic vascular disease. Considerable efforts have focused on different measures to lower elevated concentrations of LDL cholesterol, such as dietary and pharmacologic measures.

Plant sterols, structurally resembling cholesterol, reduce serum cholesterol concentrations by inhibiting the absorption of both dietary and biliary cholesterol from the small intestine (1, 2). Sitostanol, the saturated form of sitosterol, has been shown to be most effective in this respect (2, 3). Because sitostanol is virtually unabsorbable, it has been considered a safe way to reduce elevated serum cholesterol concentrations. Several studies have shown that 2.0–3.0 g sitostanol from full-fat sitostanol ester–containing margarines or mayonnaises significantly reduces serum total and LDL-cholesterol concentrations without affecting HDL-cholesterol or serum triacylglycerol concentrations (4–9). However, the effect of plant stanols delivered in low-fat margarines on elevated cholesterol concentrations as part of a recommended low-fat, low-cholesterol diet (10) has not been studied.

Therefore, we investigated to what extent the 2 low-fat margarines enriched with wood or vegetable oil–based plant stanols would reduce serum total and LDL-cholesterol concentrations as part of a low-fat, low-cholesterol diet and whether or not these 2 low-fat plant stanol ester–containing margarines would lower serum cholesterol concentrations equally.

SUBJECTS AND METHODS

Subjects

Altogether, 91 subjects were screened for the study from the occupational health care system and former studies carried out at the Department of Clinical Nutrition, University of Kuopio, Kuopio, Finland. To be included in the study, subjects had to have a serum total cholesterol concentration of 5.4–7.5 mmol/L; to have a serum triacylglycerol concentration <3.0 mmol/L; to

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TABLE 1
Baseline characteristics of the subjects in the 3 study groups[1]

Variables	WSEM group (n = 8 M, 10 F)	VOSEM group (n = 6 M, 14 F)	Control group (n = 6 M, 11 F)
Age (y)	43.2 ± 8.2	40.8 ± 9.3	46.0 ± 8.2
Weight (kg)			
Men	83.0 ± 9.5	80.5 ± 12.8	80.4 ± 7.5
Women	64.6 ± 11.0	61.9 ± 7.5	68.1 ± 12.2
Body mass index (kg/m^2)	25.6 ± 4.0	24.2 ± 3.0	25.7 ± 3.5
Waist circumference (cm)			
Men	93.4 ± 8.1	94.4 ± 9.3	94.8 ± 8.4
Women	81.2 ± 12.1	78.1 ± 9.4	83.8 ± 12.8
Lipids (mmol/L)			
Total cholesterol	6.36 ± 0.76	6.15 ± 0.79	5.93 ± 0.64
LDL cholesterol	4.36 ± 0.76	4.21 ± 0.89	4.10 ± 0.60
HDL cholesterol	1.36 ± 0.38	1.37 ± 0.32	1.27 ± 0.27
Triacylglycerols	1.42 ± 0.67	1.25 ± 0.39	1.24 ± 0.66
Blood pressure (mm Hg)			
Systolic	123 ± 8	121 ± 11	127 ± 17
Diastolic	79 ± 6	79 ± 9	81 ± 9

[1] $\bar{x}$ ± SD. There were no significant differences among groups. WSEM, wood stanol ester–containing margarine; VOSEM, vegetable oil stanol ester–containing margarine.

be aged 20–60 y; to have normal liver, kidney, and thyroid function; to not be taking any lipid-lowering drugs or other drugs that might affect lipid concentrations; to be willing to participate; and to not be an abuser of alcohol. On the basis of these criteria, 60 subjects were selected for the study. Five subjects dropped out at the beginning of the run-in period for personal reasons. These subjects did not differ in initial serum lipid concentrations, weight, or lifestyle habits from the 55 subjects who completed the study. Five subjects used low-estrogen oral contraceptives, 6 used postmenopausal estrogen medication, and 3 used calcium channel blockers, diuretics, or both for the treatment of hypertension or ischemic heart disease. Ten of the subjects were smokers. The subjects were requested to maintain their weight, alcohol consumption, smoking habits, and physical activity during the study. Baseline characteristics of the subjects are shown in **Table 1**. The study protocol was approved by the Ethics Committee of the University of Kuopio and all subjects gave their informed consent.

Study design

This double-blind, parallel, randomized study consisted of a 4-wk run-in period (high-fat diet) and an 8-wk experimental period (low-fat, low-cholesterol diet). In 2 subjects, the experimental diet period lasted only 6 wk because of a trip abroad.

Routine laboratory measurements were taken at the screening visit and at the last visit of the study to ensure normal health status. In addition, medical history, drug use, smoking habits, alcohol consumption, and physical activity were reviewed with a questionnaire at the same time points. The subjects started the study by following a high-fat diet for 4 wk. At the end of the run-in period, the subjects were randomly assigned into 3 experimental groups: wood stanol ester–containing margarine (WSEM), vegetable oil stanol ester–containing margarine (VOSEM), and control margarine. Smoking and the phase of menstrual cycle were taken into account in the randomization. After randomization, the subjects followed a low-fat diet for the next 8 wk. Fasting blood samples were taken at the beginning of the run-in (−4 wk) and the experimental diet (0 wk) periods and at weeks 2, 4, and 8. Body weight and side effects were recorded at each visit.

Diets

The composition of the low-erucic acid rapeseed oil–based low-fat margarines (Raisio Group, Raisio, Finland) is presented in **Table 2**. The control margarine contained 35% of energy as fat and no added plant stanols. The 2 test margarines contained 40% of energy as fat and were prepared with use of commercially available plant sterols (wood sterols: Ultra sitosterol, Kaukas Oy, Finland; vegetable sterols: derived principally from soy oil, Archer Daniels Midland Co, Decatur, IL) by recrystallization, hydrogenation to form plant stanols, and esterification to produce fatty acid esters of the obtained plant stanols. The subjects consumed 25 g low-fat margarine/d as part of their low-fat, low-cholesterol diet. The theoretical daily intake of stanols was 2.34 g (2.15 g sitostanol and 0.19 g campestanol) in the WSEM group and 2.20 g (1.50 g sitostanol and 0.70 g campestanol) in the VOSEM group. Vitamin A (5.5 μg/g) and vitamin D (0.07 μg/g) were added to all 3 spreads. The subjects received coded tubs of the test margarines when visiting the laboratory and they were asked to record daily the consumption of the test margarines.

During the run-in period, dietary goals were to consume 36–38% of energy as fat (16–18% as saturated, 14% as monounsaturated, and 6% as polyunsaturated fat), 20% as protein, and 40–44% as carbohydrate. During the experimental period, the diet resembled the Step I diet of the National Cholesterol Education Program (10) and provided 28–30% of energy as fat (8–10% as saturated, 12% as monounsaturated, and 8% as polyunsaturated fatty acids), 20% as protein, and 50–52% as carbohydrate. The goal for cholesterol intake was 35.7 and 23.8 mg/MJ during the run-in and experimental periods, respectively. Except for the 3 test margarines, the diets were composed of normal Finnish food items. The fatty acid compositions were adjusted by changing the quality of spreads, vegetable oils, and liquid milk products during the different study periods. During the run-in

TABLE 2
Composition of low-fat wood stanol ester–containing (WSEM), vegetable oil stanol ester–containing (VOSEM), and control margarines[1]

Nutrients	WSEM margarine	VOSEM margarine	Control margarine
		g	
Fat	10.0	10.0	8.8
Total stanols	2.34	2.20	—
Total unsaturated sterols	0.10	0.15	0.05
Fatty acids			
Polyunsaturated	2.05	2.13	2.10
trans Polyunsaturated	0.03	0.05	0.05
Monounsaturated	4.18	4.08	4.13
trans Monounsaturated	0	0	0.03
Saturated	1.10	1.13	2.03

[1] Values are per 25 g spread.

period, a milk-fat based spread (a blend of 0.6 g milk fat and 0.2 g vegetable oil/g spread), a small amount of rapeseed oil, and 1.5%-fat milk were consumed. During the experimental period, a low-fat test margarine, sunflower oil, and skim or 1.0%-fat milk were used. The compliance of the subjects was improved by providing the spreads, vegetable oils, and liquid milk products free of charge.

The subjects received detailed written and oral instructions about the diets, including the precise amounts of food to be eaten and the quality of food, by main food groups. The diets were calculated for 9 energy intakes: 6.7, 7.6, 8.4, 9.2, 10.1, 10.9, 11.8, 12.6, and 13.4 MJ/d. The energy requirement of each subject was estimated from a 4-d food record that subjects completed before the study and by using the Harris-Benedict formula (11), to which energy needs as a result of physical activity were added.

Adherence to the diets was monitored by examining a 4-d (completed on 3 weekdays and 1 weekend day) food record once during the run-in period and 3 times during the experimental period. The subjects recorded their food consumption after consulting a booklet containing photographs of food portions (12) aimed to help them estimate portion sizes. At every study visit, the subjects met a dietitian who advised them on the practical management of the diets and checked their food records. The diets were planned and the nutrients in the food records were calculated by using the MICRO-NUTRICA dietary analysis program (Finnish Social Insurance Institute, Turku, Finland). The values for the food-composition database were taken from Finnish food analyses and international food-composition tables (13).

Laboratory measurements

Venous blood samples were obtained after a 12-h overnight fast. After ultracentrifugation and precipitation (14), enzymatic colorimetric methods were used to determine cholesterol and triacylglycerols from whole serum and separated lipoproteins by using commercial kits (Monotest Cholesterol and Triacylglycerol GPO-PAP; Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) with a Kone Specific Clinical Analyzer (Kone Ltd, Espoo, Finland).

Serum samples for β-carotene, fat-soluble vitamins, apolipoprotein (apo) A-I, apo B, and plant sterols were stored at −70°C until analyzed at the end of the study. A Kone Specific Clinical Analyzer and apo A-I and apo B reagents from Kone Corporation were used to analyze apolipoproteins based on immunoprecipitation enhanced by polyethylene glycol at 340 nm. β-Carotene and fat-soluble vitamins were analyzed by HPLC (Perkin-Elmer, Norwalk, CT) on a C_{18} column (Waters, Milford, MA) (15, 16). Serum plant sterols were measured by gas-liquid chromatography (model 5890A; Hewlett-Packard, Palo Alto, CA) equipped with a (0.25 mm internal diameter) 25-m fused silica CP-Sil 5-CB capillary column (Chrompack, Raritan, NJ) (17).

Statistical analyses

Statistical analyses were performed with SPSS for WINDOWS 6.0 statistics program (SPSS Inc, Chicago). Normal distribution of variables was checked with the Shapiro-Wilks test (18). Differences in serum lipid variables were analyzed with repeated-measures multivariate analysis of variance (MANOVA) followed by Student's *t* test in between-group analyses and paired *t* test in within-group analyses. Statistical significance for the continuous response variables (serum lipids, fat-soluble vitamins, apolipoproteins, and mean plant sterols) were tested with a single-measurement, simple-factorial ANOVA followed by Student's *t* test. Logarithmic transformations were used when appropriate. If the initial concentration differed significantly among groups, the concentration was adjusted in the between-groups comparisons by dividing the response variable by the initial concentration. In addition, variables that were not normally distributed, even after logarithmic transformation, and noncontinuous variables were tested with the Kruskal-Wallis test, the chi-square test, or Wilcoxon's matched-pairs signed-rank test. Bonferroni adjustment was used to control the overall α level. The results are expressed as means ±SDs.

RESULTS

Baseline characteristics

There were no significant differences in baseline characteristics among the study groups (Table 1). Body weight decreased marginally during the study in all groups (1.2 ± 1.1, 1.2 ± 1.0, and 1.1 ± 1.3 kg in the WSEM, VOSEM, and control groups, respectively; NS among groups). Physical activity and smoking habits remained stable and no side effects were reported.

Feasibility of the diets

The mean consumption of the test margarines was 98.9%, 98.0%, and 98.0% of the scheduled amount in the WSEM, VOSEM, and control groups, respectively. Thus, the actual daily mean stanol intakes were 2.31 ± 0.03 g (2.13 ± 0.03 g sitostanol and 0.19 ± 0.00 g campestanol) in the WSEM group and 2.16 ± 0.12 g (1.47 ± 0.08 g sitostanol and 0.69 ± 0.04 g campestanol) in the VOSEM group. The small differences in the sitostanol and campestanol intakes were significant between the experimental groups ($P < 0.001$).

There were no significant differences in habitual nutrient intakes before the study among the groups. Nutrient intakes during the experimental diet periods remained stable and were not significantly different among the 3 groups (**Table 3**). Furthermore, the dietary goals were well achieved by all groups. In fact, the mean intake of fat, saturated fatty acids, and dietary cholesterol during the experimental period was even lower than the dietary goals. Energy intake was 44–54 kJ/d lower on average during the experimental than during the run-in period.

TABLE 3
Actual composition of the diets during the study in the 3 study groups[1]

Nutrients	Run-in period			Experimental period[2]		
	WSEM group (n = 18)	VOSEM group (n = 20)	Control group (n = 17)	WSEM group (n = 18)	VOSEM group (n = 20)	Control group (n = 17)
Energy (MJ/d)	8.7 ± 2.0	8.4 ± 1.4	8.0 ± 1.9	7.8 ± 1.4	7.7 ± 1.5	7.1 ± 1.3
Fat (% of energy)	40.6 ± 3.6	39.9 ± 4.6	41.1 ± 3.0	26.4 ± 3.3	25.6 ± 3.9	26.5 ± 3.1
Saturated fatty acids (% of energy)	16.8 ± 1.8	16.9 ± 2.1	16.9 ± 1.5	7.0 ± 1.4	6.8 ± 1.7	7.3 ± 1.6
Monounsaturated fatty acids (% of energy)	14.6 ± 1.6	14.3 ± 2.1	15.0 ± 1.4	8.9 ± 1.8	8.1 ± 1.5	8.6 ± 1.4
Polyunsaturated fatty acid (% of energy)	6.4 ± 1.0	5.9 ± 0.8	6.3 ± 0.7	8.3 ± 0.7	8.3 ± 1.2	8.5 ± 1.2
Protein (% of energy)	16.7 ± 1.9	16.0 ± 1.5	17.0 ± 1.5	18.4 ± 1.6	18.1 ± 2.1	19.2 ± 2.1
Carbohydrate (% of energy)	40.1 ± 3.7	40.7 ± 5.0	39.2 ± 2.6	51.2 ± 4.1	51.8 ± 4.9	50.8 ± 4.8
Alcohol (% of energy)	1.4 ± 1.9	2.2 ± 3.2	1.4 ± 1.8	2.6 ± 3.4	3.1 ± 3.9	2.1 ± 2.3
Cholesterol (mg/MJ)	26 ± 7	34 ± 5	38 ± 7	21 ± 7	18 ± 5	19 ± 5
Fiber (g/MJ)	2.9 ± 0.7	2.9 ± 0.8	2.7 ± 0.5	3.7 ± 0.6	4.0 ± 1.1	4.0 ± 1.0
Vitamin A (μg RE/d)	1258 ± 983	1187 ± 635	1140 ± 548	880 ± 394	928 ± 260	972 ± 323
β-Carotene (μg/d)	3725 ± 3437	4142 ± 3320	3804 ± 1826	3259 ± 2201	3388 ± 1384	3056 ± 920
Vitamin E (mg/d)	11.7 ± 2.2	11.0 ± 2.1	11.2 ± 2.5	16.8 ± 3.0	16.7 ± 3.4	16.8 ± 4.1
Vitamin D (μg/d)	3.1 ± 2.5	3.0 ± 2.4	2.4 ± 1.5	4.9 ± 2.3	4.2 ± 2.0	4.1 ± 1.2

[1] $\bar{x}$ ± SD. There were no significant differences among groups. WSEM, wood stanol ester-containing margarine; VOSEM, vegetable oil stanol ester–containing margarine; RE, retinol equivalents.
[2] Nutrient intakes are presented as the mean from the 3 food records.

Serum lipids and lipoproteins

There were no significant differences between baseline (−4 wk) and 0-wk (at randomization) serum lipids and lipoproteins among the 3 groups. During the run-in period, serum total or lipoprotein lipid concentrations did not change significantly in any of the 3 groups. No significant differences were found between men and women and therefore the results are presented for both sexes combined.

Serum total and LDL-cholesterol concentrations decreased significantly within all study groups during the experimental period. Most of the reduction in serum total and LDL-cholesterol concentrations was achieved after 2 wk. The serum total cholesterol concentration decreased by 18.3%, 15.7%, and 7.7% in the WSEM, VOSEM, and control groups, respectively. The reduction was significantly greater in the WSEM (10.6%, $P < 0.001$) and VOSEM (8.1%, $P < 0.05$) groups than in the control group, but no significant differences were found between the 2 experimental groups (**Table 4**). The serum LDL-cholesterol concentration decreased by 23.6%, 18.4%, and 9.9% in the WSEM, VOSEM, and control groups, respectively. There were significant differences only in the absolute (0.73 mmol/L, $P < 0.01$) and percentage (13.7%, $P < 0.01$) reductions in LDL-cholesterol concentrations between the WSEM and the control groups. The difference in percentage reduction in LDL-cholesterol concentration (8.6%) between the VOSEM and control groups was almost significant after Bonferroni correction ($P = 0.072$). Furthermore, there were no significant differences in absolute or percentage changes between the WSEM and VOSEM groups.

Serum HDL-cholesterol concentrations did not change significantly from baseline in any of the study groups, whereas VLDL cholesterol decreased significantly at 8 wk only in the VOSEM group (Table 4). However, there were no significant differences in VLDL-cholesterol concentrations among the groups at the end of the study. Serum VLDL triacylglycerols decreased significantly from baseline only in the WSEM group (Table 4) and serum HDL-triacylglycerol concentrations did not change significantly in any of the groups (data not shown). LDL triacylglycerols at 8 wk (0.30 ± 0.08 mmol/L) were significantly greater than those at baseline (0.27 ± 0.06 mmol/L) in the control group. There were no significant differences in total, VLDL, or LDL triacylglycerols among the groups at the end of the study.

The decrease from baseline in apo B concentrations at 8 wk in the WSEM (by 0.23 ± 0.16 g/L, 19.2%; $P < 0.001$), VOSEM (by 0.15 ± 0.14 g/L, 13.7%; $P < 0.001$), and control (by 0.06 ± 0.01 g/L, 5.2%; $P < 0.05$) groups was significant and paralleled the decrease in LDL-cholesterol concentrations in all groups. Although HDL cholesterol remained unchanged, apo A-I decreased significantly from baseline in the WSEM (by 0.17 ± 0.17 g/L, 9.0%; $P < 0.01$), VOSEM (by 0.15 ± 0.16 g/L, 8.6%; $P < 0.01$), and control (by 0.10 ± 0.16 g/L, 6.1%; $P < 0.05$) groups at 8 wk. Furthermore, the ratio of apo A-I to apo B increased by 14.3% and 8.3% in the WSEM and VOSEM groups, respectively, but the increase was significant ($P < 0.001$) only in the WSEM group.

Serum β-carotene and fat-soluble vitamins

Serum retinol concentrations did not change significantly in the 3 groups. The absolute concentration of serum β-carotene and α-tocopherol concentrations decreased significantly in the WSEM and VOSEM groups, but in the control group the change in serum β-carotene and α-tocopherol concentrations was not significant (**Table 5**). There was a significant difference in the absolute change in serum β-carotene between the experimental groups and the control group; however, the difference in the absolute change in serum α-tocopherol was significant only between the WSEM and control groups. However, there were no significant changes in serum β-carotene or α-tocopherol concentrations among the groups when the values were related to the serum total cholesterol concentration, ie, when vitamin concentrations were divided by serum total cholesterol concentrations. In fact, the ratio of serum α-tocopherol to total cholesterol increased significantly in all groups.

Serum 25-hydroxyergocalciferol (ercalcidiol) concentrations did not change significantly, whereas the absolute concentration of 25-hydroxycholecalciferol (calcidiol) increased significantly in all groups, but the increase was significantly smaller in the WSEM than in the VOSEM group (Table 5). However, there was

TABLE 4
Serum lipids in the 3 study groups during the experimental period[1]

	WSEM group (n = 18)	VOSEM group (n = 20)	Control (n = 17)
Total cholesterol (mmol/L)[2]			
0 wk	6.55 ± 0.78[3]	6.13 ± 0.81	6.06 ± 0.54
4 wk	5.34 ± 0.74	5.38 ± 0.85	5.69 ± 0.56
8 wk	5.34 ± 0.76[4]	5.15 ± 0.78[4]	5.57 ± 0.49[5]
P (MANOVA)[6]	<0.001	<0.001	<0.01
Change (from 0 to 8 wk)[7]	−1.21 ± 0.61[8]	−0.98 ± 0.59[9]	0.48 ± 0.49
LDL cholesterol (mmol/L)[10]			
0 wk	4.54 ± 0.72	4.25 ± 0.85	4.27 ± 0.59
4 wk	3.50 ± 0.69	3.54 ± 0.69	3.89 ± 0.62
8 wk	3.48 ± 0.77[4]	3.45 ± 0.76[4]	3.82 ± 0.56[5]
P (MANOVA)[6]	<0.001	<0.001	<0.01
Change (from 0 to 8 wk)[7]	1.06 ± 0.45[11]	−0.80 ± 0.50	−0.45 ± 0.59
HDL cholesterol (mmol/L)			
0 wk	1.44 ± 0.38	1.41 ± 0.38	1.36 ± 0.26
4 wk	1.38 ± 0.30	1.32 ± 0.37	1.35 ± 0.27
8 wk	1.41 ± 0.33	1.36 ± 0.31	1.37 ± 0.26
P (MANOVA)[6]	NS	NS	NS
Change (from 0 to 8 wk)	−0.03 ± 0.17	−0.05 ± 0.18	0.01 ± 0.15
VLDL cholesterol (mmol/L)			
0 wk	0.57 ± 0.35	0.47 ± 0.24	0.42 ± 0.27
4 wk	0.46 ± 0.22	0.51 ± 0.30	0.46 ± 0.26
8 wk	0.45 ± 0.34	0.34 ± 0.18[12]	0.38 ± 0.28
P (MANOVA)[6]	NS	<0.01	NS
Change (from 0 to 8 wk)	−0.13 ± 0.40	−0.13 ± 0.21	−0.04 ± 0.28
Total triacylglycerols (mmol/L)[10]			
0 wk	1.45 ± 0.70	1.24 ± 0.50	1.25 ± 0.68
4 wk	1.16 ± 0.54	1.26 ± 0.61	1.19 ± 0.59
8 wk	1.26 ± 0.67	1.13 ± 0.45	1.33 ± 0.80
P (MANOVA)[6]	<0.05	NS	NS
Change (from 0 to 8 wk)	−0.20 ± 0.55	−0.11 ± 0.41	0.08 ± 0.31
VLDL triacylglycerols (mmol/L)			
0 wk	0.96 ± 0.62	0.76 ± 0.49	0.82 ± 0.63
4 wk	0.73 ± 0.52	0.88 ± 0.56	0.76 ± 0.55
8 wk	0.77 ± 0.64[12]	0.63 ± 0.37	0.85 ± 0.75
P (MANOVA)[6]	<0.05	NS	NS
Change (from 0 to 8 wk)	−0.19 ± 0.50	−0.13 ± 0.38	0.03 ± 0.28

[1] WSEM, wood stanol ester–containing margarine; VOSEM, vegetable oil stanol ester–containing margarine; MANOVA, repeated-measures multivariate analysis of variance.
[2,10] Significant group-by-time interaction (MANOVA): [2] $P < 0.001$, [10] $P < 0.01$.
[3] $\bar{x}$ ± SD.
[4,5,12] Significantly different from 0 wk (paired t test): [4] $P < 0.001$, [5] $P < 0.01$, [12] $P < 0.05$.
[6] Significant difference in overall within-group changes.
[7] Significant difference among groups, $P < 0.01$ (ANOVA).
[8,9,11] Significantly different from control group (Student's t test and Bonferroni correction): [8] $P < 0.001$, [9] $P < 0.05$, [11] $P < 0.01$.

no significant difference in the percentage increase in calcidiol concentrations among the study groups.

Plant sterols

Baseline concentrations of serum sitostanol and campestanol did not change significantly over the 8-wk study period in the WSEM, VOSEM, and control groups: sitostanol (from 4.6 ± 4.3 to 4.8 ± 7.4 μmol/L, 4.3 ± 5.5 to 3.8 ± 5.3 μmol/L, and 5.5 ± 5.3 to 3.8 ± 4.8 μmol/L, respectively); campestanol (from 3.5 ± 3.0 to 3.2 ± 3.2 μmol/L, 4.5 ± 7.4 to 2.7 ± 5.2 μmol/L, and 4.7 ± 7.9 to 5.7 ± 9.2 μmol/L, respectively). Serum campesterol concentrations did not change significantly in the control group but decreased significantly from baseline ($P < 0.001$) in both experimental groups: from 21.7 ± 6.5 to 14.2 ± 6.0 μmol/L (34.5% change) in the WSEM group and from 27.2 ± 18.7 to 16.0 ± 9.5 μmol/L (41.3% change) in the VOSEM group. Furthermore, serum campesterol concentrations were still significantly decreased in both experimental groups after correction for the reduction in serum cholesterol. In addition, serum sitosterol concentrations tended to decrease in both the experimental groups, but not significantly so.

DISCUSSION

In the present study, the wood- and vegetable oil–based plant stanol ester–containing margarines (WSEM and VOSEM groups, respectively), as part of a low-fat diet, reduced more markedly both serum total and LDL-cholesterol concentrations than did

TABLE 5

Serum β-carotene, retinol, α-tocopherol, calcidiol, and ercalcidiol concentrations and ratios of β-carotene to total cholesterol and of α-tocopherol to total cholesterol in the 3 study groups during the experimental period[1]

	WSEM group (n = 18)	VOSEM group (n = 20)	Control group (n = 17)
β-Carotene (μmol/L)[2]			
0 wk	1.66 ± 1.10	1.47 ± 0.79	1.00 ± 0.37
8 wk	1.22 ± 0.97[3]	1.07 ± 0.54[3]	1.06 ± 0.42
Change (from 0 to 8 wk)[4,5]	−0.44 ± 0.57[4]	−0.40 ± 0.54[7]	0.05 ± 0.26
Retinol (μmol/L)			
0 wk	2.50 ± 0.72	2.21 ± 0.81	2.30 ± 0.66
8 wk	2.36 ± 0.68	2.12 ± 0.82	2.21 ± 0.70
Change (from 0 to 8 wk)	−0.14 ± 0.45	−0.09 ± 0.40	−0.09 ± 0.29
α-Tocopherol (μmol/L)[2]			
0 wk	51.49 ± 8.17	45.10 ± 9.68	44.58 ± 9.86
8 wk	45.27 ± 6.97[4]	41.51 ± 9.30[3]	43.45 ± 9.46
Change (from 0 to 8 wk)[4,9]	−6.22 ± 5.04[7]	−3.59 ± 4.29	−1.13 ± 3.34
Calcidiol (nmol/L)[7,8]			
0 wk	67.47 ± 23.73	62.48 ± 21.57	73.66 ± 44.26
8 wk	80.62 ± 22.96[11]	96.23 ± 33.68[8]	103.19 ± 43.30[8]
Change (from 0 to 8 wk)[9]	13.15 ± 22.75[12]	33.75 ± 25.72	29.53 ± 24.12
Ercalcidiol (nmol/L)			
0 wk	38.14 ± 42.39	41.19 ± 28.99	63.44 ± 32.76
8 wk	49.06 ± 33.58	42.70 ± 34.65	72.00 ± 41.47
Change (from 0 to 8 wk)	10.92 ± 27.87	1.51 ± 22.42	8.56 ± 32.30
β-Carotene:total cholesterol			
0 wk	0.27 ± 0.21	0.24 ± 0.13	0.17 ± 0.07
8 wk	0.24 ± 0.23	0.21 ± 0.10	0.19 ± 0.08
Change (from 0 to 8 wk)	−0.03 ± 0.12	0.03 ± 0.08	0.02 ± 0.05
α-Tocopherol:total cholesterol			
0 wk	7.86 ± 0.81	7.40 ± 1.41	7.38 ± 1.55
8 wk	8.50 ± 0.90[4]	8.06 ± 1.35[8]	7.80 ± 1.58[11]
Change (from 0 to 8 wk)	0.65 ± 0.50	0.66 ± 0.66	0.42 ± 0.75

[1] $\bar{x}$ ± SD. WSEM, wood stanol ester–containing margarine; VOSEM, vegetable oil stanol ester–containing margarine; MANOVA, multivariate analysis of variance.

[2,10] Significant group-by-time interaction (MANOVA): [2] $P < 0.01$, [10] $P \leq 0.05$.

[3,8,11] Significantly different from 0 wk (paired t test): [3] $P < 0.01$, [8] $P < 0.001$, [11] $P < 0.05$.

[4] Initial concentrations were nearly significantly different by ANOVA (β-carotene, $P = 0.063$; α-tocopherol, $P = 0.053$) among the study groups; therefore, initial concentrations were taken into account in the between-groups comparisons by dividing the response variable by the initial value.

[5,9] Significant difference among groups (ANOVA): [5] $P < 0.01$, [9] $P < 0.05$.

[6,7] Change significantly different from change in control group (Student's t test with Bonferroni correction): [6] $P < 0.05$, [7] $P < 0.01$.

[12] Significantly different from VOSEM group, $P < 0.05$ (Student's t test with Bonferroni correction).

the low-fat diet alone in subjects with elevated serum total cholesterol concentrations. The cholesterol-lowering effects of the 2 plant stanol ester–containing margarine diets did not differ significantly. These findings indicate that low-fat plant stanol ester–containing margarines, when part of a low-fat diet (10), can reduce serum cholesterol concentrations almost as much as cholesterol-lowering drugs (19, 20).

There have been no studies of the effects on serum cholesterol concentrations of plant stanols as part of a strictly and frequently monitored low-fat, low-cholesterol diet. Moreover, earlier studies used full-fat margarines and mayonnaises (4–9), whereas the present study used low-fat stanol ester–containing margarines (40% of energy as fat, including 9% nonabsorbable stanols). In contrast with Denke's study (21), we found that stanol esters can significantly lower serum cholesterol concentrations even in those with a low cholesterol intake. Note that nonesterified sitostanol suspended in safflower oil and packed into gelatin capsules was used in Denke's study.

The novel finding that plant stanols can reduce serum cholesterol concentrations, even in conjunction with a markedly low dietary cholesterol intake, indicates that plant stanols must inhibit not only the absorption of dietary cholesterol but also that of biliary cholesterol. This is supported by the findings of earlier studies of plant stanol (2, 4, 7, 9), in which the fecal excretion of neutral sterols increased despite a constant dietary cholesterol intake. In addition, in the present study the serum campesterol concentration, which is known to reflect intestinal cholesterol absorption (22, 23), decreased significantly in both stanol ester groups, which agrees with the findings of earlier studies (2, 4, 7, 22, 23). In some studies of plant stanol in diabetic subjects (4, 24), the biliary secretion of cholesterol, which normally ranges from 600 to 1000 mg/d (25), was found to increase significantly (11–16%) (4, 24). An average of 50% of the cholesterol that enters the small intestine is reabsorbed (25). Cholesterol absorption was shown to decrease by 60% in diabetic patients with a daily intake of 3 g sitostanol delivered as fatty acid esters (4, 24).

Sitostanol has been shown to be virtually unabsorbable (26–28), but 12.5% of campestanol was found to be absorbed in a study of intestinal perfusion in humans (29). However, the results from the present study indicate that the absorption of campestanol was also negligible when campestanol was fed as part of a stanol blend containing substantial amounts of sitostanol

(65%). In the present study, the serum campesterol concentration decreased significantly and campestanol decreased nonsignificantly in both stanol ester groups. Therefore, the vegetable oil–based sterol blend can be used after saturation to stanol without an increase in serum campestanol concentration. The absorption of campestanol might be possible when it is not ingested as part of a blend containing competitive components like sitostanol. When campestanol is used as part of the stanol blend that contains substantial amounts of sitostanol, as was used in the present study, campestanol seems not to be absorbed at all (30).

The 2 low-fat test margarines were intended to differ from each other only with respect to the origin of the plant stanols, with the VOSEM margarine containing more campestanol and less sitostanol than the WSEM margarine. However, the actual daily intake of total plant stanol was 6.5% higher in the WSEM than in the VOSEM group. The cholesterol-lowering effect of sitostanol is well documented in the literature, but the effects of campestanol have not been studied, probably because of practical problems in obtaining pure campestanol in reasonable amounts. However, it has been shown in rats (31) that the oleate ester of campesterol can decrease the absorption of dietary cholesterol with the same efficacy as free β-sitosterol, stigmasterol, or the oleate ester of β-sitosterol. Furthermore, recent data from free-living humans indicate that rapeseed oil–derived campesterol could reduce cholesterol absorption and thus reduce serum cholesterol concentration (32). On the basis of these data, campestanol can also be expected to reduce cholesterol absorption. Thus, the difference in stanol compositions is not likely to have an effect on the present results.

On the basis of the food records during both study periods the adherence to the diets was good. Actually, during the low-fat diet the intakes of fat, saturated fatty acids, and dietary cholesterol were even lower than the dietary goals. Note that the intake of dietary cholesterol achieved the goal of the Step II diet of the National Cholesterol Education Program (<200 mg/d) (10), and the intake of saturated fatty acids was close to these goals (<7% of energy) in all study groups. Despite the frequent monitoring, there was a slight decrease in body weight in all study groups during the experimental period. The decrease in weight was primarily due to the lower intake of energy during the experimental than during the run-in period. However, because the weight change was marginal in all groups and because there were no significant differences in weight change among the groups, the decrease in weight cannot explain the findings of the present study.

Low-fat stanol ester–containing margarines appeared to have little effect on serum concentrations of retinol and crealcidiol. The serum absolute concentration of β-carotene and α-tocopherol decreased significantly in both the stanol ester–containing margarine groups, but this would be expected because β-carotene and α-tocopherol are transported in serum in lipoproteins, whose concentrations decreased during the experimental diet periods. When the serum β-carotene concentrations were related to the serum total cholesterol concentrations, the decrease was not significant in either of the low-fat stanol ester–containing margarine groups. In addition, the decrease in serum α-tocopherol concentration was ascribed to the changes in serum cholesterol concentrations because the ratio of serum α-tocopherol to total cholesterol actually increased significantly in all of the test margarine groups. These findings agree with the findings of Gylling et al (33). The increase in calcidiol concentrations was significantly smaller in the WSEM than in the VOSEM group. However, there were no significant differences among the groups in percentage changes in calcidiol or absolute calcidiol concentrations at the end of the study.

In conclusion, both the low-fat WSEM and VOSEM margarines when used as part of a low-fat, low-cholesterol diet are effective in reducing serum cholesterol concentrations with apparently equal efficacy in subjects with elevated serum cholesterol concentrations. In addition, these margarines offer an additional, clinically significant reduction in serum cholesterol concentrations to that obtained with a low-fat diet alone.

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Retinol, vitamin D, carotenes and α-tocopherol in serum of a moderately hypercholesterolemic population consuming sitostanol ester margarine☆

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Abstract

We have shown earlier that sitostanol ester margarine lowers serum cholesterol by inhibiting cholesterol absorption so that, theoretically, there could be interference with the absorption of fat-soluble vitamins. Accordingly, we investigated whether sitostanol ester margarine affects the serum levels of vitamin D, retinol, α-tocopherol and α- and β-carotenes during 1-year treatment in 102 subjects and 49 controls with moderate hypercholesterolemia. The vitamins were assayed at baseline on home diet, on margarine alone, after 1 year's consumption of sitostanol ester margarine and after an additional 2 months on home diet. In the sitostanol group, serum plant sterols, indicators of cholesterol absorption efficiency, were reduced up to −38% in relation to controls from home diet ($P < 0.01$) indicating that cholesterol absorption was markedly reduced. Vitamin D and retinol concentrations and the ratio of α-tocopherol to cholesterol were unchanged by sitostanol ester. Serum β-carotenes and α-carotene concentration but not proportion were reduced in the sitostanol group from baseline and in relation to controls ($P < 0.01$). Retinol and vitamin D were unassociated with serum cholesterol, plant sterols or other vitamins, whereas α-tocopherol and carotenes were significantly associated with serum plant sterols suggesting that the higher cholesterol absorption efficiency, the higher the α-tocopherol and carotene levels in serum. We conclude that sitostanol ester did not affect vitamin D and retinol concentrations and α-tocopherol/cholesterol proportion, but reduced serum β-carotene levels. α-Tocopherol and carotenes, but not vitamin D and retinol, were related to serum cholesterol and cholesterol absorption.

Keywords: Vitamin D; Retinol; α-Tocopherol; Carotenes; Sitostanol; Cholesterol absorption

1. Introduction

Plant sterols inhibit cholesterol absorption, and, accordingly, they have been studied since the 1950s as potential hypocholesterolemic agents [1–6]. These studies have been mainly performed with tall oil sterols, which contain in addition to relatively large amounts of sitosterol and less of campesterol, also saturated plant sterols, stanols. The preparations used in earlier studies were insoluble in fats and oils and in crystalline, homogenized or microhomogenized form. Sitostanol is a 5α-saturated derivative of sitosterol, and it is virtually unabsorbable [7–9]. We have shown earlier that when sitostanol has been made fat soluble by esterification with rapeseed oil fatty acids and dissolved in mayonnaise or margarine, it lowered serum total and LDL cholesterol at least by 10 and 15% in hypercholesterolemic subjects [10–12]. Since cholesterol reduction is mainly due to inhibition of cholesterol absorption, absorption efficiency of cholesterol being reduced by up to 65% [9], sitostanol ester might also interfere with absorption of fat-soluble vitamins. Accordingly, the aim of this study was to investigate whether serum levels of vitamin D, retinol, α-tocopherol and α- and β-carotene are altered by 1-year sitostanol ester margarine diet. In addition, the measurements of serum

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plant sterols (campesterol and sitosterol) were performed because they are known to reflect cholesterol absorption [13].

2. Subjects and methods

2.1. Subjects

The study population ($n = 153$) was recruited from a random population sample of about 1500 people from the province of North Karelia, Finland. The study population, the study design and major lipid results have been described in detail previously [11]. In short, of the population sample, subjects with serum cholesterol concentration ≥ 5.58 mmol/l, triacylglycerol concentration < 3.0 mmol/l, age between 25 and 64 years and BMI (weight/height^2) < 30 kg/m^2, and not having thyroid, renal, liver or alcohol problems were eligible to participate in the study. All subjects volunteered for the study, and the study protocol was approved by the Ethics Committee of Second Department of Medicine, University of Helsinki.

2.2. Study design

The subjects were advised to replace 24 g of their normal dietary fat with a rapeseed oil margarine for 6 weeks, after which they were randomized double-blind to two groups. The first group (controls, $n = 49$, because two subjects discontinued the study) continued to use rapeseed oil margarine for 1 year, and the second group (sitostanol group, $n = 102$) used the same margarine with added sitostanol ester so that the daily intake of sitostanol was intended to be 3 g/day. After 6 months, the subjects of the sitostanol group were rerandomized either to continue the intake of 3 g of sitostanol/day ($n = 51$) or to reduce the intake to 2 g/day ($n = 51$) for 6 months. The amount of rapeseed oil margarine was kept unchanged. After the 1 year, the subjects returned to their regular baseline, ad libitum home diet and were restudied after 2 months.

No vitamins A or D were added to the rapeseed oil margarines. The principal fatty acid composition of margarines was as follows: 16:0 = 16.7%; 18:1 = 47.3%; 18:2 = 17.7%; and 18:3 = 8.9%. The sitosterol blend was obtained from tall oil soap, and it was saturated to stanols. Free stanols were transesterified with rapeseed oil fatty acids (Raisio Group, Raisio, Finland) [9,10,14].

The margarine was portion-packed with 8 g of margarine per portion pack. The subjects used one portion pack three times a day, i.e. at breakfast, lunch and dinner, on a slice of bread. The sitostanol ester margarine contained 1 g of sitostanol in 8 g of margarine, and after reassignment in the second treatment group, 0.7 g of sitostanol in 8 g of margarine. The intake of margarine was confirmed by the number and emptiness of the portions returned during every visit; the daily consumption of the margarines was similar in the two groups [11].

Blood samples for the vitamin and plant sterol measurements were taken from the fasting subjects at baseline, at the end of the rapeseed oil margarine and the 12-month intervention period and after 2 months on regular ad libitum home diet. We present the average data from the two specimens taken 2 weeks apart. The treatment samples were taken in September and the regular diet samples in August (baseline) and in November (2 months after the intervention). Serum for the vitamin analyses was taken into dark test tubes and all analyses proceeded in subdued light. The samples were immediately frozen at -70°C and for each subject all samples were analyzed in the same run.

Serum vitamin D concentration was analyzed from the 3-g sitostanol group ($n = 51$) and from the controls once at home diet and after 12 months of treatment. Other vitamins were quantified from the whole study population and during each intervention period. A total of 19 random subjects from the control group and 36 from the sitostanol treatment groups kept a 3-day food record, from which the dietary constituents were calculated according to a computer-based quantitation [15], and are shown in Table 1 at home diet and during the study.

A total of 49 subjects of the control group and all 102 of the sitostanol group completed the study. The 2- and 3-g sitostanol groups are combined in the following, because the results concerning the vitamins and plant sterols were identical between the groups (data not shown). Measurement of sitostanol in the margarine and the actual intake of the margarine revealed that the mean sitostanol intake was 2.6 and 1.8 g/day in the two sitostanol groups.

2.3. Methods

Serum total and lipoprotein cholesterol and triacylglycerols were determined enzymatically with commercial kits (Boehringer Diagnostica, Germany). Vitamin D was analyzed by quantitating 25(OH)cholecalciferol in serum [16,17]. Retinol, α-tocopherol and α- and β-carotenes were analyzed with reverse-phase high pressure liquid chromatography (HPLC) according to the method described by Shäfer Elinder and Walldius [18] using α-tocopherol acetate as internal standard. Serum plant sterol concentrations were analyzed with gas-liquid chromatography on a 50-m HP Ultra 1 column (Hewlett Packard) [19,20].

Statistical significances were tested using Biomedical Data Program (BMDP) [21]. The difference between the groups was analyzed with analysis of variance and covariance with repeated measures and Student's two-

tailed t-test, and the intraindividual changes with paired t-test. Correlations were analyzed by calculating the Pearson's product-moment correlation coefficients. The serum values of α-tocopherol, α- and β-carotene and plant sterols were also standardized and expressed as proportions or ratios to serum cholesterol, because cholestanol [22], plant sterols [23], α-tocopherol [24] and the carotenes [25] are transported by lipoproteins in serum. P-value <0.05 was considered significant.

3. Results

The dietary intakes of cholesterol and fats were similar in the two groups (Table 1). Serum total cholesterol level was reduced from baseline home diet by $-0.3 \pm 0.6\%$ (S.E.M.) (NS) by rapeseed oil margarine only, and by $-9.4 \pm 0.9\%$ ($P < 0.001$) by sitostanol ester margarine (Fig. 1). The sitostanol ester-induced reduction of serum cholesterol was -9% when compared with the controls ($P < 0.001$). After the treatment ıen switched back to home diet, serum cholesterol

Table 1
Age and weight in the total study group and dietary intake of cholesterol and fatty acids in a random sample of 55 subjects during the study diet and on ad libitum home diet[a]

Variables	Controls ($n = 49$)	Sitostanol ester ($n = 102$)
Age, years	51 ± 1	50 ± 1
Weight, kg		
Home diet	73 ± 2	71 ± 1
Treatment	72 ± 2	71 ± 1
BMI, kg/m²		
Home diet	26.1 ± 0.5	25.5 ± 0.6
Treatment	25.6 ± 0.4	25.3 ± 0.2
Cholesterol intake, mg/day	($n = 19$)	($n = 36$)
Home diet	284 ± 28	335 ± 24
Treatment	314 ± 27	311 ± 21
Fat intake, g/day	($n = 19$)	($n = 36$)
ome diet	77 ± 8	79 ± 4
Treatment	85 ± 6	85 ± 4
Saturated fatty acids, g/day	($n = 19$)	($n = 36$)
Home diet	33 ± 4	36 ± 2
Treatment	34 ± 3	35 ± 2
Monounsaturated fatty acids, g/day	($n = 19$)	($n = 36$)
Home diet	28 ± 3	28 ± 1
Treatment	32 ± 2*	32 ± 1*
Polyunsaturated fatty acids, g/day	($n = 19$)	($n = 36$)
Home diet	13 ± 1	12 ± 1
Treatment	15 ± 1*	15 ± 1*

[a] Mean ± S.E.M.
* $P < 0.05$ from home diet.

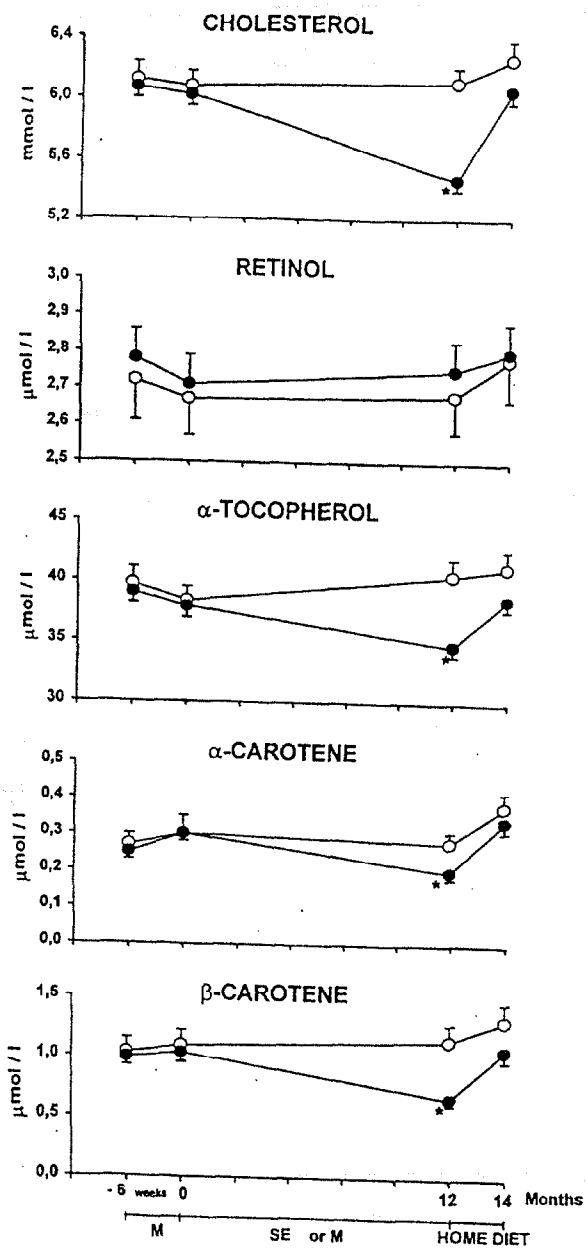

Fig. 1. Serum cholesterol and vitamin concentrations during consumption of margarine without (○) and with (●) sitostanol ester. Consumption was as follows: −6 weeks to 0, margarine (M); 0–12 months, M (controls) and M with sitostanol ester (SE); and 12–14 months, home diet. Mean ± S.E.M. *$P < 0.05$, between the groups and within sitostanol ester group, analysis of variance and covariance for repeated measures.

level was increased in both groups (Fig. 1). The detailed lipid-lowering effect of sitostanol ester margarine is given in Ref. [11].

Serum campesterol and sitosterol proportions, indicators of cholesterol absorption efficiency, were signifi-

cantly lowered by −26 and −12% from baseline home values ($P < 0.001$ for both) by sitostanol ester, and when compared with the controls, by 38 and 14%, respectively (Table 2).

Serum retinol concentration was unchanged by sitostanol ester (Fig. 1). α-Tocopherol concentration was significantly reduced by $-10 \pm 1\%$ by sitostanol (Fig. 1), but its proportion to cholesterol was unchanged (Table 2). The α- and β-carotene concentrations (Fig. 1) and β-carotene proportion, but not that of α-carotene (Table 2), were significantly reduced in the sitostanol group from baseline and the margarine period and in relation to the controls ($P < 0.01$ for all). Sitostanol had no effect on the serum levels of calcium, and the levels of vitamin D were significantly higher and alkaline phosphatase lower in both groups during the intervention from the home periods (Table 2).

Table 2
The effects of sitostanol ester on the serum lipid and plant sterol levels during baseline home diet and treatment periods

Variables[a]	Controls ($n = 49$)[b]	Sitostanol ester ($n = 102$)[b]
Campesterol/cholesterol[c]		
Home diet	283.9 ± 17.1	279.9 ± 12.8
Treatment	317.5 ± 18.1	200.3 ± 8.8*,†
Sitosterol/cholesterol[c]		
Home diet	148.1 ± 7.5	149.9 ± 5.8
Treatment	150.1 ± 8.3	126.9 ± 4.2*,†
α-Tocopherol/cholesterol[d]		
Home diet	6.51 ± 0.20	6.43 ± 0.12
Treatment	6.60 ± 0.18	6.34 ± 0.13
α-Carotene/cholesterol[d]		
Home diet	0.045 ± 0.006	0.041 ± 0.003
Treatment	0.046 ± 0.004	0.037 ± 0.003
β-Carotene/cholesterol[d]		
Home diet	0.17 ± 0.02	0.16 ± 0.01
Treatment	0.18 ± 0.02	0.12 ± 0.01*,†
Serum calcium, mmol/l		($n = 51$)
Home diet	2.36 ± 0.01	2.38 ± 0.01
Treatment	2.34 ± 0.01	2.35 ± 0.01
Alkaline phosphatase, IU/l		($n = 51$)
Home diet	109.4 ± 3.8	117.8 ± 4.4
Treatment	92.6 ± 3.2*	102.7 ± 3.9*
Vitamin D, nmol/l		($n = 51$)
Home diet	82.1 ± 4.2	65.7 ± 3.7†
Treatment	91.0 ± 3.8*	75.2 ± 3.6*,†

[a] Normal values for serum calcium 2.25–2.65 mmol/l, alkaline phosphatase 60–275 U/l, vitamin D, October–May: 22–72 nmol/l (home diet period); June–September: 26–120 nmol/l (treatment period).
[b] Mean ± S.E.M.
[c] $10^2 \times$ μmol/mmol of cholesterol.
[d] μmol/mmol.
* $P < 0.05$ or less from home diet.
† $P < 0.05$ or less, sitostanol group versus controls.

Serum α-tocopherol and the α- and β-carotene concentrations were significantly positively related to each other and to serum cholesterol on home diet (Table 3), but not consistently to serum triacylglycerols ($r = 0.226$, $P < 0.01$, $r = -0.046$ and -0.135, respectively). In addition, α-tocopherol and α- and β-carotenes were significantly positively related to serum plant sterol concentrations, and negatively to BMI indicating that the higher the cholesterol absorption efficiency and the higher BMI, the higher and lower were the respective serum α-tocopherol and carotene concentrations (Table 3).

Sitostanol-induced change in serum retinol concentration was significantly correlated with those of α-tocopherol and cholesterol, whereas the changes in serum α-tocopherol and β-carotene concentrations were interrelated and correlated with those of serum cholesterol and plant sterols (Table 4). The change in serum vitamin D concentration was significantly correlated with that of retinol.

4. Discussion

It appears that serum vitamin concentrations have not been previously studied during sitostanol ester treatment. Thus, the new observations in the present study are that, in spite of the significant lowering of serum cholesterol by 9% and up to 38% lowering of plant sterols by sitostanol ester dissolved in margarine, the serum concentrations of vitamin D and retinol, and the α-tocopherol/cholesterol ratio were not changed during 12 months' sitostanol ester margarine consumption. Serum β-carotene levels but not the α-carotene to cholesterol proportion were significantly reduced in the sitostanol group. Also, this study shows for the first time that the serum levels of α-tocopherol and carotenes were tightly associated with indicators of cholesterol absorption, viz. the plant sterols, but those of retinol and vitamin D did not exhibit this association suggesting that they might be absorbed independently of cholesterol, apparently by a somewhat different mechanism. Sitosterol, the indicator of cholesterol absorption, and the serum levels of α-tocopherol and α- and β-carotenes and their changes by sitostanol ester were negatively associated as well with BMI.

The homeostasis of vitamin D is dependent on both absorption from the intestine and from endogenous synthesis. Vitamin D is absorbed in the distal ileum by a bile acid-requiring process. Fat malabsorption reduces vitamin D absorption, but sitostanol ester feeding does not cause fat malabsorption [9,12]. From the enterocyte, vitamin D is transported in chylomicrons mainly in free form in contrast to cholesterol [26]. At least in rats, it is absorbed also in mid-jejunum [27], and it may also have a specific receptor-mediated absorp-

Table 3
Correlation coefficients between BMI and the serum levels of vitamins, cholesterol and plant sterols on home diet ($n = 153$) and on sitostanol ester ($n = 102$)

Variables	Retinol, μmol/l	α-Tocopherol, μmol/l	α-Carotene, μmol/l	β-Carotene, μmol/l	Vitamin D, nmol/l	BMI, kg/m^2
Home diet ($n = 153$)						
α-Tocopherol, μmol/l	0.089					
α-Carotene, μmol/l	−0.056	0.248*				
β-Carotene, μmol/l	0.059	0.581*	0.515*			
Vitamin D, nmol/l	0.065	0.086	0.081	0.057		
Cholesterol, mmol/l	0.116	0.604*	0.276*	0.386*	0.075	−0.001
Campesterol, μmol/l	−0.054	0.286*	0.267*	0.296*	0.095	−0.162
Sitosterol, μmol/l	−0.040	0.334*	0.283*	0.412*	0.053	−0.215*
BMI, kg/m^2	0.057	−0.161	−0.297*	−0.232*	−0.092	1.000
Sitostanol ester ($n = 102$)						
α-Tocopherol, μmol/l	0.039					
α-Carotene, μmol/l	−0.014	0.224				
β-Carotene, μmol/l	0.006	0.479*	0.648*			
Vitamin D, nmol/l	0.080	−0.121	0.119	−0.004		
Cholesterol, mmol/l	0.026	0.507*	0.270*	0.247	−0.098	0.027
Campesterol, μmol/l	−0.188	0.229	0.292*	0.387*	0.085	−0.170
Sitosterol, μmol/l	−0.204	0.284*	0.334*	0.399*	−0.018	−0.195
BMI, kg/m^2	0.114	−0.259*	−0.283*	−0.322*	−0.128	1.000

* $P < 0.01$.

tion pathway. These mechanisms possibly explain the complete unassociation between vitamin D and cholesterol absorption and metabolism observed in this study.

β-Carotene, having a non-polar chemical structure with two β-ionone rings connected by an isoprene chain, was the only vitamin serum level which was significantly reduced by sitostanol, yet its metabolite retinol exhibited unchanged serum concentration. This is in agreement with the earlier findings that β-carotene feeding does not increase serum retinol levels [28–31]. Retinol is chemically more polar than β-carotene, because the latter is oxidized to two molecules of retinol. α-Carotene produces only one molecule of retinol, because it has only one β-ionone and one non-β-ionone ring. The serum levels of β-carotene are regulated by dietary supplementation [28,29,32,33]. The present study shows that its absorption is related to cholesterol absorption efficiency since its serum level and change were related to those of plant sterols. β-Carotene is absorbed from 9 to 22% [34–36], and the absorbed β-carotene is mostly converted to retinol in the enterocyte [34,35]. In a recent study, 57% of β-carotene was converted to retinoids in enterocytes and 43% in liver [36]. All serum β-carotene is transported in lipoproteins, mainly in LDL [25,37]. However, we feel that because of the recent reports of harmful effects following β-carotene supplementation [38,39] and the unaffected serum retinol concentration in the present study, the β-carotene reduction was not of essential concern. Sucrose polyester, the non-absorbable fat analogue, reduces significantly the plasma concentration of α-tocopherol, β-carotene and the other carotenoids [40–42]. Sucrose polyester inhibits cholesterol absorption probably by altering partition of cholesterol out of the micellar phase into an oily sucrose polyester phase resulting subsequently in serum cholesterol lowering [40,41]. Fecal output of cholesterol as cholesterol itself and bile acids is increased and stool frequency is also increased [41,42]. Sitostanol ester does not cause diarrhoea, fecal urgency or gastrointestinal side-effects, nor have we seen any changes in fecal fat or bile acids, but cholesterol elimination in stools increases consistently [9,12].

The concentration of serum retinol, similarly to that of vitamin D, was unassociated with the serum cholesterol and plant sterol levels, even though the change in serum retinol was associated with that of serum cholesterol. Orally fed retinol is absorbed by 75–90% [34,35,43], esterified in the enterocyte to retinyl esters and transported in chylomicrons through lymph and taken up as their remnant particles by the liver. Quantitation of retinyl esters in serum has been frequently used as an indicator of the clearance of postprandial lipoproteins [44]. Lack of association between serum retinol and plant sterol levels suggests that serum retinol does not reflect cholesterol absorption, probably partly owing to its better solubility.

The serum concentrations of α-tocopherol and carotenes, on the other hand, seemed to depend on cholesterol absorption. The correlation equations showed that if for example serum sitosterol concentration was reduced by 100 μmol/l, α-tocopherol was reduced by 2 μmol/l. In addition, the serum levels of

Table 4
Correlation coefficients between changes of serum vitamin, cholesterol and plant sterol concentrations caused by sitostanol ester from home diet ($n = 102$)

Variables	Δ Vitamin D, nmol/l	Δ Retinol, μmol/l	Δ α-Tocopherol, μmol/l	Δ β-Carotene, μmol/l
Δ Retinol, μmol/l	0.361*			
Δ α-Tocopherol, μmol/l	0.155	0.461*		
Δ β-Carotene, μmol/l	0.173	0.154	0.427*	
Δ Cholesterol, mmol/l	0.208	0.399*	0.714*	0.379*
Δ Campesterol, μmol/l	0.077	0.155	0.370*	0.358*
Δ Sitosterol, μmol/l	0.043	0.153	0.390*	0.398*

* $P < 0.01$.

carotenoids were inversely dependent on BMI. Since the latter is negatively associated with cholesterol absorption efficiency [45,46], the results suggest that obese subjects may have low serum α-tocopherol and carotenoid concentrations.

α-Tocopherol is absorbed in free form by a non-saturable passive process, and transported in chylomicrons into the lymphatics [24]. The absorption efficiency of α-tocopherol is around 40% [24]. It is mainly transported in LDL and HDL, and only a small fraction (5–11%) in VLDL [24,25,47–50]. This explains the significant interrelation between serum α-tocopherol and cholesterol, but not triacylglycerols. It also indicates that the serum levels of α-tocopherol should be standardized to serum cholesterol levels. Accordingly, in the present study, sitostanol ester lowered serum concentrations of both α-tocopherol and cholesterol similarly by 10%, so that the α-tocopherol/cholesterol ratio was unchanged. The serum level of α-tocopherol reflects its dietary intake [24,32], and can be used as an indicator of overall body supply.

In conclusion, sitostanol ester, even though inhibiting cholesterol absorption and reducing serum levels of total and LDL cholesterol, does not affect the serum levels of vitamin D, retinol or α-tocopherol/cholesterol ratio, but reduces that of β-carotene.

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PART I

Cholesterol Reduction by Different Plant Stanol Mixtures and With Variable Fat Intake

Helena Gylling and Tatu A. Miettinen

Our aim was to investigate (1) whether different campestanol/sitostanol mixtures in margarine differ in reducing serum cholesterol, and (2) whether sitostanol ester in butter decreases serum cholesterol and alters cholesterol absorption and metabolism. Twenty-three postmenopausal women replaced 25 g dietary fat with (1) sitostanol ester–rich (campestanol to sitostanol ratio 1:11) and (2) campestanol ester–rich (campestanol to sitostanol ratio 1:2) rapeseed oil margarine, (3) butter, and (4) sitostanol ester–rich (campestanol to sitostanol ratio 1:13) butter. The respective scheduled stanol intake was 3.18, 3.16, and 2.43 g/d. The 6-week margarine periods and, after an 8-week washout, 5-week butter periods were double-blind and in random order. Serum cholesterol precursor sterols (indicators of cholesterol synthesis) and plant sterols (indicators of cholesterol absorption) were quantified with gas-liquid chromatography (GLC). Low-density lipoprotein (LDL) cholesterol was reduced by 8% and 10% with the sitostanol and campestanol ester–rich margarines versus baseline ($P < .05$ for both) and high-density lipoprotein (HDL) cholesterol was increased by 6% and 5% ($P < .05$), so the LDL/HDL cholesterol ratio was reduced by 15% ($P < .05$ for both). Sitostanol ester–rich butter decreased LDL cholesterol 12% and the LDL/HDL cholesterol ratio 11% ($P < .05$ for both) versus the butter period. The serum proportions of plant sterols and cholestanol were similarly reduced and those of cholesterol precursor sterols were similarly increased during all periods ($P < .05$ for all). Serum proportions of sitostanol and campestanol were slightly increased, indicating that their absorption related to their dietary intake. During all stanol interventions, serum vitamin D and retinol concentrations and α-tocopherol to cholesterol ratios were unchanged, whereas those of α- and β-carotenes were significantly reduced. We conclude that varying the campestanol to sitostanol ratio from 1:13 to 1:2 in margarine and in butter similarly decreased cholesterol absorption, LDL cholesterol, and the LDL/HDL cholesterol ratio such that the serum lipids became less atherogenic.

SERUM CHOLESTEROL is regulated by the interplay of cholesterol absorption, cholesterol synthesis, and low-density lipoprotein (LDL) receptor activity. However, dietary factors also affect cholesterol homeostasis such that dietary cholesterol and saturated fatty acids independently elevate[1,2] and dietary plant sterols decrease[3-10] serum cholesterol in humans. Plant sterols have been studied since the 1950s as potential hypocholesterolemic agents.[3-10] These studies have been performed mainly with tall oil sterols containing a relatively large amount of sitosterol and less campesterol and saturated stanols. However, sitosterol and especially campesterol are absorbed about 5% and 16%,[11-15] but the saturated derivative of sitosterol, sitostanol, is virtually unabsorbable,[16,17] whereas campestanol may be absorbed to some extent.[14]

We have previously shown that sitostanol (with small amounts of campestanol), when made fat-soluble by transesterification with rapeseed oil fatty acids and dissolved in mayonnaise or margarine, decreases serum total and LDL cholesterol by at least 10% and 14% in mildly hypercholesterolemic populations,[18-20] women with coronary artery disease,[21] children with familial hypercholesterolemia,[22] and type 2 diabetics.[23,24] Sitostanol and its esters decrease serum cholesterol by inhibiting the absorption and increasing the synthesis of cholesterol.[15,18-25] These changes are also reflected in decreased serum plant sterols and increased precursor sterols of cholesterol.[21,22] It has been demonstrated that plant sterols reduce serum cholesterol effectively in subjects consuming a saturated fatty acid–enriched diet.[26] Dietary saturated fatty acids elevate serum cholesterol mainly by enhancing LDL production and downregulating LDL receptor activity,[27,28] with no[29,30] or some[31] effect on cholesterol absorption.

Now, several questions arise to be answered in the present stanol ester mixture feeding study: (1) Is a reduction in serum cholesterol dependent on the campestanol to sitostanol ratio in margarine?; (2) Is absorption of campestanol and sitostanol detectable by their serum values?; (3) Are the serum levels of campestanol and sitostanol and their parent plant sterols dependent on their dietary intake?; and finally, (4) How effective is sitostanol ester in butter to reduce serum cholesterol and alter the absorption and synthesis of cholesterol?

SUBJECTS AND METHODS

Patients

The study population consisted of 24 moderately hypercholesterolemic postmenopausal women aged 50 to 55 years, with a mean of 52.7 ± 1.2 (mean ± SE) years. They were recruited from a random age cohort based on the population register of the Helsinki area. The inclusion criteria for this study were as follows: serum cholesterol between 5.5 and 8.0 mmol/L, serum triglycerides less than 2.5 mmol/L, and body mass index less than 28 kg/m^2. Postmenopausal status was determined by the absence of menstruation and serum follicle-stimulating hormone greater than 30 μmg/L. Eight women had postmenopausal hormone replacement therapy, four with tablets and four with transdermal estrogen, and they had no change in the therapy during the intervention. The study subjects had no prior hypolipidemic treatment or thyroid, gastrointestinal, or hepatic disease or diabetes mellitus. All volunteered for the study and provided informed consent. The study was approved by the Ethics Committee of our hospital.

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Study Design

After two baseline blood samples 1 week apart on the ad libitum home diet, the subjects were advised to replace 25 g of their normal dietary fat with (1) sitostanol ester–rich (campestanol to sitostanol ratio 1:11) and (2) campestanol ester–rich (campestanol to sitostanol ratio 1:2) rapeseed oil margarine and (3) butter without and (4) with sitostanol (campestanol to sitostanol ratio 1:13) ester. We did not have a margarine-only period because we were not investigating the efficacy of plant stanols per se, but only in combination with margarine or butter. The margarines contained 3.18 and 3.16 g stanols/25 g margarine, and the butter 2.43 g stanols/25 g butter. The margarines contained no *trans*-fatty acids. However, the daily intake of plant sterols was different (Table 1). Sitostanol ester–rich margarine contained 82.8% sitostanol and 7.5% campestanol of the tall oil–based plant sterols, whereas the campestanol-rich margarine of the vegetable oil–based sterols contained 65.2% sitostanol and 28.1% campestanol, respectively. In addition, the sitostanol ester–rich margarine contained two times more sitosterol than the other margarine, but the campesterol intake was similar. The sitostanol butter contained 92.0% sitostanol and 7.3% campestanol of the total plant sterols. The margarine interventions lasted 6 weeks and the butter periods 5 weeks. The margarine periods were double-blind in random order with a crossover design. After a washout of 8 weeks, the same women were randomly and double-blindly assigned the butter without or with sitostanol ester.

Neither vitamin A nor vitamin D were added to the margarine. β-Carotene was used as a coloring agent. The butter products contained normal levels of carotenoids and vitamins. The margarine stanols were transesterified with rapeseed oil fatty acids, and butter stanols with butter fatty acids (Raisio Group, Raisio, Finland).

Two blood samples for lipid, vitamin, cholesterol precursor sterol (indicators of cholesterol synthesis),[32] and plant sterol and cholestanol (indicators of cholesterol absorption efficiency)[32] measurements were taken from the fasting subjects at baseline during the home diet and 1 week apart at the end of each intervention period. The mean value for the two specimens is presented. During the home diet and the margarine and butter periods, nutrients were analyzed from a 7-day food record according to a computer program.[33] Serum for the vitamin analyses was taken in dark test tubes, and all analyses were performed in subdued light. All samples were immediately frozen to −70°C.

Methods

Serum total cholesterol and triglycerides were determined enzymatically with commercial kits (Boehringer Diagnostica, Mannheim, Germany). High-density lipoprotein (HDL) cholesterol was determined enzymatically after apolipoprotein B (apo B) containing lipoproteins were precipitated. LDL cholesterol was calculated.[34] Serum noncholesterol sterol concentrations were analyzed with gas-liquid chromatography (GLC) on a 50-m SE-30 capillary column[32,35] (Ultra 1; Hewlett Packard). However, sitostanol and campestanol were quantified by GLC on an Ultra 2 capillary column. Identification of campestanol and sitostanol was based on their retention time relative to their parent compounds campesterol and sitosterol, respectively. The fact that the peaks with these retention times contained respective stanols was evidenced by mass spectrometry for larger respective peaks from a patient with phytosterolemia. Owing to low concentrations of campestanol and sitostanol in normal serum, no mass spectrometric evidence was obtained for the presence of these two stanols. Vitamin D was analyzed by quantifying 25(OH)cholecalciferol in serum.[36] Retinol, α-tocopherol, and α- and β-carotenes were analyzed with reverse-phase high-performance liquid chromatography[37] using α-tocopherol acetate as an internal standard.

Table 1. Scheduled Daily Intake (mg/d) of Plant Sterols in the Margarine and Butter Diets

Variable	Sitostanol Ester–Rich Margarine	Campestanol Ester–Rich Margarine	Sitostanol Ester Butter
Campesterol	79	84	8
Campestanol	264	952	202
Sitosterol	262	143	11
Sitostanol	2,914	2,206	2,232
Total stanols	3,178	3,158	2,434
Total sterols	3,519	3,385	2,453

Table 2. Weight, Body Mass Index, and Dietary Cholesterol and Fat Intake During the Different Diets

Variable	Home Diet (n = 23)	Margarines (n = 23)	Butter (n = 21)
Weight (kg)	66.7 ± 1.5	66.9 ± 1.6	66.7 ± 1.6
Body mass index (kg/m²)	25.7 ± 0.7	25.7 ± 0.7	25.6 ± 0.7
Dietary cholesterol (mg/d)	269 ± 19	262 ± 19	323 ± 19*
Dietary fat (g/d)	80 ± 7	93 ± 6*	97 ± 6*
Polyunsaturated/saturated fatty acid ratio	0.40 ± 0.03	0.58 ± 0.03*	0.26 ± 0.02*

NOTE. Results are the mean ± SE.
*Significantly different *v* home diet.

Statistical Analysis

Statistical significance was tested with ANOVA and covariance with repeated measures (BMDP Statistical Software, Los Angeles, CA) and a paired *t* test. Logarithmic transformations were used with skewed distributions. Serum values for noncholesterol sterols, α-tocopherol, and α- and β-carotene were standardized and also expressed in proportion to serum cholesterol, because the noncholesterol sterols, α-tocopherol, and carotenes are transported by lipoproteins, mainly LDL, in serum. A P value less than .05 was considered significant.

RESULTS

Twenty-four subjects participated in the two margarine interventions, and 21 subjects completed the whole study. One subject had to be excluded because of violation of the protocol, and two subjects withdrew for reasons not connected with the study.

The weight and body mass index were unchanged throughout the study (Table 2). Daily cholesterol intake was low during the margarine period but increased during butter consumption, and fat intake was increased by 13 ± 1 g/d during the margarine period and 17 ± 1 g/d during the butter period. Scheduled plant sterol intake was variable (Table 1). However, total stanol intake was similar in the sitostanol ester–rich and campestanol ester–rich margarine periods, but lower during the butter period.

Sitostanol and Campestanol Ester–Rich Margarines

Serum total and LDL cholesterol were significantly reduced by 4% ± 2% and 8% ± 3% with sitostanol ester–rich margarine and by 6% ± 2% and 10% ± 2% with campestanol ester–rich margarine versus the baseline home values (Table 3). HDL cholesterol levels were significantly increased by 6% ± 2% and 5% ± 2% and the LDL/HDL cholesterol ratio was reduced by 15% with both stanol ester margarines. The baseline data and the changes in lipids were not related to estrogen treatment.

The proportion of serum campesterol, sitosterol, and choles-

Table 3. Serum and Lipoprotein Lipids (mmol/L) During the Different Diets

Variable	Home Diet (n = 23)	Sitostanol Ester–Rich Margarine (n = 23)	Campestanol Ester–Rich Margarine (n = 23)	Butter (n = 21)	Sitostanol Ester Butter (n = 21)
Serum cholesterol	6.06 ± 0.16	5.79 ± 0.17*	5.71 ± 0.18*	6.34 ± 0.21*	5.88 ± 0.18†
LDL cholesterol	3.98 ± 0.14	3.62 ± 0.14*	3.58 ± 0.17*	4.15 ± 0.18	3.70 ± 0.16†
HDL cholesterol	1.54 ± 0.09	1.63 ± 0.10*	1.62 ± 0.09*	1.63 ± 0.10*	1.64 ± 0.10
Serum triglycerides	1.21 ± 0.14	1.18 ± 0.13	1.15 ± 0.12	1.26 ± 0.17	1.18 ± 0.13
LDL/HDL cholesterol	2.80 ± 0.20	2.44 ± 0.19*	2.42 ± 0.20*	2.77 ± 0.23	2.46 ± 0.19†

NOTE. Results are the mean ± SE and were analyzed by ANOVA and analysis of covariance for repeated measures.
*Significantly different *v* home diet.
†Significantly different *v* butter.

tanol was reduced with the stanol ester margarines by 6% to 21% versus the baseline values (Table 4). The sitosterol proportion was even more effectively reduced by campestanol versus sitostanol ester–rich margarine, probably due to the higher sitosterol intake with the latter (Table 1). Compared with the home diet, the serum campestanol proportion (Table 4) was slightly increased by both stanol ester margarines, significantly more so by the campestanol ester–rich period, most likely due to a higher dietary intake of campestanol (264 *v* 952 mg/d). The serum sitostanol proportion was also slightly but significantly increased, more so by the sitostanol ester–rich margarine with a higher sitostanol intake (2,914 *v* 2,206 mg/d). The increase seems smaller for campesterol versus sitosterol in each stanol mixture. The serum cholesterol precursor sterols Δ^8-cholestenol, desmosterol, and lathosterol were compensatorily similarly increased by +12% to +19%, respectively.

Serum concentrations of vitamin D and retinol were unchanged from baseline values during both periods (Table 5). The serum concentration of α-tocopherol was significantly reduced during both margarine periods, but the α-tocopherol to cholesterol ratio was unchanged. The serum concentration and proportion of α- and β-carotenes were significantly reduced by both stanol ester margarines.

Butter Versus Sitostanol Ester Butter

Butter alone increased serum total and LDL cholesterol by 4% and HDL cholesterol by 6% (Table 3) without any constant changes in the serum noncholesterol sterol proportions (Table 4). The addition of sitostanol ester to butter significantly decreased serum total and LDL cholesterol by 8% ± 2% and 12% ± 2% versus butter alone and decreased the LDL/HDL ratio. The serum plant sterol and cholestanol proportions were decreased by 12% to 29% ($P < .05$ for both) compared with butter alone and the proportion of serum precursor sterols was compensatorily increased. The proportion of campestanol was slightly increased and sitostanol was doubled, yet the final values were only about one fourth of the respective sitosterol value.

The vitamin D concentration was increased versus the home values similarly by the two butter preparations, but the α-tocopherol proportion and retinol concentration were unchanged by the butters (Table 5). The α-carotene concentration and proportion were decreased by butter versus the home diet, but the sitostanol ester decreased the β-carotene concentration and proportion versus the butter-alone period.

DISCUSSION

This study shows for the first time that campestanol ester–rich margarine with 28% campestanol decreases serum total and LDL cholesterol as effectively as sitostanol ester–rich margarine with a low campestanol content (7.5%). We were not interested in the pure plant stanol effect per se, because we have described it previously[21] and it is not used alone; for this reason, we did not have a margarine-only study period. Sitostanol esterified with butter fat fatty acids and dissolved in butter decreased serum cholesterol as effectively as the respective stanol mixture in margarine, despite a slightly smaller stanol dose. These results suggest that the stanol and fatty acid composition of stanol ester can be varied and dissolved either in monoene-enriched margarine or in butter without diminishing the cholesterol-lowering effect. In addition, all stanol ester–containing products increased HDL cholesterol followed by a 15% decrease of the LDL/HDL cholesterol ratio such that the serum lipids became less atherogenic during both margarine-

Table 4. Serum Noncholesterol Sterol to Cholesterol Proportion (10^2 μmol/mmol cholesterol) During the Different Diets

Variable	Home Diet (n = 23)	Sitostanol Ester–Rich Margarine (n = 23)	Campestanol Ester–Rich Margarine (n = 23)	Butter (n = 21)	Sitostanol Ester Butter (n = 21)
Δ^8-Cholestenol	19.2 ± 1.5	22.1 ± 1.2*	22.0 ± 1.3*	17.6 ± 1.4	20.1 ± 1.4†
Desmosterol	73.7 ± 5.2	80.6 ± 3.9*	80.3 ± 4.0*	73.8 ± 4.6	84.8 ± 5.1*†
Lathosterol	182.8 ± 10.1	204.9 ± 10.9*	207.1 ± 11.4*	175.1 ± 14.8	202.3 ± 12.9*†
Campesterol	210.9 ± 17.0	164.9 ± 13.0*	164.7 ± 12.7*	209.4 ± 17.8	151.2 ± 15.3*†
Campestanol	1.84 ± 0.18	3.44 ± 0.30*	8.52 ± 0.66*‡	2.35 ± 0.27*	2.52 ± 0.14*
Sitosterol	125.0 ± 7.8	107.8 ± 6.1*	97.0 ± 5.9*‡	125.8 ± 9.6	90.2 ± 6.2*†
Sitostanol	11.1 ± 0.6	23.2 ± 1.1*	20.9 ± 0.9*‡	11.2 ± 0.5	23.1 ± 1.2*†
Cholestanol	127.9 ± 6.6	114.2 ± 5.6*	119.6 ± 5.5*	122.8 ± 6.9	111.9 ± 6.1*†

NOTE. Results are the mean ± SE and were analyzed by ANOVA and analysis of covariance for repeated measures.
*Significantly different *v* home diet.
†Significantly different *v* butter.
‡Significantly different *v* sitostanol ester–rich margarine.

Table 5. Serum Levels of Vitamin D, Retinol, α-Tocopherol, and α- and β-Carotene During the Different Diets

Variable	Home Diet (n = 23)	Sitostanol Ester–Rich Margarine (n = 23)	Campestanol Ester–Rich Margarine (n = 23)	Butter (n = 21)	Sitostanol Ester Butter (n = 21)
Vitamin D (nmol/L)‡	52.7 ± 5.1	58.0 ± 6.2	56.2 ± 5.9	65.4 ± 5.6*	65.5 ± 6.0*
Retinol (μmol/L)	2.25 ± 0.08	2.27 ± 0.07	2.33 ± 0.09	2.23 ± 0.08	2.33 ± 0.08
α-Tocopherol (μmol/L)	41.7 ± 1.9	38.1 ± 1.7*	38.3 ± 1.6*	42.7 ± 2.0	39.8 ± 1.8*†
α-Tocopherol/cholesterol	6.94 ± 0.33	6.62 ± 0.25	6.79 ± 0.30	6.78 ± 0.28	6.84 ± 0.32
α-Carotene (μmol/L)	0.46 ± 0.06	0.32 ± 0.04*	0.31 ± 0.04*	0.35 ± 0.04*	0.32 ± 0.05*
α-Carotene/cholesterol	0.07 ± 0.01	0.06 ± 0.01*	0.05 ± 0.01*	0.06 ± 0.01*	0.05 ± 0.01*
β-Carotene (μmol/L)	1.63 ± 0.17	1.10 ± 0.13*	1.10 ± 0.15*	1.57 ± 0.19	1.19 ± 0.13*†
β-Carotene/cholesterol	0.27 ± 0.03	0.19 ± 0.02*	0.19 ± 0.02*	0.25 ± 0.03	0.21 ± 0.02*†

NOTE. Results are the mean ± SE and were analyzed by ANOVA and analysis of covariance for repeated measures.
*Significantly different *v* home diet.
†Significantly different *v* butter.
‡Normal range, 22-72 nmol/L.

and butter-based stanol ester diets. The 8% and 10% LDL cholesterol decreases represent the combined effect of margarine and stanol, a smaller effect versus some earlier studies,[18-24] especially in coronary women,[21] but is now associated with increased HDL cholesterol, found only infrequently in our prior experiments.[22,23] Serum sitostanol and campestanol, being less than one tenth of the sitosterol and campesterol concentrations at baseline, were significantly increased during the margarine- and butter-based stanol ester diets even though the final serum concentrations remained one fifth to one seventh of the respective parent-compound values. The stanol esters, despite inhibiting cholesterol absorption, had no effect on the serum concentrations of vitamin D and retinol and the α-tocopherol to cholesterol proportion, whereas serum carotene levels and proportions were reduced.

The stanol esters were obtained from different sources. The tall oil–based stanol product contained 83% sitostanol and 7.5% campestanol of the total sterol mixture, with the respective values being 65% and 28% for the campestanol ester–rich sterol mixture obtained from vegetable oil sterols. The two stanol mixtures, the dietary intake of which was similar in the two groups, seemed equally effective in inhibiting cholesterol absorption efficiency and compensatorily upregulating cholesterol synthesis and reducing serum cholesterol. Thus, the reduction in serum cholestanol and plant sterols and the increase in cholesterol precursors were similar during the two sterol group feedings. The dietary intake of plant sterols seems to be associated with their serum level.[38] Thus, the changes in serum stanols and sterols depended on their scheduled dietary intake. Accordingly, campestanol seemed to be absorbed especially from the campestanol-rich sterol mixture, and sitostanol especially from the sitostanol-rich margarine. The present study actually evidences for the first time that these stanols are also absorbed slightly in normal subjects. It is known from previous studies that sitostanol is only slightly absorbed from the intestine, such that 2% of orally fed sitostanol is recovered from rat lymph in 24 hours[39] and it has been used in human studies as a nonabsorbable marker.[40] The absorption of campestanol is less well known, but it can be presumed to be less well absorbed than campesterol because, in general, stanols seem to be less absorbable than the respective parent sterols. In an intestinal perfusion study, 12.5% of infused campestanol was absorbed in humans,[14] but the overall quantities were small. In the present study, the relative increase of the campestanol proportion was about 36%, whereas for sitostanol it was about 110%. The smaller increase of campestanol versus sitostanol, even on the campestanol-rich diet, may be due to an effective inhibitory action of large amounts of sitostanol on smaller amounts of campestanol in the intestinal sterol mixture. Previous studies have shown that an alteration in dietary plant sterol composition sensitively changes their serum levels.[38] The serum campestanol concentration increased by 11 μg/100 mL during the campestanol ester–rich margarine treatment but the amount remained small, suggesting that these minor serum concentrations are probably meaningless, especially since other serum plant sterol levels were markedly decreased. However, there is one group of patients in whom even small amounts of stanols may accumulate in the body: homozygous patients with phytosterolemia. This extremely rare disease is characterized by increased absorption[41,42] and decreased biliary secretion[41] of plant sterols and accelerated atherosclerosis.[42]

The serum concentrations of vitamin D and retinol and the α-tocopherol proportion were unaffected by the stanol ester margarine consumption. β-Carotene, having a nonpolar chemical structure with two β-ionone rings connected by an isoprene chain, was the only vitamin derivative for which the serum level was significantly reduced by sitostanol yet its metabolite retinol exhibited an unchanged serum concentration. This is in agreement with previous findings that β-carotene feeding does not increase serum retinol levels.[43-44] Retinol is chemically more polar than β-carotene, so the latter is oxidized to two molecules of retinol. α-Carotene produces only one molecule of retinol because it has only one β-ionone and one non–β-ionone ring. Serum levels of β-carotene reflect dietary supplementation,[43-45] but the present findings suggest that its absorption was related to the cholesterol absorption efficiency. β-Carotene is absorbed from 9% to 22%,[46-48] with the absorbed fraction mostly converted to retinol in the enterocyte.[46,47] In a recent study, 57% of absorbed β-carotene was converted to retinoids in enterocytes and 43% in the liver.[48] All serum β-carotene is transported in lipoproteins, mainly LDL.[49] The clinical importance of the carotene decrease remains obscure, since β-carotene treatment has resulted in harmful clinical effects.[50,51]

Diets high in saturated fatty acids increase serum cholesterol

by increasing cholesterol absorption, diminishing cholesterol synthesis and LDL apo B receptor activity, and increasing LDL apo B production.[35] However, the present study revealed that stanol esters are able to decrease serum total and LDL cholesterol and the LDL/HDL cholesterol ratio even in a saturated environment, ie, when stanol is esterified with butter fatty acids and then dissolved in butter. By inhibiting cholesterol absorption, a probable mechanism, despite upregulation of cholesterol synthesis, is an activation of LDL apo B transport due to reduced intestinal flow of cholesterol to the liver without altered LDL apo B removal.[23,24] A lack of hepatic cholesterol decreases apo B synthesis, resulting in reduced transport of VLDL apo B to LDL.[52] Accordingly, the stanol esters are effective cholesterol-lowering agents despite the type of dietary fat intake.

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EFFECTS OF PLANT STEROL-ENRICHED REDUCED-FAT SPREADS ON SERUM CAROTENOID AND FAT-SOLUBLE VITAMIN LEVELS. Maki KC, Davidson MH, Umporowicz D, Dicklin MR, Ingram KA, Gebhart B, Chicago Center for Clinical Research, Chicago, IL; Schaefer E, Tufts University, Boston, MA; Chen S, Franke WC, Lipton, Englewood Cliffs, NJ

Research regarding plant sterols has demonstrated their ability to decrease levels of total and low-density lipoprotein (LDL) cholesterol. Because phytosterols reduce cholesterol absorption, there is concern that they may also decrease absorption of lipophilic nutrients. This double-blind, randomized, controlled clinical trial assessed the impact of two doses of phytosterol-esters (1.1 or 2.2 g/d), delivered in a reduced-fat spread as part of the National Cholesterol Education Program Step I diet, on serum lipids and indicators of lipophilic nutrient status in free-living men and women with hypercholesterolemia. During the 5-week treatment period, mean compliance with study product consumption exceeded 90% in all study groups. In the 1.1 and 2.2 g/d groups, respectively, LDL-cholesterol was reduced from 4.08 (SEM=0.06) to 3.89 (0.06) mmol/L (-5.9% vs. control, p=0.003) and from 4.11 (0.09) to 3.91 (0.09) mmol/L (-6.9% vs. control, p=0.008). Blood concentrations of all fat-soluble vitamins (subset of 71 subjects) and carotenoids (n=224) were within normal reference ranges at baseline and following treatment. There were no significant differences between control and treatment group responses in retinol, 25-hydroxycholecalciferol, alpha- and gamma-tocopherol, or phylloquinone. After total cholesterol correction, trans-β-carotene levels in the 1.1 and 2.2 g/d groups, respectively, were reduced from 0.102 to 0.084 (median, μg/mg) (-13.7% vs. control, p<0.001) and from 0.084 to 0.075 (-19.7% vs. control, p<0.001). Cholesterol-corrected trans-lycopene levels were significantly reduced in the 2.2 g/d group from 0.099 to 0.079 (-15.8% vs. control, p=0.027). There were no significant reductions in cholesterol-corrected α-carotene, lutein, and zeaxanthine levels. Results of this study indicate that a phytosterol-ester containing spread, which is easily incorporated into a low-fat diet, is a useful adjunct in the management of hypercholesterolemia and does not reduce fat-soluble vitamin levels. Carotenoid concentrations were significantly decreased, however, they remained within normal ranges.

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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

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Objective: To investigate cholesterol-lowering effects of stanol ester (STAEST) and sterol ester (STEEST)-enriched margarines as part of a low-fat diet.
Design: According to a Latin square model randomized double-blind repeated measures design with three test margarines and three periods.
Setting: Outpatient clinical trial with free-living subjects.
Subjects: Thirty-four hypercholesterolaemic subjects completed the study.
Interventions: Subjects consumed three rapeseed oil-based test margarines (STAEST, STEEST and control (no added stanols or sterols)) as part of a low-fat diet each for 4 weeks.
Results: Mean daily intake of total plant sterols plus stanols was 2.01–2.04 g during the two test margarine periods. In reference to control, serum total cholesterol was reduced by 9.2 and 7.3% with the STAEST and STEEST margarine, respectively ($P<0.001$ for both). The respective reductions for low-density lipoprotein (LDL) cholesterol were 12.7 and 10.4% ($P<0.001$). The cholesterol-lowering effects of the test margarines did not differ significantly. The presence of apolipoprotein E4 allele had a significant effect on LDL cholesterol response during the STAEST margarine only. Serum sitosterol and campesterol increased by 0.83 and 2.77 mg/l with the STEEST ($P<0.001$), respectively and decreased by 1.18 and 2.60 mg/l with the STAEST margarine ($P<0.001$). Increases of serum sitostanol and campestanol were 0.11 and 0.19 mg/l with the STAEST margarine ($P<0.001$), repsectively. No significant changes were found in serum fat-soluble vitamin and carotenoid concentrations when related to serum total cholesterol.
Conclusions: STAEST and STEEST margarines reduced significantly and equally serum total and LDL cholesterol concentrations as part of a low-fat diet.
Sponsorship: Grant to the University of Kuopio by Raisio Benecol Ltd, Raisio, Finland.
Descriptors: cholesterol-lowering diet; hypercholesterolaemic; plant sterols; plant stanols; carotenoids; vitamins; apolipoprotein E
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Introduction

Great interest has been focused on the cholesterol-lowering properties of plant sterols and stanols (Jones *et al*, 1997; Pollak & Kritchevsky, 1981), and there are now commercial products available aimed at helping to reduce elevated serum total cholesterol concentrations. Based on previous studies 2.0–3.0 g/day plant stanols from full-fat sitostanol ester margarine or mayonnaises as part of a moderately rich or high-fat diet significantly reduces serum total and low-density lipoprotein (LDL) cholesterol concentrations without affecting high-density lipoprotein (HDL) cholesterol or triglyceride concentrations (Gylling *et al*, 1995,1997; Gylling & Miettinen, 1994; Miettinen *et al*, 1995; Niinikoski *et al*, 1997; Vanhanen *et al*, 1994). In our own study (Hallikainen & Uusitupa, 1999), low-fat stanol ester margarines, consumed as part of a recommended low-fat, low-cholesterol diet, reduced serum LDL cholesterol by 18.4–23.6% as compared to the high-fat baseline diet, the additional effect of stanol ester margarine being 8.6–10.6%. Sitostanol has been suggested to have greater hypocholesterolaemic activity than sitosterol (Becker *et al*, 1993; Heinemann *et al*, 1986). In one single study with normolipidaemic subjects, a soybean sterol ester margarine, based on unhydrogenated soysterols with an esterification degree of 65%, was found to be as effective as a stanol ester margarine in lowering plasma cholesterol concentrations (Weststrate & Meijer, 1998). In that trial, subjects followed their own habitual diet except that the habitually used spreads were replaced by test margarines. However, efficacy of plant sterol ester margarine in comparison to stanol ester margarine with matching fatty acid compositions, equal esterification degree (>98.5%) and equal daily intake of total

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Contributors: MAH was responsible for the study design, gave nutrition counselling, analysed and interpreted the data and wrote the manuscript. MIJU and ESS contributed to the planning of study design and diets, interpreting the data and writing the manuscript. HG analysed serum cholesterol precursors and plant sterols, and ATE analysed fatty acid composition of serum lipids and, in addition, both also contributed to interpreting the data and writing the manuscript.

sterols (unsaturated + saturated sterols) has not been studied in connection with a low-fat, cholesterol-lowering diet.

Plant stanols have been suggested to be almost non-absorbable (Hassan & Rampone, 1979; Lütjohann *et al*, 1995), but recent studies indicate that they may be poorly absorbed from the small intestine (Gylling *et al*, 1999a; Gylling & Miettinen, 1999). Plant sterols are absorbed from 5% (sitosterol) to 15% (campesterol) (Heinemann *et al*, 1993; Salen *et al*, 1970). Furthermore, plant sterols and stanols may interfere with the absorption of carotenoids, as indicated by reduced serum carotenoid concentrations (Gylling *et al*, 1999b; Hallikainen *et al*, 1999, 2000; Hallikainen & Uusitupa, 1999; Hendriks *et al*, 1999; Weststrate & Meijer, 1998). This has to be taken into account in long-term use of plant sterol- and stanol-containing products emphasizing the use of a generally recommended diet.

Therefore, in the present study we investigated whether or not the two margarines enriched with plant sterol esters or stanol esters are equal in lowering serum total and LDL cholesterol concentrations and to what extent these two margarines would reduce serum cholesterol concentrations as part of a low-fat diet in reference to the low-fat diet alone. Additionally serum plant sterol and stanol concentrations were studied. As a *post-hoc* analysis we also investigated the effects of the main apolipoprotein E genotypes on lipid responses.

Methods

Subjects

Altogether 42 subjects (20 men/22 women) v
moderate hypercholesterolaemia were recruited
from the former studies carried out at the De
Clinical Nutrition, University of Kuopio ar
occupational health care system. In addition
of the city of Kuopio were recruited to the stuc
inclusion criteria were as follows: serum tota
4.8–7.0 mmol/l and total triglycerides below 2
screening. Other inclusion criteria were a
normal liver, kidney and thyroid function, no li
medication, no unstable coronary heart disease
no gastrointestinal diseases, no alcohol abuse
nol/day) and no irregular eating habits. E
dropped out during the study: one at the beg
pre-trial period due to poor compliance; four
the pre-trial period due to personal reasons or poor compliance; and three during the first period due to personal reasons. Altogether 34 subjects completed the study. In the beginning of the study their mean age was 48.8 ± 8.1 y (mean ± s.d.), their mean body mass index (BMI) was 24.9 ± 2.4 kg/m^2, and their mean systolic and diastolic blood pressure was 124 ± 14 and 81 ± 9 mmHg, respectively. Baseline total-, LDL-, HDL- and VLDL-cholesterol and triglyceride concentrations were 6.24 ± 0.83, 4.43 ± 0.81, 1.60 ± 0.31, 0.22 ± 0.21 and 1.11 ± 0.52 mmol/l, respectively.

One subject used low-oestrogen oral contraceptives, two subjects had hormone-releasing intra-uterine devices (IUD), one subject used hormone replacement therapy, three subjects used postmenopausal oestrogen therapy, one had thyroxin therapy for hypothyroidism, one used a calcium channel blocker, one used a renin–angiotensin system-affecting medication and two used anti-thrombolytic medication. Two subjects were smokers. The subjects were requested to maintain their medication, weight, alcohol consumption, smoking habits and physical activity constant during the study.

The subjects gave written consent for the study and the study protocol was approved by the Ethics Committee of the University of Kuopio.

Study design

The study was carried out from September to December 1998 applying a randomized double-blind repeated measures design with three test spreads (stanol ester (STAEST), sterol ester (STEEST) and control margarine). Each period lasted for 4 weeks. The randomization was made according to the model of Latin square design. Before randomization during the first 2 weeks the subjects followed a standardized low-fat diet and their diet was monitored. The pre-trial period made it possible to evaluate the inclusion criteria and compliance with the study.

Routine laboratory measurements were taken to ensure normal health status at the first and last visits of the study. In addition, previous and present diseases, current medication, alcohol and tobacco consumption, physical activity, use of vitamins or other nutrient supplements were interviewed by a structured questionnaire at the first visit of the study. Alcohol and tobacco consumption and physical
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Duplicate

apeseed oil (LEAR)-
sio, Finland) is pre-
of fat was 80% and
excluding sterols and
argarines and control
rgarine was prepared
(DRT, Les Derives
S.A. Dax Cedex,
France and Archer Daniels Midland Co, Decatur, IL, respectively) by recrystallization, hydrogenation to form plant stanols, and esterification to produce low erucic acid rapeseed oil based fatty acid esters of plant stanols. In turn STEEST margarine was prepared from vegetable oil based sterols (Archer Daniels Midland Co, Decatur, IL) by recrystallization, and esterification with low erucic acid rapeseed oil based fatty acid esters to produce fatty acid esters of plant sterols. The daily dose of the test margarine was 20 g taken in two to three portions with meals. The theoretical daily amount of total sterols and stanols was 2.02 g (0.10 g total sterols and 1.92 g total stanols) in the STAEST margarine and 2.06 g (1.98 g total sterols and 0.09 g total stanols) in the STEEST margarine. The control margarine and the margarine consumed during the pre-trial period contained naturally small amounts of sterols (about 0.09 g/daily dose of margarine). All three margarines were fortified with vitamin A (870 μg RE/100 g) and vitamin D

Table 1 Composition of daily dosage (20 g) of test margarines

Nutrients (g/20 g margarine)	*Control margarine*	*STAEST margarine*	*STEEST margarine*
Total fat	14.2	16.2	16.0
Absorbable fat[a]	14.2	14.0	14.0
Fatty acids:			
Saturated	3.2	3.3	3.3
Lauric (C 12:0)	0.3	0.4	0.5
Myristic (C 14:0)	0.2	0.2	0.3
Palmitic (C 16:0)	3.3	3.3	3.3
Stearic (18:0)	0.6	0.5	0.5
Monounsaturated	7.1	7.0	6.9
Oleic (C 18:1 *cis*)	10.2	10.1	9.9
Polyunsaturated	3.1	3.1	3.2
Linoleic (C 18:2 *cis*)	3.2	3.3	3.3
Linolenic (C 18:3 *cis*)	1.3	1.3	1.4
Total stanols	0	1.92	0.09
Sitostanol	0	1.43	0.06
Campestanol	0	0.49	0.02
Total sterols	0.09	0.10	1.98
Brassicasterol	0.01	—	0.06
Campesterol	0.03	0.04	0.57
Sitosterol	0.04	0.06	1.00
Stigmasterol	—	—	0.34
Total sterols and stanols	0.09	2.02	2.06

STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]Absorbable fat = total fat − total sterol and stanols.

(7 μg/100 g). This kind of fortification of margarines is a normal procedure in Finland.

The subjects received the coded tubs of test margarines when visiting the study unit. To verify the precise daily dose of test spread, 142 g, ie weekly dose of the test and control margarines, was packed into one tub and subjects were advised to mark the fat spread with a knife into seven equal parts in advance. One tub of test spread per each week was delivered and one extra tub for occasions of unexpected loss or damage of test products. The subjects were asked to record the use of test fats daily, and to return the empty and partly empty tubs and the extra tub of test spread to the study unit at the end of each period. The packages and the test spread left over were weighed and recorded.

Subjects followed a low-fat diet (step 1) of the National Cholesterol Education Program (1994) throughout the study. The planned composition of the diet was: <30 of energy percent (E%) from fat including 8–10 E% saturated, 12–14 E% monounsaturated and 5–7 E% polyunsaturated fat, and <300 mg/day dietary cholesterol. The diet was composed of normal Finnish food items. All subjects received individual oral and written instructions on the diet, including the precise amounts and quality of foods as main food groups: fats, dairy products, meat and meat products, cereals, fruits and berries, and vegetables and roots. The diet plan was made for eight energy levels: 6.7–12.6 MJ/day. The energy requirement of a subject was estimated according to the Harris Benedict formula with the energy requirement due to physical activity added (Alpers *et al*, 1986). If necessary the energy intake level was changed in order to ensure unchanged body weight during the study. The feasibility of the diet was improved by providing test margarines, rapeseed oil, salad dressing and low-fat cheese for the participants free of charge.

Adherence to the low-fat diet was monitored by 4-day food records kept at the end of each period, four times altogether during the study. One of the recording days was a weekend day or the person's day off from work. The subjects recorded their food consumption using a portion-size booklet with photos to estimate the portion size (Haapa *et al*, 1985). At study visits the amounts and qualities of foods in the records were checked by the nutritionist for completion, filling in data that were lacking. Fatty acid composition of serum lipids was determined as an objective marker of dietary adherence.

The diet was planned and the nutrients in the food records were calculated using the Micro-Nutrica® dietary analysis program (version 2.0, Finnish Social Insurance Institute, Turku, Finland). The food composition database is based on analyses of the Finnish food and international food composition tables (Rastas *et al*, 1993). In addition, the database was updated for the purposes of the present study.

Laboratory measurements

Systolic and diastolic blood pressure was measured by a mercury sphygmomanometer (Mercurius Stator, Spiedel + Keller, Germany) after subjects had rested for 5–10 min. Two measurements were taken and the mean of them used in the analyses. Body weight was measured with a digital scale. All measurements were done and venous blood samples were obtained after a 12 h overnight fast using standardized methods. Since the phase of the menstrual cycle may have an effect on serum cholesterol concentration (Cullinane *et al*, 1995), the end measurements were performed at days 5–10 of the cycle in those women with the menstrual cycle.

Routine laboratory samples were analysed with standardized methods at the Kuopio University Hospital. Plasma glucose was analysed by enzymatic photometric method using reagent Granutest 100 (Merck, Damstadt, Germany) with a Kone Specific Clinical Analyser (Kone Ltd, Espoo, Finland).

Lipoproteins were separated by ultracentrifugation for 18 h at density 1.006 to remove very low density lipoprotein (VLDL). HDL in the infranatant was separated from LDL by precipitation of LDL with dextran sulphate and magnesium chloride (Penttilä *et al*, 1981). LDL cholesterol was calculated as a difference between the mass of cholesterol in the infranatant and HDL, and VLDL cholesterol was calculated as a difference between the whole serum and the infranatant. Enzymatic photometric methods were used for the determination of cholesterol and triglycerides from whole serum and separated lipoproteins using commercial kits (Monotest® Cholesterol and Triglyceride GPO-PAP, Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) with a Kone Specific Clinical Analyser (Kone Ltd, Espoo, Finland). The coefficient of variance (CV) between measurements for serum total cholesterol was 1.3–1.4%, for triglycerides 1.7–1.9% and for HDL cholesterol 1.1–1.2%.

Serum samples for analysis of apolipoprotein (apo) A-I and B, carotenoids and fat soluble vitamins, cholesterol precursors, plant sterols and cholestanol, and fatty acid composition were stored at −70°C until analysed at the end of the study. Analyses of apolipoproteins were based on the measurement of immunoprecipitation enhanced by polyethylene glycol (PEG) at 340 nm. A Kone Specific Clinical Analyser and apo A-I and apo B reagents from Kone Corporation (Espoo, Finland) were used. Serum carotenoids, retinol and tocopherols were analysed by the high-performance liquid chromatography system (Perkin-Elmer, Norwalk, CT) on a C_{18} column (Waters, Milford, MA)

(Kaplan *et al*, 1987; Parviainen, 1983) using an external standard. Serum 25-hydroxyvitamin D_3 was analysed with a radioimmunoassay method (25-Hydroxyvitamin D 1125 RIA KIT, DiaSorin, Stillwater, MN) using an external standard.

Serum cholesterol precursors (Δ8-cholestenol, Δ7-lathosterol, desmosterol and squalene), plant sterols (sitosterol, sitostanol, campesterol, campestanol and avenasterol) and cholestanol, a metabolite of cholesterol, were quantified from nonsaponifiable serum materials by capillary gas–liquid chromatograph (GLC, HP 5890 Series II, Hewlett Packard, Delaware) equipped with a 50 m long Ultra 1 capillary column (methyl-polysiloxane) (Hewlett Packard, USA) for cholestanol, squalene, Δ8-cholestenol, Δ7-lathosterol, campesterol and sitosterol, and equipped with a 50 m long Ultra 2 capillary column (5% phenyl-methyl siloxane, Hewlett Packard, USA) for sitostanol and campestanol (Miettinen 1988, Miettinen & Koivisto 1983). Serum cholesterol precursors, plant sterols and cholestanol were determined in duplicate from the same samples and the mean value of two measurements was used in the statistical analyses.

In the analysis of serum fatty acid composition lipids were extracted with chloroform–methanol (2 : 1) (Ågren *et al*, 1992), and lipid fractions (cholesteryl esters, triglycerides and phospholipids) were separated with an aminopropyl column. Fatty acids were analysed with a gas-chromatograph (Hewlett-Packard 5890 series II, Hewlett-Packard Company, Waldbronn, Germany) equipped with FFAP-column (length 25 m, inner diameter 2 mm and film thickness 0.3 μm). Fatty acids are presented as molar percentage of total fatty acids.

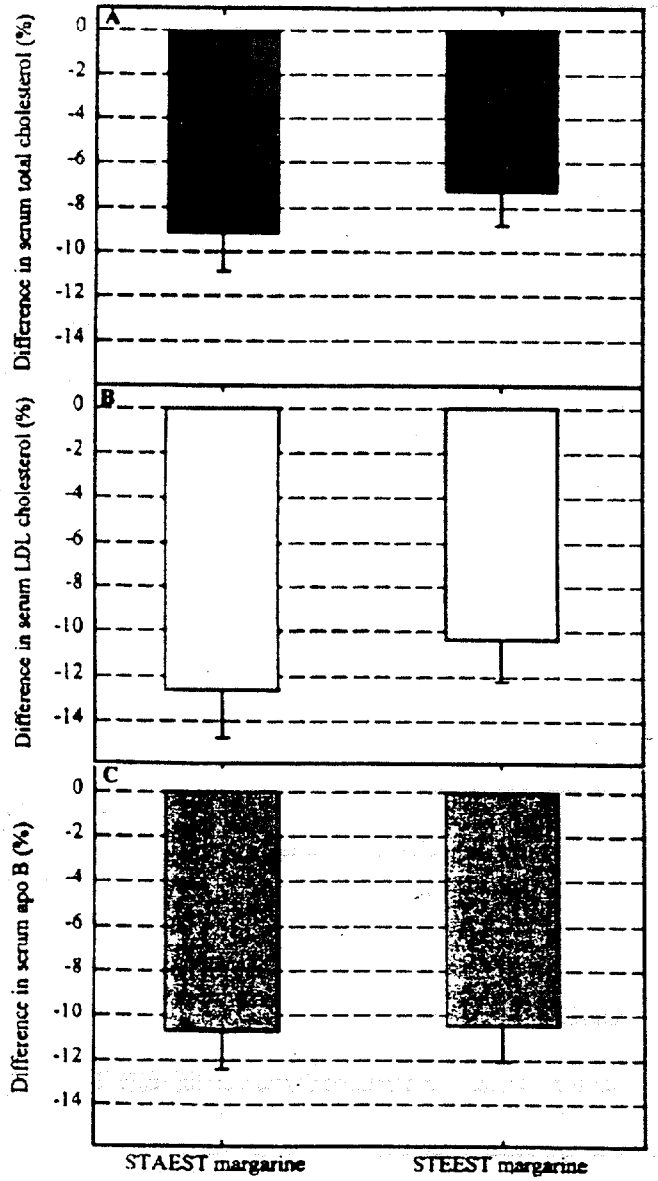

Figure 1 Differences in serum total (A) and LDL cholesterol (B), and apo B (C) concentrations (%) in reference to the control margarine period; $n = 34$, values are means ± s.e.m. There were no significant differences in serum lipid and lipoprotein responses between the two test margarine periods analysed with analysis of variance for repeated measurements (GLM). STAEST = stanol ester margarine and STEEST = sterol ester margarine.

Apolipoprotein E genotypes were determined by polymerase chain reaction (PCR) using primers described previously by Tsukamoto *et al* (1993). PCR amplification of apolipoprotein E polymorphism was conducted in a 16 µl volume containing 50 ng of genomic DNA, 0.4 pmol/l of each primer, 10 mmol/l Tris–HCl (pH 8.8), 50 mmol/l KCl, 1.5 mmol/l of $MgCl_2$, 0.1% Triton X-100, 113 µmol/l dNTPs, 0.7 units of DNA polymerase (DynaZyme DNA polymerase, Finnzymes, Espoo, Finland), and 8.5% glycerol to enhance amplification and annealing of GC-rich primers. Conditions for amplification were denaturation at 96°C for 4 min, followed by 35 cycles of denaturation at 96°C for 45 s, annealing at 64°C for 45 s, and extension at 72°C for 60 s with final extension at 72°C for 4 min. The PCR product was digested with *Hha*I (New England Biolabs, Beverly, MA). The digested DNA fragments were separated on 12% polyacrylamide gel. Finally, separated DNA fragments were visualized by ethidium bromide staining.

Statistical analyses

All statistical analyses were performed with SPSS for Windows 7.5 statistics program (SPSS, Chicago, IL, USA). The results are given as means ± s.d., except in Figure 1, where they are given as means ± s.e.m.

The main comparison was made among the mean values at the end of each experimental period. In the results and discussion sections only the end measurements, and their absolute or percentage changes, are presented. The percentage changes were computed comparing the end measurements of both test margarine periods to the end measurement of the control margarine period. To eliminate the effects of changes in lipoprotein concentrations, serum carotenoid, tocopherol, cholesterol precursor, plant sterol and cholestanol values are given, besides the crude concentrations, also in terms mmol/mol of cholesterol or 10^{-3} mg/mg of cholesterol, which express the ratios to total cholesterol.

Normal distribution was checked with Shapiro Wilks test, and homogeneity of variance was checked with analysis of variance for repeated measurement (GLM) before further analyses. If a variable was not normally distributed the statistical analysis was made after logarithmic transformation. GLM was used to compare the overall changes and the effect of the order of spread consumption periods, carry-over effect and gender on the main end-point variables among the different experimental margarine periods. GLM was also used in the further analyses. Confidence intervals (CI) presented in the text for the percentage changes of total and LDL cholesterol and apo B were not corrected for the above-mentioned factors.

Intake of nutrients and serum fatty acid composition among the experimental periods was analysed with GLM and a paired *t*-test, or if nutrient data or fatty acid data was not normally distributed even after the logarithmic transformation Friedman two-tailed ANOVA test was used. To control the overall α level, Bonferroni adjustment was used. The Wilcoxon matched-paired signed rank test was used to compare the change of alcohol consumption, smoking habits and physical activity. Routine laboratory examinations were tested with paired *t*-test or Wilcoxon matched-paired signed rank test.

The number of subjects recruited for the study was based on an assumption of having 0.2 mmol/l difference in serum LDL cholesterol response among the test spreads significant with an α level of 0.05 ($P<0.05$), and with 0.80 statistical power (Cohen, 1988).

Results

Baseline characteristics

There were no significant changes in BMI (24.9 ± 2.3, 24.8 ± 2.4 and 24.8 ± 2.4 kg/m^2 during the control, STAEST and STEEST margarine periods, respectively) or systolic and diastolic blood pressure during the study. Physical activity, alcohol consumption and smoking habits remained stable. A woman who used low-oestrogen oral contraceptives stopped use at the end of the first study period. Excluding her from the statistical analyses did not affect the results.

To ensure normal health status during the study, samples for routine laboratory measurements were drawn in the beginning (−2 weeks) and at the end (12 weeks) of the study. There was a small but significant decrease in the mean fasting plasma glucose (from 5.53 ± 0.41 to 5.35 ± 0.41 mmol/l) and serum γ-glutamyltransferase (from 25.8 ± 20.3 to 21.3 ± 12.5 U/l) values during the study. The mean serum TSH concentration increased slightly (from 1.7 ± 1.3 to 1.9 ± 1.2 mU/l, $P<0.05$). One subject had a marginally elevated serum TSH concentration (7.3 and 6.1 mU/l, at the beginning and end of the study, respectively). Excluding him from statistical analyses did not affect the results. The changes in routine laboratory measurements cannot be connected with the test margarines. In men the mean blood haemoglobin concentration decreased slightly (from 144.4 ± 8.1 to 141.2 ± 6.3 g/l) but significantly due to the fact that seven of the men also participated in a fat-loading test with many blood samples at the end of all test margarine periods. In women blood haemoglobin concentration remained unchanged during the entire study. Other routine measurements, blood thrombocytes, serum alanine aminotransferase and creatinine, did not change significantly during the study.

Feasibility of the diet

The mean daily consumption of margarine according to the weighed returned tubs was 19.2–19.9 g during the different margarine periods. The actual mean daily intake of total sterols and stanols was 0.09 ± 0.03 g (0.09 ± 0.03 g total sterols and 0 g total stanols), 2.01 ± 0.06 g (0.10 ± 0.03 g total sterols and 1.91 ± 0.05 g total stanols) and 2.04 ± 0.14 (1.96 ± 0.13 g total sterols and 0.09 ± 0.06 g total stanols) during the control, STAEST margarine and STEEST margarine periods, respectively.

The actual composition of the diet during the different test margarine periods is presented in Table 2. The goal for the composition of the low-fat diet was well achieved (Table 2). Generally, there were no significant differences in the nutrient intakes among the periods.

