

KUOPION YLIOPISTON JULKAISUJA D. LÄÄKETIEDE 251
KUOPIO UNIVERSITY PUBLICATIONS D. MEDICAL SCIENCES 251

MAARIT HALLIKAINEN

**Role of plant stanol ester- and sterol ester-enriched
margarines in the treatment of hypercholesterolemia**

Doctoral dissertation

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on Saturday 29th September 2001, at 12 noon.

Department of Clinical Nutrition
University of Kuopio and Kuopio University Hospital

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Series editors: Professor Esko Alhava, M.D., Ph.D.
Department of Surgery

Professor Martti Hakumäki, M.D., Ph.D.
Department of Physiology

Author's address: Department of Clinical Nutrition
University of Kuopio
P.O.Box 1627
FIN-70211 KUOPIO
FINLAND

Supervisors: Professor Matti Uusitupa, M.D.
Department of Clinical Nutrition
University of Kuopio

Docent Essi Sarkkinen, Ph.D.
Oy Foodfiles Ltd. and
Department of Clinical Nutrition
University of Kuopio

Reviewers: Acting Professor, Docent Hannu Vanhanen, M.D.
Department of Medicine,
University of Helsinki

Docent Terho Lehtimäki, M.D.
Department of Clinical Chemistry,
University of Tampere

Opponent: Emeritus Professor Tatu Miettinen, M.D.
Department of Medicine,
University of Helsinki

Hallikainen, Maarit. Role of plant stanol ester- and sterol ester-enriched margarines in the treatment of hypercholesterolemia. Kuopio University Publications D. Medical Sciences 251. 2001. 90 p.
ISBN 951-781-851-3
ISSN 1235-0303

ABSTRACT

The effects of plant stanol esters or sterol esters on serum lipids and lipoprotein lipids, serum fat-soluble vitamins and carotenoids, serum cholesterol precursors as well as serum plant sterols and stanols were examined in mildly or moderately hypercholesterolemic men and women. Study I/II utilized a parallel study design, studies III/IV and V involved a repeated measures design. In study I/II 55 subjects were randomized after a 4-week baseline, high-fat, diet period into three experimental groups ingesting three low-fat margarines: wood stanol ester (WSEM), vegetable oil stanol ester (VOSEM) and control. The groups consumed the margarines for eight weeks as part of a low-fat, low-cholesterol diet. In study III/IV, each of 22 subjects consumed five different doses of plant stanol [target (actual) intake 0 (0), 0.8 (0.8), 1.6 (1.6), 2.4 (2.3), 3.2 (3.1) g/day] added as stanol esters to margarine for four weeks as part of a standardized habitual diet. The order of dose periods was randomly determined. In study V, 34 subjects consumed stanol ester (STAEST), sterol ester (STEEST) and control margarines as part of a cholesterol-lowering diet each for four weeks. The randomization was performed according to the Latin square model.

In study I, the low-fat WSEM and VOSEM margarines reduced serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) as part of a cholesterol-lowering diet significantly by 16-18% and 18-24%, respectively, from a high-fat baseline diet. An additional approximately 10% cholesterol-lowering effect of these margarines compared with the low-fat diet (control) was noted (I). There was no significant difference in the cholesterol-lowering efficacy between these test margarines (I). Study III showed that the effect of plant stanol esters on serum TC and LDL-C is dose-dependent. A significant reduction in serum TC and LDL-C was achieved with the stanol dose of 1.6 g/d, and increasing the dose from 2.4 g/d to 3.2 g/d did not offer additional cholesterol-lowering effect. In study V, no significant differences between the STAEST and STEEST margarines with respect to efficacy in reducing serum TC (9.2% vs. 7.3%, compared with control) and LDL-C (12.7% vs. 10.4%) in short-term were found.

Plant stanol esters or sterol esters did not affect serum fat-soluble vitamins (I, III, V). Their impact on serum carotenoids was minor (I/II, III, V) when the dietary intake of vegetables was ensured.