The results of the fatty acid composition of serum cholesteryl esters paralleled with the food records (Table 3). In general, there were no major differences in the fatty acid composition during the different test margarine periods. However, the proportion of oleic acid was slightly, but

Table 2 Actual composition of the diets during the different test margarine periods

Nutrients	Period			
	Control margarine (n = 34)	*STAEST margarine (n = 34)*	*STEEST margarine (n = 34)*	*P-values*[a]
Energy (MJ/day)	8.3 ± 1.9	8.3 ± 1.8	8.1 ± 1.7	0.318
Fat (percentage total energy intake, E%)	30.0 ± 3.7	30.5 ± 3.2	31.1 ± 3.9	0.100
Saturated fatty acids (E%)	8.2 ± 1.9	8.4 ± 1.8	8.8 ± 2.0	0.347
Monounsaturated fatty acids (E%)	12.4 ± 1.6	12.5 ± 1.5	12.7 ± 1.9	0.648
Polyunsaturated fatty acids (E%)	6.7 ± 0.9	6.6 ± 0.8	6.9 ± 0.9	0.069
Proteins (E%)	16.1 ± 2.3	16.9 ± 2.1	17.2 ± 2.7	0.045
Carbohydrates (E%)	49.6 ± 5.4	49.1 ± 5.2	48.3 ± 4.8	0.916
Alcohol (E%)	2.9 ± 4.1	2.1 ± 3.3	2.0 ± 2.5	0.642
Cholesterol (mg/d)	166 ± 88	173 ± 66	179 ± 66	0.098
Cholesterol (mg/MJ)	19.6 ± 7.6	20.5 ± 4.9	22.2 ± 6.6	0.061
Fibre (g/day)	30.3 ± 8.4	30.1 ± 7.5	28.9 ± 9.5	0.443
Fibre (g/MJ)	3.7 ± 1.0	3.7 ± 0.9	3.6 ± 0.9	0.445
Vitamin A (μg RE/day)[b]	1337 ± 878	1188 ± 544	1139 ± 736	0.452
β-carotene (μg/day)	4155 ± 2732	4726 ± 3120	4056 ± 2638	0.413
Vitamin D (μg/day)	4.4 ± 2.7	5.7 ± 3.8	6.1 ± 4.9	0.318
Vitamin E (mg/day)	12.3 ± 3.3	12.5 ± 2.8	12.0 ± 3.2	0.434

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]Variables were analysed with Friedman two-way ANOVA test (energy, saturated fatty acid, carbohydrates, alcohol, vitamin A and vitamin D) or analysis of variance for repeated measurements (GLM) and paired *t*-test with Bonferroni correction. In pairwise comparisons after Bonferroni correction there were no significant differences in protein intake between any two periods.
[b]RE = retinol equivalents.

significantly lower at the end of the STEEST margarine period than at the end of the control period. In addition, the proportion of stearic acid was significantly lower at the end of both test margarine periods than at the end of the control period. There were no significant differences in the fatty acid composition of triglycerides or phospolipids during the study (data not shown).

Serum lipids and lipoproteins

The concentrations of serum lipids and lipoproteins at the end of each experimental periods are shown in Table 4. Figure 1 presents the percentage differences in serum total and LDL cholesterol and apo B compared to the control margarine period.

No significant differences were found in the concentrations of serum lipids between genders ($P = 0.095$) or among orders of spread consumption ($P > 0.1$). In addition, no carry-over effect was found ($P > 0.1$).

Serum total and LDL cholesterol concentrations were significantly lower at the end of STAEST and STEEST margarine periods than at the end of the control period (Table 4). Compared to the control period the mean decreases in serum total cholesterol were 9.2 ± 9.7% (CI −12.6, −5.8) and 7.3 ± 8.9% (CI −10.4, −4.2) during the STAEST and STEEST margarine periods, respectively. For LDL cholesterol the mean decreases were 12.7 ± 12.2% (CI −16.9, −8.4) and 10.4 ± 10.6% (CI −14.1, −6.7), respectively. There were no significant differences in the decreases of serum total and LDL cholesterol concentrations between the two test margarine periods (difference 1.9 ± 8.4% and 2.3 ± 11.7%, respectively).

Serum HDL and VLDL cholesterol, and triglyceride concentration did not change significantly during the entire study (Table 4).

There were no significant changes in serum apo AI concentration (Table 4) during the study. The changes in

Table 3 Serum fatty acid composition of cholesteryl esters during the different test margarine periods

Fatty acid (mol %)	Period			
	Control margarine (n = 34)	*STAEST margarine (n = 34)*	*STEEST margarine (n = 34)*	*P values*[a]
Myristic acid 14:0	1.17 ± 0.33	1.14 ± 0.36	1.22 ± 0.42	0.526
Palmitic acid 16:0	12.46 ± 0.87	12.37 ± 0.86	12.45 ± 0.89	0.699
Palmitoleic acid 16:1	3.80 ± 0.88	3.68 ± 0.91	3.58 ± 0.80	0.233
Stearic acid 18:0	0.78 ± 0.15	0.67 ± 0.13*	0.67 ± 0.13*	<0.001
Oleic acid 18:1 n-9 + n-7	21.22 ± 1.50	21.07 ± 1.49	20.66 ± 1.52†	0.007
Linoleic acid 18 2 n-6	51.45 ± 3.21	52.27 ± 3.27	52.20 ± 3.37	0.142
γ-linolenic acid 18:3 n-6	0.65 ± 0.29	0.63 ± 0.32	0.59 ± 0.26	0.313
α-linolenic acid 18:3 n-3	1.07 ± 0.20	1.00 ± 0.16	0.99 ± 0.23	0.282
Dihomo-γ-linolenic acid 20:3 n-6	0.52 ± 0.15	0.49 ± 0.10	0.53 ± 0.17	0.360
Arachidonic acid 20:4 n-6	4.58 ± 0.95	4.50 ± 0.89	4.63 ± 0.82	0.374
Eicosapentanoic acid 20:5 n-3	1.68 ± 1.00	1.57 ± 0.66	1.86 ± 1.04	0.065
Docosahexanoic acid 22:6 n-3	0.63 ± 0.18	0.60 ± 0.19	0.63 ± 0.20	0.486

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]The significance of the differences for overall changes during the test margarine periods analysed with analysis of variance for repeated measurements (GLM) or Friedman two-tailed ANOVA (α-linolenic acid and eicosapentanoic acid).
*$P < 0.001$; †$P < 0.01$, significant difference between either of the test margarine periods and control margarine period analysed with paired *t*-test with Bonferroni correction. There were no significant differences in serum fatty acid composition of cholesteryl esters between two test margarine periods.

Table 4 Serum lipids and lipoproteins at the end of the test margarine periods

Variables	Period			
	Control margarine (n = 34)	*STAEST margarine (n = 34)*	*STEEST margarine (n = 34)*	*P-values*[a]
Total cholesterol (mmol/l)	6.10 ± 0.69	5.52 ± 0.75*	5.64 ± 0.71*	<0.001
LDL cholesterol (mmol/l)	4.19 ± 0.61	3.65 ± 0.69*	3.74 ± 0.58*	<0.001
HDL cholesterol (mmol/l)	1.50 ± 0.27	1.50 ± 0.29	1.55 ± 0.31	0.079
VLDL cholesterol (mmol/l)	0.40 ± 0.30	0.37 ± 0.26	0.35 ± 0.14	0.768
Triglycerides (mmol/l)	1.13 ± 0.45	1.10 ± 0.53	1.03 ± 0.33	0.221
Apo AI (g/l)	1.59 ± 0.22	1.55 ± 0.23	1.59 ± 0.27	0.143
Apo B (g/l)	1.01 ± 0.18	0.90 ± 0.18*	0.90 ± 0.13*	<0.001
Apo AI/apo B	1.62 ± 0.41	1.79 ± 0.46*	1.81 ± 0.43*	<0.001

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]Significance of the difference for overall changes during the test margarine periods analysed with analysis of variance for repeated measurements (GLM).
*$P<0.001$ denotes the significance of the difference between either of the test margarine periods and control period analysed with GLM with Bonferroni correction. There were no significant differences in serum lipid and lipoprotein concentrations between the two test margarine periods.

serum apo B concentration and in apo AI/apo B lipoprotein ratio were parallel with the changes in serum LDL cholesterol concentration (Table 4). The mean decreases in apo B concentrations were 10.7 ± 9.7% (CI −14.1, −7.3) and 10.4 ± 9.3% (CI −13.7, −7.2) during the STAEST and STEEST margarine periods, respectively, compared with the control period.

In a secondary analysis we also examined whether apolipoprotein E genotype group (E4/3, $n=12$ *vs* E3/3, $n=22$) has an effect on the LDL cholesterol response during the STAEST and STEEST margarine periods. This analysis revealed that the overall reduction in LDL cholesterol was greater in subjects with apolipoprotein E4/3 than in those with apolipoprotein E3/3 ($P=0.024$, interaction term of genotype with test margarine periods, GLM). This was entirely due to a greater effect of STAEST margarine on the LDL cholesterol response in subjects having apolipoprotein E4 allele (16.8 ± 13.9% *vs* 10.4 ± 10.8%, apolipoprotein E4/3 *vs* E3/3, $P=0.141$, ANOVA). The LDL cholesterol response was almost the same in the two apolipoprotein E groups (9.6 ± 13.5% *vs* 10.8 ± 8.9%, apolipoprotein E4/3 *vs* E 3/3, $P=0.707$, ANOVA) during the STEEST margarine period.

Cholesterol precursors and plant sterols

The concentrations of serum cholesterol precursors and plant sterols during the different test margarine periods are shown in Table 5.

As expected, serum Δ8-cholestenol and Δ7-lathosterol concentrations, which are indicators of cholesterol synthesis, increased during the STAEST and STEEST margarine periods in reference to the control period (Table 5). When those concentrations were related to serum total cholesterol

Table 5 Serum cholesterol precursors, plant sterols and cholestanol (mg/l), and ratios of serum cholesterol precursors, plant sterols and cholestanol to cholesterol (10^{-3} mg/mg of cholesterol) at the end of the test margarine periods

	Period			
	Control margarine (n = 34)	*STAEST margarine (n = 34)*	*STEEST margarine (n = 34)*	*P-values*[a]
Cholestanol	2.68 ± 0.48	2.36 ± 0.52*	2.28 ± 0.42*	<0.001
Δ8-cholestenol	0.33 ± 0.12	0.36 ± 0.13	0.37 ± 0.12†	0.010
Desmosterol	1.22 ± 0.35	1.22 ± 0.29	1.17 ± 0.24	0.850
Δ7-lathosterol	3.28 ± 1.11	3.58 ± 1.21	3.60 ± 1.24†	0.008
Campesterol	7.70 ± 1.92	5.09 ± 1.58*	10.46 ± 2.44*‡	<0.001
Sitosterol	3.40 ± 0.88	2.21 ± 0.73*	4.23 ± 1.02*‡	<0.001
Squalene	0.66 ± 0.14	0.71 ± 0.17	0.70 ± 0.17	0.292
Campestanol	0.06 ± 0.03	0.16 ± 0.04*	0.05 ± 0.02‡	<0.001
Sitostanol	0.08 ± 0.04	0.27 ± 0.07*	0.06 ± 0.04‡	<0.001
Avenasterol	1.00 ± 0.22	0.70 ± 0.15*	0.83 ± 0.16*‡	<0.001
Cholestanol/TC[b]	1.36 ± 0.23	1.30 ± 0.24	1.25 ± 0.24*§	<0.001
Δ8-Cholestenol/TC	0.16 ± 0.06	0.20 ± 0.06*	0.20 ± 0.06*	<0.001
Desmosterol/TC	0.61 ± 0.15	0.66 ± 0.13†	0.64 ± 0.12¶	0.001
Δ7-lathosterol/TC	1.64 ± 0.50	1.95 ± 0.61*	1.95 ± 0.59*	<0.001
Campesterol/TC	3.88 ± 0.91	2.78 ± 0.74*	5.70 ± 1.11*‡	<0.001
Sitosterol/TC	1.72 ± 0.43	1.21 ± 0.33*	2.30 ± 0.47*‡	<0.001
Squalene/TC	0.33 ± 0.07	0.39 ± 0.10¶	0.39 ± 0.10¶	0.003
Campestanol/TC	0.03 ± 0.01	0.09 ± 0.02*	0.03 ± 0.01‡	<0.001
Sitostanol/TC	0.04 ± 0.02	0.15 ± 0.04*	0.03 ± 0.02‡	<0.001
Avenasterol/TC	0.50 ± 0.10	0.39 ± 0.06*	0.45 ± 0.06*‡	<0.001

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]Significance of the differences for overall changes during the test margarine periods analysed with analysis of variance for repeated measurements (GLM).
[b]TC = total cholesterol.
*$P<0.001$, †$P<0.01$, ¶$P<0.05$, significant difference between the either the test margarine period and the control margarine period; ‡$P<0.001$, §$P<0.05$, significant difference between the STAEST margarine and the STEEST margarine periods analysed with GLM with Bonferroni correction.

concentration the increases were more pronounced (Table 5). Serum cholestanol concentration, which reflects cholesterol absorption, decreased significantly during both test margarine periods (Table 5).

Serum campesterol and sitosterol concentrations decreased significantly during the STAEST margarine period and increased significantly during the STEEST margarine period compared to the control period (Table 5). Furthermore, serum campesterol and sitosterol concentrations were significantly higher at the end of the STEEST margarine period than at the end of the STAEST margarine period. In reference to control period, the reduction in serum campesterol and sitosterol concentrations was 2.60 ± 1.03 mg/l (33.8%) and 1.18 ± 0.47 mg/l (34.8%), respectively, during the STAEST margarine period. During the STEEST margarine period the increase in serum campesterol and sitosterol concentrations was 2.77 ± 1.49 mg/l (38.3%) and 0.83 ± 0.62 mg/l (26.6%), respectively. Changes in the ratios of serum campesterol and sitosterol to serum total cholesterol were parallel with the changes in their absolute concentrations (Table 5).

Serum campestanol (0.11 ± 0.04 and 0.11 ± 0.05 mg/l, change in reference to control and STEEST margarine period) and sitostanol concentrations (0.19 ± 0.07 mg/l and 0.21 ± 0.68 mg/l, change in reference to control and STEEST margarine period) were slightly but significantly higher at the end of STAEST margarine period than at the end of the control and the STEEST margarine periods (Table 5). There were no significant differences in serum campestanol or sitostanol concentrations between the STEEST margarine period and the control margarine period. Serum sitostanol concentration was significantly higher in women than in men at the end of the STAEST margarine period (0.24 ± 0.07 *vs* 0.30 ± 0.05 mg/l men *vs* women), but there were no differences in serum sitostanol concentrations between the genders at the end of the two other periods.

There were no significant differences in percentage changes in either serum cholesterol precursors or plant sterols between two apolipoprotein E genotype groups (3/3 and 4/3) among the different test margarine periods (data not shown).

Carotenoids and fat soluble vitamins

There were no significant changes in serum 25-hydroxyvitamin D_3, retinol, α-carotene or lycopene concentrations nor their ratios to the serum total cholesterol during the study (Table 6). Serum β-carotene concentration was significantly lower at the end of the STAEST and STEEST margarine periods than at the end of the control period. The serum $\alpha + \beta$-carotene concentration was significantly lower at the end of the STEEST margarine period as compared to the control period, but the difference between the control and the STAEST margarine periods was not significant. However, there were no significant differences in serum β-carotene/total cholesterol ratio or $\alpha + \beta$-carotene/total cholesterol ratio among the periods.

Serum γ-tocopherol concentration did not change significantly during the study, but serum α-tocopherol concentrations was significantly lower at the end of both test margarine periods than at the end of the control period (Table 6). After relating the serum α- and $\alpha + \gamma$- tocopherol to the serum total cholesterol there were no significant differences among the different periods.

Discussion

In the present study the STAEST and STEEST margarines reduced significantly serum total (9.2% and 7.3%, respectively) and LDL cholesterol (12.7% and 10.4%, respectively) concentration as part of a low-fat diet compared to a low-fat diet alone in the subjects with mild to moderate hypercholesterolaemia, but the cholesterol-lowering effects of the test margarines did not differ significantly from each other. The decreases of serum apo B were parallel with the decreases of serum LDL cholesterol concentration.

On the basis of the food records the adherence to the low-fat diet was good. The intake of saturated fatty acids achieved the goal of step 1 diet of the National Cholesterol Education Program (1994) (actual mean intake 8–9 E% *vs*

Table 6 Serum carotenoids and fat-soluble vitamins at the end of the test margarine periods

	Period			
	Control margarine (n = 34)[a]	*STAEST margarine (n = 34)*[a]	*STEEST margarine (n = 34)*[a]	*P-values*[b]
Retinol (μmol/l)	2.80 ± 1.00	2.71 ± 1.04	2.70 ± 1.01	0.494
α-Carotene (μmol/l)	0.66 ± 0.43	0.64 ± 0.42	0.61 ± 0.36	0.129
β-Carotene (μmol/l)	1.39 ± 1.03	1.23 ± 0.99*	1.16 ± 0.82*	0.004
α + β-Carotene (μmol/l)	2.05 ± 1.43	1.87 ± 1.38	1.77 ± 1.16*	0.022
Lycopene (μmol/l)	0.73 ± 0.37	0.71 ± 0.37	0.69 ± 0.39	0.677
α-Tocopherol (μmol/l)	43.55 ± 7.56	40.08 ± 6.83†	40.55 ± 7.01†	0.001
γ-Tocopherol (μmol/l)	3.00 ± 0.98	2.98 ± 0.81	2.87 ± 0.89	0.592
α + γ-Tocopherol (μmol/l)	46.55 ± 7.95	43.07 ± 7.15†	43.42 ± 7.41†	0.001
25-Hydroxyvitamin D_3 (nmol/l)	50.33 ± 23.52	49.38 ± 24.39	52.26 ± 25.18	0.659
α-Carotene/TC	0.11 ± 0.08	0.12 ± 0.08	0.11 ± 0.07	0.161
β-Carotene/TC	0.23 ± 0.17	0.23 ± 0.19	0.21 ± 0.15	0.251
α + β-Carotene/TC	0.34 ± 0.24	0.35 ± 0.26	0.32 ± 0.22	0.563
Lycopene/TC	0.12 ± 0.07	0.13 ± 0.07	0.13 ± 0.08	0.505
α-Tocopherol/TC	7.14 ± 0.89	7.27 ± 0.91	7.21 ± 1.03	0.382
γ-Tocopherol/TC	0.49 ± 0.15	0.55 ± 0.15	0.51 ± 0.15	0.086
α + γ-Tocopherol/TC	7.63 ± 0.91	7.82 ± 0.96	7.72 ± 1.06	0.249

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.

[a] $n = 33$ for results concerning lycopene and lycopene/TC (TC = total cholesterol).

[b] Significance of the difference for overall changes during the test margarine periods analysed with analysis of variance for repeated measurements (GLM).

*$P<0.05$, †$P<0.001$, significant difference between the either test margarine period and control period analysed with GLM with Bonferroni correction. There were no significant differences in serum fat-soluble and carotenoid concentrations between two test margarine periods.

goal 8–10 E%) and the mean intake of fat (30–31 E%) was close to the goal (<30 E%) in all experimental periods. In addition, the mean intake of dietary cholesterol met the goal well. The results of the fatty acid composition of cholesteryl esters paralleled with the results of the food records. There were no major differences in serum fatty acid composition among the experimental periods. Furthermore, the fatty acid composition of the STAEST and STEEST margarine, the fatty acid composition of the sterol and stanol fatty acid esters, the esterification degree of stanols and sterols, and the actual daily intake of total sterols (unsaturated and saturated) in the STAEST and STEEST margarine were identical. No significant changes in BMI, physical activity or other living habits were found during the study. Thus, the differences in lipid responses between two test margarine periods can be ascribed to the stanol and sterol fatty acid esters rather than differences in background diet or other background variables.

In the present study the subjects consumed test margarines in a randomized order according to the model of Latin square design, and each subject worked as his/her own control. The benefit of the present study design is that it eliminates the between-individual variation and the effect of time. In earlier studies it has been shown that plant sterols reduce cholesterol concentrations within 2–3 weeks of the initiation of treatment, and on the other hand, that the serum cholesterol concentration return to initial value within 2–3 weeks, upon cessation the ingestion of plant sterols (Farquhar *et al*, 1956; Heinemann *et al*, 1986; Jones *et al*, 1997; Weststrate & Meijer, 1998). Therefore, the 4 week study period can be considered long enough to demonstrate the cholesterol-lowering effect of the test margarines.

Our findings are parallel with the findings of our earlier study (Hallikainen & Uusitupa, 1999) and strengthen the view that stanol ester margarine decreases serum total and LDL cholesterol concentrations as part of a low-fat diet. Furthermore, our findings are in accordance with the study of Weststrate and Meijer (1998), in which a soybean oil sterol ester margarine based on unhydrogenated soysterols was equally effective as a stanol ester margarine (Benecol®) in lowering plasma total and LDL cholesterol concentration. The finding that plant stanols or sterols can reduce serum cholesterol concentrations even after a markedly low dietary cholesterol intake indicates that plant stanols and sterols must inhibit not only the absorption of dietary but also that of biliary cholesterol. This is supported by the findings of the earlier plant stanols/sterols studies, in which the faecal excretion of neutral sterols was increased despite the constant dietary cholesterol intake (Becker *et al*, 1993; Gylling *et al*, 1997; Gylling & Miettinen 1994).

The subjects with apolipoprotein E4 allele have been found to have a higher cholesterol absorption rate (Kesäniemi *et al*, 1996; Ordovas, 1999) and therefore those subjects might have more benefit from plant stanols and sterols. In a secondary analysis the subjects with apolipoprotein E4/3 genotype had a greater percentage reduction in LDL cholesterol during the STAEST margarine period (16.8%) than during the STEEST margarine period (9.6%). There are some studies in which the effects of sitosterol, sitostanol and sitostanol esters after combining different treatment groups (Miettinen & Vanhanen, 1994), sitostanol esters (Vanhanen *et al*, 1994) or plant stanol esters (Uusitupa & Hallikainen, 1999) on lipid responses in different apolipoprotein E phenotype or genotype groups have been investigated. Miettinen and Vanhanen (1994) and Vanhanen *et al* (1994) found that serum total and LDL cholesterol concentrations were reduced more effectively in subjects with the apolipoprotein E allele 4 than those with allele 2 or 3. However, we did not find this difference with stanol ester-enriched low-fat margarines (Uusitupa & Hallikainen, 1999). As far as we know there are no previous studies in which the effects of plant stanols on serum cholesterol concentrations in different apolipoprotein E genotype groups have been compared to that of plant sterols. Our present results are interesting, but these results need to be confirmed in a prospective study design where equal numbers of subjects with different apolipoprotein E genotypes should be allocated to different diet groups.

Serum plant sterols in very high concentrations have been found to be atherogenic (Glueck *et al*, 1991). Under normal conditions the plant sterol concentration is only on an average 3–17 mg/l in serum being roughly only 1/1000 of serum cholesterol concentration, although typical dietary intake of plant sterols is almost equal to dietary intake of cholesterol (about 160–360 mg/day, Ling & Jones 1995). Furthermore, dietary intake of plant stanols and their serum concentrations are very low compared to that of the plant sterol concentrations. In the present study serum campesterol and sitosterol concentrations were significantly higher at the end of the STEEST margarine period compared to the end of the control and STAEST margarine periods. Respectively, serum sitostanol and campestanol concentrations were significantly higher at the end of the STAEST margarine period than at the end of the control and STEEST margarine periods. However, during the entire study serum plant sterol and stanol concentrations remained very low, indicating that the absorbed amounts were very small in relation to the daily intake of plant sterols or stanols from the test margarines. Although STAEST margarine also contained a small amount of plant sterols, their serum concentrations reduced significantly when that margarine was consumed owing to the ability of the plant stanols to inhibit the absorption of plant sterols. All these findings are in line with the findings of earlier studies (Gylling *et al*, 1999a; Gylling & Miettinen 1999; Hallikainen & Uusitupa, 1999; Hallikainen *et al*, 2000; Jones *et al*, 1997,1999; Weststrate & Meijer, 1998). Besides the negligible absorption of plant stanols, the low serum concentrations could also result from fast and effective clearance of absorbed stanols (Salen *et al*, 1970).

The increased serum Δ7-lathosterol concentration and Δ7-lathosterol/total cholesterol ratio can be ascribed to compensatorily increased endogenous cholesterol synthesis due to cholesterol malabsorption during the test margarine periods. Also, in previous studies the synthesis of Δ7-lathosterol has been found to be stimulated by plant stanol esters (Gylling *et al*, 1995,1997,1999a; Gylling & Miettinen, 1994,1996; Vanhanen *et al*, 1993).

During the test margarine periods there were no significant changes in concentrations of serum 25-hydroxyvitamin D_3, retinol or in concentrations of serum lycopene, α-carotene, β-carotene and tocopherols related to the serum total cholesterol concentration. The findings of the present study are in agreement with the findings of our earlier studies (Hallikainen *et al*, 1999, 2000; Hallikainen & Uusitupa, 1999), in which we found only small effects on serum carotenoid concentrations when changes in serum carotenoids were related to the changes in serum total cholesterol. However, there are plant stanol and plant

Table I. Daily dietary intake of energy, fats, cholesterol, and vitamins A and D at baseline in children who first received plant stanol ester margarine and children who first received control margarine

Variable	Children with plant stanol ester margarine first (n = 34)	Children with control margarine first (n = 32)
Energy (kcal)	1462 (234)	1523 (262)
Fat (g)	47.6 (11.2)	49.9 (12.0)
Fat (E%)	29.4 (4.3)	29.6 (3.8)
SAFA (g)	18.1 (4.5)	19.0 (5.3)
SAFA (E%)	11.2 (2.1)	11.3 (2.0)
MUFA (g)	17.0 (4.5)	17.8 (4.4)
MUFA (E%)	10.5 (1.8)	10.6 (1.6)
PUFA (g)	8.9 (2.8)	9.0 (2.3)
PUFA (E%)	5.5 (1.3)	5.4 (1.2)
P/S-ratio	0.50 (0.14)	0.49 (0.12)
Cholesterol (mg)	136 (41)	168 (40)
Vitamin A (μg RE)	999 (1074)	990 (974)
Vitamin D (μg)	2.6 (1.0)	3.0 (1.4)

Values are means (SD).
SAFA, Saturated fatty acids; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids; *P/S ratio*, ratio of polyunsaturated to saturated fatty acids; *RE*, retinol equivalent.
Baseline dietary records of 13 children are missing.

Plant stanol ester margarine is available in many countries in Europe and now also in the United States. Thus children will have access to this margarine when other members of the family use it as a part of their diet. Accordingly, we studied how daily 3-month replacement of conventional vegetable margarine spreads with plant stanol ester margarine influences serum lipid and fat-soluble vitamin concentrations in healthy 6-year-old children already consuming a low-saturated-fat, low-cholesterol diet. In addition, we examined the safety of the margarine.

SUBJECTS

The children in the study were all participants of the STRIP project (Special Turku Coronary Risk Factor Intervention Project for children), which is a randomized, prospective trial aimed at decreasing exposure of the intervention children to known atherosclerosis risk factors. In the project, launched in 1990, 1062 infants were randomized to the intervention (n = 540) or control (n = 522) group at the age of 7 months as described.[6,11] The intervention families visited the study pediatrician and dietitian at the child's age of 7, 8, 10, and 13 months and thereafter twice a year. The intervention families were advised to supply the child with a diet low in saturated fat and cholesterol; this diet comprised, for example, changes from breast milk or formula to skim milk at 1 year of age and supplementation of the child's daily diet with 2 to 3 teaspoonfuls of vegetable oil, preferably low-erucic acid rapeseed oil, before the age of 2 years. The goal for the child's total fat intake was 30 to 35 E% before the age of 3 years. The counseling dealt mainly with quality of fat, aiming at the replacement of saturated fatty acids with polysaturated and monounsaturated fatty acids to approach a polyunsaturated/monounsaturated/saturated fat ratio of 1:1:1 and a daily cholesterol intake <200 mg. After 3 years of age the recommended amount of fat was ≤30 E%. Families in the control group visited the project pediatrician and dietitian at 7 and 13 months and then twice a year. They were counseled like families in the Finnish well-baby clinics to change the child from breast milk or formula to cow's milk with 1.9% to 2.9% fat at the age of 1 year, and they received no individualized dietary advice. The intervention and control families recorded the child's food consumption for 4 days approximately a week before each visit with a food diary. The diet data were analyzed with the Micro-Nutrica program (Research and Development Unit, Social Insurance Institution, Turku, Finland).[12]

At the 6-year visit the children in the intervention group and their parents were asked whether they were interested in participating in this plant stanol ester margarine study. Children with type 1 diabetes or FH were excluded. Recruitment of 81 children began in April 1996 and finished 14 months later. All children had a normal or mildly elevated serum cholesterol concentration with a mean (SD) 4.29 (0.61) mmol/L (166 [24] mg/dL), and all showed normal growth with mean weight (SD) and height (SD) 22.6 (2.7) kg and 118.8 (4.7) cm, respectively; at the age of 6 years, or +2.8% and +0.4 SD of the mean weight and height of Finnish children at that age.[13] Because children belonged to the STRIP intervention group, they already were consuming a diet low in saturated fat and cholesterol,[6,11] as demonstrated by the fat and cholesterol intakes at the beginning of the study (Table 1). The study was approved by the Joint Commission on Ethics of the Turku University and the Turku University Central Hospital. Informed consent was obtained from the parents of all children.

METHODS

This placebo-controlled, double-blinded, random order crossover trial

comprised two 3-month study periods with a 6-week washout period. To minimize the effect of seasons on serum cholesterol values, every second child began with the plant stanol ester margarine period, and every second child participated in the control margarine period in a blinded fashion.[14,15] During study periods the children were advised to replace 20 g of daily dietary fat with the respective margarine. The plant stanol ester margarine (Benecol, Raisio Inc, Raisio, Finland) contained 8.2 g/100 g of plant stanol (82% sitostanol), resulting in daily plant stanol intake of 1.6 g. The control margarine was rapeseed oil margarine with a fatty acid content similar to that of the plant stanol ester margarine, but vitamins A (443 μg retinol equivalent/100 g) and D (6 μg/100 g) were added only to the plant stanol ester margarine. The families used disposable spoons holding 5 g margarine to measure 4 even spoonfuls of margarine daily, at breakfast, lunch, dinner, and afternoon or evening snacks on slices of bread, or if the child was unable to eat enough margarine as spread, on porridge, and on mashed potatoes. Study margarines were used for cooking and frying only if the child would eat all the food prepared. Margarine containers were returned without washing, and the true mean daily intake was calculated by measuring the weight of remnants plus the container and the margarine container washed and dried.

Blood was drawn from an antecubital vein with the patients under cutaneous anesthesia (Emla, Astra, Södertälje, Sweden) during fasting at the beginning and end of the 2 study periods. Serum was separated by centrifugation (3400 × g, for 12 minutes) after clotting at room temperature in the dark. Samples for the measurement of serum concentrations of lipids, fat-soluble vitamins, creatinine, lactate dehydrogenase, γ-glutamyl transferase, and noncholesterol sterols were stored before analysis at -25°C for a few weeks and then at -70°C for less than a year. Serum total cholesterol, HDL cholesterol after precipitation of LDL and very low-density lipoprotein with dextran sulfate, and triglyceride concentrations were measured with fully enzymatic methods (CHOD-PAP and GPO-PAP, Merck, Darmstadt, Germany) with an Olympus AU 510 analyzer. Serum LDL cholesterol concentration was calculated according to the Friedewald formula.[16] Serum concentrations of fat-soluble vitamin A, α-tocopherol, and β-carotene were determined with high-performance liquid chromatography at the laboratory of the Research and Development Unit of the Social Insurance Institution, Turku, Finland.[17] Serum 25-OH vitamin D concentrations were measured with a radioimmunoassay kit (Incstar Corporation). Serum creatinine, lactate dehydrogenase, and γ-glutamyl transferase concentrations were measured with kinetic enzymatic methods with a Hitachi 917 Automatic Analyzer, and white and red blood cell values were measured with a hematologic analyzer in the Central Laboratory of Turku University Central Hospital. Serum noncholesterol sterols were quantified with gas liquid chromatography at the research laboratory of the Department of Medicine of the Helsinki University Hospital with a 50-m capillary SE-30 column (Hewlett Packard (Ultra I and Ultra II).[18,19]

The results are expressed as means (SD) with 95% CI. Serum triglyceride values were log-transformed for analysis because of skewness in the distribution of values. According to the intention-to-treat principle, all randomized subjects with at least 2 blood samplings were included in the analyses. For statistical analysis the SAS 6.12 program package was used (SAS Institute, Cary, NC). Multiple effects on outcome variables were studied with analysis of variance for crossover design.[20] The following effects were included in the model: treatment, period, first-order carry-over effect, and second-order carry-over effect. Differences in serum sitostanol concentration before and after the use of plant stanol ester margarine were analyzed with the nonparametric Wilcoxon signed rank test because of the markedly skewed distribution of sitostanol concentration. Linear regression analysis was used to test the association of baseline serum total and LDL cholesterol concentrations to the changes in these variables during the use of plant stanol ester margarine. Pearson's correlation coefficients were calculated for correlation between changes in serum cholesterol and the plant sterol values. P values < .05 in 2-sided tests were regarded as significant. To detect (α = .05, β = .80) a true difference of 0.4 mmol/L (15 mg/dL) (assuming an SD of 0.06 mmol/L [2 mg/dL]) in change in serum cholesterol concentration between the 2 margarine periods, the required minimum sample size in crossover design was 38 children (19 children for both treatment sequences).

RESULTS

Out of the 81 children in the STRIP intervention (36 girls) recruited, 72 children (32 girls) completed the study. The plant stanol ester margarine and control margarine periods were evenly distributed over the study period, thus eliminating the known effects of seasons on serum lipid values.[14,15] A total of 4, 3, and 2 children withdrew during the plant stanol ester margarine period, the control margarine period, and the washout period, respectively, mainly because the child refused to participate in the frequent blood draws or to eat margarine. The children consumed daily on average (SD) 18.2 g (4.8) of the plant stanol ester margarine (1.5 g of plant stanols) and 18.1 g (4.1) of the control margarine during 95 (range 79 to 132) days and 96 (84 to 113) days, respectively. Plant stanol ester margarine induced no adverse clinical symptoms or signs.

Serum total and LDL cholesterol concentrations decreased slightly from

Table II. Serum lipids (mmol/L) in children during plant stanol ester and control margarine periods

Variable	Plant stanol period		Control period		Treatment effect (95% CI)	Treatment effect %	P value
	Before	After	Before	After			
A. Above median (n = 38)							
Total cholesterol	4.32 (0.68)	3.94 (0.60)	4.37 (0.67)	4.18 (0.60)	−0.25 (−0.07 to −0.42)	−5.8	.006
HDL cholesterol	1.26 (0.28)	1.25 (0.25)	1.24 (0.24)	1.23 (0.23)	+0.01 (−0.05 to +0.07)	+0.8	.73
HDL/total cholesterol	0.29 (0.06)	0.32 (0.06)	0.29 (0.05)	0.30 (0.05)	+0.02 (+0.01 to +0.04)	+7.5	.001
Triglycerides	0.70 (0.20)	0.72 (0.28)	0.76 (0.24)	0.70 (0.29)	1.019 (0.964 to 1.069)*	+1.9	.73
LDL cholesterol	2.72 (0.57)	2.34 (0.50)	2.75 (0.56)	2.59 (0.53)	−0.27 (−0.11 to −0.42)	−9.9	.0007
B. Below median (n = 41)							
Total cholesterol	4.27 (0.55)	4.10 (0.54)	4.28 (0.64)	4.31 (0.56)	−0.22 (−0.05 to −0.38)	−5.2	.009
HDL cholesterol	1.23 (0.23)	1.24 (0.23)	1.23 (0.23)	1.27 (0.25)	−0.02 (−0.08 to +0.04)	−1.6	.51
HDL/total cholesterol	0.29 (0.06)	0.30 (0.05)	0.29 (0.05)	0.30 (0.05)	+0.01 (−0.001 to +0.02)	+3.8	.08
Triglycerides	0.71 (0.21)	0.72 (0.27)	0.74 (0.28)	0.82 (0.45)	0.958 (0.911 to 1.008)*	−4.2	.10
LDL cholesterol	2.69 (0.47)	2.50 (0.43)	2.69 (0.49)	2.63 (0.47)	−0.14 (−0.01 to −0.27)	−5.2	.03

The children have been divided into 2 groups according to whether their mean daily plant stanol ester margarine consumption was above or below the median daily intake (18.6 g). Values are means (SD). Treatment % shows the effect in percentages of the baseline values.
*Estimates for serum triglycerides are ratio of mean changes during the 2 margarine periods (plant stanol margarine vs control margarine). To convert data to mg/dL, divide cholesterol values by 0.0259 and triglyceride values by 0.0113.

Table III. Serum plant sterol and cholesterol precursor sterol to cholesterol ratios (10^2 μmol/mmol of cholesterol) before and after use of plant stanol ester margarine and control margarine

Variable	Plant stanol period		Control period		Treatment effect (95% CI)	Treatment effect %	P value
	Before	After	Before	After			
Campesterol/cholesterol	424 (150)	268 (91)	393 (122)	420 (111)	−165 (−144 to −186)	−38.9	.0001
Sitosterol/cholesterol	204 (76)	137 (46)	195 (66)	200 (61)	−66 (−54 to −75)	−31.9	.0001
Δ-8 cholesterol/cholesterol	9 (4)	12 (5)	9 (5)	10 (4)	+2 (+1 to +3)	+18.0	.002
Desmosterol/cholesterol	66 (13)	77 (16)	68 (14)	69 (13)	+7 (+4 to +11)	+11.1	.0001
Lathosterol/cholesterol	118 (36)	142 (38)	125 (41)	126 (32)	+16 (+8 to +24)	+13.3	.0001

Values are means (SD). Treatment % shows the effect in percentages of baseline values.
10^2 μmol/mmol of cholesterol = 10^2 μg/mg of cholesterol.

the baseline values during the control margarine period, but decreased 5.4% and 7.5%, respectively, during the plant stanol ester margarine period. The mean treatment effect (SE) was 0.23 (0.06) mmol/L (9 [2] mg/dL) for total cholesterol and 0.20 (0.05) mmol/L (8 [2] mg/dL) for LDL cholesterol. The decrease in serum total and LDL cholesterol concentrations during use of plant stanol ester margarine correlated positively with their baseline values: the higher the baseline values were, the greater was the decrease (regression coefficient = 0.40, r^2 = 0.24, P = .001, and 0.40, r^2 = 0.27, P = .0001, respectively). The serum HDL cholesterol concentration remained unchanged. Consequently, the HDL to total cholesterol ratio increased by 5.6% during the use of plant stanol ester margarine. Serum triglyceride values were stable during both margarine periods.

The effects of plant stanol ester margarine were then analyzed separately in the 38 and 41 children who consumed daily plant stanol ester margarine ≥18.6 g (median daily intake of all study children) or <18.6 g, respectively (Table II). Although serum total and LDL cholesterol concentrations decreased in both groups of children during the plant stanol ester margarine period, the serum HDL cholesterol to total cholesterol ratio increased only in the children with daily plant stanol margarine intake ≥18.6 g.

Serum campesterol and sitosterol concentrations are known to correlate strongly with cholesterol absorption.[21,22] Because plant sterols are mainly transported in cholesterol-containing particles in serum, the absolute concentrations were adjusted for serum cholesterol concentration to eliminate the effect of changes in serum cholesterol concentration. Daily use of plant stanol ester margarine reduced the

Table IV. Serum concentrations of 25-OH vitamin D, vitamin A, α-tocopherol, and β-carotene before and after plant stanol ester margarine and control margarine periods

Variable	n	Plant stanol period		Control period		Treatment effect (95% CI)	Treatment effect %	P value
		Before	After	Before	After			
25-OH vitamin D (nmol/L)	49	59.9 (21.3)	56.3 (17.6)	56.2 (18.5)	59.8 (21.6)	−4.77 (−12.57 to +3.15)	−8.0	.23
Vitamin A (μmol/L)	49	1.29 (0.21)	1.26 (0.24)	1.29 (0.28)	1.32 (0.24)	−0.03 (−0.10 to +0.02)	−2.3	.16
α-Tocopherol (μmol/L)	49	18.9 (3.1)	17.8 (3.2)	18.9 (3.2)	19.3 (2.8)	−1.5 (−0.7 to −2.4)	−7.9	.0009
α-Tocopherol/LDL (μmol/mmol of LDL chol)	49	7.08 (1.09)	7.62 (1.28)	7.13 (1.21)	7.57 (1.37)	+0.12 (−0.28 to +0.49)	+1.7	.58
β-Carotene (μmol/L)	74	0.86 (0.39)	0.63 (0.31)	0.82 (0.33)	0.84 (0.41)	−0.20 (−0.11 to −0.28)	−23.3	.0001
β-Carotene/LDL (μmol/mmol of LDL chol)	74	0.32 (0.15)	0.28 (0.15)	0.30 (0.15)	0.34 (0.17)	−0.06 (−0.02 to −0.07)	−18.8	.003

Values are means (SD). Treatment % shows the effect in percentages of baseline values. To convert 25-OH vitamin D values to ng/mL, divide by 2.496. To convert vitamin A values to μg/dL, divide by 0.0349. To convert α-tocopherol values to mg/dL, divide by 23.22. To convert β-carotene values to μg/dL, divide by 0.0186.

serum campesterol/cholesterol ratio and the sitosterol/cholesterol ratio by 39% and 32%, respectively, reflecting decreased cholesterol absorption (Table III). The plant stanol ester margarine-induced decreases in serum total and LDL cholesterol concentrations showed a positive correlation with the decrease in serum campesterol concentration ($r = 0.42$, $P = .0001$ and $r = 0.39$, $P = .0005$, respectively) and sitosterol concentration ($r = 0.36$, $P = .002$ and $r = 0.33$, $P = .004$, respectively).

Serum precursor sterol (Δ-8 cholestenol, desmosterol, and lathosterol)/cholesterol ratios, known to reflect cholesterol synthesis,[22] increased during the use of plant stanol ester margarine by 18%, 11%, and 13%, respectively (Table III).

To study the potential absorption of ingested sitostanol, we determined serum sitostanol concentrations before and at the end of the plant stanol ester margarine period. The concentration was below the detection limit 0.05 μmol/L (2 μg/dL) in 65 of the 77 children before the period; the mean ± SD (range) serum sitostanol concentration in the 12 children with measurable concentrations was 0.19 ± 0.08 (0.08 to 0.32) μmol/L (7 ± 3 [3 to 12] μg/dL). After the 3-month use of plant stanol ester margarine, the serum sitostanol concentration was measurable in 73 of the 74 children; 0.67 ± 0.24 (0.11 to 1.31) μmol/L (25 ± 9 [4 to 49] μg/dL); $P = .0001$ for difference in the measurable values before and after the use of plant stanol ester margarine.

No advice was given to the families regarding vitamin supplementation during the study periods, but supplement use was recorded. The effects of use of vitamin A- and D-enriched plant stanol ester margarine on serum concentrations of vitamin A, 25-OH vitamin D, and α-tocopherol were analyzed in those 49 children who received no other supplementation of fat-soluble vitamins than that added to the plant stanol ester margarine. Changes in serum β-carotene values were similarly studied in those 74 children who received no β-carotene–containing supplements. Serum 25-OH vitamin D and vitamin A concentrations were not affected by the use of plant stanol ester margarine (Table IV). The absolute concentrations of serum α-tocopherol and β-carotene decreased from the baseline values by 7.9% and 23.3%, respectively (Table IV). However, when the concentrations of these antioxidants were adjusted for serum LDL cholesterol concentrations (both antioxidants are carried in the LDL particles in plasma), α-tocopherol values showed no change during the use of plant stanol ester margarine, but the decrease (19% from baseline values) in β-carotene values was significant. The decrease in the β-carotene/LDL ratio was not greater in the children whose margarine intake exceeded the median (18.6 g) compared with others (mean [SE] 0.03 [0.03] (μmol/mmol of LDL cholesterol, $P = .21$ for children with margarine intake >18.6 g and 0.06 [0.02] mmol/mmol of LDL cholesterol, $P = .003$ for children with margarine intake <18.6 g).

Plant stanol ester margarine caused no changes in measured hematologic variables, serum creatinine concentrations, or lactate dehydrogenase and γ-glutamyl transferase activities (data not shown).

DISCUSSION

This 3-month study shows that daily replacement of 18.2 g of dietary fat with plant stanol ester margarine significantly reduces serum total and LDL cholesterol concentration in healthy children during a low-saturated-fat, low-cholesterol diet. Because serum HDL cholesterol concentration was not affected by the addition of plant stanol esters, HDL/total cholesterol ratio was increased. Furthermore the fact that serum total and LDL cholesterol concentration showed a tendency

to decrease more in the children with daily plant stanol ester margarine intake above the median compared with those with intake below the median suggests that the response is probably dose-dependent. The 5.4% and 7.5% mean reductions in serum total and LDL cholesterol concentrations during use of plant stanol ester margarine in our study are approximately a half of the percent decreases achieved by the same product in children with FH and markedly increased serum cholesterol values.[10] Because the mean amount of daily ingested plant stanol esters of the children in our study was only half of that used by the FH group (1.5 g vs 3.0 g), the differences in responses in these 2 studies may be directly caused by the differences in the amount of plant stanol esters used. Another possibility is that the response depends on the serum cholesterol concentration at the baseline; that is, responses are larger in children with markedly elevated serum cholesterol and LDL cholesterol values. In fact, the greater the baseline total and LDL cholesterol were in the children in our study, the greater were the decreases in these variables during the use of plant stanol ester margarine. In a similar manner, high serum baseline LDL cholesterol concentrations predicted large decreases in serum cholesterol concentration during the use of plant stanol ester margarine in postmenopausal women[25] and in a population of adults of both sexes with increased baseline serum cholesterol values.[9]

Plant stanol esters reduce serum cholesterol concentration mainly by inhibiting cholesterol absorption.[9] Accordingly, in our study the use of plant stanol ester margarine reduced markedly serum plant sterol (campesterol and sitosterol) to cholesterol ratios, known to reflect cholesterol absorption.[21,22] On the other hand, serum cholesterol precursor sterol (lathosterol, desmosterol, and Δ-8 cholestenol) to cholesterol ratios increased, suggesting compensatory activation of cholesterol synthesis.[22] Cholesterol absorption efficiency and absorbed dietary cholesterol regulate cholesterol synthesis.[24] Thus compensatory activation of cholesterol synthesis has been observed, when cholesterol absorption is reduced by plant stanols[9] or celiac disease,[25] although serum cholesterol concentration ultimately decreases. One study suggests that reduction in serum LDL cholesterol concentration is accompanied by enhanced cholesterol synthesis in healthy subjects by other dietary interventions (sunflower oil and rapeseed oil diets).[26]

The use of sitostanol reduces absorption of both dietary and biliary cholesterol.[27] Our data speak against the hypothesis proposed by Denke[28] that plant stanols lower serum cholesterol values only when used by subjects consuming a high-cholesterol diet. The children in the study had already been on a diet low in saturated fat and cholesterol for years. At baseline in this study the mean daily intakes of fat, saturated fat, and cholesterol were 29 E%, 11 E%, and 150 mg, respectively. The use of plant stanol ester margarine increased total fat intake to 33 E% (this value includes also the nonabsorbable plant stanol esters), but intakes of saturated fat and cholesterol remained unchanged. This resulted in dietary composition almost similar to the intervention diet of Denke's study. Yet, plant stanol ester margarine reduced markedly serum total and LDL cholesterol concentrations in the children in our study.

Animal and human studies have shown that sitostanol is virtually unabsorbable.[27,29] In our study sitostanol was detectable in serum of almost all children after the use of plant stanol ester margarine, indicating that minimal amounts of sitostanol are absorbed. However, the mean serum sitostanol concentration was only approximately 1:6000 of the concentration of serum cholesterol, even though the children ingested 10 times greater amounts of sitostanol than cholesterol.

Because fat-soluble vitamins are absorbed like cholesterol in mixed micelles, plant stanols may interfere with their absorption. In our study all children had consumed commercial margarines supplemented with vitamin D (7 μg/100g) and vitamin A (550 to 900 μg retinol equivalent/100g) before the beginning of the plant stanol ester margarine trial. Serum concentrations of vitamin A and 25-OH vitamin D remained unchanged during the use of vitamin-supplemented plant stanol ester margarine, implying that supplemented plant stanol ester margarine does not affect serum concentrations of these vitamins at least during the 3-month study period. Serum concentrations of vitamins A and 25-OH D were also unaffected after ingestion of the control margarine, which was not supplemented with these vitamins. This result may indicate that the vitamins A and D consumed in the supplemented margarine contribute less than expected to their serum values. In adults serum concentrations of vitamin A and 25-OH vitamin D were unchanged after plant stanol ester margarine was used for 1 year.[30,31]

Plant stanol esters may also interfere with the absorption of fat-soluble antioxidants, α-tocopherol and β-carotene. However, in our study LDL cholesterol-adjusted α-tocopherol values remained unchanged as previously reported in adults.[9] Serum β-carotene concentration decreased markedly during the use of plant stanol ester margarine in the children in our study even when adjusted for LDL cholesterol concentration. A similar result was seen in adults.[30,31] Oxidized LDL has an important role in atherogenesis,[33] and in epidemiologic studies plasma β-carotene concentration >0.4 to 0.5 μmol/L is considered to be related to decreased risk of ischemic heart disease.[34] In this study serum β-carotene values at the beginning and after the plant stanol ester margarine period clearly exceeded these values. Furthermore controlled intervention trials have

failed to reduce the incidence of cardiovascular disease by β-carotene supplementation.[36-37] Thus it seems unlikely that the slightly decreased β-carotene concentration during the use of plant stanol ester margarine could hamper the positive effect of reduction in LDL cholesterol concentration on atherosclerosis prevention. However, the definite importance of the reduced β-carotene concentration cannot be established.

In summary, intense dietary intervention in the STRIP trial has previously resulted in a 4.5% decrease in serum cholesterol concentrations in healthy children.[6] The addition of esterified plant stanols to the diet further reduced children's serum cholesterol concentration by 5.4%. The combined effect is a 10% reduction in serum cholesterol concentration. Reduction in serum cholesterol of this magnitude, if permanent, could reduce ischemic heart disease mortality,[38] especially if the intervention is started in childhood. The use of plant stanol ester margarine caused no adverse clinical effects, but serum absolute and LDL cholesterol-adjusted β-carotene concentrations decreased clearly.

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Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters

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Objective: To examine in humans the effects on serum lipids, lipoproteins and fat-soluble antioxidants of a daily consumption of 2.5 g plant stanols, consumed either once per day at lunch or divided over the three meals.
Design: A randomized, double-blind, placebo-controlled, cross-over design.
Subjects: Thirty-nine healthy normocholesterolemic or mildly hypercholesterolemic subjects participated.
Interventions: Each subject consumed in random order; no plant stanols; 2.5 g plant stanols at lunch; and 2.5 g plant stanols divided over the three meals (0.42 g at breakfast, 0.84 g at lunch and 1.25 g at dinner, which is proportional to dietary cholesterol intake). Each period lasted 4 weeks. Plant stanols were esterified with fatty acids from low erucic rapeseed oil (LEAR) and incorporated into margarines or shortenings.
Results: Consumption of 2.5 g plant stanols at lunch results in a similar low-density lipoprotein (LDL)-cholesterol-lowering efficacy compared to consumption of 2.5 g plant stanols divided over the three meals (−0.29 mmol/l compared with the control period ($P < 0.001$; 95% CI, −0.19 to −0.39 mmol/l) for the once per day diet and −0.31 mmol/l ($P < 0.001$; 95% CI, −0.20 to −0.41 mmol/l)) for the three times per day period). High-density Lipoprotein (HDL) cholesterol and triacylglycerol concentrations did not change. After standardization for LDL cholesterol, the sum of the most lipophylic hydrocarbon carotenoids (ie α-carotene, β-carotene and lycopene) in particular was slightly, though not significantly, lowered by -0.017 ± 0.018 μmol/mmol LDL cholesterol ($P = 0.307$) after the once per day period and by -0.032 ± 0.016 μmol/mmol LDL cholesterol ($P = 0.049$) after the three times per day period.
Conclusions: Our findings suggest that for lowering LDL cholesterol concentrations it is not necessary to consume products rich in plant stanol ester at each meal or simultaneously with dietary cholesterol.
Sponsorship: Raisio Group, Raisio, Finland.
Descriptors: plant stanols; consumption frequency; diet; serum lipids; serum lipoproteins; fat-soluble antioxidants
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Introduction

Plant stanols are useful hypocholesterolemic agents since a daily intake of 2–3 g lowers LDL cholesterol concentrations by 10–15% as found in various populations (Wester, 1999; Law, 2000). The proposed mechanism is that plant stanols reduce the micellar solubility of cholesterol and consequently lower intestinal absorption of both exogenous and endogenous cholesterol (Heinemann *et al*, 1991). This suggests that plant stanol esters should be consumed at each meal to obtain a maximal cholesterol-lowering effect. However, consuming plant stanol esters at lunch and dinner only (Weststrate & Meijer, 1998) showed a decrease in LDL cholesterol comparable to that when consumed three times daily (Miettinen *et al*, 1995; Plat & Mensink, 2000). This suggests that plant stanols are active in the intestinal tract for at least a few hours. It has, however, never systematically been evaluated whether the efficacy of plant stanols to lower serum LDL cholesterol depends on consumption frequency.

The main purpose of the present study therefore was to examine in a normocholesterolemic and mildly hypercholesterolemic population the effects on serum lipids and lipoproteins of a margarine and shortening enriched with plant stanol esters, consumed three times per day, *vs* an equal dose of plant stanol esters, consumed once per day. Also effects on plasma fat soluble antioxidant concentrations were evaluated, as these may be affected by consumption of plant sterol and stanol esters (Weststrate & Meijer, 1998; Gylling & Miettinen, 1999).

Methods

Subjects

Forty-three subjects from Maastricht and surrounding areas applied for the study. Twenty-six of these volunteers had participated in a previous study on the effects of plant stanol esters on serum lipids and lipoproteins (Plat & Mensink, 2000), while the others were recruited via posters in public buildings. Subjects were invited for a screening visit to see if they met our eligibility criteria: age 18–65 y, fasting serum total cholesterol concentration < 6.5 mmol/l

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(251 mg/dl), fasting serum triacylglycerol concentration < 3.0 mmol/l), body mass index < 30 kg/m^2, diastolic blood pressure < 95 mmHg, systolic blood pressure < 160 mmHg, no presence of proteinuria or glucosuria, no use of medication or a diet known to affect serum lipids, and no history of coronary heart disease. Volunteers had not donated blood at least 4 weeks before or during this trial, and did not participate in another biomedical study. All subjects gave their written informed consent before the start of the study. A population of normocholesterolemic and mildly hypercholesterolemic subjects was used, since the serum cholesterol lowering efficacy of plant stanol esters—expressed as a percentage—does not depend on initial serum LDL cholesterol concentrations (Wester, 1999; Law, 2000). Hypercholesterolemic subjects were not included, as many of these patients have a history of cardiovascular disease, or use medication or a diet known to affect serum lipids, which were all exclusion criteria.

One subject was excluded for a serum total cholesterol concentration >6.5 mmol/l and two subjects decided not to participate. Consequently, the study started with 40 volunteers. One subject dropped out during the first week, because she could not combine the study protocol with her lifestyle. The remaining 39 volunteers, 28 women and 11 men, completed the study successfully. These participants were 31 ± 14 y of age (mean ± s.d.) and had a body mass index of 22.7 ± 2.6 kg/m^2. Before the study started, mean serum total cholesterol and triacylglycerol concentrations were 4.74 ± 0.85 mmol/l (range 2.83–6.28 mmol/l) and 0.99 ± 0.39 mmol/l (range 0.39–1.84 mmol/l) in women and 4.94 ± 89 mmol/l (range 3.37–6.15 mmol/l) and 0.97 ± 0.53 mmol/l (range 0.44–2.02 mmol/l) in men. Seventeen women had cholesterol concentrations below 5.0 mmol/l (normocholesterolemic) and 11 women had cholesterol concentrations between 5.0 and 6.5 mmol/l (mildly hypercholesterolemic). For men, these figures were seven and four, respectively. One man and three women smoked cigarettes, 19 women used oral contraceptives and one woman was postmenopausal.

Design and diets

The study, which was approved by the Medical Ethics Committee of Maastricht University, had a double-blind, placebo-controlled cross-over design (Figure 1). Each subject received three different diets for 4 weeks in one of the six possible treatment orders. There was no washout period between the three different dietary periods. Before the start of the study, the subjects were randomly allocated to one of the six groups. The participants were instructed to maintain their customary lifestyles and home diets throughout the study. During the study, they recorded in diaries any symptoms, visits to physicians, medication used, menstrual phase, alcohol use and any deviation from the protocol. Body weight was recorded weekly.

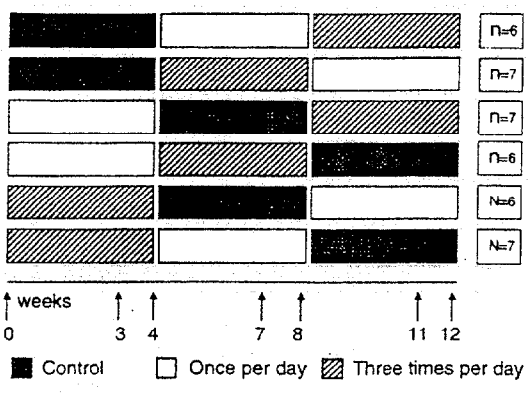

Figure 1 Experimental design of the study.

During the study, the subjects were required to replace at breakfast and at lunch their habitual margarines for an experimental margarine of which, at breakfast 10 g and at lunch 20 g, had to be consumed. Within 1 h after dinner, each participant also had to eat a cake or cookie, which contained 10 g of an experimental shortening. These cakes and cookies were prepared every week by a local bakery especially for this study. To control fat and fatty acid intake as much as possible, each participant also received during each period a shortening without plant stanol esters that had to be used for baking and cooking.

One experimental margarine contained 4.2 g/100 g plant stanols as its fatty acid (low plant stanol ester margarine), and another margarine 12.5 g/100 g (high plant stanol ester margarine). The plant stanol concentration in the experimental shortening was 12.5 g/100 g. Products provided during the control period did not contain any plant stanol esters.

The mixture of vegetable oil and pinewood-derived plant stanols contained approximately 76% sitostanol and 24% campestanol. Sitostanol was prepared from β-sitosterol and stigmasterol, and campestanol from campesterol, both by hydrogenation. Free sitostanol and campestanol were transesterified with rapeseed oil fatty acids, forming fat-soluble sitostanol and campestanol esters. The plant stanol esters were then mixed with the experimental margarines and shortening. The plant stanol esters were added to the experimental margarines at the expense of water and to the experimental shortening at the expense of absorbable fats. All the margarines and shortenings were prepared from low erucic acid rapeseed oil (LEAR) and contained 68% (margarines), 99% (control shortening) or 86% (experimental shortening) absorbable fats. All margarines and the shortening were fortified with normal amounts of vitamin A and D. β-Carotene was used as a coloring agent, while vitamin E was present as a natural compound. The margarines and shortenings were produced and provided by the Raisio Group, Raisio, Finland.

At a daily intake of 10 g margarine at breakfast, 20 g margarine at lunch, and 10 g shortening incorporated into the cakes and cookies after dinner, the aimed plant stanol intake during the experimental periods was 2.5 g. The distribution of plant stanol intake over the day, however, was different (Figure 2). During the once per day period the 2.5 g of plant stanols were consumed once per day at lunch, while during the three times per day period the plant stanols were provided in amounts proportional to cholesterol intake (Ministeries van Welzijn, Volksgezondheid en Cultuur en van Landbouw, Natuurbeheer en Visserij, 1993). Thus, 0.42 g plant stanols were consumed at breakfast, 0.84 g at lunch and 1.25 g at dinner.

The volunteers had to come at least once a week to the Department to receive a new supply of products. The experimental margarines were given in color-labeled tubs, which contained 75 g margarine (breakfast) or 145 g margarine (lunch). The cookies or cakes were provided in similarly color-labeled bags. The tubs and the bags provided margarine, cakes and cookies for one week. Parts of

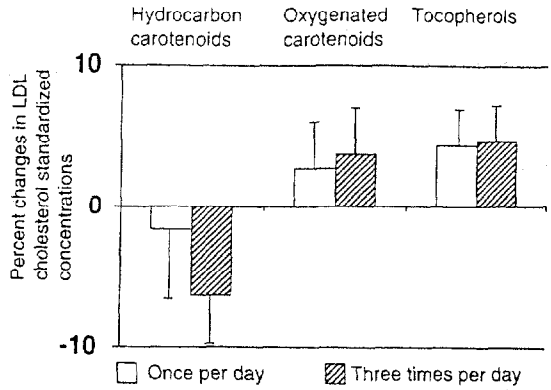

Figure 3 Percentage changes of LDL cholesterol standardized plasma hydrocarbon carotenoid, oxygenated carotenoid and tocopherol concentrations (μmol/mmol LDL cholesterol) at the end of the once per day period and the three times per day period, both compared with the concentrations at the end of the control period (means ± s.e.). Hydrocarbon carotenoids were calculated as the sum of β-carotene, α-carotene and lycopene, oxygenated carotenoids as the sum of lutein/zeaxanthin and β-cryptoxanthin, and tocopherols as the sum of α-tocopherol, β + γ-tocopherol and δ-tocopherol.

1998), lower serum total and LDL cholesterol concentrations. We have now demonstrated that a daily intake of 2.5 g plant stanols as its fatty acid esters, either consumed once per day (at lunch) or divided over three meals (0.4 g at breakfast, 0.8 g at lunch and 1.2 g at dinner), resulted in a similar decrease in serum total and LDL cholesterol. The amount of plant stanols in the latter period was divided over the three meals in such a way that the largest intake was at dinner and the lowest intake at breakfast. This differentiation is largely in correspondence with the distribution of cholesterol intake over the day (Ministeries van Welzijn, Volksgezondheid en Cultuur en van Landbouw, Natuurbeheer en Visserij, 1993; Table 2). Our findings therefore demonstrate that it is not necessary to consume plant stanol ester products simultaneously with dietary cholesterol or with each meal. This provides variety and may increase compliance for potential consumers. Like in other studies, serum HDL cholesterol and triacylglycerol concentrations were not affected. As a result, the total to HDL cholesterol ratio was significantly lower at the end of both the once per day and the three times per day period, as compared with the control period.

The mechanism by which plant stanol esters affect lipoprotein metabolism and lower serum cholesterol concentrations has only partly been elucidated. It is, however, generally assumed that the intestinal absorption of both dietary and biliary cholesterol is reduced in the presence of plant stanols, since the micellar solubility of cholesterol is lowered (Ikeda *et al*, 1989). Therefore, it has been suggested that plant sterols, which also lower the micellar solubility of cholesterol, should be consumed at each cholesterol-containing meal to achieve an optimal effect (Mattson *et al*, 1982). However, this suggestion is not supported by our findings. We therefore hypothesize that plant stanols, or plant stanol esters, remain in the intestinal lumen or in the enterocytes for a while. Indeed, only 70% of an orally administered single bolus of ^{14}C labeled sitostanol to male Wistar rats is found in the feces after 24 h (Ikeda & Sugano, 1978). After 2 and 3 days the cumulative fecal excretions were 90% and 97%, respectively. Thus, when the low absorption of sitostanol into the circulation (Hassan & Rampone, 1979) is neglected, at least 25–30% of the sitostanol is still in the intestinal tract after one day. However, when rats were fed 0.5% cholesterol and 0.5% sitostanol (W/W) for 18 days, the daily fecal excretion of sitostanol showed a recovery of approximately 100% (Sugano *et al*, 1977). This implies that in rats, at least within 18 days, a steady state was reached and sitostanol intake equaled sitostanol excretion. This still does not elucidate whether sitostanol remains in the intestinal lumen, and if so, in which part, or in the enterocytes. It also does not answer the question of how long plant stanols are active in the intestine. Studies with caco-2 cells have addressed the question whether micellar ^{14}C-labeled sitosterol could be taken up in the enterocyte and subsequently be excreted across the basolateral membrane (Field *et al*, 1997). To our knowledge no such studies with sitostanol have been published. It appeared that sitosterol was indeed associated with the caco-2 cells. It was, however, not esterified intracellular and not excreted to the basolateral medium. This implies that sitosterol can indeed remain in or can be associated with enterocytes. The functional significance of these findings, however, is unknown. Theoretically sitosterol could remain associated with the enterocytes only temporarily, be released into the lumen after several hours, and consequently affect micellar solubility of intestinal cholesterol at that moment. It can, however, also be speculated that plant sterols or stanols not only affect micellar solubility of cholesterol, but have additional effects on intestinal lipoprotein metabolism as well.

In this study, serum LDL cholesterol concentrations were significantly reduced by 9–10%, when plant stanol esters were consumed. In a previous study, also in a normocholesterolemic and mildly hypercholesterolemic population, serum LDL cholesterol concentrations decreased by 11–13%, when 3.8 or 4.0 g plant stanols as its fatty acid esters were consumed (Plat & Mensink, 2000). As already discussed (Mensink & Plat, 1998; Wester, 1999), hardly any additional benefit is obtained when daily intake of plant stanols exceeds 2.2 g.

Although total fat consumption during the three diet periods was similar, the fatty acid compositions of the diets were not entirely comparable. This was due to the slightly lower absorbable fat content of the stanol ester shortening compared with the control shortening. However, the marginal differences in the dietary fatty acid compositions were too small to have a major impact on serum lipoproteins. The LDL-cholesterol-lowering effect of the once per day period might have been overestimated by 0.02 mmol/l compared with the control period, while the LDL-cholesterol-lowering effect of the three times per day diet might have been underestimated by 0.01 and 0.04 mmol/l, when compared with the control period and the once per day period, respectively (Mensink & Katan, 1992).

Consumption of 2.5 g plant stanols three times a day significantly lowered most of the carotenoid and tocopherol isomers studied. In contrast, consumption of a similar amount of plant stanols once day at lunch only resulted in reduced absolute α-tocopherol and β-carotene concentrations. In addition, all antioxidants studied showed slightly lower concentrations at the end of the three times per day period compared with the concentrations at the end of the once per day period (Tables 4 and 5). These absolute reductions can be explained largely by a reduced number of LDL particles in the circulation, which are major carriers of the fat-soluble antioxidants. Therefore, the differences were no longer significant after standardization for LDL choles-

terol. Furthermore, we have shown that, in particular, the most lipophylic hydrocarbon carotenoid concentrations (ie α-carotene, β-carotene and lycopene) were lowered by plant stanol ester consumption. The mechanism and the biological significance of these effects, however, remain to be elucidated.

From our results we conclude that a daily consumption of 2.5 g plant stanols as fatty acid esters either at lunch or divided over the three meals does not affect its serum LDL-cholesterol-lowering efficacy. This implies that it is not necessary to consume plant stanol esters simultaneously with dietary cholesterol or with each meal. We therefore hypothesize that plant stanols, or plant stanol esters, remain in the intestinal lumen, or possibly in or associated with the enterocytes. It can also be speculated that plant stanols not only affect micellar solubility of cholesterol, but have other intestinal effects on lipoprotein metabolism as well. Therefore, further research will be necessary to elucidate the mechanism by which plant stanols lower LDL cholesterol.

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Effects of low-fat stanol ester enriched margarines on concentrations of serum carotenoids in subjects with elevated serum cholesterol concentrations

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Objective: To investigate the effects of low-fat stanol ester margarines on concentrations of serum carotenoids.
Design: A randomized parallel double-blind study design consisting of a 4-week run-in (high-fat diet) and an 8-week experimental (low-fat, low-cholesterol diet) period. During the experimental diet period subjects consumed low-fat wood stanol ester (WSEM), vegetable oil stanol ester (VOSEM) or control (no stanol esters) margarine daily. The daily mean total stanol intake was 2.31 and 2.16 g in the WSEM and VOSEM groups, respectively.
Setting: Outpatient clinical trial with free-living subjects.
Subjects: Altogether, 60 hypercholesterolaemic subjects were selected for the study out of 91 originally screened. The study was completed by 55 subjects.
Main outcomes measures: Serum α- and β-carotene and lycopene determined by the HPLC.
Results: Serum α-carotene concentration did not change significantly in either of the experimental groups, whereas β-carotene concentration decreased significantly in the WSEM and VOSEM groups ($P < 0.01$), and the change differed significantly ($P < 0.05$ and $P < 0.01$, respectively) from that of the control group. Decrease in $\alpha+\beta$-carotene concentration was significantly greater ($P < 0.05$) in both experimental groups than in the control group. However, the change in α-, β- or $\alpha+\beta$-carotene/total cholesterol ratio did not differ significantly among the groups. No significant changes were found in serum lycopene or lycopene/total cholesterol ratio in both experimental groups.
Conclusions: Low-fat stanol ester margarines appeared to have little effect on serum concentrations of α-, β- or $\alpha + \beta$-carotene, or lycopene.
Sponsorship: Grant to the University of Kuopio by Raisio Benecol Ltd, Raisio, Finland.
Descriptors: plant sterols; carotenoid; lycopene; α-carotene; β-carotene; cholesterol

Introduction

Plant sterols have been found to reduce serum cholesterol concentrations by inhibiting the absorption of both dietary and biliary cholesterol from the small intestine (Heinemann *et al*, 1991; Becker *et al*, 1993; Gylling *et al*, 1997). They may also reduce the concentrations of serum carotenoids, particularly serum β-carotene concentration (Gylling et al, 1996). In a recently published study it was observed that plant sterol-enriched margarines reduced plasma lycopene as well as $\alpha+\beta$-carotene concentrations, even if the changes in plasma lipid concentrations were taken into account (Weststrate & Meijer, 1998). Reduction in serum carotenoid concentrations can be undesirable, because there are indications that carotenoids could have beneficial effects on human health (Gerster 1993; Mayne, 1996). The most studied carotenoid in this respect is β-carotene (Gerster 1993; Mayne, 1996).

Lycopene is one of the most abundant carotenoids in human blood and tissues; it has not been found to have a provitamin A activity, but it has been found to have antioxidant properties (Clinton, 1998). Therefore, we investigated the effects of two low-fat margarines enriched with wood- or vegetable oil-based plant stanol esters on serum lycopene and α- and $\alpha + \beta$-carotene concentrations as part of a low-fat diet. Results on serum lipids have been published elsewhere (Hallikainen & Uusitupa,1999).

Methods

Subjects, study design, diets and laboratory measurements have been described in more detail elsewhere (Hallikainen & Uusitupa, 1999).

Subjects

Altogether 60 hypercholesterolaemic subjects were selected for the study out of 91 originally screened subjects. The study was completed by 55 subjects, whose baseline characteristics are shown in Table 1. The study protocol was approved by the Ethics Committee of the University of Kuopio and all subjects gave their written consent.

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Guarantor: M Uusitupa
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Table 1 Baseline characteristics of the subjects, intake of plant stanols and serum carotenoid concentrations during the study[a]

	WSEM (N=18)	VOSEM (N=20)[b]	CONTROL (N=17)	P-values[c]
Men/women	8/10	6/14	6/11	
Age (y)	43.2±8.2	40.8±9.3	46.0±8.2	NS
Body mass index (kg/m²)	25.6±4.0	24.2±3.0	25.7±3.5	NS
Intake of plant stanols from test margarines (g/d)	2.31±0.03	2.16±0.12	0	
Sitostanol	2.13±0.03	1.47±0.08	0	
Campestanol	0.19±0.0	0.69±0.04	0	
Serum concentrations of carotenoids				
α-carotene (μmol/l):				
At 0 weeks	0.33±0.23	0.33±0.20	0.35±0.39	NS
At 8 weeks	0.31±0.19	0.29±0.11	0.28±0.10	NS
Changes (from 0 to 8 weeks)	−0.02±0.16	−0.04±0.13	−0.07±0.40	NS
P-values[d]	NS	NS	NS	
β-carotene (μmol/l):				
At 0 weeks	1.66±1.10	1.47±0.79	1.00±0.37	0.063
At 8 weeks	1.22±0.97	1.07±0.54	1.06±0.42	NS
Changes (from 0 to 8 weeks)[e]	−0.44±0.57*	−0.40±0.54†	0.05±0.26	0.002
P-values[d]	0.002	0.001	NS	
α+β-carotene (μmol/l):				
At 0 weeks	2.00±1.24	1.80±0.91	1.35±0.49	NS
At 8 weeks	1.53±1.11	1.36±0.61	1.34±0.46	NS
Changes (from 0 to 8 weeks)	−0.47±0.65*	−0.44±0.59*	−0.02±0.53	0.004
P-values[d]	0.010	0.010	NS	
Lycopene (μmol/l):				
At 0 weeks	0.96±0.67	1.04±0.92	0.58±0.42	0.072
At 8 weeks	0.97±0.54	0.98±0.57	0.77±0.37	NS
Changes (from 0 to 8 weeks)[e]	0.01±0.82	−0.06±0.84	0.19±0.58	NS
P-values[d]	NS	NS	NS	
α-carotene/total cholesterol ratio:				
At 0 weeks	0.05±0.04	0.06±0.04	0.06±0.07	NS
At 8 weeks	0.06±0.04	0.06±0.03	0.05±0.02	NS
Changes (from 0 to 8 weeks)	0.01±0.03	0.00±0.02	−0.01±0.07	NS
P-values[d]	NS	NS	0.018	
β-carotene/total cholesterol ratio:				
At 0 weeks	0.27±0.21	0.24±0.13	0.17±0.07	NS
At 8 weeks	0.24±0.23	0.21±0.10	0.19±0.08	NS
Changes (from 0 to 8 weeks)	−0.03±0.12	−0.03±0.08	0.02±0.05	NS
P-values[d]	NS	NS	NS	
α+β-carotene/total cholesterol ratio:				
At 0 weeks	0.32±0.24	0.30±0.16	0.23±0.09	NS
At 8 weeks	0.30±0.26	0.27±0.12	0.24±0.09	NS
Changes (from 0 to 8 weeks)	−0.02±0.14	−0.03±0.09	0.01±0.10	NS
P-values[d]	NS	NS	NS	
Lycopene/total cholesterol ratio:				
At 0 weeks	0.15±0.11	0.18±0.18	0.10±0.06	NS
At 8 weeks	0.18±0.11	0.20±0.12	0.14±0.08	NS
Changes (from 0 to 8 weeks)	0.04±0.16	0.02±0.14	0.05±0.07	NS
P-values[d]	NS	NS	0.010	

[a]Values are means±s.d. [b]N=19 for results concerning lycopene and lycopene/total cholesterol ratio. [c]Indicates the significance of the difference among the groups analysed with a single measurement simple factorial analysis of variance (ANOVA) test (age, body mass index, β-carotene, lycopene and ratios of β-carotene/total cholesterol and lycopene/total cholesterol) or the Kruskal–Wallis one way ANOVA test (α-carotene, α+β-carotene and ratios of α-carotene/total cholesterol and α+β-carotene/total cholesterol). [d]Indicates the significance of the difference within the group during the experimental period analysed with a paired *t*-test or the Wilcoxon matched-paired signed rank test. [e]The initial concentrations were almost significantly different among the study groups analysed with ANOVA, and therefore the initial concentrations have been taken into account in the between-groups comparisons by dividing the response variable by the initial value. *$P < 0.05$, †$P < 0.01$ indicate the significances of the differences between the experimental study groups and the control group analysed with Student's *t*-test or the Mann–Whitney U-test with Bonferroni correction.

Study design

The study has carried out with a parallel double-blind study design. All subjects started the study with a 4-week run-in (high-fat diet) period. At the end of the run-in period the subjects were randomized into one of the three experimental groups: wood stanol ester margarine (WSEM), vegetable oil stanol ester margarine (VOSEM) or control margarine. After randomization the subjects followed a closely instructed, and strictly and frequently monitored low-fat, low-cholesterol diet which resembled Step 2 of the National Cholesterol Education Program (1994) for the next 8 weeks. As part of that diet the subjects consumed 25 g rapeseed oil-based low-fat margarine (Raisio Group Plc, Raisio, Finland) daily. The daily dose of the test margarines was taken in two to three portions in connection with the meals. The two test margarines contained about 31% and the control margarine about 35% absorbable fat. The theoretical daily intake of total stanol was 2.34 g (of which 2.15 g was sitostanol and 0.19 g campestanol) and 2.20 g (of which 1.50 g was sitostanol and 1.70 g campestanol) in the WSEM and VOSEM groups, respectively. The control margarine did not contain added plant stanols.

The subjects recorded the consumption of the test margarines daily in the follow-up diaries and the adherence to the diets was monitored by a 4-day (3 weekdays and 1 weekend day) food record kept three times during the experimental period.

Laboratory measurements

Venous blood samples were obtained after a 12 h overnight fast. Enzymatic colorimetric methods were used for the determination of serum total and lipoprotein lipids using commercial kits (Monotest® Cholesterol and Triglyceride GPO-PAP, Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) and a Kone Specific Clinical Analyzer (Kone Ltd, Espoo, Finland). Serum carotenoids were obtained at the beginning and the end of the experimental diet period and were analysed at the end of the study from samples stored at −70°C, using a high-performance liquid chromatography system (Perkin Elmer, USA) equipped with a C18 column (Nova-pak Waters, USA), and detected at 450 nm, using a Uvikon 735 LC detector (Germany) (Driskell *et al*, 1983; Kaplan *et al* 1987).

Statistical analyses

Statistical analyses were performed with SPSS for Windows 6.0 statistics program (SPSS, Chicago, IL, USA). Values of serum carotenoids were analysed by the analyses of variance for repeated measurements (MANOVA), followed by a single-measurement simple factorial analysis of variance (ANOVA) test and Student's *t*-test or paired *t*-test, or the Kruskal–Wallis one-way ANOVA test followed by Mann–Whitney U-test. Logarithmic transformations were used when appropriate. To control the overall α level, Bonferroni adjustment was made. The results are expressed as means ± s.d.

Results

Results on baseline characteristics, intake of nutrients, changes in serum lipids and also β-carotene have been presented elsewhere (Hallikainen & Uusitupa, 1999).

The intake of nutrients during the experimental diet periods was stable and did not differ among the three groups. The mean intakes of fat and saturated fatty acids were 25.6–26.5% of energy (E%) and 6.8–7.3 E%, respectively, in the three study groups. The mean daily intake of dietary cholesterol was 137–161 mg, achieving the goal of the Step 2 diet of the National Cholesterol Education Program (< 200 mg/d; National Cholesterol Education Program, 1994). Actual daily intakes of plant stanols as mean values are presented in Table 1.

During the experimental period serum total and LDL cholesterol decreased significantly in all three groups. The net decrease in serum total cholesterol concentration was 10.6% ($P < 0.01$) and 8.1% ($P < 0.05$), and in LDL cholesterol 13.7% ($P < 0.01$) and 8.6% ($P = 0.072$) in the WSEM and VOSEM groups, respectively, compared with the control group.

Serum β-carotene decreased significantly within both experimental groups ($P < 0.01$), but increased slightly, but non-significantly, within the control group. The changes differed significantly between the experimental groups and the control group. However, no significant differences among the groups were found in serum β-carotene after standardization for serum total cholesterol concentration.

There were no significant changes in serum α-carotene concentration or α-carotene/total cholesterol ratio within the WSEM and VOSEM groups (Table 1). Within the control group that ratio decreased slightly, but statistically significantly. Serum $\alpha+\beta$-carotene concentration decreased within all groups, but only within the WSEM and VOSEM groups did the decrease reach statistical significance (Table 1), and it differed significantly from that of the control group. However, there were no significant changes in serum $\alpha+\beta$-carotene concentrations after standardization for serum total cholesterol concentration within any of the three study groups or differences in the changes among the groups. Serum lycopene and lycopene/total cholesterol ratio did not change significantly in the experimental groups, but the ratio increased significantly in the control group (Table 1).

Discussion

In the present study the wood stanol ester margarine and the vegetable oil stanol ester margarine had only a small effect on serum concentrations of α- and β-carotene, the sum of $\alpha+\beta$-carotene or lycopene, in particular when changes were related to the changes in serum total cholesterol concentrations. Before lipid standardization serum β-carotene concentration decreased significantly; however this was not unexpected, because serum LDL-cholesterol concentrations decreased significantly in the present study and in circulation carotenoids are transported in lipoproteins, mainly in the LDL fraction, but also in HDL and to a minor extent in VLDL fraction (Clevidence & Bieri, 1993).

There are only a few studies (Gylling *et al*, 1996; Weststrate & Meijer, 1998; Hendriks, *et al*, 1999) in which the effects of plant stanols or plant sterols on serum or plasma carotenoid concentrations have been investigated. In the study of Gylling *et al* (1996), after lipid standardization serum β-carotene concentration tended to decrease more in two sitostanol ester margarine (sitostanol intake 3 g/d and 2 g/d) groups than in the control group, whereas unstandardized and lipid standardized serum α-carotene concentration decreased similarly in all three groups. In the study of Weststrate and Meijer (1998) the plant sterol enriched margarines (sterol intake 1.5–3.3 g/d) also significantly decreased lipid standardized plasma $\alpha+\beta$-carotene concentrations. Moreover, Hendriks *et al* (1999) found that the daily dose of 0.83 and 3.24 g plant sterols decreased lipid standardized plasma $\alpha+\beta$-carotene more than the 1.61 g daily dose. The effects of plant sterols and plant stanols on plasma lycopene concentrations were smaller than the effects on plasma carotenes (Weststrate & Meijer, 1998; Hendriks *et al*, 1999). In particular, the results of Hendriks *et al* (1999) indicate that other factors, i.e. nutrient density of background diet, could affect the fluctuations of serum carotenoid concentrations rather than the dose of plant stanol or plant sterol.

The differences between our findings and the findings of Gylling *et al* (1996), Weststrate & Meijer (1998) and Hendriks *et al* (1999) might be due to the differences in composition of background diets during the studies. In our study subjects received detailed written and oral instructions about the low-fat (high-nutrient density) diets, specifying amounts and quality of food by main food groups, including vegetables. The background diets in other studies were not so closely standardized since only the consumption

of the test margarines was instructed and subjects followed their habitual, moderate-fat or high-fat diet.

One reason for divergent results could be a seasonal variation in the intakes of carotenoids. However, according to earlier studies there is only a weak correlation between lycopene intake and serum lycopene concentration (Campbell *et al*, 1996; Clinton 1998).

According to our findings the effects of plant sterols on serum carotenoid concentrations were minor and clinically non-important, but additional studies will be needed to determine the long-term effects of plant sterols on serum carotenoid profile. At the moment, it seems to be reasonable to advise healthy food rich in vegetables for people using stanol ester margarine to reduce elevated serum cholesterol concentrations.

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PART 3

Spreads enriched with plant sterols, either esterified 4,4-dimethylsterols or free 4-desmethylsterols, and plasma total- and LDL-cholesterol concentrations

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In a 9-week study seventy-six healthy adult volunteers with an average age of 44 (SD 11) years, with baseline plasma total cholesterol levels below 8 mmol/l, received in a balanced, double-blind, crossover design, a total of three different table spreads for personal use. Two spreads were fortified either with free (non-esterified) vegetable-oil sterols, mainly from soyabean oil (31 g sterol equivalents/kg; 0·8 g/d) or sheanut-oil sterols (133 g sterol equivalents/kg; 3·3 g/d). One spread was not fortified (control). Average intake of spread was 25 g/d for 3 weeks. None of the spreads induced changes in blood clinical chemistry or haematology. Plasma total- and LDL-cholesterol concentrations were statistically significantly reduced by 3·8 % and 6 % (both 0·19 mmol/l) respectively, for the spread enriched with free soyabean-oil sterols compared with the control spread. The spread enriched with sheanut-oil sterols did not lower plasma total- and LDL-cholesterol levels. None of the plant-sterol-enriched spreads affected plasma HDL-cholesterol concentrations. Plasma-lipid-standardized concentrations of α- plus β-carotene were not statistically significantly affected by the soyabean-oil sterol spread in contrast to lipid-standardized plasma lycopene levels which showed a statistically significant decrease (9·5 %). These findings indicate that a daily intake of free soyabean-oil sterols as low as 0·8 g added to a spread is effective in lowering blood total- and LDL-cholesterol levels with limited effects on blood carotenoid levels. The lowering in total- and LDL-cholesterol blood levels due to consumption of the vegetable-oil-sterol-enriched spread may be helpful in reducing the risk of CHD for the population.

Plant sterols: Lipids: Carotenoids

The major manifestation of cardiovascular disease is CHD, which remains the leading cause of death in the developed world. Atherosclerosis, which damages the coronary arteries, is the primary disease mechanism of CHD (Reitsma, 1995). Hypercholesterolaemia contributes to the atherosclerosis and its clinical manifestations such as coronary artery occlusion and ischaemic myocardial infarction (Steinberg & Witztum, 1990).

Lowering blood total- and LDL-cholesterol levels has been advocated as a method to decrease the incidence of CHD (Probstfield & Rifkind, 1991; Cucherat & Boissel, 1993). The benefits of serum cholesterol concentration reduction are related to age; a 10 % reduction in total serum cholesterol concentration produces a reduction in IHD of 50 % at age 40, 40 % at age 50, 30 % at age 60, and 20 % at age 70. The benefit can be realised quickly, the greater part after 2 years and the full benefit after 5 years (Law *et al.* 1994). Reduction in the blood cholesterol concentration not only slows the process of atherosclerosis, but can also result in regression of atherosclerosis (Levine *et al.* 1995).

Dietary modification is the first step in lowering blood cholesterol levels, for example, replacing saturated fatty acids by unsaturated fatty acids (Mensink & Katan, 1992) and increasing the intake of soluble fibres (Ripsin *et al.* 1992). In addition to unsaturated fatty acids and soluble fibres, plant sterols, such as sitosterol, may also reduce blood cholesterol levels (Pollak & Kritchevsky, 1981). Sitosterol at intakes of 3–20 g/d reduces blood cholesterol levels by 5–15 % in human studies without major side-effects (Farquhar & Sokolow, 1958; Oster *et al.* 1976; Lees *et al.* 1977; Schwartzkopff & Jantke, 1978). Plant sterols are naturally occurring plant compounds. The most important dietary sources are vegetable oils and vegetable-oil-based products such as margarines (Ling & Jones, 1995). The most predominant plant sterols are sitosterol, campesterol and

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stigmasterol, and intake of these is estimated to be about 250 mg/d in non-vegetarians and about 500 mg/d for vegetarians (Ling & Jones, 1995). Plant sterols reduce the absorption of cholesterol in the gut possibly by displacing cholesterol from the micelles, by limiting the intestinal solubility of cholesterol or by decreasing the hydrolysis of cholesterol esters in the small intestine (Ling & Jones, 1995). Daily consumption of about 3 g of a mixture of vegetable-oil sterol esters, in particular esters of sitosterol, campesterol and stigmasterol, in a commonly used food, margarine, was recently reported to be effective in lowering plasma total- and LDL-cholesterol levels in normocholesterolaemic and mildly hypercholesterolaemic subjects (Weststrate & Meijer, 1998). There have been very few studies published on the effect of doses of plant sterols less than 1 g/d on plasma cholesterol levels in normocholesterolaemic subjects using commonly consumed foods (Pelletier *et al.* 1995).

The objective of the present study was to compare the effects of the consumption of two types of spreads (margarines) enriched with plant sterols, either sheanut-oil sterol (133 g sterol equivalents/kg), mainly 4,4-dimethylsterols, or non-esterified soyabean-oil sterols (31 g sterol equivalents/kg), mainly 4-desmethylsterols, in healthy volunteers on plasma total- and LDL-cholesterol levels. Flora, a spread not enriched with plant sterols but containing similar amounts and types of fatty acids, was used as the control. Our previous study (Weststrate & Meijer, 1998) had shown a substantial plasma total- and LDL-cholesterol-lowering action of a spread enriched with esterified vegetable-oil sterols, mainly derived from soyabean oil (110 g sterol equivalents/kg). Non-esterified soyabean-oil sterols might be somewhat more effective in lowering plasma total- and LDL-cholesterol levels compared with esterified soyabean-oil sterols, as sterols need to be in an unesterified or free state to inhibit cholesterol absorption (Peterson *et al.* 1953; Swell *et al.* 1956; Best & Duncan, 1958). However, solubility of free plant sterols in spread is limited to approximately 30 g/kg. In our previous study a sheanut-oil-sterol-enriched spread (101·5 g sterol equivalents/kg) was not effective in lowering blood cholesterol levels (Weststrate & Meijer, 1998). A different fatty acid composition of this spread compared with the control product (less linoleic fatty acid and more oleic fatty acid) could have contributed to this result. In the present study, in contrast to our previous study, the fatty acid composition of the sheanut-oil-sterol-enriched spread was similar to the control spread. Blood chemistry and haematology were performed to assess possible adverse effects of spread consumption. Plasma α- plus β-carotene and lycopene levels were assessed only for the spreads which gave a statistically significant reduction in plasma total- and LDL-cholesterol concentrations, as we found in our previous study (Weststrate & Meijer, 1998) a reduction of between 15 and 19 % in plasma lipid-standardized levels of these lipophilic dietary compounds after intake of spreads enriched with plant sterols.

Subjects and methods

Subjects

Volunteers were recruited by advertisement from inhabitants of Vlaardingen (The Netherlands) and its surroundings, and from employees of Unilever Research Vlaardingen, with the exception of employees of the Unilever Nutrition Centre. Subjects' characteristics are shown in Table 1. They ranged in age from 18 to 62 years and their BMI at the start of the study were between 19·2 and 29·6 kg/m^2. Alcohol drinking was below 21 units/week for females and below 28 units/week for males. Volunteers had less than 10 h/week of intense exercise. Their entry plasma total cholesterol levels were below 8 mmol/l and plasma activities of L-γ-glutamyltransferase (*EC* 2.3.2.2), alanine aminotransferase (*EC* 2.6.1.2), aspartate aminotransferase (*EC* 2.6.1.1) and alkaline phosphatase (*EC* 3.1.3.1) were all within the normal ranges. Leucocyte, erythrocyte and platelet count, haemoglobin level and packed cell volume were all within the normal ranges. The volunteers were not participating in any other biomedical trial and had not donated blood within 1 month before the start of the study. Female volunteers were non-lactating and not pregnant. The study set-up was approved by our local Medical-Ethical Committee and written informed consent was obtained from all subjects.

Study design

The study had a balanced, double-blind, crossover design with a run-in period of 1 week with the control spread so that the volunteers could become accustomed to the use of the spreads. Immediately thereafter the experimental period of 9 weeks started in which each spread was given in random

Table 1. Descriptive characteristics of the volunteers participating in the plant-sterol-enriched spread study
(Mean values and standard deviations)

	Total (*n* 76)		Males (*n* 39)		Females (*n* 37)	
Characteristic	Mean	SD	Mean	SD	Mean	SD
Age (years)	44	11	45	12	43	11
Body weight (kg)	75·2	10·7	81·7	9·6	68·4	7·1
Body height (m)	1·75	0·09	1·82	0·07	1·69	0·05
BMI (kg/m^2)	24·4	2·4	24·7	2·2	24·0	2·5
Sporting activities (h/week)	1	2	1	2	1	1
Alcoholic beverage consumption (units/week)	5	6	6	6	3	5

order for 3 weeks at an intake level of 25 g/d. Subjects were randomly allocated over the six different period × group sequences. Groups were stratified according to sex. After each experimental period of 3 weeks, a fasting venous blood sample was taken from an antecubital vein with the subject seated in a chair. Body weight was measured on a digital balance (Mettler Instruments AG, Greifensee, Switzerland), with the subjects wearing light indoor clothing, without shoes and after voiding.

Experimental spreads

The test articles were specially prepared spreads fortified with non-esterified plant sterols derived from either edible vegetable oil (mainly soyabean oil) distillates (Henkel Corporation, LaGrange, IL, USA), or a sheanut oil concentrate in which the plant sterols were esterified, mainly with cinnamate (69 %) and acetate (25 %) (Loders Croklaan, Wormerveer, The Netherlands). The distillate or concentrate was used in spread production together with other edible oils and fats in order to achieve an acceptable product. The final plant sterol concentration was 31 g/kg for the soyabean-oil sterol product and 133 g/kg for the sheanut-oil sterol product, when expressed as free sterol equivalents. Flora (VandenBergh Foods, Purfleet, Surrey, UK), a spread not fortified with plant sterols, was used as the control. Table 2 shows the composition characteristics of the spreads. Tubs containing the individual daily amounts of spread were labelled with a blind product code. The spreads were intended for personal use and meant to replace all or part of the volunteers' habitual spread or butter used for spreading, but not to be used for baking or frying. Compliance was assessed by asking the volunteers about spread consumption and by weighing left-overs from the tubs.

Lifestyle and dietary intake

The volunteers were instructed to maintain their normal lifestyle and dietary and activity patterns during the entire study. The dietary intake during each period, i.e. over 21 d, was assessed at the end of each period using a validated food-frequency questionnaire (Grootenhuis *et al.* 1995), with special emphasis on intakes of total energy, macronutrients, fatty acids, cholesterol and fibre. This questionnaire was modified for this study to give more detailed information on the intake of specific brands of spreads and dietary fats in the habitual diet in order to better evaluate fat composition of the habitual diet during each period. The answers to the questionnaire were checked for completeness by dietitians. Throughout the study volunteers recorded illnesses, medicine use, and any important deviations from their lifestyle or dietary and activity patterns.

Measurements

Blood samples. Fasting venous blood samples were taken on the last day of each period. Plasma total- and HDL-cholesterol levels were determined using test kits from Boehringer (Mannheim, Germany). Plasma LDL-cholesterol level was calculated by the Friedewald formula (Friedewald *et al.* 1972). Plasma total and free glycerol concentrations were analysed using a total glycerol test kit (Roche, Basel, Switzerland) and a free glycerol test kit (Sigma, St Louis, MO, USA). Plasma triacylglycerol level was calculated as plasma total glycerol level (mmol/l) minus plasma free glycerol level (mmol/l). In blood, activities of L-γ-glutamyltransferase and alkaline phosphatase and levels of bilirubin, creatinine, albumin and glucose were determined using test kits from Boehringer. The activities of aspartate aminotransferase and alanine aminotransferase and plasma levels of urea were determined using test kits from Roche. All determinations were done on a Cobas Mira S automated analyser (Roche). Plasma α- plus β-carotene and lycopene levels were only assessed in the blood samples which were taken after consumption of the soyabean-oil-sterol spread, which caused a statistically significant reduction in plasma total- and LDL-cholesterol concentrations, and in the blood samples which were taken

Table 2. Analysed composition of the spreads (per kg product) used in the plant-sterol-enriched spread study

Spread, ...	Flora*	Soyabean†	Sheanut‡
Total fat (g)§	710	710	720
Water (g)	290	260	110
Fatty acid composition (g):			
Saturated fatty acids	160	140	190
Monounsaturated fatty acids	210	220	190
Polyunsaturated fatty acids	340	350	340
Free sterols plus sterol esters (g)	3	33	167
Sterol equivalents (g)	3	31	133
Sterol composition (g):			
4-Desmethylsterols	2·7	30·5	4·6
4-Monomethylsterols	0·3	0·4	1·2
4,4-Dimethylsterols	0·2	0·2	127
Total vitamin E (mg)‖	430	460	300
α + β-Carotene (mg)	4·6	4·9	4·7

* Control spread, not enriched with plant sterols.
† Spread enriched with 31 g soyabean-oil sterols/kg.
‡ Spread enriched with 133 g sheanut-oil sterols/kg.
§ Total *trans* fatty acid content of spreads was less than 10 g/kg fat, sterols excluded.
‖ Includes α-, β-, and γ-tocopherol.

after consumption of Flora. This was done to assess the potential impact of the sterol-containing spread on the absorption of lipophilic dietary compounds. The plasma carotenoids were separated by HPLC on a Nucleosil 5-$N(CH_3)_2$ column (Marchery & Nagel, Düren, Germany) using *n*-heptane as eluent as described previously (Weststrate & van het Hof, 1995). Plasma carotenoids were determined from the absorption at 450 nm. Plasma carotenoid concentrations were standardized for plasma lipid (total cholesterol and triacylglycerol) levels as previously described (Weststrate & van het Hof, 1995), because the sterol-enriched spreads did affect blood lipoprotein (plasma carotenoid carriers) concentrations. Haemoglobin concentrations, leucocyte count, platelet count and calculation of the packed cell volume were all performed on a Sysmex F-800 blood cell counter (Toa Medical Electronics Co. Ltd, Kobe, Japan). Staff conducting the laboratory analyses were blind to the group assignments.

Spreads. Directly after production and in each experimental period the spreads were analysed. Mean values for the composition are given in Table 2. During the study the stability of the spreads was checked once. The homogeneity of the sterols in the spreads was good, CV were generally less than 10 % (results not shown). For fat analysis the sample was mixed with celite, freeze-dried and extracted with dichloromethane. After evaporation, the residue (fat) was weighed. The triacylglycerol content was calculated by subtracting the total sterol and/or sterol ester content from the total fat content. Fatty acid composition was analysed by methanolysis of the fatty acids. The methyl esters were extracted with heptane and analysed by GC (Hewlett Packard, Amstelveen, The Netherlands) using a CP-Sil 88 column (Chrompack, Middelburg, The Netherlands). Analysis of sterol types was performed according to previously described methods (Grob *et al.* 1989; Artho *et al.* 1993; Plank & Lorbeer, 1993; Gordon & Miller, 1997). The spreads were saponified with KOH before extracting the unsaponifiable part into cyclohexane. Thin-film chromatography (with as TLC-eluent a mix of toluene with ethyl acetate) was used for separating the 4-desmethylsterol, the 4-monomethylsterol and the 4,4-dimethylsterol fractions (10 g ultraphor/l water as detection reagent) with β-cholestanol as internal standard. The fractions were extracted with a mixture of chloroform, diethyl ether and ethanol. After silylizing with pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide this fraction was analysed by GC (Fisions Instruments, Milan, Italy) using a CP-Sil 19 column (Chrompack). Detection was by flame ionization detector (Fisions Instruments). For determination of the amounts of free and esterified sterols a portion of the isolated fat phase was silylated in order to convert the free sterols into trimethylsilyl ether derivatives. After isolation of the free sterols and steryl fatty acid esters from the triacylglycerol matrix by means of normal-phase HPLC, the free sterols and steryl fatty acids were quantified by GC using internal standards. Sterol equivalents were calculated by multiplying the weight percentages of steryl fatty acid esters by a factor 0·608 (calculated from the average molecular mass of sterols and fatty acids in soyabean oil). The total sterol equivalents were obtained by adding the concentration of free sterols and the sterol equivalents from the steryl fatty acid esters. Vitamin E was analysed by a straight-phase HPLC method, based on international standard operation procedures (American Oil Chemists' Society, 1992). Carotenoid content was assessed for Flora and the spread containing free soyabean-oil sterols. For the carotenoid analysis the sample was mixed with diethyl ether. After vortex-mixing the extract was dried under N_2 and dissolved in *n*-heptane containing the internal standard, ethyl-β-apo-8-carotenoate. The analysis was performed by straight-phase HPLC using an ET 200/8/4 nucleosil 5-$N(CH_3)_2$ column (Marchery & Nagel) and *n*-heptane–isopropanol (1000 : 24, v/v) as mobile phase at a flow rate of 1 ml/min and a column temperature of 20°. Carotenoids were detected by u.v.-visible detection at 450 nm for α- plus β-carotene and at 470 nm for lycopene.

Statistical analysis

Differences in plasma variables and dietary intake between spread groups were initially evaluated using ANOVA using as factors subject, period, spread, carry-over, sex and sex × spread. The carry-over effect was subsequently removed from the model as it was not statistically significant. Blood lipid and carotenoid data were log normalized before analysis. Geometric means are given with 95 % CI and have been back transformed (Table 4). Differences between spread groups were assessed using the Tukey multiple comparison test (Miller, 1981). A P value of 0·05 was considered statistically significant. The Statistical Analysis Systems program version 6.12 (1987; SAS Institute Inc., Cary, NC, USA) was used for analysis. All the statistical significance levels are given two-sided.

Results

Thirty-nine men and thirty-seven women completed the study. During the study two women dropped out because of medicine use and their data were not used in final analysis. Two people missed one period because of diarrhoea and one person did not give a blood sample in one period.

Compliance

The mean intakes of Flora, the vegetable-oil-sterol spread and the spread enriched with sheanut-oil sterols, estimated by weighing the left-over spread in the tubs after each period, were respectively 24·2 g/d, 24·8 g/d and 24·4 g/d with CV 1·1 %, 1·9 % and 4·0 % (results not shown). The mean daily total plant sterol intakes were respectively 0·08 g, 0·8 g and 3·2 g. The habitual dietary intake (including the test spread consumption) is given in Table 3. Except for the statistically significant but very small difference in intake of saturated fatty acids (in % energy) between the soyabean-oil-sterol spread and the sheanut-oil-sterol spread, no statistically significant differences were observed among the groups in dietary intake. The average body weight in the group with the spread containing free soyabean oil sterols was slightly higher than the average body weight in the Flora group (75·5 kg *v.* 75·2 kg). However, an analysis for males and females separately revealed no statistically

Table 3. Energy and dietary nutrient intakes (as percentage of total energy unless otherwise indicated) of the volunteers participating in the plant-sterol-enriched spread study

(Mean values and 95% confidence intervals)

Spread...	Flora†		Soyabean‡		Sheanut§	
Nutrient	Mean	95% CI	Mean	95% CI	Mean	95% CI
Energy (kJ/d)	9977	9732, 10221	10060	9816, 10304	10019	9775, 10264
Total fat	38·3	37·8, 38·8	38·3	37·8, 38·8	38·4	37·9, 38·9
Saturated fatty acids	13·9	13·7, 14·2	13·8	13·6, 14·1	14·3*	14·0, 14·6
Monounsaturated fatty acids	12·8	12·6, 13·0	12·9	12·6, 13·1	12·5	12·3, 12·7
Polyunsaturated fatty acids	9·2	9·1, 9·4	9·2	9·1, 9·4	9·2	9·1, 9·4
Protein	15·6	15·4, 15·9	15·4	15·2, 15·6	15·6	15·4, 15·8
Carbohydrates	43·2	42·7, 43·8	43·3	42·7, 43·8	43·1	42·6, 43·7
Cholesterol (mg/d)	246	236, 256	247	238, 257	242	232, 252
Fibre (g/d)	26·0	25·3, 26·8	26·7	26·0, 27·5	26·3	25·5, 27·0

Mean value was significantly different from that for soyabean spread: * $P < 0.05$.
† Control spread, not enriched with plant sterols.
‡ Enriched with 31 g soyabean-oil sterols/kg.
§ Enriched with 133 g sheanut-oil sterols/kg.

significant differences in average body weight between the groups. No important deviations from lifestyle, dietary or activity pattern occurred during the study.

Blood variables

Table 4 shows the plasma lipid, α- plus β-carotene, and lycopene concentrations based on the 3-week sampling result. There was no interaction effect between spread group and sex. The soyabean-oil-sterol spread lowered plasma total- and LDL-cholesterol levels significantly compared with Flora without an effect on plasma HDL-cholesterol levels. Consequently the ratio LDL : HDL-cholesterol was also statistically significantly reduced. The sheanut-oil-sterol spread did not statistically significantly change blood lipid levels compared with Flora. Fig. 1 shows the individual changes in plasma LDL-cholesterol concentrations after consumption of the soyabean-oil-sterol spread and the sheanut-oil-sterol spread compared with the control spread (Flora). In most subjects the soyabean-oil-sterol spread caused a decrease in plasma LDL-cholesterol concentrations, however, some subjects showed a small increase. Two subjects showed large increases in plasma total- and LDL-cholesterol levels after consumption of the soyabean-oil-sterol spread, probably due to chance. The sheanut-oil-sterol spread caused an increase in plasma LDL-cholesterol concentrations in more subjects. Plasma triacylglycerol levels were not affected by the spreads. The soyabean-oil-sterol spread caused a statistically significant reduction in plasma lycopene concentration but not in plasma α- plus β-carotene level. Standardization of the plasma lycopene concentration for decreased lipid did diminish the lowering effect of soyabean-oil-sterol spread on blood lycopene level, but the effect still remained statistically significant. All values of the blood variables during the study were within the normal ranges.

Discussion

This double-blind crossover study in healthy adults showed that daily consumption of 25 g of a spread enriched with free soyabean-oil sterols (31 g sterol equivalents/kg; 0·8 g/d),

Table 4. Plasma lipid, α- plus β-carotene and lycopene concentrations in volunteers consuming a control spread (Flora) or plant-sterol-enriched spreads (25 g/d) for 3 weeks†

(Mean values and 95% confidence intervals for seventy-five subjects)

	Flora		Soyabean‡		Sheanut§	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Total cholesterol (TC) (mmol/l)	5·06	4·99, 5·13	4·87*	4·80, 4·94	5·01	4·94, 5·08
LDL-cholesterol (mmol/l)	3·14	3·07, 3·20	2·95*	2·89, 3·01	3·08‖	3·01, 3·14
HDL-cholesterol (mmol/l)	1·28	1·26, 1·30	1·30	1·27, 1·32	1·29‖	1·27, 1·31
LDL- : HDL-cholesterol	2·45	2·39, 2·51	2·26*	2·23, 2·33	2·39‖	2·33, 2·44
Triacylglycerol (TAG) (mmol/l)	1·20	1·15, 1·25	1·14	1·09, 1·20	1·20	1·15, 1·25
α- + β-Carotene (μg/l)	226	219, 234	217	210, 224	ND	
Lycopene (μg/l)	130	122, 138	113*	106, 120	ND	
α- + β-Carotene/(TC + TAG) (μg/mmol)	35·8	34·8, 36·8	35·7	34·7, 36·8	ND	

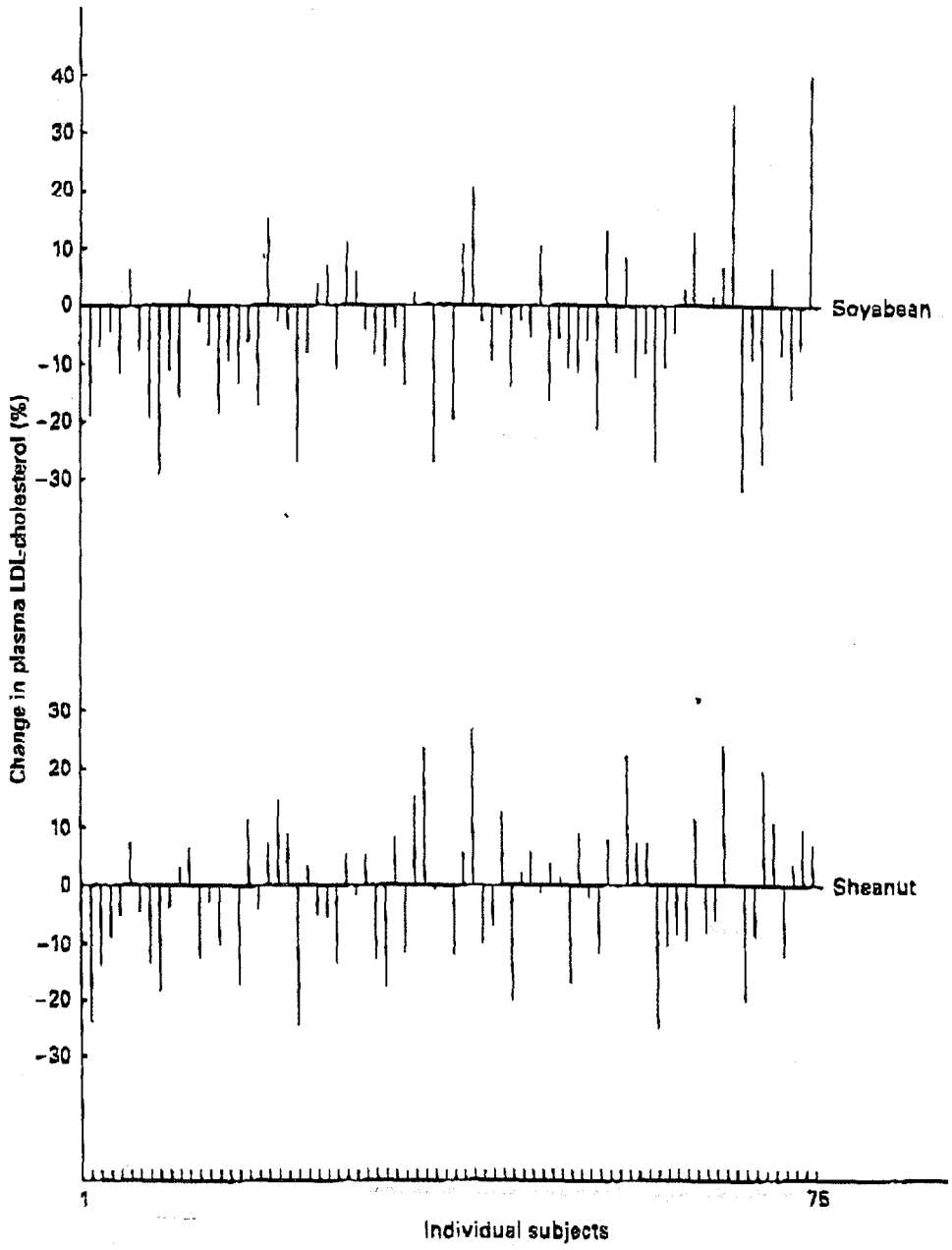

Fig. 1. Changes (%) in the plasma LDL-cholesterol levels of individual subjects after consumption of spreads enriched with soyabean-oil sterols or sheanut-oil sterols (25 g/d for 3 weeks) compared with a control spread (Flora). For details of spreads and procedures, see Table 2 and pp. 274–276.

lowered plasma total- and LDL-cholesterol concentrations respectively by 3·8 % and 6 % (both 0·19 mmol/l) compared with a spread with similar fatty acid content not enriched with these sterols. No effect on plasma HDL-cholesterol or triacylglycerol concentrations was found. A similar study with somewhat younger subjects showed reductions in plasma total- and LDL-cholesterol concentrations of respectively 4·9 % (0·26 mmol/l) and 6·7 % (0·20 mmol/l) after consumption of a spread enriched with esterified soyabean sterols at a similar intake level of 0·8 g/d (Hendriks *et al.* 1999). This indicates that, as sterols need to be in a free state to inhibit cholesterol absorption (Peterson *et al.* 1953; Swell *et al.* 1956; Best & Duncan, 1958), intestinal hydrolysis of

limiting factor, at this level of plant sterol intake, in determining the efficacy of plant sterols to lower blood cholesterol levels. Esterification of sterols is necessary to increase the solubility of sterols in fat (Jandacek *et al.* 1977). In our previous study (Weststrate & Meijer, 1998) a four-fold higher intake of esterified soyabean-oil sterols (3·3 g/d) caused a two-fold higher reduction in plasma total- and LDL-cholesterol concentrations compared with this study, respectively 8·3 % (0·43 mmol/l) and 13 % (0·44 mmol/l). This indicates that there is a non-linear relationship between plant sterol intake and blood cholesterol level reduction, which has also been found by other investigators (Lees *et al.* 1977).