Plant stanol esters reduced serum plant sterol concentrations significantly already with the stanol dose of 0.8 g/d (III/IV) indicating that cholesterol absorption was effectively inhibited already with the small stanol ester doses. The findings of serum Δ^7 -lathosterol/TC ratio (an indirect indicator of cholesterol synthesis) indicated that cholesterol synthesis was stimulated by a stanol dose of 0.8 g/d, but no further increase was observed when the stanol dose was higher than 1.6 g/d (IV). The consumption of plant stanol esters increased serum sitostanol and campestanol concentrations by about twofold, but the concentrations remained extremely low, and they plateaued with a dose equal to or greater than the 0.8 g/d (III/IV).

In conclusion, plant stanol ester- and sterol ester-enriched margarines are an effective and safe way to achieve a reduction in serum cholesterol when they are consumed as part of a low-fat, low-cholesterol diet. The optimal dose of stanol ester is 1.6-2.4 g/d of stanols.

National Library of Medicine Classification WD 200.5.H8, QU 86, WA 722

Medical subject headings: anticholesterolemic agents; carotenoids/blood; cholesterol/blood; diet, fat-restricted; dietary fats; hypercholesterolemia; lipids/blood; margarine; phytosterols/therapeutic use; vitamins/blood

ACKNOWLEDGEMENTS

This work was carried out in the University of Kuopio, Department of Clinical Nutrition.

I wish to express my deepest gratitude to my principal supervisor, Professor Matti Uusitupa, M.D., now the rector of the University of Kuopio, for the possibility to do research work and for his support and professional guidance during these years.

I am also greatly indebted to my other supervisor, Docent Essi Sarkkinen, Ph.D. Her advice, unfailing support and encouragement have been indispensable at all stages of this work.

I am very grateful to the official reviewers of this work, Acting Professor, Docent Hannu Vanhanen, M.D., and Docent Terho Lehtimäki, M.D., for their constructive criticism and valuable suggestions.

I am very grateful for Professor Helena Gylling, M.D., for her expert advice and comments regarding the manuscripts of the articles and this thesis.

I want to express my gratitude to Arja Erkkilä, Ph.D., for analyzing the fatty acid composition of serum lipids, and Virpi Lindi, M.Sc., and Raisa Valve, Ph.D., for determining apo E genotypes. In addition, I want to thank Arja and Virpi for their invaluable help in so many different ways during these years.

I wish to thank Ms. Irja Kanniainen, Kaija Kettunen and Erja Kinnunen for skillfully performing the laboratory work. In addition, I wish to thank Professor Gerald Salen, M.D., and his coworkers for analyzing serum plant sterols in study I, and Ms. Leena Kaipiainen, Pia Hoffström and Ritva Nissilä for analyzing serum plant sterols and cholesterol precursors in studies III/IV and V.

I wish to thank nutritionists Soili Lehtomäki, M.Sc., for nutrition counseling (study V) and Niina Tapola, M.Sc., for her great help in managing the food records (studies I/II and III/IV).

I owe my special thanks to Helvi Vidgren, Ph.D., with whom I have shared successes and setbacks. Her constant support and encouragement during these years have meant a lot to me. I am also grateful to Professor Hannu Mykkänen, Ph.D., Leila Karhunen, Ph.D., Marjukka Kolehmainen, M.Sc., Anne Louheranta, M.Sc., and Ursula Schwab, Ph.D., for much good advice during these years. In addition, I want to thank the entire personnel of the Department of Clinical Nutrition, University of Kuopio for the pleasant working atmosphere.

I wish to thank statistician Pirjo Halonen, M.Sc., and Veikko Jokela, M.Sc., for their help concerning statistical analyses. I also wish to thank Ewen MacDonald, Ph.D., who revised the English language of this thesis.

I express my sincere thanks to all subjects who participated in these studies and made this work possible.

My warmest thanks belong to my parents, sisters and brothers and their families, other relatives and friends for their support during this work. In addition, I want especially thank my sister, Professor Vieno Piironen, Ph.D., for good "tips" and information concerning the plant sterol content of food items.

Last, but not least I want to thank my dear husband, Lauri, for his love and support so in the good and the bad days.

This study was financially supported by Raisio Benecol Ltd., Kuopio University, Kuopio University Hospital, the Finnish Cultural Foundation of Northern Savo and Juho Vainio Foundation, all of which I acknowledge with gratitude.

Kuopio, August 2001

Maarit Hallikainen

ABBREVIATIONS

ACAT	Acyl-CoA:cholesterol acyltransferase
ANOVA	Analysis of variance
apo	Apolipoprotein
BMI	Body mass index
CAD	Coronary artery disease
DM	Diabetes mellitus
E%	Percent of energy
FCR	Fractional catabolic rate
FH	Familial hypercholesterolemia
FH-NK	Familial hypercholesterolemia - North Karelia mutation of low density lipoprotein receptor gene
FW	Fresh weight
GLC	Gas liquid chromatograph
GLM	General Linear Models
HDL-C	High density lipoprotein cholesterol
HDL-TG	High density lipoprotein triglyceride(s)
IDL-C	Intermediate density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
LDL-TG	Low density lipoprotein triglyceride(s)
MANOVA	Multivariate analysis of variance
MUFA	Monounsaturated fatty acid(s)
NCEP	National Cholesterol Education Program
P/S	Polyunsaturated to saturated fatty acids
PUFA	Polyunsaturated fatty acid(s)
RE	Retinol equivalents
SAFA	Saturated fatty acid(s)
Sitostanol	β -sitostanol
Sitosterol	β -sitosterol
STAEST	Stanol ester
STEEST	Sterol ester
TC	Total cholesterol
TG	Triglyceride(s)
TR	Transport rate
VLDL-C	Very low density lipoprotein cholesterol
VLDL-TG	Very low density lipoprotein triglyceride(s)
VOSEM	Vegetable oil stanol ester-enriched margarine
WSEM	Wood stanol ester-enriched margarine

Plant sterol and plant stanol products contain, in particular, β -sitosterol or β -sitostanol, respectively; and therefore many authors have used ' β -sitosterol' or ' β -sitostanol' when describing their products. In this thesis terminology 'plant sterols' and 'plant stanols', respectively, have been used, because preparations contain usually at least traces of other sterols. In addition, in this thesis the term 'plant sterols' is also used as a generic term to include free and esterified plant sterols as well as free and esterified plant stanols if no particular form of plant sterols was especially emphasized or specified.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals (I-V):

- I Hallikainen MA, Uusitupa MIJ. Effects of 2 low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects. *Am J Clin Nutr* 1999;69:403-410.
- II Hallikainen MA, Sarkkinen ES, Uusitupa MIJ. Effects of low-fat stanol ester margarines on concentrations of serum carotenoids in subjects with elevated serum cholesterol concentrations. *Eur J Clin Nutr* 1999;53:966-969.
- III Hallikainen MA, Sarkkinen ES, Uusitupa MIJ. Plant stanol esters affect serum cholesterol concentrations of hypercholesterolemic men and women in a dose-dependent manner. *J Nutr* 2000;130:767-776.
- IV Hallikainen MA, Sarkkinen ES, Gylling H, Uusitupa MI. Plant stanol esters affect serum plant sterols, but not in serum cholesterol precursors in a dose-dependent manner in hypercholesterolemic subjects (submitted).
- V Hallikainen MA, Sarkkinen ES, Gylling H, Erkkilä AT, Uusitupa MI. Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. *Eur J Clin Nutr* 2000;54:715-725.

In addition, some unpublished results are presented.

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1 INTRODUCTION

Dietary changes alone result usually in a modest reduction (3-6%) in serum total (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations at the population level (1). Therefore, great interest has been focused on plant sterols which have a clear hypocholesterolemic effect and which can be added to normal food items.

Plant sterols, which resemble cholesterol structurally, are essential components of all plant cells. The most common plant sterols are β -sitosterol (sitosterol), campesterol and stigmasterol. The most common saturated forms of plant sterols are β -sitostanol (sitostanol) and campestanol. Since the 1950's, plant sterols have been known to have hypocholesterolemic properties (2). This is based on their ability to inhibit intestinal absorption of both dietary and biliary cholesterol. In the 1970s, plant sterols were marketed as cholesterol-lowering agents, however, owing to high doses, poor-solubility and their chalky taste, they were gradually displaced by new and more effective drugs, the statins. In the early 1990s, an innovation to transesterify plant sterols with fatty acids made it possible to add plant sterols to fat-containing food items (e.g. margarines) in a soluble-form without affecting their sensory properties.