As in our previous study (Weststrate & Meijer, 1998) the

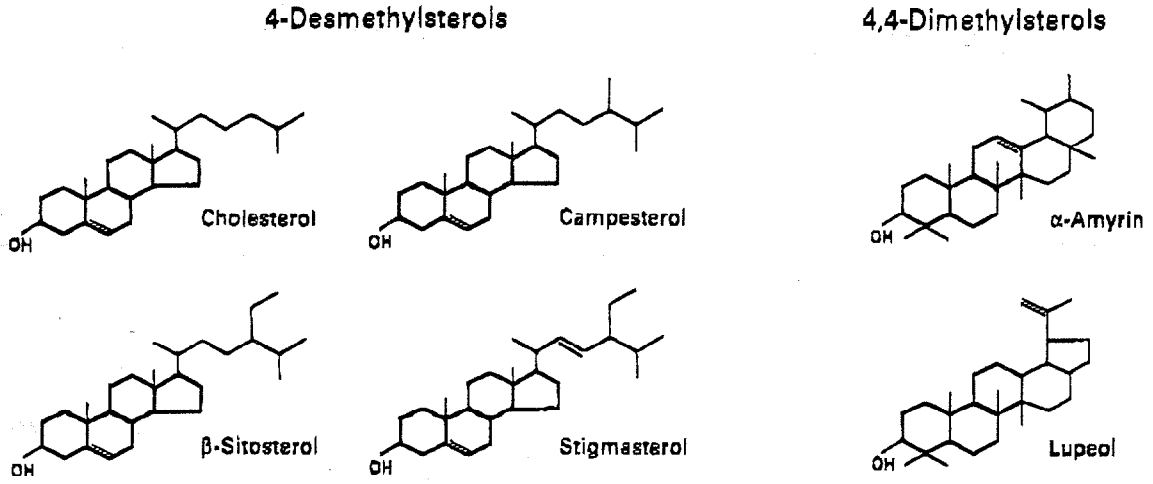

Fig. 2. Structure of 4-desmethylsterols and 4,4-dimethylsterols.

sheanut-oil-sterol spread was not effective in lowering blood cholesterol. In the current study, differences in fatty acid composition between control and sheanut-oil-sterol spreads did not confound the outcome as they may have done in our previous study (Weststrate & Meijer, 1998). The sheanut-oil sterols are primarily phenolic acid esters of 4,4-dimethylsterols, whereas the soyabean-oil product contained 4-desmethylsterols. The structure of 4-desmethylsterols is more similar to cholesterol than the structure of 4,4-dimethylsterols (Fig. 2). This may offer more opportunity for competition with cholesterol for incorporation in mixed micelles, the most likely mechanism for the blood-cholesterol-lowering action of sterols (Ling & Jones, 1995). This study indicates that sheanut-oil sterols will not be efficacious in lowering plasma cholesterol levels. The present study was not a strictly controlled feeding trial and changes in consumers behaviour may have affected the outcome. However, the study was double-blinded and compliance with the instructions to consume the spreads appeared to be excellent. Moreover nutrient and energy intakes, including those of fatty acids, cholesterol and fibre as well as body weights were very similar for the different spread groups. We believe, therefore, that the results of this trial are valid. Spread consumption was very close to the recommended use level of 25 g/d. Only two people (2·6 %) dropped out, both for reasons not related to consumption of the spread. The number of volunteers consuming a given spread was similar for all products (seventy-five). The habitual dietary intake of fatty acids, fibre and cholesterol, which may affect blood lipid levels, was not statistically significantly different between the spread groups, except for a small and irrelevant difference in energy percentage of saturated fatty acids between the soyabean-oil-sterol spread and the sheanut-oil-sterol spread (< 1 % energy).

In two earlier studies esterified plant sterols at an intake level of approximately 3 g/d reduced plasma lipid standardized concentrations of α- plus β-carotene approximately 15–20 % and of lycopene approximately 10–15 % (Weststrate & Meijer, 1998; Hendriks *et al.* 1999), whereas no reduction was observed for plasma-lipid-standardized vitamin E. Gylling *et al.* (1996) also showed an effect at a similar intake level of sitostanol ester on plasma α- and β-carotene levels. The three-fold lower level of non-esterified plant sterols used in the present study did not lead to reduction of plasma lipid-standardized concentrations of α- plus β-carotene. However, the lipid standardized plasma lycopene levels were statistically significantly reduced by 9·5 %. This reduction was not statistically significant at a similar intake level of esterified plant sterols (Hendriks *et al.* 1999). However, in the latter study a small significant effect on plasma α- and β-carotene was found. Thus, lipid-standardized plasma carotenoid levels are not or slightly affected by esterified or non-esterified plant sterols intakes of up to 1 g/d.

The clinical importance of a lowering of plasma levels of carotenoids is difficult to ascertain. Carotenoids may have positive effects on health as indicated primarily by epidemiological studies (Mayne, 1996). The role of carotenoids, other than their provitamin A activity, is however, not established with reasonable certainty. It is currently not possible to determine a minimum plasma carotenoid level, for carotenoids as a group or for individual carotenoids, below which it is conclusively established that health risks or benefits would change, neither is it possible to set limits for reductions in plasma carotenoid levels, either for carotenoids as a group or for individual carotenoids. It seems, however, prudent to aim, if at all possible, for small or negligible long-term reductions of blood carotenoid levels in consumers of sterol-enriched products. Giovannucci *et al.* (1995) showed that the relative risk for prostate cancer was about 20 % lower in men consuming relatively high amounts of lycopene compared with those with lower intakes. The mean difference in intake between the high and low quintile was about 9 mg/d. There is no information on the dose–response relationship between increased dietary lycopene intakes and changes in plasma lycopene levels. A study by Yeum *et al.* (1996) indicates that a controlled diet containing 3·3 mg lycopene/d increased plasma lycopene levels in US men and women compared with baseline by about 60 %. No baseline lycopene intake data were, however, given. Baseline lycopene intake in US men and women may be estimated to be about 2 mg/d (Nebeling *et al.*

1997). This suggests that a difference of more than 1 mg/d lycopene intake will correspond to a difference of plasma lycopene levels of about 60 %. In the present study we observed a reduction of 13 % in unstandardized plasma lycopene levels. We hypothesize that this difference in plasma lycopene levels may be expected to correspond to a difference in dietary lycopene intake of about 0·2 mg/d. The study of Giovanucci *et al.* (1995) showed that a difference in intake of about 9 mg/lycopene/d was associated with a change in relative risk for prostate cancer of about 20 %, or about a 2 % change in relative risk per 1 mg/d change in dietary lycopene intake. Even when we assume that the results of this study indicate a causal relationship between reduced lycopene intake and increased risk of prostate cancer (which is not proven) the decrease in plasma lycopene levels observed in our present study translates to a negligible change in relative risk for prostate cancer.

Because of the within-person variations in plasma total- and LDL-cholesterol levels, which are up to about 10 % (analytical variation excluded) (Smith *et al.* 1993), not all individuals showed a reduction in plasma total- and LDL-cholesterol concentrations after consumption of the spread enriched with free soyabean-oil sterols (which caused average reductions in plasma total- and LDL-cholesterol levels of respectively 3·8 % and 6 %, smaller than the within-person variations for plasma total- and LDL-cholesterol levels). These increases in plasma total- and LDL-cholesterol concentrations are thus related to chance. If more measurements had been done per individual to establish his or her 'true' blood lipid levels, the chance of observing increases would be minimal and more accurate individual values of plasma total- and LDL-cholesterol concentrations would have been obtained. Plant sterols have been part of the human diet for a very long time and for vegetarians, for example, plant sterol intake can be substantial, e.g. 500 mg/d (Ling & Jones, 1995), which is in contrast to the intake of plant stanols. The introduction of plant-sterol-enriched products would in itself not introduce new compounds into the diet: it might, however, cause an increase in the exposure to plant sterols in consumers of such products. The possible health risks associated with such an introduction need to be carefully assessed. Current evidence indicates that plant sterols are unlikely to raise severe concerns regarding possible human health risks. Many human studies with dose levels up to 25 g/plant sterols/d for up to several months have been performed without adverse effects (reviewed by Pollak & Kritschevsky, 1981). The only undesirable effect observed have been occasional gastrointestinal disturbances, e.g. constipation (Lees *et al.* 1977; Kane & Malloy, 1982) and diarrhoea, which are more related to the vehicle (large volumes of vegetable oils) used to administer the plant sterols. The only reported adverse effects of plant sterols have been in individuals with phytosterolaemia. Phytosterolaemia is a very rare inherited lipid storage disease, affecting a few individuals per million, in which a high proportion of plant sterols is absorbed from the intestine (Salen *et al.* 1992). The condition has a high risk of premature atherosclerosis. Individuals with this condition should avoid the consumption of plant sterols. However, for the general population this increased absorption of plant sterols does not occur, rather the plant sterols are poorly absorbed and, if absorbed, rapidly cleared (Ling & Jones, 1995). This is well illustrated by the results of a previous study from our group where an intake of about 3·3 g/d of esterified vegetable oil plant sterols from a spread *v.* an intake of about 100 mg/d of plant sterols from a control spread raised plasma levels of sitosterol from 3·3 mg/l to 4·6 mg/l and of campesterol from 7 mg/l to 12·1 mg/l (Weststrate & Meijer, 1998). Thus, in spite of significant increases in plant sterol intake i.e. of more than a factor of 30, the increase in plant sterol plasma levels was less than a factor of 2. We have, in addition to the present study, performed a number of studies with high dose levels of plant sterols addressing specific toxicological and physiological issues. These studies have not shown evidence of oestrogenic, toxic or adverse effects of plant sterols on bile acid and neutral sterol excretion or the gut microflora (Ayesh *et al.* 1999; Baker *et al.* 1999; Hepburn *et al.* 1999; Weststrate *et al.* 1999).

We estimate, based on a meta-analysis by Law *et al.* (1994) that the observed reductions in total and LDL-cholesterol concentrations for the soyabean-oil sterol spread could help in reducing CHD risk by about 15 % at age 40 and 6 % at age 70. According to a study of Stampfer *et al.* (1991) the predicted reduction in risk for CHD would be about 10 %.

We conclude that this study shows that consumption of a spread enriched with a low concentration of soyabean-oil sterols in unesterified form is effective in reducing mean total- and HDL-cholesterol levels in free-living subjects by about 4 and 6 % respectively. These decreases are similar to what may be expected from free-living subjects adhering to prudent diets (Tang *et al.* 1998). We have also shown that these decreases occur with limited effects on plasma levels of carotenoids. It would be of public health importance if part of the fat or water in edible fat-containing products could be replaced by plant sterols. This would be predicted to help in reducing the risk of cardiovascular disease in the population.

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Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects

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Objectives: To compare effects on plasma total-, LDL-, and HDL-cholesterol concentrations of margarines enriched with different vegetable oil sterols or sitostanol-ester.
Design: A randomized double-blind placebo-controlled balanced incomplete Latin square design with five treatments and four periods of 3.5 weeks. Margarines enriched with sterols from soybean, sheanut or ricebran oil or with sitostanol-ester were compared to a non-enriched control margarine. Sterol intake was between 1.5–3.3 g/d. Two thirds of the soybean oil sterols were esterified to fatty acids.
Setting: Unilever Research Laboratory, Vlaardingen, The Netherlands.
Subjects: One hundred healthy non-obese normocholesterolaemic and mildly hypercholesterolaemic volunteers aged 45 ± 12.8 y, with plasma total cholesterol levels below 8 mmol/L at entry.
Main outcome measures: Plasma lipid, carotenoid and sterol concentrations, blood clinical chemistry and haematology, fatty acid composition of plasma cholesterylesters and food intake.
Results: Ninety-five volunteers completed the study. None of the margarines induced adverse changes in blood clinical chemistry, serum total bile acids or haematology. Plasma total- and LDL-cholesterol concentrations were significantly reduced by 8–13% (0.37–0.44 mmol/L) compared to control for margarines enriched in soybean oil sterol-esters or sitostanol-ester. No effect on HDL-cholesterol concentrations occurred. The LDL- to HDL-cholesterol ratio was reduced by 0.37 and 0.33 units for these margarines, respectively. Effects on blood lipids did not differ between normocholesterolaemic and mildly hypercholesterolaemic subjects. Plasma sitosterol and campesterol levels were significantly higher for the soybean oil sterol margarine and significantly lower for the sitostanol-ester margarine compared to control. Dietary intake was very similar across treatments. The fatty acid composition of plasma cholesterylesters confirmed the good compliance to the treatment. All sterol enriched margarines reduced lipid-standardized plasma α- plus β-carotene levels. Plasma lycopene levels were also reduced but this effect was not significant for all products.
Conclusions: A margarine with sterol-esters from soybean oil, mainly esters from sitosterol, campesterol and stigmasterol, is as effective as a margarine with sitostanol-ester in lowering blood total- and LDL-cholesterol levels without affecting HDL-cholesterol concentrations. Incorporation in edible fat containing products of such substances may substantially reduce the risk of cardiovascular disease in the population.
Sponsorship: Unilever Research.

Introduction

It is well established that linoleic acid lowers blood cholesterol concentrations compared to other more saturated types of fatty acids or carbohydrates (Keys *et al*, 1965; Mensink & Katan, 1992). In addition to linoleic acid, other fat-soluble compounds may also lower blood cholesterol levels. Since the 1950s it has been known that plant sterols, or phytosterols, may reduce blood cholesterol levels (Pollak & Kritchevsky, 1981). Plant sterols are naturally occurring plant compounds. The most important dietary sources are vegetable oils and vegetable oil-based products such as margarines. The most predominant plant sterols are sitosterol, campesterol and stigmasterol. Their intake is estimated to be under 500 mg/d. Plant sterols have structural similarity to cholesterol, but are not synthesized by the human body. The exact mechanism for cholesterol lowering by plant sterols is not fully understood, but they appear to inhibit the uptake of dietary and biliary cholesterol from the gut (Ling & Jones, 1995).

Recently Miettinen *et al* reported that long term consumption of a margarine with sitostanol-ester, derived by hydrogenation of sitosterol from pine tree woodpulp followed by esterification of sitostanol to fatty acids, substantially reduced serum LDL-cholesterol concentrations in mildly hypercholesterolaemic subjects (Miettinen *et al*, 1995). A much shorter trial in humans also showed efficacy of sitostanol-ester to lower blood cholesterol levels (Heinemann *et al*, 1986). Miettinen *et al* suggested that substitution of sitostanol-ester margarine for a portion of normal dietary fat is suitable as a strategy to reduce plasma cholesterol levels in the population.

In nature plant sterols occur primarily in the unhydrogenated free form, which may in part be esterified to fatty acids, sugar moieties or phenolic acids. Sterols with different chemical structures may vary in potential to reduce plasma cholesterol levels. The purpose of the present study was to assess in normocholesterolaemic and mildly

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hypercholesterolaemic subjects the efficacy to lower plasma total- and LDL-cholesterol concentrations of margarines enriched with sterols derived from various oils, namely soybean oil, ricebran oil or sheanut oil. We compared these margarines to a non-enriched margarine and a margarine fortified with sitostanol-ester, which has reported cholesterol lowering action (Miettinen *et al*, 1995). Soybean oil contains sterols of the 4-desmethylsterol class, namely mainly sitosterol, campesterol and stigmasterol. These sterols were esterified to fatty acids from sunflowerseed oil to enhance their solubility in margarine, but they were not hydrogenated as the sterols present in the margarine used by Miettinen *et al*, 1995. The esterification of the free sterols enabled us to incorporate relatively large amounts of sterols into the margarine without affecting its physical and sensory properties. Ricebran oil and sheanut oil contain methylated sterols of 4,4′-dimethylsterol class, which may be in the free form or esterified to phenolic acids or fatty acids. A number of studies have suggested that the reported cholesterol lowering action of ricebran oil could be due to its sterol content (Seetharamaiah & Chandrasekhara, 1989). A study comparing the efficacy in humans of sterols derived from sheanut oil or ricebran oil added to margarines to lower blood cholesterol levels has not yet been performed.

We assessed the effects of margarine consumption on plasma lipids, routine blood chemistry and haematology, plasma α- plus β-carotene and lycopene levels to determine effects of sterols on the absorption of lipophilic nutrients, plasma phytosterol levels to obtain information on the bioavailability of the sterols, on the fatty acid composition of plasma cholesterylesters to validate the dietary intake assessment method and on habitual energy, total fat, fatty acid and cholesterol intake.

Subjects and methods

Subjects

We recruited the volunteers by advertisements in local newspapers and the weekly periodical of the laboratory. A total of 208 subjects expressed their interest in the study. A total of 141 volunteers met our eligibility criteria for a blood screening. Eligible subjects had not donated blood within one month prior to start of the study, reported to use spreads habitually, were willing to comply with the experimental procedures, did not or would not participate in another biomedical trial, were between 18 and 65 y of age, had body mass index between 19 and 30 kg/m^2, reported to be not on a slimming, medically prescribed, vegetarian or vegan diet, reported not to receive medical treatment or having a current disease or history of metabolic diseases, chronic gastrointestinal disorder, cardiovascular disease, high blood pressure or high blood cholesterol. Eligible subjects reported not to use medicines except analgesics or oral contraceptives. Eligible subjects reported intense exercise below 10 h/week and consumption of alcoholic beverages below 21 units/week when female and below 28 units/week when male. Females were in a reported non-pregnant or non-lactating state. Blood was sampled from 136 potential volunteers. Five subjects were excluded on the basis of blood lipid or haematological variable. For practical reasons 100 volunteers was the maximum for this study, we therefore excluded another thirty-one volunteers by lot. Volunteers had at entry plasma total cholesterol levels below 8 mmol/L and normal plasma activities of L-γ-glutamyltransferase, alanine aminotransferase and aspartate aminotransferase. White blood cell, red blood cell and platelet counts, haemoglobin concentration and haematocrit were all within the normal range. Reported bowel frequency was at least once per 48 h. All volunteers gave their written informed consent for participation prior to the start of the study. Volunteers received 250 DFl after completion of the trial.

The volunteers were requested to retain their normal lifestyle and dietary pattern. During the whole study the volunteers answered repeatedly (12 times) a structured questionnaire on important deviations in lifestyle as well as on intercurrent illnesses and medicine use. After each period the questionnaire was collected and checked for completeness by the dieticians. Volunteers were not allowed to donate blood except for the study and simultaneous participation in another biomedical trial was prohibited.

Experimental design

The study had a randomized double-blind placebo-controlled balanced incomplete Latin square design with five treatments and four periods. Subjects received in four consecutive periods of 24 or 25 d (3.5 weeks) 30 g/d of a margarine in a coded tub for consumption at lunch and dinner. The spread was meant to replace the spreads habitually used by the volunteers. Five types of margarines were investigated. The statistician allocated each person to one of the treatments for each period such that the design was balanced. The tubs with margarine were labelled before the start of the trial with a blind code so that volunteers nor dieticians knew what products were consumed.

Fasting blood was sampled after 2.5 and 3.5 weeks. Body weight was measured after 3.5 weeks. In each period, dietary intake was assessed with a food frequency method. Compliance and health status, medicine use and deviations from the normal lifestyle were registered by questionnaire. A short run-in period of 5 d in which the volunteers familiarized themselves with the procedures preceded the actual trial. The study was designed to have a power of 90% with a confidence of 95% to assess differences in total cholesterol levels between treatments of 3–5%, based on an expected average plasma total cholesterol concentration of 5 mmol/L and an expected within-person variation in plasma total cholesterol level of 0.35 mmol/L (Personal communication, P Zock, 1996, Wageningen Agricultural University). The study set-up was approved by the Medical and Ethical Committee of Unilever Nederland BV.

Test and control margarines

The test margarines were specially prepared spreads (VandenBergh Foods, Purfleet, UK, and Unilever Research Laboratory, Vlaardingen, The Netherlands), packed in blinded tubs and labelled with a colour code for each period of 3.5 weeks. The margarines were fortified with phytosterol (-ester) concentrates derived from either predominantly soybean oil distillates (Henkel Corporation, LaGrange, USA), rice bran oil (Tsuno, Wakayama, Japan) or sheanut oil (Loders Croklaan, Wormerveer, The Netherlands) concentrates. The soybean oil sterols were esterified with fatty acids from sunflowerseed oil to an esterification degree of 65% (Unilever Research Laboratory, Vlaardingen, The Netherlands). The ricebran oil sterols contained only sterols esterified to ferulic acid (oryzánol). The sheanut oil sterols were a mixture of sterols esterified to primarily cinnamic acid (69%), acetic acid

(25%) and fatty acids (4%). The sterol concentrates were refined and used in spread production together with other edible oils and fats (sunflowerseed oil, rapeseed oil and hardstock) in order to achieve a product as close as possible in fatty acid composition as the nonfortified control. Final free phytosterol concentration was between 5.5–11% in the different products. For technical reasons we could not increase the level of sterols derived from ricebran oil to the level obtained for the shea and soybean products. We were also not able to match the fatty acid composition of the sheanut sterol spread exactly to the control spread as the sheanut oil sterol concentrate was not available in a sufficiently high concentration. As control articles we used 'Benecol' (Raisio Inc, Finland), which is fortified mainly with sitostanol-ester, and 'Flora' (VandenBergh Foods, Crawley, UK), a spread not fortified with sterols. Total fat content, fatty acid composition, free sterols, carotenoids and tocopherol content of margarines were measured. For determination of the fat content samples of margarine were mixed with celite, cooled to $-20°C$ and freeze-dried at 10°C. Fat was extracted with dichloromethane, after evaporation, the leftover was weighed. The triglyceride content was calculated by subtracting the total sterol/sterolester content from the total fat content. The fatty acid composition was analysed by methanolysis of the fatty acids in a sample of margarine extracted with a methanolic NaOH solution using BF_3 as catalyst. The methyl esters were extracted with heptane and analysed with a gaschromatograph (Hewlett Packard, Amstelveen, The Netherlands) using a CP-Sil 88 column (Chrompack, Middelburg, The Netherlands). Analysis of sterol types was done by saponifying the margarine with KOH and extracting the unsaponifiable part into cyclohexane. Thin-film chromatography was used for separating the 4-desmethylsterol, the 4-monomethylsterol and the 4,4-dimethylsterol fractions with β-cholestane as internal standard. The fractions were extracted with a mix of chloroform, diethylether and ethanol. After silylizing with pyridine and BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) this fraction was analysed gaschromatographically (Fisions Instruments, Milan, Italy) by means of a non-polar capillary column (CP-Sil 19 CB, Chrompack, Middelburg, The Netherlands) using flame ionisation as detection. α-Tocopherol was analysed by a straight-phase HPLC method. The sample of margarine was separated chromatographically (Separations, HI Ambacht, The Netherlands) on a HPLC silica column. Elution was carried out with 0.5% isopropyl alcohol in hexane at a flow rate of 1 ml/min. α-Tocopherol was detected by means of fluorescence detection, excitation wavelength at 290 nm and emission wavelength at 330 nm (Hewlett Packard, Amstelveen, The Netherlands). For the carotenoid analysis a sample of margarine of about 100 mg was dissolved in 5 ml diethylether. One gram of waterfree sodiumcarbonate was added before vortexing. The mixture was centrifuged for 5 min at 2000 rpm. The extract was dried under nitrogen and dissolved in 1 ml of *n*-heptane containing the internal standard, ethyl-β-apo-8-carotenoate. The analysis was performed with an ET 200/8/4 nucleosil 5-N$(CH_3)_2$ column (Marchery & Nagel, Dueren, Germany). N-heptane was used as mobile phase at a flow rate of 0.5 ml/min and a column temperature of 15°C. Carotenoids were detected at 450 nm.

The homogeneity of sterols and their content in the margarines was analysed prior to the start by analysing and comparing samples taken at three different times during the spread production process. Coefficients of variation in the content of the major sterols for these samples were generally less than 5%. This indicates a good homogeneity of

Table 1 Analysed composition of spreads used in the sterol enriched margarine study

	Flora[a]	*Benecol*[b]	*Soybean*	*Ricebran*	*Sheanut*
Total fat as glycerides (g/100 g)	70	72	73	72	71
Water (g/100 g)	30	19	16	22	21
Major fatty acids[c] (g/100 g)					
Lauric (C12:0)	1.6	2.2	1.2	1.1	2.1
Myristic (C14:0)	0.7	1.1	0.6	0.5	0.9
Palmitic (C16:0)	9.3	14.2	8.7	8.2	10.9
Stearic (C18:0)	2.7	2.3	3.0	3.0	15.4
Oleic (C18:1 cis)	20.2	39.2	24.6	24.7	32.3
Linoleic (C18:2, 9c, 12c)	32.1	13.4	38.4	37.2	17.8
Linolenic (C18:3 9c, 12c, 15c)	1.5	5.5	1.8	1.6	0.3
Total sterols (g/100 g)	0.36	9.12	10.82	5.58	10.15
Major sterols (g/100 g)					
Brassicasterol	0.01	0	0	0	0
Campesterol	0.06	0.20	2.70	0.78	0.01
Campestanol		0.90	0.13	0.07	
Stigmasterol	0.01	0	1.89	0.07	0.01
Sitosterol	0.16	0.38	5.03	0.50	0.05
Sitostanol		7.45	0.17	0.37	0.14
Cycloartenol	0.01		0.02	1.57	0
24 meth. cycloartenol			0.01	2.07	0
beta-amyrin	0	0	0	0	0.58
butyrospermol	0	0	0	0	1.81
alpha-amyrin	0	0	0	0	3.25
lupeol	0	0	0	0	1.52
other shea sterols	0	0	0	0	2.31
Vitamin E (mg/100 g)	39	12	68	15	36
α- + β-carotene (mg/100 g)	0.47	0.48	0.53	0.44	0.70

[a] Control: not enriched in sterols.
[b] Containing sitostanol-ester.
[c] Total trans fatty acid content of spreads was less than 1 g/100 g.

the sterols in the margarines (data not shown). The analysed margarine composition is given in Table 1. Differences, as expected, occurred in sterol content. The sitostanol-ester product (Benecol) and the sheanut margarine contained more saturated and monounsaturated fatty acids and less linolcic acid than the other margarines. The sitostanol-ester product contained more α-linoleic acid than the other margarines. The margarines were tested for acceptability prior to the start of the study and during each period.

The margarines were intended to replace an equivalent amount of the spread(s) habitually used by the volunteers, but they were not to be used for cooking or frying. Half of the amount of margarine was intended for consumption at lunch, the other half was for consumption at dinner, for example by melting the spread over vegetables or potatoes, rice etc, or by mixing the spread in a soup or by spreading the margarine on bread or toast eaten at dinner. Compliance was checked by the dieticians using regular telephone contacts with the volunteers, by weighing the total number of tubs before and after each experimental period, by interviewing the volunteers about their use of the margarines at the end of each 3.5 week period, by evaluation of a volunteer-completed questionnaire on the use of the spread and also from analysis of the fatty acid composition of plasma cholesterylesters. Volunteers recorded daily whether they had consumed all of the spread at particular meal times. Margarines were provided in lots of 30 tubs per period; volunteers were requested to store them in the refrigerator until use. At the end of each period remaining tubs were returned to the dieticians.

Blood lipids, routine blood chemistry and haematology

At screening for the study and at 2.5 and 3.5 weeks in each period of the study blood was obtained by veinpuncture from an antecubital vein from volunteers that were at least 10 h in a fasting state. Plasma and serum were prepared by centrifuging blood for 10 min at 3000 g. All parameters were determined using commercial test kits on a Cobas Mira S analyser (Roche, Basle, Switzerland). Total cholesterol and total glycerol concentrations were determined by enzymatic methods after storage at −80°C. HDL-cholesterol was measured as described for total cholesterol after precipitation of VLDL-cholesterol and LDL-cholesterol with phosphotungstate and magnesium. LDL-cholesterol was calculated using the Friedewald formula (Friedewald *et al*, 1972). Within-run coefficient of variation for total cholesterol, HDL-cholesterol and total glycerol were below 2.5%.

We also performed routine blood chemistry in the 3.5 week blood samples to assess possible adverse effects of spread consumption. In plasma, using commercial testkits, GGT (L-γ-glutamyltransferase, EC 2.3.2.2), ALT (alanine aminotransferase, EC 2.6.1.2) and AST (aspartate aminotransferase, EC 2.6.1.1) were determined. ALP (alkaline phosphatase, EC 3.1.3.1) activity was measured in serum. Plasma albumin, glucose, urea and creatinine were also determined using commercial testkits. In serum we measured total bile acids using an enzymatic spectrophotometric 3α-hydroxy bile acid assay (Enzabile, Nycomed Pharma Oslo, Norway).

Haemoglobin concentration, white blood cell count, red blood cell count, platelet count and calculation of haematocrit were performed using a Sysmex microcell F-800 bloodcell counter (Toa Medical Electronics Co. Ltd., Kobe, Japan).

Plasma sterols, fatty acids of cholesterylesters and carotenoids

Plasma sterolesters were saponified with alkali and then extracted with *n*-heptane, 5-α-cholestane was added as internal standard. The extract was dried and the residue was silylated for analysis with gas chromatography. The analysis was performed in a random half of the samples for individuals that had consumed Flora, the soybean oil sterol margarine and the sitostanol-ester margarine ($n = 29$).

Fatty acids from plasma cholesterylesters were analysed in plasma obtained during the first seven weeks of the trial, namely in 50% of all samples. Lipid was extracted from plasma into dichloromethane (Sundler R *et al*, 1974). After removing the dichloromethane the mixture was dissolved into hexane:diethylether. The mixture was applied on a prepared silicacartridge and eluted with *n*-heptane:diethylether. Fatty acids were transesterified using methanol and acetyl chloride before gas chromatography.

Carotenoids were determined only in the 3.5 week blood samples after storage at −80°C. Carotenoids were measured to assess potential impact of the sterol containing spread on the absorption of lipophillic dietary compounds. From previous studies we knew that β-carotene and lycopene were more sensitive parameters than vitamin E to assess the impact of a largely non-absorbable lipophillic food component on the absorption of lipophillic dietary compounds (Weststrate & van het Hof, 1995). Plasma was extracted with *n*-heptane for determination of carotenoids. Ethyl-β-apo-8′-carotenoate was used as an internal standard. A normal phase Nucleosil 5N $(CH_3)_2$ column (Machery & Nagel, Dueren, Germany) was used with a flow of 1.4 ml/min using *n*-heptane/chloroform/2-propanol (1000/15/1.5 v/v) as an eluent. Carotenoids were detected at 450 nm as previously described (Weststrate & van het Hof, 1995). α-+β-Carotene and lycopene concentrations were quantified. Plasma carotenoid concentrations were standardised for plasma lipid (total cholesterol and total glycerol) levels as previously described (Weststrate & van het Hof, 1995), because some sterol enriched margarines significantly affected blood lipoprotein (carriers in the plasma of carotenoids) concentrations.

Dietary intake

Dietary intake was assessed by a modification of the Valivet method (Nutri-AKT, Rhenoy, The Netherlands). This method has been developed and validated by the Department of Human Nutrition of the Wageningen Agricultural University (Feunekes *et al*, 1993). The method is a food frequency method with 104 items specifically aimed at estimating fat intake. We modified the method for our purposes. Most importantly the number of margarines and oils was extended to cover the variation found in the study and we substituted more recent analytical data of fat composition of a variety of products for older data in the food composition database. Subjects received the method after 2.5 weeks in each 3.5 weeks period and answered the questions at the last day of each period. The dieticians then checked the questionnaires for completeness.

Statistical evaluation of treatment

Differences in plasma variables between treatments were evaluated by analysis of variance using the following model: response = gender, subject (within gender), period, diet, carry-over, diet *x* gender as factors. For blood lipids we included a factor time (2.5 weeks and 3.5 weeks) as repeated measures. The carry-over effect was removed

from the model as it was not significant. When the interaction between gender and diet was significant (only for carotenes), the analysis was performed for males and females, separately. When this interaction term was not significant it was removed from the model. Blood lipid and carotenoid data were lognormalized before analysis. Differences between treatments were assessed using the Tukey's multiple comparison test (Miller, 1981). Tukey's test adjusts the *P*-value for all possible comparisons. A *P*-value of 0.05 was considered significant. The presented means are least squares means. The means with 95% confidence interval of the log transformed data are back transformed to the original scale in order to facilitate interpretation. Back transformation of the confidence interval of difference between treatments of the log transformed data results in a minimum significant ratio, which can be expressed as a percentage (see last column in Tables 2, 3 and 4). For reasons of comparison the untransformed data, namely on dietary intake and fatty acid composition of serum cholesterylesters, is presented in a similar way. For untransformed data the minimum significant difference is an absolute value (see last column, Tables 5 and 6). The confidence interval of a difference between treatments is then equal to the difference between these treatments $\pm$ the minimun significant difference. SAS version 6.12 was used for the analysis (Statistical Analysis System Institute, 1987). Descriptive statistics are given as mean $\pm$ s.d. unless indicated otherwise.

Results

General

The study started as intended with 100 adult volunteers, 50 males and 50 females, with a mean age of 45 ± 12.8 y and a mean body mass index of 24.2 ± 2.16 kg m^{-2}. Baseline total-, LDL- and HDL-cholesterol levels were 5.35 ± 1.06, 3.54 ± 0.97 and 1.26 ± 0.35 mmol/L, respectively. There were four dropouts in the first 3.5 weeks due to illnesses and associated medicine use not related to the treatment, one volunteer was withdrawn in the third period because of a reported pregnant state. Body weight did not differ significantly between the various treatments. Average weight varied in men from 82.2 kg for the sheanut sterol spread to 82.5 kg for the ricebran sterol spread and in women from 66.7 kg for Flora to 66.8 kg for the sitostanol-ester margarine (Benecol) and the ricebran sterol product. During the study no changes were reported in the type of diet consumed. There were only minor changes reported in lifestyle: between 1 and 3 subjects reported changes in smoking behaviour and between 5 and 12 subjects reported changes in physical activity (more or less exercise) during a given treatment period. There was no treatment-related effect on routine blood chemistry, serum total bile acids and complete blood counts (data not shown). Serum total bile acids were 3.04, 3.37, 3.08, 3.01 and 3.71 µmol/L for control, sitostanol-ester, soybean oil sterol, ricebran sterol and sheanut sterol margarines, respectively, with a minimum significant difference between treatments of 0.85 µmol/L.

Compliance with the treatment was excellent. Average spread consumption varied between a minimum of 29.8 g/d for the ricebran sterol spread to a maximum of 30.4 g/d for the sheanut sterol spread with coefficients of variation varying between 3.3 (Flora) to 4.4% for the sitostanol-ester margarine. The percentage of days on which not all of a spread was eaten was 0.6% for the sitostanol ester margarine, 0.6% for the ricebran sterol spread, 0.7% for Flora, 1% for the sheanut sterol and 1.3% for the soybean sterol margarines. The very large majority of margarines were used as intended at lunch and dinner, overall for each product in more than 99.2% of all meal occurrences.

Plasma lipids

Table 2 shows blood lipid data based on the average of the 2.5 and 3.5 week sampling results, including the minimal significant difference with 95% confidence. There was no significant difference in plasma total cholesterol concentrations between 2.5 and 3.5 weeks, however, plasma total glycerol, LDL- and HDL-cholesterol concentrations at 2.5 weeks differed significantly from the concentrations at 3.5 weeks (data not shown). On average HDL-cholesterol and triglycerides were slightly higher after 2.5 weeks compared to 3.5 weeks, in contrast to LDL-cholesterol concentrations. These differences were, however, very small and not affected by the treatment. Therefore in the analyses the mean of the 2.5 and 3.5 weeks results is used. There was a significant period effect for LDL-, HDL-cholesterol and total glycerol concentrations. Generally the mean concentrations increased slightly during the trial. This effect was in particular present for the 2.5 week values. Also this period effect was not interacting with treatment. Benecol (sitostanol-ester) and the soybean spread significantly lowered total- and LDL-cholesterol concentrations by about 0.37–0.44 mmol/L compared to Flora without an effect on HDL-cholesterol level. Consequently the total- or LDL-cholesterol to HDL-cholesterol ratio was significantly reduced for Benecol and the soybean sterol product. The sheanut or ricebran sterol margarines did not differ in blood lipids compared to Flora. Total glycerol levels were not affected by the treatment. There was no significant difference between males and females in treatment effect.

Table 3 shows the treatment effects for tertiles based on the study entry values for total- or LDL-cholesterol concentrations. It is clear that responses expressed in percentage change compared to Flora are very similar in the mildly hypercholesterolaemic subjects, that is, in those with a high starting plasma total- or LDL-cholesterol level and the normocholesterolaemic subjects, that is, those with lower starting total- or LDL-cholesterol concentrations.

Plasma carotenoids

Table 4 shows blood carotenoid levels after 3.5 weeks on treatment or control, as measured and after standardisation for concentrations of plasma lipids. There was a significant period effect, plasma α- + β-carotene levels decreased from a concentration of 220 µg/L in the first period to 168 µg/L in the fourth period. Similarly plasma lycopene concentrations decreased from 85 µg/L–63 µg/L. This period effect was not affected by treatment. All sterol fortified margarines caused a lowering of blood carotenoid levels, but the decrease in lycopene concentration was
not significant for the ricebran sterol product. The decreases were greatest for the sheanut margarine. Lipid standardisation of the carotenoid concentrations diminished the lowering effect of Benecol and the soybean product with the decrease in lycopene not being statistically significant any longer for the soybean oil sterol margarine. There was an interaction between treatment and gender. The effect on α-plus β-carotene levels differed between

Table 2 Blood lipid concentrations in volunteers participating in the sterol enriched margarine study

	Flora[a]	*Benecol*[b]	*Soybean*	*Ricebran*	*Sheanut*	*Minimal significant difference* (%)
Total cholesterol (mmol/L)	5.18 (5.11–5.26)	4.81 (4.73–4.88; – 7.3)	4.75 (4.68–4.82; – 8.3)	5.13 (5.05–5.21; – 1.1)	5.15 (5.07–5.22; –0.7)	2.1
HDL-cholesterol (mmol/L)	1.25 (1.22–1.27)	1.25 (1.23–1.27; 0.1)	1.25 (1.23–1.27; 0.6)	1.23 (1.21–1.25; – 1.3)	1.23 (1.21–1.25; – 1.2)	2.5
LDL-cholesterol (mmol/L)	3.36 (3.29–3.43)	2.96 (2.89–3.02; – 13)	2.92 (2.86–2.99; – 13)	3.31 (3.24–3.38; – 1.5)	3.33 (3.26–3.40; –0.9)	3.1
LDL- to HDL-cholesterol ratio	2.70 (2.62–2.77)	2.37 (2.31–2.44; – 12)	2.33 (2.27–2.40; – 14)	2.69 (2.62–2.77; – 0.3)	2.71 (2.63–2.78; 0.3)	3.9
Total glycerol (mmol/L)	1.11 (1.06–1.16)	1.15 (1.10–1.21; 3.6)	1.10 (1.05–1.15; – 0.9)	1.14 (1.09–1.19; 3)	1.16 (1.11–1.22; 4.4)	6.7

Figures between brackets refer to confidence interval for mean and percentage change compared to Flora.
[a] Control, not enriched in sterols.
[b] Containing sitostanol-ester.

Table 3 Blood lipid concentrations in volunteers participating in the sterol enriched margarine study according to tertiles of total cholesterol or LDL-cholesterol levels at entry to the study

	Flora[a]	*Benecol*[b]	*Soybean*	*Ricebran*	*Sheanut*	*Minimal significant difference* (%)
Low Tertile ($N=32$)						
Total cholesterol (mmol/L) 4.16 ± 0.50	4.22 (4.11–4.33)	3.92 (3.83–4.01; – 7.1)	3.85 (3.77–3.94; – 8.6)	4.12 (4.03–4.21; – 2.3)	4.17 (4.07–4.28; – 1.1)	3.7
LDL-cholesterol (mmol/L) 2.45 ± 0.37	2.50 (2.39–2.61)	2.20 (2.12–2.29; – 12)	2.14 (2.06–2.23; – 14)	2.48 (2.39–2.58; – 0.7)	2.51 (2.42–2.61; 0.6)	6.5
Mid Tertile ($N=32$)						
Total cholesterol (mmol/L) 5.41 ± 0.30	5.33 (5.20–5.46)	4.97 (4.86–5.09; – 6.7)	4.93 (4.80–5.07; – 7.5)	5.31 (5.17–5.46; – 0.4)	5.33 (5.20–5.46; 0.0)	3.5
LDL-cholesterol (mmol/L) 3.52 ± 0.32	3.49 (3.38–3.60)	3.06 (2.95–3.16; – 12)	3.06 (2.96–3.18; – 12)	3.39 (3.26–3.52; – 2.9)	3.46 (3.33–3.59; – 1.0)	4.7
High Tertile ($N=31$)						
Total cholesterol (mmol/L) 6.54 ± 0.61	6.23 (6.04–6.42)	5.74 (5.54–5.95; – 7.9)	5.69 (5.53–5.86; – 8.7)	6.19 (6.01–6.38; – 0.6)	6.16 (5.98–6.34; – 1.2)	4.5
LDL-cholesterol (mmol/L) 4.62 ± 0.55	4.37 (4.19–4.56)	3.87 (3.71–4.04; – 11)	3.83 (3.68–3.99; – 12)	4.34 (4.17–4.52; – 0.7)	4.29 (4.12–4.47; – 1.8)	6.3

Figures in brackets refer to confidence interval for mean and percentage change compared to Flora.
[a] Control, not enriched in sterols.
[b] Containing sitostanol-ester.

Table 4 Plasma α- + β-carotene and lycopene levels, raw and lipid-standardized, in volunteers participating in the sterol enriched margarine study

	Flora[a]	*Benecol*[b]	*Soybean*	*Ricebran*	*Sheanut*	*Minimal significant difference* (%)
α- + β-carotene (μg/L)	243 (232–255)	189 (180–198; – 22)	186 (178–195; – 23)	223 (213–234; – 8.3)	139 (132–146; – 43)	7
Lycopene (μ g/L)	90 (81.1–99.8)	70 (62.9–77.4; – 22)	72 (65.2–80.1; – 20)	85 (76.9–94.7; – 5.1)	54 (48.8–60.1; – 40)	15.8
α- + β-carotene/ (TC[c] + TG[d]) (μg/mmol)	39.2 (37.3–41.1)	31.9 (30.4–33.5; – 19)	31.8 (30.3–33.4; – 19)	35.8 (34.1–37.6; – 8.6)	22.2 (21.1–23.3; – 43)	7.1
Lycopene/(TC + TG) (μg/mmol)	14.5 (13–16.1)	11.8 (10.6–13.1; – 19)	12.3 (11.1–13.7; – 15)	13.7 (12.3–15.2; – 5.5)	8.65 (7.78–9.60; – 40)	16

Figures between brackets refer to confidence interval for mean and percentage change compared to Flora.
[a] Control, not enriched in sterols.
[b] Containing sitostanol-ester.
[c] TC = total cholesterol.
[d] TG = total glycerol.

males and females for Benecol (higher reduction for males) and the soybean spread (higher reduction for females) (data not shown).

Plasma plant sterols

Stigmasterol or stanols were not detectable in plasma. Sitosterol levels were significantly lower for the sitostanol-ester margarine compared to the soybean oil spread and Flora, namely 2.1 mg/L (sitostanol ester) *vs* 4.6 mg/L (soybean) and 3.3 mg/L (Flora). Campesterol levels were also lower, but only the difference with the soybean oil product was significant. Levels were 5.8 mg/L (sitostanol), 12.1 mg/L (soybean) and 7 mg/L (Flora). Sitosterol and campesterol levels were significantly higher for the soybean product compared to Flora.

Dietary intake

Food consumption data are given in Table 5. These data include the contribution made to dietary intake by the margarines. On average, spread intake contributed slightly less than 8% to the total energy intake and about 18% to total fat intake. Energy intake, total fat intake (in percentage of energy intake) and dietary cholesterol intake did not differ significantly among the treatments. There were a few small differences in the intake of fatty acids between the treatments. During consumption of the sheanut and sitostanol-ester margarines overall intake of saturated and monounsaturated fatty acids was higher and intake of polyunsaturated fatty acids (mostly linoleic acid) was lower than during consumption of the other margarines. As the first two margarines contained higher amounts of saturated and monounsaturated fatty acids than the other three products this difference confirms what we expected to occur. Unsaturated fatty acid intake did not differ between Flora, the soybean and ricebran spreads. The intake of saturated fatty acids was slightly higher during Flora compared to the soybean spread. Energy, fat and cholesterol intake were higher for males than for females. However, gender did not interact with treatment.

Fatty acid composition of plasma cholesterylesters

The fatty acid composition of plasma cholesterylesters, indicated in Table 6, supports the overall similarity in fatty acid intake between the various treatments. Table 6 also shows a lower content of linoleic acid in the plasma cholesterylesters for the sitostanol-ester and sheanut sterol products compared to the other margarines. In contrast, oleic acid content was higher. This is in agreement with the data obtained by the food frequency method.

Discussion

General

This study shows that in normocholesterolaemic and mildly hypercholesterolaemic healthy males and females a margarine enriched with soybean oil sterols, primarily esters of sitosterol, campesterol and stigmasterol, lowered plasma total- and LDL-cholesterol concentrations by 8–13%, respectively compared to a spread with a similar fatty acid content and composition but not enriched in sterols. This blood cholesterol lowering effect was present within 2.5 weeks after feeding. Margarines containing sheanut or ricebran oil sterols were not effective in lowering blood cholesterol concentrations compared to the control margar-

Table 5 Dietary intake of volunteers participating in the sterol enriched margarine study

	Flora[a]	*Benecol*[b]	*Soybean*	*Ricebran*	*Sheanut*	*Minimal significant difference (%)*
Energy (kJ/d)	10103 (9770–10437)	10238 (9904–10571)	9926 (9596–10257)	10157 (9821–10493)	10122 (9789–10456)	471
Fat (energy %)	42.0 (41.2–42.8)	41.8 (41.0–42.6)	41.5 (40.7–42.2)	41.4 (40.6–42.2)	41.3 (40.5–42.1)	1.11
Saturated fatty acids (energy %)	15.9 (15.5–16.3)	16.2 (15.8–16.6)	15.3 (14.9–15.7)	15.4 (15.0–15.8)	16.9 (16.5–17.3)	0.57
Monounsaturated fatty acids (energy %)	14.0 (13.7–14.4)	15.6 (15.3–16.0)	13.9 (13.6–14.3)	14.1 (13.7–14.4)	14.7 (14.3–15.0)	0.47
Polyunsaturated fatty acids (energy %)	10.6 (10.2–10.9)	8.50 (8.15–8.84)	10.8 (10.4–11.1)	10.5 (10.1–10.8)	8.18 (7.83–8.52)	0.49
Cholesterol (mg/d)	233 (221–246)	243 (231–255)	226 (213–238)	233 (221–246)	227 (215–240)	17.3

Figures between brackets refer to confidence interval for mean.
[a] Control, not enriched in sterols.
[b] Containing sitostanol-ester.

Table 6 Fatty acid composition of plasma cholesterylesters in volunteers participating in the sterol enriched margarine study

Fatty acid (%)	*Flora*[a]	*Benecol*[b]	*Soybean*	*Ricebran*	*Sheanut*	*Minimal significant difference* (%)
C12:0–C18:0	14.9 (14.5–15.2)	14.9 (14.5–15.3)	14.8 (14.4–15.2)	14.9 (14.5–15.2)	14.9 (14.5–15.2)	0.55
C18:1 cis	13.8 (13.4–14.2)	15.4 (15.0–15.8)	13.4 (13.0–13.8)	13.7 (13.3–14.1)	15.5 (15.0–15.9)	0.60
C18:2 *n*-6	57.0 (56.1–57.8)	54.6 (53.7–55.5)	56.9 (56.0–57.8)	57.3 (56.4–58.2)	55.1 (54.3–56.0)	1.23
C18:3 *n*-3	0.47 (0.43–0.51)	0.68 (0.64–0.72)	0.44 (0.40–0.48)	0.46 (0.42–0.50)	0.44 (0.40–0.48)	0.06
Rest fatty acids	13.9 (13.5–14.4)	14.4 (14.0–14.9)	14.5 (14.0–14.9)	13.7 (13.3–14.1)	14.1 (13.7–14.5)	0.60

Figures between brackets refer to confidence interval for mean.
[a] Control, not enriched in sterols.
[b] Containing sitostanol-ester.

ine. The fatty acid composition of the shea sterol margarine was less favourable with respect to blood cholesterol lowering compared to the fatty acid composition of the control margarine, suggesting that we may have underestimated the true cholesterol lowering action of the shea sterol margarine. We confirmed the cholesterol lowering action reported by Miettinen *et al* (1995) of a spread enriched with sitostanol-ester. We did not observe an effect on plasma HDL-cholesterol or triglyceride concentrations of the sterol enriched margarines.

The ricebran oil and sheanut oil sterol margarines were not effective in lowering blood cholesterol levels. There are no reports in the peer-reviewed literature of human feeding studies with sheanut oil or ricebran oil sterols in normocholesterolaemic or mildly hypercholesterolaemic subjects, so the data of this study can not be compared to literature findings. Our findings for the ricebran oil margarine are in agreement with data of Lichtenstein *et al*, (1994) who fed volunteers in a well controlled design ricebran oil and did not observe a cholesterol lowering action compared to other feeding regimes with similar fatty acid intakes. There is, however, evidence from a well controlled study in nonhuman primates that rice bran oil can lower total- and LDL-cholesterol without affecting HDL-concentrations (Nicolosi *et al*, 1991). Further studies in humans are needed to validate claims on the cholesterol lowering action of rice bran oil or rice bran oil concentrates rich in sterols.

The ricebran and sheanut sterol margarines contained 4,4′-dimethylsterols, esterified primarily to, respectively ferulic acid and cinnamate and acetic acid, whereas the soybean oil product and Benecol contained fatty acid esters of either saturated or unsaturated 4-desmethylsterols. The structure of 4-desmethylsterols, sitostanol, sitosterol, stigmasterol and campesterol, is more similar to cholesterol than the structure of 4,4′-dimethylsterols. This may offer more opportunity for effective competition with cholesterol for incorporation in mixed micelles, which is the supposed mechanism for the cholesterol lowering action of sterols (Ling & Jones, 1995). The lower sterol content (for ricebran) and differences in fatty acid composition (for shea) may also have contributed to differences in effect between the ricebran and sheanut products on the one hand and the other sterol enriched margarines on the other hand. We estimate that we may have underestimated the LDL-cholesterol lowering action of the sheanut margarine in comparison to the control product due to differences in fatty acid intake by a maximum of 0.10 mmol/L (Mensink & Katan, 1992). This is close to the significant difference compared to control that we could have detected with 95% confidence.

We do not believe that differences in compliance can explain the observed variation in efficacy between the treatments. Compliance was excellent. Spread consumption was very close to the target dose of 30 g/d and intake occurred nearly always at lunch and evening meal. Drop out rate was small and the number of volunteers consuming a given spread was very similar for all products.

Habitual intake of energy, dietary fat (as percent of energy intake) and cholesterol was not significantly different between the margarines. The absence of difference in body weight between the treatments confirms the similarity across treatments of energy intake and energy balance. Intake of fatty acids differed among margarines reflecting differences in fatty acid composition of margarines namely a lower linoleic acid and higher oleic acid and saturated fatty acid content of Benecol and the sheanut product compared to the other margarines.

The fatty acid composition of the plasma cholesteryl-esters reflects fatty acid intake over the last few weeks and is a good variable to assess compliance to dietary fat interventions (Zock *et al*, 1997). We could support the reported differences in dietary intake of the various fatty acids with the data on fatty acid composition of plasma cholesterylesters. During consumption of the sitostanol-ester and sheanut product the level in plasma cholesterylesters of linoleic acid was lower, the level of oleic acid and α-linolenic acid (sitostanol-ester product only) was higher compared to consumption of the other margarines. These findings corroborate our other measures of compliance and they underscore the validity of the method we used to estimate dietary intake. The differences in dietary fatty acid intake during consumption of the sitostanol-ester product may have caused a slight underestimation of the LDL-cholesterol lowering effect of this product compared to Flora and the soybean spread. We estimate the maximum size of that effect to be negligible namely 0.03 mmol/L against Flora and 0.05 mmol/L against the soybean spread (Mensink & Katan, 1992)

Food intake

Overall the percentage of energy derived from fat was slightly higher than the average percentage of energy from fat in the Netherlands, which is about 38% (Voorlichtingsbureau voor de Voeding, 1993). Habitual energy intake in the Netherlands of individuals aged between 22–65 y is about 9.5 MJ/d. Energy intake in this study population was slightly higher indicating that it is very unlikely that energy or fat intake have been under reported by our study population.

Blood chemistry and haematology

We monitored blood chemistry and haematological variables and found no evidence for adverse effects of consumption of the sterol enriched spreads on plasma aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and L-γ-glutamyltransferase activities, serum total

Table 2: Study plan

	Phase I: Screening / Run in		Phase II: Active Medication				Phase III: Follow-up	
Week	-5	-1	1	5	8	9	13	17
Day			1	29	50	57	85	113
Visit	1	2	3	4	5	6	7	8
Adm. History	X							
24 h Dietary Recall	X		X			X		
Physical Exam	X	X	X	X	X	X	X	X
Adverse Event Report			X	X	X	X	X	X
Biochemical Profiling	Lipids	Screen	X	X	X	X	X	X

At visit 1 subjects underwent a selection procedure including an interview (personal and family medical history; previous medical treatments, dietary habits); a clinical examination; and a laboratory test (Total, LDL-, HDL-cholesterol, and triglycerides) under fasting conditions. If subjects complied with inclusion/exclusion criteria they received dietary counselling and underwent another clinical examination, and a laboratory test (Total, LDL-, HDL-cholesterol, and triglycerides, thyroid Function tests, full blood count) under fasting conditions (Visit 2). At visit 3 through to visit 8 patients underwent a physical examination and a laboratory test including determination of total, LDL-, HDL-cholesterol, and triglycerides as well as various safety measurements (Glucose, Urea & Electrolytes, Liver Function Tests, Calcium Profile). At visits 3, 6, and 8 vitamin A, beta-carotene, and Vitamin E were determined as additional safety parameters.

All clinical visits were performed between 7 and 10 am. The subjects were instructed not to eat 12 hours prior to the clinic visits.

Diet and Dietary intake

Randomisation of the subjects (at visit 3) was proceeded by a 4 week wash out period for cessation of current lipid lowering drugs (if applicable), with dietary recommendations to attempt to ameliorate dyslipidemia. This period was based on a low saturated/low cholesterol diet as currently recommended by the European Atherosclerosis Society and the American Heart association, step 1 diet, respectively. The advise was given by trained dieticians. Furthermore, 24h recalls to monitor food intake were collected at visit 1, visit 3 and visit 6 (see Table 2) in both treatment and placebo group. The recall was applied to assess and compare group means of macro nutrients (fat with SAFA, MUFA, PUFA; protein; carbohydrate). For calculation of the nutrient intake the software GENI (Micro6, Vandoeuvre les Nancy, France) was used.

Study products

The phytosterol enriched cereal bars were prepared by Wander AG, Neuenegg, Switzerland. Composition of active and placebo bars is shown in table 3. The cereal bars were fortified with unesterified tall oil sterols (Reducol™, Forbes Medi-Tech, Vancouver, Canada) with 52% sitosterol, 15% sitostanol, 17% campesterol, and 3% campestanol. Each bar of 25 g

contained 0.6 g tall oil sterols. Subjects consumed 3 bars per day (1.8 g sterols/d) with their main meals

Table 3: Composition of cereal bars

Variables	Placebo Bar	Phytosterol Bar
Water (g/100g)	6.4	6.5
Protein (g/100g)	3.3	3.4
Fat (g/100g)	14.3	14.1
Carbohydrates (g/100g)	69.1	71.2
Organic Acids (g/100g)	0.3	0.3
Minerals (g/100g)	0.8	0.8
Dietary fiber (g/100g)	2.4	2.5
KJ (per 100 g)	1763.5	1795.7

Analysis

At visit 1 lipid laboratory values were measured locally, however the samples for visits 2 through to 8 for all measured parameters were pooled and analysed centrally at the Royal Liverpool Hospital, Liverpool, England.

Blood samples for serum and plasma determination were centrifuged (3000 rpm x 10 min, +4°C) within 1 hour after drawing. Plasma and serum was separated and stored at -20°C until analysis.

Blood lipids
Total and HDL cholesterol and triglycerides were determined with a Hitachi 747 analyser (Hitachi Inc., Tokio, Japan) using enzymatic methods (Roche UK Ltd, Lewes, England). LDL cholesterol was calculated by using the Friedewald equation (Friedewald, Levy, Fredrickson, 1987).

(Pro)Vitamins
Vitamin A and E and beta-carotene was determined using a HPLC method as describe by Thurnham et al () and Zaman et al (). Proteins in the serum were precipitated with methanol and the vitamin/lipid complexes were extracted into hexane. The dried extract was redissolved in THF and methanol and quantification of the vitamins was achieved by reverse phase HPLC.

Statistical analysis

The primary parameter of the study was the intra-individual pre-post difference in plasma LDL cholesterol. Both values (pre-treatment and post-treatment) were calculated as mean values of two visits: visits 2 and 3 (pre-treatment) and visits 5 and 6 (post-treatment). Pre-post difference in plasma total cholesterol and HDL cholesterol, as well as plasma (pro)vitamins were secondary parameter. A two sample t-test was used with an accepted level of significance of $p<0.05$. The statistical software used is SAS®.

RESULTS

General

131 subjects were randomised and all completed the entire study. All subjects tolerated the cereal bars well although a total of 83 adverse events were reported. Non of the adverse events however were classified by the investigators as related to study products. While in the placebo group 48 adverse events were reported only 35 events occurred in the active group. All recorded adverse were mild or moderate in severity.

Patients were asked to note the actual times of product ingestion in a personal booklets. Compliance was satisfying for all the subjects expect 3 and was considered as homogeneous between groups.

The study population consisted of 100 male (76.3%) and 31 female subjects (23.7%) with a homogenous distribution between the two groups (p=0.97). All 131 subjects could be considered as healthy as full examination (including blood pressure and heart rate) and laboratory tests were normal.

Body weight and diet

Body weight did not change during the study. Bodyweight of the placebo group was 75.2 ± 13.4 kg at the randomisation visit and 75.2 ± 13.1 kg at the end of the treatment phase. For the active group values were 755 ± 11.5 kg at the randomisation vs 75.4 ± 11.5 at the end of the active treatment, respectively.

Nutrient intake during the study of both placebo and active group is shown in table 4. No difference between the 2 groups was detected at any time point measured. The dietary counselling was well accepted in both groups and lead to the expected decrease in fat intake (36% energy from fat at visit versus 29% energy from fat at visit 3). The quality of fat ingested also changed slightly with a tendency to decrease saturated fatty acids. Cholesterol intake also decreased due to counselling, however the goal of >150 mg per day could not be achieved.

Table 4: Intake of energy, protein, carbohydrate and fat as assessed by 24 hour recall at visit 1, visit 3 and visit 6. Intakes are expressed as grams (g) and as percent energy (%En).

	Placebo			Phytosterol		
	V1	V3	V6	V1	V3	V6
Energy (kcal)	1698 ± 59	1664 ± 59	1663 ± 50	1770 ± 57	1657 ± 54	1715 ± 49
Protein (g)	72 ± 3	77 ± 4	69 ± 4	74 ± 3	74 ± 3	79 ± 4
%En	17	19	17	17	18	18
Carbohydrate (g)	176 ± 8	193 ± 10	190 ± 10	192 ± 9	188 ± 8	212 ± 10
%En	41	46	46	43	45	49
Fat (g)	67 ± 4	53 ± 4	56 ± 3	67 ± 4	55 ± 4	54 ± 3
%En	36	29	30	34	30	28
SAFA (g)	22 ± 2	19 ± 2	20 ± 2	25 ± 2	21 ± 2	20 ± 1
%En	12	10	11	13	11	10
MUFA (g)	25 ± 2	18 ± 2	22 ± 2	26 ± 2	21 ± 2	18 ± 1
%En	13	10	12	13	11	9
PUFA (g)	8 ± 1	8 ± 1	9 ± 1	10 ± 1	7 ± 1	8 ± 1
%En	4	4	5	5	4	4
Cholesterol (mg)	271 ± 18	247 ± 27	226 ± 24	264 ± 18	234 ± 23	243 ± 21

Blood lipids

Total and LDL cholesterol were both decreased significantly by tall oil sterol consumption, while HDL cholesterol and triglycerides were not affected (Table 5).

Table 5: Total, LDL and HDL cholesterol and triglycerides concentrations before and after consumption of cereal bars with or without tall oil sterol fortification.

	Placebo (n=68)	**Phytosterol** (n=63)	**P**
Total cholesterol (mmol/l)			
Inclusion (V2-V3)	6.83 ± 0.12	6.60 ± 0.10	0.15
End (V5-V6)	6.95 ± 0.13	6.37 ± 0.12	**0.001**
Change[#]	0.14 ± 0.10	-0.23 ± 0.08	**0.001**
Relative change (%)	2.48 ± 1.35	-3.26 ± 1.26	**0.001**
LDL Cholesterol (mmol/l)			
Inclusion (V2-V3)	4.69 ± 0.12	4.46 ± 0.10	0.14
End (V5-V6)	4.72 ± 0.12	4.27 ± 0.12	**0.01**
Change	0.04 ± 0.09	-0.19 ± 0.08	**0.01**
Relative change (%)	2.04 ± 1.89	-3.76 ± 1.66	**0.01**
HDL Cholesterol (mmol/l)			
Inclusion (V2-V3)	1.38 ± 0.04	1.34 ± 0.04	0.49
End (V5-V6)	1.39 ± 0.05	1.31 ± 0.04	0.27
Change	0.01 ± 0.02	-0.03 ± 0.02	0.19
Relative change (%)	1.88 ± 1.70	-1.39 ± 1.65	0.18
Triglycerides (mmol/l)			
Inclusion (V2-V3)	1.74 ± 0.12	1.80 ± 0.14	0.66
End (V5-V6)	1.92 ± 0.13	1.77 ± 0.10	0.52
Change	0.18 ± 0.08	-0.04 ± 0.10	0.16

[#]Change is calculated as difference of end (mean of V5 and V6) minus inclusion (mean of V2 and V3) values.

At inclusion no statistical difference in total, LDL, and HDL cholesterol could be detected between placebo and phytosterol group. However, absolute total and LDL cholesterol concentrations in the placebo group were higher, e.g. 0.23 mmol/l for LDL cholesterol. After the 8-week treatment both total and LDL cholesterol was reduced in the phytosterol group, whereas in the placebo group cholesterol concentrations were slightly higher. Final concentration of total and LDL cholesterol, the absolute and the relative change from baseline were significantly different in the phytosterol group compared to placebo. Both HDL cholesterol and triglycerides only showed minor changes in both groups and no significant difference could be detected.

(Pro)Vitamins

Serum concentrations of vitamin A, vitamin E and beta-carotene were not affected by phytosterol ingestion (Table 6).

Table 6: Serum concentrations of vitamin A, vitamin E, and beta-carotene at randomisation (V3), end of treatment (V6), and end of follow up phase (V8).

	Placebo (n=59)	**Phytosterol** (n=52)	**P**
Vitamin A (umol/L)			
Inclusion V3	2.85 ± 0.09	2.81 ± 0.10	0.75
End of treatment V6	3.02 ± 0.09	3.32 ± 0.45	0.52
Follow up V8	2.95 ± 0.10	2.83 ± 0.10	0.39
Vitamin E (umol/L)			
Inclusion V3	30.4 ± 1.5	32.4 ± 1.5	0.45
End of treatment V6	35.6 ± 1.4	33.3 ± 1.4	0.25
Follow up V8	33.8 ± 1.3	34.5 ± 1.4	0.73
beta-carotene (umol/L)			
Inclusion V3	0.83 ± 0.08	0.88 ± 0.10	0.71
End of treatment V6	0.76 ± 0.08	0.76 ± 0.08	0.99
Follow up V8	0.85 ± 0.08	0.92 ± 0.09	0.58

In both phytosterol and placebo group vitamin A and E concentrations were slightly higher after the treatment phase then at the start of the trial, however reached again starting values at the end of the follow up phase. At non of the points measured any statistical significant difference could be detected between placebo and phytosterol group. Beta-carotene concentrations were slightly reduced in both groups after the treatment phase, however, difference between the 2 groups did not show any significance.

DISCUSSION

In the present study a low fat cereal bar enriched with TOS, as part of a low fat diet, reduced both total and LDL-cholesterol without negatively affecting serum concentrations of fat soluble (pro)vitamins.

In the past several short and long term studies (e.g. Vanhanen et al, 1993; Miettinen et al, 1995) showed that a daily intake of approx. 3 g sitostanol can significantly lower total and LDL cholesterol by up to 10% with no change in HDL cholesterol. However, in most of the studies total fat (36 - 41% of total energy) and cholesterol intake (300 - 450 mg) were high. While the mechanism of action of phytosterols on serum cholesterol level is still not known in detail, it is generally accepted that phytosterols inhibit absorption of both endo- and exogenous cholesterol from the intestine (Grundy & Mok, 1976; Heinemann et al, 1991). A high dietary fat intake and high fat foods delivering the phytosterols may therefore be of benefit to maximise the action of phytosterols. However, from a nutritional standpoint effects would be much more relevant if achieved in patients on a low fat dieted combined with a low fat food as delivery vehicle. Thurnham (1999) criticised the fact that by reducing cholesterol by the ingestion of high fat foods fortified with phytosterols will not be associated with a reduction in dietary fat or energy nor will there be an incentive to increase the intake of fiber, fruit and vegetables.
In widely discussed study Denke (1995) failed to show a significant cholesterol lowering effect of sitostanol (3 g/d) combined with a low fat (30% of total energy) and cholesterol intake (<200 mg/d). While the low fat intake may have played a role for not finding an effect the method of delivery (sitostanol was suspended in safflower oil and packed in gelatine capsules) may have been the more important factor for the negative results. In contrast to Denke's results Hallikainen and Uusitupa (1999) have successfully shown that a fat reduced

phytosterol fortified margarine is able to reduce both LDL and total cholesterol in patients following a low fat diet. The effect produced by the sterols combined with the effect of the low fat diet on cholesterol levels was significantly bigger then the effect of the diet alone.
The present study shows that a phytosterol enriched cereal bar with a fat content below 15% reduces both LDL and total cholesterol by 6% compared to placebo. This was achieved with a low daily dose of 1.8 g of free tall oil sterols on the background of a low fat diet (less then 30% energy from fat). Studies in the literature at a similar dose of phytosterols showed similar reductions in total and LDL cholesterol. Hendriks et al (1999) showed that a margarine (70% fat w/w) containing 0.83 g sterols reduced total and LDL cholesterol by 4.9% and 6.7% respectively, whereas a dose of 1.61 g reduced the 2 concentrations by 5.9% and 8.5%. Dietary fat intake in the Hendriks study was also relatively low at 33 energy%.

While the positive action of plant phytosterols is wildly accepted and regarded as a useful dietary alternative to drug treatment for mildly hypercholesterolemic subjects, there is some concern about the possible negative effect of sterols on fat soluble vitamins and provitmains. The reduction of cholesterol by plant sterols is thought mainly due to decreased cholesterol absorption, therefore sterols may also interfere with the absorption of fat soluble vitamins. Gylling et al (1996) showed that alpha-tocopherol and carotene concentrations were significantly decreased after consumption of 3 g esterified sitostanol. Lipid standardised alpha-tocpherol levels did however not change. Weststrate & Meijer (1998) recorded a significant decrease of approx. 20% of (alpha&beta)-carotene (lipid standardised or not) after consumption of 3 g esterified plant sterols. In a recent study Hendriks et al (1999) discovered the same effects as Westrated but additionally showed that the lowering effects were dose-dependant. The highest dose of 3.24 g of esterified plant sterols decreased plasma lipid soluble vitamins and carotenoids by 8 to 20%, effects were somewhat reduced a lower plant sterol doses of 1.61 g and 0.83 g per day. The plasma reductions were however only significant for carotenoids. In contrast to the above studies with esterified plant sterols Sierksam et al (1999), investigated the cholesterol lowering effects of free soy sterols, could not detect any negative effect on (alpha&beta)-carotene concentrations. This may have been an effect of the low daily intake of 0.8 g per day, however we showed no negative effect of a daily consumption of 1.8 g of free sterol on vitamin A and E, and beta-carotene concentrations. It is unclear why free sterols may have no negative effect on carotenoid concentration and further studies will be needed to verify and understand the reported finding.

CONCLUSIONS

This present study shows that the consumption of cereal bars enriched with 1.8 g tall oil sterols decreases both LDL and total cholesterol by 0.23 mmol/L and 0.36 mmol/L, respectively, as compared to placebo. This effects was seen on the background of a fat reduced diet in mildly hypercholesterolemic subject. Serum concentrations of vitamin A and E and beta-carotene was not negatively influenced by phytosterol consumption which may be due to the use of free not esterified sterols. A low fat cereal bar delivers beside the cholesterol lowering sterols other important nutrients as dietary fibers and fits well into the desired eating pattern of a patient with unhealthy cholesterol levels. If patients replace products rich in fat and/or cholesterol by a cholesterol lowering cereal bar effects may be much bigger then shown in the present study.

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8

β-Carotene and Other Carotenoids

SUMMARY

Blood concentrations of carotenoids are the best biological markers for consumption of fruits and vegetables. A large body of observational epidemiological evidence suggests that higher blood concentrations of β-carotene and other carotenoids obtained from foods are associated with lower risk of several chronic diseases. This evidence, although consistent, cannot be used to establish a requirement for β-carotene or carotenoid intake because the observed effects may be due to other substances found in carotenoid-rich food, or to other behavioral correlates of increased fruit and vegetable consumption. While there is evidence that β-carotene is an antioxidant in vitro, its importance to health is not known. The one clear function of certain carotenoids that is firmly linked to a health outcome is the provitamin A activity of some dietary carotenoids (α-carotene, β-carotene, and β-cryptoxanthin) and their role in the prevention of vitamin A deficiency. Establishment of a requirement for carotenoids based upon vitamin A activity must be done in concert with the evaluation of Dietary Reference Intakes (DRIs) for vitamin A, which was not included in this report, but will be addressed in a subsequent DRI report. Although no DRIs are proposed for β-carotene or other carotenoids at the present time, existing recommendations for increased consumption of carotenoid-rich fruits and vegetables are supported. Based on evidence that β-carotene *supplements* have not been shown to confer any benefit for the prevention of the major chronic diseases and may cause harm in certain subgroups, it is concluded that β-carotene supplements are not advisable, other than as a provitamin

A source and for the prevention and control of vitamin A deficiency in at-risk populations.

BACKGROUND INFORMATION

The most prevalent carotenoids in North American diets include the following: α-carotene, β-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin. The structures of these carotenoids are shown in Figure 8-1. Three of these carotenoids, namely α-carotene, β-carotene, and β-cryptoxanthin, can be converted into retinol and are thus referred to as provitamin A carotenoids. Lycopene, lutein, and zeaxanthin have no vitamin A activity and are thus referred to as nonprovitamin A carotenoids. Most naturally occurring carotenoids are in the *all-trans*-configuration; but under conditions of heating, for example, *cis*-isomers such as 13-*cis*-β-carotene (Figure 8-1) are formed.

Functions and Actions

The various biological effects of carotenoids can be classified into functions, actions, and associations. Carotenoids function in plants and in photosynthetic bacteria as accessory pigments in photosynthesis and protect against photosensitization in animals, plants, and bacteria. In humans, the only known function of carotenoids is vitamin A activity (provitamin A carotenoids only).

Carotenoids also are thought to have a variety of different actions, including possible antioxidant activity, immunoenhancement, inhibition of mutagenesis and transformation, inhibition of premalignant lesions, quenching of nonphotochemical fluorescence, and activity as a pigment in primate macula (Olson, 1999). Carotenoids have also been associated with various health effects: decreased risk of macular degeneration and cataracts, decreased risk of some cancers, and decreased risk of some cardiovascular events (Olson, 1999).

However, as described above, the only known function of carotenoids in humans is to act as a source of vitamin A in the diet. This function, as well as carotenoid actions and associations, is reviewed elsewhere (Krinsky, 1993; Olson, 1989) and discussed in subsequent sections.

Physiology of Absorption, Metabolism, and Excretion

Absorption

The intestinal absorption of dietary carotenoids is facilitated by

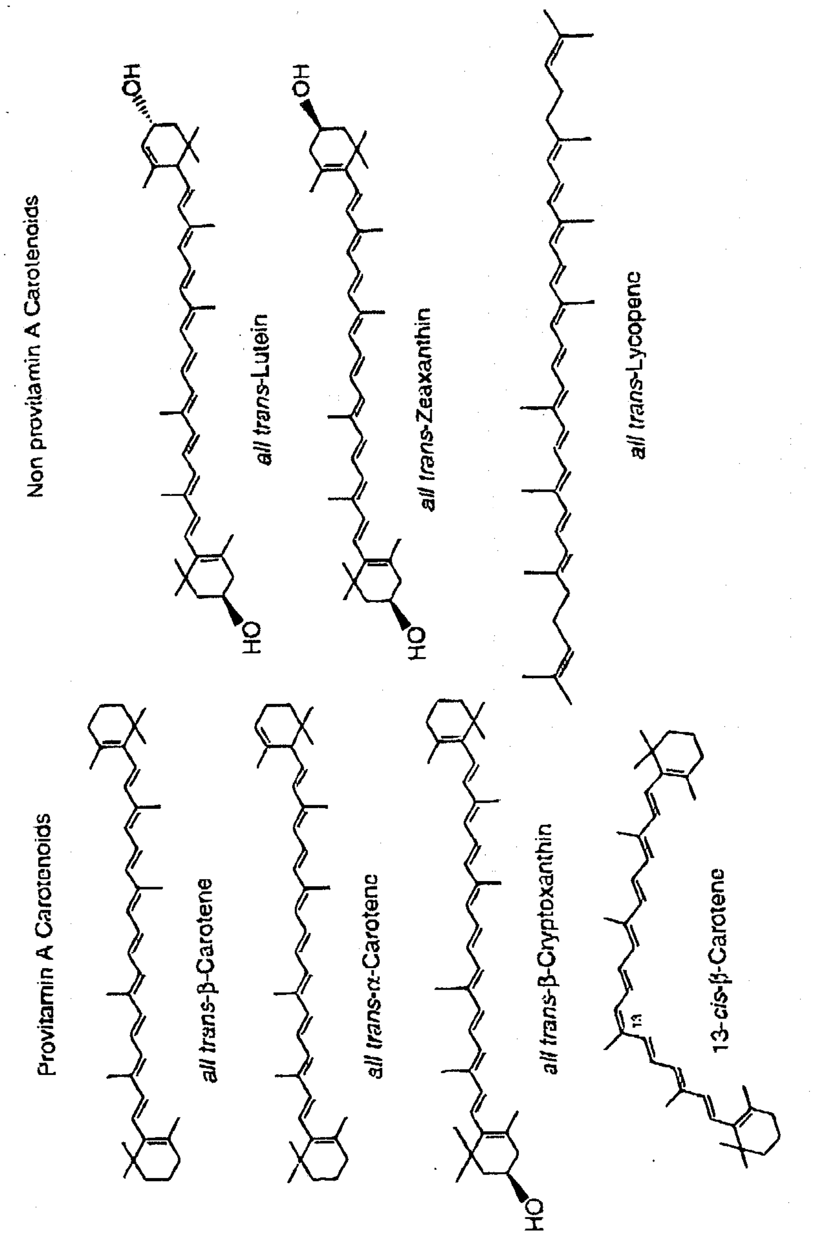


FIGURE 8-1 Structure of provitamin A and nonprovitamin A carotenoids.

formation of bile acid micelles. The hydrocarbon backbone of carotenoids makes them insoluble in water, and like other nonar lipids, they must be solubilized within micelles in the gasntestinal tract to allow for absorption. Micellar solubilization litates the diffusion of lipids across the unstirred water layer. : presence of fat in the small intestine stimulates the secretion of acids from the gall bladder and improves the absorption of ɔtenoids by increasing the size and stability of micelles, thus aling more carotenoids to be solubilized. The uptake of β-caro- : by the mucosal cell is believed to occur by passive diffusion llander and Ruble, 1978). Uptake by these cells, however, is not icient for absorption to be completed. Once inside the mucosal , carotenoids or their metabolic products (e.g., vitamin A) must be incorporated into chylomicrons and released into the lymtics. When mucosal cells are sloughed off due to cell turnover, ing their contents into the lumen of the gastrointestinal tract, ɔtenoids that have been taken up by the cells but not yet incorated into chylomicrons are lost into the lumen (Boileau et al., 9).

abolism, Transport, and Excretion

ɪrotenoids may be either absorbed intact, or in the case of those essing vitamin A activity, cleaved to form vitamin A prior to ɛtion into lymph. Portal transport of carotenoids is minimal to the lipophilic nature of their structures. Some portal trans- of more polar metabolites, such as retinoic acid, can occur on, 1999).

rotenoid cleavage is accomplished either by the intestinal mul enzyme β-carotene 15,15′-dioxygenase (EC 1.13.11.21) or by :entral cleavage mechanisms (Boileau et al., 1999; Olson, 1999; er, 1996; Wang, 1994). The extent of conversion of a highly vailable source of dietary β-carotene to vitamin A in humans ɔeen shown to be between 60 and 75 percent, with an addition- percent of the β-carotene absorbed intact (Goodman et al.,). However, absorption of most carotenoids from foods is conably lower and can be as low as 2 percent (Rodriguez and Ir- 1972). The effects of dietary and nondietary factors on the ency of carotenoid absorption are reviewed later.

ncentral (or excentric) cleavage of carotenoids yields a wide ty of metabolic products, some of which are further metabo- . These cleavage products include aldehyde, acid, alcohol, and ide derivatives (Parker, 1996; Wang, 1994). Isomerization of carotenoids or their metabolic products may occur in vivo because isomers have been found upon extraction of carotenoids from human tissues (Clinton et al., 1996). Although little attention has been given to the study of carotenoid excretion pathways, epoxides and carotenoid metabolic products with less than 15 carbon chain lengths would presumably have no vitamin A activity. It is assumed that bile and urine would be excretion routes for metabolites (Olson, 1999).

The carotenoids are transported in blood exclusively by lipoproteins. The carotenoid content of individual lipoprotein classes is not homogeneous. In the fasted state, the hydrocarbon carotenoids such as α-carotene, β-carotene, and lycopene are carried predominantly by low-density lipoprotein. The remaining carotenoids, including the more polar xanthophylls such as lutein and zeaxanthin, are carried by high-density lipoprotein (HDL) and, to a lesser extent, by very low-density lipoprotein (Johnson and Russell, 1992; Parker, 1996; Traber et al., 1994). It is thought that β-carotene and other hydrocarbon carotenoids reside in the hydrophobic core of the particles, whereas the more polar xanthophylls reside closer to the surface (Parker, 1996).

β-Carotene is the most studied carotenoid in terms of metabolism and its potential effects on health. Lycopene, lutein, zeaxanthin, and α-carotene have received increasing attention in recent years. Much remains to be learned, however, about the relative metabolic effects of these carotenoids.

Body Stores

Recently, 34 carotenoids were identified in human serum and milk (Khachik et al., 1997b). Of these, 13 were geometrical isomers of their *all-trans* parent structures and 8 were metabolites. This finding is in contrast to the up to 50 carotenoids that have been identified in the U.S. diet and the more than 600 found in nature. The most prevalent carotenoids in human serum (Khachik et al., 1997b) are the same as those most commonly found in the diet: β-carotene, lycopene, and lutein (Nebeling et al., 1997). *Cis*-isomers of lycopene are commonly found in the serum and in fact have been shown to constitute more than 50 percent of the total serum lycopene (Stahl et al., 1992). In contrast, *cis*-isomers of β-carotene are considerably less common in serum with the *trans*-isomers being more common. In addition to these forms of α-carotene, β-carotene, lycopene, and zeaxanthin are also major serum carotenoids. The concentrations of various carotenoids in human serum and

ssues are highly variable and likely depend on a number of factors ıch as food sources, efficiency of absorption, amount of fat in the iet, and so forth (Table 8-1).

The serum concentration of carotenoids after a single dose peaks : 24 to 48 hours post dose (Johnson and Russell, 1992). The earli- st postprandial serum appearance of carotenoids is in the chylomi- :on fraction. It has been proposed that the increase in carotenoids ı the triglyceride-rich lipoprotein fraction (primarily chylomi- :ons) be used for quantitating carotenoid absorption (van Vliet et ., 1995). This would provide a more direct measure of absorption ecause total serum carotenoid content is not an exclusive measure f newly absorbed carotenoids.

Data from the Third National Health and Nutrition Examination ırvey (NHANES III) demonstrate the variability of normal serum ırotenoid concentrations (Appendix Tables F-4 through F-8). This ıriability is attributed to a variety of life-style and physiological fac- ırs. In a recent population-based study, Brady et al. (1996) report- d that lower serum concentrations of α-carotene, β-carotene, β- yptoxanthin, lutein, and zeaxanthin, but not lycopene, were :nerally associated with male gender, smoking, younger age, lower on-HDL cholesterol, greater ethanol consumption, and higher ody mass index.

The delivery of carotenoids to extrahepatic tissue is accomplished ırough the interaction of lipoprotein particles with receptors and ıe degradation of lipoproteins by extrahepatic enzymes such as poprotein lipase. Carotenoids are present in a number of human ssues including adipose, liver, kidney, and adrenal, but adipose ssue and liver appear to be the main storage sites (Parker, 1996). owever, based on a wet tissue weight, the liver, adrenal gland, and stes contain the highest per-gram concentrations (Stahl et al.,)92). Similar to what is reported in serum, β-carotene, lutein, and copene are the main tissue carotenoids, although α-carotene, β- yptoxanthin, and zeaxanthin are also present (Boileau et al.,)99). In contrast to serum profiles, 9-*cis*-β-carotene is consistently esent in storage tissues. In both serum and tissue storage, lyco- :ne *cis*-isomers constitute greater than 50 percent of the total lyco- :ne present (Clinton et al., 1996; Stahl et al., 1992).

Clinical Effects of Inadequate Intake

If adequate retinol is provided in the diet, there are no known inical effects of consuming diets low in carotenes over the short rm. One study of premenopausal women consuming low-carotene diets in a metabolic ward reported skin lesions (Burri et al., 1993). However, this effect was not observed after 60 days of depletion in a subsequent β-carotene depletion study by the same group of investigators (Lin et al., 1998). These studies of carotene-deficient diets were reported to increase various measures of oxidative susceptibility (Dixon et al., 1994, 1998; Lin et al., 1998), but as discussed below, this is of uncertain relevance with regard to clinical outcomes.

SELECTION OF POSSIBLE INDICATORS FOR ESTIMATING THE REQUIREMENT FOR β-CAROTENE AND OTHER CAROTENOIDS

Vitamin A Equivalency

Vitamin A equivalency is a possible indicator for establishing requirements for provitamin A carotenoids. However, any such establishment of requirements for carotenoids based on vitamin A activity must be considered in concert with the evaluation of requirements for vitamin A. This information will be presented in a later Dietary Reference Intakes report.

Markers of Antioxidant Activity

The effect of increasing β-carotene intake on several markers of antioxidant activity has been investigated in a series of studies involving humans. These studies have examined antioxidant marker activity in apparently healthy men and women as well as in subjects who were physiologically challenged (i.e., smokers and patients with coronary disease or cystic fibrosis).

Studies of the effect of β-carotene intake on measures of antioxidant activity are summarized in Table 8-2. The dietary source of β-carotene ranged from modification of diets with normally consumed foods to giving supplements that provided as much as 120 mg/day of a highly bioavailable preparation. In general, subjects in most studies consumed β-carotene in amounts that would be difficult to achieve from foods alone and, as a result, relate to the pharmacological range of intakes.

The findings reported in Table 8-2 indicate that β-carotene supplementation did not alter, or inconsistently alter, markers of antioxidant activity, which were somewhat dependent on β-carotene intake. In studies in which subjects were fed less than 25 mg/day of β-carotene, either from foods or as a supplement, changes in the markers for antioxidant activity were minimal. Exceptions noted

ABLE 8-1 Concentrations of Selected Carotenoids in uman Serum and Tissues

otenoid	Serum (μmol/L)	Liver (μmol/g)	Kidney (μmol/g)	Lung (μmol/g)
arotene	0.02–0.47 (1.0–25.3 μg/dL)	0.075–10.8 (0.04–5.8 μg/g)	0.037–1.5 (0.02–0.80 μg/g)	0.1–1.0 (0.05–0.54 μg/g)
arotene	0.04–2.26 (2.2–122.7 μg/dL)	0.39–19.4 (0.21–6.3 μg/g)	0.093–2.8 (0.05–1.5 μg/g)	0.1–1.6 (0.05–0.86 μg/g)
yptoxanthin	0.03–0.70 (1.4–38.2 μg/dL)	0.037–20.0 (0.05–11.0 μg/g)	0.019–3.9 (0.05–2.2 μg/g)	0.1–2.5 (0.05–1.4 μg/g)
ein	0.10–1.23 (5.8–69.8 μg/dL)	0.10–3.0 (0.06–6.9 μg/g)	0.037–2.1 (0.05–5.9 μg/g)	0.1–2.3 (0.05–1.3 μg/g)
opene	0.05–1.05 (2.7–54.6 μg/dL)	0.20–17.2 (0.11–11.1 μg/g)	0.093–2.4 (0.05–1.3 μg/g)	0.1–1.0 (0.05–2.3 μg/g)

URCE: Data from Schmitz et al. (1991) and Kaplan et al. (1990) for tissues l Iowa State University Department of Statistics (1999) for serum.

re decreased deoxyribonucleic acid strand breaks observed when mg/day of β-carotene was administered as carrot juice (Poolbel et al., 1997) and lowered copper-induced oxidation of lowsity lipoprotein when 12 or 24 mg/day of β-carotene was given ng with vitamins C and E (Mosca et al., 1997). As shown in Table , feeding β-carotene in amounts greater than 25 mg/day gener- resulted in inconsistent responses of the biological markers nitored. Administration of β-carotene to subjects with increased dative stress (e.g., smoking, cystic fibrosis) was associated with re consistent evidence of decreased lipid peroxidation compared studies in which subjects without known additional oxidative ss were given β-carotene. In studies that involved depletion fol- ed by repletion of body stores of β-carotene, as indicated by sma concentrations, the biological markers that were negatively ered as a result of depleted body stores of β-carotene were re- ed to baseline values as a consequence of repletion (Table 8-2). summary, results from some studies show improvement of mea- es of antioxidant activity due to intake of relatively high levels of arotene, while studies that investigated low to modest levels of β- otene show no or inconsistent changes in the same activities. Some benefit of feeding increased amounts of β-carotene was observed for several markers of antioxidant activity when body stores were relatively low or when an oxidant-type stress was present. These observations suggest that the lack of effect in some studies may be due to study populations whose baseline β-carotene status was already adequate. Nevertheless, current data do not provide convincing evidence that substantially increasing β-carotene intake above current dietary intakes has a significant effect on measures of antioxidant status. Also, none of these markers has been validated to be predictive of any known health outcomes. Therefore, these data are inadequate for the estimation of a requirement for β-carotene.

Gap Junctional Communication

Appropriate communication among cells is essential for the coordination of biochemical functions in complex, multicellular organisms. One theory suggests that failure of signaling is one cause of cell overgrowth and eventually cancer. Two research groups have demonstrated that carotenoids stimulate gap junction communication between cells in vitro (Sies and Stahl, 1997; Zhang et al., 1991).

TABLE 8-2 β-Carotene Intake and Measures of Antioxidant Activity in Selected Studies

Reference, Country	Subjects	β-Carotene Dose	Duration	Plasma β-Carotene (μmol/L)	Findings
Richards et al., 1990 South Africa	40 smokers, average age 33 y; received placebo and 20 received treatment	40 mg/d, Roche prep	6 wk	Baseline—0.50 (27 μg/dL) Trt[a]—2.06 (111 μg/dL)	No change in leukocyte sister chromatid exchange
Mobarhan et al., 1990; Gottlieb et al., 1993 United States	15 healthy men, aged 19–30 y; randomly assigned repletion levels	Carotene-free diet (depletion); Repletion: 15 mg/d or 120 mg/d, Roche prep	2 wk 4 wk	Baseline—0.24 (13 μg/dL) Depletion—0.09 (5 μg/dL) 15 mg/d—3.32 (178 μg/dL) 120 mg/d—8.74 (469 μg/dL)	↓ Breath pentane on 120 mg/d only; ↓ Serum lipid peroxide levels, both repletion levels
Van Poppel et al., 1992a, 1992b, 1995 Holland	143 male smokers, average age 39 y; randomly assigned to placebo or treatment	40 mg/d first 2 wk 20 mg/d next 12 wk	2 wk 12 wk	Baseline—0.33 (18 μg/dL) Trt at 14 wk—4.36 (234 μg/dL)	↓ Sputum nuclei No change in lymphocyte sister chromatid exchange or urinary 8-oxodG[b]
Allard et al., 1994 Canada	38 male nonsmokers, 25 male smokers, aged 20–75 y; randomly assigned to placebo or treatment	20 mg/d, Roche prep	4 wk	Placebo/NS[c]—0.38 (20 μg/dL) Placebo/S[d]—0.27 (14 μg/dL) Trt/NS—3.50 (188 μg/dL) Trt/S—3.38 (181 μg/dL)	↓ Breath pentane in smokers No change in breath pentane in nonsmokers No change in breath ethane, RBC[e], MDA[f] or plasma Se-GSHPx[g] in either group
Galzada et al., 1995 United States	12 healthy men and 7 women, aged 21–50 y; randomly assigned to placebo or treatment	15 mg/d, Roche prep	14 d 56 d	Baseline—0.87 (47 μg/dL) Trt—3.07 (165 μg/dL)	No change in plasma Trolox equivalent antioxidant activity
Gaziano et al., 1995 United States	4 healthy men and 12 women, aged 25–47 y; randomly assigned to either synthetic (BASF) or natural (Henkel) β-carotene	100 mg/d load dose; natural treatment, 66 or 100 mg/2d; synthetic treatment, 50 mg/2 d	6 d load; followed by 21 d treatment	Baseline—0.25 (13 μg/dL) Both Trts—1.39 (75 μg/dL)	↑ Cu^{2+}-induced LDL[k] oxidation No change in AAPH[l]-induced LDL oxidation
Winklhofer-Roob et al., 1995 Switzerland	CF[m] patients, 32 boys and girls; average age 10.8 y	0.5 mg/kg BW[n]/d. 3M Medica, Ltd.	3 m	Baseline—0.09 (5 μg/dL) Trt—1.07 (57 μg/dL)	↓ Plasma MDA and Cu^{2+}-induced LDL oxidation
Clevidence et al., 1997 United States	5 healthy men and 7 women, aged 27–61 y	18 mg/d additional as foods; kale, tomato juice, sweet potato	3 wk	Baseline—0.29 (15 μg/dL) Trt—0.76 (40.2 μg/dL)	No change in plasma ORAC[o], plasma hydroperoxides, LDL TBARS, or 8-oxodG

continued

TABLE 8-2 Continued

Reference, Country	Subjects	β-Carotene Dose	Duration	Plasma β-Carotene (μmol/L)	Findings
Jininger et al., 1997 France	11 healthy men and 11 females, aged 25–45 y; 11 smokers and 11 nonsmokers	10 mg/d additional as foods (primarily carrots)	2 wk	Baseline/NS—0.95 (51 μg/dL) Baseline/S—0.58 (31 μg/dL) Trt/NS—1.13 (61 μg/dL) Trt/S—0.82 (44 μg/dL)	↑ RBC CuZn-SOD activity; No change in plasma MDA, GSH[h], GSSG, -SH groups, carbonyls, or Se-GSHPx activity
Iosca et al., 1997 United States	Coronary artery disease patients; 39 men and 6 women, aged 39–80 y	12 mg/d + vit E and vit C; or 24 mg/d + vit E and vit C	12 wk	Baseline—0.30 (16 μg/dL) 12 mg/d—1.99 (107 μg/dL) 24 mg/d—3.01 (162 μg/dL)	↓ Copper-induced LDL oxidation
ool-Zobel et al., 1997 Germany	23 healthy men, aged 27–40 y	Low carotenoid depletion food diet; followed by 22 mg/d as carrot juice	2 wk depletion; 4 wk added food containing β-carotene	Not reported	↓ DNA strand breaks (COMET) and oxidized pyrimidine bases in lymphocytes
Yang et al., 1997 Japan	192 healthy men; nonsmokers and smokers, aged 18–58 y	From foods; subjects grouped by plasma levels		0.56 (29.8 μg/dL) as criteria for establishing high/low intake	No difference in lymphocyte DNA adducts between high and low plasma β-carotene groups
ixon et al., 1998; Lin et al., 1998 United States	9 healthy women, premenopausal, aged 18–45 y	≤ 0.58 mg/d as diet or diet + small supplement for depletion; repletion 3.3 mg/d, Roche prep	100 d depletion; 20 d repletion	Baseline—0.76 (40.2 μg/dL) Depletion—0.33 (17.5 μg/dL) Repletion—1.73 (91.5 μg/dL)	Depletion ↑ plasma MDA and LDL oxidation rate (carbonyl production). Repletion ↓ LDL oxidation rate below baseline
ast et al., 1998 Austria	24 CF patients and 14 age-matched healthy children, average age 12.8 y	1 mg/kg BW/d to max of 30 mg/d	12 wk	Baseline—0.08 (4.8 μg/dL) 12 wk—0.60 (31.7 μg/dL)	↓ Plasma MDA on high-dose βC
einberg and Chait, 1998 United States	8 men and 12 women who smoked were in Trt group; average age 29 y	Vegetable-based juice + 30 mg β-Carotene/d + vit C & vit E	4 wk	Baseline—0.23 (12.3 μg/dL) Trt—1.21 (64.9 μg/dL)	↓ Breath pentane, LDL oxidation No change in plasma total peroxyl radical trapping

Conversion factor used for β-carotene = μmol/L ÷ 0.01863 = μg/dL.
[a] Trt = treatment.
[b] 8-OxodG = 8-oxo-7,8-dihydro-2′-deoxyguanosine.
[c] NS = nonsmokers.
[d] S = smokers.
[e] RBC = red blood cell.
[f] MDA = malondialdehyde.
[g] Se-GSHPx = selenium glutathione peroxidase.
[h] TBARS = thiobarbituric acid reactive substances.
[i] SOD = superoxide dismutase.
[j] GSSG = erythrocyte oxidized glutathione.
[k] LDL = low-density lipoprotein.
[l] AAPH = 2,2′-azobis[2-amidinopropane]dihydrochloride.
[m] CF = cystic fibrosis.
[n] BW = body weight.
[o] ORAC = oxygen radical absorbance capacity.
[p] GSH = glutathione.

It is not known whether the parent carotenoids or their metabolites are the active factors (Hanusch et al., 1995), nor is it known whether carotenoids influence this communication process in vivo. More study is needed to ascertain whether carotenoids play a direct role in cell-cell communication and, if so, what health outcomes are influenced by this action.

Immune Function

There has been great interest in the potential role of carotenoids n enhancement of the immune response. Children with vitamin A leficiency suffer from compromised immunity and have difficulty rotecting themselves from infections. It is important to remember, owever, that studies conducted with provitamin A carotenoids may ield results that are attributable to the conversion of carotenoids to itamin A or other retinoids, not to the effects of the intact caroenoid.

Santos et al. (1996) showed that long-term β-carotene supplemenation enhanced natural killer cell activity in men 65 to 86 years of ge, but not in men 51 to 64 years of age; enhancement by β-caroene in this age group was confirmed in a subsequent study (Santos t al., 1998). Hughes et al. (1997) evaluated mechanisms by which -carotene might enable immune cells to act more efficiently. Subcts were supplemented for 26 days with either 15 mg of β-carotene r a placebo. Subjects receiving the β-carotene treatment had ncreases in expression of adhesion molecules by monocytes, in ex vo secretion of tumor necrosis factor-α, and in the percentage of onocytes expressing major histocompatibility complex II, a cell rface molecule responsible for presenting antigen to T-helper lls.

Other immunological effects that carotenoids are reported to inease are lymphocyte response to mitogens (Kramer and Burri, 97) and total white blood cells and helper T cells in human immunodeficiency virus-infected humans (Coodley et al., 1993). hether these and the other effects noted are specific to carooids and are important in overall immunity is not confirmed. herefore the usefulness of these as markers for disease has yet to : established.

Relationship of Carotenoid Intake to Chronic Disease

A vast number of observational studies, including both case-con-ol and cohort studies, of carotenoids and chronic disease risk have been conducted. Many of the studies are based upon estimated intake of carotenoids in the diet, while many include biochemical evaluation of carotenoid concentrations in blood. Because the dietary intake data are generally obtained via food frequency questionnaires, they do not provide quantitative estimates of carotenoid intake, but rather allow for relative ranking of carotenoid intakes within a population. The blood concentration data, however, are more quantitative and generally more comparable across studies.

Prospective blood carotenoid concentration studies may be particularly informative because blood samples are generally obtained several years prior to the clinical detection of disease. Thus, for the purposes of evaluating the association between quantitative carotenoid exposure and risk of chronic disease, the prospective blood concentration studies are most useful and are given the greatest weight in the analysis that follows. The studies in which food intakes were the basis for evaluating risk of disease are less useful due to the inherent problems in adequately estimating carotenoid intake. These studies, however, may give support to the overall evaluation of the role of carotenoids in chronic disease. The following section briefly summarizes some key research findings from observational studies of the relationship between carotenoids and chronic disease risk.

Mortality

Greenberg et al. (1996) obtained blood samples from 1,188 men and 532 women enrolled in a skin cancer prevention trial and examined the relationship between plasma β-carotene concentrations at entry and subsequent mortality over a median follow-up period of 8.2 years (Table 8-3). Persons in the lowest quartile of plasma β-carotene had a significant increase in their risk of dying compared to those with higher plasma concentrations of β-carotene. The adjusted relative risk was lowest for persons with plasma β-carotene concentrations in the range of 0.34 to 0.53 μmol/L (18 to 28 μg/dL) (quartile 3), with a risk reduction (compared to the lowest quartile) of 43 percent for total deaths, 43 percent for cardiovascular disease deaths, and 51 percent for cancer deaths. The relative risk for overall mortality was 38 percent lower for persons who had plasma β-carotene concentrations in the highest quartile compared to the lowest quartile (relative risk [RR] = 0.62; 95 percent confidence interval [CI] = 0.44–0.87). Thus, these results suggest that plasma β-carotene concentrations in the range of 0.34 to 0.53 μmol/L (18 to 28 μg/dL) are associated with the lowest risk of all-cause

TABLE 8-3 Concentrations of β-Carotene and Total Carotenoids in Plasma or Serum Associated with a Lower Risk of Various Health Outcomes in Selected Studies

Author	Population	Endpoint	β-Carotene Concentration (μmol/L)[a]	Total Carotenoid Concentration (μmol/L)[a]
Nomura et al., 1985	Japanese men	Lung cancer	≥0.54 (29 μg/dL)	
Menkes et al., 1986	U.S. men and women	Lung cancer	≥0.54 (29 μg/dL)	
Connett et al., 1989	MRFIT[b] cohort men	Lung cancer	≥0.22 (12 μg/dL)[c]	≥1.84 (99 μg/dL)[c]
Greenberg et al., 1996	U.S. men and women, 24–84 y	All-cause mortality	0.34–0.53 (18–28 μg/dL)	
Jacques and Chylack, 1991	U.S. men and women, 40–70 y	Cataract		>3.3 (177 μg/dL)
Riemersma et al., 1991	British men	Angina	>0.54 (29 μg/dL)[d]	
Stahelin et al., 1991	Swiss men	Total cancers Lung cancer	≥0.34 (18 μg/dL)[d] ≥0.34 (18 μg/dL)[d]	
Batieha et al., 1993	U.S. women	Cervical cancer	≥0.26 (14 μg/dL)	≥1.73 (93 μg/dL)
EDCCSG, 1993	U.S. men and women	Macular degeneration	≥0.74 (40 μg/dL)	≥2.39 (128 μg/dL)
Eichholzer et al., 1992; Gey et al., 1993b	Swiss men	Ischemic heart disease	≥0.18 (10 μg/dL)[d]	
Zheng et al., 1993	U.S. men and women	Oropharyngeal cancers	≥0.28 (15 μg/dL)	≥1.75 (94 μg/dL)
Morris et al., 1994	U.S. men	CHD[e]		>3.16 (170 μg/dL)
West et al., 1994	U.S. men and women, ≥40 y	Macular degeneration	>0.88 (47 μg/dL)	
Sahyoun et al., 1996	U.S. men and women, >60 y	Cancer mortality CHD mortality All other causes, mortality		>3.13 (168 μg/dL) 1.73–3.13 (93–168 μg/dL) >3.13 (168 μg/dL)
Bonithon-Kopp et al., 1997	French men, >58 y French women, >58 y	Intima-media thickness Intima-media thickness		>2.1 (113 μg/dL) >3.7 (199 μg/dL)

[a] Concentration in the quartile/quantile where the risk reduction was of the greatest magnitude. For studies that only report mean or median concentrations in the diseased and disease-free groups, the concentration is the level in the group that remained free of disease. SI Conversion factor used for β-carotene and total carotenoids = 0.01863 μg/dL to μmol/L, with the exception of Greenberg et al., 1996.

[b] MRFIT = Multiple Risk Factor Intervention Trial.

[c] Samples were stored at −50°C.

[d] Assumes value given for carotene is 80% β-carotene.

[e] CHD = coronary heart disease.

mortality in U.S. adults. Note that these blood concentrations reflect levels in the absence of supplementation with β-carotene. Thus, this prospective study emphasizes the inverse association between β-carotene-rich foods and the risk of all-cause mortality.

Another cohort study of carotenoids and mortality examined both dietary intake of total carotenoids and plasma concentrations of total carotenoids as predictors of mortality (Sahyoun et al., 1996). Results indicated that mortality from cancer and all causes other than coronary heart disease (CHD) was lowest at a plasma concentration of 3.13 μmol/L (168 μg/dL) total carotenoids or greater; mortality from CHD was lowest at plasma concentrations of 1.73 to 3.13 μmol/L (93 to 168 μg/dL). Overall mortality was lowest at

etary carotenoid intake levels of 8.6 mg/day (RR = 0.68 compared those consuming 1.1 mg/day of carotenoids).
In the Western Electric cohort study, all-cause mortality was low- t for men who consumed the highest tertile of dietary β-carotene tR = 0.80 for more than 4.1 mg/day of β-carotene versus less than 9 mg/day of β-carotene; p for trend = 0.01) (Pandey et al., 1995).

ncer

Because there are literally hundreds of studies of carotenoids and ncer risk, this section emphasizes the results of epidemiological udies of all cancers combined, studies of carotenoids and lung ncer, and a few other selected tumor sites for which an inverse sociation with carotenoids is commonly seen.

Observational Epidemiological Studies. The Basel Prospective Study aluated the relationship between plasma carotene concentrations blood samples obtained in 1971–1973 and subsequent cancer ortality up to 1985 (Stahelin et al., 1991). Results showed that rsons who went on to develop any cancer had significantly lower ediagnostic carotene concentrations than persons who remained ve and free of cancer in 1985 (mean plasma total carotenoid ncentration 0.34 μmol/L [18 μg/dL] in those with cancer versus 43 μmol/L [23 μg/dL] in those free of cancer). The authors state at the reported carotene values represent approximately 80 per- nt β-carotene and 20 percent α-carotene; thus, plasma β-carotene ncentrations of approximately 0.34 μmol/L (0.43 μmol/L × 0.8) 3 μg/dL [23 μg/dL × 0.8]) were typical for the survivors of this hort. This concentration is within the range associated with lower k elsewhere as shown in Table 8-3.
Numerous epidemiological studies have shown that individuals o consume a relatively large quantity of carotenoid-rich fruits d vegetables have a lower risk of cancer at several tumor sites lock et al., 1992). The consistency of the results from observa- nal studies is particularly striking for lung cancer, where caro- noid and fruit and vegetable intake has been associated with low- lung cancer risk in 8 of 8 prospective studies and 18 of 20 rospective studies reviewed (Ziegler et al., 1996b).
Focusing on prospective blood analyses studies, the study with the gest number of cases (n = 99) was reported by Menkes et al. 986) as part of the Washington County, Maryland, cohort. The k of lung cancer increased in a linear fashion with decreasing um concentrations of β-carotene, with the greatest risk at the lowest quintile (cutpoint not stated). The mean concentration of serum β-carotene in persons who subsequently developed lung cancer was 0.47 μmol/L (25 μg/dL), compared to 0.54 μmol/L (29 μg/dL) in persons who remained free of disease.
Nomura et al. (1985) conducted a prospective study of 6,860 men of Japanese ancestry in Hawaii; 74 men subsequently developed lung cancer. Men who later developed lung cancer had lower serum β-carotene concentrations (0.37 μmol/L [20 μg/dL]) than control subjects (0.54 μmol/L [29 μg/dL]). Similar results were reported in the Basel Prospective Study. Men who later developed lung cancer (n = 68) had α- plus β-carotene serum concentrations of 0.30 μmol/L (16 μg/dL) versus 0.43 μmol/L (23 μg/dL) in survivors (Stahelin et al., 1991). The Multiple Risk Factor Intervention Trial (MRFIT) cohort study had prediagnostic serologic data on 66 lung cancer cases and 131 control subjects (Connett et al., 1989). Lung cancer cases had lower serum β-carotene concentrations (mean of 0.17 μmol/L [9 μg/dL]) and total carotenoid concentrations (1.62 μmol/L [87 μg/dL]) compared to the control subjects (0.22 μmol/ L [12 μg/dL] and 1.84 μmol/L [99 μg/dL]), respectively. The absolute carotenoid concentrations in this study are lower than those in the previous studies, which may be a consequence of long-term storage of the samples at –50°C, rather than at –70°C or colder as is recommended for carotenoids.
As for dietary studies, the majority of the studies of carotenoids and lung cancer risk have relied upon the U.S. Department of Agriculture (USDA) Nutrient Database for Standard Reference, Release 13, which does not contain estimates of the amount of carotenoids in various food items, but simply contains estimates of provitamin A activity. With the release of a new carotenoid database in 1993 (Mangels et al., 1993), quantitative studies relating consumption of individual carotenoids to lung cancer risk are now available. Le Marchand et al. (1993) found that higher dietary intake of α-carotene, β-carotene, and lutein was significantly associated with lower lung cancer risk in both men and women. Optimal levels of intake for each of these three carotenoids were as follows: β-carotene more than 4.0 mg/day for men and more than 4.4 mg/day for women; α-carotene more than 0.6 mg/day for men and more than 0.7 mg/day for women; and lutein more than 3.3 mg/day for both males and females. Ziegler et al. (1996a) also found significant inverse trends for dietary α- and β-carotene and a marginally significant effect for lutein and zeaxanthin with risk of lung cancer. Optimal levels in this study were as follows: β-carotene 2.5–5.9 mg/day; α-carotene more than 1.5 mg/day; and lutein and zeaxanthin more than 4.2 mg/day.

As reviewed elsewhere, retrospective and prospective epidemiological studies of diet and serum carotenoids strongly indicate that greater consumption of fruits, vegetables, and carotenoids is inversely associated with risk of cancers of the oral cavity, pharynx, and larynx (Mayne, 1996; Mayne and Goodwin, 1993). In a review (Block et al., 1992), 13 of 13 studies indicated that fruit and vegetable intake was associated with reduced risk of cancers of the oral cavity, pharynx, and larynx. As for prospective serologic studies, Zheng et al. (1993) conducted a nested case-control study of serum micronutrients and subsequent risk of oral and pharyngeal cancer. Blood samples were collected and stored in 1974 from a cohort of 25,802 adults in Maryland. Over the next 15 years, 28 individuals developed oral or pharyngeal cancer. Serum analyses indicated that prediagnostic serum concentrations of all the major individual carotenoids, particularly β-carotene, were lower among the case group than among control subjects selected from the same cohort. β-Carotene concentrations in persons who later developed these cancers were 0.21 μmol/L (11 μg/dL) versus 0.28 μmol/L (15 μg/dL) in control subjects (mean; $p = 0.03$). Adjustment for smoking, which is known to be associated with lower serum carotenoid concentrations, attenuated the protective association slightly. The unadjusted and adjusted relative odds of oral or pharyngeal cancer, comparing the upper tertile of serum β-carotene concentrations (cutpoints not given) versus the lower tertile, were 0.50 and 0.69, respectively.

One recent prospective cohort study (Giovannucci et al., 1995) evaluated 47,894 participants in the Health Professionals Follow-up Study, 812 of whom were diagnosed with prostate cancer during the 6 year follow-up. Intake of tomato-based foods (tomato sauce, tomatoes, and pizza—but not tomato juice) and lycopene, which is found predominantly in tomato products, was associated with significantly lower prostate cancer risk. Risk was lowest for those who were estimated to consume more than 6.46 mg/day of lycopene. The lack of association for tomato juice may reflect the fact that lycopene is more bioavailable from processed tomato products than from fresh tomatoes (Gartner et al., 1997).

A prospective study of serum micronutrients and prostate cancer in Japanese men in Hawaii, however, found no difference in prediagnostic serum lycopene concentrations in 142 cases versus 142 matched control subjects (Nomura et al., 1997). The lack of effect seen in this study could possibly relate to the fact that serum lycopene concentrations were relatively low in this population (median 0.25 μmol/L [13 μg/dL]). This is likely a consequence of the fact that tomato products are not widely consumed in the Asian diet (thus the range of exposure may have been limited). Comprehensive reviews of the relationship between lycopene and prostate cancer have been published elsewhere (Clinton, 1998; Giovannucci, 1999).

Consumption of fruits and vegetables also has been reported to be inversely associated with cervical cancer risk in a number of studies. Batieha et al. (1993) conducted a nested case-control study, analyzing a variety of carotenoids in sera stored from 50 women who had developed either invasive cervical cancer or carcinoma in situ during a 15-year follow-up and in 99 control women pair-matched to the cases. The risk of cervical cancer was significantly higher among women with the lowest prediagnostic serum concentrations of total carotenoids (odds ratio [OR] = 2.7; 95 percent CI = 1.1–6.4), α-carotene (OR = 3.1; 95 percent CI = 1.3–7.6), and β-carotene (OR = 3.1; 95 percent CI = 1.2–8.1) compared to women in the upper tertiles. Mean serum concentrations of β-cryptoxanthin were also lower among cases relative to control subjects ($p = 0.03$). Optimal concentrations of these carotenoids for reducing the risk of cervical cancer were as follows: total carotenoids greater than 1.88 μmol/L (101 μg/dL); α-carotene greater than 0.05 μmol/L (2.7 μg/dL); β-carotene greater than 0.26 μmol/L (14 μg/dL); and cryptoxanthin greater than 0.17 μmol/L (9 μg/dL).

Intervention Trials. Three major double-blind, randomized intervention trials have been conducted using high-dose β-carotene supplements, either alone or in combination with other agents, in an attempt to evaluate any protective role in the development of lung or total cancers. In none of these studies was there any evidence of a protective role for supplementary β-carotene.

In current smokers participating in the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study, supplementation with 20 mg/day of β-carotene (with or without 50 mg of α-tocopherol) for 5 to 8 years led to a higher incidence in lung cancer but had no effect on the incidence of other major cancers occurring in this population (prostate, bladder, colon or rectum, or stomach) (ATBC Cancer Prevention Study Group, 1994). In addition, the Carotene and Retinol Efficacy Trial (CARET) used a nutrient combination of β-carotene (30 mg/day) plus retinyl palmitate (25,000 international units [IU]/day) versus placebo in asbestos workers and smokers (Omenn et al., 1996a, 1996b). This study reported more lung cancer cases in the supplemented group. The Physicians' Health Study (PHS) of supplemental β-carotene versus placebo in 22,071 male

U.S. physicians reported no significant effect of 12 years of supplementation of β-carotene (50 mg every other day) on cancer or total mortality (Hennekens et al., 1996).

Summary. Higher consumption of carotenoid-containing fruits and vegetables and higher plasma concentrations of several carotenoids, including β-carotene, are associated with a lower risk of many different cancers, especially lung, oral cavity, pharyngeal, laryngeal, and cervical cancers. These prospective blood concentration studies show that β-carotene concentrations in the range of 0.28 μmol/L (15 μg/dL) or less are associated with higher risk of many cancers (Table 8-3), whereas concentrations greater than 0.28 to 0.37 μmol/L (15 to 20 μg/dL) are associated with reduced risk of many cancers. This approximate threshold for cancer risk reduction is concordant with that for the prevention of all-cause mortality, given above. Furthermore, these studies show that increased consumption of foods containing these carotenoids, including carotenoids lacking vitamin A activity, is associated with risk reduction. However, in three large randomized clinical trials using high-dose β-carotene supplements (20 or 30 mg/day or 50 mg given every other day) for 4 to 12 years, no protection was reported with respect to lung cancer, or any other cancer.

Cardiovascular Disease

Epidemiological studies, including descriptive, cohort, and case-control studies, suggest that carotenoid- and β-carotene-rich diets are associated with a reduced risk of cardiovascular disease (Gaziano and Hennekens, 1993; Kohlmeier and Hastings, 1995; Manson et al., 1993). Beginning with biochemical epidemiological studies of plasma carotenoids, Gey et al. (1993a) reported data from the Vitamin Substudy of the World Health Organization's Monitoring Cardiovascular (WHO/MONICA) Project, in which plasma was obtained from approximately 100 apparently healthy men from each of 16 study sites within Europe. A comparison between median plasma β-carotene concentrations and ischemic heart disease mortality revealed no association when all 16 study sites were considered ($r^2 = 0.04$). However, a reasonably strong inverse association was evident ($r^2 = 0.50$) when three study sites, all apparent outliers and all Finnish sites), were excluded from the analysis.

Men in the Basel Prospective Study, who had low blood concentrations of β-carotene and vitamin C initially and who were followed for 12 years, had a significantly higher risk of subsequent ischemic heart disease (RR = 1.96; $p = 0.022$) and stroke (RR = 4.17; $p = 0.002$) (Eichholzer et al., 1992; Gey et al., 1993b). Based upon these and other data, Gey et al. (1993a) proposed that more than 0.4 to 0.5 μmol/L (21 to 27 μg/dL) α-plus β-carotene or 0.3 to 0.4 μmol/L (16 to 21 μg/dL) β-carotene is needed to reduce the risk of ischemic heart disease.

Total serum carotenoids, measured at baseline in the placebo group of the Lipid Research Clinics Coronary Primary Prevention Trial, were inversely related to subsequent coronary heart disease events (Morris et al., 1994). Men in the highest quartile of total serum carotenoids (more than 3.16 μmol/L [172 μg/dL]) had an adjusted relative risk of 0.64 (95 percent CI = 0.44–0.92); among those who never smoked, the relative risk was 0.28 (95 percent CI = 0.11–0.73). Riemersma et al. (1991) reported that persons with plasma carotene concentrations in the lowest quintile (less than 0.26 μmol/L [14 μg/dL]) had 2.64 times the risk of angina pectoris. Adjustment for smoking reduced the magnitude of risk. However, because smoking may be part of the causal path, adjustment may not be appropriate.

The U.S. Health Professionals Follow-up Study of over 39,000 men reported a relative risk for coronary heart disease of 0.71 (95 percent CI = 0.55–0.92) for those at the top quintile of total carotene intake relative to the lowest quintile of intake (Rimm et al., 1993). The effect of β-carotene varied by smoking status: among current smokers, the relative risk was 0.30 (95 percent CI = 0.11–0.82); among former smokers, the risk was 0.60 (95 percent CI = 0.38–0.94), and among nonsmokers, the risk was 1.09 (95 percent CI = 0.66–1.79). A prospective cohort study of postmenopausal women found that the lowest risk of coronary heart disease was found for dietary carotenoid intakes greater than 8,857 IU/day (RR = 0.77; p = NS) (Kushi et al., 1996). A case-control study in 10 European countries found that lycopene concentrations, but not other carotenoid concentrations, in adipose tissue were inversely associated with the risk of myocardial infarction (Kohlmeier et al., 1997).

Cardiovascular epidemiology studies are now pursuing the use of intermediate endpoints, such as intima-media thickness, which can be estimated via ultrasonography as a measure of atherosclerosis. Bonithon-Kopp et al. (1997) reported a decrease in the intima-media thickness of the common carotid arteries with increasing concentrations of total plasma carotenoids in both men and women. Plasma carotenoid concentrations in excess of 2.07 μmol/L (111 μg/dL) were optimal for men; concentrations in excess of 3.73 μmol/L (200 μg/dL) were optimal for women. Salonen et al. (1993)

evaluated the change in the intima-media thickness as a measure of atherosclerotic progression and reported that progression was 92 percent greater in the lowest (less than or equal to 0.27 μmol/L [14 μg/dL]) versus the highest (more than or equal to 0.64 μmol/L [34 μg/dL]) quartile of plasma β-carotene.

Age-Related Macular Degeneration

Dietary carotenoids have been suggested to decrease the risk of age-related macular degeneration (AMD), the most common cause of irreversible blindness in people over age 65 in the United States, Canada, and Europe (Seddon et al., 1994; Snodderly, 1995). The macula lutea (macula) is a bright yellow spot in the center of the retina and is specialized and functions to maintain acute central vision. Of all the carotenoids circulating in the body, only two polar species, lutein and zeaxanthin, are contained in the macula (Bone et al., 1985; Handelman et al., 1988). Two groups of investigators have suggested pathways by which these two carotenoids are biochemically interchanged in the macula (Bone et al., 1993; Khachik et al., 1997a).

The potential role of carotenoids in the prevention of AMD has been comprehensively reviewed (Snodderly, 1995). Seddon et al. (1994) analyzed the association between carotenoid intake and advanced AMD in a large, multicenter, case-control study involving 356 cases and 520 control subjects with other ocular conditions. Those in the highest quintile of dietary carotenoid intake had a 43 percent lower risk for macular degeneration compared with those in the lowest (OR = 0.57; 95 percent CI = 0.35–0.92). Among the specific carotenoids, intake of lutein and zeaxanthin (grouped in the carotenoid food composition database) was most strongly associated with decreased risk. Those in the highest quintile of intake had a 60 percent lower risk compared to the lowest quintile of intake.

Some, but not all, studies using blood carotenoid concentrations also suggest protective effects against risk of AMD. The Eye Disease Case-Control Study (EDCCSG, 1993) measured serum carotenoids in 391 cases with neovascular AMD and 577 control subjects. The study reported protective effects of total carotenoids, α-carotene, β-carotene, β-cryptoxanthin, and lutein and zeaxanthin, with odds ratios ranging from 0.3 to 0.5 for the high group (more than the eightieth percentile) versus the low group (less than the twentieth percentile). Carotenoid concentrations associated with the lowest risk are shown in Table 8-4.

TABLE 8-4 Example of Plasma Carotenoid Concentrations Associated with Lowest Risk of Age-Related Macular Degeneration

Carotenoids	Concentrations (μmol/L)[a]
Total carotenoids	≥2.39 (128 μg/dL)
α-Carotene	≥0.19 (10 μg/dL)
β-Carotene	≥0.74 (40 μg/dL)
β-Cryptoxanthin	≥0.32 (18 μg/dL)
Lutein and zeaxanthin	≥0.67 (38 μg/dL)
Lycopene	≥0.61 (33 μg/dL)

[a] SI conversion factor used for total carotenoids, α- and β-carotene, and lycopene = 0.01863 μg/dL to μmol/L; for β-cryptoxanthin = 0.01809; and for lutein and zeaxanthin = 0.01758.
SOURCE: EDCCSG (1993).

Mares-Perlman et al. (1994) examined the association between serum carotenoid concentrations and age-related maculopathy in 167 case-control pairs and reported no association for any of the carotenoids, except lycopene, with persons in the lowest quintile of lycopene having a doubling in risk of maculopathy (cutpoint not stated). West et al. (1994) examined the relationship between plasma β-carotene concentration and AMD in 226 subjects and found the risk was lowest for the highest quartile of plasma β-carotene (more than 0.88 μmol/L [47 μg/dL]) (OR high quartile versus low = 0.62). Plasma lutein and zeaxanthin were not measured in this study.

Hammond and Fuld (1992) developed an optical system that, in situ, measures the intensity of the unique yellow color of the macula and presumably estimates the levels of lutein and zeaxanthin. This measure is known as Macular Pigment Optical Density (MPOD). Dietary intake of carotenoids, fat, and iron, as well as plasma concentrations of lutein and zeaxanthin, were positively related with MPOD in men, but only plasma concentrations of lutein and zeaxanthin were associated with MPOD values for women (Hammond et al., 1996). In the same studies, men had significantly higher MPOD readings than women despite similar plasma carotenoid concentrations and similar dietary intake, except for fat. These investigators also demonstrated that the MPOD of most subjects could be substantially increased by the addition of relatively small amounts of foods to the diet that are high in lutein (½ cup

spinach per day) or lutein and zeaxanthin (1 cup of corn per day) (Hammond et al., 1997). Interestingly, when MPOD was enhanced following dietary modification, it was maintained at that level for several months despite resumption of an unmodified diet.

In summary, results of studies that have investigated MPOD as a biological indicator of carotenoid adequacy suggest that it has substantial potential as an indicator for estimating the requirements for lutein and zeaxanthin. Because of the unique metabolism of carotenoids in the macula, this technique will be useful in associating dietary intakes of lutein and zeaxanthin with the health of the macula. However, insufficient MPOD studies have been conducted to date to make recommendations relative to the dietary intakes of lutein and zeaxanthin.

Cataracts

Cataracts are also problematic, with cataract extraction being the most frequently performed surgical procedure in the elderly (Taylor, 1993). Although the etiology of this condition is not known, oxidative processes may play a role. Cataracts are thought to result from photo-oxidation of lens proteins, resulting in protein damage, accumulation, aggregation, and precipitation in the lens (Taylor, 1993). The cornea and lens filter out ultraviolet light, but visible blue light reaches the retina and may contribute to photic damage or other oxidative insults (Seddon et al., 1994).

Higher dietary intake of carotenoids or higher blood concentrations of carotenoids have been found to be inversely associated with the risk of various forms of cataract in some, but not all, studies. Jacques and Chylack (1991) reported that subjects with low plasma carotenoid concentrations (those with concentrations less than the twentieth percentile: less than 1.7 μmol/L [90 μg/dL]) had a 5.6-fold increased risk of any senile cataract and a 7.2-fold increased risk of cortical cataract, compared with subjects with high plasma total carotenoid concentrations (greater than the eightieth percentile; more than 3.3 μmol/L [177 μg/dL]). Mares-Perlman et al. (1995) performed a cross-sectional analysis of serum α-carotene, β-carotene, β-cryptoxanthin, lutein and zeaxanthin, and lycopene versus the severity of nuclear and cortical opacities, and found that higher concentrations of individual or total carotenoids were not associated with the severity of nuclear or cortical opacities overall. However, higher serum β-carotene (highest quintile median concentration 0.32 μmol/L [17 μg/dL]) was associated with less opacity in men, and higher concentrations of α-carotene (highest quintile median 0.14 μmol/L [7.5 μg/dL]), β-cryptoxanthin (highest quintile median 0.31 μmol/L [17 μg/dL]), and lutein (highest quintile median 0.44 μmol/L [25 μg/dL]) were associated with less nuclear sclerosis in men who smoked. In women, however, higher concentrations of some carotenoids (highest quintile median 2.19 μmol/L [118 μg/dL]) were associated with an increased severity of nuclear sclerosis.

Recently, the U.S. Health Professionals Follow-up Study reported a relative risk for cataract extraction in men of 0.81 (95 percent CI = 0.65–1.01) for those at the top quintile of lutein and zeaxanthin intake (median intake of 6.87 mg/day) relative to the lowest quintile of intake (Brown et al., 1999). Similar inverse associations for dietary lutein and zeaxanthin were seen in the Nurses' Health Study cohort, with a relative risk of 0.78 (95 percent CI = 0.63–0.95) for those at the top quintile of total lutein and zeaxanthin intake (median intake of 11.68 mg/day) relative to the lowest quintile of intake (Chasan-Taber et al., 1999). This decreased risk of cataract (severe enough to require extraction) with higher intakes of lutein and zeaxanthin was not found with higher intakes of other carotenoids (α-carotene, β-carotene, lycopene, and β-cryptoxanthin) in either of these studies.

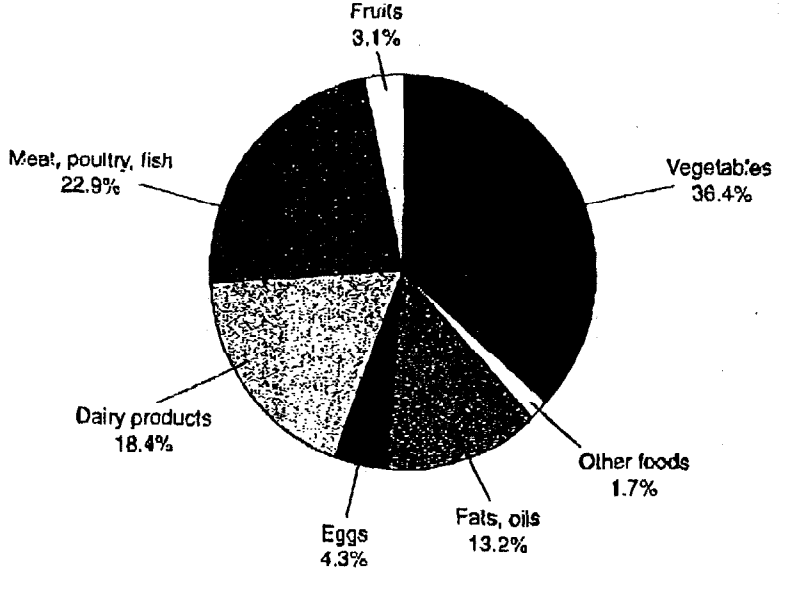
Plasma and Tissue Concentrations

As just detailed, plasma and tissue concentrations of carotenoids have been associated with a variety of health outcomes; that is, higher concentrations are associated with a lower risk of cancer, coronary heart disease, and all-cause mortality. This could be used as a possible indicator for establishing requirements for carotenoids. However, the limitation of this approach is that it is not clear whether observed health benefits are due to carotenoids per se or to other substances found in carotenoid-rich foods.

Thus, these data are suggestive of prudent intake levels, not required levels of intake. Recommendations have been made by a number of federal agencies and other organizations with regard to fruit and vegetable intake. Nutrient analysis of menus adhering to the U.S. Dietary Guidelines and the National Cancer Institute's Five-a-Day for Better Health Program, for example, indicates that persons following these diets would be consuming approximately 5.2 to 6.0 mg/day provitamin A carotenes on average if a variety of fruits and vegetables were consumed (Lachance, 1997). Similar levels would be obtained by following Canada's Food Guide for

ealthy Eating which specifies a minimum of five servings of vege-
bles and fruit (Health Canada, 1997). Other food-based dietary
tterns recommended for the prevention of cancer and other
ronic diseases would provide approximately 9 to 18 mg/day of
rotenoids (WCRF/AICR, 1997).

The current U.S. and international guidelines encourage plant-
ised dietary patterns with less emphasis on foods of animal ori-
n. With this type of dietary pattern, approximately 90 percent of
e total ingested vitamin A would be in the form of provitamin A
rotenoids (Lachance, 1997). This pattern is in stark contrast to
rrent intake patterns in the United States, where less than 40
rcent of vitamin A in the diet is derived from provitamin A caro-
noids in fruits and vegetables (Figure 8-2), or to the intake pat-
rns found in native Americans in some arctic regions of the
ited States and Canada (Kuhnlein et al., 1996).



URE 8-2 Contributors to Vitamin A intake in the U.S. food supply. The "other
ls" category includes grain products (0.5 percent) and miscellaneous foods
percent).
RCE: LSRO/FASEB (1995).

An examination of human studies using dietary interventions with carotenoid-containing foods is necessary to determine the plasma carotene concentrations that an optimal diet would be expected to produce. In a controlled diet study (Micozzi et al., 1992), plasma β-carotene concentrations in the men who received the low carotenoid diet (less than 2 mg/day) to which broccoli had been added to provide 6 mg/day of carotenoids (3 mg of β-carotene, 3 mg of lutein) were raised significantly from 0.30 μmol/L (16 μg/dL) at baseline to 0.49 μmol/L (26 μg/dL) after six weeks, as were plasma lutein concentrations (from 0.38 μmol/L [22 μg/dL] to 0.63 μmol/L [36 μg/dL]). Plasma lycopene declined with this intervention because the baseline diet as well as broccoli was low in the content of lycopene and other carotenoids.

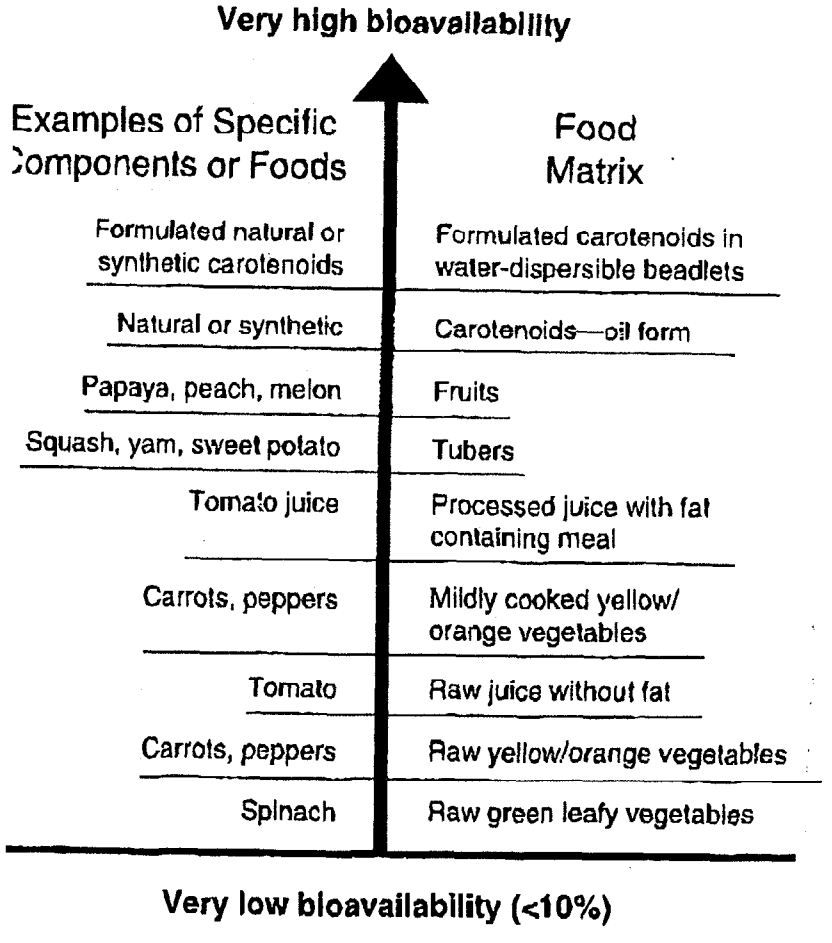
The Minnesota Cancer Prevention Research Unit feeding studies evaluated three experimental diets (two of which included carotenoids) and one control diet given for 9 days each to 23 young nonsmoking men and women. Persons on the control diet had a plasma β-carotene concentration of 0.26 μmol/L (14 μg/dL); 5 mg/day β-carotene from food increased plasma β-carotene to 0.37 μmol/L (19.5 μg/dL). When β-carotene from food was increased to 42 mg/day, plasma β-carotene increased further to 0.83 μmol/L (44 μg/dL) (Martini et al., 1995). Yong et al. (1994) studied dietary carotenoid intake and plasma carotenoids cross-sectionally in premenopausal nonsmoking women; the population had a geometric mean β-carotene intake of approximately 3 mg/day and a geometric mean plasma β-carotene concentration of 0.30 μmol/L (15.8 μg/dL). For total carotenoids, the geometric mean level of intake was 6.6 to 8.1 mg/day, with a total carotenoid concentration in plasma of approximately 1.51 μmol/L (80 μg/dL). A randomized, controlled trial on the effect of increasing fruit and vegetable intake for 8 weeks on plasma micronutrient concentrations was conducted with 87 subjects in New Zealand (Zino et al., 1997). β-Carotene intake increased from about 2.0 mg/day at baseline to 4.7 mg/day at week 4. This resulted in a mean plasma β-carotene increase from 0.34 μmol/L (18 μg/dL) at baseline to 0.48 μmol/L (25 μg/dL) at 4 weeks.

These data, although in varying populations, suggest that 3 to 6 mg/day of β-carotene from food sources is prudent to maintain plasma β-carotene concentrations in the range associated with a lower risk of various chronic disease outcomes (see Table 8-3).

FACTORS AFFECTING CAROTENOID BIOAVAILABILITY

Bioavailability

Bioavailability of carotenoids from food, concentrated extracts, or ıthetic products is quite variable (Figure 8-3) because a complex : of factors affects carotenoid bioavailability. Erdman et al. (1993) d Castenmiller and West (1998) described the events necessary r adequate absorption of carotenoids from the diet: (1) digestion the food matrix, (2) formation of lipid micelles in the gastrointes-



URE 8-3 Effect of food matrix and processing on bioavailability of carotenoids. URCE: Adapted from Boileau et al (1999).

ginal tract, (3) uptake of carotenoids by intestinal mucosal cells, and (4) transport of carotenoids and their metabolic products to the lymph or portal circulation.

Food Matrix

Of the factors that affect carotenoid bioavailability, the food matrix effects on carotenoid absorption are generally the most critical. The absorption of β-carotene supplements that are solubilized with emulsifiers and protected by antioxidants can be 70 percent or more. In contrast, less than 5 percent bioavailability of carotenes has been reported from raw foods such as carrots (Rodriguez and Irwin, 1972). Recently, van het Hof et al. (1999) reported substantial differences between the relative bioavailabilities of β-carotene (14 percent) compared to lutein (67 percent) when feeding a high-vegetable diet (490 g of vegetables without supplements) and comparing it to a low-vegetable diet (130 g of vegetables) supplemented with β-carotene (6 mg/day) or lutein (9 mg/day), both of which were assumed to be 100 percent bioavailable. These differences were based on changes in plasma concentration of β-carotene or lutein.

Daily supplementation of dark-green leafy vegetables rich in carotenoids to lactating Indonesian women with low vitamin A status did not increase vitamin A status, whereas a similar amount of β-carotene given in a wafer supplement led to a significant increase in plasma retinol (de Pee et al., 1995). More recently, the same group (de Pee et al., 1998) studied anemic school children in Indonesia and calculated the relative vitamin A equivalency of β-carotene from different food sources. The calculated equivalencies were as follows: 26 μg of β-carotene from leafy vegetables and carrots corresponded to 12 μg of β-carotene from fruit, and equaled 1 μg of preformed vitamin A in vitamin A-rich foods. In contrast, Mahapatra and Manorama (1997), in a small study with vitamin A-deficient school children in India, concluded that β-carotene from red palm oil was as bioavailable as preformed vitamin A.

β-Carotene in the form of supplements has a much higher bioavailablity than β-carotene from foods. Micozzi et al. (1992) demonstrated that 30 mg/day of supplemental *all-trans* β-carotene produced more than a fivefold increase in plasma β-carotene compared to 29 mg/day of β-carotene from carrots. The relatively low bioavailability of plant carotenoids may be due to the fact that they can be bound in carotenoproteins and are often associated with the plant matrix. Typically in green leafy vegetables, carotenoids are found bound in chloroplasts where they play roles in photosynthesis. In

arrot root, α- and β-carotene are largely in crystal forms. In both ases, the carotenoids are not easily solubilized out of these tissues y the digestive process.

Cooking

The hypothesis that cooking may improve the bioavailability of arotenoids has been tested. The bioavailability of lycopene from omato juice is vastly improved by heat treatment in the presence of il (Gartner et al., 1997; Stahl and Sies, 1992). When subjects con-med tomato juice (equivalent to a single lycopene dose of 2.5 mol/kg body weight) that had been heated at 100°C for 1 hour ith oil, they experienced a serum lycopene peak at 24 to 48 hours. n contrast, equivalent doses that were not heat treated did not sult in an increase in serum lycopene. Steaming has also been own to increase the amount of extractable carotenoids in spinach id carrots (Dietz et al., 1988). In contrast to steaming, more pro-nged exposure to high temperatures (boiling) can reduce the rotenoid availability of vegetables by increasing the production of omers or oxidation products. For example, canned carrots contain percent *all-trans* β-carotene, 19 percent 13-*cis*-β-carotene, and 8 rcent 9-*cis*-β-carotene, while fresh carrots contain 100 percent of e β-carotene in the *all-trans* configuration (Chandler and Schwartz, 87). The relative vitamin A values of *cis* isomers of β-carotene mpared to *all-trans* β-carotene is an active area of research.

etary Fat

Many research groups have shown that to optimize carotenoid sorption, dietary fat must be consumed during the same eating riod as the carotenoid. Roels et al. (1958) demonstrated that in ys with vitamin A deficiency in an African village, supplementa-n of their carotene-sufficient but low-fat diets with 18 g/day of ve oil improved carotene absorption from 5 to 25 percent. More ently, Jalal et al. (1998) studied the roles of β-carotene-rich meals ostly red sweet potatoes), extra dietary fat (15 g/day), and de-rming on serum retinol concentrations of children in Sumatra. or to the intervention, these children all had intestinal infesta-ns and were consuming diets with about 7 percent of calories m fat. A 3-week intervention of β-carotene-rich meals alone im-ved vitamin A status without added fat or deworming, but the nbination of all three measures—β-carotene meals, added fat, l deworming—provided the greatest increase in serum retinol.

Other Factors

Lipid-lowering drugs have been shown to decrease serum carotenoids dramatically (Elinder et al., 1995). In a double-blind, randomized trial, treatment with cholestyramine (a lipid-lowering resin) for 4 months and probucol (antioxidant and lipid-lowering drug) for 2 months resulted in a 65 percent reduction in serum β-carotene and a 51 percent reduction in lycopene. The reductions were attributed to reduced intestinal absorption of lipids by cholestyramine and reduced lipoprotein particle number and size by probucol. Sucrose polyester (olestra), the nonabsorbable fat substitute, lowered carotenoid absorption when consumed at the same time as carotenoids (Koonsvitsky et al., 1997; Weststrate and van het Hof, 1995). Plant sterol-enriched margarines (Weststrate and Meijer, 1998) and dietary pectin supplementation also decreased β-carotene absorption (Rock and Swendseid, 1992).

Nutrient-Nutrient Interactions

Competitive interactions among different carotenoids during the absorptive process have been studied. Recipients of daily β-carotene supplements in either 12-mg or 30-mg capsules for 6 weeks had significantly lower plasma lutein concentrations than subjects who consumed both β-carotene and lutein from food sources (Micozzi et al., 1992). In addition, plasma β-carotene was higher in the subjects receiving β-carotene as supplements rather than as food, demonstrating the greater bioavailability of this source. Interactions between β-carotene and lutein have also been described by other investigators. When subjects were given purified crystalline β-carotene and crystalline lutein in a combined dose, β-carotene significantly reduced the serum area under the curve (AUC) value (a measure of total absorption) for lutein (Kostic et al., 1995). Lutein in a combined dose with β-carotene significantly enhanced β-carotene AUC in those subjects whose AUC for β-carotene (when dosed alone) was the lowest.

These studies (White et al., 1994) indicate that two carotenoids administered concurrently in controlled settings can affect the absorption of each other. Several investigators have examined the effect of daily supplementation with high-dose β-carotene on plasma concentrations of other carotenoids in participants in multiyear cancer prevention intervention trials (Albanes et al., 1997; Mayne et al., 1998; Nierenberg et al., 1997; Wahlqvist et al., 1994). These studies suggest no overall adverse effect on other carotenoids with

high-dose supplementation of β-carotene daily for several years. This finding is not inconsistent with the results of the metabolic studies, because the trials were done in free-living individuals taking a supplement of β-carotene each day, which most likely is not consumed concurrently with an entire day's intake of other carotenoids from food.

FINDINGS BY LIFE STAGE AND GENDER GROUP

As discussed elsewhere in this document, this report does not establish a requirement for β-carotene or other carotenoids for any gender or life stage group. This issue will be considered in a subsequent report when addressing vitamin A. However, the following summarizes findings regarding carotenoid status, as measured by serum carotenoid concentrations, in different groups of the population.

Special Populations

If plasma carotenoid concentrations are considered as an indicator of adequacy with regard to reducing risk of chronic disease, it becomes apparent that certain subgroups of the population are known to have notably lower circulating concentrations of carotenoids. Thus, consumption of carotenoid-containing foods may have to be greater in these groups in order to achieve plasma carotenoid concentrations that are associated with a reduced risk of chronic disease (Table 8-3).

Adolescents

Serum carotenoid concentrations were measured in the Third National Health and Nutrition Examination Survey (NHANES III). As shown in Appendix Table F-4, serum β-carotene concentrations were lower during the period of adolescence and early adulthood in this U.S. population survey. The average concentration in children was approximately 0.34 μmol/L (18 μg/dL), which dropped to 0.28 μmol/L (15 μg/dL) or less in teenagers and did not return to childhood concentrations until the fourth decade (the thirties) for women, and the fifth decade (the forties) for men. This lower level during adolescence is also evident for α-carotene (Appendix Table F-5), β-cryptoxanthin (Appendix Table F-6), and lutein/zeaxanthin (Appendix Table F-7), but not lycopene (Appendix Table F-8). This may reflect relatively greater consumption of tomato products compared to other vegetables by adolescents in the United States.

Smoking

Many investigators have reported that those who smoke, on average, have lower plasma carotenoid concentrations compared to individuals that don't smoke (Brady et al., 1996; Chow et al., 1986; Comstock et al., 1988; Fukao et al., 1996; Herbeth et al., 1990; Margetts and Jackson, 1996; Pamuk et al., 1994; Stryker et al., 1988; Witter et al., 1982). The greater the intensity of smoking (cigarettes per day), the greater is the decrease in serum carotenoid concentrations. Fukao et al. (1996) studied 1,902 Japanese men in a cohort study and showed a dose-dependent decline in geometric mean serum β-carotene with greater smoking intensity (Table 8-5).

While smokers ingest less β-carotene than nonsmokers, it is unclear at present whether or not the lower serum concentrations seen can be fully explained by the reduced β-carotene intakes of smokers, as discussed recently by Brady et al. (1996). Many studies find differences in serum carotenoid concentrations even after adjusting for intake. However, because dietary intake is necessarily measured with some error, it is unclear whether full adjustment is possible. Tobacco smoke is known to be highly oxidative, and the gas phase of tobacco smoke has been shown to destroy β-carotene and other carotenoids in in vitro studies of human plasma (Handelman et al., 1996). As demonstrated recently by Baker et al. (1999), both smoke and gas-phase smoke oxidize β-carotene to carbonyls, epoxides, and nitro derivatives. Thus, it is possible that the smoke oxidatively degrades β-carotene in vivo and thus contributes to the reduction in circulating levels.

TABLE 8-5 Serum β-Carotene in Men in Relation to Smoking

	Mean[a] Serum β-Carotene (μmol/L)
Nonsmokers	0.39 (20.7 μg/dL)
Ex-smokers	0.31 (16.6 μg/dL)
Smokers	
1–10 cigarettes/d	0.25 (13.6 μg/dL)
11–20 cigarettes/d	0.23 (12.1 μg/dL)
21–100 cigarettes/d	0.20 (10.5 μg/dL)

[a] Geometric mean.
SOURCE: Fukao et al. (1996).

first report in the area of putative mechanisms to explain the ease in lung cancer risk observed in heavy smokers taking high- e supplements indicates that ferrets exposed to cigarette smoke supplemented with β-carotene developed squamous metaplasia heir lungs as well as altered retinoid signaling (Wang et al., 9). Another report suggests that oxidation products of β-caro- e stimulate the binding of metabolites of benzo[*a*]pyrene to xyribonucleic acid (Salgo et al., 1999). These very new data await firmation and further development.

lthough smoking may result in a need for higher intakes of di- y carotenoids to achieve optimal plasma carotenoid concentra- is, caution is warranted because β-carotene supplements, but not rotene-rich foods, have been suggested as causing adverse ef- s in smokers (see "Tolerable Upper Intake Levels"). Thus, any ommendations need to state clearly that those who smoke, in ticular, may benefit from even higher average intakes of caro- oids *from foods.*

ohol Consumption

lcohol intake, like tobacco, is inversely associated with serum β- otene and carotenoid concentrations (Brady et al., 1996; Fukao l., 1996; Herbeth et al., 1988, 1990; Stryker et al., 1988). Brady et (1996) reported that higher ethanol intake was associated with a rease in all serum carotenoids measured, with the exception of opene. The inverse association appears to be dose dependent as wn by the cohort study in men of Fukao et al. (1996) in Table 8- t should be noted that in this study, the effects of smoking and ohol consumption independently affected serum β-carotene con- trations in men.

ersons who consume large quantities of ethanol typically con- ne diets that are micronutrient deficient. Therefore, as is the e for smoking, it is not clear whether the observed decrements fully attributable to reduced intakes or also reflect metabolic isequences of chronic ethanol ingestion.

INTAKE OF CAROTENOIDS

Food Sources

. database of values for α-carotene, β-carotene, β-cryptoxanthin, ein plus zeaxanthin, and lycopene for 120 foods has been assem- d (Mangels et al., 1993) and was recently updated and released

TABLE 8-6 Serum β-Carotene in Men in Relation to Alcohol Consumption

	Mean[a] Serum β-carotene (μmol/L)
Nondrinkers	0.38 (20.1 μg/dL)
Ex-drinkers	0.32 (16.9 μg/dL)
Drinkers	
1–15 g ethanol/d	0.33 (17.9 μg/dL)
15–28 g ethanol/d	0.30 (16.2 μg/dL)
29–56 g ethanol/d	0.19 (10.0 μg/dL)
56–140 g ethanol/d	0.15 (8.2 μg/dL)

[a] Geometric mean.
SOURCE: Fukao et al. (1996).

(Holden et al., 1999). Using an expansion of the earlier database and based on the 1986 U.S. Department of Agriculture Continuing Survey of Food Intake by Individuals (CSFII), Chug-Ahuja et al. (1993) reported that carrots were the major contributor of β-carotene to the diet of women of reproductive age (25 percent) with lesser contributions from the following food categories: cantaloupe, broccoli, vegetable beef or chicken soup, and spinach or collard greens. Similarly, the major contributors for α-carotene, β-cryptoxanthin, lycopene, and lutein and zeaxanthin were, respectively, carrots, followed by the categories of orange juice and its blends, tomatoes and tomato products, and spinach or collard greens.

A summary of the carotenoid content of human milk is shown in Table 8-7. It should be noted that the β-carotene content and the concentrations of other carotenoids in human milk are highly variable and appear to be altered easily by manipulation of the carotenoid content of the mother's diet. Most infant formulas, either milk or soy based, do not have carotenoids added to them and, as a result, would be expected to contain very low levels of β-carotene and other carotenoids.

Dietary Intake

Data for intakes of carotenoids (β-carotene, α-carotene, β-cryptoxanthin, lutein and zeaxanthin, and lycopene) from the 1988–1992 Third National Health and Nutrition Examination Survey (NHANES III) based on an expanded food composition database

ABLE 8-7 Carotenoid Content in Human Milk[a]

ıthor, Year	Country	Number of Subjects	Stage of Lactation[b]	Maternal Carotenoid Intake	Carotenoid Content in Milk (μg/dL)[c]	Methods
:bre-Medbin et al., 1976	Sweden	66	0.5–1.5 mo 1.5–3.5 mo 3.5–6.5 mo	Not reported	β-Carotene: 16.3 ± 7.5 β-Carotene: 17.1 ± 7.5 β-Carotene: 20.8 ± 10.2	Spectrophotometric
tte and Calloway, 1981	U.S.[d]	23	19–62 d	Suboptimal	Carotene: 19.7 ± 6.3[e]	Spectrophotometric
appell et al., 1985	Canada	24[f]	1 d 4 d 37 d	Not reported	Carotene: 200 ± 12 Carotene: 100 ± 4 Carotene: 23 ± 5	HPLC[g]
trea et al., 986	U.S.	19[f]	1–5 d	Not reported	β-Carotene: day 1: 213 ± 167 day 2: 117 ± 112 day 3: 120 ± 63 day 4: 50 ± 20 day 5: 39 ± 35	Spectrophotometric
ton et al., 990	U.S.	11	Colostrum	Not reported	α-Carotene: 16 ± 17 β-Carotene: 66 ± 76 β-Cryptoxanthin: 71 ± 61 Lycopene: 96 ± 85	TLC[h], HPLC, and spectrophotometric
liano t al., 1992	U.S.	3	1 mo	Not reported	α-Carotene: 0.32 ± 0.02 β-Carotene: 1.01 ± 0.02 Lutein: 1.06 ± 0.03 Lycopene: 2.73 ± 0.13	HPLC
liano t al., 1994	U.S.	18	>1 mo	Not reported	α-Carotene: 0.6 ± 0.4 β-Carotene: 2.5 ± 1.6[i] β-Cryptoxanthin: 1.1 ± 0.4 Lycopene: 1.7 ± 0.9	HPLC
field al., 1997	U.S.	12	≤6 mo	Dietary intake: β-carotene: 5.08 ± 2.5 mg/d α-carotene: 13.0 ± 0.8 mg/d Lycopene: 2.8 ± 2.6 mg/d β-Carotene supplements: Group 1: 60 mg/wk × 10 wk Group 2: 210 mg/wk × 3 wk	Initial β-carotene: 1.9 ± 0.3 (group 1) 2.7 ± 0.9 (group 2) Postsupplementation: β-carotene milk concentrations were 4 times the initial for both groups. No significant increases in α-carotene, β-cryptoxanthin, lutein/zeaxanthin, or lycopene noted	HPLC

continued

TABLE 8-7 Carotenoid Content in Human Milk[a]

Author, Year	Country	Number of Subjects	Stage of Lactation[b]	Maternal Carotenoid Intake	Carotenoid Content in Milk (μg/dL)[c]	Methods
Johnson et al., 1997	U.S.	12	1–8 mo	Supplement: 64 mg *all-trans*-β-carotene + 69 mg 9-*cis*-β-carotene (1 dose/d × 7 d) Fed a low-carotenoid diet	*all-trans*-β-Carotene[j] prestudy: ≈ 42.9 d 8: ≈ 225.4 9-*cis*-β-Carotene prestudy: ≈ 1.3 d 8: ≈ 3.2	HPLC
Canfield et al., 1998	U.S.	5	>1 mo	Mean dietary intake of β-carotene at baseline = 4.0 ± 3.5 mg/d (measured by three 24-h dietary intake records) Supplemented with 30 mg β-carotene for 28 d	Initial[i,k]: α-carotene: 0.7 ± 0.2 β-carotene: 3.6 ± 1.0 β-cryptoxanthin : 1.4 ± 0.2 Lutein/zeaxanthin: 1.2 ± 0.2 Lycopene: 2.6 ± 0.3 Postsupplementation: β-carotene concentrations increased, on average, 6.4-fold over initial and remained elevated (≈ 2-fold over initial) 1 mo after supplementation	HPLC

NOTE: For Guiliano et al. (1994), Canfield et al. (1997, 1998), and Johnson et al. (1997), milk carotenoid values were converted from nmol/L or μmol/L to μg/dL. The conversion factor used for β-carotene, α-carotene, lutein/zeaxanthin: μg/dL × 0.01863 = μmol/L. The conversion factor used for β-cryptoxanthin: μg/dL × 0.01809 = μmol/L.

[a] Unless noted otherwise, milk content was based on studies of healthy women with full-term pregnancies.

[b] pp = Postpartum.

[c] Mean ± standard deviation.

[d] U.S. = United States.

[e] Mean milk volume was reported as 634 ± 113 mL/d.

[f] Milk samples were obtained from both preterm and full-term pregnancies.

[g] HPLC = high-performance liquid chromatography.

[h] TLC = thin-layer chromatography.

[i] Large intra- and interindividual variability in milk carotenoid concentration.

[j] One month after the study, milk concentrations remained higher than baseline in the supplemented women. No changes in milk concentrations were seen in the placebo group.

[k] Mean milk volume was reported as 62.9 mL per human-feeding episode (Canfield et al., 1998).

r carotenoids are presently being analyzed and are not available be included in this report. Thus, they will be included in the pendix of the next DRI report that will include vitamin A.

However, dietary recall data from 1,102 adult women participat- g in the 1986 Continuing Survey of Food Intake by Individuals dicate mean intakes of β-carotene, α-carotene, lutein, and lyco- ne of 1.8, 0.4, 1.3, and 2.6 mg/day, respectively, with total caro- noid intake from β-carotene, α-carotene, β-cryptoxanthin, lutein, xanthin, and lycopene of approximately 6 mg/day (Chug-Ahuja al., 1993). Later food frequency data from the 8,341 adults partic- ting in the 1992 National Health Interview Survey indicate that an intakes of β-carotene, lutein, and lycopene for men were 2.9, 2.2, and 2.3 mg/day, respectively, and for women 2.5, 1.9, and 2.1 mg/day, respectively (Nebeling et al., 1997). Another survey, the Nutritional Factors in Eye Disease Study, with 2,152 adults responding to a food frequency questionnaire, reported median dietary carotenoid intakes or ranges of 1.3 mg/day of β-carotene, 0.2 mg/day of α-carotene, 0.02–0.07 mg/day of β-cryptoxanthin, 0.7–0.8 mg/day of lutein and zeaxanthin, and 0.6–1.6 mg/day of lycopene (VandenLangenberg et al., 1996).

Intake levels of β-carotene for infants can be estimated using data on human milk concentrations of β-carotene (Table 8-7). Human milk β-carotene concentrations obtained at more than 1 month postpartum varied from 1 to 21 μg/dL. Assuming that infants re-

ng human milk consume 0.78 L/day on average in the first 6 ths (Chapter 2); this would result in β-carotene intake levels of 163 μg/day.

Intake from Supplements

Carotene, α-carotene, lutein and zeaxanthin, and lycopene are able as dietary supplements. There are no reliable estimates of mount of these dietary supplements consumed by individuals e United States or Canada.

TOLERABLE UPPER INTAKE LEVELS

Hazard Identification

rse Effects

adverse effects other than carotenodermia have been report- om the consumption of β-carotene or other carotenoids in . Carotenodermia is a harmless but clearly documented biolog- effect of high carotenoid intake. It is characterized by a yellow- iscoloration of the skin that results from an elevation of caro- concentrations.

Carotene is used therapeutically, at extremely high doses (ap- imately 180 mg/day), for the treatment of erythropoietic pro- rphyria, a photosensitivity disorder. No toxic side effects have observed at these doses. There is no evidence that β-carotene her carotenoids are teratogenic, mutagenic, or carcinogenic in -term bioassays in experimental animals (Heywood et al., 1985). ldition, long-term supplementation with β-carotene to persons adequate vitamin A status does not increase the concentration rum retinol (Nierenberg et al., 1997). However, two recent cal trials reported an increase in lung cancer associated with lemental β-carotene in current smokers (ATBC Cancer Preven- Study Group, 1994; Omenn et al., 1996a,b). These effects are ssed below.

Lung Cancer. The Alpha-Tocopherol, Beta-Carotene (ATBC) er Prevention Study showed a significantly higher incidence of cancer (relative risk [RR] = 1.18; 95 percent confidence inter- CI] = 1.03–1.36) and total mortality (RR = 1.08; 95 percent CI = -1.16) in current smokers supplemented with 20 mg/day β- tene (with or without 50 mg of α-tocopherol) for 5 to 8 years compared to the placebo group (ATBC Cancer Prevention Study Group, 1994). Supplemental β-carotene had no significant effect on the incidence of other major cancers occurring in this population (prostate, bladder, colon or rectum, stomach). In addition, the Carotene and Retinol Efficacy Trial (CARET), a multicenter lung cancer prevention trial of a nutrient combination versus placebo in asbestos workers and smokers (Omenn et al., 1996a,b) reported more lung cancer cases in the supplemented group. The nutrient combination used in CARET included supplemental β-carotene (30 mg/day) plus retinol (25,000 international units [IU]/day). Both CARET and ATBC included β-carotene in the intervention and reported similar effects on lung cancer. However, it should be noted that CARET used a nutrient combination, without a factorial design, and it is not clear whether the reported effects were attributable to β-carotene, retinol, or both acting in concert.

In contrast, the Physicians' Health Study of supplemental β-carotene versus placebo in 22,071 male U.S. physicians reported no significant effect of 12 years of supplementation with β-carotene (50 mg every other day) on cancer or total mortality (Hennekens et al., 1996). With regard to lung cancer, there was no indication of excess lung cancer in the β-carotene-supplemented individuals, even among smokers who took the supplements for up to 12 years.

One additional trial, which was not designed as a lung cancer prevention trial, nonetheless produced results that are of relevance to the topic of lung cancer prevention. The trial tested the efficacy of four different nutrient combinations in inhibiting the development of esophageal and gastric cancers in 30,000 men and women aged 40 to 69 years living in Linxian County, China (Blot et al., 1993). One of the nutrient supplements was a combination of β-carotene, selenium, and vitamin E. After a 5-year intervention period, those who were given this combination had a 13 percent reduction in cancer deaths (RR = 0.87; 95 percent CI = 0.75–1.00), a 9 percent reduction in total deaths (RR = 0.91; 95 percent CI = 0.84–0.99), a 4 percent reduction in esophageal cancer deaths (RR = 0.96; 95 percent CI = 0.78–1.18), and a 21 percent reduction in gastric cancer deaths (RR = 0.79; 95 percent CI = 0.64–0.99). For lung cancer, this trial had limited statistical power, with only 31 total lung cancer deaths (Blot et al., 1994). However, the relative risk of death from lung cancer was 0.55 (95 percent CI = 0.26–1.14) among those receiving the combination of β-carotene, α-tocopherol, and selenium. The smoking prevalence, including individuals who had ever smoked cigarettes for 6 or more months, was 30 percent.

At the present time, the data pertaining to a possible adverse ef-

of β-carotene in smokers are somewhat conflicting. The results ngoing studies may help resolve this issue. There also appears e a relationship between the adverse effects of β-carotene and smoking and alcohol consumption in the ATBC and CARET ls. In the ATBC trial, only those men who consumed more than g/day of alcohol (approximately one drink per day) showed an erse response to β-carotene supplementation (Albanes et al., 6). In the CARET study, adverse effects were associated with the viduals in the highest quartile of alcohol intake (Omenn et al., 6a).

Carotenodermia. Carotenodermia is characterized by yellowish dis-oration of the skin that results from an elevation of plasma caro-e concentrations. This condition has been reported in adults ng supplements containing 30 mg/day or more of β-carotene long periods of time or consuming high levels of carotenoid-foods such as carrots (Bendich, 1988) and is the primary effect xcess carotenoid intake noted in infants, toddlers, and young dren (Lascari, 1981). Carotenodermia is distinguished from ndice in that the ocular sclera are yellowed in jaundiced subjects not in those with carotenodermia. Carotenodermia is consid-l harmless and is readily reversible when carotene ingestion is ontinued.

Lycopenodermia. Lycopenodermia results from high intakes of ly-ene-rich foods such as tomatoes and is characterized by a deep ge discoloration of the skin. Lycopene is a more intensely col-l pigment than carotene and may cause discoloration at lower centrations than other carotenoids (Lascari, 1981).

Other Adverse Effects. Allergic reactions, increased incidence of tate cancer, retinopathy, leukopenia, and reproductive disor-have been associated anecdotally with high carotene consump-(Bendich, 1988; Kobza et al., 1973; Shoenfeld et al., 1982). e of these effects has been confirmed by clinical trials. There is vidence of hypervitaminosis A in individuals consuming high s of β-carotene or other carotenoids (up to 180 mg/day) (Lewis, ?; Mathews-Roth, 1986; Mathews-Roth et al., 1972, 1974).

Dose-Response Assessment

te data on the potential for β-carotene to produce increased cancer rates in smokers are conflicting and not sufficient for a dose-response assessment and derivation of a Tolerable Upper Intake Level (UL) for this endpoint. Supplements of 30 mg/day or more of β-carotene for long periods of time may be associated with carotenodermia, but this effect is more cosmetic than adverse and can be considered harmless and readily reversible. Because of the inconsistent data on adverse effects of β-carotene, a UL cannot be established at this time. ULs are not established for other carotenoids due to a lack of suitable data.

Intake Assessment

Data for intakes of carotenoids (β-carotene, α-carotene, β-cryptoxanthin, lutein and zeaxanthin, and lycopene) from the 1988–1992 Third National Health and Nutrition Examination Survey (NHANES III) based on an expanded food composition database for carotenoids are presently being analyzed and are not available to be included in this report. Thus, they will be included in the appendix of the next DRI report that will include vitamin A.

Risk Characterization

A possible increase in lung cancer incidence has been noted only in smokers taking high-dose supplements of β-carotene (20 mg/day or greater). As discussed earlier, supplemental forms of β-carotene have markedly greater bioavailability than β-carotene from foods. The bioavailability of β-carotene from supplements can also be variable depending on the formulation, nutritional status of the person or population, and dietary intake pattern (e.g., fat intake). Given these substantial differences in bioavailability, it is perhaps logical to characterize the risk as a function of plasma β-carotene concentration (see Figure 8-4). Median serum β-carotene concentrations in the participants receiving 20 mg/day of β-carotene in the Finnish trial rose from 0.32 μmol/L (17 μg/dL) at baseline to 5.66 μmol/L (300 μg/dL) at 3 years; this blood concentration was associated with an adverse effect (ATBC Cancer Prevention Study Group, 1994). In CARET, the median postintervention plasma concentration of β-carotene was 3.96 μmol/L (210 μg/dL); this blood concentration also was reported to be associated with an adverse effect (Omenn et al., 1996a). The first to ninety-ninth percentile for plasma β-carotene from NHANES III is also indicated in Appendix Table F-4. These data suggest that the concentrations associated with possible adverse effects on lung cancer are well beyond the concentrations achieved via dietary intake.

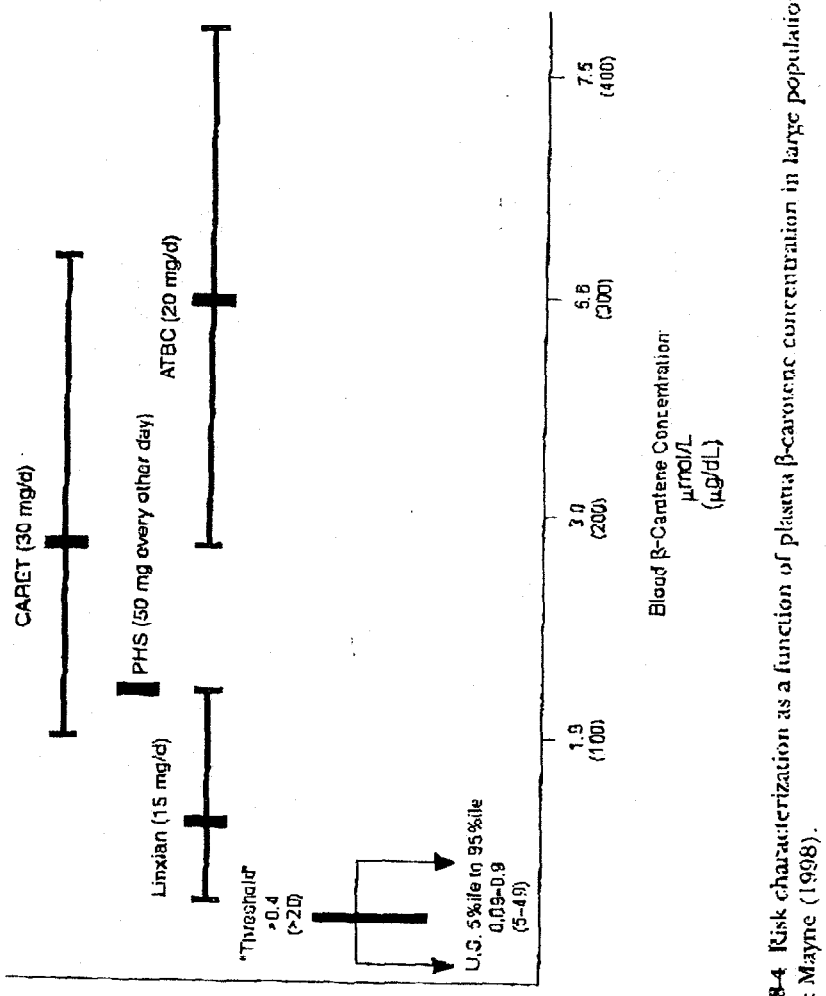


FIGURE 8-4 Risk characterization as a function of plasma β-carotene concentration in large population studies.
SOURCE: Mayne (1998).

Thus, while 20 mg/day of β-carotene in the form of a supplement is sufficient to raise blood concentrations to a range reported to be associated with an increase in lung cancer risk, the same amount of β-carotene in foods is not. Micozzi et al. (1992) demonstrated that 30 mg/day of supplemental β-carotene produced more than a five-fold increase in plasma β-carotene compared to 29 mg/day of β-carotene from carrots.

Based on these considerations, the existing recommendation for consumption of five or more servings of fruits and vegetables per day is supported because this would provide 3 to 6 mg/day of β-carotene. A UI. has not been set for β-carotene or carotenoids. Instead, it is concluded that β-carotene supplements are not advisable for the general population. This conclusion is based on a totality of evidence that includes several large-scale randomized trials of supplemental β-carotene. These trials indicate a lack of evidence of overall benefit on total cancer or cardiovascular disease and possible harm in certain subgroups such as current smokers or asbestos-exposed subjects. This advisement does not pertain to the possible use of supplemental β-carotene as a provitamin A source or for the prevention of vitamin A deficiency in populations with inadequate vitamin A nutriture or in patients suffering from erythropoietic protoporphyria.

RESEARCH RECOMMENDATIONS FOR β-CAROTENE AND OTHER CAROTENOIDS

- As described earlier, β-carotene and other carotenoids have been shown to modulate a variety of intermediate endpoints. However, studies validating that changes in an intermediate endpoint are predictive of changes in a health outcome are critically needed. As an example, macular pigment optical density (MPOD) is a promising intermediate marker for age-related macular degeneration (AMD), but human studies validating this endpoint prospectively are needed, as are studies demonstrating that changes in MPOD are predictive of changes in risk of macular degeneration.
- As a corollary, studies are needed on the effects of long-term depletion of β-carotene and subsequent repletion, with an evaluation of validated intermediate endpoints.
- Significantly more research is needed on health effects of dietary carotenoids other than β-carotene. Possible associations between lycopene and decreased prostate cancer risk, between lutein and zeaxanthin and lowered risk of AMD, and between α-carotene or lutein and various cancers have to be evaluated in additional

servational studies, in animal models, and in human intervention ls, if justified. Studies should consider not only the other caroids, but also the *cis*-versus *trans*-configuration of the carotenoid.

- Since the data from the human intervention trials of β-carote are contradictory, additional data are needed from interven- trials involving β-carotene, several of which are ongoing. An mination is needed of health effects in populations with varying eline risk profiles and, in particular, of studies evaluating interions in populations with poor baseline nutritional status. Post- follow-up of completed β-carotene trials is also needed.
- Studies aimed at the identification of correlates of higher β- otene intake and plasma concentrations, which might help to lain the lower risks of cancer associated with carotene-rich diets, needed.
- Additional research is needed that targets putative mecha- ns to explain a possible increase in lung cancer risk in heavy okers taking high-dose β-carotene supplements (animal studies, hemical studies, and molecular studies). In particular, confirion and extension of findings such as those of recent reports arding lung metaplasia (Wang et al., 1999) and carotenoid oxion products (Salgo et al., 1999), and their relevance to cancer elopment in humans, are needed.
- Surveys are needed that routinely assess and report dietary kes of individual food carotenoids from large, representative ulation samples. Intakes from both foods and dietary supplents must be considered.
- Efforts should be directed toward evaluating equivalency and onstrating efficacy of carotenoids in foods to meet vitamin A ds in vitamin A-deficient populations, in order to develop susable strategies to eradicate this worldwide public health problem.

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1. TERMS OF REFERENCE

With reference to the initial assessment carried out by the Dutch authorities, in the light of the relevant comments/objections presented by member states and pursuant to the article 11 of Regulation (EC) 258/97, the Committee is asked to assess the safety from the point of view of consumer health, of phytosterol esters in yellow fat spreads as a novel food.

2. BACKGROUND

The Commission has received the initial evaluation of a petition for an approval for the use of phytosterol esters in yellow fat spreads as a novel food. Phytosterol esters, used at levels proposed in the application, will lead to a new product with enhanced cholesterol lowering activity.
The application was made under the Novel Foods and Novel Food Ingredients Regulation 258/97/EC. The product is classified under Class1, Subclass1: a pure chemical substance or simple mixture obtained from sources already in use for food purposes in the European Community.

The petitioner first submitted an application to the Dutch competent authority for initial assessment. The Dutch Preliminary Advisory Committee on the Safety of Novel Foods performed the initial safety assessment of this new product, concluding that "...the product.....is safe for human use at the levels indicated below. The committee has assessed the full dossier and with current knowledge sees no human health concern occurring from a nutritional or toxicological point of view. However, ...:the committee advises to restrict the dosage of the phytosterols to a maximum of 8% w/w. At this level serum cholesterol levels drop practically at the same rate as with higher doses of phytosterols, but there is less or no drop in serum carotenoid levels".

During the consultation procedure following the initial assessment by the Netherlands, comments/objections were made by a number of Member States' Authorities. The European Commission therefore decided to submit this dossier to the Scientific Committee on Food (SCF) for evaluation of the safety of this product. Major questions, concerns and recommendations made by Member States were:

- It was questioned whether the product is actually free of rDNA,
- Appropriate labelling of the phytosterol esters ingredient is needed to reach the proper target groups,
- The level of phytosterols permitted to be used in the margarine should be limited to a maximum of 8% (w/w),
- Long term clinical studies are recommended to clarify whether the reduction of cholesterol absorption will be compensated by increased endogenous cholesterol synthesis,

- The assessment of the ß-carotene lowering effect should be based on the 97.5th percentile of intakes rather than the mean,
- Approval of the product will include validation of health claims,
- Time limited (1 year) permission and post-marketing information should be recommended,
- Oxidation products of cholesterol and phytosterols should be addressed,
- Health risk posed by the product to phytosterolaemic individuals should be sufficiently taken into consideration.

These questions, concerns and recommendations are addressed by the SCF in this opinion if considered relevant for the safety evaluation.

3. EVALUATION

The application submitted by the petitioner follows the Commission Recommendations (1). The product falls into Class 1, Subclass 1: a pure chemical substance or a simple mixture obtained from sources already in use for food purposes in the European Community. According to Regulation 258/97 phytosterols would fall under article 1, Paragraph 2, Section e.

The present evaluation is based on the structured schemes of the SCF as a guide to identify the different aspects required to establish the safety of the novel food (1), on the information submitted by the petitioner (2), and on the comments of the member states on the initial assessment report made by the Dutch Competent Authority.

3.1 Specification of phytosterol esters

With phytosterol esters as a novel food ingredient added to margarine, the applicant developed a yellow fat spread that enhances cholesterol-lowering activity in humans. This novel food ingredient is the esterification product of phytosterols, mainly with polyunsaturated and, in part, monoenoic fatty acids. The common or usual name of phytosterol esters is proposed to be "vegetable oil sterol esters". Phytosterols are extracted from the edible oils (soya, maize, rapeseed, sunflower) and esterified with sunflower oil fatty acids. The product tested in genotoxicity studies had the following specification (results of the analysis of five different batches) (2):

- Free fatty acids <0.2% (w/w)
- Total fatty acids 37.0 – 37.7% (w/w), mean 37.4% (w/w)
- Fatty acid composition (of total fatty acids):

C16:0	7.5 – 7.6%
C18:0	4.8 – 4.9%
C18:1	22.6 – 22.7%
C18:2	65.0 – 65.1%

- Free sterols 10.5 – 11.8% (w/w), mean 10.9% (w/w)
- Total sterols 62.3 – 63.0% (w/w), mean 62.6% (w/w)
- Sterol profile (of total sterols):

Cholesterol	0.2 – 0.3%
Brassicasterol	2.7 – 3.1%
Campesterol	26.5 – 27.0%
Stigmasterol	17.4 – 18.0%
ß-sitosterol	50.8 – 51.2%
unknown	1.2 – 1.7%

- Total volatiles <0.5% (w/w)
- Peroxide values 1.5 – 2.1 meq/kg, mean 1.9 meq/kg

Based on the variability in sourcing/seasonal variation of the plant sterols, the applicant expects the following sterol profile (2):

Cholesterol	0.0 – 2.0% of total sterols
Brassicasterol	0.0 – 9.0%
Campesterol	10.0 – 40.0%
Campestanol	0.0 – 6.0%
Stigmasterol	6.0 – 30.0%
ß-sitosterol	30.0 – 65.0%
Sitostanol	0.0 – 10.0%
D5-Avenasterol	0.0 – 4.0%
D7-Avenasterol	0.0 – 2.0%
D7-Stigmastenol	0.0 – 2.0%
Other	0.0 – 5.0%

Phytosterols occur naturally in food as free alcohol, esterified with long chain fatty acids (25 – 80% of total phytosterols) and conjugated as glucosides (usually in small amounts). The majority of plant oils contains 0.1 – 0.5%, while some germ oils (rice bran, wheat germ, oats) contain up to 4% total phytosterols (3). Reduced and low fat spreads on the market contain approximately 0.3-0.4% phytosterols.

The application for this novel food ingredient covers yellow fat spreads with increased levels of phytosterols (up to 12%, on average 8%).

3.2 Effects of production processes

Phytosterols are by-products of vegetable oil refining. Phytosterols are isolated from conventional edible oils (soya, maize, sunflower, rapeseed). The conventional caustic refining procedure comprises degumming, neutralisation, bleaching and deodorization. The last step, a mass-transfer process, by which substances are evaporated from the oil under reduced pressure (2 – 10 mbar) and elevated temperature (230 – 270°C), leads to a distillate making around 0.1 – 0.3 % of oil mass and contains 8 – 20 % sterols (details in 5).

Formulation and process rules currently used to ensure safety of conventional spreads have been used for the new products. All materials are produced according to Good Manufacturing Practices. All processing materials are of food-grade or equivalent.

Storage and distribution temperatures used are the same as conventional spreads and the same Hazard Analysis and Critical Control Point (HACCP) schemes are used to control product safety and quality.

Phytosterols from oil from already approved genetically modified plant strains might be present in the mixture. The isolated phytosterols are re-esterified with fatty acids from sunflower oil.

The production process of the spread has a history of safe use for ingredients for the food industry and it does not affect the composition and structure of the components.

3.3 History of source organism

Commercially available and used plant oils from different plants, but mainly from soya, sunflower, maize, and rapeseed, are the sources for production of the novel food ingredient. These source oils are not derived from plants especially grown for this purpose and they are generally not genetically modified, although it cannot be excluded that some mixtures contain oils or isolated sterols from GMOs. In this case these can be expected to be approved organisms and derived products like the Round-up-ready soybean and its products. Thus, whenever labelling requirements exist for the use of already approved ingredients derived from GMOs, these requirements will also apply to this novel food.

3.4 Ingredients use in food

The applicant has applied to use vegetable sterol esters in new vegetable oil-based spreads at levels up to 20% (≈ 12% free sterols). The use of the new product is intended to help maintain healthy cholesterol levels as part of a diet low in saturated fat and cholesterol. The spread base contains around 40% fat, composed of edible vegetable oils which are high in polyunsaturated (PUFA), low in saturated and very low in *trans* fatty acids. The product is not intended for use in cooking.

3.5 Anticipated intake/extent and consequences of use

With normal consumption of this kind of spreads being 20 – 30 g/d, the intake of phytosterols when using the novel product will increase to about 1.6 – 2.4 g/d, amounting to an 8-12 fold increase of the current daily intake from traditional products. In the USA plant sterol esters in plant oil-based spreads at levels up to 20% are generally recognised as safe (GRAS) (4).

The main sources of phytosterols in the basic diet are cooking oils and margarines. Bread and cereals can also contribute significantly to total phytosterol intake (6). Reduced-fat health spreads contain 0.3 – 0.4% phytosterols, corn oil margarines are highest in phytosterols (0.5%). Vegetables and fruits contain <0.05% (based on the edible portions), except seedlings of barley, beans, peas which contain 0.1 – 0.2% phytosterols. Some seeds are also rich sources: sunflower and sesame seeds contain 0.5 – 0.7%, and legumes can contain 0.22% phytosterols.

A typical U.S. diet provides approximately 250 mg of phytosterols per day (≈ 4 mg/kg bw/d) (7). In the UK and the Netherlands phytosterol intake was estimated to be about 200 mg/d. In the adult Finnish population average intakes amounted to 300 mg/d (≈ 5 mg/kg bw/d) with an upper limit of 680 mg/d (≈ 10 mg/kg bw/d). Generally the intake of adult vegetarians and their children is higher (up to 40%) than the average for the population as a whole (7-9).

Infant formulae based on cow's milk contain 0.08 – 0.20 mmol/l ß-sitosterol, 0.03 – 0.10 mmol/l campesterol and around 0.02 mmol/l stigmasterol, while the phytosterol content in human milk is negligible and can not be detected using current methods (10).

Oral phytosterol intake of about 3g/d inhibits the intestinal cholesterol absorption, probably by blocking the receptors (11). Aiming at a similar blood cholesterol modulating effect, studies with hypercholesteraemic subjects have employed dose levels of many grams (up to 25 g/d) of phytosterols per day for up to three years (8). Such limited total- and LDL-cholesterol lowering effect can be compensated by an increased endogenous cholesterol synthesis, unlike the use of drugs suppressing synthesis or perturbing the enterohepatic cycle of cholesterol and bile acids, which might lead to very low cholesterol levels. In addition, studies have shown that drug treatment with statins to reduce cholesterol levels increased the phytosterol/cholesterol ratio by increased absorption. For example, the ratio of sitosterol/cholesterol was shown to increase by 200 % (9, 13). This means, that high intake of phytosterols could be a potential problem when ingested together with cholesterol lowering/inhibiting drugs.

From marketing data on users and usage of a similar yellow fat spread containing phytostanols in the EU, the petitioner concludes that the product is used predominantly by people who are more than 50 years old and of relatively high socio-economic standing, and that the majority of households using the product consist of one or two people. The petitioner's current sales data for yellow fat spreads containing phytosterol esters in USA and Australia indicate a comparable consumer profile.

Typical daily consumption of yellow fat spreads in Europe is between 20 and 30 g/d. In order to achieve the required cholesterol lowering effects the intake of phytosterols - according to petitioner - should amount to 1.6 – 3.6 g/d (expressed as esters 2.2 – 5.0 g/d). That means that phytosterol concentrations in the yellow fat spread should be 6 – 12% (w/w). Estimates of fat spread use of males above 50 years of age in the UK and the Netherlands show that the 95th percentile of use is approximately 57 g/d and 70 g/d respectively.

The marketing of the product is focussed on the particular section of the population that is trying to control its blood cholesterol levels.

As indicated by the applicant there is only a small number of people at risk of adverse reaction. These comprise individuals with an inborn error of phytosterol metabolism (autosomal, recessive); worldwide 50 cases are known (2). Appropriate

labelling should assure that phytosterolaemic patients can avoid consuming the product.

Although it is not intended to recommend this product to healthy young adults, or for children, these individuals may consume the product when it is available in a family home. The initial assessment came to the conclusion that children in Europe are not expected to experience negative effects from possible lowered cholesterol levels.

The new product is intended to replace other yellow fat spreads. Based on the product specification the novel food differs from other fat spreads only in the phytosterol ester content at the cost of corresponding amounts of non-fat compounds (water). Thus there will be no change in intake of nutrients and/or other compounds. The new product contains a similar amount of polyunsaturated fatty acids as other so called "heart health" products.

3.6 Nutritional information

Phytosterol esters are hydrolysed by pancreatic carboxyl ester lipase. Absorption of free phytosterols in humans and experimental animals is low: 4 – 5% for β-sitosterol and stigmasterol, 9 – 10% for campesterol and brassicasterol (12). At higher dietary intake (2000 mg/d), absorption of sitosterol by humans is reduced. In healthy subjects campestanol is better absorbed than campesterol (12.5% vs. 9.6% of intake). Phytosterol absorption in women was found to be slightly higher than in men (13) and higher in children than in adults (14).

Absorbed phytosterols are transported in the triglyceride-rich lipoprotein (VLDL) and chylomicrons, taken up by the liver and then excreted into the bile (15). Circulating phytosterols are transported in the blood mainly in LDL and HDL fractions. Tissues with LDL receptors such as the liver, adrenals and testes may then take up phytosterols and convert them into steroid hormones (16). Since their concentration in these tissues is much lower than that of cholesterol, this will not significantly contribute to hormone synthesis. However, no information on the relative potency of hormones derived from either cholesterol or phytosterols is available. Unabsorbed sitosterol and campesterol is converted by the human colonic microflora to sitostanol/stigmastanone and campestanol/campestanone, respectively. Among a group of 31 normal North Americans, 23 high converters have been found converting a mean of 83 ± 9% sitosterol (17).

After ingestion of 8.6g phytosterols/d in healthy human adults, faecal concentrations of sterols and sterol metabolites increased from about 40 to 190 mg/g dry weight and 30 to 50 mg/g dry weight, respectively. The major sterol metabolites excreted were metabolites saturated at the 5,6-position in β-configuration or metabolites formed by oxidation at the 3-position. The faecal concentration of 4-cholesten-3-one was slightly but significantly increased (about 2 mg/g). Faecal secondary bile acid concentration was reduced. The formation of small amounts of oxysterols could not be excluded, but considered to be unlikely (18). Phytosterols in food can be oxidised, particularly at higher temperatures (>180 °C) (19). Oxidation products (7-hydroxy-

and 7-keto-components) are formed at very low levels which are similar to other plant oil products containing phytosterols and are also poorly absorbed (2).

If the product is consumed on regular basis (20 – 25 g phytosterol-enriched spread daily, equivalent to 1.6 – 2.0 g phytosterols/d) then the average lowering of plasma LDL-cholesterol will be 8 to 10%, relative to initial plasma levels (4.16 ± 0.5 to 6.54 ± 0.61 mM). The reduction in blood cholesterol levels of the magnitude anticipated from consumption of phytosterol-enriched spreads is safe in those individuals who do not have elevated plasma cholesterol levels. This was confirmed by the results of the three-year Dietary Intervention Study in Children (DISC). In 8 – 11 year old children a diet low in fat, saturated fatty acids and cholesterol lead to a modest decrease in LDL-cholesterol, while maintaining adequate growth, iron status, nutritional quality and psychological well-being during the critical growth period of adolescence (20). Children and adults would not be expected to experience any adverse effect on metabolism when their blood cholesterol is lowered. The novel food is intended to be used by population groups above 50 years of age, who try to control their elevated blood cholesterol.

At levels of phytosterols in spreads of 3.4, 6.5 and 11 – 13% (w/w) in short term (21) and 8% (w/w) in a one-year follow-up study (2) the new product is equally effective in lowering LDL cholesterol by 8 – 10%, relative to the initial plasma level. At 11 – 13% of phytosterols in the fat spreads no appreciable effect on the fat-soluble vitamins calciferol, tocopherols and phylloquinone was noticed, but a 10% reduction of α- and ß-carotene as well as lycopene was observed. This reduction of 10% itself seems not to be of physiological relevance, but, considering a long term exposure and taking into account the 97.5 percentile of intake, the decline of ß-carotene levels might be higher.

In the initial assessment by the Dutch competent authority it was expected that a maximum of 8% (w/w) phytosterols will cause little or no drop in serum carotenoid levels.

The applicant considered the points raised by some member states after the initial assessment and commissioned a one-year follow up study (2) with healthy subjects using fat spread containing 8% (w/w) phytosterols (the preparation contained 38g fat in 100 g spread). A report with the results of this study was forwarded to the SCF. The results can be summarised as follows:

- When adjusted for total lipids no statistically significant changes after week 26 and week 52 were found for lutein, zeaxanthin, ß-cryptoxanthin, lycopene and α-carotene. Only ß-carotene was significantly ($p=0.037$) decreased at week 52; the level dropped by 24% (21% when lipid adjusted) as compared with the initial level at time point 0. This significant drop of ß-carotene level occurred after 52 weeks despite the fact that the tested new fat spread contained a maximum of 50 mg carotenoids per kg fat (mainly ß-carotene and lycopene added for coloration). The reduction was twice that observed in the short-term studies with 12% phytosterols. Nevertheless, it can be seen from the week 0 results that there is a large variation within the normal range for

plasma ß-carotene levels, that there are also seasonal variations and that this reduction is within normal range (Table 1).

Table 1: Changes in plasma ß-carotene levels in one-year follow-up study (data from ref.2; doc. ref. D99/047)

n=64 healthy adults	ß- carotene (nmol)	ß-carotene/lipid adjust. (nmol/mmol)
Week 0	410.4±216.8	58.3±33.1
Week 26	321.9±175.6	48.0±28.1
Week 52	310.1±190.3	46.1±28.6

The reduced plasma ß-carotene levels might become more relevant when the vitamin A status is not optimal. This is the case for pregnant and lactating women as well as younger children.

- There were no significant differences in the plasma levels of retinol, 25-OH-cholecalciferol or α-tocopherol (total lipid adjusted) during 52 weeks of study. Phylloquinone status was not statistically different between test and control groups after 26 weeks of study.

- There was a significant cholesterol-lowering effect of phytosterol esters enriched margarine throughout the one-year duration of the study (2).

- There were no side effects seen in the individuals during the study.

3.7 Microbiological information

Spreads containing phytosterol esters have been tested for their microbiological stability and have been found to be similar to conventional spreads. The production process and the inherent properties of the novel products give no rise to concerns of microbiological risk. No data indicate an effect on the intestinal flora in terms of bacterial profile or metabolic activity beyond natural variations (2).

3.8 Toxicological information

The toxicological information available on phytosterols and phytosterol esters comprises data from studies on absorption, distribution, metabolism, and excretion and on (a) subchronic toxicity, (b) genotoxicity, (c) reproductive toxicity, (d) potential estrogenic activity and from (e) human studies:

(a) In a 13-week feeding study with rats, a mixture of phytosterol esters, obtained mainly from soya bean oil and re-esterified with fatty acids from sunflower oil, was tested at dosages of 0.16, 1.6, 3.2 and 8.1% in the diet. The mixture contained 62% total sterols consisting of mainly β-sitosterol (48.7%), campesterol (25.8%) and stigmasterol (21.6%) with only 1.1.% brassicasterol.

Neither D5- and D7-avenasterol nor D7-stigmastenol were present. Apart from some minor changes in haematology and clinical-chemical parameters, no relevant toxic effects up to the highest dose of 6.6 g/kg bw/day (corresponding to 4.1 g phytosterols/kg bw/day) were found (22).

(b) Phytosterols (47.9% β-sitosterol, 28.8% campesterol, 23.3% stigmasterol) and phytosterol-esters (47.3% β-sitosterol, 28.1% campesterol, 24.1% stigmasterol) show neither evidence of mutagenic activity in the bacterial mutation assay with Salmonella typhimurium (strains TA 1535, 1537, 98 and 100) nor clastogenic activity in tests on chromosomal aberrations with human peripheral blood lymphocytes *in vitro* both in the presence and absence of S-9 mix derived from rat livers.
In addition, two *in vivo* genotoxicity studies were conducted using a phytosterol ester mixture containing 0.3% cholesterol, 3.0% brassicasterol, 28.1% campesterol, 0.8% campestanol, 18.7% stigmasterol, 45.5% ß-sitosterol, 2.6% ß-sitostanol, 1.1% D5-avenasterol and 1.9% others. Using an *in vivo/in vitro* procedure the mixture did not induce unscheduled DNA synthesis (UDS) in the livers of orally dosed male rats (once with 2000 mg/kg). In another study in rats the plant sterol mixture did not induce micronuclei in the polychromatic erythrocytes of bone marrow of male rats treated up to 2000 mg/kg/d (2, doc. ref. D00/004).
Neither 4-cholesten-3-one, an oxidation product of cholesterol increased in volunteers fed phytosterols, nor 5β-cholestan-3-one showed mutagenic activity when tested in the bacterial mutation assay with five strains of *Salmonella typhimurium*. Furthermore, none of the substances showed a clastogenic potential in the *in vitro* chromosome aberration assay with human lymphocytes (23,24).

(c) A variety of effects on the reproductive system such as antiandrogenic action in rabbits and decrease in testicular weight and sperm concentration in rats have been reported for ß-sitosterol and phytosterol-rich extracts (25, 26, 27, 28). These observations have been made after administration by the subcutaneous route and/or with phytosterol preparations, the purity of which was not specified.
However, in a two-generation reproduction study in rats, phytosterol esters of the same composition as in the 13-week study had no effect on the reproduction of the F0 and F1-generations, nor on the development of the F1- and F2-pups, nor on the sexual maturation of the F1-weanlings nor on oestrous cycles. A dietary phytosterol ester concentration of 8.1% was shown to be the no-observed-adverse-effect level (NOAEL). This was equivalent to a dose of 2.5-9.1 g/kg bw/day depending on the period during the study (29).

(d) Orally administered ß-sitosterol of unknown purity increased uterine weight in rats receiving a low dose for 30 days (6.2 µg/dl in drinking water). This weak oestrogenic response was not observed at higher doses (12.4 µg and 18.6 µg/dl) (30).
In contrast to these studies, uterotrophic assays with immature female rats orally gavaged with phytosterols (47.9% β-sitosterol, 28.8% campesterol,

23.3% stigmasterol) and phytosterol esters (47.3% β-sitosterol, 28.1% campesterol, 24.1% stigmasterol) in doses of 5, 50 and 500 mg/kg bw/day for 3 days did not reveal any oestrogenic response using uterine weights as the end point. In addition, phytosterols of the same composition did not display oestrogenic activity in a recombinant yeast assay for oestrogenic potential, nor did they show binding in a rat uterine cytosol oestrogen receptor binding assay (31). These studies, together with the two-generation reproduction study, provide sufficient reassurance of absence of endocrine effects via the oral route.

(e) In a 3-week study with 12 men and 12 women who consumed 5.8 g phytosterols (in 40 g margarine) per day no changes in the sex hormone levels in females was shown (18). Two double-blind placebo-controlled 14-week tests did not provide evidence for any adverse effects on haematological and clinical parameters (21,32). These trials and the one-year follow-up study using phytosterol esters (8% w/w expressed as phytosterols) in the fat spreads were carried out primarily with the view to assessing the cholesterol lowering effect of phytosterol esters. These tests have not reported any toxic effects relating to the phytosterols.

Phytosterol preparations are used for the medical treatment of benign prostatic hyperplasia. A number of placebo-controlled, double-blind clinical trials was conducted with preparations of uncertain compositions said to be mainly β-sitosterol. With doses of 20 mg β -sitosterol three times per day (33) and 130 mg ß-sitosterol daily (34), significant improvements in symptoms and urinary flow parameters were reported (35). The mechanism of this effect and the active ingredient remains to be determined. Side effects have not been reported.

4. CONCLUSIONS

- The Committee considers that the dossier and the additional information submitted during 1999 and 2000 is complete and follows the SCF recommendations. The novel food, phytosterol esters in yellow fat spreads, has been correctly classified as Class1, Subclass1, a pure chemical substance or simple mixture obtained from sources already in use for food in the European Community.

- The new yellow fat spread differs from conventional fat spreads/margarine by its phytosterol origin (obtained from edible vegetable oils), their chemical structure (esters with long chain unsaturated fatty acids of sunflower oil) and concentration (about 16 – 24-fold higher than the conventional product). This concentration will increase the total intake of phytosterols by 8 – 12 times, compared with traditional products.

- Based on extensive toxicological testing of phytosterol preparations in a 13-week feeding study with rats, in a two-generation feeding study with rats, in studies on

oestrogenic potential and in tests on genotoxicity, no safety concerns were apparent.
The safety in use of phytosterols has been demonstrated for mixtures of predominantly ß-sitosterol, campesterol and stigmasterol and/or their esters with fatty acids, to which the specification of the new product should be restricted. The phytosterol profile of 30-65 % ß-sitosterol, 10-40 % campesterol, 6-30 % stigmasterol and a total of 5% other phytosterols, based on total sterol content (w/w), is considered acceptable by the Committee.

- The Committee considers that the very small number of people with inborn error of phytosterol metabolism (phytosterolaemia) should be made aware of the presence of higher levels of phytosterols in this product and that patients on cholesterol-lowering medication should only consume the product under medical supervision.

- Ingestion of 20g per day for one year of products containing 8 % free phytosterols reduced plasma ß-carotene concentrations by 20%. Although the ß-carotene concentration was still within the normal range and within normal seasonal variations, such a reduction in plasma ß-carotene levels might become more relevant for persons whose vitamin A status is not optimal. The Committee is therefore of the opinion that this ß-carotene lowering effect should be communicated to the consumer, together with appropriate dietary advice regarding the regular consumption of fruits and vegetables.

- Given the overall evaluation of the submitted information the Committee concludes that the use of phytosterol esters in yellow fat spreads at a maximum level corresponding to 8% free phytosterols is safe for human use.

- The Committee is of the opinion that the applicant should perform, in accordance with Chapter XI in the Annex of Commission Recommendation 97/618/EC (1), a post-marketing surveillance study to obtain data on consumption and further investigation of possible health effects, among others the effects on plasma ß-carotene levels. The Committee will wish to review this information when it becomes available.

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EFFECT OF PLANT STEROLS ON SERUM LIPIDS AND ATHEROSCLEROSIS

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INTRODUCTION

Experimental hypercholesterolemia leads to initiation and progression of intimal arterial lesions. Spontaneous hypercholesterolemia does not always lead to atherogenesis. For example, Racer Homar pigeons have the same degree of hypercholesterolemia as White Carneau pigeons, but only the latter develop arterial lesions (Lofland, 1965). For man, the relationship between hypercholesterolemia and atherogenesis is not clear-cut, except for primary, monogenic, endogenous, familial hypercholesterolemia of homozygous type which is accompanied by premature atherosclerosis.

Experimental atherogenesis can be prevented or mitigated by preventing or mitigating hypercholesterolemia. The degree of plasma cholesterol elevation can be reduced. In man, the degree of hypercholesterolemia can be lessened and progression of cholesterol elevation can be prevented or slowed.

The plasma cholesterol pool is a mix of endogenous and exogenous cholesterol. The mix is not perfect, endogenous cholesterol being absorbed more rapidly and more completely than exogenous cholesterol. This has been demonstrated in chicks by differential labeling of the two sources, using phytosterols as inhibitors of dietary cholesterol resorption (Sklan *et al.*, 1977). The observation can be safely extrapolated to man. Homeostatic regulation must never be forgotten.

In man, exogenous sources of cholesterol other than dietary play either no role at all, or a minor or a rare role, compared to alimentation. Thus, most efforts to inhibit or to reduce hypercholesterolemia are targeted against dietary cholesterol. Since all mankind ingests some cholesterol which then contributes to the pool the dietary source should be controlled universally, regardless of other sources and of the level of plasma cholesterol. One of the means for regulating dietary cholesterol is based on preventing its resorption from the jejunum. And, one of the ways to accomplish this is by ingestion of phytosterols, particularly, β-sitosterol. This justifies including a chapter on phytosterols in the Encyclopedia.

The dual title of the Encyclopedia implies that therapy need not always be pharmacal. Dietary treatment of diabetes mellitus is cited here as an example of non-medicinal therapy. Pharmaceuticals, though mostly used for treatment, are sometimes used for diagnostic purposes. An example are pituitary hormones used for differentiating between primary and secondary endocrinopathies. Beta-sitosterol can be profitably used for differentiating alimentary hypercholesterolemia from other forms, for determining the amount dietary cholesterol contributes to the pool, for estimating to what extent a sitosterol regimen would reduce an elevated plasma cholesterol level. Moreover, sitosterol can be used preventively and therapeutically for regulating plasma cholesterol. Lastly, it can be ingested in the form of medication or as a natural constituent of many foods.

1. PHYTOSTEROLS

In the course of discussion it will become clear why the terms phytosterols, plant sterols, sitosterol or β-sitosterol are being used interchangeably. At least 44 phytosterols have been

identified in seven classes of plants, with up to 20 sterols in a single plant (Bean, 1973). Beta-sitosterol is present in more plants than any other phytosterol and it is usually present in larger quantity than other sterols. It is by far the most important plant sterol—for pharmacists and dietitians, for physicians and patients, for all man.

Beneke, 1862, discovered a sterol in peas and assumed it to be cholesterol. A few years later, ergosterol was found in the ergot fungus of maize and sitosterol was found in cereal grain. It took fifty years before empirical and structural formulas were established for stigmasterol and sitosterol. For many years, identification of sterols was based on determining the melting point and the optical rotation of purified sterols, of steryl acetates and steryl benzoates. Today, sterols are identified by sophisticated methods and devices, including many types of chromatography, electrophoresis, spectrophotometry, radiology, molecular rotation and nuclear magnetic resonance. These methods are being applied to pure sterols obtained by repeated recrystallization, to steryl esters, steryl-D-glucosides and steryl digitonides.

In nature, phytosterols are present in pure form, esterified, or conjugated as glucosides. Three plant sterols, namely, β-sitosterol, campesterol and stigmasterol, are most frequently encountered in the flora and have been studied most, although a few others have also attracted attention. Phytosterols are in ornamental plants, medicinal herbs, edible plants, shrubs and trees. They are in seeds, seed oils, roots, stems and branches, leaves and blossoms, though not in equal total amounts or equal proportions in various parts of a plant or a tree.

The phytosterol content, or that of any of the sterols, is not constant. The growth of plants varies with geographic location, altitude, climate, sunshine and rain, with acidity of the soil, with photoperiods and seasons. The sterol content varies from class to class, from crop to crop, and between members of the same genus or family. Various parts, including the edible parts of plants, are not affected equally by all or some of the external factors. Added to natural fluctuations, such as occur with growing and aging, are those introduced by man. Using natural against chemical fertilizers plays a major role. Differences caused by timing of seeding and timing of harvesting have been recognized for medicinal plants 2000 years ago by Tibetan monks and to this day proper timing is being annually revised in conformity with the astrologic calendar (Pollak, 1984). The composition of food is altered by processing, transportation, refrigeration or lack of such. storage, by preservatives and coloring and, of course, by food preparation. All this has to be considered when prescribing a diet.

Although of scant practical importance, differences in phytosterol content between cell membranes, mitochondria, chloroplast, lysosomes and nuclei have been investigated far more thoroughly than some important features. References to all the variables were recorded by Pollak and Kritchevsky (1980) in a monograph on "Sitosterol". Their book allows for omission of most of its 956 references. Some have to be repeated as needed background. There is a challange not to present an abstract of the monograph or its second edition, to offer new information and new ideas to those familiar and those not acquainted with the book.

2. PHYTOSTEROLS AND ZOOSTEROLS

At the time phytosterols were discovered a sharp line was drawn between sterols in the flora and sterols in the fauna. A third type, the mycosterols, was later added to the plant sterols. Since 1957, reports were mounting on the presence of zoosterols—cholesterol. 24-methylene-cholesterol, 7,22-dehydroxycholesterol, desmosterol—in the flora. From 1962 on, the presence of β-sitosterol, stigmasterol and campesterol has been reported in the fauna. The parallel between animal steroid hormones and phytohormones has been revealed, with emphasis on β-sitosterol acting as a mild phytoestrogen.

Zoosterols, principally, cholesterol, have been found in many plants. Phytosterols, predominantly, β-sitosterol, have been found in few animal species, only. With some exceptions—the most important to be mentioned—the amounts of sterols which crossed

over from or to flora and fauna are small and insignificant. Thus, for practical reasons, continued use of the old terminology separating sterols into phyto- and zoosterols may be acceptable.

2.1. Zoosterols in the Flora

Cholesterol, to a lesser extent 24-methylenecholesterol, have been identified in many plants. They are present in microorganisms, algae, cacti, medicinal herbs, ornamental plants, weeds and shrubs, also in such edibles as spinach, oats, beans, nuts, potatoes, oranges and apple seeds. Mostly, the amounts are less than 1% of total sterols.

Of ten or more species with a relatively high cholesterol content four are singled out—for various reasons. The maximum amount of cholesterol, namely, 50% of total sterols, was found in *Hypochoeris radiata*, the "cat's ear" (Devys and Barbier, 1965). This plant is closely related to the edible *Taraxum officinale*, the "lions tooth" or "dandelion", which contains no cholesterol at all (Westerman and Roedick, 1981). In cotton bud, one-half of esterified sterols were identified as cholesteryl palmitate, whereas cholesteryl-D-glucoside and free cholesterol were present in small amounts (Thompson *et al.*, 1970). Their data are presented in condensed form in Table 1, not because of practical importance but as a representative example of sterol distribution in the flora.

Table 1. *Per cent Distribution of Sterols in Cotton Bud*

Sterols	Cholesterol	β-sitosterol	Stigmasterol & Campesterol
Free	1.6	57.3	37.2
Esterified	50.6	49.4	0
Glycosides	4.3	80.5	19.2
Esterified glycosides	4.7	78.7	7.5

Among lower plants, the light green algae, *Euglena gracilis*, have 12% of cholesterol (Brandt *et al.*, 1970), a fact which could become important if seeweeds were to become part of the western diet. In Japan, where kelp is widely consumed, the cholesterol content of algae may not play a role as part of a generally cholesterol-poor diet. Far more significant are persistent and consistent reports—of which only two are cited—on Solanaceae. In *solanum tuberosum*, the potato, there are four sterols: 12% cholesterol, 48% β-sitosterol, 36% stigmasterol and 3% campesterol (Ardenne *et al.*, 1965). Cholesterol-4-^{14}C administered to potato sprouts converted to the atherogenic metabolite 24-hydroxycholesterol and to cholest-4-en-3-one (Heftmann and Weaver, 1974). Potatoes are the main staple in the western world. As part of a high caloric diet, they could contribute to alimentary hypercholesterolemia. Pollak, in 1961, and repeatedly thereafter, has pointed out the low incidence of myocardial infarction in rice-eating populations contrasting with the high incidence in potato-eating Westerners.

2.2. Conversion of Phytosterols to Zoosterols

Conversion of sterols takes place in some plants, in many insects and in some helminths. The bibliography on this subject is large and still growing. A single reference must suffice. Allais *et al.* (1971) fed honey bees, *Apis melifica* L. ^{3}H-β-sitosterol and found that at termination of the study cholesterol had increased by the same amount by which sitosterol had decreased. Seven possible pathways by which phytosterols could be converted to desmosterol and cholesterol were cited by Pollak and Kritchevsky (1980).

2.3. Phytosterols in the Fauna

Only the three major plant sterols, β-sitosterol, stigmasterol and campesterol were found in several species. Others, namely, ergosterol, brassicasterol and α-spinasterol, were not found often. The major sterols were discovered in paramecia, trichomonas and tetrahymena species, in *amoeba histolytica*, in many phyto-, zoo- and oligophagous insects.

in lobsters and prawns. Because of the small amounts present in invertebrates, the data are of academic interest only.

To illustrate the distribution of the three main phytosterols in subhuman vertebrates, one report is being mentioned. In the green turtle of New Guinea, *Chelonia mydas mydas* Linne, Minato and Otomo (1969) found 3% β-sitosterol, 0.2% campesterol and 0.18% stigmasterol.

The presence of phytosterols in higher vertebrates, particularly, in humans, is important. Between 1974 and 1979, Mellies *et al.* (1979) assayed phytosterols in the blood serum of infants on breast milk, whole milk, or formula, and in children on a normal diet, or on a low-cholesterol and high phytosterol diet. Their important finding was lack of correlation between mother and child, in spite of good correlation between maternal serum and milk and in spite of proven transplacental passage of plant sterols. The latter was documented by sterol assays in the aorta of an abortus at 30 weeks gestation and in the aortae of two neonates. The highest mean blood serum levels were 3.53 mg/dl for β-sitosterol and 0.72 mg/dl for campesterol in nine infants fed formula. Their total for total phytosterols comes close to the 4.74/mg/dl of β-sitosterol recorded by Böhle *et al.* (1964). They examined 18 healthy adults and 12 patients with hypercholesterolemia–hyperlipidemia and atherosclerosis. In health, the β-sitosterol level was 6.66 times higher than in disease. Comparable results were reported by several other investigators.

Sterol distribution in neoplastic tissues attracted special attention. Huddad *et al.* (1970) found no differences in plasma phytosterols of 11 normal women, 7 with lactating breasts and 14 with mammary carcinoma. In normal tissues there were 7.71 n/g of phytosterols. in cancerous tissues, 7.23 n/g. However, Whitney *et al.* (1973) reported that serum phytosteryl acetates equaled 14.1 n/ml in 14 women with breast cancer, 35.6 n/ml in 4 healthy women, and 74.1 n/ml in 9 healthy men. Data of others on benign and malignant breast lesions do not add additional information.

2.4. Absorption of Phytosterols

The discrepancy between plasma cholesterol and plasma phytosterol values is considerable. Many have speculated on the reasons. Ingested in small amounts, resorption of phytosterols is negligible. Lees and Lees (1976) reported that on ingestion of 3 g sitosterol per day the plasma β-sitosterol was 0.47–2.07 mg/dl on intake of 6 g/day, it was 0.62–2.34 mg/dl. There was only vague relation between the ingested dose and the plasma level. This was confirmed when a much larger amount was given: 18 patients received daily about 18 g β-sitosterol (as 18 g "Cytellin") upon which their plasma sitosterol was between 0.37 and 0.85 mg/dl, averaging 0.48 mg/dl.

Answers to the reasons for low phytosterol levels were sought since the low absorbability of plant sterols was first reported by Schönheimer in 1929. The voluminous bibliography has been reviewed by Pollak and Kritchevsky (1980). The problem is still being investigated.

The absorbability of sterols has been studied *in vitro* and *in vivo*. Kreuter *et al.* (1981) used two-chamber diffusion cells and determined that the permeability coefficient for β-[4-^{14}C]sitosterol was four times smaller than for [4-^{14}C]cholesterol. Ikeda and Sugano (1983) found that mixed bile salt micelles solubilized both sterols to comparable extent. when applied singly, but that on simultaneous application sitosterol restricted cholesterol solubility. Uptake of β-sitosterol was one-fifth of the uptake of cholesterol. The ability of the jejunal brush-border membrane to discriminate between sterols would be a major factor for specific sterol absorption. *In vitro* experiments with rat jejunal villous cells, performed by Child and Kuksis (1983a,b), led to the observation that 7-dehydrocholesterol was absorbed four to five times faster than 7-dehydrositosterol. They concluded that β-sitosterol is unable to enter the brush border vesicles and that selective absorption of a sterol depends on the ring system having the bulk of the cholesterol nucleus though not necessarily a rigid or polar one containing OH group. The second conclusion has been challenged by others who consider the "bulkiness" of the side chain structure of plant sterols as the obstacle to penetrating the cell membrane and, thus, to their transmembrane

passage. This concept finds support of *in vivo* feeding experiments in which sterol uptake by rat small intestine was studied (Bhattacharyya, 1981), further, from dietary experiments with Rhesus monkeys (Bhattacharyya and Eggen, 1981). Of particular interest is their observation that some monkeys are low and others are high responders to cholesterol and to campesterol. After six weeks of a 2% dietary supplement, one group had 0.24 mg/dl, the other, 1.65 mg/dl plasma campesterol. Curiously, β-sitosterol was not absorbed by either group of the monkeys. Certainly, humans, too, differ with regard to sterol absorption.

2.5. Hyperphytosterolemia

The number of "high responders" to phytosterols among humans is small. The first report, on two female siblings, 23 and 21 years of age, was made by Bhattacharyya and Connor in 1974. A third instance was reported by Schulman *et al.* in 1976 about a 31 year old female, with onset of her disease dating back to age of 18 months and fully developed xanthomatosis at age of 13 years. The plasma sterol distribution in the first three patients with proven hyperphytosterolemia is presented in Table 2.

A fourth report was made by Miettinen in 1980. It concerned a male with xanthomatosis and premature coronary disease requiring a triple bypass at age 29, diagnosed at first as familial hypercholesterolemia until it was found that, while the serum cholestrol level was normal, serum phytosterols equaled 22–26% and biliary phytosterols equaled 27–30% of total sterols. The phenomenon was ascribed to unusually high absorption of plant sterols, to impaired sterol elimination and to low cholesterol synthesis. Kwiterowich *et al.* (1981) were stimulated by the death due to coronary atherosclerosis of a 13 year old Amish boy to investigate his family history. Five of the boy's 12 siblings had tendon tuberous xanthomas and phytosterolemia, especially hyper-β-sitosterolemia. In the five siblings plasma apolipoprotein of LDL, i.e. B protein, was very high, with a mean of 173 mg/dl, while LDL-cholesterol was only moderately increased, to 209 mg/dl. Four asymptomatic siblings and the parents had also increased B protein, normal or only mildly elevated total and LDL-cholesterol. Then, the two patients, first described in 1974 by Bhattacharyya and Connor were reviewed in 1981 by Kwiterowish *et al.* (one of the reviewers and co-authors being Connor). Their normocholesterolemia and hyperphytosterolemia had persisted for the six years, LDL-B protein was found to be increased. Three of their grandparents, their mother, father (suffering from angina pectoris) and two uncles had normal total serum cholesterol, normal LDL-cholesterol and increased LDL-B protein. It was concluded that the two sisters were homozygous for a mutant allel and that heterozygous could be identified by increased LDL-B protein, even in absence of phytosterolemia.

Matsuo *et al.* (1981) described the first Japanese patient with both, hyperlipoproteinemia IIa and phytosterolemia, who had tuberous xanthoma. Wang *et al.* (1981) recorded the first Chinese with the complex findings of β-sitosterolemia, mild campesterolemia, cerebrotendinous xanthomatosis, severe coronary atherosclerosis, hemolytic spherocytic anemia and defective synthesis of chenodeoxycholic acid. Co-existing phytosterolemia and cholestanonemia in a patient led Lin *et al.* (1983) to a year-long study of the turnover of intravenously administered 22,23-^{3}H-sitosterol and 4-^{14}C-cholesterol. The half-lives of exponential curves for sitosterol were abnormally long and daily turnover for β-sitosterol was ten times greater than in healthy subjects. Upon isolating three pentols from the urine and feces of a patient with sitosterolemia and xanthomatosis, Duval *et al.* (1985) suggested an abnormality in bile synthesis as causing the disease.

Table 2. *Plasma Sterols (mg dl) in the First Three Reported Instances of Familial Hyper-β-sitosterolemia*

Cholesterol	β-sitosterol	Campesterol	Stigmasterol	Phytosterols as % of total sterols
203	37	10	0.5	18.1
206	17	8	0.5	11.9
242	12	6	trace	7.1

The cited reports do not reflect the total number of families with phytosterolemia. However, the publications serve as a reminder that all patients with xanthomatosis and their families should be studied. All should have detailed sterol assays. Excessive ingestion of phytosterols should be avoided in those who are prone to hyperphytosterolemia. An interesting offshoot to such investigations is a report by Fleischmajer *et al.* (1981) on a normolipemic patient with tendon and tuberous xanthomas, without hypercholesterolemia, broad-β-disease, or β-sitosterolemia.

3. SITOSTEROL–CHOLESTEROL INTERPLAY

Successful prevention or reduction of hypercholesterolemia using a sitosterol regimen lies in recognizing the principle by which such regimen operates. For man, the selection of proper subjects based on proper classification of hypercholesterolemia is the first requirement for success, the second being compliance with instructions which have been proven when sitosterol was first introduced as plasma cholesterol depressant.

Several theories have been advanced concerning the mode by which intake of β-sitosterol leads to plasma cholesterol reduction. First, results of animal experiments in which sitosterol was fed together with cholesterol, and next, the results of balance studies in animals and in humans led to the conclusion that β-sitosterol, also, β-sitostanol, interfere with the resorption of cholesterol from the intestinal lumen. The bibliography has been reviewed by Pollak and Krichevsky (1980). There is no need to recapitulate past studies or to cite new studies lest they add new information.

The question as to whether sitosterol could combine with cholesterol to form large, unresorbable crystals has been studied but not resolved. The concept found support by reports on presence of mixed crystals of two or three sterols in plants, and by results of solubility studies of mixed crystals in oils and bile acids, *in vitro* and *in vivo*. Similar experiments concerned the formation of micells with triglycerides, fatty acids and bile acids, and on partition between micellar and emulsion phase, with sitosterol lowering cholesterol activity in the particulate fraction.

Adsorption of cholesterol on the surface of phytosterols was ruled out by experimental results. However, aggregation of sterols has not been sufficiently studied. The possibility of sitosterol promoting cholesterol excretion has been considered. Isolated reports were made on differences in the conversion of cholesterol to coprostanol in rats fed sitosterol plus bile acids. The effect of β-sitosterol on cholesterol synthesis has been studied, with negative results. Marked suppression of cholesterol biosynthesis results from marked suppression of cholesterol resorption, be it due to sitosterol or other agents.

Reports on competitive esterification outnumber reports on all the other suggested modes. Some investigators believe that esterification is essential for cholesterol resorption; others believe that esterification enhances resorption. If β-sitosterol should deviate fatty acids it would follow that sitosteryl esters would be formed instead of cholesteryl esters. This has not been proven. Be it as it may, esterification does not affect the side chain of β-sitosterol and it is the side chain structure which prevents penetration of the cell membrane. While reducing cholesterol resorption, β-sitosterol by itself is not resorbed.

Most of the recent studies focus on the influence of β-sitosterol on esterifying enzymes. Regrettably, most studies were and are being made with rats and other small animals, although it has been recognized that there are distinct species differences with regard to sterol absorption. Conclusions are not uniform.

Kritchevsky *et al.* (1975) found that rabbits fed β-sitosterol have a cholesteryl synthetase/cholesteryl hydrolase ratio in aortic tissue which was 64% higher than the ratio in control tissue and 177% higher than in presence of cholesterol. Ide *et al.* (1980) checked cholesterol synthetic activity in fasted–fed rats. Ingestion of β-sitosterol depressed liver cholesterol and enhanced cholesterol excretion as neutral steroid. It did not increase hepatic 3-hydroxy-3-methylglutaryl coenzyme-A reductase, nor cholesterol synthetase.

In vivo dual isotope ratio and observation *in situ* of rat intestinal loop was used by Shidoji *et al.* (1980). On intake of 500 mg, cholesterol absorption equaled 44% of the oral

dose while β-sitosterol absorption was only 5.4% of the dose. Cholesterol absorption decreased with increasing doses of β-sitosterol. This was ascribed to enzymatic action.

Kuroki *et al.* (1983) attributed the effect of feeding female hamsters either cholesterol or sitosterol to hepatic 12α-hydroxylase activity. This enzyme was inhibited by 63% on cholesterol feeding and by 30% on sitosterol feeding. Cholesterol caused increase in chenodeoxycholic acid and lithocholic acid in the bile, accompanied by a reciprocal decrease in biliary cholic acid. Sitosterol had no significant effect. On cholesterol intake, serum and liver cholesterol increased whereas both decreased on sitosterol intake.

Field and Mathur (1983) fed rabbits coconut oil plus/minus β-sitosterol or cholesterol, or both sterols. Acyltransferase-dependent esterification rate of β-sitosterol was slower than that of cholesterol. ACAT-Co-A-dependent esterification of cholesterol was at least 60 times greater than that of β-sitosterol. They concluded that the reason for poor absorption of sitosterol lies in its inadequate esterification. This may be questioned since sitosteryl esters are not absorbed better than free sitosterol. The fate of fatty acids not utilized for esterification of sitosterol has not been monitored. Enzyme specificity has not been considered.

Ten subjects with primary hypercholesterolemia were studied by Weisweiler *et al.* (1984) for two months during which they received 6 g β-sitosterol per day. On this regimen, molecular esterification rate of LCAT increase from 76 to 122, the fractional esterification rate, from 1.5 to 7.2. Total plasma cholesterol decreased from 8.9 to 8.02 mmol/l, free cholesterol, from 1.88 to 1.6 mmol/l, and esterified cholesterol, from 1.24 to 1.16 mmol/l. Apo-AI rose from 1.13 to 1.35 g/l and apo-B, from 1.51 o 1.53 g/l. LDL-cholesterol was affected via accellerated esterification rate by the LCAT enzyme.

4. CLASSIFICATION OF HYPERCHOLESTEROLEMIAS

Proper classification of each subject's hypercholesterolemia is a prerequisit for choice of treatment and for success. This will be further emphasized in the discussion of sources of cholesterol and of types of hypercholesterolemia. The two lists, one of the sources, the other one, of the types of hypercholesterolemia, are not identical. Not all the cholesterol entering the body leads to hypercholesterolemia. Then, plasma cholesterol can increase from endogenous sources and, lastly, in absence of an increased cholesterol supply.

4.1. Sources and Pathways of Cholesterol

Cholesterol can find its way into the body and into the blood stream by several routes. An excessive supply, whether of endo- or exogenous origin, may lead to elevation of plasma cholesterol. The degree depends not only on the quantity of cholesterol, the source and the route, on many variables influencing cholesterol transport and metabolism, but on each individual's ability to cope with cholesterol. Sources and pathways are listed in Table 3.

Table 3. *Sources and Pathways of Cholesterol*

A.	Endogenous, by biosynthesis	
B.	Endogenous, metabolic	
	(a)	Hypothyroidism, primary or secondary
	(b)	Nephrotic phase of chronic nephritis
	(c)	Cholestasis, of diverse origin
C.	Exogenous, by enteric route	
	(a)	Dietary, due to cholesterol overalimentation
	(b)	High colonic irrigation; milk-egg-sugar enema
D.	Exogenous, by parenteral route	
	(a)	Hematogenous, by blood transfusions
	(b)	Percutaneous, by "cholesterol shampoo"
E.	Exogenous, by male ejaculate	
	per vagina, per os, per rectum	

TABLE 4. *Types of Hypercholesterolemia**

I.	Endogenous
A.	Monogenic, primary, familial, "essential"
	(a) heterozygous
	(b) homozygous
B.	Polygenic, secondary, metabolic
	(a) thyroidogenic
	(b) nephrogenic
	(c) cholestatic
II.	Exogenous, secondary, polygenic
A.	Enteral
	(a) alimentary, dietary, oral
	(b) nutritional enema
	(c) medicinal, hypercholesterolemia as side effect
B.	Parenteral
	(a) hematogenous, by transfusion

*From Pollak, O. J. (1987).

4.2. TYPES OF HYPERCHOLESTEROLEMIA

In 1976, Ahrens introduced a new terminology, separating hypercholesterolemias into primary or monogenic types and secondary or polygenic types. His scheme has been adopted and adapted, resulting in an expanded list presented as Table 4 (Pollak, 1987).

Two types of hypercholesterolemia can coexist. A subject with primary hypercholesterolemia can acquire a secondary type, especially with hyperalimentation.

Sources, routes and effects are being discussed together. Excessive cholesterol biosynthesis is an inborn error of metabolism, an enzymatic defect. It leads to primary, monogenic, familial or "essential" hypercholesterolemia. This may be heterozygous or homozygous. In the latter form, it is commonly accompanied by one or another form of xanthomatosis, often by premature coronary artery atherosclerosis. The degree of hypercholesterolemia is very high, its regulation is very difficult, especially of the homozygous form. Lack of success in blocking the synthetic pathways is chiefly due to multiple adverse effects of the medications proposed until now. Multiple reports have been made on the effective use of β-sitosterol in lowering the plasma level in children and also in some adults with clinical manifestations. For sober evaluation of such reports one should have information as to cholesterol intake and to the degree to which dietary cholesterol contributes to the plasma pool.

For polygenic hypercholesterolemia of metabolic origin, whether due to a low metabolism in a hypothyroid or myxedematous patient, due to altered elimination in the patient with chronic nephritis and the nephrotic syndrome, or due to lack of conversion of cholesterol to bile acids or blockage of biliary passages, to bile stasis, the obvious course of treatment of hypercholesterolemia lies in treating the causative condition. Other metabolic diseases have been mentioned as causing hypercholesterolemia. These are diabetes mellitus, gout, and dysproteinemias. Hyperglycemia, hyperuricemia, and dysproteinemia are indeed often accompanied by dyslipidemia, including hypercholesterolemia. However, a causal relationship has never been proven. Depressing plasma glucose, uric acid, or normalizing serum proteins will not result in lowering plasma cholesterol.

All the polygenic, secondary types of hypercholesterolemia of exogenous origin are introduced by man. As such, they all are preventable or controllable. The most important of the group is the alimentary hypercholesterolemia. It also is the most widespread type, especially in the western world. It can be counteracted in many ways, one of which is by ingesting plant sterols, particularly, β-sitosterol. This is the main subject of the chapter on plant sterols in the Encyclopedia.

A high-caloric nutritive enema has been used in the past for patients who were unable to swallow. The concoction contained one liter of milk, or half-a-liter of milk plus half-a-liter of cream, six to eight whole eggs, some sugar. One liter contained up to 2000 or 2500 mg cholesterol. Patients received up to three enemas per day for three days. The effect on the patients' plasma cholesterol level has never been studied. Certainly, it could

lead to transitory hypercholesterolemia, which, if high enough, could be most damaging even if temporary only. This is of theoretical interest since the practice has been replaced by intravenous feeding. The latter, too should be closely monitored for increase of plasma triglycerides and cholesterol.

The hematogenous route of cholesterol, leading to marked increase in plasma cholesterol, has been discussed in the monograph by Pollak and Kritchevsky (1980). The danger of inducing hypercholesterolemia through multiple blood transfusions is real. It has been largely overlooked. Cholesterol is being introduced directly into the blood stream, at that, in large amounts. Sudden episodic overload can lead to cholesterol deposits in the arterial intima. One unit of whole blood represents 450 ml blood plus 20 ml of anticoagulant solution. The amount of cholesterol in whole blood is the sum of plasma and cell cholesterol. The two become equalized within four hours after blood withdrawal. Given a 46% hematocrit, three donors with 200, 240 and 300 mg/dl plasma cholesterol would have, respectively, 600, 720 and 900 mg total cholesterol per unit of whole blood. Then, the total amounts of cholesterol introduced into the circulation would have to be multiplied by the number of transfusions given. It could be considerable. The risk of iatrogenic hypercholesterolemia can be reduced. Assay of plasma or whole blood cholesterol in blood donors is hardly feasible, but some donors can be eliminated during the interview preceding the donation. Instead of whole blood, blood components should be used whenever possible. The number of transfusions should be held at a minimum. Intervals between transfusions should be stretched to a maximum.

Cholesterol derived from two other sources does not affect the plasma level. Yet, the sources should be mentioned. The percutaneous route is represented by a "cholesterol shampoo" which recently appeared on store shelves, advertised for its superior penetration of the scalp. The amount of cholesterol is not indicated on the label. An assay, at the Wistar Institute of Philadelphia, revealed that a 500 g can of the shampoo contained 40 mg cholesterol. This would make it 0.8–1.6 mg for a single application for which 10–20 ml or g are used. The dose is too small to influence plasma cholesterol levels.

Of even smaller significance is the male ejaculate as a source of cholesterol, though it can be introduced into the human body by several routes. The cholesterol content of ejaculate is 80 mg/dl. Normally, the volume of ejaculate is 3–3.5 ml. Thus, the amount of cholesterol per sample would be only 2.4–2.8 mg.

Plasma cholesterol could increase without excess supply of cholesterol. A good example is a newly introduced medication, "Accutane", for sever, cystic acne, taken orally in 40 mg capsules. The preparation does not contain cholesterol. According to the manufacturer's instructions accompanying each batch of 100 capsules, the medication can lead to hypertriglyceridemia in 25% of patients, to hypercholesterolemia in 7%, to a decrease in HDL in 15%. Roche Lab., the producer, advises blood lipid assays before a treatment course and in four weeks intervals during treatment. The listed contraindications are pregnancy, nursing, the use of oral contraceptives, intake of the chemically related vitamin A, and allergy. A pregnancy test is advised before starting treatment. This all suggests that the drug, isotretnoin, interferes with sterol metabolism. The far greater influence on triglycerides remains unexplained. Plasma lipid levels may depend on dosage and will return to starting levels on cessation of therapy. Yet, even brief episodes of hypercholesterolemia–hypertriglyceridemia must be avoided. An alert is sounded for fear that other medications, current or future, may have a similar effect.

5. RISK FACTORS

Experimental hypercholesterolemia is the only real atherogenic risk in animals. Surely, there are others, such as unequal species susceptibility, or restricted mobility of caged animals. For humans, hypercholesterolemia, regardless of its source, is the major risk factors, although the number of other factors for man exceeds by far those for lower species.

Unfortunately, all known or suspected risks are usually being lumped together. Separating risks into atherogenic and thrombogenic risks and assigning each factor or groups of factors proper weights is imperative for planning a therapeutic or prophylactic program. There is some overlap between atherogenic and thrombogenic risks, namely, in the role played by cholesterol.