Several clinical studies on plant stanol esters and sterol esters have shown the cholesterol-lowering efficacy of these agents (3). In most of the earlier studies, moderate rich or high-fat diets have been used. Two clinically relevant questions have remained; can plant stanol esters and sterol esters be effective also as part of a cholesterol-lowering diet and do they provide an additional cholesterol-lowering efficacy compared with a low-fat diet alone? Different amounts of plant stanols (0.7-4.0 g/d) have been used in evaluating the hypocholesterolemic effects of plant stanol esters. However, there are no studies in which the dose-response effect of stanol esters has been investigated with several different doses i.e. is there a dose of stanol ester beyond which no additional benefits can be obtained? The chemical structure of different plant sterols may affect cholesterol-lowering efficacy of these agents. However, comparative studies between stanol esters and sterol esters have yielded inconsistent results (4, 5).

The primary aim of the present studies was to investigate the role of stanol ester- and sterol ester-enriched margarines in lowering elevated serum cholesterol concentrations as part of a low-fat diet, and to determine the optimal dose of plant stanol esters in practice.

2 REVIEW OF LITERATURE

2.1 Plant sterols

2.1.1 Nomenclature and structure of plant sterols

Plant sterols, also called phytosterols, are steroid alcohols. They resemble cholesterol structurally in that they contain a tetracyclic cyclopenta[a]phenanthrene ring in the α -configuration, a 3β -hydroxyl group and an alkyl side chain at the C-17 carbon atom in the β -configuration (6, 7). The most common plant sterols are 4-desmethylsterols (8), which differ from cholesterol in their side-chain substitution (extra ethyl or methyl group) at the C-24 position, and/or an additional double bond in the side chain (Figure 1). The most common representatives of that structure are sitosterol (24 α -ethylcholest-5-en-3 β -ol), campesterol (24 α -methylcholest-5-en-3 β -ol) and stigmasterol (24 α -ethylcholest-5,22-en-3 β -ol). The double bond in the B ring can also be in a different position, accordingly these sterols can be categorized to Δ^5 -sterols, Δ^7 -sterols and $\Delta^{5,7}$ -sterols (9). The ring structure of plant sterols can also be saturated. The most common plant stanols are sitostanol (24 α -ethylcholest-3 β -ol), and campestanol (24 α -methylcholest-3 β -ol). Plant materials contain also minor amounts of 4 α -monomethyl sterols and 4,4-dimethyl sterols, which are the precursors of plant sterols (8, 10).

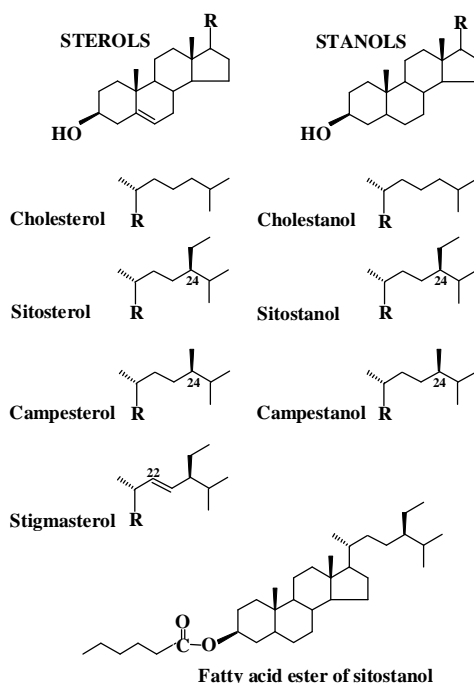


Figure 1. Structure of cholesterol and the most common plant sterols and their saturated forms, and as an example, the structure of the fatty acid ester of sitostanol is shown.