Hypercholesterolemia is a major atherogenic risk; it also plays a role in thrombogenesis. Alimentary hypercholesterolemia–hypertriglyceridemia leads to "lipid" deposits, to atherogenesis. Episodic hyperalimentation is a factor in the sludging of blood, hypercoagulability and thrombus formation.

Most known risks are operative only in conjunction with one or more other risks. The modest role of one risk may be exagerated by a pre- or co-existing second risk. A few examples are in order. Nicotine is not atherogenic, but it is thrombogenic. Its effect on blood coagulation is greatly enhanced in subjects with hypercholesterolemia or with hyperestrogenemia. The thrombogenic effect of estrogens may be debatable, but it becomes a certainty in synergism with nicotine, as recognized for women smokers using oral contraceptives.

Preventing atherosclerosis, the symptomatic clinical disease, should start with preventing atherogenesis, the anatomical arterial alteration. Preventing atherogenesis should start with preventing risks. Only two atherogenic risks can operate, more or less, independently of other risks. One of these is a high caloric diet. Total intake is more important that the calories supplied by fat. The other risk is hypercholesterolemia, regardless of its endo- or exogenous cause. At that, these two risks are often linked: Hyperalimentation includes excessive ingestion of cholesterol-rich foods.

6. CONTROL OF DIETARY CHOLESTEROL

Restricting cholesterol consumption seems the simplest and easiest way of controlling alimentary hypercholesterolemia. Indeed, there are references to the salutory effect of a strict vegetarian diet and to the negligible effect of a lacto-vegetarian diet on plasma cholesterol and its sequelae (Pollak and Kritchevsky, 1980). Lest reared on a regimen of natural foods of plant origin from the cradle, a no-fat and no-cholesterol diet is not well tolerated. Man's teeths are for biting, shredding and grinding of food of mixed origin. A low-fat and low-cholesterol diet may be helpful for the overweighed, temporarily. It could be helpful for a time, if complied with. But, how "low" is "low" with regard to diet and its effect. Adherance to any diet is difficult in a society taking many meals in public places.

The amount of cholesterol available for resorption can be reduced. This is the basis for ingestion of phytosterols and also for some medications. Intake of phytosterols is not the only tool available. Conversion of cholesterol to coprostanol can be stimulated by calcium, depressed by lactose, inhibited at pH 5.8. Evacuation of the bowel can reduce the amount of cholesterol available for reabsorbtion. Dietary fiber has a like effect. Beta-sitosterol blocks resorption and re-absorption. Saturated fatty acids enhance resorption, polyunsaturated fatty acids neutralize the action of saturated fatty acids. Elimination of cholesterol is enhanced by pancreatic enzymes and bile acids, by fiber, by stimulating peristalsis or enhancing the blood supply to the intestines.

Controlling dietary cholesterol requires controlling also total caloric intake, the amounts and types of fatty acids, of triglycerides, protein, carbohydrates, vitamins and minerals, fiber and plant sterols. In other words, a broad dietary program is required rather than simply restricting fat, cholesterol and saturated fatty acids.

7. ANIMAL EXPERIMENTS WITH PHYTOSTEROLS

Introduction of new pharmaceuticals or of a new non-medicinal treatment for man's ailments is usually based on results of animal experimentation, if for no other reasons, at least, to determine dosage and safety. This applies also to phytosterols used as plasma

cholesterol depressants and thus, indirectly, for prevention of atherogenesis and its progression.

The first pertinent report on the usefulness of plant sterols for regulating plasma cholesterol was made by Peterson in 1951. Chicken were fed a diet supplemented with 0.5–1% cholesterol (C), 0.5 to 1% mixed soy sterols (SS), or a mixture of these two sterols. On addition of cholesterol to the basic ration, plasma cholesterol rose from 196 to 942 mg/dl. On addition of soy sterols to the ration, plasma cholesterol did not rise above the normal level. Nor did it rise on supplementing the ration with both sterols. In another series, the effect of 0.05% cholesterol added to the ration was completely blocked by adding 0.25% or more of soy sterols, that is at a C:SS ratio of 1:5.

At the same time the experiments with chicken were under way, the effect of sitosterol on serum cholesterol of rabbits was tested. The experimental results were reported by Pollak (1952, 1953a). Rabbits were fed 1–1.5 g cholesterol (C) daily for 14 days, or 1 g, 2 g, 3 g, 5 g, 6 g, 7 g or 10 g sitosterol (S) per day, or, the two sterols were fed in proportions of 1:1, 3:1, 5:1, 6:1, 7:1, or 10:1 for 14 days. Whereas cholesterol supplement alone caused a 14- to 16-fold increase of serum cholesterol, sitosterol fed alone caused no increase at all. Fed in combination, the increment of serum cholesterol lessened reciprocally to increased doses of sitosterol. At a S:C = 7:1, the rise of serum cholesterol was blocked completely. This ratio has to be corrected because the preparation contained only 75–80% of β-sitosterol. Thus, the effective ratio was S:C = 5.6:1 or 5.25:1.

In the chicken and in the rabbit, atherosclerosis can be prevented by preventing hypercholesterolemia. The results of the experiments were comparable, as was the effective dose of phytosterols. The "mixed soy sterols", too, had only 75–80% of β-sitosterol. A S:C ratio of 3:1 (corrected for β-sitosterol content, a ratio of 2.4:1 or 2:25:1) allowed serum cholesterol to rise by a degree which was too small to induce atherogenesis.

Phytosterols were given to large numbers of chicken and rabbits by many investigators. Publications on experiments with these two species and many others number by now close to two hundred. "Plant sterols", soy sterols, corn oil sterols, cottonseed oil sterols and sitosterol were used in various proportions to cholesterol, either to prevent experimental hypercholesterolemia, or to reduce existing plasma cholesterol elevation. Except for instances in which insufficient amounts of phytosterols were used, the results mirrored those originally reported for chicken and rabbits. A few experiments with pigeons, guinea pigs, dogs and monkeys failed because of inadequate doses of sitosterol. Experiments with rats resulted usually in decrease of serum and liver cholesterol, but in isolated instances, only one or the other was reduced in response to sitosterol feeding.

With a single exception, sitosterol was applied in the form of powder or in suspension. Sayeed and Ahmad (1979) fed mice 12 vegetables available in Bangladesh and found that one with the highest β-sitosterol content effectively reduced serum cholesterol of mice when fed as 1% of the diet for 11 days, and serum and liver cholesterol, when fed for 22 days.

7.1. Phytosterols other than Sitosterol

Success with oral administration of β-sitosterol as an "anti-cholesterol" agent prompted trials with sitosterol derivatives and with other plant sterols. Since Pollak and Kritchevsky (1980) reviewed the pertinent bibliography, only a few more recent essays are being mentioned here. Since all the work except one was done with animals, the subject is being covered as part of experimentations.

In the past, several sitosteryl esters were found ineffective when fed to rats. Tabata *et al.* (1980) tried a different approach. Rats were fed a 3% cholesterol diet and injected intravenously with β-sitosterol, stigmasterol, ergosterol, 7-keto-cholesterol, or their respective esters. The serum cholesterol-lowering effect depended on the double bond at C5 and side chain at C17, with esters having consistently lower effect on serum and liver cholesterol than free sterols.

Beta-D-glucoside-β-sitosterol was fed to normo- and to hypercholesterolemic rats by Miromova and Kalashnikova (1982). Normocholesterolemic rats were not affected. In

hypercholesterolemic rats, plasma cholesterol decreased by 38%, β-lipoproteins, by 46%, phospholipids, by 38%. Apparently, sitosteryl glucoside activated phospholipid synthesis and normalized the cholesterol/phospholipid ratio.

A representative reference is being made, chosen from tens of publications comparing sitosterol and sitostanol fed to rats. Ikeda and Sugano (1978) found dihydro-β-sitosterol (SH) less absorbable than sitosterol (S), with HS in feces equal to 97% of the oral dose, fecal S equal to 88% of the oral dose. Upon intravenous injection of [4-^{14}C]β-sitostanol and [4-^{24}C]β-sitosterol, turnover of HS was more rapid than that of S and neutral fecal steroids were twice as high after HS than after S. The LDL/HDL ratio for both, HS and S, was lower than that for endogenous cholesterol. Rabbits were used in another study by Ikeda *et al.* (1981). On 0.5 β-sitostanol and 0.2 cholesterol, serum cholesterol decreased more than if the ratio was 0.5:0.5. HS proved more hypocholesterolemic than S, especially with regard to LDL-cholesterol.

Vahouny *et al.* (1983) administered three sterols intragastrically to rats, β-sitosterol (24-ethylcholesterol), stigmasterol (Δ^{22}-24α-cholesterol) and fucosterol (24-ethylidenecholesterol). Lymph was collected 24 hours later for analysis. Sito- and stigmasterol inhibited absorption of simultaneously administered cholesterol by 54%. Fucosterol was inactive, as noted by others. Sito- and stigmasterol were also compared by Chandler *et al.* (1979). They fed 44 one-day-old cockerels β-sitosterol for 19 days and fed 99 one-day-old birds stigmasterol for 24 days. Stigmasterol had no effect on plasma cholesterol. Uchida *et al.* (1983) supplemented the diet of mice with 1% sitosterol or spinasterol for 15 days. Both sterols increased cholesterol excretion, decreased plasma and liver cholesterol, decreased the bile acid pool size and also decreased fecal bile acid excretion, especially that of chenodeoxycholic acid.

In a solitary clinical study, Mattson *et al.* (1982) served to nine adults a meal with 500 mg cholesterol as scrambled eggs for the first period, added 1 g of β-sitosterol in a second period, 2 g β-sitosteryl oleate in a third period. Plasma cholesterol of all subjects decreased from the fifth day on, reflecting the lowered cholesterol absorption. Free sitosterol depressed cholesterol absorption by 42%, sitosteryl oleate by 35%, in spite of twice the oral dose of sitosterol.

Nearly all the studies with sitosterol derivatives or with phytosterols others than sitosterol were performed with animals, mostly, with rats and mice. Beta-sitostanol was found most useful. Extrapolation of results to man is hazardous. Regrettably, β-sitostanol alone has not been tested in clinical studies. Some commercial "sitosterol" preparations contain β-sitostanol. One of these, with 92% β-sitosterol and 8% β-sitostanol was found to have sufficient potency to allow reducing the daily dose of the product from 7 to 3 g.

7.2. Low-cholesterol Eggs

Nearly all animal experiments were designed to elucidate the effect of phytosterols on plasma cholesterol. A few investigators set different goals. They wanted to produce eggs with a low cholesterol content. Since eggs are a major source of dietary cholesterol, low-cholesterol eggs would provide a profitable approach to lessening of alimentary hypercholesterolemia.

Previously, attempts were made to produce eggs with a high content of polyunsaturated fatty acids, known to lessen the resorption of cholesterol. These attempts failed. In fact, men consuming such eggs for 18 days had a higher plasma cholesterol than they had before such diet.

The first attempts to produce low-cholesterol eggs by feeding laying hens plant sterols also failed. The failure was due to insufficient amounts of β-sitosterol used, namely of 1% added to chicken feed. In 1971, Clarenburg *et al.*, succeeded by adding ^{3}H-β-sitosterol as 1, 2, or 4% of the feed, without interfering with egg production. They obtained eggs with significantly lower cholesterol content than the amount in eggs of control hens. The results are presented in Table 5.

At a 35% reduction, the cholesterol content of an egg weighing 50 g would decrease from

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TABLE 5. *Per cent Decrease of Egg Cholesterol on Sitosterol Intake*

Sitosterol as	1%	2%	4%	of chicken feed
after 7–12 days	−5.7	−11.0	−29.5	egg cholesterol
after 15–30 days	−6.2	−35.5	−35.8	

about 150 to 50 mg. Moreover, such eggs would contain about 40 mg of sitosterol. Speculations are open as to the commercial potentials of marketing low-cholesterol eggs. The health benefits are obvious.

8. FACTORS INFLUENCING RESULTS OF CLINICAL STUDIES

Proper evaluation of results of a sitosterol regimen requires considering all the factors which influence this approach.

8.1. Chemical and Physical Properties of Sitosterol Preparations

While it is most important to understand the principle by which sitosterol interferes with cholesterol absorption, it is also important to know all the factors influencing the process.

One can refer to phytosterols by their chemical formulas or by their origin. Peterson (1951) reduced plasma cholesterol of chicken by feeding them "mixed soy sterols" and Pollak (1952, 1953a) reduced serum cholesterol of rabbits by feeding them "sitosterol". Both preparations had a 75–80% β-sitosterol content. The potency of commercial products depends on the starting material and processing. Wood shavings are a better source of β-sitosterol than tall oil, soybeans, or cotton seed oil. Over one dozen of products manufactured by at least twenty firms have been made available. They contain anywhere from 17 to 93% of β-sitosterol, the average being 68.5%. Some products underwent changes with time: for example, "Cytellin", the most widely used preparation in the USA, contained, at first 65%, later 89%, and ultimately 92% β-sitosterol.

Products were dispensed as solutions, suspensions, emulsions, elixirs, as powders, crystals, granules, micronized, as tablets, pellets, in "candy bars" or in capsules or bags. The physical character certainly influences the potency, almost as much as the actual β-sitosterol content. A spray-dried powder proved more potent than either plain powder or granules or tablets, which previously were found superior to liquid preparations.

Chemical and physical properties of the products influence dosage and ultimately the results of a sitosterol regimen.

8.2. Internal Factors

Internal factors represent a mixed bag. Not all of them are significant as far as the results of clinical studies are concerned.

The age factor has been largely ignored, at least for humans, although it is well known that the plasma cholesterol level varies with age. It also varies with emotional stress and disease. Then, there are diurnal and seasonal variations. All these are more pronounced in subjects with severe hypercholesterolemia. Conflicting reports concern adolescents who may not react as well as adults to either a vegetarian diet or to a β-sitosterol regimen. Children, however, seem to respond better than adults.

References to gender are few, mostly only to female rats responding better than male rats. A single report on humans can be dismissed because of the small numbers of males and females.

Overweight individuals could present a problem. On daily ingestion of 7.3 g sitosterol the plasma level of cholesterol decreased in overweight patients more than in underweight. However, the level tended to rise again in spite of continued medication, especially in those who were gaining weight.

Genetic factors have hardly been studied. Significant differences in the response to β-sitosterol were noted in several breeds and crossbreeds of chicks, swine and monkeys. As for man, no differences were found in patients with type IIA and type IIB hyperlipoproteinemia.

8.3. External Factors

External factors influencing the course and results of a sitosterol regimen are largely introduced by the investigator. Although ingestion of phytosterols can be safely recommended for everyone the regimen is clearly indicated for controlling alimentary hypercholesterolemia.

Individuals having comparable plasma cholesterol levels will not respond in like manner to sitosterol. In a mixed population, there will be those with normocholesterolemia and those with hypercholesterolemie of different causes. It would not be reasonable to expect uniform results to any treatment. Moreover, not all subjects comply with physician's orders to equal degree.

It has been widely acknowledged that markedly elevated cholesterol levels can be reduced easier than moderately elevated levels, certainly, more effectively than "normal" plasma levels. This applies to all measures for control of alimentary hypercholesterolemia, not just to sitosterol. There is one exception to this rule, namely, primary monogenic hypercholesterolemia, commonly of a high degree. During a panel discussion, Pollak (Wilkinson *et al.*, 1958) stated that "each individual seems to have its own 'basal' plasma cholesterol level which cannot be depressed without triggering homeostatic control." He also stated "that an individual's 'maximum' level can hardly be exceeded by an overload of dietary cholesterol." Indeed, persons with comparable fasting plasma cholesterol values respond differently to eating the same number of eggs for a meal.

Many investigators introduced a factor which affects adversely the sitosterol regimen. They did so by pre-medicating their patients, either by diet or by medications, or both, thus lowering the plasma cholesterol into normal or near normal range, into the group of mild responders.

8.4. Timing of Sitosterol Intake

Peterson (1951) experimenting with chicken and Pollak (1952, 1953a) experimenting with rabbits found that the hypocholesterolemic effect of plant sterols was much better when these were fed together with cholesterol than when phyto- and zoosterol were given separately. Pollak (1953b) suggested for humans intake of sitosterol three times a day, with meals, to assure prompt interaction between sito- and cholesterol in the intestines.

8.5. Dosage of Sitosterol

Originally, Pollak (1953b) recommended a 6 g/day dose of sitosterol powder. The preparation used contained 75–80% β-sitosterol. By increasing the purity to 92%, the daily dose could be reduced to 3 g (Lees and Lees, 1976). Many clinicians objected to the "large" dose. In fact, one-half of all the clinical investigators prescribed doses between 1 and 10 g per day; one-fourth of these used less than 7 g a day. The mean average amount used was 4 g per day, less than the recommended dose for many a medication.

The concern about the need for excess of β-sitosterol over cholesterol intake is reminiscent of past debates about the direction in which various ratios should be manipulated. Such question concerned the phospholipid/cholesterol ratio, then, the (PUFA/SFA) polyunsaturated fatty acids/saturated fatty acids ratio. More recently, the question arose whether it would prove more beneficial to increase (HDL) high density lipoprotein-cholesterol or to reduce (LDL) low density lipoprotein cholesterol.

The usefulness of a sitosterol regimen should be determined by a trial run, using 5 g day of a high purity preparation for two weeks. By that time, plasma cholesterol will have decreased by from 10 to 20% roughly by the same amount as supplied by the diet to the plasma pool. Now, the individual dose can be "titrated" (Shipley, 1957), by first increasing the daily dose in two-week intervals until consistent maximum effect has been reached, then, gradually decreasing the daily dose and a maintenance dose has been reached.

9. CLINICAL STUDIES WITH PHYTOSTEROLS

The first clinical study with sitosterol as serum cholesterol depressant was conducted in 1951 and 1952 by Pollak (1952). A daily dose of 5–7 g of crude sitosterol powder was given

to 26 unselected subjects, none of whom had clinical symptoms of atherosclerosis. Their serum cholesterol levels ranged between 126 and 414 mg/dl, with a mean average of 256 mg/dl. Within two weeks and without dietary restrictions, the range was 131–264 mg/dl, the mean average was 173.5 mg/dl. The decrease in serum cholesterol averaged for the whole group 74 mg or 28%. For those whose cholesterol was below 240 mg/dl, the decrease was 12.6%; for those whose level was above 240 mg/dl, the decrease was 22%, with up to 39% for those whose level exceeded 285 mg/dl prior to treatment. Upon cessation of the sitosterol regimen, the serum cholesterol levels reversed to the original height within two to three weeks. After a pause of 40 days, a second course of sitosterol had the same effect as the first course.

Those who ignored the plan presented in the prototype study were often disappointed with their results. Those who followed the original recommendations with regard to selection of suitable subjects, dosage and timing of sitosterol ingestion, and did not try to reduce plasma cholesterol prior to sitosterol intake, achieved results which were comparable to those of the prototype study. Often, the results were better than those reported in the model study.

The review of clinical studies has to be made in two parts. The number of publications during the first 25 years was very large, that of the last ten years was small. The older essays have been reviewed by Pollak and Kritchevsky (1980). During the first period, attention had been paid to total serum or plasma cholesterol, only in the last years was consideration given to the types of hyperlipoproteinemia, mostly of type IIa and IIb. In the last decade, attention shifted to lipoproteins of various density and to the cholesterol content of fractions. Also, in search for ways to control monogenic hypercholesterolemia, more studies on this type were conducted in recent years than in the past.

9.1. Review of Clinical Studies from 1952 to 1976

The original report triggered numerous studies. In the first period, over one hundred essays were published, referring to sitosterol administration to over 1800 subjects. About 40% of these had a "normal" plasma cholesterol, below 240 mg/dl, or had only a slightly higher level, up to 260 mg/dl. Slightly over 60% of all subjects had a higher degree of hypercholesterolemia.

Several teams of investigators separated subjects into those with levels below 240 mg/dl and those with a higher level. Some selected 260 mg/dl as the dividing point, still others, 300 mg/dl. Alimentary hypercholesterolemia, *per se*, falls mostly into the 240–300 mg/dl range, by the standards and methods used.

9.2. Review of Clinical Studies from 1977 to 1985

In view of all the variants introduced into the clinical studies, the response to sitosterol intake on plasma cholesterol was quite remarkable. A study by Kandziora (1980) is a good example of modifications of the originally proposed program. He studies 30 patients: Nine of these had been premedicated with a diet, eight, with drugs, six with diet and drugs, and three subjects continued on drugs even during the sitosterol regimen. This left just four subjects who were treated solely with sitosterol. A granular preparation containing 89% of β-sitosterol was given in six daily doses of 1.75 g (total, 10.5 g/day) for four weeks, then, in three doses of 1.75 g (total, 5.25 g day) for eight weeks. After the 12 weeks period, serum cholesterol had decreased by from 5 to 29% (average, by 14%) in spite of pre-medication and ingestion of sitosterol between meals.

The character of hypercholesterolemia has rarely been mentioned. A few referred to biliary cirrhosis or to hypothyroidism. Familial hypercholesterolemia has been mentioned far more often, in the past and more recently.

Schwartzkopff and Jantke (1978) treated one patient with essential hypercholesterolemia. The daily dose of sitosterol granules was reduced gradually from 20 to 15 g, 12 g, finally, to 6 g. Plasma cholesterol fell by 6.7% only, from 717 to 669 mg/dl. Schlierf *et al.* (1978) concluded, upon treating 15 children and adolescents with sitosterol, that the treatment was ineffective in 12 subjects who completed a six months course and

that it is not suited for juvenile hypercholesterolemia, since total cholesterol decreased by only 6%, LDL by 7% and HDL by 15%.

Prescribing a relatively palatable tall oil suspension of sitosterol, first, 18 g/day, later, only 3 g/day, for up to two years to 46 patients with type II hyperlipoproteinemia together with a diet with 40% of total calories as fat, Lees *et al.* (1977) obtained in nine patients a plasma cholesterol decrease by 34.6%. Although the mean response for the whole group amounted to only −12%, sitosterol was recommended as an adjuvant to a dietary regimen. Etminan *et al.* (1979) placed 28 stationary patients with type IIa or IIb hyperlipoproteinemia first on a low-fat and low-cholesterol diet, then, for two to three weeks, on diet plus sitosterol, modifying its daily intake from 20 to 15 to 13 g during three to four weeks, and to 3 g for another eight weeks. The combination of diet plus sitosterol proved three times as effective as diet alone, resulting in a 29.5% drop in plasma cholesterol.

Favorable results by combining a strict diet with ingestion of β-sitosterol were also reported by Drexel *et al.* (1981). A 41-year-old woman had heterozygous familial hypercholesterolemia, with multiple xanthelasmas and small tendon xanthomas. Her family history was strongly positive. Two of her four daughters had hypercholesterolemia. After three months on a sitosterol regimen, her total cholesterol fell by 14.5% and after six more months it fell by another 9.5%, making it a 24% decrease. The fall of total cholesterol was mainly due to fall in IDL-and VLDL-cholesterol. The changes were permanent. On cessation of sitosterol intake, VLDL fell further on a strict diet. The effect of diet plus sitosterol on LDL was small, questionable. However, HDL_2- and HDL_3-cholesterol increased in the patient; in one daughter, only HDL_2 increased whereas HDL_3 increased in both daughters with hypercholesterolemia. These increases were not permanent.

9.3. Duration of Clinical Studies

The reasons why clinical trials which were successful were ever terminated is obscure. Certainly, subjects who are asymptomatic are reluctant to continue on a regimen. However, those with symptoms of atherosclerosis, should not be allowed to terminate the program. Some of the studies lasted a single day during which three doses of sitosterol were taken. Some patients were studied for 48 hours after a single dose. Results of such studies are worthless. Some projects extended over weeks, months, or years. The longest study lasted 40 months, with from three to five doses of sitosterol per day. Thus, comparison of results is difficult, especially if one considers all the other modifying factors.

Those who for one reason or another failed to achieve satisfactory results had to find faults with the program. Objections were voiced to the fact that the cholesterol-lowering effect of sitosterol is not permanent and that the regimen has to be extended indefinitely. The very same objection applies to medications interfering with cholesterol absorption. It also applies to dietary restriction of cholesterol. A low fat–low cholesterol diet should be kept "forever".

9.4. Phytosterol Incorporation into Food

The section on animal experiments with phytosterols has been appended by a subsection on production of low-cholesterol eggs. The section on clinical studies with phytosterols is being supplemented by a subsection on incorporating plant sterols into butter. Both projects aim at providing new ways to reduce dietary hypercholesterolemia.

Petersen *et al.* (1956) incorporated soy sterols into butter. Their 19 subjects, divided into two groups, were healthy, had normal plasma cholesterol which apparently was not affected by ingesting three pats of butter daily. Consuming butter with soy sterols resulted in all subjects in plasma cholesterol reduction by 11%. The design and the results are presented in form of Table 6.

Cholesterol in butter amounts to about 280 mg/100 g. A bar of butter weighs 112 g (112.4 g) and contains 313.6 mg (314.7 mg) cholesterol. Usually, a bar is cut into eight pats. Each pat weighs 14 g and holds 39.2 mg cholesterol. Daily intake of three pats provides

TABLE 6. *Plasma Cholesterol Response to Butter and Butter with Soy Sterols*

No. of subjects	Period weeks	Butter g/per day	Butter g/per period	Cholesterol mg/per day	Cholesterol mg/per period	Soy sterols g/per day	Soy sterols g/per period	Cholesterol mg/dl	Cholesterol plasma
10	1	42	294	117.6	823.2	0	0	218	
	3	42	882	117.6	2469.6	5.7	359.1	194	−11%
	1	42	294	117.6	823.2	0	0	209	
	4	42	1176	117.6	3292.8	0	0	217	
9	1	42	294	117.6	823.2	5.7	119.7	194	−11%

117.6 mg cholesterol (C). Soy sterols (SS) were incorporated into a bar of butter in 15.2 g amount. Three pats of sterol-supplemented butter provide 5.7 g soy sterols per day. The SS:C ratio of about 10:1 could be reduced by 20 or 25% using β-sitosterol of high purity as additive. In a 8:1 or 7.5:1 ratio the amount of sitosterol would suffice to neutralize besides the cholesterol in butter all other cholesterol available for absorption or re-absorption.

Pollak (1953b) and co-workers participating actively in the first study ingested 7.5 g of crude sitosterol, a chalky, not very palatable product and, because of its physical appearance and taste, placed it between two pieces of lightly buttered, soft, white bread. In a way, their procedure was similar to that used by Peterson *et al.* (1956), recording an 11% cholesterol reduction in normocholesterolemic subjects with plasma levels of 217 and 218 mg/dl, while Pollak (1953b) obtained a 12.6% reduction for those with levels below 240 mg/dl and a 22% decrease for those with levels above 240 mg/dl.

10. SITOSTEROL COMPARED TO OTHER AGENTS

Objects to sitosterol intake as plasma cholesterol depressant were directed against the "large dose required", against the lack of uniform response, and against lack of permanent effect after a two-weeks course of sitosterol intake. All these criticisms have been already refuted. All of them can also be rejected on basis of comparing β-sitosterol with other means used or suggested to reduce plasma cholesterol.

10.1. ANIMAL EXPERIMENTS

The number of reports on comparison of several cholesterol-lowering agents using animals is not very large. In rats fed cholesterol, adding taurine to the diet caused inhibition of hypercholesterolemia by 50% adding glycine to the diet had no effect. In rabbits fed cholesterol-enriched chow, choline decreased the degree of hypercholesterolemia by 20%; adding lecithin decreased it by 32–50%, depending on the dosage, used. In other experiments with rabbits, phytosterols and also anetholtrithione supplement to chow had equal effect, lowering plasma cholesterol by 55%, compared to positive controls. In chicken, phenylthyramine proved to be toxic, triparanol alone or plus cottonseed oil prevented experimental hypercholesterolemia. Sitosterol was more effective, vanadium sulfate or diethanolamine were ineffective with regard to lessening aortic lesions of hypercholesterolemic pigeons.

Dogs given 5% cholesterol and ^{131}I developed hypercholesterolemia of 700–900 mg/dl. Adding 10 mg estradiol valerate lessened the cholesterol rise to levels of 500 mg/dl. Adding 43 g β-sitosterol resulted in plasma cholesterol levels below 400 mg/dl (Jordan *et al.*, 1961). In baboons with an average plasma cholesterol of 193 mg/dl, feeding beef tallow caused a rise to 391 mg/dl, Clofibrate, a rise to 337 mg/dl. On 0.3% β-sitosterol, the level was 302 mg/dl on addition to tallow (Howard *et al.*, 1967).

Collating all available data, one can conclude that, in various animal species, phytosterols, fed as β-sitosterol, soy sterols, soy phospholipids, or cottonseed oil, had either comparable or superior effect on plasma cholesterol than synthetic chemicals.

10.2. CLINICAL STUDIES

The number of reports on clinical trials comparing sitosterol with other plasma cholesterol depressing agents is small. Usually, only two or three agents were used but for few subjects. Studies on large numbers of patients regrettably do not include phytosterols among the agents tested.

In one study, β-sitosterol led to a 23% reduction and corn oil to a 23% reduction of serum cholesterol, whereas alpha-tocopherol had no effect (Beveridge *et al.*, 1958). In another study, β-sitosterol decreased serum cholesterol by 16%, while phenylbutyramine had no effect (Doorenbos *et al.*, 1958). A low-fat diet, a brain extract, or β-sitosterol reduced plasma cholesterol equally, by 10% (Jones and Keough, 1958). Nicotinic acid plus Clofibrate, or nicotinic acid plus D-thyroxine failed to influence plasma cholesterol, however, on 5.3 g/day β-sitosterol intake, the same patient's level fell by 26% (Mühlfelder *et al.*, 1976).

Ahrens (1976), in a study which did not include phytosterols, found that Cholestyramine (Questran) used alone in a 24 g/day dose, or together with Atromide S (Clofibrate) in a 2g day dose, caused plasma cholesterol reduction by 20% and 14%, respectively. He also summarized the results of the Coronary Drug Project involving 1013 subjects. In these, plasma cholesterol decreased by 13% on a strict vegetarian diet, 19% on a low-fat and low-cholesterol diet, 19% on ingestion of 100 g corn oil/day in three doses, by 19% on ingestion of 100 g safflower oil, and 19% on a diet rich in polyunsaturated fatty acids.

This assessment, made in 1976 by Ahrens, can be supplemented by another one made by Pollak in 1983 (1985) in his summary of the Eighth International Symposium on Drugs Affecting Lipid Metabolism. He marveled at the enthusiasm greeting a 10% plasma cholesterol reduction by newly developed medications which included "Gamma-oryzanol", a preparation of phytosterols plus ferulic acid.

11. COMBINING SITOSTEROL WITH OTHER AGENTS

Comparison of β-sitosterol with other agents led to the conclusion that several agents have comparable effects. The question arose as to whether the potency could be enhanced by combining two agents, or whether the daily dose of agents could be reduced by using two synergistic means. To some extent, the theme has been addressed by suggestions to combine a low-cholesterol diet with a sitosterol regimen.

11.1. ANIMAL EXPERIMENTS

Linoleic acid is an important ingredients of cholesterol-lowering vegetable oils. Pollak (1958) fed rabbits chow with a 1% cholesterol supplement. Within two weeks, serum cholesterol increased seven-fold. This increase was inhibited on adding up to .7 g crude sitosterol or on adding 0.5–2 g linoleic acid per day. Adding 3 g sitosterol plus 2 g ethyl linoleate together had the same effect.

Rabbits fed cholesterol and sitosterol in 1:1 proportion developed only half the degree of hypercholesterolemia as when cholesterol was fed alone. Adding either taurine or choline did not affect the results (Herrmann, 1959). Cholic acid at 0.5%, enhanced the effect of 0.5–1% corn oil, also the effect of 0.1, 0.2, 0.5, 1.5, or 10% sitosterol on the serum cholesterol of rats (Nath *et al.*, 1959). Lignoceryl alcohol or 2% tetracosanol failed to enforce the effect of β-sitosterol on serum cholesterol of rats (Mesherskaia *et al.*, 1961). In rats fed a diet with 0.5% cholesterol and 1% soy sterols, adding 0.05% triterpene alcohols, namely, cycloartenol and 24-methylenecycloartenol, had a synergistic effect with regard to plasma cholesterol reduction and enhanced cholesterol excretion (Kiribuchi *et al.*, 1983). In cockerels with 1200 mg/dl plasma cholesterol levels soy sterols caused a reduction to 440 mg/dl. Replacing soy sterols with 2,4-dimethylamine resulted in a 750 mg/dl level (King *et al.*, 1956).

Beta-sitostanol plus cholesterol were fed to rats, and to one series cholestyramine was added (Hassan *et al.*, 1982). Fecal cholestyramine equalled 51% of the oral dose and

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β-sitostanol equal 83% of the oral dose. When the two substances were fed together, fecal cholestyramine was 73%. In another study, β-sitosterol was fed, 20 g/kg body weight, alone, or together with cholestyramine, also, as 20 g/kg, to young guinea pigs. Plasma cholesterol increased, though bile acid and sterol excretion had increased.

Summarizing the results does not appear profitable.

11.2. Clinical Studies

Combined administration of sitosterol with another agent was far more often used for clinical studies than in animal experiments, allowing for conclusions as to usefulness of combinations. Also, the number of agents tested exceeded those used for animals.

A low-cholesterol diet plus 9 g β-sitosterol per day led to a 40% reduction of serum cholesterol, more than could be expected by either diet alone or by sitosterol intake alone (Schön, 1959). Safflower oil alone (11 g/day) or β-sitosterol alone (18 g/day) decreased serum cholesterol equally, by 20%. Ingested combined, the decrease came to 32%. However, while corn oil alone (7.5 g/day) and β-sitosterol alone (15 g/day) had comparable effect, combining the two did not enhance the results (Farquhar and Sokolow, 1958).

Pyridoxin, 1 mg, α-tocopherol, 200 mg, β-sitosterol, 0.3 g, were added to safflower oil, 9.8 g. After one to two months, this resulted in enhancing the effect of safflower oil (Engelberg, 1958). Larger amounts of these substances, i.e. 300 g plant sterols, 20 g vitamin E, 1 mg pyridoxin and 9.8 g safflower oil, decreased serum cholesterol by 40%, far more than could be expected from any component of the mixture, alone (Meltzer *et al.*, 1958).

Cholagogic mineral waters plus β-sitosterol, taken for three weeks, led to 13% decrease of serum cholesterol for subjects with levels below 330 mg/dl and a 21% decrease for those with levels above 330 mg/dl (Mugler, 1962). Gallogen, a choleretic, in 255 mg dose, plus 18 g sitosterol depressed plasma cholesterol by 16% whereas sitosterol in 25 g/day dose decreased it by 25% in eight weeks (Farquhar and Sokolow, 1957).

Nicotinic acid, a potent inhibitor of cholesterol biosynthesis, led to uneven results when combined with other agents. Neither adding 3 g/day niacin or adding 2 g dessicated thyroid to the diet for 28 days had any effect on plasma cholesterol levels which had been previously lowered by 25% on ingestion of 4.6 g sitosterol per day (Breslaw, 1958). Berge *et al.* (1959) lowered plasma cholesterol by 10% using 4.8 g niacin alone or 4.8 g sitosterol alone; in combination, the decrease was 21%. The same research team recorded a 17% reduction in plasma cholesterol on 4.3 g niacin a day, a 12% reduction on ingestion of 3 fl. oz. of a sitosterol emulsion, and a 21% reduction by combining the two agents for three months. Yet, in another study, niacin had no effect when given alone, a minimal effect of a 6% drop in combination with safflower oil, a 12% drop of plasma cholesterol in combination with β-sitosterol.

One study in which sitosterol was given together with Clofibrate, a popular drug, is being singled out. In combination, the two agents stimulated cholesterol biosynthesis, nullifying any hypocholesterolemic effect (Horlick, 1971).

For analysis, results of clinical studies have to be separated into two groups. On one side of the ledger are the negative results of trials to potentiate the effect of sitosterol by adding pyridoxine, tocopherols, cholagogic mineral waters or choleretic gallogen. Reasons for uneven response on combining sitosterol and niacin are due to lack of selectivity. Triggering homeostatic response to major depression of plasma cholesterol must be underscored. On the second side of the ledger is the potentiation of the effect of β-sitosterol by a low-cholesterol diet or by intake of safflower oil—curiously, not by corn oil. Such combination allows for a smaller dose of sitosterol and for a less strict diet. Campagnoli (1961) was the first to suggest combining β-sitosterol ingestion with a diet rich in vegetables, cereals and cellulose as the best available course.

12. SIDE EFFECTS OF SITOSTEROL

A side effect is not always an adverse effect. The only undesirable side effect of a sitosterol regimen, reported so far, is an occasional bout of diarrhea. It is more likely due

to the vehicle used for sitosterol or to simultaneous intake of large quantities of vegetable oils than to sitosterol itself.

The target and the effect of phytosterol ingestion are preventing or reducing hypercholesterolemia and hereby retarding or lessening atherogenesis. Several researchers reported notable improvement in the symptomatology of atherosclerosis and in electrocardiograms of patients treated with sitosterol.

Sporadic claims have been made and are being made to this day about a large variety of beneficial effects of sitosterol, in *in vitro* and *in vivo* studies for animals and humans. Sitosterol was said to counteract experimental gastric ulcers in mice and guinea pigs, prevent experimental colonic cancer in rats, reduce hyperglycemia in rats and hypertension in rabbits. Sitosterol has also been recommended as plasminogen activator in endothelial cells. For man, it has been used for treatment of psoriasis and of rheumatoid arthritis, allegedly, with success. Sitosterol has also been hailed as an effective anti-pyretic, as anti-edematous and anti-inflammatory agent. None of all these effects, whether ultimately proven right or wrong, can be called adverse.

Sitosterol has two well documented side effects. The first it its enhancing cholesterol solubility. It has been proven *in vitro* and *in vivo* in animals and in man. The effect of β-sitosterol medication on the solubility gall stones has been monitored radiologically. The bibliography on medical treatment of cholelithiasis is steadily increasing, as is the number of patients medicated successfully with β-sitosterol $\pm$ litholytic bile acids. A brief review on this topic by Pollak in 1982 can already be called obsolete or, at least, incomplete. By and large, ingestion of 12 g β-sitosterol for from six to eight weeks will result in the reduction of the number and size of calculi by 10–15%. The results depend on numbers and size and on the chemical composition of the calculi. Long term treatment has a sustained cholelitholytic effect. Sitosterol ingestion alone, or potentiated by simultaneous intake of cholic acid, causes partial or complete dissolution or fragmentation of concretions. It may be anticipated that ingestion of sitosterol plus cholic acid will eventually be replaced by intrabiliary instillation. The colic accompanying expulsion of fragments or of whole calculi could be called an adverse effect, but not the phenomenon itself.

The second side effect of a sitosterol regimen is also of great interest. Several sitosterol preparations have been made available by several German firms under different names, including "Sitosterol Prostate Capsules". Taken orally as 10 mg capsules three times a day, i.e. 30 mg/day, for 12 weeks, or by others, in 1.76/day dose for only 5 weeks, the regimen had an undisputedly favorable effect on patients with prostate adenoma or prostatitis. The number of reports made to date is large. They differ only with regard to the number of patients and the dosage and length of treatment. They agree with regard to the results. One urologist reports that 85% of patients were free of symptoms, had no nocturia or dysuria and no residual urine after the treatment. Another specialist claims good results in 86% of his patients. Dörner and Fritsche (1982) reported on over 6000 patients treated successfully with β-sitosterol. The mode of action of sitosterol on prostatic hypertrophy is being debated. On the basis of tissue analysis, of needle biopsy specimens, which indicate a reduced amount of extractable PgE2, many believe that β-sitosterol blocks the synthesis of prostaglandins. So far, nobody has suggested that β-sitosterol may act as a low-potency phytoestrogen. At any rate, the demand for β-sitosterol for treatment of prostate adenoma and prostatitis may—at least, in Europe—exceed its use as cholesterol depressant. In fact, its hypocholesterolemic action may become a desirable side effect of treating the prostate.

12.1. Comparing β-sitosterol and Cholestyramine

In sharp contrast to β-sitosterol as plasma cholesterol depressant stands Cholestyramine, a resin which affects cholesterol metabolism by combining with bile acids. It is being prescribed in three to four daily doses of 9 g packets which contain 4 g of the anhydrous resin, making it a daily supply of 27–36 g of the medication, or 12–16 g of the resin.

Far more worrisome than the large dose are the numerous adverse side effects of the bile acid sequestrant. The *Physicians' Desk Reference* of 1983 lists not fewer than 60 adverse effects. Not all are equally important. A note that cholestyramine increased the incidence of experimental cancer in rats—contrasting with multiple reports on β-sitosterol having the opposite effect—is most disturbing, since analogous findings led the US Food and Drug Administration to banning many a food and many a drug. Added to the list of adverse effects is a shorter list of drugs, including some antibiotics, the resorption of which is interfered with by cholestyramine. The listing is surely incomplete. A report is being cited because it refers directly to comparison of β-sitosterol with cholestyramine. Nineteen patients with hypercholesterolemia received for 14 days three times a day 4 or 8 g cholestyramine or 3.5–8.8 g β-sitosterol, both, in addition to labeled Digoxin (β-acetyldigoxin). Sitosterol did not interfere with digoxin resorption whereas cholestyramine reduced resorption by 12–14% (Schwartzkopff and Jantke, 1980).

The paucity of side effects, acknowledged by those who used it as hypocholesterolemic and by those who used it for treatment of prostate adenoma, places β-sitosterol in a separate class. In this respect, it differs not only from cholestyramine but from all other chemicals proposed as plasma cholesterol depressants. Many synthetic products were tried in animals. Most had to be discarded because of toxicity. Hepatotoxicity for rats seems to be a common denominator for these chemicals. Nevertheless, a fairly large number of drugs have been introduced into clinical practice. Many had to be discarded for adverse side effects. Some, including bile sequestrants, Cholestyramine and Colestipol, are still being used, in spite of all their adverse effects and contraindications. New products are constantly being developed, tested in research laboratories, by the pharmaceutic industry and by clinicians. Those presented at the 1983 Symposium on Drugs Affecting Lipid Metabolism seem in no way superior to older drugs, neither with regard to potency nor to safety (Pollak, 1985).

13. PHARMACAL USE OF PHYTOSTEROLS

After all that has been said about ingesting sitosterol as a chemical, be it as powder, granules or in emulsion, about its safety and its potency comparing favorably with other cholesterol lowering agents, it seems odd that sitosterol has been displaced in clinical practice by bile acid sequestrants with comparable potency but known for multiple adverse effects.

The first reports on phytosterols, soy sterols and sitosterol, issued in the early fifties, were followed by a flood of publications on the usefulness of β-sitosterol for regulating plasma cholesterol in animals and in man. After a 25 year period, the flood receded. Some years later, several essays appeared recommending β-sitosterol as plasma cholesterol depressant in type IIa and IIb hypercholesterolemia. This suggested a rivival of sitosterol (Pollak, 1978). However, it did not materialize and today β-sitosterol is being used mainly in studies of sterol metabolism, not therapeutically for man. Actually, in clinical practice, it has become more popular for non-surgical treatment of cholelithiasis and prostate adenoma than as hypocholesterolemic agent.

Reasons for lack of utilization of sitosterol in hypercholesterolemia have been analyzed and refuted in preceeding sections. Many problems are applicable to sitosterol and to cholesterol, too, such as the question of purity of sterols. Problems with purity of sitosterol preparations are matched by problems with purity of cholesterol products. The latter influence not only the results of experiments but also the results of cholesterol assays in plasma and tissues.

There are two principal reasons for the lack of popularity of β-sitosterol application in the form of a drug. It is a fact that phytosterols and β-sitosterol are not listed in recent editions of important reference texts. The *Essential Guide to Prescription Drugs* (1982) and the *Physicians' Desk Reference* (1983) make no mention of plant sterols. *The Merck Index* (1984) has two single-word entries for β-sitosterol and γ-sitosterol under "therapeutic category", referring to them, respectively as "antihyperlipoproteinemic" and "anti-

cholesterolemic". Corrections are to be made in the next edition. The *Merck Manual of Diagnosis and Therapy* (1982) does not list phyto- or sitosterol under medications, but has five lines of β-sitosterolemia and xanthomatosis as a rare recessive familial disease. The anonymous author of 14 lines on "sitosterols" (note the plural) in the *AMA Drug Evaluation* (1984) has not the faintest knowledge about β-sitosterol, writing about a 12–24 g daily dose instead of 3 g/day and ignoring the effect of β-sitosterol on IDL- and VLDL-cholesterol.

The second reason for lack of utilization of β-sitosterol as a plasma cholesterol lowering medication lies in the fact that none of the multiple, broad cooperative studies on prevention of hypercholesterolemia, coronary atherosclerosis and myocardial infarction used phytosterols for clinical studies. This applies to the NHLBI type II Coronary Intervention Study, the Coronary Drug Project, the Lipid Research Clinics Prevalence Study, the American Heart Association and the Atherosclerosis Study Group of the Inter-Society Commission for Heart Research and other groups. The reasons for excluding β-sitosterol from clinical trials is not known.

14. DIETARY USE OF PHYTOSTEROLS

The list of texts and committee reports, incomplete as it may be, failing to pay attention to phytosterols is quite long, though not longer than the list of books on foods and nutrition which ignore phytosterols. Not all can be named. In *Human Nutrition* (1982), carbohydrates, fats and proteins are listed as if each of these would be an indivisible entity. Fibers and minerals of foods are listed but neither cholesterol nor any phytosterols is being listed in 23 tables. The *Food and Nutrition Encyclopedia* (1983) has two hefty volumes. It lists food and food products, fresh, frozen, variously prepared, it includes data on total calories, fat, carbohydrates, proteins, fiber, vitamins and minerals—but not the cholesterol or sitosterol content.

The most recent report straddles three topics, that of hypercholesterolemia as risk factor of heart attacks, the subject of "Diet Therapy" and that of "Drug Therapy" of hypercholesterolemia. It is a *Concensus Development Conference of the National Institutes of Health Dealing with Lowering Cholesterol to Prevent Heart Disease.* The conference was held late in 1984, the report was issued in 1985. Omission of reference to phytosterols either under diet or under drug therapy is the least of the deficiencies of the report. Lacking details and specific conclusions, the report is reminiscent of a communiqué issued on termination of a political summit. The first comments concerns cholesterol as a risk of heart attack. Notwithstanding media advertising, ischemic myocardial infarction is not caused by cholesterol blocking a coronary artery but either by a thrombus or by hemorrhage from an ulcerous atheroma, less often, in diabetics, by encroachment upon the lumen in medicalcinosis, only rarely by an embolus of cholesterol crystals. Hypercholesterolemia is a major atherogenic factor, only a minor thrombogenic factor. The concensus refers to "blood cholesterol", a term long discarded since whole blood contains about 25% more cholesterol than plasma. The report does not disclose the method by which the panel arrived at "normal" cholesterol ranges for subjects 20–29, 30–39, and 40 years and over. In the past, criteria of normalcy were arrived at by broad studies of subjects without and with symptoms of atherosclerosis. Results of assays based on the Schoeheimer–Sperry method led to general acceptance of 240 mg/dl as the maximum "normal" level for the western world. In May 1985, Statland wrote that of 20 laboratories, 14 reported as normal values up to 279 mg dl and four reported values up to 300 mg dl for men 30–39, 16 reported values up to 300 mg dl and one reported 312 mg/dl for men 40–49 years old. Goldbourt *et al.* (1985) studied 9902 males age 40 years and older and measured HDL-cholesterol in 6547 of them, in addition to total serum cholesterol. They concluded that decreased HDL-cholesterol is the major risk factor within the cholesterol complex. From the results of assays for 618 death from coronary disease they concluded that increased risk of mortality related only in those with over 241 mg/dl serum cholesterol.

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No dietary intervention is supported for those with a total serum cholesterol level below 240 mg/dl and certainly not for those with levels below 220 mg/dl. The *Lipid Clinics Research Primary Prevention Trial Result* (1984) is based on seven years' incidence of fatal myocardial infarction in men whose total plasma cholesterol was above 265 mg/dl. There is a gap between statistical and optimal normality. The concensus that levels of 200–230 mg/dl are associated with increased risk of developing premature coronary artery disease and that such levels represent about 50% of adults in the USA lacks credibility. How many of the panelists have levels in this range, how many people have lower and how many have higher levels? Certainly, those with levels above 230 mg/dl outnumber by far those with levels below 200 mg/dl. If the concensus would be right, the incidence of myocardial infarction, high as it is, would truly be staggering. The role of the HDL/LDL ratio is being acknowledged but not the role of other subfractions or that of apoproteins. No criteria of normalcy, actual or desired, are given for HDL. The goal of reducing the plasma cholesterol of the entire population to less than 200 mg/dl and to less than 180 mg/dl for those under 30 years old is unrealistic, ignoring homeostasis. The part of the concensus dealing with "Drug Therapy" is most discouraging. While it is true that, for the time being, niacin is the only drug available for depressing cholesterol biosynthesis, there are alternatives available to bile sequestrants which the panel recommends, ignoring all the adverse effects and contraindications. The part of the consensus dealing with "Diet Therapy" also calls for scrutiny. "Caloric restriction for weight normalization in the overweight" should be based on tables considering caloric requirements based on age, gender, height and weight. Recommending "a diet composed of approximately 30% of the intake as fat, reducing saturated fat to 10% and increasing all polyunsaturated fat up to 10% of the caloric intake" is meaningless without absolute figures for total caloric supply. Shifts in the PUFA/SFA ratio are unnecessary for those whose dietary cholesterol consumption is kept within acceptable limits. Suggesting that no more than 200–300 mg, or, better, only 150 mg of cholesterol by consumed per day seems most desirable—until one realizes that a single fresh whole egg weighing about 50 g contains 235–325 mg cholesterol (Kritchevsky, 1958), an amount which comes close to or exceeds the recommended daily intake. Suggesting labeling of foods as to cholesterol and fatty acid content has severe limitations, as it cannot be used for every cut of meat and every fish displayed on the counter. Implementation is difficult and acceptance is negligible. Suggesting a National Cholesterol Education Program based on the Concensus is doomed to failure from the start.

Detailed criticism of the Concensus is justified because it is already being quoted as authoritative by the *FDA Drug Bulletin* of April 1985 and, repeatedly, in *Dyslipoproteinemias and Diabetes* (1985).

Replacing the pharmacal approach by a dietary regimen can be accomplished either by reducing cholesterol intake or by increasing sitosterol intake. Reducing cholesterol is comparatively easy since the cholesterol content of most foods is well known. Increasing sitosterol consumption is, at present, very difficult because of lack of knowledge about the sitosterol content of most foods. Thus, while generalities are out of place when recommending a low-cholesterol diet, they are unavoidable when suggesting a high-sitosterol diet.

Both, cholesterol and sitosterol have been mostly used in form of chemicals or pharmaceuticals for animal experiments and for clinical studies. Curiously, the first experiments to induce experimental atherosclerosis were performed by feeding rabbits egg and milk. For β-sitosterol, there is a single report available on feeding mice sitosterol in the form of food, namely, feeding them colocasia leaves, with the intent to lower serum cholesterol (Sayeed and Ahmad, 1979). Animals and humans consume zoo- and phytosterols, though not under their labels, during administration of the sterols in chemical form. Such food consumption influences the results of studies. Supplementation of chow by carrots or lettuce alters the results of experimentally induced hypercholesterolemia in rabbits.

At least two foods which, in the past, have been suggested for lowering plasma cholesterol contain β-sitosterol. The California variety of the avocado pear has 2–4% of

unsaponifiables, with 80% β-sitosterol and 20% campesterol. The Egyptian variety of the artichoke contains 0.2% β-sitosterol, also, some stigmasterol and minor plant sterols.

Vegetable oils, used more widely than avocado pears and artichokes, are another source of sitosterol. Consumption of the oils has been popularized through aggressive advertising, stressing absence of cholesterol and presence of polyunsaturated fatty acids. The active principle of the cholesterol lowering effect of vegetable oils has been variously ascribed to phosphatides, essential fatty acids, polyunsaturated fatty acids, and to β-sitosterol. Of a series of over 20 assays by one research team on the amounts and the role of β-sitosterol in vegetable oils one is cited as representative (Beveridge *et al.*, 1958). Borgström (1976) in a review of plant sterols, attributed to plasma cholesterol depressing effect of vegetable oils solely to their β-sitosterol content. Weihrauch and Gardner (1978), reviewing available data from 1960 to 1978, stated that β-sitosterol is present in amounts of 100–500 mg/100 g in 39 vegetable oils, contrasting with a 4% cholesterol content. However, the β-sitosterol content was reported as 120–436 mg/100 g for corn oil and as 39–58 mg/100 g for coconut oil by Melton and Plerksophon (1982). Very likely, the hypocholesterolemic effect of vegetable oils, especially, of germ oils, is due to both their linoleic acid and β-sitosterol content (Hasegawa and Shibuya, 1983).

By and large, vegetable oils have been analyzed for the major phytosterols far more often and to much greater details than any other foods. The β-sitosterol content of margarines depends on the source: Corn oil margarine has 430–580 mg/100 g, soybean oil, 136–430 mg/100 g. Processing and refining—degumming, neutralizing, blanching, hydrogenation and deodorizing—have an impact on the end-product, be it oil or margarine (Kochhar, 1983). By refining, for example, soybean oil loses 24% of phytosterols, on hydrogenation, it loses 50% (Kinematsu *et al.*, 1973).

The sitosterol content of oils has never been advertised, neither has the fact that hydrogenation during cooking converts polyunsaturated fatty acids into monosaturated acids.

Since it is known that oxidation of cholesterol occurs on heating and with aging, increasing its hypercholesterolemic effect, it would be of interest to learn whether β-sitosterol undergoes a similar change which then would alter its anti-cholesterol effect. A single report was made by Ghavami and Morton in 1984. Deodorized soy bean oil, with 60% β-sitosterol, 35.5% campesterol and 5.5% stigmasterol content, was heated to frying temperature of 180 ± 10°C for 24, 48, 72 and 96 hours. Gradually, the sterol content decreased, without change in the proportion of the three sterols, by 4.5, 11, 15.4 and 22.4%, respectively, for each 24-hour interval. Of course, no food is being fried for more than a few minutes.

In 1958, Cooper suggested that sitosterol could be prescribed either as medication or as dietary component. There is an analogy with vitamins. For example, one can get an adequate supply of Vitamin C by eating citrus fruit or by swallowing tablets of concentrated ascorbic acid. Differences between drugs and natural products apply equally to vitamins and phytosterols. Pharmaceuticals have the advantage of having a measured amount of active ingredients. Foods are more palatable but have the disadvantage of great variability as to sterol content. Cholesterol and phytosterols are influenced by many factors already mentioned, and many more. Processing, preservation, refrigeration and preparation for consumption alter sterols quantitatively and qualitatively.

Well over 200 reports have been issued on phytosterols in foods. Most reports acknowledge the presence of phytosterols without data on proportions or absolute amounts. The list of references—albeit, superficial—is far too long to be cited. Rarely has the distribution of sterols between edible and non-edible parts of a plant been considered. This is important. For example, turnip leaves have 160 mg/100 g of phytosterols, the edible root has only 63 mg/100 g. Colocasia leaves, which are being eaten in Bangladesh, contain 3200 mg/100 g β-sitosterol. The tuberous root, the β-sitosterol of which is not known but is probably lower than that of the leaves of Caladium colocasia, is consumed widely as taro in the Pacific islands and is the starting material for the popular Hawaian poi.

Data on differences between fresh, stored, frozen, canned, parboiled or cooked

vegetables are not available. Such data, especially if expressed in measures used in the kitchen, in portions or cups, rather than in mg/100 g, would be welcome by those interested in prescribing a phyto- or sitosterol diet. Most essays refer to plant sterols, not to the three main members, and report the total as percent of unsaponifiable matter. Only on the basis of sporadic reports on sterol distribution in edible plants or part of plants can we assume that β-sitosterol amounts to about 80% of total phytosterols. There are many exceptions to this. Cinnamon has 33 mg/100 g and sesame seeds have 714 mg/100 g of phytosterols: in both, β-sitosterol amounts to 65% of the total. Barley seeds have only 41% of β-sitosterol and buckwheat has 84.5%. Both are consumed in larger quantities than black tea, with 1083 mg/100 g β-sitosterol. The latter seems impressive until one realizes that a tea bag or its equivalent as loose tea, sufficient to make two cups of tea, weighs only 250 mg. There is great need for more and better assays.

Two good reviews on phytosterols in foods are available, none on β-sitosterol. Lange (1950) collated data on cholesterol, phytosterols and tocopherol (mg/100 g) in food products and animal tissues. Weihrauch and Gardner (1978) covered the bibliography for the 1960–1978 period for the US Department of Agriculture. Selected data from the two reviews are presented in Table 7. A few recent reports on β-sitosterol content of foods are to be added.

Most of the tabulated data refer to foods with a relatively high phytosterol content. Some foods are being included in spite of a low content. One of these is garlic, included because it is being considered by some as anti-atherosclerotic. If it has any such effect it is not because of its sitosterol content.

Cereals are near the top on the list, with regard to phytosterol content in mg/100 g. They offer an additional advantage of providing a high supply of fiber. At any rate, they must be included, next to fruits, vegetables and nuts, in any "phytosterol diet" or "sitosterol diet". Such diets, based on reproducible information about the actual amounts of plant sterols in foods available in the western world, are most desirable. The American diet supplies about 500 mg cholesterol per day, but only about 180 mg β-sitosterol (Conner, 1968).

Concerned about the lack of information on phytosterols in foods, one may be looking forward to pertinent new reports. Under the promising title of "sterols in foods" (Sheppard, 1977) one finds data on 15 foods, mainly, pie crust mix, pound cake, doughnuts, begels and mayonnaise. In duplicate analysis, a jar of mayonnaise contained 103 and 107 mg/100 g of β-sitosterol, contrasting with only 26 and 28 mg/100 g in imitation mayonnaise. Marked differences between two sources of the same food have been reported by others, as indicated in Table 7.

The source of foods and food products is not the only determining factor with regards to results of assays. This has been discussed in connection with data on vegetable oils and margarines. Methodology plays an important role. Lanzani *et al.* (1979) found that soya protein isolate has 10 μg/g phytosterols, with 4.8 μg/g β-sitosterol, and that soya meal had 110 μg/g phytosterols, with 55 μg/g β-sitosterol. Different results were reported by Agater and Llewellyn (1982), namely, mean averages of 9.2 μg/g β-sitosterol in three tests of five samples of soya protein isolate and 6 μg/g in soya flour. Their data for β-sitosterol in soya protein concentrate and in extracted soya flour were, respectively, 15.2 and 20.6 μg g. The two reports also differed with regard to β-sitosterol in "meats". Lanzani *et al.* (1979) found 0.4 μg/g, that is, only one-third of the amount found by Agater and Llewellyn (1982) in shin of beef, who also found 0.19 μg/g in lamb, 1.6 μg/g in ham, and 3.6 μg/g in chicken. Discrepancies in results were ascribed to methodology. Thin layer chromatography, with 39% variation coefficients, was used for isolation of sterols from soya protein isolates, concentrates and flour; gas liquid chromatography was used for separation and identification of component sterols.

It is gratifying that in the USA the public has been "sold" on the "salad bar". However, it is being used as a supplement to the usual lunch or supper rather than a substitute for meat. It is of interest to learn that 17 farmers in the state of New Jersey are growing dandelions for salad, vegetable stew, adding them to corn chowder, making dandelion

TABLE 7. *Phytosterols (mg/100 g) in Edible Plants and Other Foods*

Cereals, grains, flour		a	Legumes, pulses		a
Rice bran	1325		Common peas	272	b
Corn gluten	940		Snow peas	222	
Buckwheat	197	b	Peanuts	220	
Corn	178		Chick-pea	70–170	
Sorghum	178		Soybeans	67.5–161	
Durum flour	154		Lima beans	121–144	
Wheat bran	144		Kidney beans	127	
Corn bran	138		Pea, seedlings	108	
Corn flour	128		Beets, top	96	
Peanut flour	90	c	Common beans	76–90	
Oats	58–62.5		Lentils	63	
Barley, hull	60		Broad beans	47.5	

a mostly, β-sitosterol
b incl. 164 mg β-sitosterol
c as β-sitosterol-D-glucoside

a mostly, β-sitosterol
b all, β-sitosterol

Oils, margarines		a	Flavorings, fruits, spices, etc.		
Wheat germ oil	3600–6700		Black tea	1400	a
Corn oil	580–1600		Orange juice, Valencia	385	b
Safflower oil	500–1000		Green tea	315	
Palm oil	120–500		Cloves	256	
Olive oil	317–475		Mayonnaise	107–218	
Cottonseed oil	470		Peanut butter	102	
Soybean oil	136–430		Artichoke, Jerusalem	100	
Peanut oil	190–300		Avocado, California	45	c
Margarine; corn oil	430–500		Apple; pear	40	
Margarine; soybean oil	136–430		Cinnamon	37	d
Margarine; base, stock L	260		Garlic	8–30	

a all, β-sitosterol

a incl. 1083 mg β-sitosterol
b incl. 300 mg β-sitosterol
c incl. 25 mg β-sitosterol
d incl. 15.2 mg β-sitosterol

Green-leaf vegetables			Nuts, seeds		
Colocasia, leaves	3200	a	Sesame seeds	714	a
Kale	464	b	Sunflower seeds	538	b
Broccoli	329	b	Barley seeds	234	c
Spinach	184	c	Peanuts	220	d
Turnip, leaves	160	d	Cashew nuts	158	e
Celery, top	144	e	Almonds	50–148	f
Cabbage	125	b	Walnuts	108	b
Carrot, top	90.5–116	b	Peacan nuts	109	b
Alfalfa	80	f	Chestnuts	19.5–57.5	g

a all, β-sitosterol
b mostly, β-sitosterol
c mostly, α-spinasterol
d root, 65 mg β-sitosterol
e root, 65 mg β-sitosterol
f mostly, brassicasterol

a incl. 464 mg β-sitosterol
b all, β-sitosterol
c incl. 230 mg β-sitosterol
d incl. 54 mg β-sitosterol
e incl. 120 mg β-sitosterol
f incl. 122 mg β-sitosterol

PS ranges indicate smallest and largest amounts reported

sausage and a potent dandelion wine. It remains to be seen whether such foods will become popular. Tofu, the protein-rich soybean curds, widely eaten in the Far East, is being advertised as tofu ice cream and many other dishes. Its popularity is ahead of that of dandelions. Incidentally, the β-sitosterol content of neither is known. The American public is still endulging in a high caloric diet, starting with the traditional breakfast of two fried eggs, bacon and toast. French fries are eaten with every lunch and supper consists mostly of meat and potatoes, with a pat of butter or sour cream, a buttered roll and a slice of pie. And, hardly a patron enters a movie theatre without carrying an oversized container of buttered popcorn. To re-educate the public with regard to diet is no mean task. Generalities must be replaced by concrete suggestions. It is essential to replace forbidden foods by palatable alternatives.

A sitosterol diet offers an alternative to sitosterol medication. Certainly, the two ways can be combined. Asymptomatic subjects may be reluctant to accept perennial medication and may prefer a dietary regimen. Some expect their physician to prescribe medications rather than a diet.

15. THE PROPHYLACTIC APPROACH

The tribulations of β-sitosterol being accepted in medicinal form and the obstacles to using it in dietary form to lowering plasma cholesterol have been discussed to some length. A third approach is available.

There are classical precedents to prophylactic supplementation of foods. To prevent endemic goitre, potassium iodide is being added to table salt. For preventing beri-beri, thiamine is being added to polished rice. These examples are being discussed to focus attention on the sequence of events which led to the desired goal. All projects require individual initiative for using a certain substance for treatment of a specific condition, next, a proposal to use the same substance for prevention of the condition. A persistent and concerted effort by the public and private sector is required before, at last, official approval is obtained for implementation of recommendations. The time lapse between the several stages is very long. At that, the evolution of the two cited projects fell into an era before creation of authorities which often interfere and delay such programs.

Iodine was first suggested for treatment of goitre by Coindet, in 1820. A full century later, Marine and Kimball (1921) suggested that ingestion of iodine would prevent the development of goitre. This led to a series of studies and to recommendations by the Goitre Society of Michigan, in 1937, and the American Public Health Association (Detroit), in 1941, at first, to add 0.02% sodium iodide, then, to add 0.01% potassium iodide to table salt. Cooperative efforts led to world-wide acceptance of the last recommendation and to eradication of endemic goitre.

The original observation that beri-beri results from a faulty diet in which rice bulged too large was made in 1886 by Takaki (Funk, 1912). Studies with fowl and pigeons developing polyneuritis when fed polished rice, but not when fed unpolished rice, were conducted by Ejkman in 1897 (Braddon, 1907) and by Vedder and Clark in 1912. Their observations were found to be applicable to man. Williams synthesized vitamin B_1 in 1937 (Williams and Spies, 1938) and in 1946 he proposed to the Baguio Committee, the Philippine Medical Association, the Chemical Society of the Philippines and the Philippine Pharmaceutical Society a plan to combat endemic beri-beri by adding 2 mg thiamine to each pound of decorticated rice. The proposal was accepted by the Philippine Government, the US Public Health Service and the Philippine National Rice and Corn Corporation. Large-scale distribution of enriched rice started late in 1948. Within a year, the incidence of beri-beri declined by 76 to 94%, on the average, by 89%, in seven provinces of Bataan. The mortality reached the zero point within 18 months. Once more, an individual initiative and a cooperative effort of several agencies led to unqualified success.

Woods, reporting in 1952 on the "Philippine Experiment", wrote that "large scale application of nutritional principles is not less beneficial than large scale use of other major advances in public health, such as vaccination, insect control, water purification and pasteurization of milk". Sanitation, specifically, waste disposal, should be added to the list.

The time is ripe to learn a lesson from the iodine and thiamine projects and to apply the principle of food additives to the problem of alimentary hypercholesterolemia. The suggestion of adding β-sitosterol to foods is being made with full awareness of all the differences. The cause and effect relationship is clearcut for endemic goitre and for beri-beri. It is far more complex for hypercholesterolemia. The first two are deficiency diseases. Atherosclerosis can be broadly labeled as due to oversupply of cholesterol, or of oversupply of dietary cholesterol. It could be also considered as being due to deficiency of sitosterol. Certainly, a proper balance between dietary cholesterol and dietary sitosterol can be recognized as "sound nutritional principle".

In 1804, Scarpa (Paolantonio, 1951), noticing the fatty character of arterial lesions, coined the term "steatomatosis". In 1913, over one hundred years later, Anitchkow described "cholesterol steatosis" ("Cholesterinsteatose") in rabbits fed cholesterol-rich food. This initiated an era of ongoing experimental and clinical studies on the relation of hypercholesterolemia, especially, of alimentary hypercholesterolemia to atherogenesis and atherosclerosis. Sitosterol was discovered in cereal grain by Burián in 1897. In 1952, Pollak reported on successful prenetion of induced hypercholesterolemia in rabbits and on reduction of hypercholesterolemia in man ingesting sitosterol powder. Three decades have passed since.

Beta-sitosterol in chemical forms offers a good alternative to other hypocholesterolemic drugs. In dietary form, it offers a good alternative to alimentary restriction of cholesterol. Whether used as pharmaceutical or as a diet, β-sitosterol has proven itself as a valuable plasma cholesterol depressant. Instead of prohibiting cholesterol ingestion, a negative measure, one can recommend sitosterol ingestion, a positive measure. Of the ten commendments, the eight forbidding certain thoughts and deeds are far more often violated than the two recommending positive behavior.

Prophylactic supplementation of foods with β-sitosterol offers a third and positive alternative to therapy of hypercholesterolemia. Certainly, sitosterol could be incorporated into many foods. For practical reasons, the additive should be restricted to butter and margarines, be these of vegetable oil origin or not. The vegetable oil margarines are practically cholesterol-free. It would complicate matters to use different amounts of sitosterol for each type. The β-sitosterol additive has to counteract not only the cholesterol in butter but all other dietary cholesterol and cholesterol from non-dietary source available for absorption and re-absorption.

On the basis of successful clinical studies in which the daily intake of sitosterol could be reduced to a 3 g maintenance dose, the amount of β-sitosterol to be blended into butter and margarines should be 8 g per bar, providing 1 g per pat. Three pats of butter or margarine consumed per day would provide the daily 3 g dose.

Alimentary hypercholesterolemia can be prevented. Its degree can be reduced. The proposed program of prophylaxis by incorporating β-sitosterol into butter and margarines is economically feasible and unobtrusive. As such it deserves broad attention by public and private health organizations, institutions and agencies, by pharmaceutical, food and dairy industries, by physicians and by the public at large.

The voices correlating dietary hypercholesterolemia with atherogenesis, morbidity and mortality from atherosclerosis—whether exaggerated or not—are getting louder and are getting more numerous. They deserve to be heard.

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MINIREVIEW
DIETARY PHYTOSTEROLS: A REVIEW OF METABOLISM, BENEFITS AND SIDE EFFECTS

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Summary

Most animal and human studies show that phytosterols reduce serum /or plasma total cholesterol and low density lipoprotein (LDL) cholesterol levels. Phytosterols are structurally very similar to cholesterol except that they always contain some substitutions at the C_{24} position on the sterol side chain. Plasma phytosterol levels in mammalian tissue are normally very low due primarily to poor absorption from the intestine and faster excretion from liver compared to cholesterol. Phytosterols are able to be metabolized in the liver into C_{21} bile acids via liver other than normal C_{24} bile acids in mammals. It is generally assumed that cholesterol reduction results directly from inhibition of cholesterol absorption through displacement of cholesterol from micelles. Structure-specific effects of individual phytosterol constituents have recently been shown where saturated phytosterols are more efficient compared to unsaturated compounds in reducing cholesterol levels. In addition, phytosterols produce a wide spectrum of therapeutic effects in animals including anti-tumour properties. Phytosterols have been shown experimentally to inhibit colon cancer development. With regard to toxicity, no obvious side effects of phytosterol have been observed in studies to date, except in individual with phytosterolemia, an inherited lipid disorder. Further characterization of the influence of various phytosterol subcomponents on lipoprotein profiles in humans is required to maximize the usefulness of this non-pharmacological approach to reduction of atherosclerosis in the population.

Key Words: phytosterol, hypocholesterolemia, absorption, therapeutics

Phytosterols produce a wide spectrum of biological activities in animals and humans. Particularly phytosterols are considered an efficacious cholesterol-lowering agent. In the last decade there have been a series of studies concerning the therapeutic effects of phytosterols in animals. The present review will examine phytosterol metabolism, summarize recent findings concerning phytosterols' therapeutic action and consider their possible toxic effects.

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Phytosterol Metabolism

Phytosterols are structurally related to cholesterol, but differ in their side chain configuration (Figure 1). There is a wide variety of phytosterol structures but the most frequent phytosterols in nature are campesterol, ß-sitosterol, stigmasterol. 5α-hydrogenation of phytosterols form saturated phytosterols, such as campestanol and sitostanol.

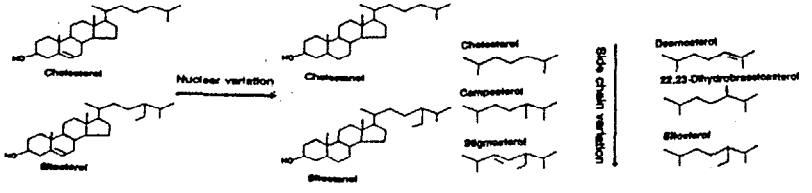

Fig. 1. Conventional chemical configuration of sterols

Dietary sources of phytosterols usually come from corn, bean and plant oil in humans since they contribute greater amount of dietary phytosterols. It is obvious that vegetarian diets contain higher amount of phytosterols as compared to conventional western diet. In the United States 250 mg/day of phytosterols are consumed (1); in contrast, a vegetarian diet would provide two times of this amount (2). Conventional diets contain saturated phytosterols in trace amounts (1, 2).

Phytosterols are not endogenously synthesized in the body, therefore, are derived solely from the diet entering the body only via intestinal absorption. The preferential absorption of cholesterol over phytosterol is a characteristic feature of intestinal sterol absorption in mammals. For healthy humans, the absorption rate of phytosterols is usually less than 5% of dietary levels, which is considerably lower than that of cholesterol which is over 40% (3, 4). Thus, approximately 95% of dietary phytosterols enter the colon. Only 0.3-1.7 mg/dl of phytosterols are found in human serum under normal conditions compared with daily dietary intakes of 160-360 mg/day (3-5), but plasma levels have been showed to increase up to two-fold by dietary supplementation (5). Solubilization of phytosterols in micelles is required for efficient intestinal absorption. It was suggested that phospholipid may play an important role in the absorptive recognition of cholesterol (6). The addition of phospholipid into bile micelle promoted the preferential absorption of cholesterol over 24-ethyl analogue, sitosterol, in the isolated jejunal villus cells (6). This phenomenon suggests that the intestinal selection against phytosterol may be a general property of phospholipid structures (6). In addition, it has been proposed that inadequate esterification of phytosterols may play a role in the poor absorption of phytosterols by gut (7). ß-sitosterol esterification was determined by incubating intestinal microsomal membranes with radiolabelled sterols. Acyltransferase dependent esterification rate of ß-sitosterol was slower than cholesterol. Acylcoenzyme A:cholesterol acyltransferase (ACAT) dependent esterification of cholesterol was at least 60 times greater than that of ß-sitosterol.

It has been demonstrated by Miettinen et al. (4), that serum levels of ß-sitosterol and campesterol were positively associated with the fractional absorption of dietary cholesterol, and negatively with cholesterol synthesis and biliary and faecal excretion in randomly selected human males. The study also suggests that serum phytosterols were significant indicators of cholesterol absorption and synthesis even under basal conditions. However, the factors that determine the serum

phytosterol levels in normal subjects are still unknown.

Intestinal phytosterol absorption is selective; campesterol is absorbed better than ß-sitosterol in the animal (8), stigmasterol is absorbed minimally. The components and interactions responsible for the absorptive discrimination may occur during the initial entry of the sterols into the intestinal wall (9). Variation in side chain could exert differential effect on the uptake of phytosterols from micellar solution by villus cells and brush border membranes. The phytosterol uptake decreased with an increasing number of carbon atoms at C_{24} of the sterol chain (10). It is assumed that hydrogenization of the nucleus double bond of a sterol results in decreased absorption (11). Sitostanol (the 5α saturated derivative of ß-sitosterol) is not absorbed at all (11, 12). However, campestanol, a saturated phytosterol, was recently shown to have higher absorbability compared to its unsaturated form (13), which is in contrast to the early assumption.

Sitosterol and campesterol intake produce an increase in plasma sitosterol and campesterol concentrations (12). However, this increase is higher for campesterol than sitosterol (12). The discrepancy is not only due to higher campesterol absorption, but also to different elimination rate from the liver (12, 14-15). Since hepatic esterification of ß-sitosterol is significantly lower than that of campesterol (16), its biliary elimination may exceed that of campesterol.

Phytosterol elimination takes place via the biliary route and appears to be more rapid than that of cholesterol (17). Correspondingly, endogenous phytosterol pool size is low compared to cholesterol, due to poor absorption in the intestine as previously stated, and faster excretion via bile.

It has been demonstrated that insects and prawns are able to transform phytosterols to cholesterol, then synthesized into steroid hormones or bile acids (18-20). To date, it has not been proven that phytosterols can be converted to endogenous cholesterol and related sterol hormones in vertebrates. Attempts have been given to demonstrate conversion of ß-sitosterol into normal C_{24}-bile acids in mammals. Salen and his colleagues (5) reported an efficient formation of cholic chenodeoxycholic acid from intravenously administered [22,23-^{3}H] sitosterol in humans. However, such attempts for demonstrating the conversion of phytosterols into bile acid have failed in rats (21) and monkey (22). Also in marked contrast to the results of previous investigation with [22,23-^{3}H] sitosterol (5), no any significant conversion into labelled C_{24}-bile acid was identified for reinvestigation with [4-^{14}]C sitosterol (23) in humans. These results suggest that human subjects, like other mammalian species studied, have little or no capacity to convert ß-sitosterol into the normal C_{24}-bile acid. Several studies have shown that ß-sitosterol is converted into polar compounds in the bile acid fraction of rat bile (21, 24-26). This polar products were di- and trihydroxylated C_{21}-bile acid.

Sitosterolemia, a rare inherited lipid storage disease, characterized chemically by the increased plant sterols and 5α-saturated stanols in plasma and tissue with premature atherosclerosis (27, 28). The absorption rate of phytosterol is very high in the patients. As diet contains 5α-dihydro phytosterol derivatives, 5α-campestanol and 5α-sitostanol in trace amounts, the saturated phytosterol at high level in plasma are probably produced endogenously in large amount. When plasma lipoproteins were measured in sitosterolemic subject, phytosterols and 5α-stanols were distributed in about the same proportion among the various lipoprotein fractions, including high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) cholesterol (29). Sterol composition in bile in sitosterolemia is different from controls. Not only is less cholesterol secreted into the bile, but sitosterol appears in the same or lower proportion relative to cholesterol in bile as compared in plasma (29). Normally, the liver preferentially

secretes sitosterol into bile so there is three times enrichment of sitosterol to cholesterol as compared to blood in control subjects (5). The large quantities of sitosterol and cholestanol in sitosterolemic liver competitively inhibited cholesterol 7α-hydroxylase for bile acid synthesis, which may eventually lead to decrease bile acid production and deficient pool size (30).

There is little information available regarding the distribution of phytosterols in various tissues of the body. It is now well established that the liver is the major organ concerning cholesterol homeostasis. Whole liver and liver microsomes of normal subjects contain only trace amounts of phytosterols (31). The amount of phytosterols is about 5% that of cholesterol in liver microsomes of rat (32). High phytosterol levels were found in liver microsomes in subjects with sitosterolemia, the larger proportion of phytosterol being sitosterol (50%) and campesterol (24%) (31).

Because phytosterols are so similar to cholesterol in structure, consideration of the membrane effects of cholesterol will aid in understanding the structure-function relationships of phytosterols. Cholesterol interacts with membrane phospholipids and affect membrane lipid composition (33, 34). High intakes of dietary phytosterol (3% β-sitosterol + 2% campesterol) for 21 days in rats lead to an increase in phytosterol incorporated into liver microsomes and a decrease in liver cell membrane fluidity (32). There was no change in either the phospholipid or in the total sterol content of liver cell membranes by high phytosterol intake. However, the phytosterol/cholesterol ratio was increased (32). Since cholesterol has been shown to affect the activities of many membrane-bound enzyme (35), phytosterol composition of membranes of some vertebrates could be an important factor governing cellular excitability (35). However, the physiological function of phytosterols in the cells is not clearly understood.

Health Effect of Phytosterols

1) Influence of Phytosterols on Circulating Cholesterol

A) Total cholesterol: Phytosterols have been regarded as cholesterol-lowering agents since the early 1950's. The original literature concerning the cholesterol reduction of phytosterols including β-sitosterol, campesterol and stigmasterol has been reviewed by Pollak and Kritchevsky (18). Recently, most human studies have focused the action of β-sitosterol and sitostanol in reducing circulating cholesterol and LDL cholesterol levels (Table 1). These results suggest that sitosterol and sitostanol might be useful hypolipidemic agents in mild hypercholesterolemia. Furthermore, phytosterol was able to alter lipid metabolism, for example, reducing liver acetyl-CoA carboxylase and malic enzyme activities (36).

There are large variations in content and ratio of individual constituents of phytosterol extracts. The physical and chemical characteristics of phytosterol preparations are not uniform, even after purification. Also, other non-sterol agents present in small quantities may exert a compounding effect on phytosterol potency. Structure-specific effects of individual constituents in phytosterol mixtures have been identified (11, 37, 38). Rats were fed a 3% cholesterol diet and injected intravenously with β-sitosterol, stigmasterol, or their respective esters (37). The serum cholesterol-lowering effect depended on the double bond at C_5, and side chain at C_{17}, with esters having consistently lower effect on serum and liver cholesterol than free sterol (37). Recently, saturated phytosterols have received more attention in the field of cholesterol-lowering agents. Ring-saturated phytosterol or corresponding esters appear to inhibit cholesterol levels more effectively than unsaturated phytosterols (11, 38, 39, 40). A small amount of saturated phytosterol is needed to reduce circulating cholesterol compared to the unsaturated compound. Heinemann and co-workers (38) reported that sitotanol at a level of 1.5 g/day was needed to lower serum cholesterol

significantly compared to up to 20 g/day of sitosterol in humans.

Table 1. Influences of sitosterol and sitostanol on circulating cholesterol levels

Sterols	Dose	Subject	Changes in cholesterol or LDL (%)	Ref
β-sitosterol	6 g/day	hypercholesterolemia	-9.0	48
Sitosterol	722 mg/day	hypercholesterolemia	-11	13
Sitosterol	2 g/day	familial hypercholesterolemia	-20 (LDL-chol)	41
Sitostanol	0.5 g/day	familial hypercholesterolemia	-33 (LDL-chol)	41
Sitostanol	630 mg/day	hypocholesterolemia	-12	13
Sitostanol	1.5 g/day	hypercholesterolemia	-10--15	12
Sitostanol ester	3.4 g/day	hypercholesterolemia	-8.0	43
Sitostanol	1.0 g/day	hypercholesterolemia	-7.0	39

The studies strongly suggest that the saturated phytosterols be further explored as agents of choice for reducing elevated circulating cholesterol levels. However, dietary cholesterol may affect the effectiveness in reduction of cholesterol levels by saturated phytosterol treatment (41). Denke (41) recently reported that lack of efficacy of low-dose (3 g/day) sitostanol in cholesterol-lowering diet (220 mg/day) for cholesterol-lowering activity in men with moderate hypercholesterolemia. This study suggested the effectiveness for reducing cholesterol level may be attenuated when the diet is low in cholesterol. The extent of effect of saturated phytosterol as a more efficient cholesterol-depressing agent is therefore a relevant area for further study.

B) Lipoproteins and Apolipoproteins: Levels of individual circulating lipoproteins are considered to be more important than that of total cholesterol for assessment of risk of coronary heart disease (42). It has been demonstrated that low density lipoprotein cholesterol is directly associated with the development of cardiovascular disease, whereas HDL cholesterol has an inverse relationship with cardiovascular disease development (42). Apolipoprotein AI is the major component in HDL cholesterol, while apolipoprotein B is a principal component of LDL cholesterol. It has been suggested that the ratio of apo B to apo AI is an indication of propensity to develop atherosclerosis (42).

A series of studies were carried out to examine the effect of phytosterols on lipoproteins levels. Laraki and colleagues (43) reported that mixtures of phytosterols significantly reduced not only the total concentration of cholesterol but also LDL cholesterol levels in rats fed a cholesterol supplemented diet. In a human study by Vanhanen et al. (44), supplementation of soluble sitostanol esters reduced total circulating cholesterol and LDL cholesterol levels by 7.5% and 10%, respectively. In addition, another study in familial hypercholesterolemia in childhood showed that sitostanol, even with the dose four-fold lower than that of sitosterol, was significantly more effective in reducing elevated LDL cholesterol, and the reduction in serum lipid levels was of the same magnitude as that observed with sitosterol (40). With the significant decrease of LDL cholesterol, reduction of atherogenic index LDL/HDL cholesterol was seen with dietary intake of phytosterol (39, 44, 45).

There have been reports on the association of dietary phytosterol and circulating HDL cholesterol and its major protein apo A. Sutherland and his colleagues (46) investigated the levels of plasma phytosterols and HDL cholesterol level in long distance runners. Plasma phytosterol concentrations were correlated positively and significantly with plasma HDL cholesterol

concentrations. The mechanism of positive association between phytosterol and HDL cholesterol level in long distance runners is not clearly understood. There were several reports regarding the influence of dietary phytosterols on the HDL cholesterol level in animals and humans. Circulating HDL cholesterol levels remained unchanged with the addition of phytosterols (43, 44, 47). However, Zak et al. (45) reported that a three week administration of phytosterol caused an increase in the cholesterol fraction in HDL cholesterol, although not significantly.

Similar to changes in LDL cholesterol, the levels of apo B have been shown to decrease with the administration of dietary phytosterols (39, 40, 44, 45). In contrast, Weisweiler and colleagues (48) reported that the addition of phytosterol did not cause any change in apo B and apo AI. With the reduction of apo B by the treatment of dietary phytosterols, a significant reduction of atherogenic index apo B/apo AI was also seen (45).

C) Lecithin:Cholesterol Acyltransferase (LCAT): Lecithin:cholesterol acyltransferase (LCAT), a major component in HDL cholesterol, facilitates the sequestration of cholesterol within the hydrophobic core of HDL cholesterol through an increase of esterification of sterol, generating in the process a chemical potential gradient which leads to continuing uptake of the sterol, a process involved in reverse cholesterol transport (47). Additions of dietary phytosterols were found to increase LCAT level in blood (43, 44, 48). Molecular esterification rate of LCAT increased by approximately 50% and the fraction of esterification rate by approximately 100% (48). In another experiment, three weeks of treatment with phytosterol raised LCAT activity in plasma and cholesterol fraction in HDL cholesterol (45). The changes of LCAT could potentially lead to redistribution of cholesterol between HDL cholesterol and other lipoproteins. The elevated molar esterification rate of the LCAT may indicate an increased synthesis and secretion of the enzyme by the liver. This change could be associated with the depressed liver cholesterol content after phytosterol treatments. Whether phytosterols could accelerate body cholesterol turnover remains to be determined.

D) Mechanism of Blood Cholesterol Reduction by Phytosterols: Multiple theories have been advanced concerning the mechanism by which phytosterol functions as a plasma cholesterol depressant. It is generally accepted that phytosterols inhibit intestinal absorption of cholesterol. Figure 2 illustrates the possible mechanisms by which phytosterol lowers circulating cholesterol levels.

i) Inhibition of Cholesterol Absorption in the Intestine: The potential mechanisms by which phytosterol inhibits cholesterol absorption include inhibition of bile salt cholesterol micellar formation and competition with the brush border for cholesterol uptake (8, 49), changing micellar solubilization, intracellular esterification and/or incorporation into chylomicron (10).

At present, reduced cholesterol solubilization in bile salt micelles is proposed as a major factor in inhibited absorption of cholesterol by phytosterols (49, 50). It has been reported that the incorporation of phytosterols into cholic acid micelles are 34, 30, 23, 15 and 3% for campesterol, sitosterol, cholesterol, stigmasterol and ergosterol, respectively (51). Since campesterol and sitosterol are more hydrophobic than cholesterol, they have a high affinity than cholesterol for micelles, thus restricts the solubility of cholesterol. Therefore, phytosterols can displace cholesterol from micelles with a favourable free energy change. A study in vitro revealed that the rate of sitosterol movement from the micellar phase to triolein was 3.5 fold higher than cholesterol (8). Sitostanol was shown to be more effective than sitosterol in reducing cholesterol absorption when higher doses of each were infused into the intestine of humans (52). Cholesterol absorption declined during sitosterol or sitostanol infusion by almost 50% and 85%, respectively

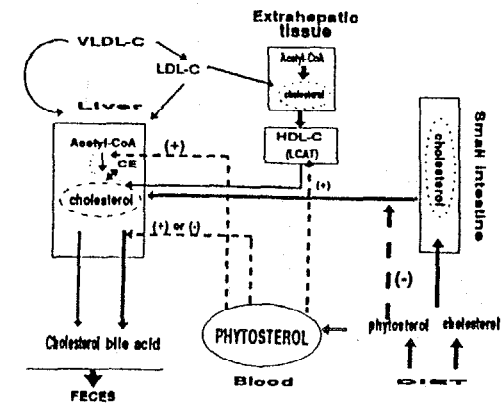

Fig. 2. A model showing the regulation of phytosterol on cholesterol metabolism in an animal. In this model the body is divided into three functionally distinct tissue groups, the small intestine, other extrahepatic tissue, and the liver, all of which are capable of synthesizing cholesterol from acetyl-CoA. Dietary phytosterols consumed reach the intestine, playing a significant inhibitory role on cholesterol absorption. Dietary phytosterol reaching the liver even at small amounts may alter the cholesterol synthesis and excretion.

(52). The fact that sitostanol was more hydrophobic than sitosterol may explain why sitostanol was more effective than sitosterol in reducing circulating cholesterol and LDL cholesterol in children (40).

Inhibition of cholesterol absorption has been demonstrated to increase cholesterol, coprostanol and bile acid excretion in rats (53). These effects were dose-dependent, and likely related to the cholesterol lowering effect of phytosterol. Phytosterols and cholesterol in the colon act as substrates for metabolizing enzymes such as cholesterol dehydrogenase, a bacterial enzyme. Catabolism of dietary phytosterol to its metabolites by these enzymes, results in increased excretion of faecal cholesterol and lower levels of its metabolites (54). With elevated faecal cholesterol, hepatic conversion of cholesterol to bile acids is promoted, consequently blood cholesterol level decreases.

ii) Synthesis and Excretion of Cholesterol by Phytosterol: Inhibition of cholesterol absorption leads to reduce feed-back regulation of enterohepatic cholesterol circulation, and produces a compensatory increase in cholesterol synthesis (39, 44). Administration of phytosterol intraperitoneally or subcutaneously to animals has been reported to lead to depressed levels of plasma cholesterol (55, 56). These studies suggested phytosterols may have an intrinsic hypocholesterolemic effect via mechanisms other than those involving cholesterol absorption. Discrimination of cholesterol absorption could not completely explain the hypocholesterolemic effect of non-intestinal administration of phytosterol, even though elevated blood phytosterols by intraperitoneally or subcutaneously treatments could be secreted from the liver via bile into the intestine, then inhibit cholesterol absorption (57). Investigators have attempted to clarify the mechanism for hypocholesterolemic action of phytosterols bypassing the intestinal mucosa. Efforts have been given to investigate the influence of phytosterol on cholesterol synthesis by a series of studies. Gerson et al. (58) reported the intraperitoneal injection of ß-sitosterol into rats lowered plasma cholesterol, while stimulating cholesterol synthesis from acetate. Konlande and Fisher (56) made similar observation in chicks. In contrast, Subbiah and Kuksis (21) were unable to demonstrate any effect from intravenous bolus injection of 4-8 mg ß-sitosterol upon

cholesterogenesis in the rats. A more recent study showed that accumulation of sitosterol in tissue of rats has no influence on 3-hydroxyl-3-methyl-glutaryl CoA (HMG CoA) reductase (a limiting enzyme of cholesterol synthesis) activity (59). Only a much large dose of phytosterol (100 mg) brought about a partial inhibition of hepatic cholesterogenesis (60). The effects of the large doses of phytosterols were attributed to the displacement of increasing amount of cholesterol from the vascular tissues (60). It is generally assumed that the presence of an alkyl group in the side chain prevents the phytosterols from an effective cholesterogenesis with critical sites of feed-back regulatory system of cholesterogenesis (60).

The results on the influence of phytosterols on conversion of cholesterol into bile acids are conflicting. Tabata (37) has shown that intravenous injection of 0.5-4 mg/day phytosterol for 5 days resulted in a significantly increased conversion of cholesterol into bile acids in the rat. Shefer et al. reported (61) that sitosterol at a level of 2% of diet for 1 week produced an increase of activity of cholesterol-7α hydroxylase (a limiting rate enzyme of conversion of cholesterol into bile acids) in the rat. Other investigators (21) failed to detect the changes. However, more recent studies showed that elevated plasma sitosterol by intraperitoneal and intravenous injection decreased cholesterol-7-alpha-hydroxylase activity in animals (57, 59).

Studies with sitosterolemia could provide more information concerning phytosterol's influence on cholesterol synthesis and its excretion via bile acids. Cholesterol synthesis in sitosterolemic patients was shown to be extremely low (27), and removal of sterol from liver was also slower (28). Remarkably, reduced HMG CoA reductase was characterized in sitosterolemic liver. However, higher tissue sitosterol concentration do not inhibit hepatic HMG CoA reductase activity or steady state message RNA levels (59). There was no difference in the efficiency of HMG CoA reductase catalytic activity detected between control and sitosterolemic liver (32). Further study with hybridized RNA has shown that the deficiency of microsomal HMG CoA reductase in sitosterolemic livers can be attributed to the very low levels of HMG CoA reductase mRNA available for enzyme translation (31). The deficiency of HMG CoA reductase in the liver of sitosterolemia is inherited and not due to the hepatic accumulation of sitosterol (59). High tissue concentration of sitosterol in patients competitively block cholesterol 7α-hydroxylase activity, and raise plasma cholesterol levels (30, 59).

2) Other Therapeutic Effects

In addition to cholesterol lowering effect, phytosterols have been suggested to possess several other therapeutic activities, based primarily on animal experiments. ß-sitosterol supplementation is associated with a series of biological activities (62). Sitosterol may possess potent antiinflammatory, antibacterial and antifungal activities (63).

ß-sitosterol insolate from pollen Typhae has been shown to possess anti-atherogenic effects through inhibition of platelet aggregation (64). ß-sitosterol-ß-D-glucoside and its aglycone have exhibited anti-ulcerative activity in chronic acetic acid-induced gastric ulcer of rats (64). Similarly, result showed that ß-sitosterol aglycone appeared better than the glucoside's in anti-ulcerative activity (65). Administration of ß-sitosterol alone or in combination with oestradiol caused a marked increase in uterine weight, and RNA, DNA and protein concentrations in ovariectomized animals. The maximum influence was evident only after median and high dose treatments (66).

3) Anti-Tumour Properties

Within the last decade it has been suggested that phytosterols may possess anti-tumour properties in both animals and humans. Phytosterols showed inhibition of tumours induced by chemicals in animals (67). There are currently a series of reports on the anti-colonic tumour activities derived from phytosterols. Epidemiological studies have shown that populations consuming high level of phytosterols may be at decreased risk for colon cancer (68, 69). Experimental studies by Raicht and co-workers (70) showed that rats administered methylnitrosourea (MNU), a direct-acting carcinogen, produced a significantly higher incidence of tumours after 28 weeks compared to rats administered MNU and fed a diet containing 0.2% ß-sitosterol. Feeding dietary ß-sitosterol, at a level of 0.3%, to rats induced with MNU significantly decreased the rate of colonic epithelial cell proliferation and compressed the crypt's proliferative compartment (71), thus suppressing expression of the altered genome. A further study investigated the dose response effect of dietary phytosterol addition on colonic mucosal cell proliferation in mice not receiving an administered carcinogen (72). Phytosterols significantly reduced the colonic epithelial cell proliferation induced by supplementation of cholic acid in diet in a dose-dependent manner. The findings accord well with epidemiological studies, which show an increased intake of phytosterol in populations at lower risk for development of colonic cancer.

Epidemiological and experimental studies in general have implicated dietary cholesterol as a factor in colon carcinogenesis, primarily through the production of its end products (coprostanol and other neutral sterol and bile acids) by colonic microflora (68). Secondary bile acid probably play a major role in the development of colonic tumours (73). It has been suggested that a high ratio of secondary to primary bile acids may increase the risk of colon cancer (74). Additionally, it has been proposed that cholesterol and its metabolites may be involved in the aetiology of colon cancer (75). Studies have shown that dietary phytosterol significantly alters levels of faecal cholesterol, cholesterol breakdown products and bile acids (53, 54).

Phytosterol may decrease epithelial cell proliferation by suppressing the bacterial metabolism of cholesterol and/or secondary bile acids in the colon and by increasing excretion of cholesterol itself. Dietary phytosterols appear to inhibit colon cancer development at an early stage (i.e. before adenoma formation); however, the mechanism by which they may decrease cell proliferation is unknown (71).

Toxicity of Phytosterols

No obvious side effects have been observed after long-term feeding of phytosterols in animals and humans (40, 55, 76). Chronic (60 days) subcutaneous administration of ß-sitosterol in rats was well tolerated and evidence of gross or microscopic lesions either in the liver or kidney was not observed. Furthermore, liver and kidney function tests were assessed by determining blood/serum parameters such as haemoglobin, blood glucose, serum protein, serum bilirubin, serum GPT and GOT (55). All clinical biochemical parameters were in the normal range with exception of serum cholesterol. Serum cholesterol was the only variable which was markedly depleted in both sexes in a dose-dependent manner, suggesting the intrinsic hypocholesterolemic effect of the sterol (55).

Phytosterol treatments were reported to cause certain side effects at very high doses. Dietary phytosterols have been occasionally reported to cause diarrhoea in animals and humans (77). Furthermore, sitosterol has been shown to affect reproductive tissue in that ß-sitosterol may act as an abortifacient in animals (78). As well, high doses (0.5-5mg/kg per day subcutaneously) of ß-sitosterol in rats reduced sperm concentrations as well as weights of testis and accessory sex

tissues in a time-dependent manner. Thirty day withdrawal from treatment restored the weight of accessory sex tissues to near-normal conditions (76). The mechanism of antifertility of phytosterols remains unknown. Recent experiment by Hirano et al. 1994 (79) revealed that phytosterols, stigmasterol and campesterol, inhibited the membrane N_a+, K+-ATPase activity of benign prostatic hyperplasia, which may subsequently suppress prostate-cell metabolism and growth. To study the pathogenesis of early development of atherosclerosis in sitosterolemia, the effect of sitosterol exposure on vascular endothelial cells in vitro was investigated in cultured human umbilical vein endothelial cell (80). Exposure to 0.7 mmol of sitosterol for 72 h caused contraction of endothelial cells and increased the release of intracellular lactate dehydrogenase. After 96 h incubation the cell were partly detached from the substrate. At this timepoint 0.35 mmol of sitosterol also caused perturbation of the endothelial cell (80).

In conclusion, evidence from studies over the past 40 years has shown that circulating total and LDL cholesterol levels can be reduced by ingestion of phytosterols. This reduction is more obvious with consumption of saturated phytosterols, such as sitostanol versus less saturated ß-sitosterol or compesterol. The mechanisms of hypocholesterolemic action include inhibition of cholesterol absorption and decreased excretion from the liver. It has been suggested that supplementation of phytosterols have anti-colonic cancer property in addition to other beneficial actions in the animals. High amounts of phytosterol may possess negative influence on reproductive system, also occasionally cause diarrhoea in animals. However, presently it can be assumed that at moderate levels of plant sterols offer advantages as safe and inexpensive primary cholesterol lowering agents for use in humans.

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THE EFFECT OF AZELASTIN HYDROCHLORIDE ON PRURI LEUKOTRIENE B4 IN HEMODIALYSIS PATIENTS

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Summary

Pruritus is a very common complication in chronic hemodialysis (H however the exact mechanism for this affliction is still not known. An usually failed to alleviate uremic pruritus. In others, an anti-allergic inhibits the release of chemical mediators, such as leukotrienes or histami cells, was reported to be effective. We evaluated the values of leukot interleukin 6 in HD patients with pruritus and the effect of an anti-allergic factors. Leukotriene B4, interleukin-6, C3a, C5a, the number of eosinopl 0, 15 and 180 minutes after the start of regular HD in 11 HD patients st pruritus and as well as in 11 HD patients without pruritus were examine patients in both groups showed significantly higher ($p<0.001$) values of le and C3a compared to healthy non-HD subjects. There was no diffe leukotriene B4, interleukin-6, IgE, C3a and C5a levels between the patie without pruritus.Two mg/day of azelastin hydrochloride, an anti-allerg orally given to the pruritus group for 3 weeks. In 5 of 11 patients, symptoms disappeared, while in 4 of 11 they improved. Independent of the drug on pruritus, leukotriene B4 levels significantly decreased compar before the administration of this drug in the pruritus group ($p<0.01$). I C3a, C5a and the number of eosinophils demonstrated no significant conclusion, although azelastin hydrochloride was effective in treating prur suppressed leukotriene B4 levels in hemodialysis patients, the high le activity itself did not seem to be related to the development of pruritus in th

Key Words: azelastin hydrochloride, hemodialysis, interleukin-6, kidney failure, leukotriene

Pruritus is a very common complaint and important issue related to the the chronic hemodialysis (HD) patients. Pruritus, by itself, is not a serious com cause a variety of problems such as sleep disturbance, irritability, skin infecti problems.

There have been few studies concerning this complication, in which the freq

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β-Sitosterolemia with Generalized Eruptive Xanthomatosis

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Abstract. The clinical features of the first case of a patient with sitosterolemia and generalized eruptive xanthomatosis are described. A six-year-old girl with generalized eruption was referred to the lipid clinic because of the high plasma cholesterol levels determined by the enzymatic method. Neither clinical signs nor results of laboratory examinations appeared to be abnormal, except for the eruption and the increase in the plasma cholesterol concentration. A family survey revealed high plasma cholesterol concentrations in the mother and one of two other siblings. Histological examination showed the eruption to be a xanthoma. Plasma sterol analysis by high-performance liquid chromatography revealed a noticeable increase in plasma plant sterol as well as cholestanol concentrations in the proband and the hypercholesterolemic sibling. The other family members had slightly high plasma sterol concentrations. This is the first case of a sitosterolemic patient with eruptive xanthomatosis. The case indicates that the clinical features of the xanthoma in sitosterolemia are not only tuberous or tendon but also eruptive, and also suggests that sitosterolemia should be considered in the differential diagnosis of hypercholesterolemia in almost every case with tuberous or eruptive xanthoma. The diagnosis is clinically important, since the disease can be treated successfully by diet therapy and bile acid binding resins.

Key words: β-Sitosterolemia, Eruptive xanthoma, Tuberous xanthoma, Hyperlipidemia, Familial
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SITOSTEROLEMIA is a rare inherited disorder of lipid metabolism, and has been reported to show many clinical symptoms and signs such as multiple xanthomas of tendon and subcutaneous tissues, premature atherosclerosis, intravascular hemolytic anemia, splenomegaly, and platelet abnormality, and others [1]. One of the significant signs is xanthomatosis, and xanthoma has been reported to be tuberous or tendon. Previously we reported a very rare case of the disease showing signs of multiple spinal xanthomas which had induced neurological symptoms and signs due to compression of the spinal cord [2, 3]. We encountered and report here the first case of sitosterolemia with generalized eruptive xanthomatosis.

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Case Report and Family Study

A six-year-old girl was referred to our lipid clinic because of generalized xanthomatosis with high serum cholesterol levels. She had had localized eruption on both shoulders and forearms since the age of 4 years, and the eruptions had become general at the age of 5 years. The eruptions were yellowish and approximately 1 mm in diameter. She had been treated as having atopic dermatitis with steroid ointments. No clinical improvements, however, were observed with these treatments. She

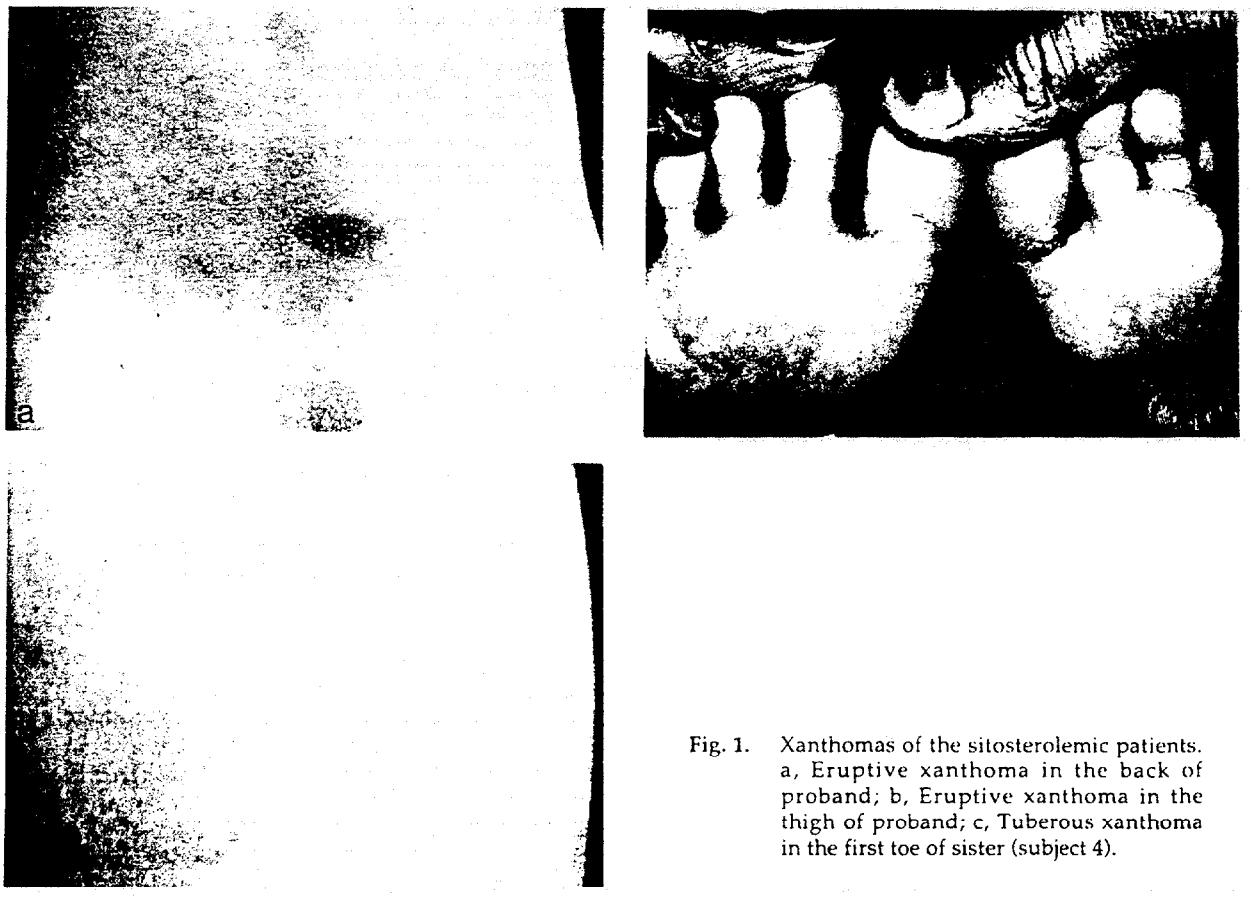

Fig. 1. Xanthomas of the sitosterolemic patients. a, Eruptive xanthoma in the back of proband; b, Eruptive xanthoma in the thigh of proband; c, Tuberous xanthoma in the first toe of sister (subject 4).

has had no arthralgia. Laboratory examination revealed a noticeable increase in serum lipid, total cholesterol 500 mg/dl and triglycerides 250 mg/dl after breakfast. There was no family history of sudden death or premature atherosclerosis, but one of the siblings (elder sister) had tuberous xanthoma in the hands, feet and buttocks.

The patient's height was 108 cm, and weight 18 kg. No physical abnormalities except for eruptive xanthoma (Fig. 1a, b) were observed. Neither tuberous xanthoma nor thickening of the Achilles' tendon were seen. Laboratory examination revealed moderate hypercholesterolemia as determined by the enzymatic method, but no other major abnormal findings including urinalysis, thyroid function tests (serum levels of TSH and free T4), and serum creatinine and fasting plasma glucose levels. Serum lipid levels of her family members at fasting are summarized in Table 1. Subject 2 (the mother) presented moderate hypertriglyceridemia, but serum triglyceride levels were only mildly increased in subjects 4 and 5. Neither anemia nor thrombocytopenia was observed in any family member studied. Histological diagnosis of the eruption (subject 5) and tuberous tumor of the skin (subject 4) was xanthoma of the skin (Fig. 2).

Plasma sterol analysis was performed as described by Kasama *et al.* [4]. Plasma levels of plant sterols as well as cholestanol were noticeably increase in subjects 4 and 5 (Fig. 3), and a diagnosis of sitosterolemia was made. The plasma concentrations of sterols are shown in Table 2. A mild increase in plasma concentrations of plant sterols as well as cholestanol was observed in the mother and father (subjects 1 and 2) who should be obligatory heterozygotes of the disease as well as the brother (subject 3).

After establishing the diagnosis of sitosterolemia, a low cholesterol and low plant sterol diet was prescribed for the patient as well as the affected sibling. Diet therapy successfully decreased the plasma levels of plant sterols as well as cholesterol (Table 3). Serum triglyceride levels in both subjects

Table 1. Serum lipid levels in family members of sitosterolemic patient with eruptive xanthomatosis

Subject		Age (yrs)	Sex	Serum lipid levels at fasting			
				Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL cholesterol (mg/dl)	Apo E phenotype
1	Father	36	M	220	78	59	N.E.
2	Mother	34	F	230	535	28	E2/3
3	Brother	10	M	176	162	32	E3/3
4	Sister	8	F	431	195	24	E2/3
5	Proband	6	F	260	150	36	E3/3

N.E., not examined.

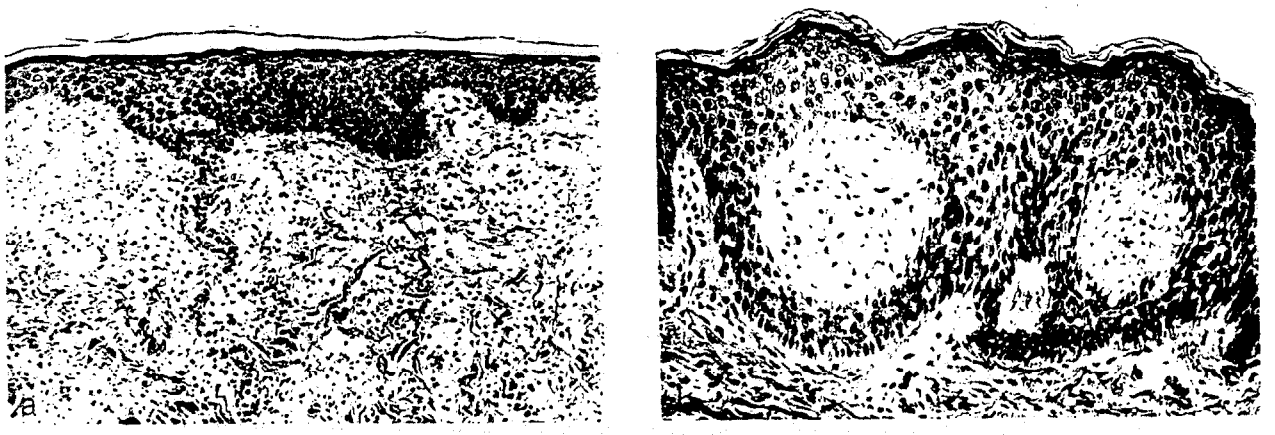

Fig. 2. Microscopic view of the xanthoma of the skin. a, At tuberous tumor of buttock in subject 4, foamy cells had accumulated around perivascular spaces. b, At the eruptive sites in subject 5, small aggregations of foamy phagocytes were observed in the papillary dermis, i.e., just beneath the epidermal layer (Hematoxylin and eosin, × 200).

decreased slightly after the treatment (195 mg/dl to 165 mg/dl in subject 4, 150 mg/dl to 112 mg/dl in subject 5). The sterol levels of plasma lipoprotein fractions separated by the ultracentrifugation method [5] in the two sitosterolemic subjects after diet therapy for 4 months are also summarized in Table 3. The sterol compositions of lipoproteins were similar in all lipoprotein fractions, in contrast to the original report [6] which described the predominant distribution of plant sterols in low density lipoprotein fraction. The xanthomas (tuberous in subject 4, and eruptive in subject 5) diminished in size during the diet therapy, and eruptive xanthoma in the face in subject 5 as well as tuberous xanthoma on the plantar side of proximal end of the first toe (Fig. 1c) disappeared almost completely.

Discussion

Xanthomatosis is an important clinical sign in familial hyperlipidemic diseases. Tuberous and tendon xanthomatosis is characteristic of hypercholesterolemic diseases such as familial hypercholesterolemia [7]. Eruptive xanthomatosis, on the other hand, is characteristic of moderate or severe hypertriglyceridemic hyperlipidemia such as type I, III and V hyperlipidemia in the WHO classification.

Sitosterolemia is a rare inherited disease in which plant sterols are accumulated in the whole body. Since the first report of two cases of the disease [6], 31 patients from 20 families have been described in the literature [1, 8–11]. Clinical

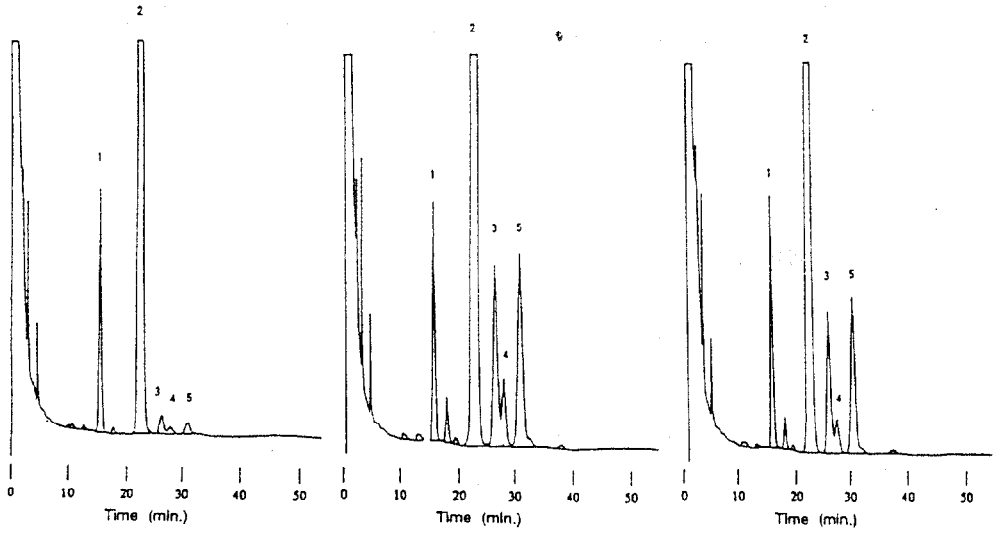

Fig. 3. Elution pattern of benzoate derivatives of plasma sterols in siblings of subject with sitosterolemia and generalized eruptive xanthomatosis. Left, subject 3 (brother); middle, subject 4 (sister); right, subject 5 (proband). Peak 1, desmosterol (internal standard); peak 2, cholesterol; peak 3, campesterol; peak 4, cholestanol; peak 5, sitosterol.

Table 2. Plasma sterol concentrations in family members of a sitosterolemic patient with eruptive xanthomatosis determined by HPLC (mg/dl)

Subject	Cholesterol	Sitosterol	Campesterol	Cholestanol
1	195	0.9	1.1	0.6
2	225	0.9	1.5	0.9
3	159	2.2	1.4	0.6
4	388	25.3	20.1	6.5
5	233	18.6	14.6	3.1
Control (n=15)	236 ± 38	1.05 ± 0.26	0.74 ± 0.36	0.43 ± 0.12
Range	193–323	0.78–1.73	0.24–1.7	0.20–0.76
Normal (n=10)	187 ± 25	0.71 ± 0.13	0.48 ± 0.23	0.39 ± 0.18
Range	140–240	0.51–0.99	0.20–0.87	0.10–0.79

The sterol levels were determined after derivatization of extracted nonsaponifiable fractions of plasma with desmosterol as an internal standard. Normal values were obtained from healthy subjects, and control values from hyperlipidemic subjects without xanthoma. The values are the mean ± SD.

presentation includes xanthomas, premature atherosclerosis, hemolysis, thrombocytopenia, splenomegaly, and arthralgia [12]. Tuberous or tendon xanthoma has been reported as a characteristic feature of the disease as seen in subject 4 in this family, but no case of eruptive xamthomas has previously been reported. The case presented here is the first reported incidence of sitosterolemia with generalized eruptive xanthomatosis. Eruptive xanthomatosis has been reported to be associated with severe hypertriglyceridemia such as chylomicronemia syndrome including familial lipoprotein lipase deficiency, in which serum triglyceride levels are more than 2,000 mg/dl [13]. It is therefore difficult to speculate on the etiology of the eruptive

Table 3. Sterol concentrations after dietary treatment in plasma lipoprotein fractions of two sitosterolemic patients determined by the HPLC

	Before treatment	After 4 month of diet therapy				
	Plasma	Plasma	VLDL	IDL	LDL	HDL
Subject 4						
Cholesterol	388	173	17.5	11.5	111	24.9
Sitosterol	25.3	12.2	0.8	0.8	8.9	2.1
Campesterol	20.1	9.6	0.8	0.6	6.9	1.4
Cholestanol	6.5	2.6	0.2	0.2	1.9	0.3
Subject 5						
Cholesterol	233	191	8.5	8.0	144	30.8
Sitosterol	18.6	14.2	0.4	0.5	10.6	2.7
Campesterol	14.6	11.6	0.4	0.5	8.7	2.0
Cholestanol	3.1	2.5	0.1	0.1	1.9	0.4

Samples were taken at fasting after low-cholesterol and low-plant sterol diet therapy for 4 months.

xanthoma in this case since serum lipid levels were only mildly hypertriglyceridemic (less than 300 mg/dl even after meals). Although we could not determine the sterol composition of the eruptive and tuberous xanthoma in this family because of the difficulty of obtaining the consent to re-biopsy the xanthomas, the eruptive xanthoma persisted for more than one year without significant fasting hypertriglyceridemia. The xanthomatosis may therefore be attributable to the inherited sitosterolemia, even though this unique clinical sign should in future be confirmed in other cases of this rare disease by not only histological examination but also a chemical composition study. Questions also remain as different types of xanthoma were observed in the same family of sitosterolemia (eruptive in subject 5 and tuberous in subject 4). It is possible that other congenital or environmental factors may contribute to the pathogenesis of eruptive xanthoma, since the mother of the proband had hypertriglyceridemia as well as mild hypercholesterolemia, as shown in Table 1.

It has been reported that a low plant sterol diet improves the clinical symptoms and signs of sitosterolemia especially xanthomas in young patients [9, 14]. In the subjects presented here, xanthoma as well as plasma lipid levels were improved by diet therapy. We have reported three middle-aged patients with sitosterolemia apart from this family [14]. The tuberous and tendon xanthoma of three patients were not clinically improved despite the successful reduction of plasma sterol concentrations by diet and cholestyramine therapy for more than five years. It is therefore important that the lipid abnormalities be treated at a young age to prevent not only the persistence of the xanthoma but also the progression of premature atherosclerosis in sitosterolemia.

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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

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To assess relationships of serum phytosterols (plant sterols [P]) to serum cholesterol (C), P were measured by gas-liquid chromatography (GLC) in 595 hypercholesterolemics (top C quintile in screening of 3,472 self-referred subjects). A second specific aim was to determine whether high serum P would track over time and whether they would predict familial aggregation of high C, high low-density lipoprotein cholesterol (LDLC), high apolipoprotein (apo) B, and increased premature coronary heart disease (CHD) in hyperphytosterolemic probands and their first-degree relatives. Mean ± (SD) C was 260 ± 56 mg/dL, campesterol (CAMP) was 2.10 ± 1.6 μg/mL, stigmasterol (STIG) 1.71 ± 1.67, sitosterol (SIT) 2.98 ± 1.61, and total P 6.79 ± 3.66 μg/mL. Serum C correlated with CAMP ($r = .15, P \leq .001$), STIG ($r = .10, P \leq .02$), SIT ($r = .34, P \leq .0001$), and total P ($r = .29, P \leq .0001$). High serum CAMP and STIG were associated with a personal or family history of CHD in subjects less than or equal to age 55 years (premature CHD). In 21 hyperphytosterolemic probands who initially had at least one P at or above the 95th percentile and a second P at or above the 75th percentile, P were remeasured 2 years later. Initial and 2-year follow-up CAMP, STIG, and SIT did not differ ($P > .7$). Initial and follow-up CAMP were correlated ($r = .47, P = .03$). There was a history of premature CHD in 42% of the hyperphytosterolemic probands' kindreds, more than twice that (19%) in the full cohort ($\chi^2 = 6.2, P = .013$). Of 34 first-degree relatives of the 21 high-P probands, 11 (32%) had top decile C, 3.2 times expected, $\chi^2 = 5.8, P = .016$, and 79% had top decile apo B, 7.9 times expected, $\chi^2 = 33.4, P < .00001$. Probands' CAMP, SIT, and P were significantly positively correlated with their 34 first-degree relatives' C, LDLC, and triglyceride levels. Probands' CAMP and P correlated with relatives' apo B. In the 34 first-degree relatives, P were correlated with C, high-density lipoprotein cholesterol (HDLC), and apo A1; CAMP and SIT correlated with HDLC, and with apo A1. High P tracks, correlates closely with and predicts high C, high LDLC, and high apo B in families, and appears to be associated with increased premature CHD, independent of cholesterol. Increased absorption of all dietary sterols with resultant high P and high C may be a heritable, atherogenic trait separate from the rare, recessive familial sitosterolemia.

SERUM PLANT STEROLS (phytosterols [P]), campesterol (CAMP), stigmasterol (STIG), and sitosterol (SIT), come entirely from dietary intake, are not endogenously synthesized, and are excellent indicators of cholesterol (C) absorption and synthesis.[1-7] Approximately 250 mg of P are ingested daily, with 65% as SIT, 30% as CAMP, and 5% as STIG.[8] A gastrointestinal gatekeeper function prevents absorption of all but a tiny fraction of dietary P (<5%), while allowing absorption of 40% to 60% of dietary C.[3,5] Total serum P range from 0.3 to 1.7 mg/dL in adults.[8] Increasing dietary P sevenfold to 10-fold in adults leads to less than a twofold increase in serum P.[3] Serum P correlate with the percentage absorption of dietary cholesterol, but are independent of the amount of dietary C and P.[5] Very large doses of P (several grams per day), by transiently blocking absorption of C, enhance C biosynthesis and lower serum C levels, but increase serum levels of P only very modestly in adults.[3,9-12] Commercial infant formulas, enriched with vegetable oils and low in C, generally elevate infants' serum mean total P from normal levels of less than 2 mg/dL[13] to approximately 9 mg/dL,[13] higher than in adult populations.[1,2,8] We previously speculated[13] that . . . "during the progression from infancy to childhood to adulthood that there is an ongoing 'maturation' in the ability of the subject to reject absorption of the phytosterols, so that, by adulthood, even major changes in dietary phytosterols result in minimal changes in plasma levels."

Colorimetric methods for measuring serum C mistakenly measure P as C,[8] since both have cyclopentanophenanthrene rings with 3-β-hydroxy substitution and a 5-6 double bond. P can be specifically quantitated in serum by gas-liquid chromatography (GLC).[1-8,13]

C absorption appears to regulate both C synthesis and serum C levels.[1,2,14-17] C absorption overloads hepatocytes with C and thus de novo C synthesis in the liver cells is down-regulated.[2,18,19]

In the rare, recessively transmitted familial hyperphytosterolemia, absorption of both P and C is high,[8,20-24] and serum P are high. Some, but not all subjects are hypercholesterolemic, and premature atherosclerosis is common.[8,20-24] In familial hyperphytosterolemia, the predominant sterol accumulated in xanthomas is C.[20,22] Lipoproteins rich in P may be unusually vulnerable to tissue uptake and degradation, with increased atherogenic transfer[20,22] of sterols to tissues at relatively low lipoprotein C levels. Reduced C synthesis may be the primary biochemical abnormality in hereditary phytosterolemia, with compensatory enhanced intestinal P absorption, increased lipoprotein sterol transport, and enhanced receptor-mediated low-density lipoprotein (LDL) degradation.[23,24]

Our specific aim in the current study was to assess the relationship of serum levels of P to C in 595 hypercholesterolemic subjects. A second specific aim was to determine whether high serum P levels would track over time and

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whether they might predict familial aggregation of high serum C, high apolipoprotein (apo) B, and increased premature coronary heart disease (CHD) in hyperphytosterolemic probands and their first-degree relatives.

MATERIALS AND METHODS

Subjects

Population-based blood sampling for the current study extended from November 7, 1987 through December 5, 1987 at four different suburban shopping center sites and at the Jewish Hospital on 4 consecutive weekends for a total of 8 days of sampling.[25] The screening included 1,986 women and 1,486 men for a total of 3,472 subjects, following a protocol approved by the Jewish Hospital I.R.C., and with signed informed consent. Given the public locations for the high-volume, population-based sampling, we did not obtain measures of height, weight, or skinfolds.

As previously described,[25] at the initial screening (November 7, 1987 through December 5, 1987) subjects could select either a measurement of serum C alone or a full lipid profile (total cholesterol, triglyceride, high-density lipoprotein cholesterol [HDLC], LDLC), which was performed only in the fasting state in subjects who were confirmed by venesectors to have had at least a 12-hour fast. Each participant received a questionnaire that focused on premature CHD events, asking the following question: "Did you or any parent, brother, or sister have a heart attack before age 55?"[25]

The cohort included in the current study incorporated the hypercholesterolemic[25] top quintile subset (n = 595) of the original 3,472 screenees, where adequate serum was available for P measurement. Of these 595 hypercholesterolemic subjects, 140 (24%) had elected at their initial sampling to get a lipid profile after a 12-hour fast.

Twenty-one individuals having at least one serum P at or above the population 95th percentile and one level at or above the 75th percentile (Table 1) were recalled for resampling in December 1989 to February 1990, 2 to 2.25 years after their first sampling. At this resampling, a detailed family history was obtained from these 21 hyperphytosterolemic probands and information was gathered on their first-degree relatives' names, addresses, and phone numbers so that they could be approached for blood sampling and evaluation. In five kindreds, the probands refused to allow or facilitate blood sampling in their first-degree relatives. In 16 kindreds, of 77 living first-degree relatives, 34 (44%), 83% of those living in southwestern Ohio, were subsequently studied, either at the Cholesterol Center or by home visits, from December 1989 to the present. Of the 43 first-degree relatives not studied to date, 36 (84%) live outside of southwestern Ohio, and attempts to obtain their blood samples by mail have, so far, been unsuccessful.

Table 1. Mean (±SD), Median, and Percentile Distributions of Serum Phytosterols (μg/mL) and Cholesterol (mg/dL) in 595 Hypercholesterolemic Subjects Studied in 1987

Percentile	C (mg/dL)	CAMP (μg/mL)	STIG (μg/mL)	SIT (μg/mL)	Total P (μg/mL)
10th	196	0.76	0.59	1.20	3.51
25th	223	1.10	0.79	1.86	4.56
50th	254	1.64	1.31	2.65	6.03
90th	328	2.56	2.11	3.77	7.95
95th	357	5.07	3.97	5.98	13.76
99th	424	7.82	7.74	8.36	19.33
Mean ± SD	260 ± 56	2.10 ± 1.6	1.71 ± 1.67	2.98 ± 1.61	6.79 ± 3.66
Median	253	1.64	1.31	2.65	6.03

Quantitative Measures of Serum C and P

Serum C, HDLC and LDLC, and triglycerides were measured in our laboratory following previously published methodology.[25] After collecting the serum samples in late 1987, and after selecting the 595 hypercholesterolemic top quintile subset by serum C,[25] the serum was frozen at −70°C (three aliquoted tubes per subject). All follow-up samples were obtained in 1989 to 1990 after a 12-hour fast in the morning to avoid diurnal variation effects on serum P levels.[17]

Serum P levels were quantitated by GLC, as follows.[26] One-milliliter samples were saponified in 5 mL of ethanolic NaOH (10 mL 10N NaOH + 90 mL 95% ethanol) for 1 hour at 100°C. The resulting sterols were extracted using petroleum ether. Samples were analyzed on Perkin-Elmer Sigma 1 Gas Chromatograph (Norwalk, CT) using 5-α cholestane as an internal standard. The conditions were injector temperature 275°C, detector temperature 280°C, column temperature 250°C isothermal. The column was a 15-m DB-17 megabore (J&W Scientific, Folsom, CA). Carrier gas was helium at 40 mL/min flow. Between-day and within-day coefficients of variation for SIT, CAMP, and STIG were less than 2%, and accuracy was excellent, with 98% of P standards being quantitated.[27] When standards were added to sera and the mixture was carried through the analytic process,[26] 92% of the added standard was recovered.[27]

Apo A1 and B were measured in the first-degree relatives of the hyperphytosterolemic probands by rate immunonephelometry.[28]

Statistical Analysis

Using the Shapiro-Wilk test,[29] none of the C or P distributions in the 595 hypercholesterolemic subjects were normally distributed, thus mandating Spearman rank-order correlations[30] and Wilcoxon nonparametric paired tests of differences.[30] Chi-square and Fisher's exact chi-square analyses[30] and ANOVA[30] were used to assess relationships of P to personal or family history of CHD at age 55 years or less (premature CHD).

RESULTS

Distributions of Serum C, P, and Age and Sex in the 595 Hypercholesterolemic Subjects

Table 1 displays percentile distributions, mean ± SD, and median levels for C, CAMP, STIG, SIT, and total P in the 595 subjects. As can be seen, by selection, the C distribution was skewed toward high values (Table 1). Of the P, SIT had the highest mean serum concentration, followed closely by CAMP, and then STIG (Table 1).

As displayed in Table 2, of the 595 subjects in the hypercholesterolemic top quintile, serum P were uniformly higher in the 140 subjects known to be fasting for 12 hours than in the 455 subjects in whom fasting status was not documented. However, the fasting and nonfasting subjects did not differ in regard to serum total C (Table 2).

Table 3 displays sex, age, and total C and P distributions in the 595 hypercholesterolemic subjects, the 21 hyperphytosterolemic, hypercholesterolemic probands who were resampled, and the 574 hypercholesterolemic subjects not resampled. The hyperphytosterolemic subset (n = 21) did not differ from the not-resampled hypercholesterolemic subjects (n = 574) in regard to percentage of men and

Table 2. Serum Phytosterols (μg/mL) and Cholesterol (mg/dL) in 1987 in 595 Hypercholesterolemic Subjects, 140 Fasting and 455 Whose Fasting Status Was Not Known

Variable	n	Mean	SD	Median	P (Wilcoxon)
CAMP	140	2.18	1.26	1.89	.029
	455	2.08	1.71	1.61	
STIG	140	1.72	1.01	1.5	.022
	455	1.71	1.82	1.2	
SIT	140	3.28	1.62	3.21	.003
	455	2.88	1.59	2.55	
Total P	140	7.18	3.0	6.42	.003
	455	6.67	3.8	5.86	
Total C	140	264	24	261	.86
	455	267	32	261	

women, age, or median total C, but, by selection, had significantly higher serum SIT, CAMP, and STIG (Table 3). There were also no significant correlations between age or sex and serum P in the 595 hypercholesterolemic subjects, the 21 hyperphytosterolemic probands, or the 574 hypercholesterolemic subjects not resampled. Thus, neither age nor gender were significant predictive variables for P levels, and the hyperphytosterolemic subset did not differ demographically from the whole sample population.

Relationships Between a Personal or Family History of CHD at Age 55 or Less and Serum P

Of the 506 subjects answering the question on personal or family history of CHD at age 55 or less (premature CHD), there were no significant differences ($P > .1$) in serum SIT, STIG, or total C between the 19% with premature CHD and the 81% without. Median CAMP (1.9 μg/mL) was marginally higher ($P = .097$) in the 19% of subjects with premature CHD than in the 81% without (1.6 μg/mL). When covariance was adjusted for total C, a less significant association between CAMP and CHD emerged ($P = .135$).

Premature CHD was nearly twice as common (27%, $P = .096$) in subjects in the top STIG decile than (14%) in the subjects in the bottom STIG decile. This difference in premature CHD was independent of serum C, with median C levels not differing ($P > .1$) between the top and bottom decile STIG groups.

There was a history of premature CHD in six of 25 (24%) subjects in the top 5% for CAMP versus 0 of 24 subjects in the bottom 5% ($P = .022$, Fisher's exact χ^2), with median serum C not differing ($P = .8$) between the top and bottom 5% CAMP groups.

In the 19 hyperphytosterolemic probands providing information on personal or family history of CHD, 42% of kindreds had a history of premature CHD, more than twice the 19% in the full cohort ($\chi^2 = 6.2$, $P = .013$). However, median C in the hyperphytosterolemic probands did not differ from that of the cohort (Table 3).

Relationships Between Serum C and CAMP in the 595 Subjects

As displayed in Table 4, serum C was significantly correlated with CAMP, STIG, SIT, and total P, with the closest relationship being with SIT ($r = .34$, $P = .0001$). CAMP levels were significantly related to STIG and SIT, as were STIG levels to SIT (Table 4). Calculating partial R^2 from the simple correlation coefficients of Table 4, 11% of the variance of serum C could be accounted for by SIT, 2% by CAMP, and 1% by STIG.

Of the 595 subjects, at their initial sampling, 140 had a full lipid profile after a 12-hour fast. In this group of 140 subjects, median total C was 261 mg/dL, triglyceride 152 mg/dL, HDL 53 mg/dL and LDL 176 mg/dL, and median CAMP was 1.89 μg/mL, STIG 1.5 μg/mL, SIT 3.21 μg/mL, and total P 6.42 μg/mL. In this group of 140 subjects, the only statistically significant correlation between serum P and lipoprotein C levels was between SIT and LDL C ($r = .18$, $P = .03$).

Follow-up of Individuals With Elevated Levels of Serum P (1987-1989)

Twenty-one hyperphytosterolemic probands from the initial cohort of 595 subjects were selected for follow-up studies in 1989 to 1990 by virtue of having at least one serum P (at the initial 1987 sampling) at or above the 95th percentile, and a second phytosterol at or above the 75th percentile (Table 5). We did not obtain quantitative dietary information on P, C, or fat intake in these 21 probands or in any of the 595 subjects in 1987.

Nine of this cohort of 21 subjects at the first (1987) sampling had at least one serum P at or above the 99th percentile (Table 5). Seventeen had at least one P at or above the 95th percentile, but below the 99th percentile (Table 5). Eight had at least one phytosterol at or above the 90th percentile, but below the 95th percentile (Table 5). There were no significant differences between the first and

Table 3. Sex, Age, Total Cholesterol (mg/dL), and Phytosterol (μg/mL) in the 595 Hypercholesterolemic Subjects Studied in 1987, the 574 Hypercholesterolemic Subjects Studied in 1987 and Not Resampled in 1989-1990, and the 21 Hypercholesterolemic, Hyperphytosterolemic Probands Resampled in 1989-1990

Group	M/F	Age (yr)		Total C	SIT	CAMP	STIG	Total P
		Mean ± SD	Median	Median	Median	Median	Median	Median
n = 595	231/364	57.8 ± 14.4	61	261	2.65	1.64	1.31	6.03
n = 574	222/352	57.7 ± 14.5	61	260	2.62	1.61	1.28	5.92
n = 21	9/12	59.2 ± 13.4	62	262	6.28	5.94	3.74	14.65
χ^2 (574:21)	NS	NS	—	—	—	—	—	—
Wilcoxon (574:21)	—	—	—	$P = .63$	$P = .0001$	$P = .0001$	$P = .0001$	$P = .0001$

Table 4. Simple Spearman Correlations, Serum Cholesterol, and Phytosterols in the 595 Hypercholesterolemic Subjects

	C		CAMP		STIG		SIT		TOTAL P	
	r	P	r	P	r	P	r	P	r	P
C	—		.148	.0003	.095	.021	.335	.0001	.292	.0001
CAMP			—		.245	.0001	.398	.0001	.719	.0001
STIG					—		.188	.0001	.575	.0001
SIT							—		.782	.001
Total P									—	

second samplings for serum CAMP, STIG, SIT, or total P (Table 5). There were highly significant correlations between the first CAMP measurements and those 2 years later, but no significant associations between the first and second STIG, SIT, and total P levels (Table 5).

At the second sampling in 1989, the distributions of serum CAMP, STIG, and SIT in the 21 hyperphytosterolemic probands remained skewed to very high percentiles, comparable to the original sample (Table 5). There were no significant differences ($P > .1$) between the percentile categories for serum P levels in the 21 hyperphytosterolemic subjects, comparing their 1987 and 1989 to 1990 distributions.

There were significant correlations between changes in serum P levels between the first and second measurements (Table 6). The change in CAMP was significantly associated with change in SIT and change in total P (Table 6). Change in STIG was associated with change in total P (Table 6). Change in SIT was associated with change in total P (Table 6).

Correlations Between Serum P and C in Hyperphytosterolemic Probands and Their First-Degree Relatives

In 16 of the 21 hyperphytosterolemic probands' kindreds, 34 first-degree relatives (44% of living relatives) were sampled. In these 16 kindreds, probands' serum CAMP, SIT, and total P were significantly correlated with their first-degree relatives' serum C and LDLC levels (Table 7). Moreover, probands' serum CAMP and total P were correlated with their first-degree relatives' apo B (Table 7). Also, probands' CAMP, SIT, and total P were associated with their first-degree relatives' triglyceride levels (Table 7). Finally, there was a significant association between probands' SIT and first-degree relatives' STIG (Table 7).

Hypercholesterolemia and Hyperapobetalipoproteinemia in 34 First-Degree Relatives of the Hyperphytosterolemic Probands

Of 34 first-degree relatives, 11 (32%) had top decile total C, 3.2 times that expected ($\chi^2 = 5.8, P = .016$), and 26 of 33

Table 5. Initial (First, 1987) and Follow-up (Second, 1989-90) Serum CAMP, STIG, and SIT (μg/mL)

Subject No.	Initial Total C (mg/dL)	CAMP		STIG		SIT		Total P	
		First	Second	First	Second	First	Second	First	Second
PHY-01	293‡	9.72§	8.49§	4.00‡	0.71	10.59§	6.89‡	24.31§	16.09‡
PHY-02	289‡	17.54§	26.37§	12.82§	1.29	6.84‡	20.11§	37.20§	47.77§
PHY-03	293†	7.82§	14.65§	1.93	2.58*	7.21‡	12.51§	16.96‡	29.74§
PHY-04	234‡	6.92‡	1.30	3.07*	0.52	8.12‡	6.85‡	18.11‡	8.67*
PHY-05	258	4.00†	6.41‡	3.74	3.75	9.44§	6.33‡	17.18§	16.49‡
PHY-06	241*	8.67§	7.41‡	0.65	1.91	8.64‡	6.71‡	17.96‡	16.03‡
PHY-07	282†	3.42	7.39‡	5.44‡	2.72*	5.30†	8.61§	14.16‡	18.72‡
PHY-09	285†	2.15	8.43§	9.80§	2.64*	1.81	5.40†	13.46†	16.47‡
PHY-10	226†	3.66	8.82§	4.84‡	4.40‡	3.32	6.64‡	11.82†	19.86§
PHY-11	334‡	6.88‡	3.02*	0.97	3.01*	5.98‡	9.81§	13.83‡	15.84‡
PHY-12	291‡	1.22	1.58	7.74§	6.61‡	1.85	3.30	10.81†	11.49†
PHY-13	266†	2.03	3.21*	4.86‡	6.62‡	1.15	2.31	8.04*	12.14†
PHY-14	300‡	2.96*	4.47*	11.56§	3.81*	2.22	6.08‡	16.74‡	14.36‡
PHY-15	262*	6.34‡	7.02‡	2.05	4.07‡	5.38†	7.84‡	13.77‡	18.93‡
PHY-16	251*	7.11‡	7.81‡	2.54*	4.28‡	6.91‡	8.40§	16.56‡	20.49§
PHY-17	248*	1.30	3.80*	1.53	6.98§	8.01‡	4.00*	10.84†	14.78‡
PHY-18	256*	6.22‡	13.88§	1.37	3.92*	6.97‡	10.18§	14.56‡	27.98§
PHY-19	252*	1.81	12.15§	7.45‡	4.58‡	6.26‡	10.34§	15.52‡	27.07§
PHY-20	248	3.29*	6.83‡	24.84§	5.78‡	1.90	3.18	30.03§	15.79‡
PHY-21	276‡	5.94‡	5.36‡	1.26*	17.75§	7.45‡	18.37§	14.65‡	41.48§
PHY-22	245	7.74‡	23.27§	0.39	4.37‡	6.28‡	17.51§	14.41‡	45.15§
Mean	268.1	5.56	8.65	5.36	4.40	5.79	8.64	16.71	21.28
Paired Wilcoxon (first visit v second visit)		NS		NS		NS		NS	
Correlation of first and second tests, Spearman		$r = .47$	$P = .034$	$r = -.02$	$P = .95$	$r = .39$	$P = .083$	$r = .19$	$P = .42$

NOTE. Total C percentile levels used were those specified by the Lipid Research Clinics.[38]

* ≥ 75th percentile.

† ≥ 90th percentile.

‡ > 95th percentile.

§ ≥ 99th percentile.

Table 6. Correlations of Changes (Δ) in Phytosterol Levels Between First (1987) and Second (1989-1990) Measurements (2.25 Years Later), Spearman Correlations (r, P) in 21 Hyperphytosterolemic Probands

	ΔCAMP	ΔSTIG	ΔSIT	Total ΔP
ΔCAMP	—	−.16	.52	.61
		.50	.016	.004
ΔSTIG		—	−.03	.50
			.91	.021
ΔSIT			—	.66
				.001
				—
ΔTotal P				

(79%) had top decile apo B, 7.9 times expected, $\chi^2 = 33.4$, $P < .00001$.

Correlations in First-Degree Relatives of Hyperphytosterolemic Probands: P and Lipids, Lipoproteins, and Apolipoproteins

As displayed in Table 8, in the 34 first-degree relatives, serum total P were significantly associated with total C, with HDLC, and with apo A1 levels. Serum CAMP and SIT were significantly correlated with HDLC and with apo A1 (Table 8).

DISCUSSION

Serum P are significant indicators of C absorption and synthesis.[1,2] When serum P are high, then presumably dietary C is efficiently absorbed, sterol absorption efficiency as a whole is high, hepatic C synthesis is low, and serum C concentrations are high.[1,2,6-9,14-19]

Mean levels of CAMP in the current study (2.1 ± 1.6 μg/mL) were comparable to those reported in 63 adult Finnish subjects by Miettinen et al[2] (3.2 ± 1.47 μg/mL). SIT in the current study (2.98 ± 1.61 μg/mL) was similar to that reported by Miettinen et al (2.57 ± 0.92).[2] Total serum C in the study by Miettinen et al.[2] (2,500 ± 464 μg/mL) was also comparable to C in the current study (2,600 ± 560 μg/mL). The range of total serum P in the current study (0.35 to 1.93 mg/dL) was similar to the stated normal range of Kuksis et al, 0.3 to 1.7 mg/dL.[8]

Table 8. Correlations in First-Degree Relatives of Hyperphytosterolemic Probands: Phytosterols Versus Lipids, Lipoproteins, and Apolipoproteins (n = 34)

	CAMP	STIG	SIT	Total P
Total C	0.29	0.25	0.24	0.37†
Triglyceride	−0.06	0.31*	−0.22	−0.03
HDLC	0.49†	−0.02	0.46†	0.41†
LDLC	0.13	0.23	0.18	0.25
Apo A1	0.37†	0.14	0.39†	0.36*
Apo B	−0.08	0.24	−0.01	0.06

*$P < .1, > .05$.
†$P \leq .05$.

Serum C levels were closely associated with serum SIT, which accounted for 11% of its variance, with CAMP, which accounted for 2% of the variance, and with STIG, which accounted for 1% of the variance. Total serum P were closely correlated with total serum C, both in the cohort of 595 hypercholesterolemic subjects ($r = .29$, $P = .0001$), and in the 34 relatives of the hyperphytosterolemic probands ($r = .37, P \leq .05$). The correlation of total serum C to SIT in the current study ($r = .335$, $P = .0001$) was nearly the same as that reported by Miettinen et al[2] ($r = .388$). However, Miettinen et al[2] reported a closer correlation of CAMP to C ($r = .425$) than we did ($r = .148$).

Our finding of significant associations of serum CAMP and SIT with HDLC and apo A1 in the 34 first-degree relatives of the hyperphytosterolemic probands is not surprising. In contrast to C, serum P preferentially accumulate in HDL, where P to C ratios are almost 40% higher than in very–low-density lipoproteins or LDL.[5] Our P-HDLC-LDLC correlations were similar to those of Miettinen et al,[2] where serum CAMP and SIT correlated with HDLC and LDLC. It has been speculated[31] that the enrichment of HDL with SIT is associated with reverse C transport and reverse P transport by HDL.

In 21 hyperphytosterolemic subjects at their initial screening in 1987, all remained hyperphytosterolemic 2 years later, and all were hypercholesterolemic, consistent with

Table 7. Pairwise Correlations Between Serum Phytosterols, Total Cholesterol, HDLC and LDLC, Triglyceride Apo A1 and Apo B in Hyperphytosterolemic Probands (n = 16) and Their First-Degree Relatives (n = 34)

	Probands				
First-Degree Relatives	CAMP	STIG	SIT	Total P	Total C
CAMP	−0.043	0.132	0.160	0.119	0.066
STIG	0.120	−0.134	0.331*	0.280	−0.140
SIT	0.156	0.177	0.164	0.143	0.091
Total P	0.060	0.146	0.222	0.196	0.055
Total C	0.416‡	−0.079	0.483§	0.536§	0.285
Triglyceride	0.344†	−0.305*	0.302*	0.335*	0.126
HDLC	−0.053	0.130	0.130	0.074	0.221
LDLC	0.412‡	−0.084	0.413‡	0.431‡	0.239
Apo A1	0.124	−0.013	0.312*	0.199	0.336*
Apo B	0.32*	−0.027	0.283	0.318*	0.362*

*$P < .1, > .05$.
†$P \leq .05$.
‡$P \leq .02$.
§$P \leq .01$.

the suggestion by Miettinen et al[1,2] that hyperabsorbers of P are also hyperabsorbers of all sterols, including C. Mean P levels in the 21 hyperphytosterolemic subjects 2 years after their initial screening did not differ from those at their initial screening, and there was considerable tracking of CAMP levels.

In the current study, hyperphytosterolemia appeared to be a heritable marker in kindreds for hypercholesterolemia, hyperapobetalipoproteinemia, and increased premature CHD. Thus, of 34 first-degree relatives of the hyperphytosterolemic probands, a significant proportion (32%) had top decile total C, 3.2 times that expected, and 79% of the first-degree relatives had top decile apo B, 7.9 times expected. Moreover, in these 34 first-degree relatives, CAMP and total P were correlated with serum total C.

In the 16 hyperphytosterolemic probands' kindreds where first-degree relatives could be sampled, the probands' serum CAMP, SIT, and total P levels were significantly correlated with their first-degree relatives' total C, LDLC, and triglyceride levels. Probands' CAMP and total P were correlated with relatives' apo B. Thus, the hypercholesterolemic probands' P significantly predicted their first-degree relatives' atherogenic LDLC and apo B. This is (to our knowledge) an original finding. Obligate heterozygotes from families with the rare, recessively inherited familial sitosterolemia, a disorder separate from the familial aggregations in our current report, usually[20-24] but not always[32] have normal serum C and P levels.

Our current report suggests that mild hyperphytosterolemia may be a familial marker for hyperabsorption of P and C in probands and their first-degree relatives. Since we did not measure P absorption, we cannot rule out the possibilitythat the high serum P might reflect slower in vivo P clearance.

Hyperphytosterolemia identifies kindreds with elevated total C and LDLC levels, presumably due to increased absorption of C. A variety of other hypotheses to explain the data can also be considered. Could high-C, high-fat diets produce hypercholesterolemia while also increasing P absorption? High-fat, high-C diets have been shown not to alter the efficacy of P absorption.[5] Although serum and lipoprotein P levels are significantly correlated with the percentage absorption of dietary C, they are entirely independent of the amount of dietary C and P,[5] and hence, the sera P:C correlations do not reflect an epiphenomenon. Could the correlation between serum C and P simply reflect an increase in lipoprotein transport capacity in serum? We are not aware of studies that show that increased lipoprotein production rates influence gastrointestinal sterol absorption. Moreover, in familial sitosterolemia,[23,24,33] it has been proposed that the primary biochemical abnormality is reduced C synthesis (reduced formation of HMG-CoA reductase), leading first to compensatory high P absorption and then to increased lipoprotein sterol transport, as well as to augmented expression of tissue LDL receptors.[33]

We postulated that our mildly hyperphytosterolemic, hypercholesterolemic subjects should be sensitive to major reductions of dietary C and P.[34] After 8 weeks of a diet low in P (mean, 94 mg/d), low in C (mean, 129 mg/d), and low in total fat (23% of calories), in seven hyperphytosterolemic probands,[34] mean serum CAMP was reduced 58% ($P \leq .05$), mean STIG decreased 98% ($P \leq .01$), and mean SIT decreased 69% ($P \leq .05$), coupled with a 10% lowering of serum C ($P = .057$).[34]

Since familial hyperphytosterolemia is associated with premature atherosclerosis,[20-24,32,35] identification of hyperphytosterolemia in hypercholesterolemic subjects may be a useful marker for individuals and their families at relatively high risk for premature CHD. P appear to initiate the development of xanthomas at otherwise normal C levels.[20] It has also been speculated[22] that there is increased tissue uptake and degradation of P-rich lipoproteins, with enhanced net transfer of sterols to tissues occurring at relatively low plasma lipoprotein levels. Within this frame of reference,[20,22] the persistent mild hyperphytosterolemia associated with hypercholesterolemia in the current study may well reflect a subcohort of hypercholesterolemic individuals at substantially increased risk for premature CHD by virtue of increased sterol transport into tissues. Our current study showed an augmented risk of premature CHD associated with mild hyperphytosterolemia. In the full cohort, median CAMP was higher in those subjects with a personal or family history of premature CHD. Premature CHD was more common in kindreds in the top than in the bottom decile for serum STIG (independent of serum C). Twenty-four percent of subjects in the top 5% for serum CAMP had a personal or family history of premature CHD, compared with no subjects in the bottom 5% for CAMP ($P = .022$), a difference independent of serum C. Moreover, also independent of serum C, 42% of the hyperphytosterolemic probands' kindreds had a history of premature CHD versus 19% of the full cohort ($P = .013$).

Since hypercholesterolemic patients are usually treated by diet or diet and drugs,[37] how might the additional identification of mild hyperphytosterolemia alter therapy? First, hyperphytosterolemia, as in the current report, may identify a subset of hypercholesterolemic subjects at more than the "usual," expected high CHD risk by virtue of high total C and LDLC. These mildly hyperphytosterolemic, hypercholesterolemic subjects have increased levels of atherogenic, P-rich lipoproteins more likely to transfer sterols to tissues,[20,22] and usually have elevated apo B levels. In familial sitosterolemia,[35] high LDL apo B levels accompany high SIT levels, and are thought to contribute to increased risk of premature CHD. It might be advisable, in mildly hyperphytosterolemic, hypercholesterolemic subjects, to provide not only conventional low-fat, low-C diets, but also diets relatively low in enriched sources of P such as vegetable oils, with a goal, realized in our study of seven of our hyperphytosterolemic probands,[34] to ameliorate the mild hyperphytosterolemia. Of the C-lowering drugs, bile acid-binding resin therapy might particularly benefit hyperphytosterolemic, hypercholesterolemic subjects.[34]

We speculate that the mildly hyperphytosterolemic, hypercholesterolemic kindreds of the current report may also have a heritable, atherogenic trait, separate from the rare, recessively transmitted, familial sitosterolemia[20-24,32,33,35] where serum P levels usually are much higher than those in our kindreds.

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minireview

Sitosterolemia

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It is almost 18 years since the first reports on sitosterolemia appeared (1). Two sisters with tendon xanthomas and normal plasma cholesterol levels were found to have elevated plant sterol concentrations in the plasma. A high percentage of dietary sitosterol was absorbed from the intestine, as measured by the sterol balance technique, and was believed to account for the plant sterol accumulation. Since the original report, 27 patients from 16 families have been detected (1–14). The clinical presentation includes tendon xanthomas, accelerated atherosclerosis particularly affecting males at a young age, hemolytic episodes, and arthritis and arthralgias. The risk of premature atherosclerosis was observed in several young male subjects who died because of acute myocardial infarctions associated with extensive coronary and aortic arteriosclerosis. The youngest was a 13-year-old Amish male who had four other homozygous siblings (3). In addition, a 17-year-old male, personally followed by the authors, developed angina pectoris, showed an abnormal cardiac stress test with decreased coronary artery perfusion, and died suddenly of an acute myocardial infarction while exercising (15). Examination of his coronary arteries at post mortem revealed 60% occlusion of the left anterior descending coronary artery (**Fig. 1**). However, multiple microinfarctions were noted in the myocardium which suggested that the atherosclerotic process had begun earlier and was chronic and progressive.

Sitosterolemia is inherited as a recessive trait (14). Heterozygotes are clinically and biochemically normal, although plasma sitosterol levels of some heterozygous subjects may be slightly but significantly increased over controls. These values still differ quantitatively from homozygotes by 10- to 20-fold (9, 12). Of interest is the high degree of inheritance of the homozygous state. In two unrelated families, homozygous sitosterolemia was present in 4/4 and 2/4 siblings respectively, from each family.

Biochemical features

The hallmark biochemical feature of the disease is the demonstration of elevated concentrations of sitosterol (24-ethyl cholesterol) in the plasma (1, 16). Actually all dietary sterols are found in plasma (17), but since sitosterol is usually the most abundant in the diet, proportionately greater quantities are present in plasma and tissues (**Fig. 2**). For this reason, the condition has been named sitosterolemia (17). In addition, the respective 5α-dihydro derivative of cholesterol (cholestanol) and the 5α-dihydro plant sterol derivatives, 5α-campestanol and 5α-sitostanol, are present in increased amounts in plasma and tissues (**Fig. 3**) (18, 19). As diets contain only small amounts of cholestanol, 5α-campestanol, and 5α-sitostanol, the 5α-dihydro derivatives probably are produced endogenously in larger amounts (19).

The diagnosis of sitosterolemia is established by demonstrating increased amounts of plant sterols (campesterol, sitosterol, stigmasterol, and avenosterol) and 5α-stanols in plasma and tissues (1, 15, 19). The usual colorimetric assay that depends on a double bond between carbons 5 and 6, or enzymatic method that detects the 3β-hydroxy group do not distinguish sitosterol from cholesterol. Therefore, to find plant sterols and 5α-stanols and establish the diagnosis, gas–liquid chromatography using a capillary column is necessary (**Fig. 4**), although high performance liquid chromatography can also be applied (12). In one family with four homozygous siblings, the unsaturated sterols represented about 16% of the total plasma sterols with cholestanol and 5α-dihydro plant stanols making up about 4% (2, 8). In other families, plant sterols and 5α-stanols may account for as little as 11% to as much as 25% of the plasma sterols (2, 3, 19). Thus, cholesterol represents only about 80% of the total plasma sterols in sitosterolemic homozygotes. The concentration and distribution of sterols and stanols from a number of sitosterolemic homozygotes from five families and their heterozygous relations are presented in **Table 1**.

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein; CTX, cerebrotendinous xanthomatosis; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

Fig. 1. Section of coronary artery from a 17-year-old sitosterolemic male who died of an acute myocardial infarction. Sixty percent occulsion of the vessel lumen by atherosclerotic thickening of the vessel wall.

For controls, values from 20 healthy subjects are given; they show that cholesterol normally represents 99.6% of the total sterols with about 0.2% cholestanol and 0.2% plant sterols. In some heterozygotes, cholestanol and sitosterol levels are similar to controls (20–22). However, in several obligate heterozygotes, cholestanol and sitosterol levels were slightly but significantly higher than the control means, but still substantially less than those found in their homozygous offsprings (9, 12). Plasma cholesterol concentrations may also vary considerably in homozygotes. As illustrated in Table 1, cholesterol levels may be low but are usually increased over age-matched controls. However, some subjects (LBU) show extremely high cholesterol concentrations that resemble the levels found in LDL receptor-deficient hypercholesterolemic subjects.

Plasma lipoproteins have been measured in homozygous sitosterolemic subjects and the increased amounts of unsaturated plant sterols and saturated 5α-stanols are distributed in about the same proportion among the various lipoprotein fractions (HDL, LDL, IDL and VLDL)

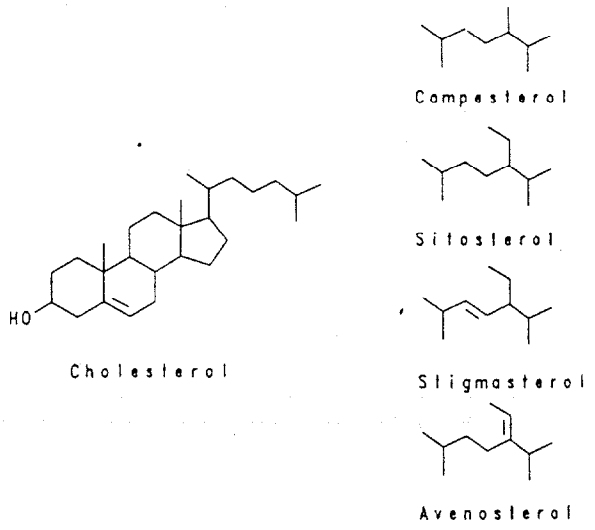
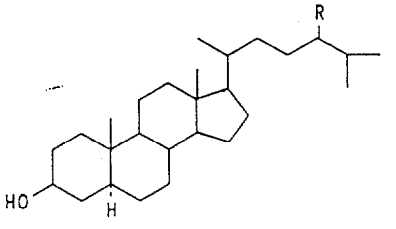


Fig. 2. Structure of plant sterols found in plasma of sitosterolemic subjects. These sterols have the same ring nucleus as cholesterol, but differ by the addition of substituents on the side chain at carbon 24, the presence of a double bond at carbon 22 in stigmasterol, and a double bond between carbons 24 and 28 in avenosterol.

(1, 6, 23). However, low density lipoprotein (LDL) concentrations tend to be elevated, reflecting higher total sterol concentrations as compared to age- and sex-matched controls. Despite the incorporation of increased amounts of plant sterols and 5α-saturated stanols, preparative density gradients for each lipoprotein class were similar to that of controls (23). The major proportion of the total low density lipoproteins was isolated in the subfraction of d 1.034 g/ml. The mean particle diameter, 25.7 ± 2.8 nm, for sitosterolemic LDL was not unusual as determined by electron microscopy, and the sitosterolemic LDL was not distinguishable morphologically from normal LDL. High density lipoprotein (HDL) concentrations tended to be normal or low in the homozygous sitosterolemic subjects (23). Electron microscopy of HDL from a male sitosterolemic subject with severe symptomatic atherosclerosis showed that the particles were round with a mean diameter of 8.5 ± 1.7 nm, consistent with the predominance of small, dense HDL (23).

Plasma concentrations of apolipoprotein B are usually increased and apolipoprotein A-I decreased for sitosterolemic homozygotes (23). However, normal plasma apoB and A-I levels were present in heterozygotes (20, 24). Thus, apolipoprotein values reflect the increased LDL and usually low HDL concentrations detected in these patients as determined by analytical and preparative ultracentrifugation (23).

Tissue sterol concentrations were measured in a 17-year-old sitosterolemic homozygote male who died unexpectedly of an acute myocardial infarction, and showed about 16% plant sterols and 5α-stanols in plasma (15). The total sterol concentrations in red blood cells, liver, lung, and heart were not different from control, but the cholesterol concentrations in these tissues were reduced and offset by the increased amounts of plant sterols and 5α-saturated stanols. Of importance, the individual plant sterols and 5α-saturated stanols were deposited in the tissues in about the same proportion that they were present in LDL. This suggested that the tissue sterols originated



R-H Cholestanol

R-CH_3 24-methyl Cholestanol

R-C_2H_5 24-ethyl Cholestanol

Fig. 3. Structures of 5α-saturated stanols found in plasma of sitosterolemic subjects. Cholestanol is the 5α-dihydro derivative of cholesterol; 5α-campestanol and 5α-sitostanol are the 5α-dihydro derivatives of campesterol and sitosterol, respectively.

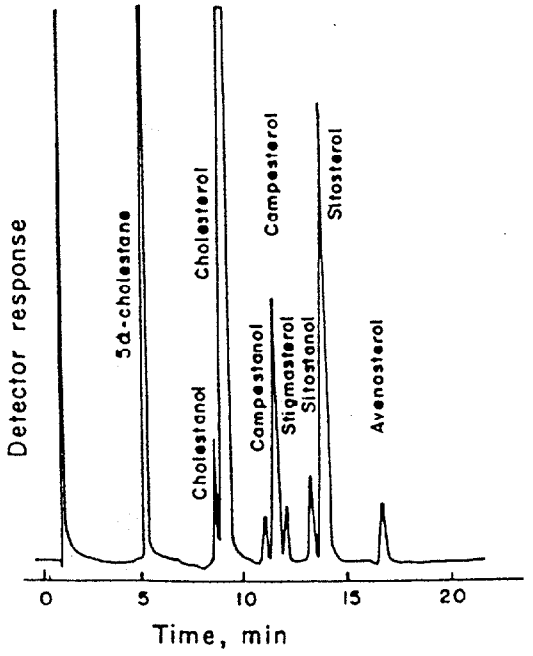


Fig. 4. Capillary gas-liquid chromatogram of plasma sterols and 5α-stanols from a homozygous sitosterolemic subject. In normal plasma only cholesterol and trace amounts (< 1%) of cholestanol and sitosterol are detected. 5α-Cholestane is added as an internal standard. Separation was performed on Chrompack CPWax-57CB capillary column.

TABLE 1. Plasma sterols and stanols

Patients	Age	Cholesterol	Sitosterol	Campesterol	5α-Cholestanol	5α-Sitostanol	5α-Campestanol
	yr			*mg/dl*			
Homozygotes							
KCN[a] (10)[b]	29	184 ± 25	15 ± 2	7.5 ± 1.1	2.1 ± 1.0	2.9 ± 1.0	1.3 ± 0.9
KC[a] (10)	23	202 ± 25	14 ± 4.1	8 ± 3.1	4.7 ± 1.0	2.2 ± 1	1.4 ± 0.2
TC[a] (10)	27	233 ± 12	21 ± 8.3	10 ± 0.5	3.8 ± 1.4	5.4 ± 1.2	1.9 ± 1.0
RC[a] (10)	18	249 ± 39	20 ± 5.5	13 ± 1.5	7.5 ± 2.4	3.9 ± 1.0	2.6 ± 0.9
GB[c] (10)	28	292 ± 8	13 ± 0.9	8 ± 0.2	1.8 ± 0.1	1.9 ± 1.0	0.8 ± 0.4
CL	42	134	27	13	1.6	3.0	2.6
JBR[d]	14	256	30	14.5	4.0	2.7	2.5
LBU	24	482	56	24.0	11.0	6.0	4.0
Heterozygotes							
AC[a] (5)	50	210 ± 26	0.95 ± 0.17	ND[e]	0.65 ± 0.21	ND	ND
VC[a] (5)	56	194 ± 14	0.36 ± 0.09	ND	0.34 ± 0.19	ND	ND
DB[c] (3)	25	204 ± 27	0.66 ± 0.05	ND	0.34 ± 0.13	ND	ND
RBR[d]	48	254	0.8	ND	1.2	ND	ND
Controls, n = 20	17–62	180 ± 5	0.22 ± 0.20	ND	0.20 ± 0.20	ND	ND

[a]C family, includes heterozygous parents.
[b]Number of samples analyzed in parentheses.
[c]B family, includes heterozygous sister.
[d]R family, includes heterozygous mother.
[e]ND, not detected.

from plasma. In contrast, brain sterols in the sitosterolemic subject were composed almost entirely of cholesterol (15). Thus, despite the presence of large amounts of plant sterols and 5α-stanols, the blood–brain barrier in sitosterolemia remains intact and is not permeable to circulating LDL. This is in contradistinction to cerebrotendinous xanthomatosis (CTX), a lipid storage disease, where increased amounts of cholestanol deposit in the brain and suggests that the blood brain–barrier is damaged and more permeable to circulating LDL (2, 15). Interestingly, atheromas in the aorta of this sitosterolemic subject contained increased amounts of esterified sterols, about 50% of the cholesterol and sitosterol were esterified as compared with only 10% esterified sterols in visceral organs (15).

Sterol composition in bile is different from controls in sitosterolemia. Not only is less cholesterol secreted into the bile, but sitosterol appears in the same or lower proportion relative to cholesterol in bile as compared in plasma (1, 2, 5, 6, 25). Normally the liver preferentially secretes sitosterol into bile so there is a 3-fold enrichment of sitosterol relative to cholesterol as compared to blood in control subjects (26). Biliary bile acids include cholic acid, deoxycholic acid, and lesser amounts of chenodeoxycholic acid and are secreted into bile in amounts adequate to prevent steatorrhea (1, 2, 4, 5, 27). No unusual biliary bile acids were detected, although it has not been established whether sitosterol and other plant sterols can be converted to primary bile acids in homozygotes. Recently, Bhattacharyya et al. (25) reported radioactive bile acids derived from [^{14}C]sitosterol in the feces of three sitosterolemic subjects, but the precise indentification of these compounds was not carried out (28, 29). However, it was noted that the large quantities of sitosterol and cholestanol in sitosterolemic liver competitively inhibited cholesterol 7α-hydroxylase, the rate-determining enzyme for bile acid synthesis, which may eventually lead to decreased bile acid production and deficient pool size (27). Thus, sitosterolemic liver has lost both the capacity to recognize sitosterol and the ability to preferentially secrete the 24-ethyl sterol into the bile. In addition, cholesterol secretion into bile is markedly diminished. Also, since biliary cholesterol secretion (lithogenicity) relative to bile acids and phospholipids is decreased (4), gallstones have not been detected in sitosterolemic subjects. (G. Salen, unpublished observation).

Monocyte (mononuclear leukocytes) sterol composition has been measured in four sitosterolemic homozygotes (30). The sterols and stanols are similar in composition (sitosterol, campesterol, cholestanol, 5α-campestanol, and 5α-sitostanol) as found in LDL indicating that the plant sterols and stanols originate from plasma. However, total sterol concentrations in monocytes from the sitosterolemic homozygotes were 2 to 3-times larger than in control monocytes. Thus, monocytes, which are precursors to foam cells, contain increased quantities of cholesterol, plant sterols, and 5α-stanols that may contribute to the accelerated atherosclerotic process in this disease.

Sitosterol metabolism

It has long been known that sitosterol is poorly absorbed from the intestine (26, 31). The low plasma concentrations found in animals and humans fed large amounts of dietary plant sterols attest to restricted intesti-

nal absorption (26). However, early sitosterol balance studies, where fecal outputs were measured and subtracted from dietary inputs, gave confusing high values (between 30 to 50% of intake) for absorption (26). To overcome potential errors in the balance technique, sitosterol absorption has been measured by two independent isotopic methods. The isotope kinetic method estimates absorption by mathematical analysis of specific activity decay curves after intravenous pulse-labeling with a tracer dose of radioactive sitosterol (26, 32–34). In normal and hyperlipidemic subjects, the plasma specific activity decay of sitosterol is much more rapid than the specific activity decay of cholesterol when both isotopic sterols were injected intravenously (**Fig. 5**) (5, 23–26). These decay curves can be divided into two exponentials and analyzed mathematically according to the two-pool model. **Table 2** lists values for controls and three homozygous sitosterolemic subjects from two unrelated families. Since sitosterol is not synthesized endogenously in normal humans and sitosterolemic subjects (5, 26), the production rate is equivalent to absorption and in the control subjects amounted less than 10 mg per day or about 5% of daily intake. Mean total sitosterol body pool size was also calculated and amounted to about 130 mg in controls. In contrast, sitosterol turnover in the three homozygous sitosterolemic subjects was much slower as compared to controls. Sitosterol production rates were 5 to 10 times larger than the control mean, confirming the enhanced absorption found in homozygotes with this disease. Total body pools were also tremendously enlarged and ranged from 3500 to 9500 mg. Although absorption was increased in these subjects, the extraordinary body pool size did not relate linearly to absorption (23, 24). The discrepancy could be explained by the fact that the elimination contant from pool A (K_A) was 2 to 10 times more rapid in control subjects than in sitosterolemic homozygotes. Thus, sitosterolemic homozygotes hyperabsorb sitosterol from the intestine but also retain the plant sterol in body tissues (5, 23, 24). This finding of very slow sitosterol turnover associated with increased absorption and very delayed removal has recently been noted in three additional sitosterolemic subjects from two unrelated families (25) who received radioactive sitosterol intravenously. Body pool sizes were extraordinarily expanded as noted previously. Sitosterol turnover has also been studied in two sitosterolemic heterozygotes (parents of homozygotes). The results show that sitosterol absorption was increased 2 to 3 times over controls but body pool sizes were not increased because sitosterol removal was rapid (24). Thus, heterozygotes still retain the ability to excrete sitosterol normally.

Enhanced sitosterol absorption in homozygotes and heterozygotes has been confirmed independently by absorption measurements obtained by adapting the plasma dual-isotope ratio method used to study cholesterol absorption (**Table 3**) (23, 24). In this technique, [^{14}C]sitosterol is fed and [^{3}H]sitosterol is administered intravenously at the same time. The $^{3}H/^{14}C$ ratio is then determined in plasma sitosterol and compared to the ideal

TABLE 2. Sitosterol turnover

	Controls (n-5)	Sitosterolemic Homozygotes*		
		CL	KCN	KEC
$t_{½A}$ (days)	3.8 ± 0.2	7.0	2.7	11
$t_{½B}$ (days)	13.8 ± 2.4	91	24	112
K_A (day^{-1})	0.17 ± 0.04	0.015	0.067	0.014
M_A (mg)	80 ± 36	5100	2400	1700
M_B (mg)	46 ± 22	4400	2400	1800
$M_A + M_B$ (mg)	126 ± 32	9500	4800	3500
PR_A (mg/day)	7.9 ± 2.3	80	162	52

Results from references 5, 23, 26.
*Patients KCN and KEC are sisters and unrelated to CL.

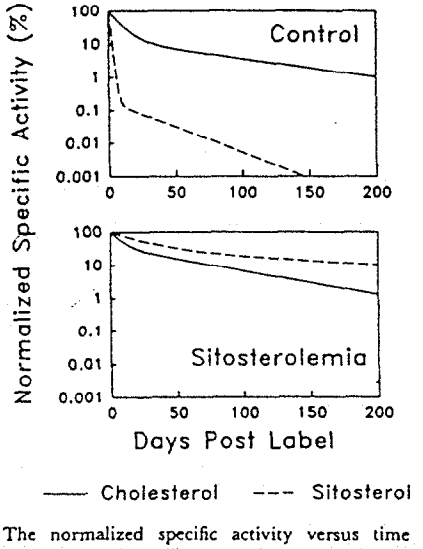


Fig. 5. The normalized specific activity versus time curves for cholesterol and sitosterol are illustrated for a control and homozygous sitosterolemic subjects. In the control subject, sitosterol decays more rapidly than cholesterol as contrasted with slower decay of sitosterol than cholesterol in the homozygote.

TABLE 3. Sitosterol and cholesterol absorption*

	Controls		Sitosterolemic Homozygotes	
	1	2	KCN	KEC
	%		%	
Sitosterol absorption	4	5	63	28
Cholesterol absorption	44	48	49	69

*Plasma dual-isotope ratio method, data from reference 23.

ratio which is calculated by dividing the total oral dose by total injected dose and is equivalent to 100% absorption (23). In two homozygotes, 28% and 63% of dietary sitosterol were absorbed which is in good agreement with the absorption values calculated by the independent isotope kinetic method (Table 2). Two healthy control subjects who consumed the same diet absorbed 4% and 5% of dietary sitosterol, respectively, while two obligate heterozygotes absorbed 15% and 17% of dietary sitosterol, respectively. Thus, sitosterol absorption is enhanced in homozygotes.

Cholesterol absorption and turnover

Cholesterol absorption as measured by the plasma dual-isotope ratio method tended to be at the high end of the normal range (49% and 69% of intake) in sitosterolemic homozygotes as reported in Table 3 (23). Thus, increased sitosterol absorption does not interfere with cholesterol absorption in sitosterolemic homozygotes although it is believed that sitosterol and cholesterol share the same intestinal absorption pathway. Also, it is important to realize that cholesterol is absorbed about 10 times more efficiently than sitosterol in healthy control subjects, but that percent sitosterol absorption approaches cholesterol absorption in some sitosterolemic homozygotes (Table 3) (23). There was no difference in cholesterol absorption between controls and heterozygotes (24). Although it has been suggested that the limited absorption of sitosterol compared to cholesterol in normal subjects may relate to greater affinity of sitosterol for intestinal bile acid micelles (35), diminished intestinal sitosterol esterification (36), and reduced sitosterol enterocyte transport (37), the upregulation of these mechanisms seems unlikely to explain the increased absorption of sitosterol in sitosterolemic homozygotes.

An important, new biochemical finding observed in the sitosterolemic subjects is reduced cholesterol turnover (Table 4) (4, 5, 23). Not only is the plasma specific activity decay of cholesterol much slower in homozygotes than control subjects, but turnover (PR_A = synthesis plus absorbed cholesterol) is markedly reduced. Calculations of cholesterol turnover by the isotope kinetic method revealed values 50–70% smaller in homozygotes than similarly fed controls (5, 23–25). Moreover, since cholesterol absorption tended to be large in sitosterolemic subjects, the diminished daily production must result from decreased cholesterol synthesis. When turnover values were corrected by subtracting absorbed cholesterol, average cholesterol synthesis was about 50% lower in sitosterolemic homozygotes than in healthy controls (23–25). In support, Miettinen (4) found cholesterol synthesis as measured by the sterol balance technique 50% and 80% lower in a homozygous sitosterolemic subject than in similarly fed control subjects when studied on two occasions 4 years apart. In contrast, cholesterol turnover and synthesis in sitosterolemic heterozygotes resembled control subjects and was not decreased (24).

TABLE 4. Cholesterol turnover[a]

	Controls (n = 4)	Sitosterolemic Homozygotes		
		KL	KCN	KEC
$t_{½A}$, days	6.7 ± 0.6	8.6	2.4	5.0
$t_{½B}$, days	53 ± 6	81	24	74
K_A, day^{-1}	0.045 ± 0.006	0.023	0.077	0.024
M_A, g	29 ± 8	31	11	21
M_B, g	48 ± 16	34	13	41
$M_A + M_B$, g	77 ± 9	65	24	65
PR_A, mg/day	1450 ± 560	670	860	710
Synthesis, mg/kg/day	14.6 ± 6.0[c]	[b]	9.5[c]	5.9[c]

[a]Data from references 4, 5, 23.
[b]Not available.
[c]Synthesis estimated by subtracting absorbed cholesterol (Table 3) from turnover (PR_A).

Mechanism of reduced cholesterol synthesis

A major discovery from balance and isotopic turnover studies was that cholesterol synthesis in sitosterolemic homozygotes was extremely low (Table 4) (4, 5, 23, 25). In order to better understand this observation, HMG-CoA reductase, the rate-controlling enzyme for cholesterol biosynthesis, was measured in liver microsomes from two sitosterolemic homozygotes (38). For comparison, liver specimens were obtained from 11 liver transplant donors whose livers became available when appropriate recipients could not be located. In the control livers (Table 5), mean HMG-CoA reductase activity was 5.3 and 8.2 times greater, respectively, than the values from the two sitosterolemic liver specimens. About 72% of the HMG-CoA reductase was expressed (active) in the sitosterolemic livers compared to 49% in the controls.

HMG-CoA reductase protein concentrations were determined in these same microsomal specimens by immunoblotting and densitometric scanning (Table 5). In the control liver microsomes, the mean relative mass of HMG-CoA reductase per mg of microsomal protein was 6.8 and 8.9 times larger, respectively, than the values for the two sitosterolemic livers. Thus, markedly reduced HMG-CoA reductase activity and enzyme protein characterize sitosterolemic liver. However, when the catalytic efficiency of HMG-CoA reductase (activity per unit protein) was calculated by dividing the enzyme specific activity by the enzyme mass, no difference was detected between control and sitosterolemic livers. This suggests that although reduced quantities of HMG-CoA reductase are produced by the sitosterolemic livers, catalytic function of the enzyme is normal. To further explore the severe deficiency of HMG-CoA reductase, poly A$^+$ RNA was isolated from liver specimens obtained from two control and one homozygous sitosterolemic subjects and hybridized

TABLE 5. Hepatic microsomal HMG-CoA reductase activity and mass*

Subjects	Activity	HMG-CoA Reductase Mass	Catalytic Efficiency
	pmol/mg/min	*peak area/mg*	*pmol/min/peak area*
Controls (n = 11)	98.1 ± 28.8	1.4 ± 0.14	68.6
Sitosterolemic homozygotes			
KCN	11.9	0.16	74.3
TC	18.4	0.21	76.2

*From reference 38.

with pRED 227 and pHRED 102, which are full-length sequence cDNA probes for hamster and human HMG-CoA reductase, respectively, and pCAT 10, a probe for human catalase mRNA. The Northern blots (Fig. 6, A and B) showed virtually no signals for sitosterolemic HMG-CoA reductase mRNA, as contrasted with signals from the HMG-CoA reductase mRNA from the control specimens. Both control and sitosterolemic specimens gave signals for catalase mRNA that indicated that the RNA isolated from control and sitosterolemic livers was intact. Thus, the deficiency of microsomal HMG-CoA reductase in sitosterolemic livers can be attributed to the very low levels of HMG-CoA reductase mRNA that are available for enzyme translation (38).

LDL receptor binding was also measured in twelve control and two sitosterolemic liver membrane preparations. Total binding (assayed in the absence of unlabeled LDL) was 54% and 80% higher, respectively, in the two sitosterolemic liver membrane preparations than the mean for the control measurements (Table 6). Similarly, high affinity, receptor-mediated LDL binding recorded as the difference between total binding and nonspecific binding (assayed in the presence of abundant unlabeled LDL) was 2.2 and 3.3 times greater, respectively, in the sitosterolemic than in the control livers. Therefore, sitosterolemic livers express increased numbers of LDL receptors, so that a much higher proportion of LDL was receptor-bound and more circulating LDL was taken up than by control liver membranes (38).

In a separate experiment, Biel et al. (39) measured in vivo LDL turnover and found greater production associated with rapid catabolism consistent with the expression of more LDL receptors in a sitosterolemia subject as compared with three matched controls.

In two sitosterolemic liver specimens, lipofuscin-like pigment was distributed in the liver cytosol (38). The nature of this pigment has not been determined at this time (Fig. 7).

Treatment

Bile acid malabsorption produced by either binding resins (cholestyramine or colestipol) or ileal bypass surgery is an effective treatment of sitosterolemia (2, 4, 7, 11, 19). Plasma cholesterol concentrations decline dramatically (decrease 25% to 50%) and the percent reduction in plasma sterol concentrations obtained with these drugs or surgery is greater than similarly treated hypercholesterolemic subjects. In most sitosterolemic patients, plant sterols usually decrease proportionally to cholesterol, and

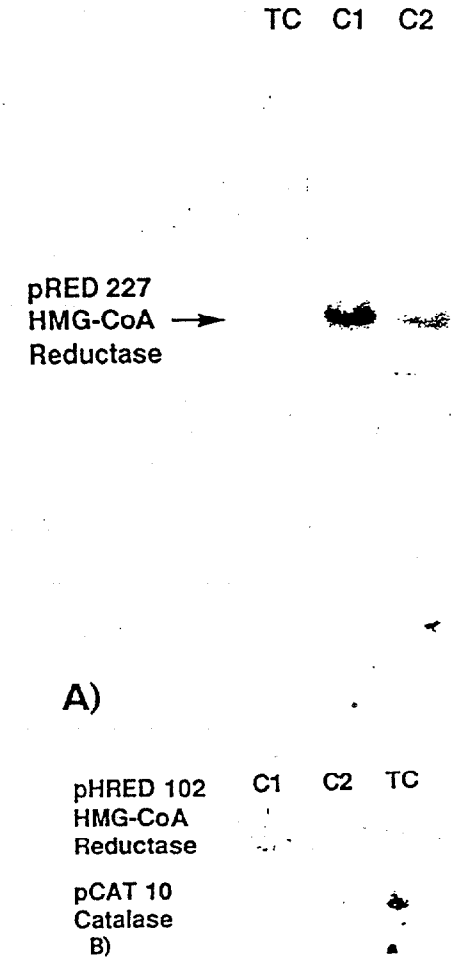


Fig. 6. Northern blot analysis of sitosterolemic hepatic mRNA. Northern blots of liver poly A+ RNA from a sitosterolemic homozygote (TC) and two control subjects that were probed with pRED 227 (A) and pHRED 102 for HMG-CoA reductase mRNA and pCAT 10 for catalase mRNA (B). Virtually no signal from sitosterolemic HMG-CoA reductase mRNA was detected (38).

TABLE 6. Hepatic LDL receptor binding*

Subjects	Total	Receptor-Mediated
	ng/mg protein	
Control, n = 12	204.0 ± 10.0	95 ± 8.2
Sitosterolemic homozygotes		
TC	315.3	193.2
KCN	336.8	312.8

*From reference 38.

cholestanol and 5α-saturated stanols were virtually eliminated from the blood (19). However, it is important to realize that not all patients respond similarly, and cholestyramine treatment produced less reduction of plasma plant sterols than cholesterol in a Japanese sitosterolemic family (12). Of note, clinical improvement including disappearance of xanthomas, elimination of aortic stenosis murmur, and decreased frequency in angina pectoris and arthritic attacks have been noted in several subjects treated with either cholestyramine or ileal bypass surgery (7, 11, 20).

Lovastatin, a competitive inhibitor of cholesterol biosynthesis that is widely used in the treatment of hypercholesterolemia has been tried but has not been an effective treatment in sitosterolemia. Plasma cholesterol, plant sterols, or 5α-saturated stanols were not reduced in two homozygous sitosterolemic subjects (20, 22).

The effect of the various treatments can be explained by examining cholesterol biosynthesis and LDL receptor function in freshly isolated peripheral mononuclear leukocytes (monocytes). These cells synthesize cholesterol and express HMG-CoA reductase activity and LDL receptors in parallel to the liver. In five homozygous sitosterolemic subjects from three unrelated families, mononuclear leukocyte cholesterol synthesis as measured by the conversion of acetate to cholesterol was 30–70% below the mean value from 16 healthy control subjects (20, 22). Subnormal monocyte cholesterol synthesis in the sitosterolemic subjects was supported by measurements of HMG-CoA reductase activity which were 50–70% lower in the homozygotes than the control mean. In contrast, LDL receptor function in monocytes from four of the five homozygous patients was increased 60% over the control mean. Thus, sitosterolemic mononuclear leukocytes manifest the same defect in cholesterol biosynthesis as the liver and compensate by the increased expression of LDL receptors.

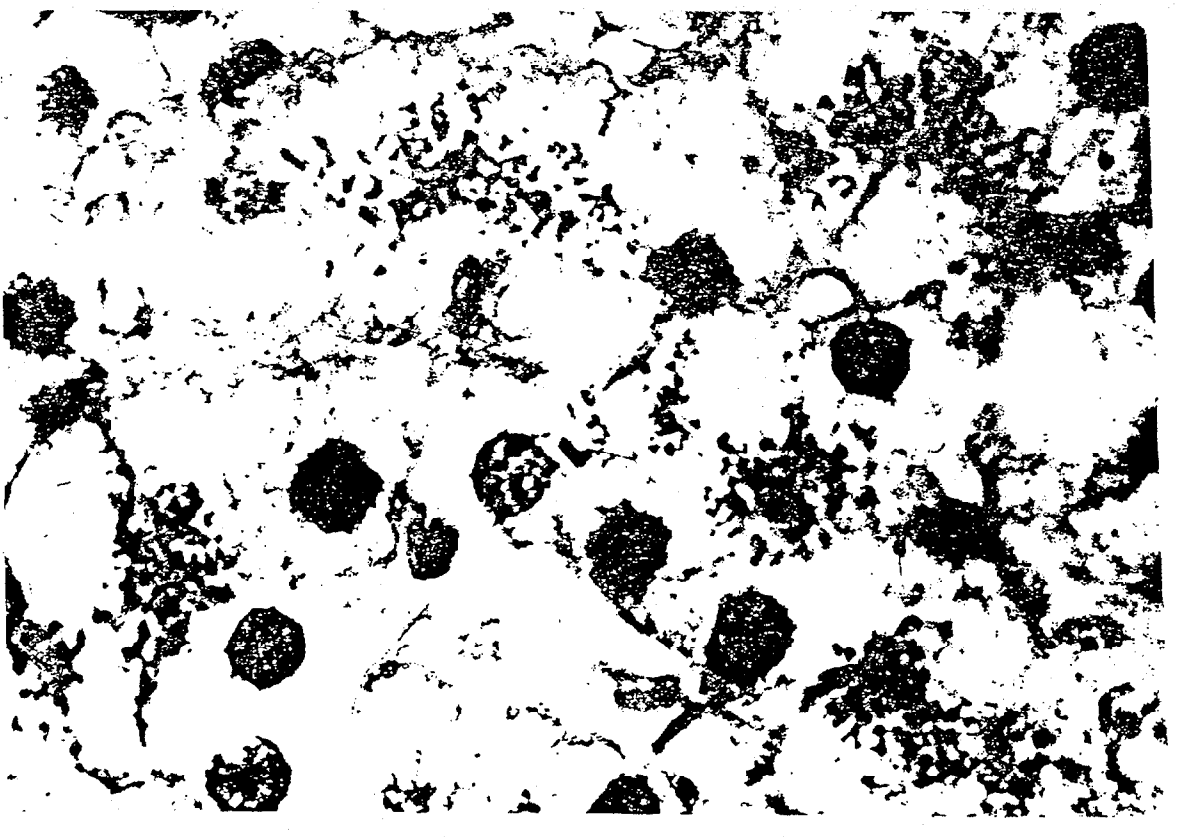


Fig. 7. Light microscopy of liver from sitosterolemic homozygote that shows lipofuscin-like granules deposited randomly in cytosol of hepatocyte. Hemotoxylin and eosin × 250 (38).

When the enterohepatic circulation of bile acids is interrupted, reducing the hepatic bile acid flux, HMG-CoA reductase activity increased 13% and LDL receptor function rose 40% in freshly isolated monocytes from healthy control subjects (20). In contrast, monocytes from four homozygous sitosterolemic subjects (from three unrelated families) failed to up-regulate either cholesterol synthesis (conversion of acetate to cholesterol) or HMG-CoA reductase activity when treated similarly (20, 22). In fact, HMG-CoA reductase activity paradoxically declined. Monocyte LDL receptor function responded normally to bile acid malabsorption by increasing between 20% and 30% in the sitosterolemic mononuclear cells (20, 22, 40, 41).

Lovastatin treatment produced no change in plasma sterol concentrations in two unrelated sitosterolemic homozygous subjects and caused only a small rise in monocyte HMG-CoA reductase activity compared with a 28% reduction in plasma sterol concentrations and a 38% increase in monocyte HMG-CoA reductase activity in control and hypercholesterolemic heterozygous subjects (20, 22). Although lovastatin competitively inhibits mevalonic acid synthesis, HMG-CoA reductase activity normally increases. Apparently, the block in cholesterol production produces gene expression for the synthesis of HMG-CoA reductase. LDL receptor function also did not change in the homozygous sitosterolemic monocytes as compared to a 41% increase in receptor-mediated LDL binding in control cells from subjects treated with lovastatin (20, 22).

These results indicate a major abnormality in cholesterol homeostasis in sitosterolemic subjects. Depressed cholesterol biosynthesis is due to a pronounced deficiency of the rate-controlling enzyme, HMG-CoA reductase, caused by virtual absence of HMG-CoA reductase mRNA. Interruption of the enterohepatic circulation of bile acids reduces the hepatic bile acid flux and, normally stimulates bile acid synthesis (42), but fails to increase cholesterol production in sitosterolemic homozygotes. Cholesterol 7α-hydroxylase activity (rate-controlling for bile acid synthesis) rises in response to bile acid malabsorption (27) so that more bile acids are formed (4). Cholesterol biosynthesis (HMG-CoA reductase activity) should increase and more LDL receptors expressed to provide additional cholesterol as substrate for bile acid synthesis. In normal subjects, the decrease in plasma cholesterol reflects the balance between input of new cholesterol (synthesis) and the removal and catabolism of LDL. Because sitosterolemic subjects cannot up-regulate HMG-CoA reductase, the demand for more substrate for bile acid synthesis can only be met by the catabolism of LDL. Thus, there is a greater than expected fall in plasma sterol concentrations (cholesterol and plant sterols) in sitosterolemic subjects.

Lovastatin, which normally lowers plasma cholesterol by competitively inhibiting HMG-CoA reductase activity and in turn stimulates expression of LDL receptors, was ineffective treatment for sitosterolemia (20, 22). Apparently, sitosterolemic cholesterol synthesis is so low that further inhibition of HMG-CoA reductase does not increase LDL receptor function. With this in mind, hypercholesterolemic patients who do not respond to therapeutic doses of lovastatin should have their plasma sterols tested by gas–liquid chromatography as the failure to respond to lovastatin may indicate sitosterolemia (20, 22).

Inherited abnormality

At the present time, the principal inherited defect has not been established with certainty. However, three abnormal mechanisms, hyperabsorption of sitosterol, decreased sitosterol elimination, and reduced cholesterol synthesis, have been linked to the pathogenesis of sitosterolemia and predisposition to atherosclerosis. The hyperabsorption of sitosterol and other dietary sterols from the intestine is well documented, but by itself will not cause the enormous sitosterol pools in this disease. Sitosterolemic heterozygotes also hyperabsorb sitosterol but do not accumulate the plant sterol (24). Not until the intestinal pathway for cholesterol absorption is elucidated will the mechanism for sitosterol hyperabsorption be understood.

To date, all sitosterolemic homozygotes show diminished hepatic secretion of sitosterol and cholesterol. Bile contains reduced amounts of both sitosterol and cholesterol, and biliary sterol excretion is further decreased when dietary intake is restricted (25). Clearly, the combination of decreased removal with increased absorption accounts for the gigantic sitosterol and other plant sterol pools.

A third key feature of the disease is abnormal regulation of cholesterol biosynthesis. We have demonstrated that reduced cholesterol synthesis results from a deficiency of HMG-CoA reductase in the liver and mononuclear cells of sitosterolemic subjects (38, 43), and that HMG-CoA reductase mRNA is barely detected in the liver. LDL receptor function is enhanced in most sitosterolemic homoyzgotes to provide cellular sterols. Attempts to stimulate cholesterol synthesis (HMG-CoA reductase) by inducing bile acid malabsorption (cholestyramine or ileal bypass surgery) or a low sterol diet did not increase HMG-CoA reductase activity in freshly isolated mononuclear cells (20, 22). Thus, the up-regulation of cholesterol synthesis is prevented in sitosterolemia.

Moreover, it is important to emphasize that HMG-CoA reductase activity and the expression of LDL receptors are normally regulated in the same direction. In other words, factors stimulating HMG-CoA reductase increase the number of LDL receptors (41, 43). In contrast, sitosterolemic mononuclear cells and liver show diminished HMG-CoA reductase activity and enzyme mass in combination with increased LDL receptor expression (20, 22, 38, 43). These observations lead us to believe that the

inherited defect in sitosterolemia involves an abnormality of the HMG-CoA reductase gene.

However, at this time it is still not possible to establish which mechanism is primary. It still is possible that enhanced sitosterol absorption and accumulation are primary events. Therefore, low cholesterol synthesis and enhanced receptor function may conceivably relate to the accumulated sterols and stanols and/or an oxygenated derivative. However, Boberg, Åkerlund, and Björkhem (44) have reported that sitosterol is not an effective feedback inhibitor of HMG-CoA reductase, and Shefer et al. (45) noted that cholestanol feeding actually increases HMG-CoA reductase activity in rat liver. Thus, neither cholestanol nor sitosterol are down-regulators of cholesterol biosynthesis.

Summary

Sitosterolemia is a rare inherited lipid storage disease characterized chemically by the accumulation of plant sterols and 5α-saturated stanols in plasma and tissues. Very low cholesterol synthesis due to a deficiency of HMG-CoA reductase associated with increased intestinal plant sterol absorption and slow hepatic sterol removal are major biochemical features. Because cholesterol synthesis cannot up-regulate, bile acid malabsorption mobilizes body sterols for bile acid synthesis and dramatically lowers plasma and monocyte sterol concentrations and may halt the progression of the atherosclerotic process.

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Increased plasma cholestanol and 5α-saturated plant sterol derivatives in subjects with sitosterolemia and xanthomatosis

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Abstract We have measured plasma sterol composition in 14 subjects with sitosterolemia and xanthomatosis. In addition to elevated plasma phytosterol (campesterol 16 ± 7 mg/dl and sitosterol 35 ± 16 mg/dl) and normal to moderately high cholesterol levels (258 ± 96 mg/dl), concentrations of 5α-saturated stanols, cholestanol, 5α-campestanol, and 5α-sitostanol were at least 10 times greater than controls. Diets contained plentiful quantities of cholesterol and plant sterols, but only trace amounts of cholestanol (<2 mg/day) and no detectable 5α-campestanol and 5α-sitostanol, which indicated that the 5α-saturated stanols were formed endogenously. Treatment with cholestyramine reduced plasma cholesterol and phytosterol levels by 45% and 5α-saturated stanols by 55%. These results indicate that abnormally high plasma concentrations of cholestanol, 5α-campestanol, and 5α-sitostanol are found in subjects with sitosterolemia and xanthomatosis, and that treatment with cholestyramine effectively reduced elevated plasma sterol levels. —**Salen, G., P. O. Kwiterovich, Jr., S. Shefer, G. S. Tint, I. Horak, V. Shore, B. Dayal, and E. Horak.** Increased plasma cholestanol and 5α-saturated plant sterol derivatives in subjects with sitosterolemia and xanthomatosis. *J. Lipid Res.* 1985. **26:** 203–209.

Supplementary key words 5α-campestanol • 5α-sitostanol • cholestyramine

Sitosterolemia with xanthomatosis is a rare inherited lipid storage disease that was described first in 1974 by Bhattacharyya and Connor (1). The major clinical manifestations include tendon and tuberous xanthomas that involve the Achilles tendons, extensor tendons of the hand and the skin of the elbows and knees, recurrent arthritis and arthralgias of the knees and ankle joints, and premature atherosclerosis. Chemically, increased amounts of plant sterols, such as campesterol and sitosterol, (**Fig. 1**) are present in the plasma, erythrocytes, and xanthomas, while plasma cholesterol levels are normal to slightly elevated (2). Hyperapobetalipoproteinemia is often present (3). Although plant sterols are present in abundant amounts in most American diets, only small quantities of sitosterol (less than 1 mg/dl) can be detected in normal plasma (4). Limited intestinal absorption combined with enhanced biliary excretion presumably keeps plasma plant sterol concentrations low in normal subjects (4). In contrast, intestinal absorption of sitosterol in patients with sitosterolemia and xanthomatosis was from 5 to 10 times greater than in controls and was associated with reduced clearance of sitosterol and cholesterol from the plasma (1, 5, 6). Thus two factors, increased absorption coupled with reduced removal, apparently lead to the enhanced tissue deposits of plant sterols and cholesterol in this disease. However, not all of the biochemical defects are known.

Recently, uncertainty has arisen concerning plasma sterol composition in patients with sitosterolemia with xanthomatosis. In the original description, Bhattacharyya and Connor (1) found only cholesterol and unsaturated sterols, campesterol, stigmasterol, and sitosterol in the plasma of two subjects. Further, only Δ^5-unsaturated sterols were detected in the plasma of the patient reported by Shulman, et al. (7) and the five patients recently studied by Kwiterovich et al. (3). However, other investigators (Whitington et al. (8), Khachadurian and Salen (9), and Wang et al. (10)) have discovered that subjects with typical clinical and chemical features of sitosterolemia and xanthomatosis (tendon xanthomas, arthritis, premature atherosclerosis, increased plasma cholesterol

Abbreviations: GLC, gas-liquid chromatography; RRT, relative retention time; LDL, low density lipoproteins; HDL, high density lipoproteins.

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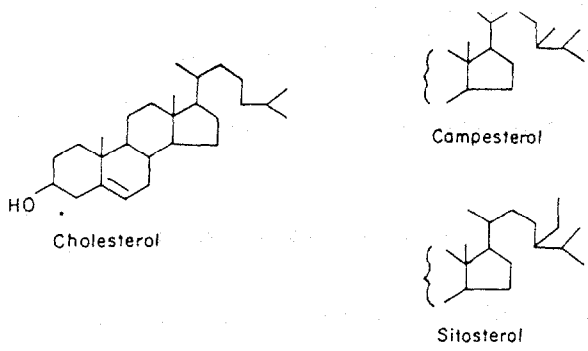

Fig. 1 Structures of major unsaturated sterols present in sitosterolemic plasma.

and plant sterol concentrations) have, in addition, markedly elevated plasma levels of cholestanol, 5α-campestanol, and 5α-sitostanol (**Fig. 2**), the respective 5α-saturated derivatives of cholesterol, campesterol, and sitosterol.

Therefore, to determine whether increased 5α-saturated stanols are a constant biochemical abnormality in this disease, plasma sterols were measured by gas-liquid chromatography in fourteen affected subjects. It has been previously reported that no cholestanol or 5α-saturated plant sterol derivatives were detected in the plasma of eight of these subjects although previous analytic methods may not have been sufficiently sensitive (1, 5, 7).

In addition, the effect of cholestyramine, a resin that binds bile acids and promotes their excretion in feces, was evaluated on plasma sterol concentrations in four subjects. Cholestyramine treatment enhances the conversion of cholesterol to bile acids in liver and often diminishes plasma cholesterol levels (11). Our results indicate that increased amounts of cholestanol, 5α-campestanol, and 5α-sitostanol were present in the plasma of *all* subjects with sitosterolemia and xanthomatosis. Cholestyramine treatment reduced elevated plasma sterol concentrations in this disease.

METHODS

Subjects

Studies were conducted in fourteen subjects (**Table 1**) in whom a diagnosis of sitosterolemia and xanthomatosis was established by clinical and biochemical criteria. Complete clinical and biochemical descriptions have been presented elsewhere (2). There were five male and nine female subjects. Three subjects (R. C., C. L., and L. B.) have died of acute myocardial infarctions. At postmortem, R. C. had extensive coronary and thoracic aorta atherosclerosis.

All subjects ate regular food. Blood was collected into tubes containing solid EDTA after an overnight fast of at least 12 hr. No subject was taking medication except during the cholestyramine treatment period. Plasma sterol concentrations were determined in ten normolipidemic individuals of the same age range and served as control values.

Chemical

Plasma sterol concentrations were measured by gas-liquid chromatography (GLC) according to the method of Ishikawa et al. (12). Briefly, plasma (1 ml) was saponified in 1 N NaOH for 1 hr. The neutral sterols were extracted with hexane and the solvent was evaporated. Neutral sterols (approximately 100 μg) were redissolved in 200 μl of hexane containing 140 μg of 5α-cholestane as an internal standard, and 3 μl was analyzed by GLC. The underivatized free sterols were separated on 180 cm × 4 mm glass columns packed with 1% SP-1000 on 80/100 mesh Gas Chrom Q (Supelco Inc., Bellefonte, PA) without prior purification by argentation thin-layer chromatography. A Hewlett Packard, Model 5830, gas chromatograph equipped with a flame ionization detector was operated at the following conditions: column temperature 230°C, flame detector 260°C, flash heater 250°C, N_2 flow 30 cc/min. The retention times (RRT) relative to 5α-cholestane for ten consecutive determinations were: cholestanol 6.3 ± 0.1, cholesterol 7.1 ± 0.1, 5α-campestanol 8.3 ± 0.1, campesterol 9.2 ± 0.1, 5α-sitostanol 10.2 ± 0.1, and sitosterol 11.3 ± 0.2. Continuous use of the SP-1000 column at these conditions is associated with shortening of the sterol retention times and periodic monitoring with reference standards was necessary. The identity of the sterols was checked by co-chromatography with authentic reference sterols and was confirmed by mass spectroscopy according to Dayal et al. (13). The mass of each sterol was corrected for analytic losses by recovery of known quantities of reference standards. A typical chromatogram that illustrates the separation of the sterols on SP-1000 is given in **Fig. 3**.

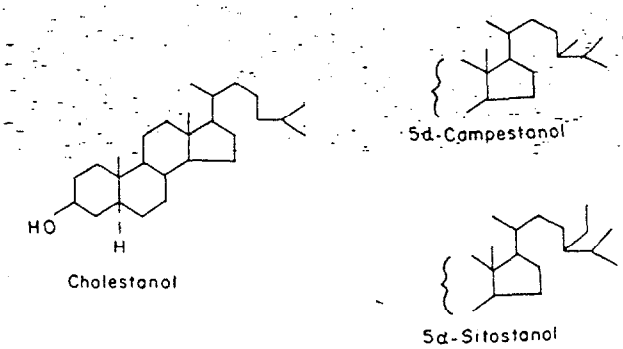

Fig. 2 Structures of 5α-saturated stanols present in plasma of sitosterolemic subjects.

TABLE 1. Plasma sterol concentration

Patient	Age	Sex	Cholesterol	Cholestanol	Campesterol	Campestanol	Sitosterol[b]	Sitostanol
	yr				*mg/dl*			
Ki. C. (n = 10)[a]	24	F	245 ± 39	6.7 ± 1.1	12 ± 1.1	2.3 ± 0.4	20 ± 2.3	4.2 ± 1.1
Ke. C. (n = 10)	18	F	202 ± 25	4.7 ± 1.0	8 ± 3.1	1.4 ± 0.2	14 ± 4.1	2.2 ± 0.7
T. C. (n = 10)	22	F	233 ± 12	3.8 ± 1.4	10 ± 5.1	1.9 ± 1.0	21 ± 8.3	5.4 ± 2.5
R. C. (n = 10)	16	M	249 ± 39	7.5 ± 2.4	13 ± 1.5	2.6 ± 0.9	20 ± 5.5	3.9 ± 1.1
C. L.	52	M	134	1.6	13	1.5	27	3.0
M. M.	7	M	202	1.2	12	1.6	26	3.1
L. H. O.	32	F	207	2.5	10	1.9	28	2.0
R. H.	30	F	368	3.6	29	9.0	65	8.0
P. M.	41	F	169	1.2	18	7.0	42	4.0
P. Z.	22	M	324	3.9	27	3.0	60	6.0
M. Z.	20	M	271	3.8	19	2.0	42	4.0
R. S.	32	F	256	3.1	15	1.0	29	3.0
L. B.	24	F	482	11	24	4.0	56	6.0
J. B.	38	F	336	4.9	20	1.2	45	3.2
Mean ± SD			258 ± 96	4.2 ± 2.7	16 ± 7	2.9 ± 2.3	35 ± 16	4.1 ± 1.7
% of Total sterols			80.5	1.3	5.0	0.9	11	1.3
% of Respective Δ^5-derivative				1.6		18		13
Control (10)			187 ± 29	0.4 ± 0.2			0.3 ± 0.3	

[a]Ten consecutive monthly determinations; average of two measurements given for remaining subjects.
[b]Small quantities of stigmasterol (24-ethyl-5,22-cholestadien-3β-ol) accompanied campesterol and sitosterol but only amounted to 1–3% of the sitosterol mass.

In separate experiments, the Δ^5-sterols, cholesterol, campesterol, and sitosterol were separated from their 5α-dihydro derivatives cholestanol, 5α-campestanol, and 5α-sitostanol by argentation thin-layer chromatography, and were then quantitated as their trimethylsilyl ether derivatives by gas–liquid chromatography on 180 cm × 4 mm glass columns packed with 3% QF-1 (Applied Science Lab, State College, PA). The retention times relative to 5α-cholestane of the trimethylsilyl ether derivatives are: cholesterol 1.73, cholestanol 1.85, campesterol 2.52, 5α-campestanol 2.66, sitosterol 3.03, and 5α-sitostanol 3.17. It is necessary to separate the unsaturated sterols from their 5α-saturated derivatives by argentation thin-layer chromatography because only small differences exist between the unsaturated and 5α-saturated sterol peak retention times on QF-1 (2). However, quantitative results by the two independent methods agreed within ± 10%.

Lipoproteins were separated by the method of Havel, Eder, and Bragdon (14). After separation of the low density and high density lipoprotein fractions, the proportions of free and esterified sterols were measured in each fraction (11).

RESULTS

Plasma concentrations of campesterol and sitosterol (Table 1) were markedly elevated in all fourteen clinically affected subjects, consistent with previous findings that this is the major biochemical determinant in establishing the diagnosis of this condition (1–10). Plasma cholesterol levels were elevated in seven of fourteen subjects, which was a finding of importance. Approximately 80% of the unsaturated sterols were cholesterol and 16% were plant sterols. Of the remaining plasma sterols, cholestanol concentrations were increased in all subjects and the mean

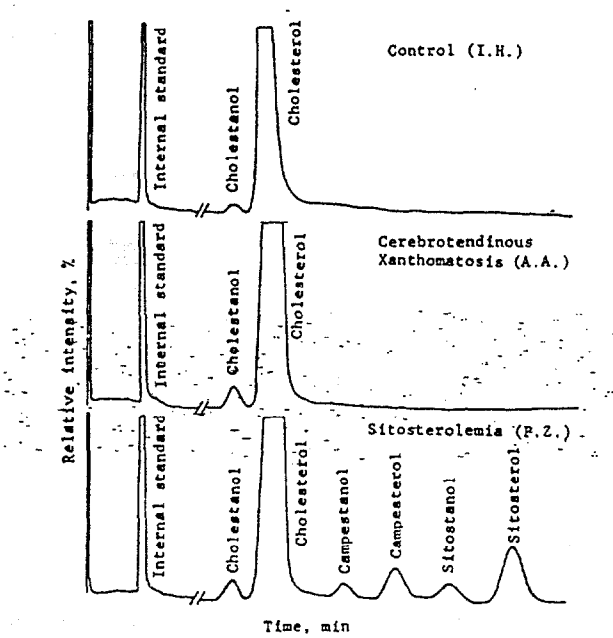

Fig. 3 Gas–liquid chromatograms of underivatized sterols present in plasma of control, cerebrotendinous xanthomatosis, and sitosterolemic patients that illustrate the separation of the Δ^5-unsaturated sterols from their 5α-saturated analogs on SP 1000 column.

value was 10-fold higher than the mean for plasma cholestanol determined in ten normolipidemic subjects. Similarly, high concentrations of 5α-campestanol and 5α-sitostanol were present in all subjects. These 5α-saturated stanols were not found in the plasma of the normolipidemic controls. Interestingly, cholestanol, although increased, amounted to only 1.6% of the plasma cholesterol level, while 5α-campestanol and 5α-sitostanol represented 18% and 13% of their respective unsaturated derivatives.

In three affected and two control subjects, plasma lipoproteins were fractionated by sequential ultracentrifugation and the sterol composition was determined. The results are given for low density lipoproteins (LDL) in Table 2 and for high density lipoproteins (HDL) in Table 3, along with the proportions of esterified sterols. In the control LDL, only small amounts of cholestanol (0.4%) were found. Plant sterols or their saturated analogs were not detected. In contrast, substantial amounts of campesterol, sitosterol, and the 5α-saturated stanols, cholestanol, 5α-campestanol, and 5α-sitostanol were present in sitosterolemic LDL. Of interest, the proportion of esterified plant sterols and 5α-saturated stanols was lower than for cholesterol in this fraction (Table 2). In sitosterolemic HDL, cholesterol concentrations were lower while cholestanol levels were similar to controls. Both campesterol and sitosterol were present, but only trace quantities of their 5α-saturated analogs were detected. The proportion of sterol esters was comparable to controls. Thus, increased amounts of plant sterols and 5α-saturated stanols are carried mainly unesterified in LDL and are a constant biochemical abnormality in patients with sitosterolemia. High density lipoproteins transport proportionally less plant sterols and 5α-saturated stanols than LDL in these subjects.

The average daily intake of dietary sterols and calories consumed by members of the C-family are listed in Table 4. The cholesterol and plant sterol intakes were determined by GLC on food that was set aside, mixed together with water, and homogenized; aliquots were analyzed for sterol composition. Cholesterol and the plant sterols were present in plentiful amounts since no attempt was made to restrict the diet during this period of observation. However, cholestanol constituted only about 0.5% of the dietary cholesterol consumed while 5α-saturated plant sterol analogs could not be detected. Thus, only trace quantities of cholestanol and no 5α-saturated plant sterol derivatives were found in the diet of this family.

In separate experiments, the effect of cholestyramine was evaluated on plasma sterol concentrations in four sitosterolemic subjects and the results are given in Table 5. Prior to treatment, plasma sterol concentrations were typical for these subjects with elevated levels of cholesterol, campesterol, sitosterol, and their respective 5α-saturated analogs cholestanol, 5α-campestanol, and 5α-sitostanol. After treatment with up to 12 g/day of cholestyramine for at least 1 month, plasma sterol concentrations declined markedly in all subjects; cholesterol and phytosterol levels decreased 45%, while 5α-saturated sterols decreased 55%. In two subjects (Ki. C. and Ke. C), plasma sterol concentrations rose substantially 1 month after cholestyramine was discontinued.

DISCUSSION

The results of these studies confirm and extend our knowledge regarding the biochemical defects in the lipid disorder sitosterolemia with xanthomatosis. All symptomatic subjects had increased campesterol and sitosterol concentrations associated with normal to moderately elevated plasma cholesterol levels, which are expected in this disease (1). In addition, plasma concentrations of the 5α-saturated stanols, cholestanol, 5α-campestanol, and 5α-sitostanol, which are the respective 5α-dihydro analogs of cholesterol, campesterol, and sitosterol (Fig. 1), were also in high concentration and were present in eight subjects where previously these stanols were not detected (1, 3, 7). However, the earlier measurements of plasma sterols in these subjects were made by GLC on columns (1% SE 30) that would not separate 5α-saturated stanols from their respective unsaturated sterol derivatives, and thus these sterols would be missed.

TABLE 2. Low density lipoproteins sterol composition

Patient	Cholesterol	Cholestanol	Campesterol	Campestanol	Sitosterol	Sitostanol
			mg/dl (% ester)			
Ke. C.	120(71)	7.1(54)	12.0(10)	2.0(10)	16(14)	4.0(11)
R. C.	220(70)	7.0(13)	12.0(40)	1.9(11)	21(46)	3.2(9)
M. M.	90(71)	3.5(51)	16.0(26)	1.6(13)	32(20)	3.5(17)
Mean	143(71)	5.9(39)	13.0(25)	1.8(11)	23(27)	3.6(12)
Control (n = 2)	76(70)	0.3(66)				

TABLE 3. High density lipoproteins sterol composition

Patient	Cholesterol	Cholestanol	Campesterol	5α-Campestanol	Sitosterol	5α-Sitostanol
			mg/dl (% ester)			
Ke. C.	45(82)	0.9(62)	0.2		3.3(88)	
R. C.	26(76)	0.7(63)	0.6		1.9	
M. M.	37(79)	0.6(62)	0.3		6.3(83)	
Mean	36(79)	0.7(62)	0.4		3.5(85)	
Control (n = 2)	49(84)	0.5(60)				

The absolute identification of the 5α-saturated stanols was based upon co-chromatographic comparison with known reference standards that were prepared chemically from their respective unsaturated derivatives by a hydroboration-protonolysis sequence as described by Dayal et al. (13). The structures of the sterols also were confirmed by mass spectroscopy (13, 15). Thus, elevated plasma levels of cholestanol, 5α-campestanol, and 5α-sitostanol are additional biochemical abnormalities in this rare lipid storage disease and these 5α-stanols along with phytosterols are deposited in virtually every tissue in the same proportion as plasma.

It was noted that, like cholesterol and unsaturated plant sterols, the 5α-saturated stanols were transported mainly by low density lipoproteins. Further, plant sterols and 5α-saturated stanols in sitosterolemic low density lipoproteins were less esterified than cholesterol, which suggests that hepatic acylcoenzyme A:cholesterol acyltransferase and plasma lechithin:cholesterol acyltransferase may be sterol-specific. In contrast, sitosterolemic high density lipoproteins transported less cholesterol with only slightly increased amounts of cholestanol and plant sterols. However, the relative proportion of cholesterol and cholestanol esters was similar to control. These results suggest that less sterol may be transported by high density lipoproteins.

In regard to the mechanism of sterol accumulation in this condition, it is well-established that enhanced intestinal absorption of the unsaturated plant sterols (campesterol and sitosterol) occurs and, perhaps in combination with reduced removal, leads to their retention in the body (1, 5, 7). Since most diets include a plentiful supply of vegetables that contain these sterols, the availability for absorption is widespread. Indeed, analysis of the diet of the C-family (Table 4) showed that almost 200 mg of phytosterols and 400 mg of cholesterol per 2000/calories were consumed each day. Thus, these individuals (C-family) ate food rich in cholesterol and plant sterols that were similar in quantity to that found in most American diets. Importantly, only about 2 mg of cholestanol and trace quantities of the saturated plant sterol derivatives, 5α-campestanol and 5α-sitostanol, were present in the daily diet. Furthermore, in previous studies, we have determined that normal individuals synthesize about 12 mg of cholestanol each day (16). Therefore, the majority of the cholestanol and virtually all of 5α-campestanol and 5α-sitostanol that are found in these subjects probably were produced endogenously.

It is now established that cholestanol is formed from cholesterol via the ketonic intermediate, 4-cholesten-3-one (17, 18), and that liver microsomes contain the 3β-hydroxy $\Delta^{4,5}$ steroid dehydrogenase that catalyzes this reaction. In contrast, skin fibroblasts from both normal subjects and those with cerebrotendinous xanthomatosis when grown in sterol-deficient media do not produce cholestanol, although cholesterol biosynthesis is normal (19). Thus, the liver appears to be essential for the formation of cholestanol. It is very likely that 5α-campestanol and 5α-sitostanol are derived from campesterol and sitosterol via an analogous pathway.

Treatment with cholestyramine reduced saturated and unsaturated plasma sterol concentrations in all treated subjects. Plasma concentrations of unsaturated sterols declined about 44%, while the corresponding 5α-saturated stanols diminished about 57%. Thus, treatment produced a greater effect on saturated sterol levels. Cholestyramine acts by promoting the intestinal loss of bile acids, thereby increasing hepatic bile acid formation from cholesterol (11). As a result, more plasma cholesterol is utilized for bile acid synthesis and plasma levels decline.

TABLE 4. Diet sterol composition

Substance	Amount/Day
Diet	
Calories	2000
Carbohydrate	225 g
Protein	75 g
Fat	90 g
Sterols	
Cholesterol	400 mg
Campesterol	65 mg
Stigmasterol	5 mg
Sitosterol	130 mg
Cholestanol	2 mg
5α-Campestanol	not detected
5α-Sitostanol	not detected

TABLE 5. Effect of cholestyramine on plasma sterol concentrations

Patient	Treatment (Duration)	Cholesterol	Cholestanol	Campesterol	5α-Campestanol	Sitosterol	5α-Sitostanol
				mg/dl			
Ki. C.	None	363	4.0	22	6	44	5
	Cholestyramine (1 month)	192	1.6	13	4	28	1
	None	270	2.2	19	5	36	4
Ke. C.	None	250	2.5	20	5	38	3
	Cholestyramine (1 month)	172	1.0	9	1.4	25	2
	None	234	2.2	18	4.4	34	4
L. H. O.	None	207	2.5	10	2	28	2
	Cholestyramine (12 months)	126	0.5	6.1	0.7	20	1.7
R. H.	None	368	3.6	29	9	65	8
	Cholestyramine (12 months)	125	0.8	8	2	24	2.0

Further, because of the enhanced fecal losses, bile acid concentrations in the upper intestine may fall sufficiently to interfere with intestinal sterol absorption. Both mechanisms may play a role in reducing the plasma concentrations of cholesterol, plant sterols, and 5α-stanols. In addition, the activation of cholesterol 7α-hydroxylase, the rate-controlling enzyme in bile acid synthesis which occurs with cholestyramine treatment, may increase the formation of 7α-hydroxylated sterols committed for bile acid synthesis, thereby reducing the intrahepatic pool of sterols available for conversion into 5α-stanols.

It is of considerable interest that subjects with both sitosterolemia and xanthomatosis and cerebrotendinous xanthomatosis exhibit common features: xanthomatosis, premature atherosclerosis, and high plasma cholestanol levels (2). However, in sitosterolemia, increased plant sterols and 5α-saturated stanols are found, no neurologic dysfunction has been noted, and cholestyramine treatment lowered plasma sterol levels, including cholestanol, markedly. In contrast, during cholestyramine treatment, plasma cholestanol levels in cerebrotendinous xanthomatosis increased 4-fold. Clearly, these two conditions in which plasma cholestanol is high are different.

In summary, increased plasma cholestanol and 5α-saturated plant stanol derivatives, 5α-campestanol and 5α-sitostanol, were found in the plasma of all fourteen subjects with sitosterolemia and xanthomatosis. Since diet contained little cholestanol and virtually no saturated stanols, absorption of these compounds probably was not responsible for their plasma accumulation. It is proposed that increased endogenous synthesis from the corresponding unsaturated sterols, cholesterol, campesterol, and sitosterol occurs. Treatment with cholestyramine reduced plasma sterol concentrations. The importance of this abnormality is not known. Whether its significance is limited to a biochemical marker of this disease or whether 5α-stanols contribute to the development of the accelerated atherosclerosis which is the major clinical feature remains to be determined.

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Clinical and Laboratory Pearl

Sitosterolemia

Comparative Effect of Dietary Sitosterol on Plasma Sterols and Cholesterol and Bile Acid Synthesis in a Sitosterolemic Homozygote and Heterozygote Subject

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Key words: sitosterolemia, dietary sitosterol, steroid output

Objective: Sitosterolemia is a genetic disorder characterized by an increased plasma plant sterol concentration due to enhanced sterol absorption coupled with reduced steroid excretion. The purpose of the present investigation was two-fold; first to assess the effects of a "basal" low sitosterol metabolic diet on plasma sterols and sterol balance, and, secondly, to quantify the relative influence of graduated increases in dietary sitosterol intake on a metabolic diet in a sitosterolemic homozygote, obligate heterozygote, and controls.

Methods: Patients were studied under strict metabolic conditions and fed a "basal" 30% fat, low-sitosterol (33 mg per 2000 kcal) diet. The level of dietary sitosterol was increased by addition of oils and resulted in final dietary sitosterol intakes of 1.8 mg/kg, 2.6 mg/kg and 3.5 mg/kg/day intakes of dietary sitosterol in the homozygote. These sitosterol dosages were selected based on sitosterol intakes equivalent to 2.6 mg/kg/day in the average American diet. Plasma cholesterol, sitosterol, and apolipoprotein A were measured, and stool collections assayed for sterol balance.

Results: Fecal sterol excretion and cholesterol synthesis were depressed markedly by 50% in the homozygote compared to the heterozygous parent, whereas plasma sitosterol levels were increased over 50-fold. When the sitosterol content of the diet was increased three-fold and dietary cholesterol was maintained in the homozygous and hypercholesterolemic control, plasma sterol levels did not increase in the homozygote. Plasma cholesterol and sitosterol levels were unaffected in the hypercholesterolemic control.

Conclusions: Plasma sterol levels remained elevated with the dietary sitosterol changes in the sitosterolemic homozygote. These findings were associated with a low fecal sterol excretion rate and depressed endogenous cholesterol synthesis. In this sitosterolemic patient, a very low sitosterol diet to curtail sterol input was of minimal therapeutic benefit. These results have important implications regarding the selection of therapy for this patient under these experimental conditions, but cannot be generalized to other homozygotes.

INTRODUCTION

Sitosterolemia is a recessively inherited disorder characterized biochemically by increased plasma levels of plant sterol and 5-α-stanol and clinically by xanthomatous lesions, aortic stenosis and premature coronary heart disease (CHD) [1–7]. The hallmark of this extremely rare disease is elevated plasma levels of sitosterol, the major dietary plant sterol. Studies suggest that an important biochemical defect in sitosterolemia is indiscriminate, and thus increased intestinal absorption of sterol (plant, animal and shellfish) is coupled with reduced hepatic sterol excretion [1–3,8–10]. Hepatic plant sterol levels in sitosterolemic patients are increased 10 to 100-fold greater then controls, while HMG-CoA reductase MRNA was barely detected in the liver of a sitosterolemic homozygote [10–11]. Low levels of HMG-CoA reductase protein has been proposed as a primary inherited molecular defect in sitosterolemia [11]. However, other investigators [9,10] maintain that sterol hyperabsorption coupled with depressed hepatic sitosterol excretion accounts for the sitosterol accumulation in these patients. As the American diet contains 185 mg sitosterol per day, a rigorous low-sterol diet to curtail input, combined with bile acid

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resins to enhance sterol excretion, are current treatment recommendations for this disorder [5].

Studies of dietary management of sitosterolemia have involved predominantly sterol-free, or sitosterol-free formula diets fed for short periods of time. Though effective in lowering sterol levels, in some subjects, these dietary regimens are difficult to follow due to severe restriction of common dietary fats [1,5]. Belamarich et al [12] demonstrated rapid reductions in plasma sterol of up to 60% in one 11-year-old sitosterolemic boy following therapy with low fat, low-cholesterol and sitosterol diet. However, not all sitosterolemics are as responsive to dietary-induced reductions in plasma sterol [10,13]. This heterogeneity in responsiveness could be due to the additive effects of a reduction in dietary fat, cholesterol or plant sterol producing cumulative decreases in plasma sterol levels. The influence of dietary sitosterol intakes alone on plasma sterol levels, independent of dietary cholesterol has not been previously studied in a sitosterolemic homozygote under metabolic conditions.

Therefore, we studied under inpatient, metabolically-controlled conditions, first, the comparative influence of a 30% fat, low sitosterol, natural food diet on: a) plasma sterol levels and distribution, and apolipoprotein B (apo B) levels in a sitosterolemic homozygote, obligate heterozygote (patient's father), and an unrelated hypercholesterolemic control; and, b) sterol balance and urinary mevalonate excretion in this same homozygous patient and heterozygous parent. Secondly, we investigated the relative effects of tripling dietary sitosterol intake on these plasma sterol parameters on a metabolic diet in our homozygote and respective hypercholesterolemic control patient.

MATERIALS AND METHODS

Table 1 shows the clinical characteristics and ad libitum lipoprotein profiles for the homozygote patient, obligate heterozygote and hypercholesterolemic control, measured on their *ad libitum* diets.

Sitosterolemic Pedigree

The index case was a 9 year-old white female whose case history has been previously reported [13,14]. Previously reported blood group studies confirmed parentage of the patient [14]. The homozygous patient's father ("sitosterolemic heterozygote"), age 53 years, volunteered as the obligate heterozygote for the "basal" low sterol diet phase and participant in the sterol balance portion of the study. The father, presented with a history of Type IIb hypercholesterolemia, vascular disease and a lipoprotein profile as shown in Table 1.

Control Subjects

A 43-year-old nun, with mild Type IIa hypercholesterolemia ("hypercholesterolemic control"), served as the control for assessing the impact of increasing dietary sitosterol intakes on plasma sterol parameters (Table 1). Ten hypercholesterolemic control subjects, (ranging from age 14 to 58 years; weight 47 to 58 kg) served as unrelated hypercholesterolemic controls for the fecal balance comparisons. They were maintained at the Rockefeller University Metabolic Unit and fed a strictly-controlled 40% fat, liquid-formula, low sterol diet as studied by the senior investigator [15]. All study patients were inpatients on the metabolic unit throughout the study. Twenty-three unrelated hyperlipidemic subjects, studied at the Oregon Health Science Center, Department of Medicine, Portland, OR, served as dietary controls for assessment of urinary mevalonate excretion.

Study Design

These studies were conducted under strict, inpatient, metabolic diet treatments at The Rockefeller University Hospital. The identical "basal" 30% fat diet was fed through all diet periods to the sitosterolemic patient. Body weight was maintained within less then ±1 kg during all periods. The metabolic diet consisted of natural foods, was prepared in the Clinical

Table 1. Patient Characteristics and *Ad libitum* Lipid and Lipoprotein Distribution of Sitosterol Homozygote, Sitosterolemic Heterozygote, and Hypercholesterolemic Control*†

Patient†	Age, Year	Wt, kg	Plasma cholesterol (mg/dl)‡	Plasma sitosterol (mg/dl)‡	Ad libitum profile, mg/dl†				
					Total lipids		Sterol distribution		
					Total-sterol	TG	VLDL	LDL	HDL
Sitosterolemic homozygote (index case)	10	35	303±71	37±8	348±14	69±9	27±16	264±4	56±7
Sitosterolemic heterozygote (case's father)	53	110	280±14	0.7±0.1	286±20	208±55	47±9	208±17	30±4
Hypercholesterolemic control (unrelated control)	43	64	264±5	0.2±0.4	259±16	86±7	39±20	160±9	61±4

* Lipid profile represents MEAN±SD of at least three determinations determined enzymatically [23–25].