2.1.2 Occurrence of plant sterols in different plants

Plant sterols are not synthesized in the human body (11). Therefore, plant sterols are obtained only from the diet. Over 250 plant sterols and related compounds have been described in varying amounts in different plants and marine materials (8). Plant sterols can exist as free sterols, steryl esters (sterol esters) of fatty or phenolic acids, steryl glycosides or acylated steryl glycosides (12). The different fractions are thought to be located in different parts of the plant cell and to have several biological functions in plants, analogous to those of cholesterol in mammalian cells (12, 13). It has been hypothesized that free sterols, and to some extent steryl glycosides and acylated steryl glycosides, are incorporated into cell membranes and thus have structural and functional roles in cell membranes (12, 14). Plant steryl esters are believed to be located intracellularly and to represent mostly a storage and transport form of sterols (12, 13).

The plant sterol content in plants is not constant. Many factors, such as genetic factors, growth circumstances and time of plant harvest, as well as subsequent processing, may affect the concentration of sterols present in plants (15, 16). In addition, different analytical methods and sample preparation techniques may result in differences of sterol concentrations (17, 18).

Vegetable oils and vegetable oil-based products are regarded as the richest sources of plant sterols, followed by cereal and cereal-based products, nuts, seeds and legumes. The average plant sterol content of some foodstuffs is presented in Table 1. In plants, the predominant sterol is sitosterol followed by campesterol and stigmasterol. The other major plant sterols are avenasterol, stigmasterol and brassicasterol (19-22).

The total plant sterol content in the most frequently consumed vegetable oils has been reported to vary between 62 and 731 mg/100 g of oil (19, 21-25) (Table 1); rapeseed oil has the richest plant sterol content, whereas olive oil has the lowest content. Furthermore, small amounts of sitostanol have been found in hydrogenated coconut oil and soybean oil (19). The plant sterol content of vegetable oil-based margarines varies widely due to their different fat contents as well as source and proportion of vegetable oil in margarines (22, 26). Predominantly rapeseed oil based on soft margarines with a fat content of 40 to 80% have been reported to contain plant sterols 130-540 mg/100 g (26).

Although cereals and cereal products contain less plant sterols than vegetable oils (Table 1), they are nonetheless important sources of plant sterols due to their high daily dietary consumption. The plant sterol content in cereal grains has been reported to range from 23 to 178 mg/100 g of fresh weight (FW) (16, 19, 22, 27). Corn, rye and wheat are good sources of plant sterols, but oats are a poor source. Cereal grains, germ and bran fractions contain the most of the plant sterols, therefore whole grain flours are better sources of plant sterols than refined flours (16). The sterol content of rye breads has been reported to be 80-90 mg/100 g, whereas that of white bread has been reported to be only 40 mg/100 g (26). Moreover, bran fractions of rye, wheat and corn have been found

to contain appreciable amounts of plant stanols (16, 19, 28).

Vegetables, fruits and berries are generally not regarded as good sources of plant sterols (Table 1). In vegetables, the total sterol content has been reported to range from 3.8 to 50 mg/100 g FW (16, 22, 29). In general, cabbage is a good and potato is poor source of plant sterols. In fruits, the total sterol content has been reported to vary from 1.3 to 75 mg/100 g FW (22, 26, 29). Raspberry, lingonberry and blueberry have been found to contain moderate amounts of plant sterols (20-30 mg/100 g FW) (26).

Seeds, nuts and legumes, whose plant sterol content has been reported to vary between 22 mg and 714 mg/100 g (22, 30), are important sources of plant sterols in some diets. In addition, spices, coffee, cocoa and tea have also been reported to contain plant sterols, but they are not major dietary sources of plant sterols (22).

Table 1. Average plant sterol content in some foodstuffs.