† Abbreviations: Wt=weight, TG=triglycerides, VLDL=very low density lipoprotein, LDL=low density lipoprotein, HDL=high density lipoprotein.

‡ Plasma cholesterol and sitosterol determined by gas liquid chromatography [20,21].

Research Kitchen, weighed out to 0.1 g, and protocol compliance monitored daily on the Unit. During the final week of each diet period, 3 to 4 blood samples were drawn and samples averaged for determination of plasma sterol levels, distribution and Apo B concentrations. Equilibrium was reached based on a consistency of plasma sterol levels. Informed consent was obtained from all participants, after review by the Rockefeller University Institutional Review Board.

Diets

Ad Libitum Diets. By diet history, the homozygote was consuming *ad libitum*, a low-fat (30% Kcal) and low-sitosterol (<50 mg/day) diet [16]. The heterozygous father and hypercholesterolemic patients ate a 38% fat diet containing 400 to 500 mg dietary cholesterol and 220 mg sitosterol per day. The experimental period consisted of two phases, I and II.

Basal Metabolic Diet Phase. During Phase I (Table 2), an initial "basal" metabolic diet, consisting of 30% fat, 53% carbohydrate and 17% protein [16], tailored after the homozygote's home diet, was fed to our case patient, the heterozygote and hypercholesterolemic control to equalize baseline dietary and treatment conditions for 28 days. An aliquot of the metabolic diet composition was homogenized and analyzed for "actual" nutrient composition by an independent laboratory (Industrial Chemical Analysis Laboratory, 50 Madison Avenue, NY, NY). The "actual" diet consisted of 29.7% fat, 51.9% carbohydrate, 18.4% protein, and contained 223 mg cholesterol/2000 kcal; it was deemed similar to the calculated diet. This "basal" metabolic diet, contained 26 mg sitosterol, 5 mg campesterol, and 2 mg "minor plant sterol," (i.e., 24-methylcholestanol + stigmasterol + 24-ethylcholestanol) per 2000 kcal and was fed for an additional 56 consecutive days (Phase II) during oil supplementation. During this phase, the basal diet was supplemented with oils. Caloric intakes were 2000, 3300 and 2200 kcals/day, respectively, for the homozygote, heterozygote and hypercholesterolemic control throughout the study.

Sitosterol Supplemented Phase. During Phase II (Table 2), we investigated the effects of increasing dietary sitosterol on plasma sterol levels by feeding the "basal" diet with graduated increases in dietary sitosterol, added isocalorically as dietary oils. Soybean and sesame oils were selected as a respective low- and high-sitosterol oil, with similar P:S ratios. An isocaloric substitution of oil was made, maintaining the total fat and dietary saturated fat content constant. The basal diet was supplemented first with soybean oil only, then a mixture of soybean and sesame oil (1:1 ratio), followed by sesame oil only, for 21, 14, and 21 days, respectively. The combined "basal" dietary sitosterol and added oils resulted in final dietary sitosterol intakes of 57, 84 and 110 mg per 2000 kcal, respectively, which corresponded to 1.8 mg/kg, 2.6 mg/kg and 3.5 mg/kg/day intakes of dietary sitosterol in the homozygote. (These dosages were selected based on sitosterol intakes of 185 mg/day in a typical American diet [5], which is equivalent to 2.6 mg/kg/day.

Soybean and sesame oil were analyzed for fatty acid composition and plant sterol content as described in Laboratory Analysis. The soybean fatty acid composition (% total measured fatty acids) was 18% saturated (5% stearate), 19% monounsaturated, and 63% polyunsaturated fatty acids. Further, the soybean oil contained, by analysis, 80 mg, 26 mg and 16 mg per 100 ml of sitosterol, campesterol, and "minor plant sterol," respectively. The sesame oil was comprised of 15% saturated (6% stearate), 40% monounsaturated, and 45% polyunsaturated fatty acids. The sesame oil contained 260 mg sitosterol (over 3-fold higher compared with soybean), with 70 mg and 43 mg

Table 2. Basal and Sitosterol-Supplemented Natural, Metabolic Diets*

Dietary Period	Basal diet (Phase I)	Sitosterol-supplemented diet (Phase II)		
	"Basal"	Low*	Intermediate	High*
	28 days	21 days	14 days	21 days
Total dietary fat, % total kcals	30%	30%	30%	30%
Fatty acid composition, % total fat				
Saturated fatty acids	40%	37	37	37
Monounsaturated fatty acids	45%	42	43	44
Polyunsaturated fatty acids	15%	21	20	19
P:S ratio	0.5	0.6	0.5	0.5
Carbohydrate, % total kcal	53%	53	53	53
Protein, % total kcal	17%	17	17	17
Total sterol, mg/2000 kcal	256	292	325	356
Dietary cholesterol, mg/2000 kcal	223	223	223	223
Total plant sterol,† mg/2000 kcal	33	69	102	133
Dietary sitosterol, mg/2000 kcal	26	57	84	110

* Olive oil isocalorically replaced by soybean and sesame oil see materials and methods section.

† Total plant sterol content is equal to Σ sitosterol+campesterol+stigmasterol+24-methyl-cholestanol+24-ethyl-cholestanol.

per 100 ml of campesterol and "minor plant sterol," respectively.

Fecal Sterol Balance

Four-day stool collections were collected daily on the metabolic unit at the end of the 4-week basal diet period, weighed, homogenized with an equal volume of water and frozen at −70°C as described by Grundy and Ahrens [17]. Chromic oxide was used as an internal marker of fecal steroid excretion with corrections made for day-day variations in fecal flow as previously validated at the Rockefeller University Metabolic Unit [18]. Neutral and acidic steroids were calculated and corrected; cholesterol balance was calculated as the difference between daily intake of cholesterol and total daily excretion of neutral and acidic steroids [9,17,18]. Balances are negative on a low cholesterol diet due to continuing endogenous cholesterol synthesis. A comparison was made between sterol balance data collected on the Rockefeller University Metabolic Unit derived from the homozygote, her heterozygous father, and ten hypercholesterolemic control subjects fed a 40% fat, metabolic low-sterol diet [18].

Urinary Mevalonate Excretion

Urinary mevalonate acid concentrations were assayed by the radio-enzymatic method of Popjak et al [19] with an intra-assay coefficient of <5%. Urinary mevalonic acid excretion as determined in three 24-hour urine specimens under metabolic conditions on the metabolic unit in the homozygote, her heterozygous father and compared to normocholesterolemic controls (n=23). Completeness of urine collection was determined by measuring 24-hour urinary creatinine clearance and restriction of subjects to the unit.

Laboratory Analysis

Blood Sample Collections. Antecubital blood samples were collected after a 12-hour fast, and plasma separated at 4°C by centrifugation at 2500 rpm for 20 minutes.

Dietary and Plasma Plant Sterol Levels. The dietary sterol distribution from aliquots of diet homogenate (1 g) or the plasma sterol distribution (1 ml) were determined by capillary gas liquid chromatography (GLC) [20,21]. After saponification, neutral sterol were extracted, dried and trimethylsilylated as previously described. Aliquots were redissolved along with 70 μg 5α-cholestane and 50 μg of coprostanol as internal standards, and injected into a Hewlett-Packard, Model 5890 gas chromatograph equipped with a capillary column (CPWAX 57 DB) (Chrompak, Bridgewater, NJ).

Diet Nutrient Composition and Oil Fatty Acid Composition. An aliquot of the diet was homogenized and analyzed by standard methods for fat, carbohydrate and protein content [22]. The soybean and sesame oils were analyzed by GLC for fatty acid composition following a diethylether extraction [22].

Plasma Sterol Lipoprotein Distribution, Apo B Levels and Apolipoprotein E Phenotyping. The total sterol concentration (cholesterol combined with plant sterols) was assayed enzymatically utilizing reagents from Boehringer Mannheim Biochemicals, Indianapolis, IN, as described previously [23,24]. The HDL-sterol concentration was quantified following a dextran-sulfate-magnesium precipitation. The LDL- and very low-density lipoprotein (VLDL-sterol) sterol concentrations were determined by difference as previously reported [23,24]. Frozen aliquots of plasma were assayed for apo B levels by sandwich enzyme-linked immunosorbent assay [25]. Total-cholesterol, HDL-cholesterol and apo B levels were standardized by the Lipid Standardization Program of the Centers for Disease Control, Atlanta, GA.

Fecal Neutral and Acidic Steroid Analysis. The fecal homogenate was thawed, an aliquot lyophilized and analyzed for fecal neutral and acidic steroids by the methods previously described [17,18]. One g of lyophilized feces was saponified in 1 N ethanolic NaOH and the neutral sterol extracted with hexane. The water layer from the feces was acidified and bile acids extracted with ethyl ether. The trimethylsilyl ether derivatives of the bile acid methyl esters and neutral sterol were injected on CP-Sil-5 CB and CP-Wax-57 CB fused silica capillary columns (Chrompack Inc., Bridgewater, NJ) and retention times compared with standards. Neutral and acidic sterols were measured and corrected for fecal losses and flow using markers; cholesterol synthesis was calculated.

Statistical Analysis

Descriptive statistics and a one-way analysis of variance with repeat measures was calculated using Biomedical Computer Programs (BMDP). A Tukey post-hoc procedure [26], using the 5% significance level, was used to test for differences among the diet-drug periods on plasma sterol parameters. Comparisons are made with respective hyper- and normocholesterolemic control subjects for sterol balance and urinary mevalonate excretion.

RESULTS

Ad Libitum Lipid Profiles

Table 1 shows the patient characteristics and ad libitum profile of the sitosterolemic homozygote, obligate heterozygote, and hypercholesterolemic control. Compared with the heterozygote and hypercholesterolemic control, the homozygote maintained markedly increased plasma sitosterol levels and LDL sterol concentrations during the ad libitum period.

"Basal" Metabolic Diet Without Oil Supplementation

Plasma Sterol Parameters. Table 3 presents the effects of the "basal" metabolic diet without oil supplementation on plasma sterol parameters in the homozygote, heterozygote and hypercholesterolemic control during Phase I. In the homozygous patient, as expected, plasma sterol levels on the *ad libitum* diet (Table 1) and the "basal" metabolic diet (Table 3) were similar, as the metabolic diet was tailored after the case patient's home diet to maximize dietary compliance. On the metabolically controlled diet, the plasma plant sterol concentration, mainly as sitosterol and campesterol but including minor plant sterol, remained markedly elevated compared with the father and controls (Table 3), with sitosterol accounting for 11% of total plasma sterol. In addition, the metabolic products of these sterols, i.e., sitostanol and campestanol, were increased in the homozygote relative to the heterozygote and control.

Plasma cholesterol levels were increased beyond the 99th percentile rank in the homozygous patient compared with age-sex matched standards [27] on the metabolic "basal" diet during Phase I. The plasma sterol (plant sterol plus cholesterol) were distributed predominantly in the LDL and HDL subfractions. Plasma apo B levels and enhanced ratios of LDL-sterol/HDL and LDL-sterol/apo B in our case remained elevated compared with *ad libitum* concentrations (Table 1) in both the heterozygote and control subject fed the same diet.

On the "basal" diet, the sitosterolemic heterozygote father, meanwhile, maintained negligible levels of plasma sitosterol (<1% of total sterol), and, an elevated plasma cholesterol level based on standards [27] and compared with the hypercholesterolemic control subject fed the same diet (Table 3). The plasma cholesterol, constituting >99% of total sterol in the heterozygote, was distributed mainly within the LDL subfraction. Apo B concentrations and the LDL/HDL-C ratio remained elevated on the "basal" diet compared with *ad libitum*. The hypercholesterolemic control, on the "basal" diet, also exhibited negligible sitosterol levels, but showed normalized levels of plasma cholesterol, LDL-C, compared with *ad libitum* diet based on age-sex matched standards [27].

Sterol Balance, acidic sterol and neutral sterol excretion. Table 4 shows the effect of the basal metabolic diet on acidic, neutral and total steroid output and sterol balance in the homozygote and heterozygote father. Sitosterol balance (dietary sitosterol—fecal sitosterol) served as an internal measure of sterol recovery. Sitosterol recovery was 90% and 98%, respectively, and chromium oxide recovery 97% and 98%, respectively, in the case and heterozygote subject. It is important to emphasize that a metabolic steady state was achieved in the present study as evidenced by the fact that sitosterol output approximated sitosterol input in both the homozygote and heterozygote subjects with a constant weight. The apparent retention in the homozygous patient is small on a very low sitosterol diet, but may contribute to the enlarged sitosterol pool [21].

No unusual bile acids, bacterial metabolites or bile alcohols were detected in the pooled stool specimens from the homozygous patient or her heterozygous father. Nearly equal proportions of the primary bile acids and their secondary products were excreted by both subjects (data not presented). The homozygous patient excreted less fecal bile acids and cholesterol, in mg/kg/day, by 52% and 64%, respectively, compared with the heterozygous control. Thus, total steroid (neutral + acidic) excretion was depressed by 59% in the homozygote as compared with her heterozygous father fed the same basal metabolic diet. The net cholesterol output (balance) was greater then dietary input due to endogenous production of cholesterol in both homozygote and heterozygote. The homozygote patient showed a net cholesterol balance of 107 mg/day, compared with 1342 mg/day and 817 mg/day in the heterozygous subject and hypercholesterolemic controls, respectively. However, the homozygous patient excreted nearly 75% less cholesterol, on a mg/kg/day basis, compared with the heterozygous subject (3.1 vs. 12.2 mg/kg/day); and hyperlipidemic controls (13.5 mg/kg/day). The difference is attributed to both diminished fecal cholesterol output and bile acid excretion in the homozygote compared to her father and unrelated hyperlipidemic controls.

Table 3. Effects of Basal Metabolic Diet on Plasma Sterols, Distribution and Ratios*†

Subject	Plasma sterol distribution, mg/dl					Lipid, lipoprotein distribution and apo B levels, mg/dl						
	Cholesterol	Sitosterol	Campesterol	Sitostanol	Campestanol	Cholestanol	Total-sterol	TG	VLDL-sterol	LDL-sterol	HDL-sterol	Apo B
Sitosterolemic homozygote	290±30	38±4	18±14	5±1	3±1	4±1	353±10	83±1	30±4	270±11	54±2	168±9
Sitosterolemic heterozygote	249±13	0.5±0.4	–	–	–	0.2±0.1	251±8	155±8	45±11	180±6	25±2	157±2
Hypercholesterolemic control	200±6	0.3±0.1	–	–	–	0.3±0.2	209±15	92±7	27±6	122±14	60±2	115±19

* Values represent mean±SD of at least three determinations obtained during final week of diet period.
† abbreviations: as per Table 1.

Table 4. Effects of Low Sterol Metabolic Diet on Neutral and Acidic Fecal Sterol Excretion and Sterol Balance*†

Subject		Fecal sterols					Sterol balance	
		Acidic‡	Neutral			Total steroids¶	Cholesterol	Sitosterol
			Total§	Cholesterol	Sitosterol			
Sitosterolemic homozygote	mg/day	47	175	149	26	222	−107	3
	mg/kg/day	1.3	5.0	4.3	0.7	6.3	−3.1	0.1
Sitosterolemic heterozygote	mg/day	298	1342	1300	42	1640	−1342	<1
	mg/kg/day	2.7	12.2	11.8	0.4	14.9	−12.2	<0.1
Hyperlipidemic controls‖ (n=10)	mg/day (mean±SD)	154±179	781±59	656±17	125±42	1055±205	−817±33	71±44
	mg/kg/day (mean±SD)	4.3±2.3	13.1±1.4	11.0±1.3	2.1±0.6	17.4±2.2	−13.5±5.2	1.2±0.7

* Abbreviations as per Table 1 and not measurable is denoted by − sign.

† Values represent mean of at least 3 determinations obtained during basal diet (Table 2).

‡ Total neutral sterols = fecal (cholesterol + sitosterol)

§ Total acidic sterols = fecal (colic acid + chenodeoxycholic acid + metabolites);

¶ Total steroid excreted = fecal (Σ total neutral + total acidic sterols).

‖ Hyperlipidemic patients fed a low sterol, formula diet [17,18].

Urinary Mevalonate Excretion. Urinary mevalonate excretion, another parameter of endogenous cholesterol synthesis, supported the sterol balance data. By this separate measure of total body cholesterol synthesis, urinary mevalonate excretion was depressed by 43% in the homozygote compared to both the heterozygote (13.2 vs. 23.0 umols/kg/day), or unrelated normocholesterolemic subjects (13.2 vs. 23.0 ± 2).

"Basal Diet" Supplemented With Dietary Sitosterol

Table 5 shows the influence of increasing dietary intakes of sitosterol on plasma sterol levels, apo B concentrations and distribution in the homozygote and hypercholesterolemic control. In the homozygote patient, plasma sitosterol levels were not significantly affected through all dietary sitosterol intakes. No qualitative changes in the other plant sterol (i.e., campesterol, stigmasterol, or sterol metabolites) or lipoprotein distribution were found (data not presented). The effect of the low and intermediate levels of dietary sitosterol on plasma cholesterol levels, sterol lipoprotein distribution and ratios was similar in the homozygote. Intermediate intakes of dietary sitosterol reduced apo B levels by 24% compared with low sitosterol intakes without significantly affecting plasma cholesterol, LDL-sterol concentrations or ratio in the homozygote. However, the highest dietary intakes of sitosterol resulted in modest reductions in plasma cholesterol by 10% ($p < 0.05$), reducing both the LDL- and HDL-sterol content by 9% and 13%, respectively, in the sitosterolemic homozygote. With the decrease in LDL-sterol, apo B levels remained reduced by 25%, ($p < 0.05$) compared with the lowest dietary sitosterol intakes but unchanged compared with the intermediate level.

In the hypercholesterolemic control patient fed the same diet, the effect of graduated increases in dietary sitosterol through the three dietary periods did not significantly alter existing negligible plasma sitosterol levels. Plasma cholesterol levels, the subfraction lipoprotein distribution, apo B concentrations, and LDL/HDL-C and LDL/apo B ratios were all largely unaffected in the control.

DISCUSSION

The results of the present investigation demonstrated extremely depressed endogenous cholesterol synthesis (3.1 mg/kg/day) and elevated plasma cholesterol and sitosterol levels in a sitosterolemic homozygote in response to a metabolically-strictly controlled, low sitosterol diet. A graduated 3-fold increase in sitosterol intake corresponded with modest reductions in plasma cholesterol, sitosterol, and apo B of 5%, 12%, and 25%, respectively. In contrast, on the same low-sitosterol diet, cholesterol synthesis remained normal (12.2 mg/kg/day) in an obligate sitosterolemic heterozygote and compared to hyperlipidemic controls (13.5 mg/kg/day). Tripling the sitosterol intake did not significantly change plasma cholesterol, sitosterol or apo B concentrations in a hypercholesterolemic control subject.

The difference between dietary cholesterol intake and the sum of fecal cholesterol output plus fecal acid sterol (bile acids) approximates daily endogenous cholesterol synthesis [21,28]. In the steady state, the loss of fecal endogenous neutral sterol and bile acids must equal amounts produced to maintain the same body pool. The constancy of plasma sterol and lipoprotein concentrations, daily weight, and fecal sitosterol outputs, especially in the heterozygote, attest to the metabolic equilibrium on the low-sterol diet in these patients. It was, therefore, noteworthy that both fecal neutral sterol and bile acid outputs were

Table 5. Effects of Low, Intermediate and High Sitosterol Metabolic Diets on Plasma Sterol Parameters*†‡

Subject	Diet treatments	Plasma lipid distribution, mg/dl					Plasma Sitosterol cholesterol			Apolipoprotein B	
	Dietary sitosterol	Total-sterol	TG	VLDL-sterol	LDL-sterol	HDL-sterol	(mg/dl)	(%)‡	(mg/dl)	Apo B (mg/dl)	LDL-sterol Apo B
Sitosterolemic homozygote	Low sitosterol diet	348±3	97±21	16±7	270±3	61±1	42±2	(11%)	282±13	166±12	1.7±0.2
	Intermediate sitosterol diet	356±7	77±5	18±5	260±8	60±2	42±2	(11%)	297±2	126±5[c]	2.1±0.1
	High sitosterol diet	318±11[ab]	74±1	20±2	245±7[ab]	53±5[a]	37±1	(11%)	268±11[ab]	124±6[c]	2.0±0.2
Hypercholesterolemic control	Low sitosterol diet	202±8	104±12	22±3	119±5	62±4	0.2±0.1	(<.2%)	219±13	98±20	1.2±0.3
	Intermediate sitosterol diet	197±8	89±10	22±2	117±6	58±3	0.2	(<.1%)	212±4	121±3	1.0±0.1
	High sitosterol diet	201±7	89±5	23±4	115±6	63±4	0.2	(<.1%)	209±7	107±6	1.1±0.2

* Values represent mean±SD of at least three determinations obtained during final week of diet period; see Table 2 for dietary composition of sitosterol-supplemented diet. Abbreviations as per Table 1.

† (%)=% of total plasma sterol levels.

‡ Overall diet effect: sitosterolemic homozygote ($p<0.02$); hypercholesterolemic control (NS).

[a] Significantly different from low sitosterol diet $p<.05$.

[b] Significantly different from intermediate sitosterol diet $p<.05$.

[c] Significantly different from low sitosterol, $p<.025$.

extremely low in the sitosterolemic homozygote, indicating severely reduced daily cholesterol synthesis even on a low sitosterol diet. Similar measurements showing reduced cholesterol synthesis in three sitosterolemic homozygotes fed a sterol-free diet that averaged 2.8 mg/kg/day vs. 10.7 ± 2.2 mg/kg/day in six controls have been reported by others [10]. Reduced endogenous cholesterol synthesis in our homozygote was supported by a dramatic 43% reduction in measured urinary mevalonate output compared to her heterozygous father and normolipidemic controls. As in other sitosterolemic patients [10,11,13,29], this homozygote patient appears unable to up-regulate HMG-CoA reductase activity (and cholesterol biosynthesis) in response to a low sterol diet.

Bile acid formation was also subnormal in our homozygous patient and in four other sitosterolemic subjects [5,9,10] compared with age-matched children fed a low cholesterol diet [30]. Shefer et al [31] have demonstrated that in sitosterolemic homozygotes, plant sterol and 5-α-stanols competitively block cholesterol 7-α-hydroxylase, the rate-determining enzyme for bile acid synthesis in liver microsomes. Inducement of intestinal bile acid malabsorption through treatment with binding resins (cholestyramine or colestipol) or ileal bypass surgery stimulate bile acid synthesis and increase cholesterol 7-α-hydroxylase activity [31], and has been demonstrated to reduce plasma sterol levels in this patient.

In contrast, cholesterol homeostasis was preserved in our sitosterolemic heterozygote [13]. Plasma sitosterol and cholestanol concentrations were less than 1% of total sterol compared to more than 11% in the homozygote, also, cholesterol synthesis, as measured by the balance method or urinary mevalonate excretion, was normal as reported in another heterozygote [8]. Further cholestyramine treatment, which stimulated bile acid synthesis, increased HMG-CoA reductase activity in mononuclear leukocytes in this heterozygous subject [13]. As a consequence, plasma cholesterol declined only slightly. Thus, one distinguishing characteristic in the sitosterolemic homozygote is the very low rate of cholesterol synthesis and HMG-CoA reductase that cannot be up-regulated either by a low sterol diet or stimulation of bile acid synthesis.

In our homozygote, the increase in dietary sitosterol may have blocked cholesterol absorption, possibly leading to inducement of hepatic LDL receptors, more rapid clearance of LDL-sterol, and small decreases on plasma sterols. It is unlikely that the dietary changes induced this effect since dietary cholesterol, total and saturated fat content, and P:S ratios were nearly identical throughout Phase II. The reduction in plasma sitosterol is noteworthy since sitosterolemic homozygote patients typically hyperabsorb and retain dietary plant sterol, enriching their plasma compartment. The balance data (Table 4) suggests a small continuous retention of sitosterol by the homozygote. Further, virtually eliminating sitosterol from the diet may produce only a slight effect on the plasma pool because of a further decrease in sitosterol output in the homozygotes as previously reported [10].

In our patient, against a background of constant dietary cholesterol intake, the minimal sterol response to manipulation of only one dietary component, dietary sitosterol, suggests that dietary intake of cholesterol, rather than sitosterol, may be more important in regulation of plasma lipid levels in sitosterolemia. A high cholesterol diet reportedly contributed to the

severe hyperlipidemia in an 11 year old sitosterolemic homozygote, and the subsequent exaggerated response to a low cholesterol diet [12].

These results have important implications regarding the selection of therapy for this patient but cannot be generalized to other homozygotes. Total fecal steroid excretion and urinary mevalonate outputs are small and may diminish even further on a low sterol diet with little or no reduction in plasma sterol concentrations. Thus, limiting the sitosterol intake of the diet is minimally effective therapy in this patient. In contrast, bile acid malabsorption lowers plasma cholesterol and sitosterol levels markedly by increasing LDL receptor expression [32,33]. More importantly, clinical abnormalities have been shown to improve in a homozygous subject [33]. Thus, in those sitosterolemic patients unresponsive to a low sterol diet, stimulation of sterol excretion by a resin may lower plasma sterol levels and further diminish the risk of atherosclerosis.

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Phytosterolaemia in a Norwegian family: diagnosis and characterization of the first Scandinavian case

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Lütjohann D, Björkhem I, Ose L. Phytosterolaemia in a Norwegian family: diagnosis and characterization of the first Scandinavian case. Scand J Clin Lab Invest 1996; 56: 229–240.

Phytosterolaemia (sitosterolaemia) is a very rare inherited sterol storage disease characterized by tendon and tuberous xanthomas and by a predisposition to atherosclerosis. We here describe the first Scandinavian case. The 14-year-old female patient was found to have markedly elevated circulating levels of plant sterols (sitosterol, sitostanol, campesterol, stigmasterol), and the levels of these sterols were 20–50 times higher than in her healthy sister and heterozygous parents. In addition to the usual serum plant sterols we found a new major sterol in the patient tentatively identified as episterol or fecosterol (24-methylidencholest-7(or 8)-en-3β-ol). A newly developed method based on the use of deuterium labelled cholesterol and plant sterols was used to measure sterol absorption in the patient and her relatives. Absorption of sitosterol averaged 20% in the patient and ranged from 4 to 8% in the relatives. Absorption of campesterol averaged 31% in the patient and ranged from 15 to 18% in her relatives. Absorption of cholesterol averaged 63% in the patient and ranged from 35 to 45% in the relatives. Cholesterol synthesis appeared to be reduced in the patient and was 46–52% of that of her relatives.

Key words: cholesterol synthesis; plant sterols; stable isotopes

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Phytosterolaemia (sitosterolaemia) is a rare sterol storage disease characterized by tendon and tuberous xanthomas and by a strong predisposition to premature coronary arteriosclerosis (for a review, see ref. [1]). Markedly increased levels of plant sterols, such as sitosterol and campesterol and their 5α-saturated analogues, are found in the circulation and different tissues. The basic biochemical defect has not yet been defined in this autosomal recessive disease. It is however well established that there is an increased absorption of plant sterols from the intestine and a decreased excretion of these compounds in the bile and faeces [1]. The link between premature atherosclerosis and high circulating levels of plant sterols is also not known. It has however been speculated that the high content of plant sterols in the circulating lipoproteins might promote their deposit in the arterial wall [2, 3].

Fewer than 40 cases of this disease have hitherto been described [1]. We here report on the first Scandinavian patient with phytosterolaemia. With the use of a newly developed method based on deuterated cholesterol and plant sterols [4], we have measured absorption of both plant sterols and cholesterol in the patient, her heterozygous parents, and her sister. Results of treatment of the patient with cholestyramine are also reported.

MATERIALS AND METHODS

Human subjects

A 14-year-old girl (BSL, weight 56 kg) with phytosterolaemia, her obligate heterozygous parents (father, KL, 43 years, 60 kg; mother, GL, 42 years, 65 kg), and her sister (EL, 8 years, 29 kg) participated in the study. The study was in accordance with the principles of the Helsinki Declaration for Human Studies, the protocol was approved by the local ethical committee, and informed consent was given by the parents.

Case history

The patient was referred to the Lipid Clinic when she was 9 years old. The patient had developed eruptive xanthomatosis since the age of 5. Biopsy showed cholesterol deposits and no veruccae as suggested. Serum total cholesterol was then found to be 16.2 mmol l^{-1} and the patient was subsequently referred to the Lipid Clinic.

When first seen in the clinic the patient demonstrated xanthomas on her back, arms and on her thumbs. Cardiac status, liver and spleen were normal. Repeated serum lipid profiles showed total cholesterol 15.8 mmol l^{-1}, HDL-cholesterol 0.7 mmol l^{-1}, triglycerides 2.0 mmol l^{-1}, apolipoprotein B (apoB) 3.49 g l^{-1} (normal range: 0.40–1.30 g l), and apolipoprotein AI (apoAI) 0.89 g l^{-1} (0.95–2.05 g l^{-1}).

She was initially started on lovastatin, 40 mg per day, and total cholesterol was reduced to 10.8 mmol l^{-1} within 4 weeks. Lovastatin medication was continued further for 4 weeks, but the cholesterol remained unchanged at 9.3 mmol l^{-1}. Combined therapy with cholestyramine (16 g per day) and lovastatin was initiated and 8 weeks later her total cholesterol had dropped to 4.0 mmol l^{-1} and apoB dropped to 0.79 g l^{-1}. All medication was stopped and her total cholesterol increased to 9.5 mmol l^{-1}. Therapy was thereafter continued with 8 or 4 g per day of cholestyramine and cholesterol remained below 5 mmol l^{-1} on this therapy. Her xanthomas disappeared and she developed normally until the diagnosis was made at the age of 14 years.

Experimental design

Before the last treatment was interrupted for 7 weeks, serum samples for analysis of lipoproteins, triglycerides, total steroids, HDL-, and LDL-steroids, and individual neutral steroids were taken.

After 6 weeks without medication absorption rates of cholesterol, campesterol, and sitosterol together with faecal excretion of neutral and acidic steroids were measured by a modified, recently developed method that has been carefully evaluated. Each subject received a mixture of 3 mg [26,26,26,27,27,27-$^2H_6$]-cholesterol, 3 mg [6,7,7-$^2H_3$]-sitosterol and 2 mg [6,7,7-$^2H_3$]-campesterol t.i.d. for 7 days together with two different faecal markers. The first marker was [$^2H_4$]-sitostanol (3 mg t.i.d.) for measurement of sterol absorption rates [4, 5], and the second was sitostanol (30 mg t.i.d.) for faecal cholesterol balance analysis [6]. Faecal samples were collected on day 0, for correction of sitostanol baseline, and on days 5 and 7. Serum samples were taken on day 0 and days 5 and 7 for enzymatic analysis of total serum steroids and total steroids in various lipoprotein fractions (HDL, LDL), triglyceride levels [7–10], and analysis of individual neutral steroids [5].

Lipoprotein(a) (Lp(a)) was measured using the Pharmacia RIA assay (Pharmacia Diagnostics, Uppsala, Sweden). ApoAI and apoB were measured by rate nephelometry with commercially available antisera and standards (Behring BNA, Marburg, Germany). When the samples reached the laboratory, 50 µg butylated hydroxytoluene (BHT) were added per millilitre serum as antioxidant, and the samples were then kept frozen at −20 °C until analysis.

During the study the subjects were on a low cholesterol and plant sterol diet. The parents kept a 7-day food diary of the consumption of food for each member of the family. The food records were evaluated with the aid of FIBER, a computer program based on Norwegian food tables.

Chemicals

Unlabelled compounds. 5α
chased from Serva Feinbi
Germany), coprostanol a
Steraloids (Whilton, Ne
hyodeoxycholic acid and c
Chemical (St Louis, Miss
tanol from Delalande A
Germany). The purity
checked by thin layer chr
and by combined gas–lic
mass spectrometry (GLC-
silyl-(TMSi)-ethers. Purit
except for sitostanol, wh
campestanol and for sitost
which contained 40%
24-ethyl-/methylcoprostar
methylcoprostanone wer
24-ethyl-/methylcholester
terol) within two steps.
using aluminium-tert.-but
Buchs, Switzerland) in dri
to the corresponding 4-ch
[11]. Hydrogenation of
ladium catalyst in the pre
[12], yielded 24-ethyl

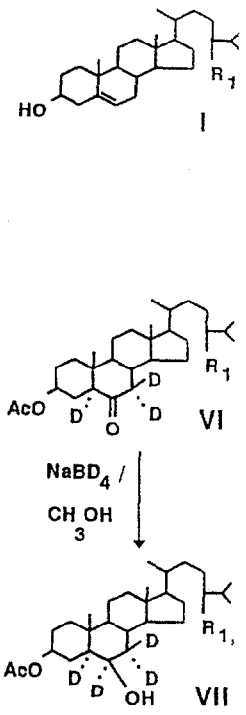

FIG. 1. The synthesis of [6,

Chemicals

Unlabelled compounds. 5α-Cholestane was purchased from Serva Feinbiochemica (Heidelberg, Germany), coprostanol and coprostanone from Steraloids (Whilton, New Hampshire, USA), hyodeoxycholic acid and cholesterol from Sigma Chemical (St Louis, Missouri, USA), and sitostanol from Delalande Arzneimittel (Cologne, Germany). The purity of the steroids was checked by thin layer chromatography (TLC), and by combined gas–liquid chromatography-mass spectrometry (GLC-MS) as their trimethylsilyl-(TMSi)-ethers. Purity was better than 98%, except for sitostanol, which contained 8% of campestanol and for sitosterol (Sigma Chemical) which contained 40% of campesterol. The 24-ethyl-/methylcoprostanol and 24-ethyl-methylcoprostanone were synthesised from 24-ethyl-/methylcholesterol (sitosterol/campesterol) within two steps. Oppenauer oxidation using aluminium-tert.-butoxide (Fluka Chemie, Buchs, Switzerland) in dried toluene/acetone led to the corresponding 4-cholesten-3-one products [11]. Hydrogenation of the ketone, with palladium catalyst in the presence of hydrochloride [12], yielded 24-ethyl-/methylcoprostanone. After purification by TLC an aliquot was hydrogenated with platinum catalyst in acetic acid to get the corresponding coprostanols. The purity of the substances was ascertained by combined GLC-MS. All other chemicals and solvents used for sample preparation were of grades recommended in the literature.

Labelled compounds. [26,26,26,27,27,27-$^2H_6$]-cholesterol and [5,6,22,23-$^2H_4$]-sitostanol were obtained from Medical Isotopes (Concord, New Hampshire, USA). Isotopic purity for [$^2H_6$]-cholesterol was higher than 88% and contained less than 0.1% unlabelled compound. Isotopic purity for [$^2H_4$]-sitostanol was 30% and contained 5% of unlabelled compound.

Synthesis of [6,7,7-$^2H_3$]-sitosterol/campesterol. A modified procedure previously described for introduction of a deuterium atom at the 7 position in cholesterol was used [13] (Fig. 1). Treatment of acetylated sitosterol/campesterol (compound II, in Figure 1, $R_1=C_2H_5/R_2=CH_3$) in ether solution at 5°C with fuming nitric acid afforded pure 6-nitrositosteryl/campesteryl acetate (III)[14]. Reaction of III with zinc and acetic acid gave 24-ethyl/methylcholestane-3β-

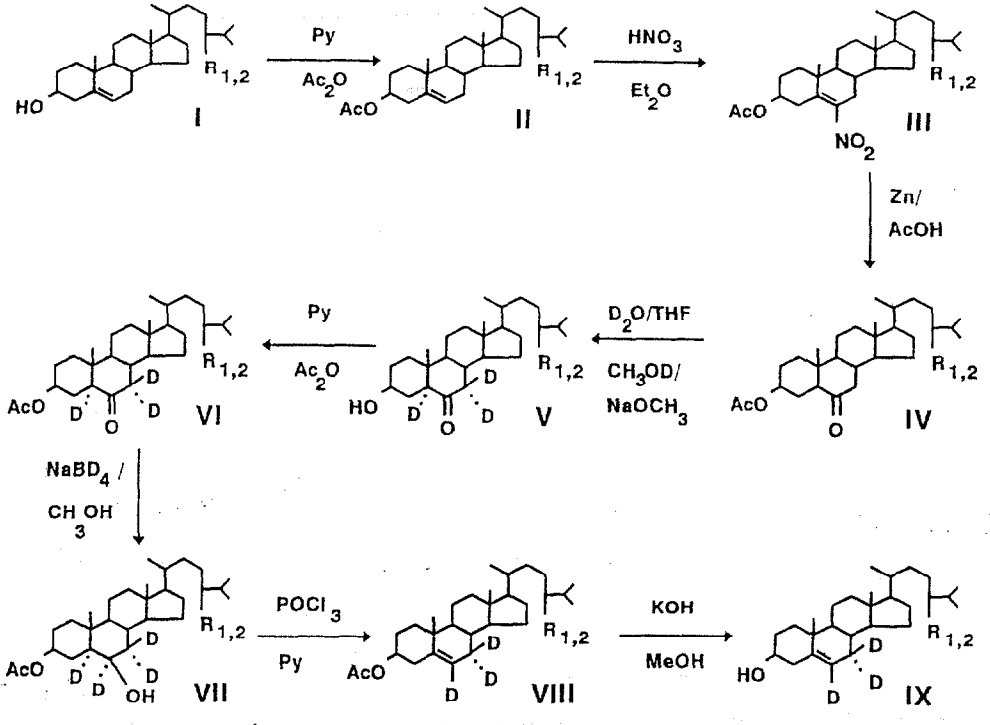

FIG. 1. The synthesis of [6,7,7-$^2H_3$]-sitosterol/campesterol ($R_1=C_2H_5/R_2=CH_3$).

ol-6-one acetate (IV). The product was purified by aluminium oxide chromatography (Al_2O_3, active, neutral, 70–230 mesh, Merck, Darmstadt, Germany), using first increasing concentrations of toluene in hexane and thereafter increasing concentrations of ethylacetate in hexane. The combined fractions containing intermediate IV were evaporated under vacuum to dryness, and dissolved in tetrahydrofurane. Sodium methoxylate, deuterated water and deuterated ethanol were added and the mixture was stirred at room temperature overnight to yield [5,7,7-$^2H_3$]-24-ethyl/methylcholestane-3β-ol-6-one (V). After acetylation to intermediate VI, sodium tetradeuterioborane in methanol was used to reduce the compound to [5,6,7,7-$^2H_4$]-24-ethyl/methylcholestane-6α-hydroxy-3β-hydroxyl acetate (VII). Dehydration with phosphooxo-trichloride in pyridine led to [6,7,7-$^2H_3$]-sitosteryl/campesteryl acetate (VIII). Then [6,7,7-$^2H_3$]- sistosterol/campesterol was obtained after alkaline hydrolysis (IX). Each step of the synthesis was controlled by TLC and GLC-MS of the single products using unlabelled reference substances. GLC-MS analysis of the products, corrected for natural abundance [15], showed an isotopic distribution pattern with 0.7% on +1, 18.9% on M+2, 79.9% on M+3, and 0.6% on M+4.

Steroid capsules. A mixture (1:1:1.5:10; w/w/w/w) of [$^2H_6$]-cholesterol, [$^2H_4$]-sitostanol, [$^2H_3$]-sitosterol/campesterol (60/40; w/w), and sitostanol was dissolved in a commercial plant oil with a plant sterol content lower than all similar preparations. After complete dissolution 0.4 ml (containing each 3 mg of [$^2H_6$]-cholesterol, [$^2H_4$]-sitostanol, [$^2H_3$]-sitosterol, 2 mg of [$^2H_3$]-campesterol, and 30 mg of sitostanol) were filled into gelatine capsules. The capsules were kept at +4°C until use.

Analysis of neutral and acidic steroids

The sample preparation for analysis of neutral and acidic steroids was the same as described previously [5, 6]. Gas liquid chromatography (GLC) of neutral serum and faecal steroids and faecal acidic steroids was performed on a Hewlett Packard (HP5890) gas chromatograph equipped with an automatic sample injector (HP 3673 A). The injector was operated in the splitless mode at 280 °C and the flame ionization detector (FID) was kept at 280 °C. Peak area integration was done by an electronic integrator (model SP 4290, Spectra Physics, San José, California, USA), equipped with the WINner software from Spectra Physics. For optimal separation of the relevant compounds, the gas chromatograph was equipped with different fused silica capillary columns, different temperature gradients were chosen, and the inlet pressures for the carrier gas, hydrogen, varied.

All neutral steroids were separated as TMSi-ethers using 5α-cholestane as internal standard. Briefly, neutral steroids were separated on a FS-OV-101 capillary column (50 m length × 0.32 mm inner diameter, 0.25 μm phase thickness, Chromatographie Service, Langerwehe, Germany), whereas the phase thickness for separation of acidic steroids was 0.17 μm. The oven temperature programmes were as follows: neutral plasma and faecal steroids: initially 3 min at 150 °C, increased at the rate of 20 °C min^{-1} to a final temperature of 280 °C kept for 26 min, inlet pressure 15 psi; acidic faecal steroids: 3 min at 150 °C, increased at the rate of 30 °C min^{-1} to 240 °C kept for 40 min, then at 3 °C min^{-1} to a final temperature of 270°C kept for 6 min, inlet pressure 20 psi.

The retention times (minutes) for the neutral serum steroids were: 5α-cholestane (ISTD), 13.52; cholesterol, 17.20; cholestanol, 17.35; Δ8-lathosterol, 17.50; desmosterol, 17.95; Δ7-lathosterol, 18.20; 24-methyliden-isocholesterol, 18.96; campesterol, 19.13, campestanol, 19.33 min; stigmasterol, 19.73; sitosterol, 21.04; sitostanol 21.29; 24-ethyliden-isocholesterol (avenasterol), 21.42.

The retention times for the neutral faecal steroids were: 5α-cholestane, 13.52; coprostanol, 15.79; coprostanone, 16.67; cholesterol, 17.19; methylcoprostanol, 17.36; methylcoprostanone, 18.47; ethylcoprostanol 18.93; campesterol, 19.13; ethylcoprostanone, 20.27; sitosterol, 21.05; sitostanol 21.23.

The main faecal acidic steroids were separated as follows (minutes): isolithocholic acid, 26.31; lithocholic acid 26.57; isodeoxycholic acid 27.55, deoxycholic acid, 28.17; chenodeoxycholic acid 28.91 min; cholic acid, 29.16; hyodeoxycholic acid (ISTD), 29.45; ursodeoxycholic acid 30.27; 7-ketolithocholic acid, 31.11.

Gas–liquid chromatography–mass spectrometry

Gas–liquid chromatography–mass spectrometry (GLC-MS) was performed on a Hewlett Packard (HP) 5 chromatograph equipped capillary column (30m l diameter, 0.25 μm phase to an HP 5972 mass sele HP 7673A automatic sam temperature was as follo increased at a rate of 2(and then at a rate of temperature of 300 °C w was kept for 8 min. Heliu gas. The injector was op mode with an inlet pressu kept at 270 °C, and the det kept at 280 °C.

The mass spectrometer selected ion monitoring m were monitored for at lea mum sampled over the compound.

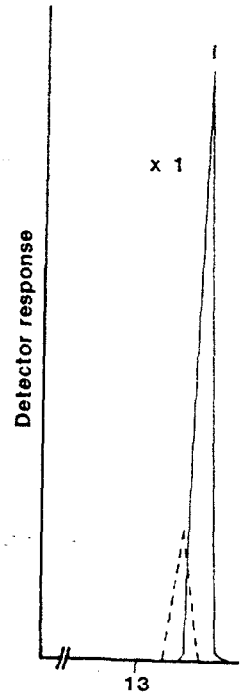

FIG. 2. Ion chromatograms [26,26,26,27,27,27-$^2H_6$]-choles peaks correspond to authenti the following order: I, copros stanone; VI, ethylcoprostanol monitored and the retention t The amplitudes of the origina

Hewlett Packard (HP) 5890 Series II Plus gas chromatograph equipped with an HP-5 MS capillary column (30m length × 0.25 mm inner diameter, 0.25 μm phase thickness), connected to an HP 5972 mass selective detector and an HP 7673A automatic sample injector. The oven temperature was as follows: 180 °C for 1 min, increased at a rate of 20 °C min^{-1} to 250 °C, and then at a rate of 5 °C min to a final temperature of 300 °C where the temperature was kept for 8 min. Helium was used as carrier gas. The injector was operated in the splitless mode with an inlet pressure of 84 kpa and was kept at 270 °C, and the detector transfer line was kept at 280 °C.

The mass spectrometer was operated in the selected ion monitoring mode. The selected ions were monitored for at least 25 scans as a minimum sampled over the peak of the eluting compound.

The ions used for analysis (m z^{-1}, mass per unit charge) and typical retention times (minutes) for the compounds were as follows (Fig. 2): [$^2H_6$]-coprostanol, 376, 13.28; coprostanol, 370, 13.36; [$^2H_6$]-coprostanone, 392, 13.90; coprostanone, 386, 13.94; [$^2H_6$]-cholesterol, 464, 14.18; cholesterol, 458, 14.26; [$^2H_3$]-methylcoprostanol, 387, 14.38; methylcoprostanol, 384, 14.41; [$^2H_3$]-methylcoprostanone, 403, 15.06; methylcoprostanone, 400, 15.10; [$^2H_3$]-ethylcoprostanol, 401, 15.39; ethylcoprostanol, 398, 15.44; [$^2H_3$]-campesterol, 475, 15.46; campesterol, 472, 15.48; [$^2H_3$]-ethylcoprostanone, 417, 16.17; ethylcoprostanone, 414, 16.19; [$^2H_3$]-sitosterol, 489, 16.50; sitosterol, 486, 16.53; [$^2H_4$]-sitostanol, 492, 16.70; sitostanol, 488, 16.73.

The electron ionization energy was 70 eV. Mass spectra of all steroids (TMSi-ethers of 3β-hydroxyl steroids) were obtained by scanning

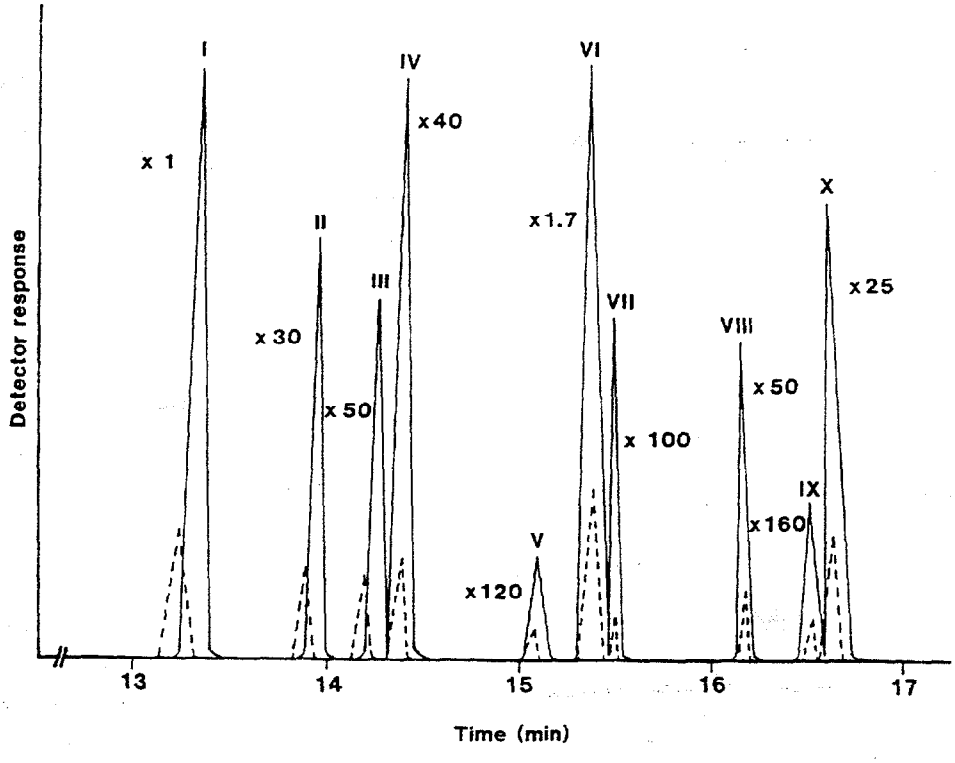

FIG. 2. Ion chromatograms from an analysis of a typical faecal sample after the oral intake of [26,26,26,27,27,27-$^2H_6$]-cholesterol, [6,7,7-$^2H_3$]-sitosterol/campesterol, and [5,6,22,23-$^2H_4$]-sitostanol. The pairs of peaks correspond to authentic (solid line), and deuterated (broken line) compounds. The compounds eluted in the following order: I, coprostanol; II, coprostanone; III, cholesterol; IV, methylcoprostanol; V, methylcoprostanone; VI, ethylcoprostanol; VII, campesterol; VIII, ethylcoprostanone; IX, sitosterol; X, sitostanol. The ions monitored and the retention times for the different compounds are given in the Materials and Methods section. The amplitudes of the original signals are magnified by the factors shown.

TABLE I. Calculation of sterol absorption.

$$\text{Percentage } S_{1-3} \text{ absorption}^{a} = 100 \times \left[1 - \frac{\text{faecal}\left(\frac{A_{1-3}+B_{1-3}+C_{1-3}}{D}\right)}{\text{dietary}\left(\frac{A_{1-3}}{D}\right)} \right]^{b}$$

[a] S_1 = cholesterol, S_2 = campesterol (24-methylcholesterol), S_3 = sitosterol (24-ethylcholesterol).
[b] A to D are the concentrations of:
$A_1 = [^2H_6]$-cholesterol, $B_1 = [^2H_6]$-coprostanol, $C_1 = [^2H_6]$-coprostanone;
$A_2 = [^2H_3]$-campesterol, $B_2 = [^2H_3]$-methylcoprostanol, $C_2 = [^2H_3]$-methylcoprostanone;
$A_3 = [^2H_3]$-sitosterol, $B_3 = [^2H_3]$-ethylcoprostanol, $C_3 = [^2H_3]$-ethylcoprostanone; $D = [^2H_4]$-sitostanol.

between 100 and 500 m z^{-1} at a rate of 0.9 scan s^{-1}.

Calculation of sterol absorption

Absorption of cholesterol, campesterol, and sitosterol was measured by a recently developed method using $[^2H_4]$-sitostanol as a non-absorbable marker [5]. For this reason, deuterated cholesterol, sitosterol, and campesterol were quantified by GLC-MS together with their corresponding coprostanols and coprostanones. The absorption of the different sterols was then calculated by the equation shown in Table I.

Statistics

Simple descriptive statistics were used. The results were expressed as mean (SD), or mean (range).

RESULTS

Serum lipids and lipoproteins

The levels of serumlipoproteins are given in Table II. The young patient with phytosterolaemia had higher concentrations of total serum steroids, LDL-steroids, and triglycerides than her obligate heterozygous father and her healthy sister. Serum total steroids and triglycerides were in the same range for the phytosterolaemic child as for her heterozygous mother. The serum LDL/HDL-steroid ratio averaged 5.9 in the patient, and was 2.2 times and 3.6 times higher than in her parents and sister, respectively. The apoB was almost in the same range as in her mother, but 1.5 and 1.9 times higher than in the father and sister, respectively. Lp(a) was markedly (2.7 times) higher in the mother than in the rest of the family.

Analysis of the sterol fraction

The results of GLC analysis of the main serum steroids are given in Table III.

In addition to the common plant sterols such as campesterol, stigmasterol, and sitosterol, and their corresponding 5α-saturated compounds i.e. campestanol and sitostanol, we were able to find two more plant sterols, one of which was 24-ethyliden-isocholesterol (avenasterol), first described by Miettinen [3]. The other plant sterol, characterized by GLC-MS, had a molecu-

TABLE II. Serum lipoprotein concentrations, mean (SD)*, in one patient with phytosterolaemia (BSL), her heterozygous parents (GL and KL) and her sister (EL).

Subject	Total steroids† mmol l^{-1}	LDL-steroids mmol l^{-1}	HDL-steroids mmol l^{-1}	Triglycerides mmol l^{-1}	ApoAI g l^{-1}	ApoB g l^{-1}	Lp(a) mg l^{-1}
BSL	6.6 (0.2)	4.7 (0.2)	0.8 (0.1)	1.6‡	0.9§	1.5§	111§
GL	6.3 (0.2)	3.9 (0.1)	1.7 (0.5)	1.5 (0.3)	1.4	1.3	318
KL	5.6 (0.2)	4.1 (0.1)	1.3 (0.1)	0.5 (0.1)	1.1	1.0	134
EL	4.7 (0.2)	2.6 (0.2)	1.6 (0.1)	1.0 (0.2)	1.4	0.8	115

* Each value represents the mean of measurements on days 0, 5, and 7.
† The concentrations of 'steroids' reflect the sum of all 3β-OH-steroids measured enzymatically.
‡ Triglycerides in the patient were measured on days 5 and 7.
§ ApoAI, ApoB, and Lp(a) were measured on day 7.

TABLE III. Serum cholesterol and non-cholesterol steroids, mean (SD)*, in one patient with phytosterolaemia (BSL), her heterozygous parents (GL and KL), and her sister (EL) measured by gas–liquid chromatography. Units are μmol l^{-1}, except for cholesterol (mmol l^{-1}).

Subject	Cholesterol	Cholestanol	Δ8-Lathol†	Desmosterol	Δ7-Lathol†	24-Me-isochol†	Campesterol	Campestanol	Stigmasterol	Sitosterol	Sitostanol	Avenasterol†
BSL	4.8 (0.3)	54 (4)	3.9 (1.0)	7.6 (0.5)	3.1 (0.7)	31 (3)	313 (17)	43 (3)	54 (1)	483 (21)	85 (3)	78 (7)
GL	5.0 (0.1)	15 (1)	3.3 (1.5)	4.8 (1.5)	3.2 (0.4)	4.9 (0.2)	18 (1)	ND‡	1.7 (0.4)	15 (3)	3.3 (0.9)	3.2 (0.9)
KL	4.6 (0.3)	16 (1)	3.3 (0.7)	4.5 (0.9)	3.6 (0.3)	6.7 (0.4)	26 (2)	ND	2.0 (0.3)	21 (3)	2.6 (0.3)	5.5 (0.9)
EL	3.7 (0.2)	10 (1)	2.9 (0.8)	4.5 (0.5)	3.2 (0.4)	3.1 (0.3)	10 (1)	ND	1.0 (0.1)	9.2 (1.0)	1.6 (0.2)	2.4 (0.3)

* Each value represents the mean of measurements on days 0, 5, and 7.

TABLE III. Serum cholesterol and non-cholesterol steroids, mean (SD)*, in one patient with phytosterolaemia (BSL), her heterozygous parents (GL and KL), and her sister (EL) measured by gas–liquid chromatography. Units are μmol l^{-1}, except for cholesterol (mmol l^{-1}).

Subject	Cholesterol	Cholestanol	Δ8-Lathot	Desmosterol	Δ7-Lathot	24-Me-isocholt	Campesterol	Campestanol	Stigmasterol	Sitosterol	Sitostanol	Avenasterolt
BSL	4.8 (0.3)	54 (4)	3.9 (1.0)	7.6 (0.5)	3.1 (0.7)	31 (3)	313 (17)	43 (3)	54 (1)	483 (21)	85 (3)	78 (7)
GL	5.0 (0.1)	15 (1)	3.3 (1.5)	4.8 (1.5)	3.2 (0.4)	4.9 (0.2)	18 (1)	ND‡	1.7 (0.4)	15 (3)	3.3 (0.9)	3.2 (0.9)
KL	4.6 (0.3)	16 (1)	3.3 (0.7)	4.5 (0.9)	3.6 (0.3)	6.7 (0.4)	26 (2)	ND	2.0 (0.3)	21 (3)	2.6 (0.3)	5.2 (0.9)
EL	3.7 (0.2)	10 (1)	2.9 (0.8)	4.5 (0.5)	3.2 (0.4)	3.1 (0.3)	10 (1)	ND	1.0 (0.1)	9.2 (1.0)	1.6 (0.2)	2.4 (0.3)

* Results represent the mean of measurements on days 0, 5, and 7.
† Δ8-Latho, Δ8-lathosterol (5α-cholest-8-en-3β-ol); Δ7-Latho, Δ7-lathosterol (5α-cholest-7-en-3β-ol); 24-Me-isochol, 24-methyliden-5α-cholest-7(8)-en-3β-ol; Avenasterol, 24-ethyliden-5α-cholest-7(8)-en-3β-ol.
‡ND, not detectable.

lar weight as TMSi-ether of 470 m z^{-1} (Fig. 3). With the exception of the shift on the highest ions with 14 mass units, the mass spectrum was very similar to that of the TMSi-ether of avenasterol. We believe that this compound has a methyliden group at C_{24} instead of an ethyliden group as in avenasterol. Further attempts to identify the substance were not made and it was assumed to be 24-methyliden-isocholesterol (24-methyliden-cholest-7(or 8)-en-3β-ol, episterol or fecosterol) [12].

The steroid fraction contained 22.3% plant steroids in the phytosterolaemic patient, whereas in the parents and in the sister they were lower than 3%. Sitosterol had the highest concentration in the patient and was 1.5 times higher than campesterol, whereas in the parents and the healthy sister the mean campesterol concentration was 1.2 times higher than sitosterol. The amounts of 5α-saturated plant sterols, e.g. campestanol and sitostanol, were also markedly increased in the patient.

Serum Δ8- and Δ7-lathosterol, often used as serum indicators for cholesterol synthesis, showed no difference in concentration compared with the results obtained in the parents and sister. However, desmosterol, another cholesterol precursor was 1.6 times higher in the patient than in the rest of the family. Serum cholesterol concentration was about the same for the phytosterolaemic child and her parents, but slightly higher than her sister's level.

Faecal excretion of neutral and acidic steroids

Faecal excretion of cholesterol and steroids metabolized from cholesterol by the bacterial flora of the gut, e.g. coprostanol and coprostanone, was only 50% in the phytosterolaemic patient compared to the excretion in her parents or her sister (Table IV).

Faecal excretion of major bile acids (lithocholic acid, deoxycholic acid, cholic acid, and chenodeoxycholic acid) was about 50% of that of her parents, but the same as in her younger sister. When corrected for body weight the patient had an excretion of bile acids of 10 μmol kg^{-1} day^{-1}, the parents 20 μmol kg^{-1} day^{-1} and the sister also 20 μmol kg^{-1} day^{-1}.

Cholesterol synthesis appeared to be about half of that of her parents and her sister (Table IV). Cholesterol synthesis expressed per kg body weight in the patient (19 μmol kg^{-1}

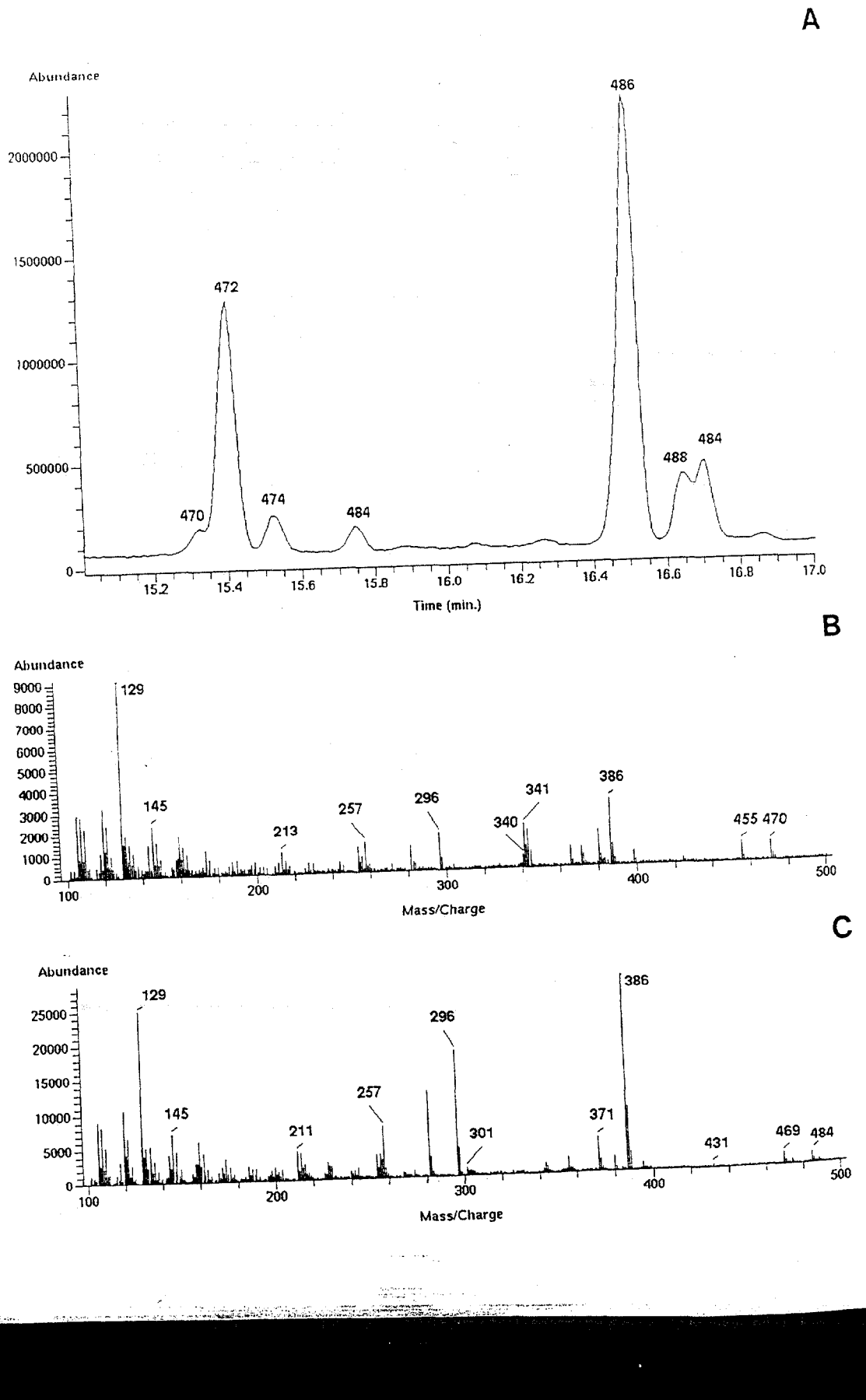

TABLE IV. Results of faec
cholesterol synthesis in one
her sister (EL). Units are n

Subject	Cholester
BSL	0.79 (0.0
GL	1.48 (0.1
KL	1.55 (0.1
EL	1.71§

* Each value represents t
† Faecal excretion of 'Ch
‡ 'Cholesterol synthesis' i
§ Only one sample could

day^{-1}) also appeared to
of her parents' synthes
day^{-1}), and about 25%
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Sterol absorption

Rates of absorption
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Cholesterol absorption i
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parents or sister. Absorp
sitosterol was higher in
family, by 90 and 220% r
differences could be see
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TABLE V. Comparison of
and sitosterol absorption,
patient with phytosterolaem
parents (GL and KL), and

	Abs	
	Cholesterol	Ca
BSL	63 (60–66)	3
GL	35 (29–41)	1
KL	45 (43–47)	1
EL	42†	1

* Results represent the m
during one week in duplicat
† Only one sample could

FIG. 3. A Gas–liquid chro
trimethylsilyl- (TMSi-) deri
fragmentation of the GLC
are molecular weights (MW
sterol spectrum which revea
for campesterol, 474 for ca
for avenasterol (24-ethylide

TABLE IV. Results of faecal excretion of steroids, mean (SD)*, derived from cholesterol and bile acids, and cholesterol synthesis in one patient with phytosterolaemia (BSL), her heterozygous parents (GL and KL), and her sister (EL). Units are mmol day^{-1}.

Subject	Cholesterol†	Bile acids	Cholesterol intake	Cholesterol synthesis‡
BSL	0.79 (0.06)	0.56 (0.08)	0.29	1.06 (0.14)
GL	1.48 (0.15)	0.95 (0.13)	0.36	2.06 (0.28)
KL	1.55 (0.18)	1.05 (0.12)	0.31	2.29 (0.30)
EL	1.71§	0.57	0.26	2.02

* Each value represents the mean of measurements on days 5 and 6 in duplicate work-up.
† Faecal excretion of 'Cholesterol' is the sum of cholesterol, coprostanol, and coprostanone.
‡ 'Cholesterol synthesis' is the sum of 'Cholesterol' and 'Bile acids' minus 'Cholesterol intake'.
§ Only one sample could be collected on day 5.

day^{-1}) also appeared to be about half of that of her parents' synthesis (mean 35 μmol kg^{-1} day^{-1}), and about 25% of that of her 6 years younger sister (70 μmol kg^{-1} day^{-1}).

Sterol absorption

Rates of absorption of cholesterol, campesterol, and sitosterol are given in Table V. Cholesterol absorption in the patient was markedly (60%) higher in the patient than in her parents or sister. Absorption of campesterol and sitosterol was higher in the patient than in her family, by 90 and 220% respectively. No marked differences could be seen in the comparison of cholesterol or plant sterol absorption between the obligate heterozygous parents and thehealthy sister.

TABLE V. Comparison of cholesterol, campesterol, and sitosterol absorption, mean (range)*, in one patient with phytosterolaemia (BSL), her heterozygous parents (GL and KL), and her sister (EL).

	Absorption, %		
	Cholesterol	Campesterol	Sitosterol
BSL	63 (60–66)	31 (27–35)	20 (17–23)
GL	35 (29–41)	18 (15–21)	8 (5–11)
KL	45 (43–47)	17 (13–21)	7 (5–9) †
EL	42†	15	4

* Results represent the mean of two measurements during one week in duplicate work-up.
† Only one sample could be measured on day 5.

Effects of treatment with cholestyramine

The therapeutic effect of the oral administration of 4 g cholestyramine in the phytosterolaemic patient is shown in Table VI. After stopping the therapy for 6 weeks a marked increase of cholesterol (+85%) and its saturated metabolite 5α-cholestanol (+145%) was followed by a mean increase of the main plant sterols of 50%.

The effects on serum cholesterol precursors were different. As could have been expected from a previous report [3], the serum concentration of Δ8-lathosterol decreased markedly (−42%). However, Δ7-lathosterol decreased by 9%, and the concentration of desmosterol increased by 29%.

DISCUSSION

The present case showed all the typical characteristics of phytosterolaemia: xanthomas, markedly elevated circulating levels of phytosterols, increased absorption of phytosterols and reduced cholesterol synthesis (cf. ref. [1]).

In our patient, serum campesterol was 313 μmol l^{-1} and serum sitosterol was 483 μmol l^{-1}. In a group of 14 patients the mean level of

FIG. 3. A Gas–liquid chromatographic (GLC) pattern between 15.0 and 17.0 min obtained in the analysis of trimethylsilyl- (TMSi-) derivatives of serum steroids of the patient with phytosterolemia; B mass spectrometric fragmentation of the GLC peak at 15.3 min; C the last GLC peak at 16.7 min. Figures on the top of each peak are molecular weights (MW) of the TMSi-derivatives of the steroids (Fig. 3A). MW 470 stands for the unknown sterol spectrum which reveals major ions at m e^{-1} 386, 296, 257, and 129 (main ion) (Fig. 3B). MW 472 stands for campesterol, 474 for campestanol, 484 for stigmasterol, 486 for sitosterol, 488 for sitostanol, and 484 stands for avenasterol (24-ethyliden-isocholesterol); its mass spectrum is shown in Fig. 3C.

TABLE VI. Serum steroid concentrations, mean (SD)*, in one patient with phytosterolaemia (BSL) during therapy, and 6 weeks after stopping oral administration of cholestyramine 4 g per day (control).

	Therapy†	Control
Cholesterol, mmol day^{-1}	2.6	4.8 (0.3)
Cholestanol, μmol day^{-1}	22	54 (4)
Δ8-Lathosterol, μmol day^{-1}	6.7	3.9 (1.0)
Desmosterol, μmol day^{-1}	5.2	7.6 (0.5)
Δ7-Lathosterol, μmol day^{-1}	3.4	3.1 (0.7)
24-Me-isocholesterol‡, μmol day^{-1}	20	31 (3)
Campesterol, μmol day^{-1}	199	313 (17)
Campestanol, μmol day^{-1}	28	43 (3)
Stigmasterol, μmol day^{-1}	28	54 (1)
Sitosterol, μmol day^{-1}	345	483 (21)
Sitostanol, μmol day^{-1}	55	85 (3)
Avenasterol‡, μmol day^{-1}	56	78 (7)

* Values represent the mean of three measurements during one week.

† One sample was analyzed in duplicate work-up.

‡ 24-Me-isocholesterol, 24-methyliden-5α-cholest-7(8)-en-3β-ol; Avenasterol, 24-ethyliden-5α-cholest-7(8)-en-3β-ol.

plasma campesterol has been reported to be 400 μmol l^{-1}, with a range of 200–725 μmol l^{-1}, whereas the mean level of sitosterol was 850 μmol l^{-1} with a range of 350–1570 μmol l^{-1} [17]. A notable finding in our patient was the presence of a new phytosterol in serum, tentatively identified as episterol or fecosterol (24-methyliden-cholest-7(or 8)-en-3β-ol, 24-methyliden-isocholesterol). The existence of this steroid has not previously been proved in connection with phytosterolaemia. In accordance with previous reports [1, 16], serum cholestanol was elevated in the patient and was about four times higher than in her parents and her sister.

Using a newly developed stable isotope method for measurement of intestinal absorption of cholesterol and plant sterols [5], we could confirm previous reports that patients with phytosterolaemia have increased absorption of both cholesterol and plant sterols [3, 17–19]. A merit of the present method, compared to previous methods utilizing a single dose of a radioactive marker, is that we could measure sterol absorption over several days. The girl with phytosterolaemia absorbed 31% of ingested deuterated campesterol and 20% of deuterated sitosterol. This is in accordance with the results of a recent study on three patients with phytosterolaemia [5]. In that study the phytosterolaemic subjects had a campesterol absorption of about 24% and a sitosterol absorption of about 16%. In previous investigations, absorption of sitosterol has been reported to range from 19 [3] to about 68% [18] in phytosterolaemic subjects. Cholesterol absorption (about 63%) was significantly higher in the present phytosterolaemic patient than in her relatives. This is in accord with the results obtained in our previous study [5].

To our knowledge, absorption of phytosterols has not previously been studied in heterozygotes of phytosterolaemia. The degree of absorption of both plant sterols and cholesterol in the present heterozygotes was however not different from that of healthy controls (cf. ref. [1]). Whether or not the sister of the present patient is heterozygous or a non-carrier is not known and cannot be evaluated from the present results.

It is evident that the approximately 30-fold higher circulating levels of sitosterol in the patient, compared to her parents and sister, cannot be explained by the threefold higher intestinal absorption. It has been shown by Salen *et al.* [18] and Bhattacharyya *et al.* [20] that patients with phytosterolaemia, in addition to increased absorption, also have a slow turnover of sitosterol and a reduced secretion of sterols from the liver.

Patients with phytosterolaemia have been reported to have normal or slightly elevated levels of serum cholesterol [1, 21]. The elevation of cholesterol might in part be due to the inhibitory effect of plant sterols on the cholesterol 7α-hydroxylase and a resulting decrease in the degradation of cholesterol into bile acids [22–4]. The present patient had a normal serum level of cholesterol at the time of the investigation. It is noteworthy, that the serum HDL/LDL-steroid ratio was increased in the patient, indicating an elevated atherogenic risk.

Most of the patients v
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Most of the patients with phytosterolaemia seem to have a reduced cholesterol synthesis, and there may be a link between cholesterol synthesis and sitosterol absorption [1]. Nguyen *et al.* even suggest that the primary metabolic defect in phytosterolaemia is inadequate cholesterol biosynthesis due to reduced synthesis of HMG-CoA reductase [25, 26]. The possibility cannot be excluded, however, that the defect in cholesterol synthesis is secondary to the accumulation of plant sterols. Using different *in vivo* and *in vitro* techniques, cholesterol synthesis has been reported to be reduced by 50–80% in patients with phytosterolaemia [1]. The cholesterol synthesis in the present phytosterolaemic patient was about half of that of her parents and healthy sister, as calculated from dietary intake and faecal excretion of cholesterol, coprostanol, coprostanone, and bile acids. Expressed by kg of body weight she had a lower excretion of bile acids in faeces than her heterozygous parents and her sister. However, the circulating level of lathosterol, believed to be a suitable marker for cholesterol synthesis [27], was normal in the patient after stopping therapy for 6 weeks.

The high circulating levels of plant sterols in patients with phytosterolaemia may be reduced markedly by a diet restricted in plant sterols [1, 24, 28]. Cholestyramine has been used as a therapeutic drug in addition to the diet [3, 20 29, 30]. This bile acid binding resin promotes bile acid secretion and increases the output of biliary plant sterols [3]. In previous studies cholestyramine treatment of phytosterolaemic patients (up to 12 g per day) has been reported to reduce circulating levels of cholesterol, plant sterols, and their 5α-saturated analogues by about 50% [20]. The xanthomas may regress and the arthritis may decrease as a consequence of such treatment. In fact, treatment of our patient resulted in regression of the xanthomas from the age of 9 to 14 years.

Interruption of treatment with cholestyramine for 6 weeks resulted in an increase of circulating cholesterol by 85%, campesterol by 57%, and sitosterol by 40% compared to post-treatment values. The corresponding 5α-analogues, cholestanol, campestanol, and sitostanol were increased to a similar extent. This shows that interruption of the therapy immediately leads to a return to the former situation.

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Tuberous Xanthomas in Sitosterolemia

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Abstract: Sitosterolemia is an autosomal recessive lipid disorder in which plasma plant sterol levels are extremely elevated and cholesterol levels are often elevated but may be normal. Clinically sitosterolemia is characterized by xanthomas, premature vascular disease, and arthritis. Adolescent boys and girls with sitosterolemia are susceptible to fatal cardiac events. Dermatologists may have a vital role in the diagnosis of this rare but serious condition because early detection and treatment are important in preventing the associated atherosclerotic heart disease. We present a 7-year-old girl with sitosterolemia and tuberous xanthomas.

Among the causes of tuberous and tendon xanthomas in children and adolescents is a rare and relatively recently described hyperlipidemia known as sitosterolemia. We present a patient with sitosterolemia and tuberous xanthomas.

CASE REPORT

A 7-year-old girl was referred to the pediatric cardiology division for evaluation of hypercholesterolemia. One year earlier she had been referred to a dermatologist who had noted nodules on her elbows that clinically appeared to be xanthomas (Fig. 1) and were confirmed as such by histology. Total cholesterol at that time was 391 mg/dl and triglycerides were 91 mg/dl. A repeat lipid profile while she was on a regular diet 3 months later showed that the total cholesterol was 441 mg/dl, with triglycerides of 49 mg/dl, an HDL cholesterol of 57 mg/dl, and an LDL cholesterol of 374 mg/dl. The 95th percentiles for total and LDL cholesterol levels at this age are 200 mg/dl and 130 mg/dl, respectively. The triglycerides and HDL cholesterol levels were normal.

Within 1 month of beginning a moderately low cholesterol and low fat diet (≤30% of calories from fat) her total cholesterol fell to 195 mg/dl. After 3 months of this low fat diet, the lipid values were total cholesterol 201 mg/dl, triglycerides 61 mg/dl, HDL cholesterol 36 mg/dl, and LDL cholesterol 153 mg/dl. Capillary gas liquid chromatography demonstrated that plant sterol levels were elevated, with sitosterol 16.5 mg/dl, campesterol 9.75 mg/dl, and cholestanol 2.0 mg/dl. The actual cholesterol level as determined by capillary gas liquid chromatography was 171 mg/dl. Thus approximately 15% of the total cholesterol measured by routine laboratory techniques was derived from plant sterols. The patient's parents, who were first cousins, had relatively normal cholesterol levels, and there was no maternal or paternal history of premature heart disease. Plant sterol levels were low in both parents.

On examination the patient was in good health and her height and weight were appropriate. No corneal arcus was observed. There were no carotid bruits. The heart examination was normal. Three tuberous xanthomas were present on the right elbow and four on the left, and all were less than 1 cm in diameter. There were no tendinous xanthomas. A biopsy specimen from a xanthoma

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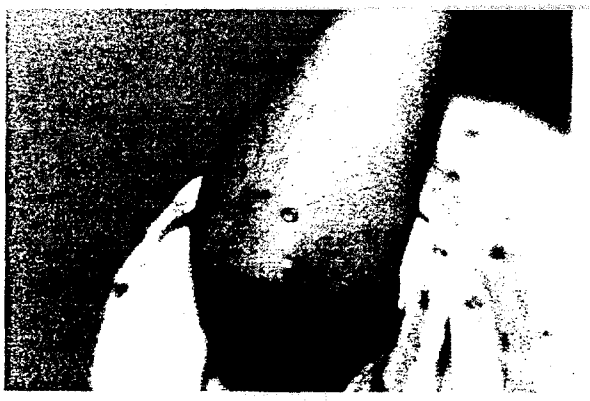

Figure 1. Tuberous xanthomas on the right elbow 1 year before treatment.

from the left elbow taken 1 year prior to the initiation of dietary modification revealed discrete collections of foamy histiocytes throughout the dermis in association with slight fibroplasia (Fig. 2). She was treated with a low fat, low cholesterol diet and 2.0 g cholestyramine daily. After 6 months of treatment, total cholesterol fell further to 179 mg/dl, with triglycerides 102 mg/dl, HDL cholesterol 43 mg/dl, and LDL cholesterol 116 mg/dl. The xanthomas appeared slightly smaller and paler.

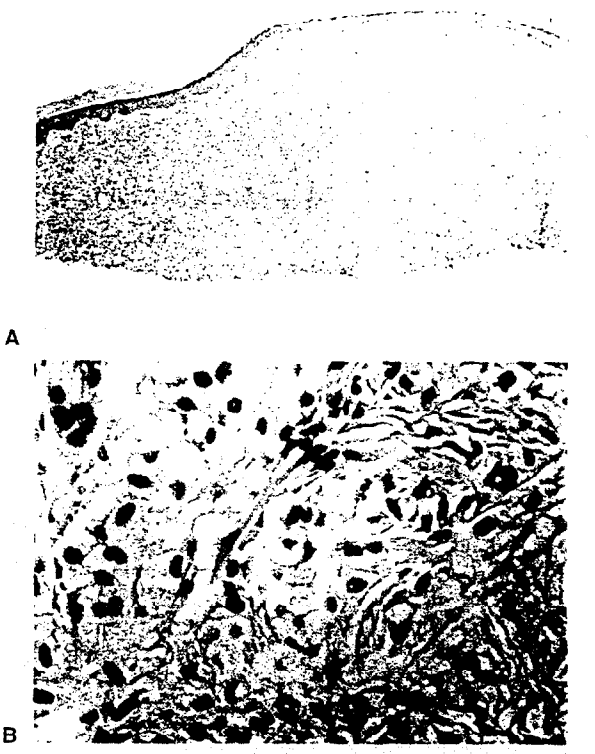

Figure 2. Discrete collections of foamy histiocytes in the dermis with slight fibroplasia seen at (A) low and (B) high magnification.

DISCUSSION

Sitosterolemia is a lipid disorder first described by Bhattacharyya and Connor in 1974 (1). The name sitosterol refers to the major plant sterol species, but since many other plant sterols are also elevated in the plasma in this disease, "phytosterolemia" may be a better name (2). Sitosterolemia is inherited as an autosomal recessive trait (2), with the heterozygotes clinically and biochemically normal, although plasma sitosterol levels may be slightly elevated in some heterozygotes compared to controls (3). The genetic defect responsible has been localized to chromosome 2p21 (2). More than 40 affected individuals have been reported worldwide.

Biochemically the key feature of sitosterolemia is an elevated concentration of sitosterol (24-ethyl cholesterol) in plasma (3). Levels of other plant sterols, including campesterol, stigmasterol, and avenosterol, and the 5-alpha-dihydro derivative of cholesterol, cholestanol, may also be extremely high (3). Plasma cholesterol levels are often markedly elevated but may be within normal limits (2,4). Sitosterol levels in normal individuals are less than 0.4 mg/dl (5) and are not identified in routine cholesterol measurement. Routine calorimetric and enzymatic methods do not distinguish sitosterol and other sterols from cholesterol. Therefore sitosterolemia may be underreported because the diagnosis requires either high-performance liquid chromatography (HPLC) or gas-liquid chromatography using a capillary column (capillary GLC) to differentiate cholesterol from the other sterols and 5-alpha-dihydro sterol derivatives (2,3). Typically cholesterol comprises 99.6% of plasma sterols, and cholestanol and plant sterols, approximately 0.2% each. By contrast, in sitosterolemic homozygotes, cholesterol represents only 80% of plasma sterols (3).

Normal diets contain equivalent amounts of cholesterol and plant sterols, but only 30–60% of cholesterol and less than 5% of plant sterols remain in the body after processing (2,4). In unaffected individuals, the liver preferentially excretes unesterified noncholesterol sterols into the bile so that almost no net absorption results. In sitosterolemia, increased intestinal absorption is coupled with reduced hepatic sterol secretion. Thus hepatic plant sterol levels are elevated 10- to 100-fold over baseline (6). Dysregulation of lipid metabolism in sitosterolemia also results in enhanced LDL receptor function and low HMG-CoA reductase activity (5).

Clinically sitosterolemia is characterized by xanthomas, premature vascular disease, and arthritis. Tendon xanthomas may affect the patellar, plantar, and Achilles

tendons, as well as the extensor tendons of the hand; tuberous xanthomas occur predominantly at the elbows and knees (1,7). While xanthomas are the primary cutaneous sign of sitosterolemia, not all sitosterolemic patients have xanthomas. Premature atherosclerotic disease, often in the absence of a family history of such, may present as fatal myocardial infarction or aortic stenosis in the first several decades of life. The xanthomas and coronary artery disease seen in sitosterolemia are similar to the features of homozygous familial hypercholesterolemia due to LDL-receptor deficiency. Adolescent boys with homozygous sitosterolemia are particularly susceptible to fatal cardiac events, and a 13-year-old Amish boy is the youngest reported case (3). Girls are also affected, with a successful coronary bypass graft described in a 16-year-old girl with homozygous disease (5). Recurrent arthritis and arthralgias of the knee and ankle joints are also seen (3). Solitary instances of sitosterolemia associated with neurologic lesions, paraplegia, and spinal cord compression secondary to xanthomatosis (7), and generalized eruptive xanthomatosis have been reported (8). However, it is important to emphasize that sitosterol and other sterols and stanols do not pass the blood–brain barrier and accumulate in either the white or gray matter of the brain (3). Since biliary secretion relative to bile acids is decreased, gallstones have not been found (3).

Initial treatment for sitosterolemia can be a cholesterol-lowering diet, which may lead to a concomitant rapid decline in plasma sterols of as much as 35% (9). Bile acid malabsorption can be achieved by administering binding resins, such as cholestyramine and colestipol, or via ileal bypass surgery (3). Both the medical and surgical options result in dramatic lowering (25–50%) of plasma sterol concentrations. Treatment can produce prompt clinical improvement, including the disappearance of xanthomas, elimination of aortic stenosis murmur, and decreased frequency of angina pectoris and arthritic episodes. In severe cases, coronary bypass grafts may be necessary (5).

The differential diagnosis of tendon and tuberous xanthomas in adults includes primary lipid abnormalities such as heterozygous and homozygous familial hypercholesterolemia secondary to LDL-receptor deficiency, broad beta disease (type III hyperlipoproteinemia), and hypertriglyceridemia. Other causes include cerebrotendinous xanthomatosis, secondary hyperlipidemia due to diabetes mellitus (10), hypothyroidism, multiple myeloma, and obstructive liver disease (7,11,12).

Dermatologists who are asked to evaluate children with xanthomas should recognize that the more limited differential diagnosis includes homozygous familial hypercholesterolemia, sitosterolemia, cerebrotendinous xanthomatosis, and obstructive cholestatic liver disease such as Alagille syndrome (13,14). Homozygous hypercholesterolemia usually presents with much higher plasma cholesterol levels than sitosterolemia, and Alagille syndrome typically includes hepatic disease. If a patient with sitosterolemia were on a low fat, low cholesterol diet, xanthomas might present in association with essentially normal plasma lipids, the so-called normolipemic xanthomas, and would go undetected unless specific tests for plant sterols were performed. Pediatric dermatologists can have a vital role in the diagnosis of this rare but serious and treatable condition and should consider this diagnosis in all children with xanthomas. Since cardiac disease can present in childhood, early detection and treatment are important in preventing the atherosclerotic heart disease associated with the condition.

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HMG-CoA reductase is not the site of the primary defect in phytosterolemia

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Abstract Phytosterolemia is an autosomal recessive disorder characterized by the excessive absorption, reduced excretion, and consequent high tissue and plasma levels of plant sterols, by the presence of tendon xanthomas, and by premature atherosclerosis. Low HMG-CoA reductase (HRase) activity and mass have been reported in liver and mononuclear leucocytes and low mRNA levels in liver from phytosterolemic subjects. These results led to the proposal that the primary defect in this condition involves the HRase gene locus. We examined this hypothesis in phytosterolemic subjects and heterozygous parents from four unrelated families. A variable number tandem repeat (VNTR) polymorphism of the HRase gene in the three informative families and a ScrFI restriction fragment length polymorphism (RFLP) within intron 2 of the gene in one of these families, segregated independently of the disease phenotype. Biological parentage was confirmed in the family in whom both polymorphisms failed to segregate with the disorder. These results conclusively exclude the HRase gene locus as the site of the primary defect in phytosterolemia. The study was extended by examining plasma levels of mevalonic acid and lathosterol, both markers of cholesterol biosynthesis, in response to cholestyramine, a bile acid sequestrant that is known to up-regulate HRase. Oral administration of cholestyramine resulted in a substantial (7.7-fold) increase in mevalonic acid levels in two phytosterolemic subjects, compared with a 2.2-fold rise in their obligate heterozygote parents and a 2.3-fold increase in three healthy control subjects. The lathosterol/cholesterol (L/C) ratio showed a quantitatively similar response. Baseline levels of mevalonate and the L/C ratio were low in the phytosterolemic patients in conformity with reports of reduced cholesterol biosynthesis and HRase activity in this disorder. These functional data provide support for the concept that the primary defect in phytosterolemia does not affect a *trans* gene locus responsible for the constitutive expression or regulation of HMG-CoA reductase.—Berger, G. M. B., R. J. Pegoraro, S. B. Patel, P. Naidu, L. Rom, H. Hidaka, A. D. Marais, A. Jadhav, R. P. Naoumova, and G. R. Thompson. HMG-CoA reductase is not the site of the primary defect in phytosterolemia. J. Lipid Res. 1998. 39: 1046–1054.**

Supplementary key words sitosterolemia • mevalonic acid • cholestyramine • family linkage studies • HRase gene • cholesterol synthesis • plant sterols • lathosterol

Phytosterolemia (sitosterolemia) is an autosomal recessive disorder characterized by excessive absorption of plant sterols (PS), early xanthomatosis, and premature coronary artery disease (1–3). To date at least 34 cases have been reported in the literature (3–5). Studies have demonstrated that absorption of sitosterol in phytosterolemic patients is 20–30% in contrast to approximately 5% in normal subjects (3, 6, 7). Shellfish sterols are also absorbed in excess (8). The concentration of PS in plasma from phytosterolemic patients varies between 15 and 27% of total sterol content (3) and increased concentrations are also found in cell membranes, xanthomas, and atherosclerotic plaques, although cholesterol remains the predominant lipid (3, 8–10). Isotopic studies indicate a marked (up to 80-fold) expansion of the plant sterol body pool in phytosterolemic subjects but very little difference between obligate heterozygotes and normals (6, 11, 12).

The pathophysiological basis for these findings involves abnormalities of both the absorption and excretion of PS. In addition to the markedly enhanced absorption of PS in phytosterolemic patients and to a lesser extent in heterozygotes, several studies have shown a significantly reduced rate of sitosterol excretion in homozygotes (6, 11, 12). The ability to concentrate sitosterol in bile is reduced (8, 11) and sitosterol is unable to undergo 7α-hydroxylation, the first step in bile acid synthesis (13–15). In heterozy-

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HRase, HMG-CoA reductase; PS, plant sterols; MVA, mevalonic acid; RFLP, restriction fragment length polymorphism; VNTR, variable number tandem repeats; PCR, polymerase chain reaction; LDL, low density lipoprotein; L/C, lathosterol/cholesterol.

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gotes, however, excretion is almost normal, thus accounting for the absence of PS accumulation and clinical manifestations (16).

In vivo and in vitro studies have uniformly demonstrated diminished rates of cholesterol synthesis in this disorder (6, 11, 12). Nguyen and co-workers (17, 18) have reported low levels of HMG-CoA reductase (HRase) mass and activity in mononuclear leucocytes, and in liver biopsy and postmortem material. Levels of HRase mRNA were also reduced in liver (18). Catalytic efficiency was normal or increased, with a greater proportion of the total HRase activity in the active form in phytosterolemic microsomes, suggesting reduced enzyme synthesis rather than an abnormal enzyme protein. HRase in mononuclear leucocytes anomalously decreased in response to cholesterol lowering by cholestyramine in phytosterolemic patients (17). These results suggested the possibility that HRase regulation may be the site of the primary defect in phytosterolemic patients but to date no mutational basis for the disease has been reported. A number of alternative explanations have been proposed (3) but conclusive evidence is lacking.

We examined the basis for the reduced HRase activity using two different approaches. In one we determined the segregation of the disease phenotype with DNA polymorphisms within the HRase gene in linkage studies performed on three informative phytosterolemic families. The second approach involved administration of cholestyramine, a bile acid sequestrant, to two phytosterolemic sisters, to four obligate heterozygotes, and to three healthy, unrelated control subjects. Fasting plasma mevalonic acid (MVA) and lathosterol were measured prior to and during the administration of bile acid sequestrant to assess indirectly changes in the rate of cholesterol biosynthesis and hepatic HRase activity. Additionally, the diurnal rhythm and response to an HRase inhibitor, simvastatin, were monitored by determining plasma MVA levels in a phytosterolemic patient and her mother.

METHODS

Subjects

Of the five phytosterolemic families investigated (3 from South Africa, 1 Japanese, and 1 American) in this study, four (families C, K, N and R) have been reported previously (5, 19). The phytosterolemic subjects had sitosterol values ranging between 0.35 and 1.21 mmol/l whereas levels in their obligate heterozygote parents were undetectable or less than 0.05 mmol/l. Xanthomatosis was prominent in most of the homozygous phytosterolemic subjects. Some of the patients were being treated with cholestyramine in addition to a low phytosterol diet with a substantial fall in plasma sitosterol levels and regression of xanthomatosis. **Table 1** summarizes the biochemical and clinical data on the three families and controls involved in the physiologic studies.

Family linkage study

Segregation of the HRase loci was studied using previously described polymorphisms (20, 21): a variable number tandem repeat (VNTR) Alu sequence-related polymorphism situated 10 kb 3′ of exon 2, and a ScrFI restriction fragment length polymorphism (RFLP) in intron 2 of the gene. Genomic DNA was salt-extracted from the white cell fraction of EDTA-blood collected from members of four families, three of which were informative. The R kindred was not studied as it comprised only mother and daughter. The VNTR region was PCR amplified as described by Zuliani and Hobbs (20); one of the primers (GZ-1) was ^{32}P-endlabeled by polynucleotide kinase. The products were denatured with formamide, separated by denaturing 8% acrylamide–urea gels, and subjected to autoradiography. Allele sizes were assigned relative to each run and all three families (14 individuals) were analyzed on the same gel. To determine the RFLP alleles in family K, DNA was amplified using previously reported PCR conditions and primers (21). The product was restricted with the enzyme ScrFI, and the multiple fragments ranging from 430 to 42 base pairs were separated on a 12% polyacrylamide gel. The two polymorphic alleles, **a** and **b**, were identified by bands of 165 (absence of the cutting site, **a**) or 120 + 45 (presence of the cutting site, **b**) base pairs. Parentage in this family was confirmed using a set of five polymorphic markers.

Cholestyramine provocation study

At least 2 weeks prior to the commencement of this study bile acid sequestrant therapy was stopped in the homozygote subjects, HK and ZK, and in the heterozygote, AK. Baseline blood samples were taken from the homozygotes and a second set of baseline blood samples 2 weeks later. The four obligate heterozygote parents (families K and C) were sampled on a single occasion only in the baseline period, whereas the three controls were sampled twice at an interval of 1 week. Immediately after the baseline collections, cholestyramine (Questran®, Mead Johnson) was given twice daily (8 g/day) to all subjects. After 3 weeks blood samples were collected and the intake of bile acid sequestrant was reduced to 6 g/day before taking a further blood sample 2 weeks later from all subjects except BeC. These studies were not carried out under metabolic ward conditions but both homozygotes remained on their low plant sterol diets throughout.

HMG-CoA reductase inhibition study

Acute mevalonic acid suppression test with simvastatin (Zocor, MSD). Patient JR and her heterozygote mother (PR) had plasma MVA levels measured at 09:00, 10:00, 12:00, 14:00, and 16:00 h to analyze diurnal rhythm (day 1). The next day (day 2) the same protocol was followed except that 40 mg simvastatin was administered immediately after the 09:00 h sample. Both subjects were

TABLE 1. Personal and clinical data on experimental subjects

	Age	Sex	CHOL	TRIG	SITO	Xanthomas
	yr		*mmol/l*	*mmol/l*	*mmol/l*	
Phytosterolemics						
HK	12	F	8.30	0.88	1.15	yes
ZK	8	F	6.14	0.83	0.63	no
JR	19	F	6.60	0.90	1.21	yes
Heterozygotes						
AK	45	M	8.63	1.66	0.05	no
MK	35	F	5.95	0.98	ND	no
BaC	40	M	5.61	1.64	0.03	no
BeC	37	F	4.88	1.25	ND	no
PR	53	F	7.90	1.90	0.01	no
Controls						
DP	28	M	4.04	0.43	—	no
BC	21	M	3.61	0.27	—	no
ED	23	M	4.37	0.71	—	no

CHOL, cholesterol; TRIG, triglyceride; SITO, sitosterol.

kept on a cholesterol-free diet containing less than 5% fat on each of these days.

Short-term administration of simvastatin in obligate heterozygotes and normolipidemic subjects. After baseline blood samples were collected, simvastatin (20 mg/daily) was administered for 8 days to the four obligate heterozygote parents in the K and C families and to four normolipidaemic subjects. At the same time plant sterol intake was increased to roughly 400 mg daily by including a variety of foodstuffs rich in plant sterols in the diet of the participants. Blood samples were collected after 4 and 8 days for analysis.

Blood was sampled between 8:00 and 9:00 h after an overnight fast in all the experimental subjects. Samples for cholesterol and PS assay were collected into tubes without anticoagulant and allowed to clot for 1 h before centrifugation at 3000 rpm for 15 min. The serum was stored at −70°C prior to analysis. Blood for MVA and lathosterol assay was collected into EDTA-containing tubes on ice, spun in a refrigerated centrifuge, and the plasma was stored at −70°C or on dry ice prior to analysis.

The methods used for cholesterol and plant sterol assay were essentially as described previously (5), except that the sensitivity for the detection of plant sterols was increased 6-fold by altering the attenuation on the gas chromatograph and by increasing the injection volume of the methylene chloride extract 3-fold. Mevalonic acid was determined by gas chromatography electron capture mass spectrometry. The method is fully detailed elsewhere (22, 23) and is summarized briefly below. After addition of [$^2H_3$]MVA to each sample as internal standard, MVA was converted into the lactone form using Dowex 50 (H^+) and then extracted into dichloromethane-propan-2-ol. After purification and conversion back to the acid form, MVA was esterified to the 3,5 *bis* (trifluor-methyl)benzylester and the trimethylsilyl derivative was prepared using *bis* (trimethylsilyl)-trifluoroacetamide. Derivatized samples were analyzed on a Finnigan 4500 quadropole mass spectrometer. This instrument uses electron capture and selected ion monitoring of ions at m/z ratios of 291 and 294 for detection of the derivatized MVA and [$^2H_3$]MVA, respectively; the intra-assay and the interassay coefficients of variation were 3.5% and 6.0%, respectively.

Serum concentrations of 7-lathosterol were determined by the method described by Wolthers et al. (24), using a PYE 4500 analytical Gas Chromatograph (Pye Unicam, Cambridge, UK) equipped with a fused capillary column (SAC-5, Sigma-Aldrich, Poole, Dorset). Helium was used as carrier gas. Lathosterol is reported in μmol/l and is also expressed as the L/C ratio so as to correct for changes in serum levels attributable to decreases in low density lipoprotein during treatment with cholestyramine (25).

Statistical analysis

Analysis of variance was used to compare results obtained in the phytosterolemic patients, heterozygotes and control subjects. Results within each group were analyzed using the paired *t*-test.

Ethical approval

Approval for this study was obtained from the Ethics Committee of the Faculty of Medicine, University of Natal. The subjects participated on a voluntary basis and were free to withdraw at any time.

RESULTS

Family linkage study

When the VNTR bands were ranked in terms of mobility, five distinct size alleles were detected in the three informative families (**Fig. 1**). In all three families the VNTR alleles segregated independently of the disease phenotype. In family K this was confirmed by an informative ScrFI RFLP which also segregated independently of the disease phenotype (**Fig. 2**). The one phytosterolemic sister inherited the **a** allele from her father and the **b** allele from her mother whereas the second affected sib, HK, was homozygous (**b/b**) which implies that she must have inherited the **b** allele from her father. Biological parentage in this kindred was confirmed by tracing the segregation of five polymorphic loci: the apolipoprotein E locus on chromosome 19 (26), the myotonic dystrophy locus (a trinucleotide repeat) on chromosome 19 (27), the platelet glycoprotein IIIa polymorphism on chromosome 17 (28), and the p53 Hae III and p53 MspI RFLPs on chromosome 17 (29, 30). In four of the five loci, at least one of the parents was heterozygous. The distribution of the polymorphic alleles was compatible with biological parentage in each case. The fourth family was uninformative for both polymorphisms.

Cholestyramine study

The baseline MVA levels and changes in response to cholestyramine are shown in **Fig. 3**. In the two phytosterolemic patients, the mean baseline level was 2.4 μg/l, in the

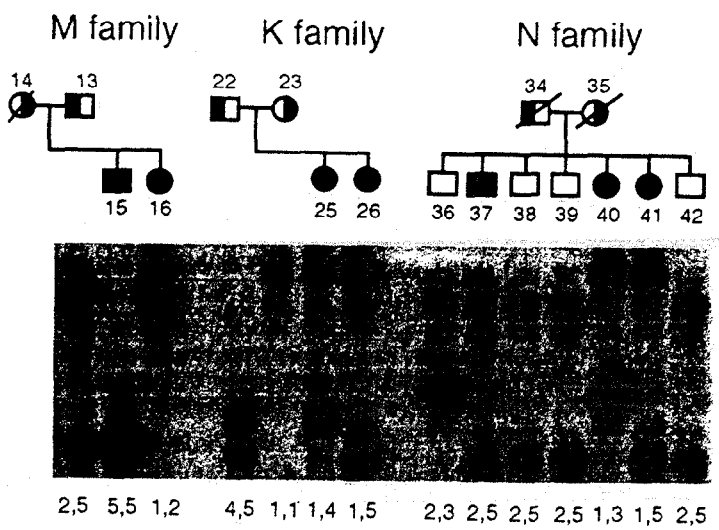

Fig. 1. Autoradiograph showing segregation of a VNTR in intron 2 of the HRase gene in three families. A total of five alleles differing in size were detected; in each family the affected children, M15 and M16, K25 and K26, and N37, N40 and N41, inherited different alleles. Although the parents were not available for genotyping in families M and N, the presence of at least three differing alleles within each family allows for informative segregation and exclusion.

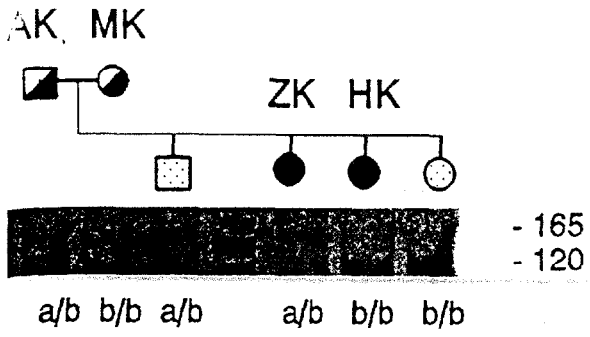

Fig. 2. Polyacrylamide gel (12%) electrophoresis showing segregation of ScrFI digested products of an intron 2 segment of the HRase gene in the K family. Heterozygosity for the restriction site within the 165 base pair fragments is indicated by **a/b** while **b/b** indicates homozygosity for the presence of this cutting site. The two affected siblings are ZK and HK.

four obligate heterozygotes the mean was 5.4 μg/l (range 2.5–8.3 μg/l), and in the three control subjects the MVA level was 4.5 μg/l (range 4.1–4.9 μg/l). The mean MVA concentration in 21 healthy adults was 6.1 μg/l (SD, 2.6 μg/l), as reported previously (31). Administration of cholestyramine resulted in an increase in MVA in all groups: 7.7-fold in the homozygote patients (range 7.2–8.2), 2.2-fold (range 0–3.0) in the heterozygotes, and 2.3-fold (range 1.6–2.7) in the control subjects. The relative changes in MVA were more pronounced ($P = 0.0009$) in the two phytosterolemics, who had lower baseline concentrations. The changes in the other two groups when pooled were less marked, but statistically significant ($P = 0.019$). Treatment with cholestyramine (Fig. 3) also induced increases in the L/C ratio in the two sitosterolemic patients (3.8- and 7.2-fold), in the three controls (2.1- to 2.8-fold), and in the four heterozygotes (0- to 1.5-fold) which were comparable to the changes observed in plasma MVA. As with the latter, the changes in the L/C ratio were more marked in the patients than in the other subjects ($P = 0.017$). The treatment was continued for a further 2 weeks using a lower concentration of cholestyramine (see Methods) resulting in a fall in MVA levels, which, however, remained above baseline in the two homozygotes and heterozygotes.

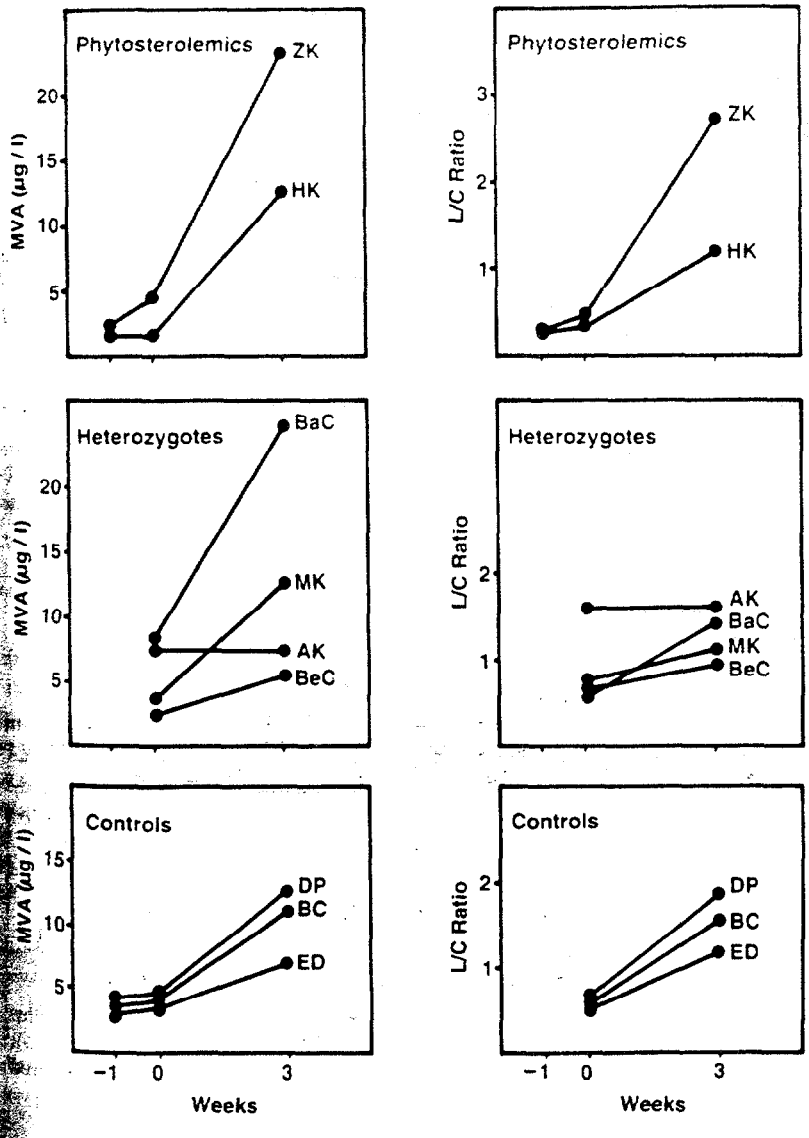

Fig. 3. Changes in baseline MVA levels (μg/l) and L/C ratios in response to treatment with cholestyramine in patients with phytosterolemia, heterozygotes and controls.

TABLE 2. Results of cholestyramine provocation study

	Lathosterol			Cholesterol			Sitosterol			
	Basal		Week 3	Basal		Week 3	Week 5	Basal		Week 3	Week 5
	μmol/l			mmol/l				mmol/l			
Phytosterolemics											
HK	0.4	0.8	3.8	5.05	6.20	3.20	3.10	0.91	1.01	0.69	0.62
ZK	1.6	2.4	9.6	4.95	5.56	3.52	3.78	0.57	0.60	0.50	0.50
Heterozygotes											
AK	—	12.4	11.3	—	7.60	7.00	6.40				
MK	—	4.2	5.7	—	5.42	5.00	5.24				
BaC	—	4.3	8.4	—	7.47	5.80	6.30				
BeC	—	3.4	4.1	—	5.10	4.45	—				
Controls											
DP	—	2.7	6.5	—	4.17	3.47	3.96				
BC	—	2.5	5.7	—	4.23	3.60	3.76				
ED	—	2.6	4.6	—	4.50	3.87	4.19				

Cholesterol levels fell in all subjects after 3 weeks of cholestyramine administration (**Table 2**), indicating reasonable compliance with the protocol. The relative fall in cholesterol was greater in the phytosterolemic patients than in the other subjects (as previously reported). Sitosterol concentrations diminished markedly in HK but only slightly in ZK. In the heterozygote and control subjects, baseline sitosterol levels were extremely low and the changes after cholestyramine intake were inconsistent.

HMG CoA reductase inhibition study

Results of the acute MVA suppression test with simvastatin in patient JR and her heterozygote mother are presented in **Fig. 4**. The mother showed normal fasting plasma MVA levels and a normal diurnal rhythm (day 1) whereas patient JR had low baseline levels. The single 40-mg dose of simvastatin reduced plasma MVA levels by 70% in the mother but had no effect in JR. Eight days administration of simvastatin to heterozygotes and controls caused a slight decrease in cholesterol levels ($P = 0.04$) but no change in either sitosterol or campesterol (**Table 3**).

DISCUSSION

The polymorphism linkage data conclusively establish that in the three informative families studied phytosterolemia was not inherited through a genetic defect at the HRase gene locus. The VNTR data were further strengthened in family K using a second polymorphism (ScrFI RFLP), together with biological proof of parentage. It is reasonable to assume that the consistency of the data across all three families renders this interpretation applicable to the majority of phytosterolemic kindreds in South Africa, Japan, and the United States.

The implications of the genetic data were further studied and extended by measuring changes in MVA and lathosterol concentrations in response to cholestyramine administration. Measurement of plasma levels and urinary excretion of MVA have been shown to be good indices of the in vivo rate of cholesterol synthesis (32, 33). A diurnal rhythm of plasma MVA has been described in humans (34) that correlated closely with the incorporation of deuterium into plasma free cholesterol (35). Whole body cholesterol synthesis as measured by sterol balance has been shown to correlate closely with fasting plasma MVA (32) under metabolic ward conditions and with urinary MVA

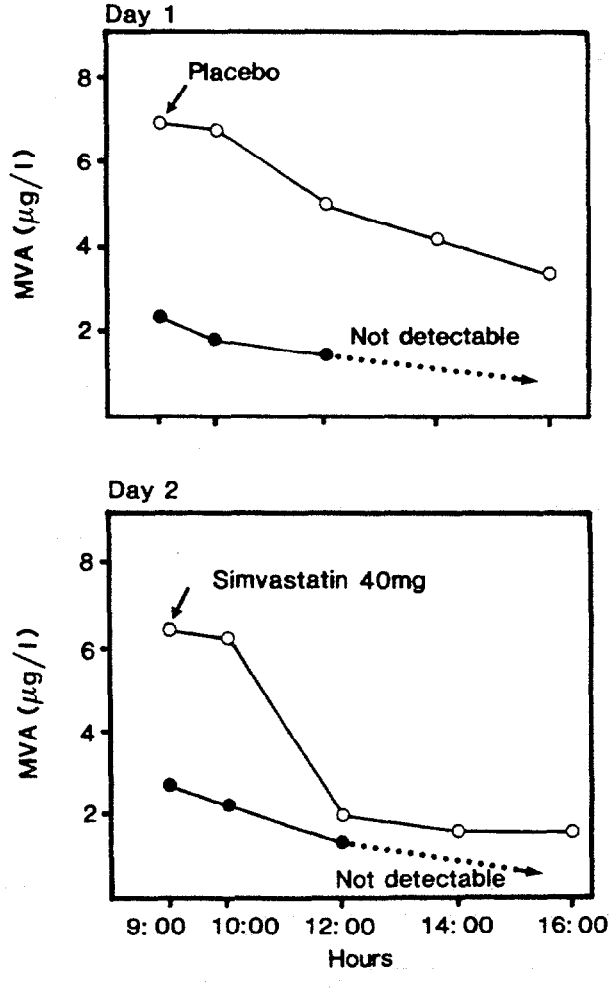

Fig. 4. MVA levels (μg/l) after suppression with simvastatin. Phytosterolemic patient (●) and her obligate heterozygote mother (○). Day 1, placebo; day 2, single dose of simvastatin 40 mg administered at 09:00 h.

TABLE 3. Effect of simvastatin on plasma sterol levels

	Baseline			Day 4			Day 8		
	Chol	Sito	Camp	Chol	Sito	Camp	Chol	Sito	Camp
	mmol/l	*μmol/l*	*mmol/l*	*mmol/l*	*μmol/l*	*mmol/l*	*mmol/l*	*μmol/l*	*mmol/l*
Heterozygotes									
AK	7.52	40.7	34.4	7.82	45.5	39.5	6.39	41.2	39.5
MK	6.24	25.4	22.1	5.63	22.3	19.3	4.67	25.3	22.8
BaC	5.32	12.0	5.5	5.03	13.2	0.0	4.15	15.7	8.5
BeC	5.21	4.6	3.9	4.91	9.6	7.7	4.39	11.5	7.5
Mean	6.07	20.7	16.5	5.85	22.6	16.6	4.90	23.4	19.6
Controls									
SM	5.55	13.3	23.0	5.05	12.0	20.1	4.74	8.5	20.9
PN	6.3	9.7	9.3	5.19	15.1	6.8	4.36	15.3	15.9
RR	4.28	12.4	11.4	3.63	6.2	7.7	3.77	9.6	10.7
GMB	5.39	11.3	10.1	4.67	11.0	10.3	4.29	11.1	7.5
Mean	5.38	11.7	13.4	4.63	11.1	11.2	4.29	11.1	13.7

excretion (36). In addition, plasma MVA levels correlate well with HRase activity in human liver (37). Furthermore, plasma levels and urinary excretion of MVA have been shown to decrease after treatment with HMG-CoA reductase inhibitors and to increase after bile acid sequestrants (33, 38). Thus there is good evidence that fasting plasma MVA levels provide a valid albeit semi-quantitative index of cholesterol synthesis. The concentration of 7-lathosterol in plasma is another well-established index of whole body cholesterol synthesis in humans (25, 39, 40) that has been shown to correlate closely with hepatic HRase activity (41). Serum lathosterol concentration and the L/C ratio both increase during treatment with cholestyramine or colestipol (42, 43), which stimulate cholesterol biosynthesis in the liver, and decrease during treatment with an HMG-CoA reductase inhibitor (25, 42, 44).

In the present study intra-individual variation in plasma MVA levels was minimized by sampling at the same time in the morning to avoid the effects of the diurnal rhythm previously reported (32, 34), and by maintaining the same diet over the period of investigation. The increase in MVA was considerably greater in the phytosterolemic patients (Fig. 3) than in either the obligate heterozygote or control group. The within-subject variability of plasma MVA estimated from data obtained in 20 hypercholesterolemic patients, from whom fasting blood samples were obtained at 9 AM on three separate occasions in 1 week (45), was 12.4% (range 5.7–18.5%). By comparison, plasma MVA increased during treatment with cholestryramine by 7.2- to 8.2-fold in the phytosterolemic patients and up to 3-fold in heterozygous relatives and normolipidemic controls. Lathosterol levels (Table 2) and especially the L/C ratio (Fig. 3) increased pari passu with the plasma MVA changes. The changes in the MVA levels and the L/C ratio in both phytosterolemic subjects were considerably greater than could be accounted for by intra-individual variability.

Plasma MVA and lathosterol, measured in our study as markers of early and late steps of the cholesterol biosynthesis pathway, respectively, were both comparably increased. This excludes the unlikely event that MVA levels increased in response to inhibition of one or more steps in the conversion of mevalonate to cholesterol. In particular, both indices reflect predominantly hepatic HRase activity (37, 41). These results are compatible with the genetic data that conclusively demonstrate that the primary defect in sitosterolemia does not involve the HRase locus. They also support, but do not unambiguously prove, the stronger hypothesis that the primary defect does not down-regulate HRase synthesis at a genetic site remote from (*trans* to) the gene locus. The regulation of HRase activity is complex and is exerted at transcriptional and various post-transcriptional levels (46). Whatever the mechanism underlying the observed reduction in HRase mRNA and enzyme mass levels in sitosterolemia, it did not prevent the apparent increase in HRase activity elicited by cholestyramine in this study. As cholestyramine normally exerts its well-documented stimulation of HRase synthesis by reducing the intrahepatic level of the free sterol pool or pools responsible for the negative feedback control of HRase gene expression, it is reasonable to conclude that the same mechanism is operative in the two sitosterolemic homozygotes. Cobb et al. (47) reported a paradoxical decrease in the 24-h urinary excretion of MVA in a 10-year-old sitosterolemic girl given cholestyramine. As the subject also suffered from heterozygous familial hypercholesterolemia, the functional significance of this observation is difficult to determine. Considering the most plausible interpretaion of our data, two further questions arise. First, what further evidence exists in favor of suppression of basal HRase activity and cholesterol biosynthesis in phytosterolemia, secondary to the biochemical consequences of the primary defect? Second, what role, if any, does the diminished HRase activity play in accounting for the accumulation of plant sterols characteristic of this disorder?

It is not clear whether increased quantities of sitosterol alone can account for the suppression of HRase activity. In rat ileum and rat liver, increased sitosterol concentrations induced by feeding and infusion experiments, respectively, failed to inhibit HRase activity or reduce mRNA levels at concentrations similar to those found in phytosterolemic homozygotes (48–50). At these concentrations 7α-hydrox-

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Effect of plant sterol-enriched margarine on plasma lipids and sterols in subjects heterozygous for phytosterolaemia

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Abstract. Stalenhoef AFH, Hectors M, Demacker PNM (University Medical Centre Nijmegen, Nijmegen, The Netherlands). Effect of plant sterol-enriched margarine on plasma lipids and sterols in subjects heterozygous for phytosterolaemia. *J Intern Med* 2001; **249:** 163–166.

Objective. Margarine with added plant sterols lowers plasma cholesterol levels. It is of importance to know whether these margarines can be used safely in carriers of a hereditary disorder with increased absorption of plant sterols.

Design. In an open feeding study of 8 weeks with a 2-week run-in period and 2 final weeks as a washout period on control margarine (0.3% plant sterols), two obligate heterozygous parents of a patient with classical sitosterolaemia were subjected for 4 weeks to a diet containing margarine enriched with plant sterols (8%). Fasting blood samples were taken weekly. Primary outcomes were plasma lipid and lipoprotein levels and plant sterol levels.

Results. Both parents were hyperlipidaemic. Total plasma cholesterol levels were decreased by 11 and 12%, respectively, after 4 weeks of the consumption of 40 g day^{-1} of plant sterol-enriched margarine. This was mainly due to changes in LDL-cholesterol, whereas the other lipoproteins, including lipoprotein(a), were unaffected. Total plant sterol levels increased maximally 139% from 0.31 to 0.82% of total sterols in the father, and maximally 83% from 0.32 to 0.66% of total sterols in the mother.

Conclusion. An intake of around 3 g day^{-1} of plant sterols by subjects heterozygous for phytosterolaemia increased campesterol or sitosterol levels in blood to similar levels as found in normal subjects. In addition, plasma cholesterol levels were reduced to the same extent as in normal or hypercholesterolaemic individuals.

Keywords: cholesterol, margarine, phytosterolaemia, plant sterols.

Introduction

Phytosterolaemia (also known as sitosterolaemia) is a rare, recessively inherited disorder characterized by extremely high plasma levels of plant sterols, xanthomas and premature coronary heart disease [1]. Under normal circumstances, the Western diet contains almost equal amounts of cholesterol and plant sterols. However, 30–60% of total dietary cholesterol and only <5% of total plant sterols are normally absorbed in the gut [2]. Affected patients have uncontrollable sterol absorption, higher plasma and tissue cholesterol and phytosterol concentrations and low endogenous cholesterol synthesis [3]. Lowering dietary cholesterol and plant sterol intake is one of the first obvious approaches. Heterozygous carriers of this disorder are clinically and biochemically normal [4].

Recently, a new cholesterol-lowering spread has been introduced that contains 8% of plant sterols (Becel pro-aktiv®, Unilever, Vlaardingen). Such a concentration will increase the individual daily intake of plant sterols by five- to 10-fold [5]. Thus, the question arises about the impact of such elevated intake of plant sterols on subjects heterozygous for phytosterolaemia. We therefore investigated the effect of feeding margarine enriched with plant sterols on plasma lipoproteins and sterols in two heterozygous parents of a patient with classical phytosterolaemia.

Materials and methods

The proband (female, age 28 years) was identified at 16 years old on the basis of the presence of tendon and tuberous xanthomas and extremely high (50- to 100-fold increased) plasma sterol levels [4]. Her parents participated in this study. They were both 52 years of age, hypercholesterolaemic and obese (BMI, 33.9 and 35.0 kg m^{-2}, respectively). The feeding study consisted of 8 weeks with a 2-week run-in period and 2 final weeks as a washout period. They first consumed control margarine (40 g day^{-1}, 35% fat and 0.3% plant sterols) for 2 weeks; after that they consumed the margarine enriched with plant sterols (8.2%) with no modification to their usual diet. Fatty acid composition of both margarines was similar (48% polyunsaturated fatty acids). The chemical composition of the margarines has been described in detail [5]. Blood samples were collected at −2, 0, 1, 2, 3, 4 and 6 weeks. Subjects gave signed written informed consent. Full-week dietary records were filled out by the two subjects. Compliance evaluated by counting the tubs was 100%; the body weights did not change during the study period.

Plasma cholesterol and triglycerides were determined enzymatically in fasting samples. High-density lipoprotein (HDL) cholesterol was determined by the phosphotungstic acid/Mg^{2+} precipitation method [6]. Very low density cholesterol (VLDL) was isolated by ultracentrifugation at $d = 1.006$ g mL^{-1} (Beckman, Palo Alto, CA). LDL cholesterol was calculated by subtraction. ApoB and apoA1 were quantified by immunonephelometry [7]. Lipoprotein(a) was measured by a specific radioimmunoassay (Mercodia AB, Uppsala, Sweden). Plant sterol levels were measured using gas chromatography following established methodology [8, 9]. Briefly, 0.1 mL plasma was saponified with KOH for 1 h at 37 °C. Extracted samples were derivatized with BSTFA/1% TMCS and injected into a Varian 3400 gas chromatograph (Sunnyvale, CA) equipped with a flame ionization detector and a data processing system, using coprostanol as an internal standard. Campesterol, stigmasterol and sitosterol were identified using authenticated standards (Sigma, St Louis, MO).

Results

The father exhibited mixed hyperlipidaemia, which was known for several years; the mother was also hypercholesterolaemic (Table 1). The consumption of plant sterol-enriched margarine did not cause side-effects. Total plasma cholesterol levels were decreased by 11 and 12% in the parents, respectively, after 4 weeks of the consumption of 40 g day^{-1}

Table 1 Plasma lipids, lipoprotein and apolipoprotein levels and lipoprotein(a) in heterozygous subjects consuming margarine enriched in plant sterols

	Cholesterol (mM)	VLDL chol. (MM)	LDL chol. (MM)	HDL chol. (MM)	Triglycerides (mM)	ApoAI (mg L^{-1})	ApoB (mg L^{-1})	Lp(a) (Mg L^{-1})
Father								
Start run-in	8.50	2.08	5.58	0.84	3.90	1360	1620	< 16
Start (0)	8.46	2.62	5.03	0.81	3.89	1345	1570	< 16
T = 1 week	7.59	1.80	4.95	0.84	3.46	1295	1450	< 16
T = 2 weeks	7.41	1.74	4.79	0.88	3.45	1395	1390	< 16
T = 3 weeks	7.67	2.09	4.74	0.84	3.49	1285	1495	< 16
T = 4 weeks	7.53	2.18	4.48	0.88	3.80	1325	1405	< 16
After 2 weeks washout	7.88	2.21	4.86	0.81	4.12	1255	1450	< 16
Mother								
Start run-in	6.17	0.38	4.70	1.09	1.57	1470	1295	92
Start (0)	6.19	0.55	4.37	0.91	1.97	1400	1285	94
T = 1 week	5.36	0.34	4.04	0.98	1.42	1325	1145	95
T = 2 weeks	5.25	0.55	3.79	0.91	2.06	1290	1120	90
T = 3 weeks	5.56	0.45	4.13	0.98	1.68	1295	1160	86
T = 4 weeks	5.46	0.55	3.90	1.01	1.91	1335	1150	82
After 2 weeks washout	5.81	0.61	4.32	0.88	2.10	1285	1265	78

Table 2 Plasma total cholesterol and plant sterol in heterozygous subjects consuming margarine enriched in plant sterols

	Cholesterol		Campesterol	Stigmasterol	Sitosterol	Total plant sterols		
	mM	% change	(μM)	(μM)	(μM)	μM	% change	% total sterols
Father								
Start run-in	8.50	–	9.80	1.10	11.15	22.05	–	0.26
Start (0)	8.46	0.0	12.25	1.35	12.90	26.50	0	0.31
T = 1 week	7.59	−10.3	22.35	1.30	17.15	40.80	54	0.62
T = 2 weeks	7.41	−12.4	27.20	1.25	17.60	46.05	74	0.65
T = 3 weeks	7.67	−9.3	37.80	2.00	23.45	63.25	139	0.82
T = 4 weeks	7.53	−11.0	34.25	1.60	20.10	55.95	111	0.74
After 2 weeks washout	7.88	−6.9	16.50	1.70	14.00	32.20	22	0.41
Mother								
Start run-in	6.17	–	8.35	0.50	10.70	19.55	–	0.32
Start (0)	6.19	0.0	9.15	0.00	10.60	19.75	0	0.32
T = 1 week	5.36	−13.4	13.85	1.20	12.25	27.30	38	0.51
T = 2 weeks	5.25	−15.2	16.80	1.30	13.35	31.45	58	0.60
T = 3 weeks	5.56	−10.2	15.75	1.35	11.65	28.75	46	0.51
T = 4 weeks	5.46	−11.8	19.25	1.60	15.20	36.05	83	0.66
After 2 weeks washout	5.81	−6.1	10.50	0.50	10.55	21.55	9	0.37

plant sterol-enriched margarine (Table 1). This was mainly due to changes in LDL cholesterol, whereas the other lipoproteins, including Lp(a) were unaffected. The decrease in apoB was in the same order as that in serum cholesterol. Total plant sterol levels increased maximally 139% from 0.31 to 0.82% of total sterols in the father, and maximally 83% from 0.32 to 0.66% of total sterols in the mother (Table 2). After 3 weeks of consumption of plant sterols, their blood levels seemed to have reached equilibrium with no further increase. In 33 healthy control subjects, fasting plasma campesterol levels on their normal diet ranged from 3.8 to 24.2 μmol L^{-1} (mean 13.1) and plasma sitosterol from 2.1 to 16.5 μmol L^{-1} (mean 8.9).

Discussion

Daily intake of plant sterol-enriched margarine lowered blood total cholesterol levels in the subjects heterozygous for phytosterolaemia by an average of 10.2 and 11.0%, respectively (average of weeks 3 and 4). This is in accordance with what has been reported earlier in normal and hypercholesterolaemic subjects [5, 10]. Of importance in this study was that an intake of around 3 g day^{-1} of plant sterols in the heterozygotes did not increase campesterol or sitosterol levels in blood to levels higher than reported in normal subjects consuming similar margarine [11, 12]. In addition, the increase in blood plant sterols observed in the two heterozygotes was slightly higher than observed in our healthy subjects on a normal diet and was less than 1% of blood total sterols. After 3 weeks of a steady consumption of plant sterols, blood levels of the subjects seemed to have reached equilibrium without further increase, but long-term follow-up studies are necessary to confirm this.

In conclusion, plant sterol-enriched margarine appears to be as efficacious in reducing cholesterol in subjects heterozygous for phytosterolaemia as in normal or hyperlipidaemic subjects, without strong accumulation of plant sterols in their plasma. As pointed out by Law [13], consumption of margarines containing plant sterols as well as stanols can be expected to reduce the risk of heart disease of about 25%.

Acknowledgements

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metabolic syndrome and associated lipid risk factors. The majority of persons with these latter abnormalities are overweight or obese and sedentary. Weight reduction therapy for overweight or obese patients will enhance LDL lowering and will provide other health benefits including modifying other lipid and nonlipid risk factors. Assistance in the management of overweight and obese persons is provided by the *Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults* from the NHLBI Obesity Education Initiative (1998). Additional risk reduction can be achieved by simultaneously increasing physical activity.

At all stages of dietary therapy, physicians are encouraged to refer patients to registered dietitians or other qualified nutritionists for *medical nutrition therapy*, which is the term for the nutritional intervention and guidance provided by a nutrition professional.

DRUG THERAPY TO ACHIEVE LDL CHOLESTEROL GOALS

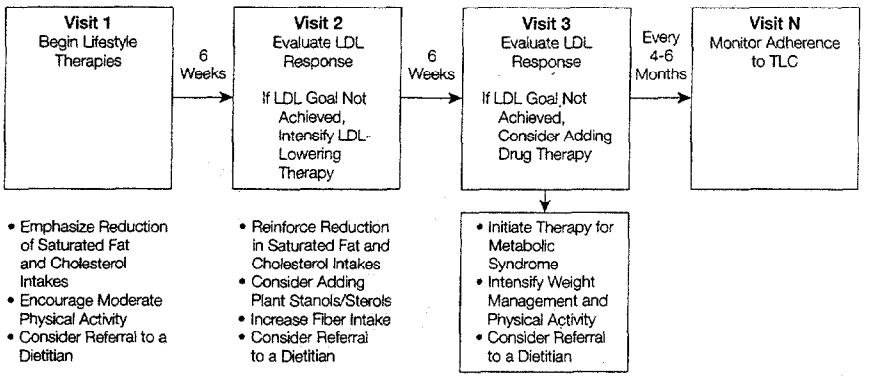
A portion of the population whose short-term or long-term risk for CHD is high will require LDL-lowering drugs in addition to TLC to reach the designated goal for LDL cholesterol (see Table 5). When drugs are prescribed, attention to TLC should always be maintained and reinforced. Currently available drugs that affect lipoprotein metabolism and their major characteristics are listed in TABLE 7.

Some cholesterol-lowering agents are currently available over-the-counter (OTC) (eg, nicotinic acid), and manufacturers of several classes of LDL-lowering drugs (eg, statins, bile acid sequestrants) have applied to the Food and Drug Administration (FDA) to allow these agents to become OTC medications. At the time of publication of ATP III, the FDA has not granted permission for OTC status for statins or bile acid sequestrants. If an OTC cholesterol-lowering drug is or becomes available, patients should continue to consult with their physicians about whether to initiate drug treatment, about setting the goals of therapy, and about monitoring for therapeutic responses and side effects.

Secondary Prevention: Drug Therapy for CHD and CHD Risk Equivalents

For persons with CHD and CHD risk equivalents, the goal is to attain an LDL cholesterol level of <100 mg/dL. The cutpoints for initiating lifestyle and drug

Figure 1. Model of Steps in Therapeutic Lifestyle Changes (TLC)



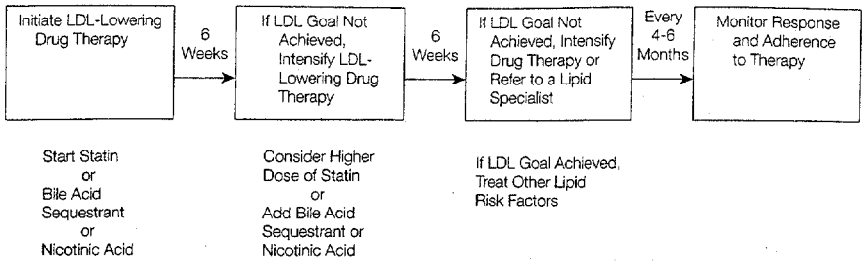
LDL indicates low-density lipoprotein.

Table 7. Drugs Affecting Lipoprotein Metabolism*

Drug Class, Agents, and Daily Doses	Lipid/Lipoprotein Effects	Side Effects	Contraindications	Clinical Trial Results
HMG-CoA reductase inhibitors (statins)†	LDL ↓ 18%-55% HDL ↑ 5%-15% TG ↓ 7%-30%	Myopathy; increased liver enzymes	Absolute: active or chronic liver disease Relative: concomitant use of certain drugs§	Reduced major coronary events, CHD deaths, need for coronary procedures, stroke, and total mortality
Bile acid sequestrants‡	LDL ↓ 15%-30% HDL ↑ 3%-5% TG No change or increase	Gastrointestinal distress; constipation; decreased absorption of other drugs	Absolute: dysbetalipoproteinemia; TG >400 mg/dL Relative:TG >200 mg/dL	Reduced major coronary events and CHD deaths
Nicotinic acid‖	LDL ↓ 5%-25% HDL ↑ 15%-35% TG ↓ 20%-50%	Flushing; hyperglycemia; hyperuricemia (or gout); upper gastrointestinal distress; hepatotoxicity	Absolute: chronic liver disease; severe gout Relative: diabetes; hyperuricemia; peptic ulcer disease	Reduced major coronary events, and possibly total mortality
Fibric acids¶	LDL ↓ 5%-20% (may be increased in patients with high TG) HDL ↑ 10%-20% TG ↓ 20%-50%	Dyspepsia; gallstones; myopathy; unexplained non-CHD deaths in WHO study	Absolute: severe renal disease; severe hepatic disease	Reduced major coronary events

*HMG-CoA indicates 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; ↓, decrease; ↑, increase; and CHD, coronary heart disease.
†Lovastatin (20-80 mg), pravastatin (20-40 mg), simvastatin (20-80 mg), fluvastatin (20-80 mg), atorvastatin (10-80 mg), and cerivastatin (0.4-0.8 mg).
‡Cholestyramine (4-16 g), colestipol (5-20 g), and colesevelam (2.6-3.8 g).
§Cyclosporine, macrolide antibiotics, various antifungal agents, and cytochrome P-450 inhibitors (fibrates and niacin should be used with appropriate caution).
‖Immediate-release (crystalline) nicotinic acid (1.5-3 g), extended-release nicotinic acid (1-2 g), and sustained-release nicotinic acid (1-2 g).
¶Gemfibrozil (600 mg twice daily), fenofibrate (200 mg), and clofibrate (1000 mg twice daily).

Figure 2. Progression of Drug Therapy in Primary Prevention



LDL indicates low-density lipoprotein.

therapies are shown in Table 5. Most patients with CHD will need LDL-lowering drug therapy. Other lipid risk factors may also warrant consideration of drug treatment. Whether or not lipid-modifying drugs are used, nonlipid risk factors require attention and favorable modification.

In patients admitted to the hospital for a major coronary event, LDL cholesterol should be measured on admission or within 24 hours. This value can be used for treatment decisions. In general, persons hospitalized for a coronary event or procedure should be discharged on drug therapy if the LDL cholesterol is ≥130 mg/dL. If the LDL is 100-129 mg/dL, clinical judgment should be used in deciding whether to initiate drug treatment at discharge, recognizing that LDL cholesterol levels begin to decline in the first few hours after an event and are significantly decreased by 24 to 48 hours and may remain low for many weeks. Thus, the initial LDL cholesterol level obtained in the hospital may be substantially lower than is usual for the patient. Some authorities hold that drug therapy should be initiated whenever a patient hospitalized for a CHD-related illness is found to have an LDL cholesterol >100 mg/dL. Initiation of drug therapy at the time of hospital discharge has 2 advantages. First, at that time patients are particularly motivated to undertake and adhere to risk-lowering interventions; and second, failure to initiate indicated therapy early is one of the causes of a large "treatment gap," because outpatient follow-up is often less consistent and more fragmented.

LDL-Lowering Drug Therapy for Primary Prevention

Table 5 shows the cutpoints for considering drug treatment in primary prevention. The general approach to management of drug therapy for primary prevention is outlined in FIGURE 2.

When drug therapy for primary prevention is a consideration, the third visit of dietary therapy (see Figure 1) will typically be the visit to initiate drug treatment. Even if drug treatment is started, TLC should be continued. As with TLC, the first priority of drug therapy is to achieve the goal for LDL cholesterol. For this reason, an LDL-lowering drug should be started. The usual drug will be a statin, but alternatives are a bile acid sequestrant or nicotinic acid. In most cases, the statin should be started at a moderate dose. In many patients, the LDL cholesterol goal will be achieved, and higher doses will not be necessary. The patient's response should be evaluated about 6 weeks after starting drug therapy. If the goal of therapy has been achieved, the current dose can be maintained. However, if the goal has not been achieved, LDL-lowering therapy can be intensified, either by increasing the dose of statin or by combining a statin with a bile acid sequestrant or nicotinic acid.

After 12 weeks of drug therapy, the response to therapy should again be assessed. If the LDL cholesterol goal is still not achieved, consideration can be given to further intensification of drug therapy. If the LDL goal cannot be attained by standard lipid-lowering therapy, consideration should be given to seeking consultation from a lipid specialist. Once the goal for LDL cholesterol has been attained, attention can turn to other lipid risk factors and nonlipid factors. Thereafter, patients can be monitored for response to therapy every 4 to 6 months, or more often if considered necessary.

BENEFIT BEYOND LDL LOWERING: THE METABOLIC SYNDROME AS A SECONDARY TARGET OF THERAPY

Evidence is accumulating that risk for CHD can be reduced beyond LDL-lowering therapy by modification of other risk factors. One potential secondary target of therapy is the metabolic syndrome, which represents a constellation of lipid and nonlipid risk factors of metabolic origin. This syndrome is closely linked to a generalized metabolic disorder called *insulin resistance* in which the normal actions of insulin are impaired. Excess body fat (particularly abdominal obesity) and physical inactivity promote the development of insulin resistance, but some individuals also are genetically predisposed to insulin resistance.

The risk factors of the metabolic syndrome are highly concordant; in aggregate they enhance risk for CHD at any given LDL cholesterol level. For purposes of ATP III, the diagnosis of the metabolic syndrome is made when 3 or more of the risk determinants shown in TABLE 8 are present. These determinants include a combination of categorical and borderline risk factors that can be readily measured in clinical practice.

Management of the metabolic syndrome has a 2-fold objective: (1) to reduce underlying causes (ie, obesity and physical inactivity) and (2) to treat associated nonlipid and lipid risk factors.

Management of Underlying Causes of the Metabolic Syndrome

First-line therapies for all lipid and nonlipid risk factors associated with the metabolic syndrome are weight reduction and increased physical activity,

which will effectively reduce all of these risk factors. Therefore, after appropriate control of LDL cholesterol, TLC should stress weight reduction and physical activity if the metabolic syndrome is present.

Weight Control. In ATP III overweight and obesity are recognized as major, underlying risk factors for CHD and identified as direct targets of intervention. Weight reduction will enhance LDL lowering and reduce all of the risk factors of the metabolic syndrome. The recommended approaches for reducing overweight and obesity are contained in the clinical guidelines of the Obesity Education Initiative.

Physical Activity. Physical inactivity is likewise a major, underlying risk factor for CHD. It augments the lipid and nonlipid risk factors of the metabolic syndrome. It further may enhance risk by impairing cardiovascular fitness and coronary blood flow. Regular physical activity reduces very low-density lipoprotein (VLDL) levels, raises HDL cholesterol, and in some persons, lowers LDL levels. It also can lower blood pressure, reduce insulin resistance, and favorably influence cardiovascular function. Thus, ATP III recommends that regular physical activity become a routine component in management of high serum cholesterol. The evidence base for this recommendation is contained in the US Surgeon General's Report on Physical Activity.

Specific Treatment of Lipid and Nonlipid Risk Factors

Beyond the underlying risk factors, therapies directed against the lipid and nonlipid risk factors of the metabolic syndrome will reduce CHD risk. These include treatment of hypertension, use of aspirin in patients with CHD to reduce the prothrombotic state (guidelines for aspirin use in primary prevention have not been firmly established), and treatment of elevated triglycerides and low HDL cholesterol as discussed below under "Management of Specific Dyslipidemias."

SPECIAL ISSUES

Management of Specific Dyslipidemias

Very High LDL Cholesterol (≥190 mg/dL). Persons with very high LDL cholesterol usually have genetic forms of hypercholesterolemia: monogenic familial hypercholesterolemia, familial defective apolipoprotein B, and polygenic hypercholesterolemia. Early detection of these disorders through cholesterol testing in young adults is needed to prevent premature CHD. Family testing is important to identify similarly affected relatives. These disorders often require combined drug therapy (statin + bile acid sequestrant) to achieve the goals of LDL-lowering therapy.

Elevated Serum Triglycerides. Recent meta-analyses of prospective studies indicate that elevated triglycerides are also an independent risk factor for CHD. Factors contributing to elevated (higher than normal) triglycerides in the general population include obesity and overweight, physical inactivity, cigarette smoking, excess alcohol intake, high-carbohydrate diets (>60% of energy intake), several diseases (eg, type 2 diabetes, chronic renal failure, nephrotic syndrome), certain drugs (eg, corticosteroids, estrogens, retinoids, higher doses of β-adrenergic blocking agents), and genetic disorders (familial combined hyperlipidemia, familial hypertriglyceridemia, and familial dysbetalipoproteinemia).

In clinical practice, elevated serum triglycerides are most often observed in persons with the metabolic syndrome, although secondary or genetic factors can heighten triglyceride levels. ATP III adopts the following classification of serum triglycerides:

- Normal triglycerides: <150 mg/dL
- Borderline-high triglycerides: 150-199 mg/dL
- High triglycerides: 200-499 mg/dL
- Very high triglycerides: ≥500 mg/dL

(To convert triglyceride values to mmol/L, divide by 88.6.)

The finding that elevated triglycerides are an independent CHD risk factor suggests that some triglyceride-rich lipoproteins are atherogenic. The latter are partially degraded VLDL, commonly called *remnant lipoproteins*. In clinical practice, VLDL cholesterol is the most readily available measure of atherogenic remnant lipoproteins. Thus, VLDL cholesterol can be a target of cholesterol-lowering therapy. ATP III identifies the sum of LDL+VLDL cholesterol (termed *non-HDL cholesterol* [total cholesterol – HDL cholesterol]) as a secondary target of therapy in persons with high triglycerides (≥200 mg/dL). The goal for non-HDL cholesterol in persons with high serum triglycerides can be set at 30 mg/dL higher than that for LDL cholesterol (TABLE 9) on the premise that a VLDL cholesterol level ≤30 mg/dL is normal.

The treatment strategy for elevated triglycerides depends on the causes of the elevation and its severity. For all persons

Table 8. Clinical Identification of the Metabolic Syndrome

Risk Factor	Defining Level
• Abdominal obesity* (waist circumference)†	
Men	>102 cm (>40 in)
Women	>88 cm (>35 in)
• Triglycerides	≥150 mg/dL
• High-density lipoprotein cholesterol	
Men	<40 mg/dL
Women	<50 mg/dL
• Blood pressure	≥130/≥85 mm Hg
• Fasting glucose	≥110 mg/dL

*Overweight and obesity are associated with insulin resistance and the metabolic syndrome. However, the presence of abdominal obesity is more highly correlated with the metabolic risk factors than is an elevated body mass index (BMI). Therefore, the simple measure of waist circumference is recommended to identify the body weight component of the metabolic syndrome.

†Some male patients can develop multiple metabolic risk factors when the waist circumference is only marginally increased, eg, 94-102 cm (37-40 in). Such patients may have strong genetic contribution to insulin resistance and they should benefit from changes in life habits, similarly to men with categorical increases in waist circumference.

Table 9. Comparison of LDL Cholesterol and Non-HDL Cholesterol Goals for 3 Risk Categories*

Risk Category	LDL Goal (mg/dL)	Non-HDL Goal (mg/dL)
CHD and CHD risk equivalent (10-year risk for CHD >20%)	<100	<130
Multiple (2+) risk factors and 10-year risk ≤20%	<130	<160
0-1 Risk factor	<160	<190

*LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; and CHD, coronary heart disease.

with borderline high or high triglycerides, the primary aim of therapy is to achieve the target goal for LDL cholesterol. When triglycerides are borderline high (150-199 mg/dL), emphasis should also be placed on weight reduction and increased physical activity. For high triglycerides (200-499 mg/dL), non-HDL cholesterol becomes a secondary target of therapy. Aside from weight reduction and increased physical activity, drug therapy can be considered in high-risk persons to achieve the non-HDL cholesterol goal. There are 2 approaches to drug therapy. First, the non-HDL cholesterol goal can be achieved by intensifying therapy with an LDL-lowering drug; second, nicotinic acid or fibrate can be added, if used with appropriate caution, to achieve the non-HDL cholesterol goal by further lowering VLDL cholesterol. In rare cases in which triglycerides are very high (≥500 mg/dL), the initial aim of therapy is to prevent acute pancreatitis through triglyceride lowering. This approach requires very low-fat diets (≤15% of calorie intake), weight reduction, increased physical activity, and usually a triglyceride-lowering drug (fibrate or nicotinic acid). Only after triglyceride levels have been lowered to <500 mg/dL should attention turn to LDL lowering to reduce risk for CHD.

Low HDL Cholesterol. Low HDL cholesterol is a strong independent predictor of CHD. In ATP III, low HDL cholesterol is defined categorically as a level <40 mg/dL, a change from the level of <35 mg/dL in ATP II. In the present guidelines, low HDL cholesterol both modifies the goal for LDL-lowering therapy and is used as a risk factor to estimate 10-year risk for CHD.

Low HDL cholesterol levels have several causes, many of which are associated with insulin resistance, ie, elevated triglycerides, overweight and obesity, physical inactivity, and type 2 diabetes. Other causes are cigarette smoking, very high carbohydrate intakes (>60% of calories), and certain drugs (eg, β-blockers, anabolic steroids, progestational agents).

ATP III does not specify a goal for HDL raising. Although clinical trial results suggest that raising HDL will reduce risk, the evidence is insufficient to specify a goal of therapy. Furthermore, currently available drugs do not robustly raise HDL cholesterol. Nonetheless, a low HDL should receive clinical attention and management according to the following sequence. In all persons with low HDL cholesterol, the primary target of therapy is LDL cholesterol; ATP III guidelines should be followed to achieve the LDL cholesterol goal. Second, after the LDL goal has been reached, emphasis shifts to weight reduction and increased physical activity (when the metabolic syndrome is present). When a low HDL cholesterol is associated with high triglycerides (200-499 mg/dL), secondary priority goes to achieving the non-HDL cholesterol goal, as outlined earlier. Also, if triglycerides are <200 mg/dL (isolated low HDL cholesterol), drugs for HDL raising (fibrates or nicotinic acid) can be considered; however, treatment for isolated low HDL is mostly reserved for persons with CHD and CHD risk equivalents.

Diabetic Dyslipidemia. This disorder is essentially atherogenic dyslipidemia in persons with type 2 diabetes. Although elevated triglycerides, low HDL cholesterol, or both are common in persons with diabetes, clinical trial results support the identification of LDL cholesterol as the primary target of therapy, as it is in those without diabetes. Since diabetes is designated a CHD risk equivalent in ATP III, the LDL cholesterol goal of therapy for most persons with diabetes will be <100 mg/dL. Furthermore, when LDL cholesterol is ≥130 mg/dL, most persons with diabetes will require initiation of LDL-lowering drugs simultaneously with TLC to achieve the LDL goal. When LDL cholesterol levels are in the range of 100-129 mg/dL at baseline or on treatment, several therapeutic options are available: increasing intensity of LDL-lowering therapy, adding a drug to modify atherogenic dyslipidemia (fibrate or nicotinic acid), or intensifying control of other risk factors including hyperglycemia. When triglyceride levels are ≥200 mg/dL, non-HDL cholesterol becomes a secondary target of cholesterol-lowering therapy. Several ongoing clinical trials (eg, Antihypertensive and Lipid Lowering Heart Attack Trial [ALLHAT]) will better quantify the magnitude of the benefit of LDL-lowering treatment in older individuals with diabetes. In older persons (≥65 years) with diabetes but no additional CHD risk factors other than age, clinical judgment is required for how intensively to apply these guidelines. A variety of factors, including concomitant illnesses, general health status, and social issues, may influence treatment decisions and may suggest a more conservative approach.

Special Considerations for Different Population Groups

Middle-Aged Men (35-65 Years). In general, men have a higher risk for CHD than do women. Middle-aged men in particular have a high prevalence of the major risk factors and are predisposed to abdominal obesity and the metabolic syndrome. A sizable fraction of all CHD in men occurs in middle age. Thus, many middle-aged men carry a relatively high risk for CHD, and for those who do, intensive LDL-lowering therapy is needed.

Women Aged 45-75 Years. In women, onset of CHD generally is delayed by some 10 to 15 years compared with that in men; thus, most CHD in women occurs after age 65 years. All risk factors contribute to CHD in women, and most premature CHD in women (<65 years) occurs in those with multiple risk factors and the metabolic syndrome. Despite the previous belief that the sex difference in risk for CHD reflects a protective effect of estrogen in women, recent secondary and primary prevention trials cast doubt on the use of hormone replacement therapy to reduce CHD risk in postmenopausal women. In contrast, the favorable effects of statin therapy in women in clinical trials make a cholesterol-lowering drug preferable to hormone replacement therapy for CHD risk reduction. Women should be treated similarly to men for secondary prevention. For primary prevention, ATP III's general approach is similarly applicable for women and men. However, the later on-

Table 10. Interventions to Improve Adherence

Focus on the Patient
• Simplify medication regimens
• Provide explicit patient instruction and use good counseling techniques to teach the patient how to follow the prescribed treatment
• Encourage the use of prompts to help patients remember treatment regimens
• Use systems to reinforce adherence and maintain contact with the patient
• Encourage the support of family and friends
• Reinforce and reward adherence
• Increase visits for patients unable to achieve treatment goal
• Increase the convenience and access to care
• Involve patients in their care through self-monitoring
Focus on the Physician and Medical Office
• Teach physicians to implement lipid treatment guidelines
• Use reminders to prompt physicians to attend to lipid management
• Identify a patient advocate in the office to help deliver or prompt care
• Use patients to prompt preventive care
• Develop a standardized treatment plan to structure care
• Use feedback from past performance to foster change in future care
• Remind patients of appointments and follow up missed appointments
Focus on the Health Delivery System
• Provide lipid management through a lipid clinic
• Utilize case management by nurses
• Deploy telemedicine
• Utilize the collaborative care of pharmacists
• Execute critical care pathways in hospitals

set of CHD for women in general should be factored into clinical decisions about use of cholesterol-lowering drugs.

Older Adults (Men ≥65 Years and Women ≥75 Years). Overall, most new CHD events and most coronary deaths occur in older persons (≥65 years). A high level of LDL cholesterol and low HDL cholesterol still carry predictive power for the development of CHD in older persons. Nevertheless, the finding of advanced subclinical atherosclerosis by noninvasive testing can be helpful for confirming the presence of high risk in older persons. Secondary prevention trials with statins have included a sizable number of older persons, mostly in the age range of 65 to 75 years. In these trials, older persons showed significant risk reduction with statin therapy. Thus, no hard-and-fast age restrictions appear necessary when selecting persons with established CHD for LDL-lowering therapy. For primary prevention, TLC is the first line of therapy for older persons. However, LDL-lowering drugs can also be considered when older persons are at higher risk because of multiple risk factors or advanced subclinical atherosclerosis.

Younger Adults (Men 20-35 Years; Women 20-45 Years). In this age group, CHD is rare except in those with severe risk factors, eg, familial hypercholesterolemia, heavy cigarette smoking, or diabetes. Even though clinical CHD is relatively rare in young adults, coronary atherosclerosis in its early stages may progress rapidly. The rate of development of coronary atherosclerosis earlier in life correlates with the major risk factors. In particular, long-term prospective studies reveal that elevated serum cholesterol detected in young adulthood predicts a higher rate of premature CHD in middle age. Thus, risk factor identification in young adults is an important aim for long-term prevention. The combination of early detection and early intervention on elevated LDL cholesterol with life-habit changes offers the opportunity for delaying or preventing onset of CHD later in life. For young adults with LDL cholesterol levels of ≥130 mg/dL, TLC should be instituted and emphasized. Particular attention should be given to young men who smoke and have a high LDL cholesterol (160-189 mg/dL); they may be candidates for LDL-lowering drugs. When young adults have very high LDL cholesterol levels (≥190 mg/dL), drug therapy should be considered, as in other adults. Those with severe genetic forms of hypercholesterolemia may require LDL-lowering drugs in combination (eg, statin+bile acid sequestrant).

Racial and Ethnic Groups. African Americans have the highest overall CHD mortality rate and the highest out-of-hospital coronary death rates of any ethnic group in the United States, particularly at younger ages. Although the reasons for the excess CHD mortality among African Americans have not been fully elucidated, it can be accounted for, at least in part, by the high prevalence of coronary risk factors. Hypertension, left ventricular hypertrophy, diabetes mellitus, cigarette smoking, obesity, physical inactivity, and multiple CHD risk factors all occur more frequently in African Americans than in whites. Other ethnic groups and minority populations in the United States include Hispanics, Native Americans, Asian and Pacific Islanders, and South Asians. Although limited data suggest that racial and ethnic groups vary somewhat in baseline risk for CHD, this evidence did not appear sufficient to lead the ATP III panel to modify general recommendations for cholesterol management in these populations.

ADHERENCE TO LDL-LOWERING THERAPY

Adherence to the ATP III guidelines by both patients and providers is a key to approximating the magnitude of the benefits demonstrated in clinical trials of cholesterol lowering. Adherence issues have to be addressed to attain the highest possible levels of CHD risk reduction. Thus, ATP III recommends the use of state-of-the-art multidisciplinary methods targeting the patient, clinicians, and health delivery systems to achieve the full population effectiveness of the guidelines for primary and secondary prevention (TABLE 10).

National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

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National Cholesterol Education Program Coordinating Committee: The Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults was approved by the National Cholesterol Education Program Coordinating Committee, which comprises the following organizational representatives:

Member Organizations: *National Heart, Lung, and Blood Institute:* Claude Lenfant, MD (Chair), James I. Cleeman, MD (Coordinator); *American Academy of Family Physicians:* Theodore G. Ganiats, MD; *American Academy of Insurance Medicine:* Gary Graham, MD; *American Academy of Pediatrics:* Ronald E. Kleinman, MD; *American Association of Occupational Health Nurses:* Pamela Hixon, BSN, RN, COHN-S; *American College of Cardiology:* Richard C. Pasternak, MD; *American College of Chest Physicians:* Gerald T. Gau, MD, *American College of Nutrition:* Harry Preuss, MD; *American College of Obstetricians and Gynecologists:* Thomas C. Peng, MD; *American College of Occupational and Environmental Medicine:* Ruth Ann Jordan, MD; *American College of Preventive Medicine:* Lewis H. Kuller, MD, DrPH; *American Diabetes Association, Inc:* Alan J. Garber, MD, PhD; *American Dietetic Association:* Linda Van Horn, PhD, RD; *American Heart Association:* Scott M. Grundy, MD, PhD; *American Hospital Association:* Sandra Cornett, RN, PhD; *American Medical Association:* Yank D. Coble, Jr, MD; *American Nurses Association:* to be named; *American Osteopathic Association:* Michael Clearfield, DO; *American Pharmaceutical Association:* James M. McKenney, PharmD; *American Public Health Association:* Stephen Havas, MD, MPH, MS; *American Red Cross:* Donald Vardell, MS; *Association of Black Cardiologists:* Karol Watson, MD, PhD; *Association of State and Territorial Health Officials:* Joanne Mitten, MHE; *Citizens for Public Action on Blood Pressure and Cholesterol, Inc:* Gerald J. Wilson, MA, MBA; *National Black Nurses Association, Inc:* Linda Burnes-Bolton, DrPH, RN, MSN; *National Medical Association:* Luther T. Clark, MD; *Society for Nutrition Education:* Darlene Lansing, MPH, RD; *Society for Public Health Education:* Donald O. Fedder, DrPH, MPH.

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APPENDIX

Shared Features of ATP III and ATP II

Adult Treatment Panel (ATP) III shares a set of core features with ATP II, shown in Table A.

Table A. Shared Features of ATP III and ATP II*

- Continued identification of LDL cholesterol lowering as the primary goal of therapy
- Consideration of high LDL cholesterol (≥160 mg/dL) as a potential target for LDL-lowering drug therapy, specifically as follows:
  - For persons with multiple risk factors whose LDL levels are high (≥160 mg/dL) after dietary therapy, consideration of drug therapy is recommended
  - For persons with 0-1 risk factor, consideration of drug therapy (after dietary therapy) is optional for LDL 160-189 mg/dL and recommended for LDL ≥190 mg/dL
- Emphasis on intensive LDL-lowering therapy in persons with established CHD
- Identification of 3 categories of risk for different LDL goals and different intensities of LDL-lowering therapy:
  - CHD and CHD risk equivalents† (other forms of clinical atherosclerotic disease)
  - Multiple (2+) risk factors‡
  - 0-1 risk factor
- Identification of subpopulations, besides middle-aged men, for detection of high LDL cholesterol (and other lipid risk factors) and for clinical intervention. These include:
  - Young adults
  - Postmenopausal women
  - Older persons
- Emphasis on weight loss and physical activity to enhance risk reduction in persons with elevated LDL cholesterol

*ATP indicates Adult Treatment Panel; LDL, low-density lipoprotein; and CHD, coronary heart disease.
†A CHD risk equivalent is a condition that carries an absolute risk for developing new CHD equal to the risk for having recurrent CHD events in persons with established CHD.
‡Risk factors that continue to modify the LDL goal include cigarette smoking, hypertension, low HDL cholesterol, family history of premature CHD, age (male ≥45 years and female ≥55 years), and diabetes (in ATP III diabetes is regarded as a CHD risk equivalent).

Estimating 10-Year Risk for Men and Women

Risk assessment for determining the 10-year risk for developing CHD is carried out using Framingham risk scoring (Table B1 for men and Table B2 for women). The risk factors included in the Framingham calculation of 10-year risk are age, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension, and cigarette smoking. The first step is to calculate the number of points for each risk factor. For initial assessment, values for total cholesterol and HDL cholesterol are required. Because of a larger database, Framingham estimates are more robust for total cholesterol than for LDL cholesterol. Note, however, that the LDL cholesterol level remains the primary target of therapy. Total cholesterol and HDL cholesterol values should be the average of at least 2 measurements obtained from lipoprotein analysis. The blood pressure value used is that obtained at the time of assessment, regardless of whether the person is on antihypertensive therapy. However, if the person is on antihypertensive treatment, an extra point is added beyond points for the blood pressure reading because treated hypertension carries residual risk (Tables B1 and B2). The average of several blood pressure measurements, as recommended by the Joint National Committee (JNC), is needed for an accurate measure of baseline blood pressure. The designation "smoker" means any cigarette smoking in the past month. The total risk score sums the points for each risk factor. The 10-year risk for myocardial infarction and coronary death (hard CHD) is estimated from total points, and the person is categorized according to absolute 10-year risk as indicated above (see Table 5).

Table B1. Estimate of 10-Year Risk for **Men** (Framingham Point Scores)

Age, y	Points
20-34	−9
35-39	−4
40-44	0
45-49	3
50-54	6
55-59	8
60-64	10
65-69	11
70-74	12
75-79	13

Total Cholesterol, mg/dL	Points				
	Age 20-39 y	Age 40-49 y	Age 50-59 y	Age 60-69 y	Age 70-79 y
<160	0	0	0	0	0
160-199	4	3	2	1	0
200-239	7	5	3	1	0
240-279	9	6	4	2	1
≥280	11	8	5	3	1

	Points				
	Age 20-39 y	Age 40-49 y	Age 50-59 y	Age 60-69 y	Age 70-79 y
Nonsmoker	0	0	0	0	0
Smoker	8	5	3	1	1

HDL, mg/dL	Points
≥60	−1
50-59	0
40-49	1
<40	2

Systolic BP, mm Hg	If Untreated	If Treated
<120	0	0
120-129	0	1
130-139	1	2
140-159	1	2
≥160	2	3

Point Total	10-Year Risk, %
<0	<1
0	1
1	1
2	1
3	1
4	1
5	2
6	2
7	3
8	4
9	5
10	6
11	8
12	10
13	12
14	16
15	20
16	25
≥17	≥30

Table B2. Estimate of 10-Year Risk for **Women** (Framingham Point Scores)

Age, y	Points
20-34	−7
35-39	−3
40-44	0
45-49	3
50-54	6
55-59	8
60-64	10
65-69	12
70-74	14
75-79	16

Total Cholesterol, mg/dL	Points				
	Age 20-39 y	Age 40-49 y	Age 50-59 y	Age 60-69 y	Age 70-79 y
<160	0	0	0	0	0
160-199	4	3	2	1	1
200-239	8	6	4	2	1
240-279	11	8	5	3	2
≥280	13	10	7	4	2

	Points				
	Age 20-39 y	Age 40-49 y	Age 50-59 y	Age 60-69 y	Age 70-79 y
Nonsmoker	0	0	0	0	0
Smoker	9	7	4	2	1

HDL, mg/dL	Points
≥60	−1
50-59	0
40-49	1
<40	2

Systolic BP, mm Hg	If Untreated	If Treated
<120	0	0
120-129	1	3
130-139	2	4
140-159	3	5
≥160	4	6

Point Total	10-Year Risk, %
<9	<1
9	1
10	1
11	1
12	1
13	2
14	2
15	3
16	4
17	5
18	6
19	8
20	11
21	14
22	17
23	22
24	27
≥25	≥30

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Table 2 Plasma total cholesterol and plant sterol in heterozygous subjects consuming margarine enriched in plant sterols

	Cholesterol		Campesterol (μM)	Stigmasterol (μM)	Sitosterol (μM)	Total plant sterols		
	mM	% change				μM	% change	% total sterols
Father								
Start run-in	8.50	–	9.80	1.10	11.15	22.05	–	0.26
Start (0)	8.46	0.0	12.25	1.35	12.90	26.50	0	0.31
T = 1 week	7.59	−10.3	22.35	1.30	17.15	40.80	54	0.62
T = 2 weeks	7.41	−12.4	27.20	1.25	17.60	46.05	74	0.65
T = 3 weeks	7.67	−9.3	37.80	2.00	23.45	63.25	139	0.82
T = 4 weeks	7.53	−11.0	34.25	1.60	20.10	55.95	111	0.74
After 2 weeks washout	7.88	−6.9	16.50	1.70	14.00	32.20	22	0.41
Mother								
Start run-in	6.17	–	8.35	0.50	10.70	19.55	–	0.32
Start (0)	6.19	0.0	9.15	0.00	10.60	19.75	0	0.32
T = 1 week	5.36	−13.4	13.85	1.20	12.25	27.30	38	0.51
T = 2 weeks	5.25	−15.2	16.80	1.30	13.35	31.45	58	0.60
T = 3 weeks	5.56	−10.2	15.75	1.35	11.65	28.75	46	0.51
T = 4 weeks	5.46	−11.8	19.25	1.60	15.20	36.05	83	0.66
After 2 weeks washout	5.81	−6.1	10.50	0.50	10.55	21.55	9	0.37

plant sterol-enriched margarine (Table 1). This was mainly due to changes in LDL cholesterol, whereas the other lipoproteins, including Lp(a) were unaffected. The decrease in apoB was in the same order as that in serum cholesterol. Total plant sterol levels increased maximally 139% from 0.31 to 0.82% of total sterols in the father, and maximally 83% from 0.32 to 0.66% of total sterols in the mother (Table 2). After 3 weeks of consumption of plant sterols, their blood levels seemed to have reached equilibrium with no further increase. In 33 healthy control subjects, fasting plasma campesterol levels on their normal diet ranged from 3.8 to 24.2 μmol L^{-1} (mean 13.1) and plasma sitosterol from 2.1 to 16.5 μmol L^{-1} (mean 8.9).

Discussion

Daily intake of plant sterol-enriched margarine lowered blood total cholesterol levels in the subjects heterozygous for phytosterolaemia by an average of 10.2 and 11.0%, respectively (average of weeks 3 and 4). This is in accordance with what has been reported earlier in normal and hypercholesterolaemic subjects [5, 10]. Of importance in this study was that an intake of around 3 g day^{-1} of plant sterols in the heterozygotes did not increase campesterol or sitosterol levels in blood to levels higher than reported in normal subjects consuming similar margarine [11, 12]. In addition, the increase in blood plant sterols observed in the two heterozygotes was slightly higher than observed in our healthy subjects on a normal diet and was less than 1% of blood total sterols. After 3 weeks of a steady consumption of plant sterols, blood levels of the subjects seemed to have reached equilibrium without further increase, but long-term follow-up studies are necessary to confirm this.

In conclusion, plant sterol-enriched margarine appears to be as efficacious in reducing cholesterol in subjects heterozygous for phytosterolaemia as in normal or hyperlipidaemic subjects, without strong accumulation of plant sterols in their plasma. As pointed out by Law [13], consumption of margarines containing plant sterols as well as stanols can be expected to reduce the risk of heart disease of about 25%.

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RENAL HYPERPLASIA: A CASE

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GENOTYPING IS A VALUABLE DIAGNOSTIC COMPLEMENT TO NEONATAL SCREENING FOR CONGENITAL ADRENAL HYPERPLASIA DUE TO STEROID 21-HYDROXYLASE DEFICIENCY.

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Screening for congenital adrenal hyperplasia (CAH) started in Sweden in 1986. 17-Hydroxy-progesterone (17-OHP) analyzed in filter paper bloodspots is used as a marker for the disease. More than 95% of the patients have 21-hydroxylase deficiency and the molecular genetics have been extensively studied. There are good genotype-phenotype correlations with very few exceptions.
In this study we have compared the 17-OHP screening value to the genotype for 90 infants with CAH. 21-Hydroxylase mutation analysis was carried out with allele specific PCR. Overall, we found good correlations between the genotypic groups and the screening values. However, a single screening value for 17-OHP can not be used to predict the severity of a childs disease. In addition, for 8% of the infants with false positive tests the diagnose could not be excluded on the basis of a second sample, ie they had inconclusive screening results.
We conclude that genotyping is a valuable complement to screening for CAH both for prediction of clinical manifestations and for confirmation or exclusion of the diagnose in unclear cases with inconclusive screening results.

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ASSESSMENT OF STEROL-TURNOVER IN A CHILD WITH PHYTOSTEROLEMIA

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Background: In phytosterolemia ccumulation of plant sterols in plasma and tissues is associated with severe premature atherosclerosis, but the relative contributions of enhanced absorption or reduced biliary excretion are controversial. We studied sterol turnover with stable isotope methods. **Patient and method:** We diagnosed phytosterolemia in a 4 year old girl previously considered as hypercholesterolemia, based on early xanthomas, mild splenic enlargement, hemolysis and thrombopenia. Plasma phytosterol concentrations were markedly elevated. At age 6 yrs. 3 mon. phytosterolemia turnover was assessed following a baseline period of 4 weeks without drugs and a diet with all plant oil from sunflower oil (C3 plant) and no corn (C4 plant naturally enriched in ^{13}C) or corn products (e.g. corn flakes, starch, oil). Then the child was admitted as an inpatient, and a balance study was performed over one week with a strictly controlled diet based on corn oil, with an average daily phytosterol intake of 445 mg from corn oil and 96 mg from C3 plant oil. Fasted plasma samples were obtained in the mornings of study days 0 through 6 for measurements of phytosterol concentrations (GC-MS) and ^{13}C-enrichments (GC-C-IRMS). **Results:** We determined lower natural ^{13}C-enrichments of phytosterols in sunflower than in corn oil ($\delta^{13}C$ of campesterol –29.6 vs. –17.3 ‰, sitosterol –29.9 vs. –16.1 ‰, stigmasterol –32.3 vs. –20.8 ‰). On day 6 delta over baseline values (DOB) had remained unchanged for plasma cholesterol but were increased for campesterol (DOB +1.05 ‰), sitosterol (DOB +1.32 ‰) and stigmasterol (DOB +3.32 ‰). We estimate the contribution of absorbed dietary sterols to the plasma pool with a stable isotope balance equation as 7.9 % for campesterol (oil content 179 mg/100g), 9.1 % for sitosterol (595 mg/100g) and 26 % for stigmasterol (51 mg/100 g). **Conclusions:** 1. Using GC-C-IRMS, small variations in natural ^{13}C-enrichments of foods can be utilised for monitoring sterol absorption. 2. Absorption is unevenly enhanced for different sterols. with a particulary high retention of stigmasterol in our patient. 3. Based on these data dietary restriction of phytosterols with particular emphasis on stigmasterol intake appears useful.

Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults

THE THIRD REPORT OF THE EXPERT Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III, or ATP III) constitutes the National Cholesterol Education Program's (NCEP's) updated clinical guidelines for cholesterol testing and management. The full ATP III document is an evidence-based and extensively referenced report that provides the scientific rationale for the recommendations contained in the executive summary. ATP III builds on previous ATP reports and expands the indications for intensive cholesterol-lowering therapy in clinical practice. It should be noted that these guidelines are intended to inform, not replace, the physician's clinical judgment, which must ultimately determine the appropriate treatment for each individual.

BACKGROUND

The third ATP report updates the existing recommendations for clinical management of high blood cholesterol. The NCEP periodically produces ATP clinical updates as warranted by advances in the science of cholesterol management. Each of the guideline reports—ATP I, II, and III—has a major thrust. ATP I outlined a strategy for primary prevention of coronary heart disease (CHD) in persons with high levels of low-density lipoprotein (LDL) cholesterol (≥160 mg/dL) or those with borderline high LDL cholesterol (130-159 mg/dL) and multiple (2+) risk factors. ATP II affirmed the importance of this approach and added a new feature: the intensive management of LDL cholesterol in persons with established CHD. For patients with CHD, ATP II set a new, lower LDL cholesterol goal of ≤100 mg/dL. ATP III adds a call for more intensive LDL-lowering therapy in certain groups of people, in accord with recent clinical trial evidence, but its core is based on ATP I and ATP II. Some of the important features shared with previous reports are shown in Table A in the APPENDIX.

While ATP III maintains attention to intensive treatment of patients with CHD, its major new feature is a focus on primary prevention in persons with multiple risk factors. Many of these persons have a relatively high risk for CHD and will benefit from more intensive LDL-lowering treatment than recommended in ATP II. TABLE 1 shows the new features of ATP III. (Note: To convert cholesterol to mmol/L, divide values by 38.7).

See also p 2508 and Patient Page.

LDL CHOLESTEROL: THE PRIMARY TARGET OF THERAPY

Research from experimental animals, laboratory investigations, epidemiology, and genetic forms of hypercholesterolemia indicate that elevated LDL cholesterol is a major cause of CHD. In addition, recent clinical trials robustly show that LDL-lowering therapy reduces risk for CHD. For these reasons, ATP III continues to identify elevated LDL cholesterol as the primary target of cholesterol-lowering therapy. As a result, the primary goals of therapy and the cutpoints for initiating treatment are stated in terms of LDL.

RISK ASSESSMENT: FIRST STEP IN RISK MANAGEMENT

A basic principle of prevention is that the intensity of risk-reduction therapy should be adjusted to a person's absolute risk. Hence, the first step in selection of LDL-lowering therapy is to assess a person's risk status. Risk assessment requires measurement of LDL cholesterol as part of lipoprotein analysis and identification of accompanying risk determinants.

In all adults aged 20 years or older, a fasting lipoprotein profile (total cholesterol, LDL cholesterol, high-density lipoprotein [HDL] cholesterol, and triglyceride) should be obtained once every 5 years. If the testing opportunity is nonfasting, only the values for total choles-

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The Full Report of ATP III is available online on the NHLBI Web site at www.nhlbi.nih.gov.

Members of the NCEP Expert Panel are listed at the end of this article.

terol and HDL cholesterol will be usable. In such a case, if total cholesterol is ≥200 mg/dL or HDL is <40 mg/dL, a follow-up lipoprotein profile is needed for appropriate management based on LDL. The relationship between LDL cholesterol levels and CHD risk is continuous over a broad range of LDL levels from low to high. Therefore, ATP III adopts the classification of LDL cholesterol levels shown in TABLE 2, which also shows the classification of total and HDL cholesterol levels.

Risk determinants in addition to LDL cholesterol include the presence or absence of CHD, other clinical forms of atherosclerotic disease, and the major risk factors other than LDL (TABLE 3). (LDL is not counted among the risk factors in Table 3 because the purpose of counting those risk factors is to modify the treatment of LDL.) Based on these other risk determinants, ATP III identifies 3 categories of risk that modify the goals and modalities of LDL-lowering therapy. TABLE 4 defines these categories of risk and shows corresponding LDL cholesterol goals.

The category of highest risk consists of CHD and CHD risk equivalents. The latter carry a risk for major coronary events equal to that of established CHD, ie, >20% per 10 years (ie, more than 20 of 100 such individuals will develop CHD or have a recurrent CHD event within 10 years). CHD risk equivalents comprise:

- Other clinical forms of atherosclerotic disease (peripheral arterial disease, abdominal aortic aneurysm, and symptomatic carotid artery disease)
- Diabetes
- Multiple risk factors that confer a 10-year risk for CHD >20%.

Diabetes counts as a CHD risk equivalent because it confers a high risk of new CHD within 10 years, in part because of its frequent association with multiple risk factors. Furthermore, because persons with diabetes who experience a myocardial infarction have an unusually high death rate either immediately or in the long term, a more intensive prevention strategy is warranted. Persons with CHD or CHD risk equivalents have the lowest LDL cholesterol goal (<100 mg/dL).

The second category consists of persons with multiple (2+) risk factors in whom 10-year risk for CHD is ≤20%. Risk is estimated from Framingham risk scores (see Appendix). The major risk factors, exclusive of elevated LDL cholesterol, are used to define the presence of multiple risk factors that modify the goals and cutpoints for LDL-lowering treatment, and these are listed in Table 3. The LDL cholesterol goal for persons with multiple (2+) risk factors is <130 mg/dL.

The third category consists of persons having 0-1 risk factor; with few exceptions, persons in this category have a 10-year risk <10%. Their LDL cholesterol goal is <160 mg/dL.

Table 1. New Features of ATP III*

Focus on Multiple Risk Factors
• Raises persons with diabetes without CHD, most of whom have multiple risk factors, to the risk level of CHD risk equivalent
• Uses Framingham projections of 10-year absolute CHD risk (ie, the percent probability of having a CHD event in 10 years) to identify certain patients with multiple (2+) risk factors for more intensive treatment
• Identifies persons with multiple metabolic risk factors (metabolic syndrome) as candidates for intensified therapeutic lifestyle changes
Modifications of Lipid and Lipoprotein Classification
• Identifies LDL cholesterol <100 mg/dL as optimal
• Raises categorical low HDL cholesterol from <35 mg/dL to <40 mg/dL because the latter is a better measure of a depressed HDL
• Lowers the triglyceride classification cutpoints to give more attention to moderate elevations
Support for Implementation
• Recommends a complete lipoprotein profile (total, LDL, and HDL cholesterol and triglycerides) as the preferred initial test, rather than screening for total cholesterol and HDL alone
• Encourages use of plant stanols/sterols and viscous (soluble) fiber as therapeutic dietary options to enhance lowering of LDL cholesterol
• Presents strategies for promoting adherence to therapeutic lifestyle changes and drug therapies
• Recommends treatment beyond LDL lowering for persons with triglycerides ≥200 mg/dL

*ATP indicates Adult Treatment Panel; CHD, coronary heart disease; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.

Table 2. ATP III Classification of LDL, Total, and HDL Cholesterol (mg/dL)*

LDL cholesterol	
<100	Optimal
100-129	Near or above optimal
130-159	Borderline high
160-189	High
≥190	Very high
Total cholesterol	
<200	Desirable
200-239	Borderline high
≥240	High
HDL cholesterol	
<40	Low
≥60	High

*ATP indicates Adult Treatment Panel; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.

Table 3. Major Risk Factors (Exclusive of LDL Cholesterol) That Modify LDL Goals*

- Cigarette smoking
- Hypertension (blood pressure ≥140/90 mm Hg or on antihypertensive medication)
- Low HDL cholesterol (<40 mg/dL)†
- Family history of premature CHD (CHD in male first-degree relative <55 years; CHD in female first-degree relative <65 years)
- Age (men ≥45 years; women ≥55 years)

*Diabetes is regarded as a coronary heart disease (CHD) risk equivalent. LDL indicates low-density lipoprotein; HDL, high-density lipoprotein.
†HDL cholesterol ≥60 mg/dL counts as a "negative" risk factor; its presence removes 1 risk factor from the total count.

Table 4. Three Categories of Risk That Modify LDL Cholesterol Goals

Risk Category	LDL Goal (mg/dL)
CHD and CHD risk equivalents	<100
Multiple (2+) risk factors*	<130
0-1 risk factor	<160

*Risk factors that modify the low-density lipoprotein (LDL) goal are listed in Table 3. CHD indicates coronary heart disease.

Method of Risk Assessment: Counting Major Risk Factors and Estimating 10-Year CHD Risk

Risk status in persons without clinically manifest CHD or other clinical forms of atherosclerotic disease is determined by a 2-step procedure. First, the number of risk factors is counted (Table 3). Second, for persons with multiple (2+) risk factors, 10-year risk assessment is carried out with Framingham scoring (see Appendix) to identify individuals whose short-term (10-

year) risk warrants consideration of intensive treatment. Estimation of the 10-year CHD risk adds a step to risk assessment beyond risk factor counting, but this step is warranted because it allows better targeting of intensive treatment to people who will benefit from it. When 0-1 risk factor is present, Framingham scoring is not necessary because 10-year risk rarely reaches levels for intensive intervention; a very high LDL level in such a person may nevertheless warrant consideration of drug therapy to reduce long-term risk. Risk factors used in Framingham scoring include age, total cholesterol, HDL cholesterol, blood pressure, and cigarette smoking. Total cholesterol is used for 10-year risk assessment because of a larger and more robust Framingham database for total than for LDL cholesterol, but LDL cholesterol is the primary target of therapy. Framingham scoring divides persons with multiple risk factors into those with 10-year risk for CHD of >20%, 10%-20%, and <10%. It should be noted that this 2-step sequence can be reversed with essentially the same results. (If Framingham scoring is carried out before risk factor counting, persons with <10% risk are then divided into those with 2+ risk factors and 0-1 risk factor by risk factor counting to determine the appropriate LDL goal [Table 4].) Initial risk assessment in ATP III uses the major risk factors to define the core risk status. Only after the core risk status has been determined should any other risk modifiers be taken into consideration for adjusting the therapeutic approach.

Role of Other Risk Factors in Risk Assessment

ATP III recognizes that risk for CHD is influenced by other factors not included among the major, independent risk factors (Table 3). Among these are life-habit risk factors and emerging risk factors. The former include obesity, physical inactivity, and atherogenic diet; the latter consist of lipoprotein(a), homocysteine, prothrombotic and proinflammatory factors, impaired fasting glucose, and evidence of subclinical atherosclerotic disease. The life-habit risk factors are direct targets for clinical intervention but are not used to set a lower LDL cholesterol goal of therapy. The emerging risk factors do not categorically modify LDL cholesterol goals; however, they appear to contribute to CHD risk to varying degrees and can have utility in selected persons to guide intensity of risk-reduction therapy. Their presence can modulate clinical judgment when making therapeutic decisions.

Metabolic Syndrome

Many persons have a constellation of major risk factors, life-habit risk factors, and emerging risk factors that constitute a condition called the metabolic syndrome. Factors characteristic of the metabolic syndrome are abdominal obesity, atherogenic dyslipidemia (elevated triglyceride, small LDL particles, low HDL cholesterol), raised blood pressure, insulin resistance (with or without glucose intolerance), and prothrombotic and proinflammatory states. ATP III recognizes the metabolic syndrome as a secondary target of risk-reduction therapy, after the primary target—LDL cholesterol. Diagnosis and treatment of the metabolic syndrome is described below under "Benefit Beyond LDL Lowering: The Metabolic Syndrome as a Secondary Target of Therapy."

The Link Between Risk Assessment and Cost-effectiveness

In ATP III, a primary aim is to match intensity of LDL-lowering therapy with absolute risk. Everyone with elevated LDL cholesterol is treated with lifestyle changes that are effective in lowering LDL levels. Persons at relatively high risk are also candidates for drug treatment, which is very effective but entails significant additional expense. The cutpoints for drug treatment are based primarily on risk-benefit considerations: those at higher risk are likely to get greater benefit. However, cutpoints for recommended management based on therapeutic efficacy are checked against currently accepted standards for cost-effectiveness. Lifestyle changes are the most cost-effective means to reduce risk for CHD. Even so, to achieve maximal benefit, many persons will require LDL-lowering drugs. Drug therapy is the major expense of LDL-lowering therapy and it dominates cost-effectiveness analysis. However, the costs of LDL-lowering drugs are currently in flux and appear to be declining. This report recognizes that as drug prices decline it will be possible to extend drug use to lower-risk persons and still be cost-effective. In addition, ATP III recognizes that some persons with high long-term risk are candidates for LDL-lowering drugs even though use of drugs may not be cost-effective by current standards.

PRIMARY PREVENTION WITH LDL-LOWERING THERAPY

Primary prevention of CHD offers the greatest opportunity for reducing the burden of CHD in the United States. The clinical approach to primary prevention is founded on the public health approach that calls for lifestyle changes, including (1) reduced intakes of saturated fat and cholesterol, (2) increased physical activity, and (3) weight control, to lower population cholesterol levels and reduce CHD risk, but the clinical approach intensifies preventive strategies for higher-risk persons. One aim of primary prevention is to reduce long-term risk (>10 years) as well as short-term risk (≤10 years). LDL goals in primary prevention depend on a person's absolute risk for CHD (ie, the probability of having a CHD event in the short term or the long term)—the higher the risk, the lower the goal. Therapeutic lifestyle changes are the foundation of clinical primary prevention. Nonetheless, some persons at higher risk because of high or very high LDL cholesterol levels or because of multiple risk factors are candidates for LDL-lowering drugs. Recent primary prevention trials show that LDL-lowering drugs reduce risk for major

coronary events and coronary death even in the short term.

Any person with elevated LDL cholesterol or other form of hyperlipidemia should undergo clinical or laboratory assessment to rule out secondary dyslipidemia before initiation of lipid-lowering therapy. Causes of secondary dyslipidemia include:

- Diabetes
- Hypothyroidism
- Obstructive liver disease
- Chronic renal failure
- Drugs that increase LDL cholesterol and decrease HDL cholesterol (progestins, anabolic steroids, and corticosteroids).

Once secondary causes have been excluded or, if appropriate, treated, the goals for LDL-lowering therapy in primary prevention are established according to a person's risk category (Table 4).

SECONDARY PREVENTION WITH LDL-LOWERING THERAPY

Recent clinical trials demonstrate that LDL-lowering therapy reduces total mortality, coronary mortality, major coronary events, coronary artery procedures, and stroke in persons with established CHD. As shown in Table 2, an LDL cholesterol level of <100 mg/dL is optimal; therefore, ATP III specifies an LDL cholesterol level of <100 mg/dL as the goal of therapy in secondary prevention. This goal is supported by clinical trials with both clinical and angiographic end points and by prospective epidemiological studies. The same goal should apply for persons with CHD risk equivalents. When persons are hospitalized for acute coronary syndromes or coronary procedures, lipid measures should be taken on admission or within 24 hours. These values can guide the physician on initiation of LDL-lowering therapy before or at discharge. Adjustment of therapy may be needed after 12 weeks.

LDL-LOWERING THERAPY IN 3 RISK CATEGORIES

The 2 major modalities of LDL-lowering therapy are therapeutic lifestyle changes (TLC) and drug therapy. Both are described in more detail later. The TLC Diet stresses reductions in saturated fat and cholesterol intakes. When the metabolic syndrome or its associated lipid risk factors (elevated triglyceride or low HDL cholesterol) are present, TLC also stresses weight reduction and increased physical activity. TABLE 5 defines LDL cholesterol goals and cutpoints for initiation of TLC and for drug consideration for persons with 3 categories of risk: CHD and CHD risk equivalents; multiple (2+) risk factors (10-year risk 10%-20% and <10%); and 0-1 risk factor.

CHD and CHD Risk Equivalents

For persons with CHD and CHD risk equivalents, LDL-lowering therapy greatly reduces risk for major coronary events and stroke and yields highly favorable cost-effectiveness ratios. The cutpoints for initiating lifestyle and drug therapies are shown in Table 5.

If baseline LDL cholesterol is ≥130 mg/dL, intensive lifestyle therapy and maximal control of other risk factors should be started. Moreover, for most patients, an LDL-lowering drug will be required to achieve an LDL cholesterol level of <100 mg/dL; thus an LDL-cholesterol lowering drug can be started simultaneously with TLC to attain the goal of therapy.

If LDL cholesterol levels are 100-129 mg/dL, either at baseline or on LDL-lowering therapy, several therapeutic approaches are available:

- Initiate or intensify lifestyle and/or drug therapies specifically to lower LDL.
- Emphasize weight reduction and increased physical activity in persons with the metabolic syndrome.
- Delay use or intensification of LDL-lowering therapies and institute treatment of other lipid or nonlipid risk factors; consider use of other lipid-modifying drugs (eg, nicotinic acid or fibric acid) if the patient has elevated triglyceride or low HDL cholesterol.

If baseline LDL cholesterol is <100 mg/dL, further LDL-lowering therapy is not required. Patients should nonetheless be advised to follow the TLC Diet on their own to help keep the LDL level optimal. Several clinical trials are currently under way to assess benefit of lowering LDL cholesterol to well below 100 mg/dL. At present, emphasis should be placed on controlling other lipid and nonlipid risk factors and on treatment of the metabolic syndrome, if present.

Multiple (2+) Risk Factors and 10-Year Risk of ≤20%

For persons with multiple (2+) risk factors and 10-year risk ≤20%, intensity of therapy is adjusted according to 10-year risk and LDL cholesterol level. The treatment approach for each category is summarized in Table 5.

Multiple (2+) Risk Factors and a 10-Year Risk of 10%-20%. In this category, the goal for LDL cholesterol is <130 mg/dL. The therapeutic aim is to reduce short-term risk as well as long-term risk for CHD. If baseline LDL cholesterol is ≥130 mg/dL, TLC is initiated and maintained for 3 months. If LDL remains ≥130 mg/dL after 3 months of TLC, consideration can be given to starting an LDL-lowering drug to achieve the LDL goal of <130 mg/dL. Use of LDL-lowering drugs at this risk level reduces CHD risk and is cost-effective. If the LDL falls to less than 130 mg/dL on TLC alone, TLC can be continued without adding drugs. In older persons (≥65 years), clinical judgment is required for how intensively to apply these guidelines; a variety of factors, including concomitant illnesses, general health status, and social issues, may influence treatment decisions and may suggest a more conservative approach.

Multiple (2+) Risk Factors and a 10-Year Risk of <10%. In this category, the goal for LDL cholesterol also is <130 mg/dL. The therapeutic aim, however, is primarily to reduce longer-term risk. If baseline LDL cholesterol is ≥130 mg/dL, the TLC Diet is initiated to reduce LDL cholesterol. If LDL

Table 5. LDL Cholesterol Goals and Cutpoints for Therapeutic Lifestyle Changes (TLC) and Drug Therapy in Different Risk Categories*

Risk Category	LDL Goal (mg/dL)	LDL Level at Which to Initiate Therapeutic Lifestyle Changes (mg/dL)	LDL Level at Which to Consider Drug Therapy (mg/dL)
CHD or CHD risk equivalents (10-year risk >20%)	<100	≥100	≥130 (100-129: drug optional)†
2+ Risk factors (10-year risk ≤20%)	<130	≥130	10-year risk 10%-20%: ≥130
			10-year risk <10%: ≥160
0-1 Risk factor‡	<160	≥160	≥190 (160-189: LDL-lowering drug optional)

*LDL indicates low-density lipoprotein; CHD, coronary heart disease.
†Some authorities recommend use of LDL-lowering drugs in this category if an LDL cholesterol level of <100 mg/dL cannot be achieved by therapeutic lifestyle changes. Others prefer use of drugs that primarily modify triglycerides and HDL, eg, nicotinic acid or fibrate. Clinical judgment also may call for deferring drug therapy in this subcategory.
‡Almost all people with 0-1 risk factor have a 10-year risk <10%; thus, 10-year risk assessment in people with 0-1 risk factor is not necessary.

Table 6. Nutrient Composition of the Therapeutic Lifestyle Changes (TLC) Diet

Nutrient	Recommended Intake
Saturated fat*	<7% of total calories
Polyunsaturated fat	Up to 10% of total calories
Monounsaturated fat	Up to 20% of total calories
Total fat	25%-35% of total calories
Carbohydrate†	50%-60% of total calories
Fiber	20-30 g/d
Protein	Approximately 15% of total calories
Cholesterol	<200 mg/d
Total calories‡	Balance energy intake and expenditure to maintain desirable body weight/prevent weight gain

**Trans* fatty acids are another LDL-raising fat that should be kept at a low intake.
†Carbohydrates should be derived predominantly from foods rich in complex carbohydrates including grains, especially whole grains, fruits, and vegetables.
‡Daily energy expenditure should include at least moderate physical activity (contributing approximately 200 kcal/d).

is <160 mg/dL on TLC alone, it should be continued. LDL-lowering drugs generally are not recommended because the patient is not at high short-term risk. On the other hand, if LDL cholesterol is ≥160 mg/dL, drug therapy can be considered to achieve an LDL cholesterol level of <130 mg/dL; the primary aim is to reduce long-term risk. Cost-effectiveness is marginal, but drug therapy can be justified to slow development of coronary atherosclerosis and to reduce long-term risk for CHD.

0-1 Risk Factor

Most persons with 0-1 risk factor have a 10-year risk <10%. They are managed according to Table 5. The goal for LDL cholesterol in this risk category is <160 mg/dL. The primary aim of therapy is to reduce long-term risk. First-line therapy is TLC. If after 3 months of TLC the LDL cholesterol is <160 mg/dL, TLC is continued. However, if LDL cholesterol is 160-189 mg/dL after an adequate trial of TLC, drug therapy is optional depending on clinical judgment. Factors favoring use of drugs include:

- A severe single risk factor (heavy cigarette smoking, poorly controlled hypertension, strong family history of premature CHD, or very low HDL cholesterol)
- Multiple life-habit risk factors and emerging risk factors (if measured)
- 10-year risk approaching 10% (if measured; see Appendix). If LDL cholesterol is ≥190 mg/dL despite TLC, drug therapy should be considered to achieve the LDL goal of <160 mg/dL.

The purpose of using LDL-lowering drugs in persons with 0-1 risk factor and elevated LDL cholesterol (≥160 mg/dL) is to slow the development of coronary atherosclerosis, which will reduce long-term risk. This aim may conflict with cost-effectiveness considerations; thus, clinical judgment is required in selection of persons for dru therapy, although a strong case can b made for using drugs when LDL cho lesterol is ≥190 mg/dL after TLC.

For persons whose LDL cholesterc levels are already below goal levels upo first encounter, instructions for appro priate changes in life habits, periodi follow-up, and control of other risk fac tors are needed.

THERAPEUTIC LIFESTYLE CHANGES IN LDL-LOWERING THERAPY

ATP III recommends a multifaceted life style approach to reduce risk for CHD This approach is designated therapeu tic lifestyle changes (TLC). Its essen tial features are:

- Reduced intakes of saturated fat (<7% of total calories) and choles terol (<200 mg/d) (see TABLE 6 fo overall composition of the TLC Diet)
- Therapeutic options for enhanc ing LDL lowering such as plant stanols sterols (2 g/d) and increased viscou (soluble) fiber (10-25 g/d)
- Weight reduction
- Increased physical activity.

A model of steps in TLC is shown ir FIGURE 1. To initiate TLC, intakes o saturated fats and cholesterol are reduced first to lower LDL cholesterol. Tc improve overall health, ATP III's TLC Diet generally contains the recommendations embodied in the Dietary Guidelines for Americans 2000. One exception is that total fat is allowed to range from 25%-35% of total calories provided saturated fats and *trans* fatty acids are kept low. A higher intake of total fat, mostly in the form of unsaturated fat, can help to reduce triglycerides and raise HDL cholesterol in persons with the metabolic syndrome. In accord with the Dietary Guidelines, moderate physical activity is encouraged. After 6 weeks, the LDL response is determined; if the LDL cholesterol goal has not been achieved, other therapeutic options for LDL lowering such as plant stanol/sterols and viscous fiber can be added.

After maximum reduction of LDL cholesterol with dietary therapy, emphasis shifts to management of the

metabolic syndrome and associated lipid risk factors. The majority of persons with these latter abnormalities are overweight or obese and sedentary. Weight reduction therapy for overweight or obese patients will enhance LDL lowering and will provide other health benefits including modifying other lipid and nonlipid risk factors. Assistance in the management of overweight and obese persons is provided by the *Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults* from the NHLBI Obesity Education Initiative (1998). Additional risk reduction can be achieved by simultaneously increasing physical activity.

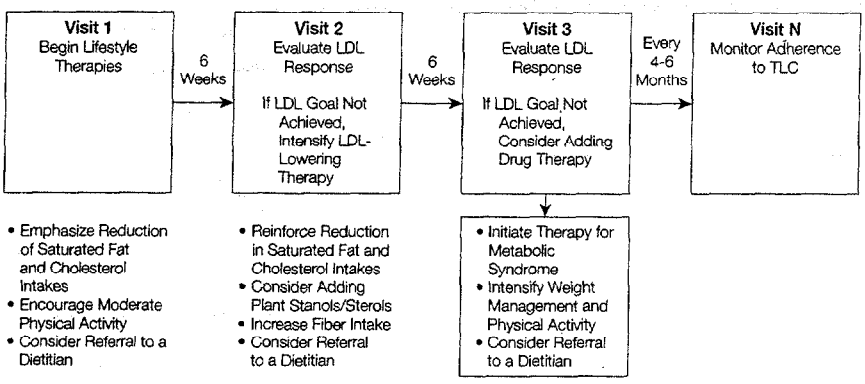
At all stages of dietary therapy, physicians are encouraged to refer patients to registered dietitians or other qualified nutritionists for *medical nutrition therapy*, which is the term for the nutritional intervention and guidance provided by a nutrition professional.

DRUG THERAPY TO ACHIEVE LDL CHOLESTEROL GOALS

A portion of the population whose short-term or long-term risk for CHD is high will require LDL-lowering drugs in addition to TLC to reach the designated goal for LDL cholesterol (see Table 5). When drugs are prescribed, attention to TLC should always be maintained and reinforced. Currently available drugs that affect lipoprotein metabolism and their major characteristics are listed in TABLE 7.

Some cholesterol-lowering agents are currently available over-the-counter (OTC) (eg, nicotinic acid), and manufacturers of several classes of LDL-lowering drugs (eg, statins, bile acid sequestrants) have applied to the Food and Drug Administration (FDA) to allow these agents to become OTC medications. At the time of publication of ATP III, the FDA has not granted permission for OTC status for statins or bile acid sequestrants. If an OTC cholesterol-lowering drug is or becomes available, patients should continue to consult with their physicians about whether to initiate drug treatment, about setting the goals of therapy, and about monitoring for therapeutic responses and side effects.

Figure 1. Model of Steps in Therapeutic Lifestyle Changes (TLC)



LDL indicates low-density lipoprotein.

Secondary Prevention: Drug Therapy for CHD and CHD Risk Equivalents

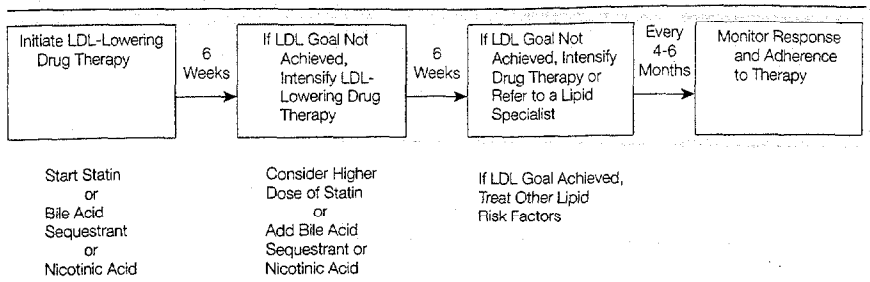
For persons with CHD and CHD risk equivalents, the goal is to attain an LDL cholesterol level of <100 mg/dL. The cutpoints for initiating lifestyle and drug

Table 7. Drugs Affecting Lipoprotein Metabolism*

Drug Class, Agents, and Daily Doses	Lipid/Lipoprotein Effects	Side Effects	Contraindications	Clinical Trial Results
HMG-CoA reductase inhibitors (statins)†	LDL ↓ 18%-55% HDL ↑ 5%-15% TG ↓ 7%-30%	Myopathy; increased liver enzymes	Absolute: active or chronic liver disease Relative: concomitant use of certain drugs§	Reduced major coronary events, CHD deaths, need for coronary procedures, stroke, and total mortality
Bile acid sequestrants‡	LDL ↓ 15%-30% HDL ↑ 3%-5% TG No change or increase	Gastrointestinal distress; constipation; decreased absorption of other drugs	Absolute: dysbetalipoproteinemia; TG >400 mg/dL Relative:TG >200 mg/dL	Reduced major coronary events and CHD deaths
Nicotinic acid‖	LDL ↓ 5%-25% HDL ↑ 15%-35% TG ↓ 20%-50%	Flushing; hyperglycemia; hyperuricemia (or gout); upper gastrointestinal distress; hepatotoxicity	Absolute: chronic liver disease; severe gout Relative: diabetes; hyperuricemia; peptic ulcer disease	Reduced major coronary events, and possibly total mortality
Fibric acids¶	LDL ↓ 5%-20% (may be increased in patients with high TG) HDL ↑ 10%-20% TG ↓ 20%-50%	Dyspepsia; gallstones; myopathy; unexplained non-CHD deaths in WHO study	Absolute: severe renal disease; severe hepatic disease	Reduced major coronary events

*HMG-CoA indicates 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; ↓, decrease; ↑, increase; and CHD, coronary heart disease.
†Lovastatin (20-80 mg), pravastatin (20-40 mg), simvastatin (20-80 mg), fluvastatin (20-80 mg), atorvastatin (10-80 mg), and cerivastatin (0.4-0.8 mg).
‡Cholestyramine (4-16 g), colestipol (5-20 g), and colesevelam (2.6-3.8 g).
§Cyclosporine, macrolide antibiotics, various antifungal agents, and cytochrome P-450 inhibitors (fibrates and niacin should be used with appropriate caution).
‖Immediate-release (crystalline) nicotinic acid (1.5-3 g), extended-release nicotinic acid (1-2 g), and sustained-release nicotinic acid (1-2 g).
¶Gemfibrozil (600 mg twice daily), fenofibrate (200 mg), and clofibrate (1000 mg twice daily).

Figure 2. Progression of Drug Therapy in Primary Prevention



LDL indicates low-density lipoprotein.

therapies are shown in Table 5. Most patients with CHD will need LDL-lowering drug therapy. Other lipid risk factors may also warrant consideration of drug treatment. Whether or not lipid-modifying drugs are used, nonlipid risk factors require attention and favorable modification.

In patients admitted to the hospital for a major coronary event, LDL cholesterol should be measured on admission or within 24 hours. This value can be used for treatment decisions. In general, persons hospitalized for a coronary event or procedure should be discharged on drug therapy if the LDL cholesterol is ≥130 mg/dL. If the LDL is 100-129 mg/dL, clinical judgment should be used in deciding whether to initiate drug treatment at discharge, recognizing that LDL cholesterol levels begin to decline in the first few hours after an event and are significantly decreased by 24 to 48 hours and may remain low for many weeks. Thus, the initial LDL cholesterol level obtained in the hospital may be substantially lower than is usual for the patient. Some authorities hold that drug therapy should be initiated whenever a patient hospitalized for a CHD-related illness is found to have an LDL cholesterol >100 mg/dL. Initiation of drug therapy at the time of hospital discharge has 2 advantages. First, at that time patients are particularly motivated to undertake and adhere to risk-lowering interventions; and second, failure to initiate indicated therapy early is one of the causes of a large "treatment gap," because outpatient follow-up is often less consistent and more fragmented.

LDL-Lowering Drug Therapy for Primary Prevention

Table 5 shows the cutpoints for considering drug treatment in primary prevention. The general approach to management of drug therapy for primary prevention is outlined in FIGURE 2.

When drug therapy for primary prevention is a consideration, the third visit of dietary therapy (see Figure 1) will typically be the visit to initiate drug treatment. Even if drug treatment is started, TLC should be continued. As with TLC, the first priority of drug therapy is to achieve the goal for LDL cholesterol. For this reason, an LDL-lowering drug should be started. The usual drug will be a statin, but alternatives are a bile acid sequestrant or nicotinic acid. In most cases, the statin should be started at a moderate dose. In many patients, the LDL cholesterol goal will be achieved, and higher doses will not be necessary. The patient's response should be evaluated about 6 weeks after starting drug therapy. If the goal of therapy has been achieved, the current dose can be maintained. However, if the goal has not been achieved, LDL-lowering therapy can be intensified, either by increasing the dose of statin or by combining a statin with a bile acid sequestrant or nicotinic acid.

After 12 weeks of drug therapy, the response to therapy should again be assessed. If the LDL cholesterol goal is still not achieved, consideration can be given to further intensification of drug therapy. If the LDL goal cannot be attained by standard lipid-lowering therapy, consideration should be given to seeking consultation from a lipid specialist. Once the goal for LDL cholesterol has been attained, attention can turn to other lipid risk factors and nonlipid factors. Thereafter, patients can be monitored for response to therapy every 4 to 6 months, or more often if considered necessary.

BENEFIT BEYOND LDL LOWERING: THE METABOLIC SYNDROME AS A SECONDARY TARGET OF THERAPY

Evidence is accumulating that risk for CHD can be reduced beyond LDL-lowering therapy by modification of other risk factors. One potential secondary target of therapy is the metabolic syndrome, which represents a constellation of lipid and nonlipid risk factors of metabolic origin. This syndrome is closely linked to a generalized metabolic disorder called *insulin resistance* in which the normal actions of insulin are impaired. Excess body fat (particularly abdominal obesity) and physical inactivity promote the development of insulin resistance, but some individuals also are genetically predisposed to insulin resistance.

The risk factors of the metabolic syndrome are highly concordant; in aggregate they enhance risk for CHD at any given LDL cholesterol level. For purposes of ATP III, the diagnosis of the metabolic syndrome is made when 3 or more of the risk determinants shown in TABLE 8 are present. These determinants include a combination of categorical and borderline risk factors that can be readily measured in clinical practice.

Management of the metabolic syndrome has a 2-fold objective: (1) to reduce underlying causes (ie, obesity and physical inactivity) and (2) to treat associated nonlipid and lipid risk factors.

Management of Underlying Causes of the Metabolic Syndrome

First-line therapies for all lipid and nonlipid risk factors associated with the metabolic syndrome are weight reduction and increased physical activity,

which will effectively reduce all of these risk factors. Therefore, after appropriate control of LDL cholesterol, TLC should stress weight reduction and physical activity if the metabolic syndrome is present.

Weight Control. In ATP III overweight and obesity are recognized as major, underlying risk factors for CHD and identified as direct targets of intervention. Weight reduction will enhance LDL lowering and reduce all of the risk factors of the metabolic syndrome. The recommended approaches for reducing overweight and obesity are contained in the clinical guidelines of the Obesity Education Initiative.

Physical Activity. Physical inactivity is likewise a major, underlying risk factor for CHD. It augments the lipid and nonlipid risk factors of the metabolic syndrome. It further may enhance risk by impairing cardiovascular fitness and coronary blood flow. Regular physical activity reduces very low-density lipoprotein (VLDL) levels, raises HDL cholesterol, and in some persons, lowers LDL levels. It also can lower blood pressure, reduce insulin resistance, and favorably influence cardiovascular function. Thus, ATP III recommends that regular physical activity become a routine component in management of high serum cholesterol. The evidence base for this recommendation is contained in the US Surgeon General's Report on Physical Activity.

Specific Treatment of Lipid and Nonlipid Risk Factors

Beyond the underlying risk factors, therapies directed against the lipid and nonlipid risk factors of the metabolic syndrome will reduce CHD risk. These include treatment of hypertension, use of aspirin in patients with CHD to reduce the prothrombotic state (guidelines for aspirin use in primary prevention have not been firmly established), and treatment of elevated triglycerides and low HDL cholesterol as discussed below under "Management of Specific Dyslipidemias."

SPECIAL ISSUES

Management of Specific Dyslipidemias

Very High LDL Cholesterol (≥190 mg/dL). Persons with very high LDL cholesterol usually have genetic forms of hypercholesterolemia: monogenic familial hypercholesterolemia, familial defective apolipoprotein B, and polygenic hypercholesterolemia. Early detection of these disorders through cholesterol testing in young adults is needed to prevent premature CHD. Family testing is important to identify similarly affected relatives. These disorders often require combined drug therapy (statin + bile acid sequestrant) to achieve the goals of LDL-lowering therapy.

Elevated Serum Triglycerides. Recent meta-analyses of prospective studies indicate that elevated triglycerides are also an independent risk factor for CHD. Factors contributing to elevated (higher than normal) triglycerides in the general population include obesity and overweight, physical inactivity, cigarette smoking, excess alcohol intake, high-carbohydrate diets (>60% of energy intake), several diseases (eg, type 2 diabetes, chronic renal failure, nephrotic syndrome), certain drugs (eg, corticosteroids, estrogens, retinoids, higher doses of β-adrenergic blocking agents), and genetic disorders (familial combined hyperlipidemia, familial hypertriglyceridemia, and familial dysbetalipoproteinemia).

In clinical practice, elevated serum triglycerides are most often observed in persons with the metabolic syndrome, although secondary or genetic factors can heighten triglyceride levels. ATP III adopts the following classification of serum triglycerides:

- Normal triglycerides: <150 mg/dL
- Borderline-high triglycerides: 150-199 mg/dL
- High triglycerides: 200-499 mg/dL
- Very high triglycerides: ≥500 mg/dL

(To convert triglyceride values to mmol/L, divide by 88.6.)

The finding that elevated triglycerides are an independent CHD risk factor suggests that some triglyceride-rich lipoproteins are atherogenic. The latter are partially degraded VLDL, commonly called *remnant lipoproteins.* In clinical practice, VLDL cholesterol is the most readily available measure of atherogenic remnant lipoproteins. Thus, VLDL cholesterol can be a target of cholesterol-lowering therapy. ATP III identifies the sum of LDL + VLDL cholesterol (termed *non-HDL cholesterol* [total cholesterol − HDL cholesterol]) as a secondary target of therapy in persons with high triglycerides (≥200 mg/dL). The goal for non-HDL cholesterol in persons with high serum triglycerides can be set at 30 mg/dL higher than that for LDL cholesterol (TABLE 9) on the premise that a VLDL cholesterol level ≤30 mg/dL is normal.

The treatment strategy for elevated triglycerides depends on the causes of the elevation and its severity. For all persons

Table 8. Clinical Identification of the Metabolic Syndrome

Risk Factor	Defining Level
• Abdominal obesity* (waist circumference)†	
Men	>102 cm (>40 in)
Women	>88 cm (>35 in)
• Triglycerides	≥150 mg/dL
• High-density lipoprotein cholesterol	
Men	<40 mg/dL
Women	<50 mg/dL
• Blood pressure	≥130/≥85 mm Hg
• Fasting glucose	≥110 mg/dL

*Overweight and obesity are associated with insulin resistance and the metabolic syndrome. However, the presence of abdominal obesity is more highly correlated with the metabolic risk factors than is an elevated body mass index (BMI). Therefore, the simple measure of waist circumference is recommended to identify the body weight component of the metabolic syndrome.

†Some male patients can develop multiple metabolic risk factors when the waist circumference is only marginally increased, eg, 94-102 cm (37-40 in). Such patients may have strong genetic contribution to insulin resistance and they should benefit from changes in life habits, similarly to men with categorical increases in waist circumference.

Table 9. Comparison of LDL Cholesterol and Non-HDL Cholesterol Goals for 3 Risk Categories*

Risk Category	LDL Goal (mg/dL)	Non-HDL Goal (mg/dL)
CHD and CHD risk equivalent (10-year risk for CHD >20%)	<100	<130
Multiple (2+) risk factors and 10-year risk ≤20%	<130	<160
0-1 Risk factor	<160	<190

*LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; and CHD, coronary heart disease.

with borderline high or high triglycerides, the primary aim of therapy is to achieve the target goal for LDL cholesterol. When triglycerides are borderline high (150-199 mg/dL), emphasis should also be placed on weight reduction and increased physical activity. For high triglycerides (200-499 mg/dL), non-HDL cholesterol becomes a secondary target of therapy. Aside from weight reduction and increased physical activity, drug therapy can be considered in high-risk persons to achieve the non-HDL cholesterol goal. There are 2 approaches to drug therapy. First, the non-HDL cholesterol goal can be achieved by intensifying therapy with an LDL-lowering drug; second, nicotinic acid or fibrate can be added, if used with appropriate caution, to achieve the non-HDL cholesterol goal by further lowering VLDL cholesterol. In rare cases in which triglycerides are very high (≥500 mg/dL), the initial aim of therapy is to prevent acute pancreatitis through triglyceride lowering. This approach requires very low-fat diets (≤15% of calorie intake), weight reduction, increased physical activity, and usually a triglyceride-lowering drug (fibrate or nicotinic acid). Only after triglyceride levels have been lowered to <500 mg/dL should attention turn to LDL lowering to reduce risk for CHD.

Low HDL Cholesterol. Low HDL cholesterol is a strong independent predictor of CHD. In ATP III, low HDL cholesterol is defined categorically as a level <40 mg/dL, a change from the level of <35 mg/dL in ATP II. In the present guidelines, low HDL cholesterol both modifies the goal for LDL-lowering therapy and is used as a risk factor to estimate 10-year risk for CHD.

Low HDL cholesterol levels have several causes, many of which are associated with insulin resistance, ie, elevated triglycerides, overweight and obesity, physical inactivity, and type 2 diabetes. Other causes are cigarette smoking, very high carbohydrate intakes (>60% of calories), and certain drugs (eg, β-blockers, anabolic steroids, progestational agents).

ATP III does not specify a goal for HDL raising. Although clinical trial results suggest that raising HDL will reduce risk, the evidence is insufficient to specify a goal of therapy. Furthermore, currently available drugs do not robustly raise HDL cholesterol. Nonetheless, a low HDL should receive clinical attention and management according to the following sequence. In all persons with low HDL cholesterol, the primary target of therapy is LDL cholesterol; ATP III guidelines should be followed to achieve the LDL cholesterol goal. Second, after the LDL goal has been reached, emphasis shifts to weight reduction and increased physical activity (when the metabolic syndrome is present). When a low HDL cholesterol is associated with high triglycerides (200-499 mg/dL), secondary priority goes to achieving the non-HDL cholesterol goal, as outlined earlier. Also, if triglycerides are <200 mg/dL (isolated low HDL cholesterol), drugs for HDL raising (fibrates or nicotinic acid) can be considered; however, treatment for isolated low HDL is mostly reserved for persons with CHD and CHD risk equivalents.

Diabetic Dyslipidemia. This disorder is essentially atherogenic dyslipidemia in persons with type 2 diabetes. Although elevated triglycerides, low HDL cholesterol, or both are common in persons with diabetes, clinical trial results support the identification of LDL cholesterol as the primary target of therapy, as it is in those without diabetes. Since diabetes is designated a CHD risk equivalent in ATP III, the LDL cholesterol goal of therapy for most persons with diabetes will be <100 mg/dL. Furthermore, when LDL cholesterol is ≥130 mg/dL, most persons with diabetes will require initiation of LDL-lowering drugs simultaneously with TLC to achieve the LDL goal. When LDL cholesterol levels are in the range of 100-129 mg/dL at baseline or on treatment, several therapeutic options are available: increasing intensity of LDL-lowering therapy, adding a drug to modify atherogenic dyslipidemia (fibrate or nicotinic acid), or intensifying control of other risk factors including hyperglycemia. When triglyceride levels are ≥200 mg/dL, non-HDL cholesterol becomes a secondary target of cholesterol-lowering therapy. Several ongoing clinical trials (eg, Antihypertensive and Lipid Lowering Heart Attack Trial [ALLHAT]) will better quantify the magnitude of the benefit of LDL-lowering treatment in older individuals with diabetes. In older persons (≥65 years) with diabetes but no additional CHD risk factors other than age, clinical judgment is required for how intensively to apply these guidelines. A variety of factors, including concomitant illnesses, general health status, and social issues, may influence treatment decisions and may suggest a more conservative approach.

Special Considerations for Different Population Groups

Middle-Aged Men (35-65 Years). In general, men have a higher risk for CHD than do women. Middle-aged men in particular have a high prevalence of the major risk factors and are predisposed to abdominal obesity and the metabolic syndrome. A sizable fraction of all CHD in men occurs in middle age. Thus, many middle-aged men carry a relatively high risk for CHD, and for those who do, intensive LDL-lowering therapy is needed.

Women Aged 45-75 Years. In women, onset of CHD generally is delayed by some 10 to 15 years compared with that in men; thus, most CHD in women occurs after age 65 years. All risk factors contribute to CHD in women, and most premature CHD in women (<65 years) occurs in those with multiple risk factors and the metabolic syndrome. Despite the previous belief that the sex difference in risk for CHD reflects a protective effect of estrogen in women, recent secondary and primary prevention trials cast doubt on the use of hormone replacement therapy to reduce CHD risk in postmenopausal women. In contrast, the favorable effects of statin therapy in women in clinical trials make a cholesterol-lowering drug preferable to hormone replacement therapy for CHD risk reduction. Women should be treated similarly to men for secondary prevention. For primary prevention, ATP III's general approach is similarly applicable for women and men. However, the later on-

Table 10. Interventions to Improve Adherence

Focus on the Patient
• Simplify medication regimens
• Provide explicit patient instruction and use good counseling techniques to teach the patient how to follow the prescribed treatment
• Encourage the use of prompts to help patients remember treatment regimens
• Use systems to reinforce adherence and maintain contact with the patient
• Encourage the support of family and friends
• Reinforce and reward adherence
• Increase visits for patients unable to achieve treatment goal
• Increase the convenience and access to care
• Involve patients in their care through self-monitoring
Focus on the Physician and Medical Office
• Teach physicians to implement lipid treatment guidelines
• Use reminders to prompt physicians to attend to lipid management
• Identify a patient advocate in the office to help deliver or prompt care
• Use patients to prompt preventive care
• Develop a standardized treatment plan to structure care
• Use feedback from past performance to foster change in future care
• Remind patients of appointments and follow up missed appointments
Focus on the Health Delivery System
• Provide lipid management through a lipid clinic
• Utilize case management by nurses
• Deploy telemedicine
• Utilize the collaborative care of pharmacists
• Execute critical care pathways in hospitals

set of CHD for women in general should be factored into clinical decisions about use of cholesterol-lowering drugs.

Older Adults (Men ≥65 Years and Women ≥75 Years). Overall, most new CHD events and most coronary deaths occur in older persons (≥65 years). A high level of LDL cholesterol and low HDL cholesterol still carry predictive power for the development of CHD in older persons. Nevertheless, the finding of advanced subclinical atherosclerosis by noninvasive testing can be helpful for confirming the presence of high risk in older persons. Secondary prevention trials with statins have included a sizable number of older persons, mostly in the age range of 65 to 75 years. In these trials, older persons showed significant risk reduction with statin therapy. Thus, no hard-and-fast age restrictions appear necessary when selecting persons with established CHD for LDL-lowering therapy. For primary prevention, TLC is the first line of therapy for older persons. However, LDL-lowering drugs can also be considered when older persons are at higher risk because of multiple risk factors or advanced subclinical atherosclerosis.

Younger Adults (Men 20-35 Years; Women 20-45 Years). In this age group, CHD is rare except in those with severe risk factors, eg, familial hypercholesterolemia, heavy cigarette smoking, or diabetes. Even though clinical CHD is relatively rare in young adults, coronary atherosclerosis in its early stages may progress rapidly. The rate of development of coronary atherosclerosis earlier in life correlates with the major risk factors. In particular, long-term prospective studies reveal that elevated serum cholesterol detected in young adulthood predicts a higher rate of premature CHD in middle age. Thus, risk factor identification in young adults is an important aim for long-term prevention. The combination of early detection and early intervention on elevated LDL cholesterol with life-habit changes offers the opportunity for delaying or preventing onset of CHD later in life. For young adults with LDL cholesterol levels of ≥130 mg/dL, TLC should be instituted and emphasized. Particular attention should be given to young men who smoke and have a high LDL cholesterol (160-189 mg/dL); they may be candidates for LDL-lowering drugs. When young adults have very high LDL cholesterol levels (≥190 mg/dL), drug therapy should be considered, as in other adults. Those with severe genetic forms of hypercholesterolemia may require LDL-lowering drugs in combination (eg, statin+bile acid sequestrant).

Racial and Ethnic Groups. African Americans have the highest overall CHD mortality rate and the highest out-of-hospital coronary death rates of any ethnic group in the United States, particularly at younger ages. Although the reasons for the excess CHD mortality among African Americans have not been fully elucidated, it can be accounted for, at least in part, by the high prevalence of coronary risk factors. Hypertension, left ventricular hypertrophy, diabetes mellitus, cigarette smoking, obesity, physical inactivity, and multiple CHD risk factors all occur more frequently in African Americans than in whites. Other ethnic groups and minority populations in the United States include Hispanics, Native Americans, Asian and Pacific Islanders, and South Asians. Although limited data suggest that racial and ethnic groups vary somewhat in baseline risk for CHD, this evidence did not appear sufficient to lead the ATP III panel to modify general recommendations for cholesterol management in these populations.

ADHERENCE TO LDL-LOWERING THERAPY

Adherence to the ATP III guidelines by both patients and providers is a key to approximating the magnitude of the benefits demonstrated in clinical trials of cholesterol lowering. Adherence issues have to be addressed to attain the highest possible levels of CHD risk reduction. Thus, ATP III recommends the use of state-of-the-art multidisciplinary methods targeting the patient, clinicians, and health delivery systems to achieve the full population effectiveness of the guidelines for primary and secondary prevention (TABLE 10).

National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

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Member Organizations: *National Heart, Lung, and Blood Institute:* Claude Lenfant, MD (Chair), James I. Cleeman, MD (Coordinator); *American Academy of Family Physicians:* Theodore G. Ganiats, MD; *American Academy of Insurance Medicine:* Gary Graham, MD; *American Academy of Pediatrics:* Ronald E. Kleinman, MD; *American Association of Occupational Health Nurses:* Pamela Hixon, BSN, RN, COHN-S; *American College of Cardiology:* Richard C. Pasternak, MD; *American College of Chest Physicians:* Gerald T. Gau, MD, *American College of Nutrition:* Harry Preuss, MD; *American College of Obstetricians and Gynecologists:* Thomas C. Peng, MD; *American College of Occupational and Environmental Medicine:* Ruth Ann Jordan, MD; *American College of Preventive Medicine:* Lewis H. Kuller, MD, DrPH; *American Diabetes Association, Inc:* Alan J. Garber, MD, PhD; *American Dietetic Association:* Linda Van Horn, PhD, RD; *American Heart Association:* Scott M. Grundy, MD, PhD; *American Hospital Association:* Sandra Cornett, RN, PhD; *American Medical Association:* Yank D. Coble, Jr, MD; *American Nurses Association:* to be named; *American Osteopathic Association:* Michael Clearfield, DO; *American Pharmaceutical Association:* James M. McKenney, PharmD; *American Public Health Association:* Stephen Havas, MD, MPH, MS; *American Red Cross:* Donald Vardell, MS; *Association of Black Cardiologists:* Karol Watson, MD, PhD; *Association of State and Territorial Health Officials:* Joanne Mitten, MHE; *Citizens for Public Action on Blood Pressure and Cholesterol, Inc:* Gerald J. Wilson, MA, MBA; *National Black Nurses Association, Inc:* Linda Burnes-Bolton, DrPH, RN, MSN; *National Medical Association:* Luther T. Clark, MD; *Society for Nutrition Education:* Darlene Lansing, MPH, RD; *Society for Public Health Education:* Donald O. Fedder, DrPH, MPH.

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APPENDIX

Shared Features of ATP III and ATP II

Adult Treatment Panel (ATP) III shares a set of core features with ATP II, shown in Table A.

Table A. *Shared Features of ATP III and ATP II**

- Continued identification of LDL cholesterol lowering as the primary goal of therapy
- Consideration of high LDL cholesterol (≥160 mg/dL) as a potential target for LDL-lowering drug therapy, specifically as follows:
  - For persons with multiple risk factors whose LDL levels are high (≥160 mg/dL) after dietary therapy, consideration of drug therapy is recommended
  - For persons with 0-1 risk factor, consideration of drug therapy (after dietary therapy) is optional for LDL 160-189 mg/dL and recommended for LDL ≥190 mg/dL
- Emphasis on intensive LDL-lowering therapy in persons with established CHD
- Identification of 3 categories of risk for different LDL goals and different intensities of LDL-lowering therapy:
  - CHD and CHD risk equivalents† (other forms of clinical atherosclerotic disease)
  - Multiple (2+) risk factors‡
  - 0-1 risk factor
- Identification of subpopulations, besides middle-aged men, for detection of high LDL cholesterol (and other lipid risk factors) and for clinical intervention. These include:
  - Young adults
  - Postmenopausal women
  - Older persons
- Emphasis on weight loss and physical activity to enhance risk reduction in persons with elevated LDL cholesterol

*ATP indicates Adult Treatment Panel; LDL, low-density lipoprotein; and CHD, coronary heart disease.
†A CHD risk equivalent is a condition that carries an absolute risk for developing new CHD equal to the risk for having recurrent CHD events in persons with established CHD.
‡Risk factors that continue to modify the LDL goal include cigarette smoking, hypertension, low HDL cholesterol, family history of premature CHD, age (male ≥45 years and female ≥55 years), and diabetes (in ATP III diabetes is regarded as a CHD risk equivalent).

Estimating 10-Year Risk for Men and Women

Risk assessment for determining the 10-year risk for developing CHD is carried out using Framingham risk scoring (Table B1 for men and Table B2 for women). The risk factors included in the Framingham calculation of 10-year risk are age, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension, and cigarette smoking. The first step is to calculate the number of points for each risk factor. For initial assessment, values for total cholesterol and HDL cholesterol are required. Because of a larger database, Framingham estimates are more robust for total cholesterol than for LDL cholesterol. Note, however, that the LDL cholesterol level remains the primary target of therapy. Total cholesterol and HDL cholesterol values should be the average of at least 2 measurements obtained from lipoprotein analysis. The blood pressure value used is that obtained at the time of assessment, regardless of whether the person is on antihypertensive therapy. However, if the person is on antihypertensive treatment, an extra point is added beyond points for the blood pressure reading because treated hypertension carries residual risk (Tables B1 and B2). The average of several blood pressure measurements, as recommended by the Joint National Committee (JNC), is needed for an accurate measure of baseline blood pressure. The designation "smoker" means any cigarette smoking in the past month. The total risk score sums the points for each risk factor. The 10-year risk for myocardial infarction and coronary death (hard CHD) is estimated from total points, and the person is categorized according to absolute 10-year risk as indicated above (see Table 5).

Table B1. Estimate of 10-Year Risk for Men (Framingham Point Scores)

Age, y	Points
20-34	−9
35-39	−4
40-44	0
45-49	3
50-54	6
55-59	8
60-64	10
65-69	11
70-74	12
75-79	13

Total Cholesterol, mg/dL	Points				
	Age 20-39 y	Age 40-49 y	Age 50-59 y	Age 60-69 y	Age 70-79 y
<160	0	0	0	0	0
160-199	4	3	2	1	0
200-239	7	5	3	1	0
240-279	9	6	4	2	1
≥280	11	8	5	3	1

	Points				
	Age 20-39 y	Age 40-49 y	Age 50-59 y	Age 60-69 y	Age 70-79 y
Nonsmoker	0	0	0	0	0
Smoker	8	5	3	1	1

HDL, mg/dL	Points
≥60	−1
50-59	0
40-49	1
<40	2

Systolic BP, mm Hg	If Untreated	If Treated
<120	0	0
120-129	0	1
130-139	1	2
140-159	1	2
≥160	2	3

Point Total	10-Year Risk, %
<0	<1
0	1
1	1
2	1
3	1
4	1
5	2
6	2
7	3
8	4
9	5
10	6
11	8
12	10
13	12
14	16
15	20
16	25
≥17	≥30

Table B2. Estimate of 10-Year Risk for Women (Framingham Point Scores)

Age, y	Points
20-34	−7
35-39	−3
40-44	0
45-49	3
50-54	6
55-59	8
60-64	10
65-69	12
70-74	14
75-79	16

Total Cholesterol, mg/dL	Points				
	Age 20-39 y	Age 40-49 y	Age 50-59 y	Age 60-69 y	Age 70-79 y
<160	0	0	0	0	0
160-199	4	3	2	1	1
200-239	8	6	4	2	1
240-279	11	8	5	3	2
≥280	13	10	7	4	2

	Points				
	Age 20-39 y	Age 40-49 y	Age 50-59 y	Age 60-69 y	Age 70-79 y
Nonsmoker	0	0	0	0	0
Smoker	9	7	4	2	1

HDL, mg/dL	Points
≥60	−1
50-59	0
40-49	1
<40	2

Systolic BP, mm Hg	If Untreated	If Treated
<120	0	0
120-129	1	3
130-139	2	4
140-159	3	5
≥160	4	6

Point Total	10-Year Risk, %
<9	<1
9	1
10	1
11	1
12	1
13	2
14	2
15	3
16	4
17	5
18	6
19	8
20	11
21	14
22	17
23	22
24	27
≥25	≥30

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