Food item	Total plant sterol (mg/100 g)	References
Vegetable oils ^a		
Corn oil	472-952	22-24
Olive oil	62-232	22, 23, 25
Rapeseed oil	250-731	21-24
Soybean oil	221-328	19, 22-25
Sunflower oil	203-302	23, 25
Cereal grains ^b		
Barley	59-83	19 ^c , 26
Corn	178	22
Oats	23-52	19 ^c , 26, 27
Rye	91-110	19 ^c , 26
Wheat	60-76	19 ^c , 26
Seeds, nuts and legumes ^b		
Almonds	143	22
Peanuts	220	22
Sesame seeds	714	22
Soybeans	161	22
Vegetables and fruits ^b		
Brussels sprouts	24-43	22, 29
Carrot	12-16	22, 29
Cauliflower	18-40	22, 26, 29
Potato	3.8	29
Apple	12-13	22, 29
Avocado	75	26
Orange	24	29

^a refined, except for olive oil which is virgin oil.

^b per fresh weight

^c calculated from reported sterol content of free and bound lipid of cereals.

Origin of plant sterols in plant sterol products

At present, the plant sterols used in clinical studies on plant sterol products are wood-based, derived predominantly from pine wood (tall oil) and/or vegetable oil-based, derived predominantly from soybeans, but also from rapeseed and sunflower oils (31). The plant sterol content differs depending on the source: plant sterols derived from wood contain approximately 90% sitosterol and 10% campesterol, while plant sterols derived from vegetable oils contain about 70% sitosterol and 30% campesterol (32-34). In addition, commercially available 'tall oil' sterols contain 15-20% by weight stanols (35, 36). Plant stanols can also be produced by hydrogenation of commercially available plant sterols (37).

2.1.3 Dietary intake of plant sterols

Food composition databases for plant sterols are still incomplete. Therefore, the calculations of the dietary intake of plant sterols are not accurate. This should be kept in mind when examining the published intake levels.

Vegetable oils, fats and cereal products are the most important sources of plant sterols in the average Western diet (38). The daily intake of plant sterols has been estimated to range from 150 to 400 mg/d (38-42) when the intake of plant stanols is estimated to be roughly 10% of the intake of plant sterols (43). However, the dietary intake of plant sterols seems to vary greatly among different populations depending primarily on the type and amount of plant food that is consumed. In some vegetarians, the intake of plant sterols has been reported to be almost 1 g/d (44), although also very low intake levels have been reported; in pure vegetarian Seventh Day Adventists, the intake of plant sterols has been reported to be only 89 mg/d, while in lacto-ovo-vegetarian and non-vegetarian Seventh Day Adventists it has been reported to be 344 mg/d and 231 mg/d, respectively (45).

2.1.4 Physical and technological properties of plant sterols

The physical properties of plant sterols may be critical in determining their ability to reduce cholesterol absorption and thus reduce serum cholesterol concentrations (described in more detail 2.5). Non-palatable and non-saponifiable plant sterols have high melting points (8, 46), therefore at room temperature plant sterols are in a solid, crystalline form and their solubility in edible fats and oils is less than 1% (37, 47). The larger their side chains, the more hydrophobic the sterols become (46). Therefore, campesterol and sitosterol (C28 and C29) are more hydrophobic than cholesterol (C27). Furthermore, a double bond in side chain increases the hydrophilicity of sterols (46).

Transesterification of plant sterols with fatty acids of vegetable oils transforms crystalline plant sterol powder into a soluble form with fat-like properties (32). In this esterified form, plant sterols are readily incorporated into different foodstuffs such as margarines in sufficient amounts without changing their original texture and feel in the

mouth. Plant stanol esters and sterol esters can replace the hard fat used in the production of margarines and other spreads, and thus improve fatty acid composition as well as reduce the amount of fat of end products (37).

2.2 Plant sterols and serum lipids

2.2.1 Effects of plant sterols on serum total cholesterol and LDL cholesterol

In the following sections, first the cholesterol-lowering effects of free plant sterols and plant stanols and then corresponding effects of plant stanol esters and sterol esters are reviewed. Later, the results of comparison studies of plant sterols and plant stanols and dose-response studies are reviewed. Finally, factors affecting the cholesterol-lowering abilities of plant sterols are discussed.

2.2.1.1 Effects of free plant sterols

Since the early 1950's, plant sterols have been known to reduce serum cholesterol concentrations significantly in animals and humans (2). In 1951 Peterson (48) and in 1952 Pollak (49) reported in chickens and in rabbits, respectively, that simultaneous feeding with cholesterol and mixed soybean sterols reduced serum TC concentrations. Subsequently, this finding has been confirmed in many animal studies (2, 50).

Pollak was the first to show that plant sterols significantly reduce serum TC concentration in humans (51). In 1953, in his study with 26 healthy subjects, the consumption of 5-10 g/d of plant sterol mixture powder (75-80% of sitosterol) as part of a habitual diet resulted in a mean reduction of 28% in serum TC compared with the habitual diet alone. Since that study, and in particular, during the next fifteen years, numerous clinical studies on the hypocholesterolemic effects of plant sterols were carried out. Those studies have been reviewed by Pollak and Kritchevsky (2) and by Pollak (50). In summary, the studies have been controlled or non-controlled and have lasted from a single day to 45 months, with typical duration of 2-8 weeks. Subjects of both genders have mainly been normocholesterolemic, mildly to severely hypercholesterolemic or hypercholesterolemic with clinical evidence of atherosclerosis. The number of subjects has varied between 1 and 118, with an average of 20 subjects per trial. Subjects have mainly followed their habitual diet or a diet modified with regard to the intake of fat and cholesterol. Doses of plant sterol have been very large, up to 53 g/d, but a typical dose has been 5-18 g/d with a mean reduction of 10-20% in serum TC concentrations. However, the lipid responses to the intake of plant sterols seemed to be varied greatly within studies as well as among studies. Factors affecting the lipid responses are discussed in later. The assumption that large amounts of plant sterols are required to achieve a sufficient lipid response prevailed until the middle of the 1970s. Then Lees and Lees (52, 53) re-evaluated the effective dose of plant sterols and revealed that in most adult patients with type 2 hyperlipoproteinemia the maximal cholesterol-lowering effect

(9-12%) could be obtained with a dose of 3 g/d of tall oil sterols containing up to 95% sitosterol. The cholesterol-lowering efficacy of smaller doses of plant sterols has also been examined (54-57). The findings of these studies are described in the section of dose-response effect of plant sterols.

2.2.1.2 Effects of free plant stanols

In general, only a few studies have been made with free plant stanols. Interest in plant stanols arose when, in rat and rabbit studies, free plant stanols were found to be more effective in lowering serum TC than free plant sterols (58-61). In 1986, Heinemann et al. (62) reported that in 6 patients with hypercholesterolemia a daily dose as low as 1.5 g of plant stanol consumed as part of a diet containing <300 mg/d cholesterol and 35 percent of energy (E%) fat [polyunsaturated to saturated fatty acids (P/S)=1] reduced serum TC and LDL-C concentrations by up to 15% compared with the control period. Plant stanols were administered as capsules containing the stanols dispersed and partly dissolved in sunflower oil (62). In contrast to the findings of Heinemann et al. (62), in 33 men with mild to moderate hypercholesterolemia Denke (63) found that the consumption of 3 g/d of plant stanols did not reduce plasma TC and LDL-C significantly when these men had consumed plant stanols as gelatin capsules as part of a low-fat, low-cholesterol diet for 3 months. The most probable reason for this unexpected finding was that the capsules contained plant stanols suspended in sunflower oil i.e. not dissolved, and they were thus in a poorly soluble, less effective, form.

2.2.1.3 Effects of plant stanol esters and plant sterol esters

During the last decade, major interest has been focused on plant stanol esters and sterol esters and their efficacy in decreasing serum cholesterol concentrations. To date, the hypocholesterolemic effect of plant stanol esters or sterol esters has been shown in over 20 publications (4, 5, 31, 33, 34, 47, 54, 64-79). These intervention studies are described in Table 2 in which the percentage reductions in serum TC and LDL-C are presented mainly compared with a control group or period, but also in some studies they are related to baseline (33, 71, 74, 75, 78, 79). Most of these studies have been done in Finnish populations. When the results of various studies are compared, however, there do not seem to be differences in the cholesterol-lowering efficacy of stanol esters or sterol esters among populations in different countries.

The controlled studies have been carried out using a cross-over or parallel study design. The duration of trials has been short, typically 4-8 weeks, except in one study, which lasted for one year (68). The daily dose of plant stanols or sterols obtained from stanol ester or sterol ester products has ranged from 0.7-0.8 g to 8.6 g, a typical dose being 2-3 g/d. The number of subjects has varied between 7 and 318 per study. Most published studies in adults have been conducted in individuals with mild to moderate hypercholesterolemia, whereas normocholesterolemic individuals have participated in

Table 2. Intervention studies on the effects of stanol esters and sterol esters on serum TC and LDL-C concentrations.

First author (Ref)	Subjects	N(M/F)	Age, mean (range), y	Study design	Study diet		Dose (g/d) ^a	Duration (wk)	Results		
					SAFA (E%)	Chol. (mg/d)			Control TC/LDL-C (mmol/l)	Net reduction with test spreads (%)	
										TC	LDL-C
Adults											
Vanhanen (47)	HC	67(47/20)	46(25-60)	Parallel	12	270	3.1 (STA)	6	5.9/3.7	-7	-9
Miettinen (54)	HC	31(22/9) ^b	45	Parallel	12	326	0.7-0.8 (STA)	9	6.5/4.4	P=NS	-8 (calc.)
Vanhanen (64)	HC	15(11/4) ^c	48(33-60)	Parallel	..	295	0.7-0.8 (STA)	9	6.5/4.4	P=NS	P=NS
							1.8-1.9 (STA)	6	6.6/4.5	-9	-15
Gylling (65)	HC+DM type 2	11(11/-)	58	Cross-over	..	341	3 (STA)	6	6.0/3.8	-6	-9
Gylling (67)	HC+DM type 2	7(7/-)	60	Cross-over	..	233	3 (STA)	7	6.6/..	-11	-14
Miettinen (68)	HC	141	50(25-64)	Parallel	14	321	1.8 (STA)	24	6.1/4.1	-9 (calc.)	-9 (calc.)
							2.6 (STA)	52		-10	-13
Niinikoski (69)	NC	24(8/16)	37(24-52)	Parallel	2.2 (STA)	5	4.8/3.6 ^d	-10	-13 ^d
Gylling (70)	CAD	22(-/22)	51(48-56)	Cross-over	<15	207	3 (STA)	7	6.0/3.7	-8	-15
Weststrate (4)	NC,HC	95	45(18-65)	Cross-over	16	234	2.5 (STA) ^e	3.5	5.2/3.4	-7	-13
							3.3 (STE)			-8	-13
Gylling (33)	HC	23(-/23)	53(50-55)	Cross-over	P/S 0.6	262	3.18 (STA)	6	6.1/4.0 ^f	-4	-8
							(camp:sito 1:11)				
							3.16 (STA)			-6	-10
							(camp:sito 1:2)				
	HC	21(-/21)	53(50-55)	Parallel	P/S 0.3	323	2.43 (STA)	5	6.3/4.2	-8	-12
							(camp:sito 1:13) ^g				
Hendriks (31)	NC,HC	80	37(19-58)	Cross-over	13	250	0.83 (STE)	3.5	5.2/3.1	-5	-7
							1.61 (STE)			-6	-9
							3.24 (STE)			-7	-10
Andersson (71)	HC	61(28/33) ^h	55(30-65)	Parallel	8	240	2 (STA) ^h	8	6.6/4.5	-7	-7

					12	279	2 (STA) ^h		6.6/4.6 ^f	-9	-12
Nguyen (72)	HC	318(157/ 161)	53	Parallel	10	234	EU 2.2 (STA) ⁱ US 2.7 (STA) ⁱ US 1.6 (STA) ⁱ	8	6.0/4.0	-5 -7 -5	-5 -10 -4
Ayesh (73)	NC,HC	21	36(30-40)	Parallel	8.6 (STE)	3 or 4 ^j	5.2/3.3	-18	-23
Plat (34)	NC,HC	112(41/ 71)	33(18-65)	Parallel	14	233	3.8 (STA) ^k 4.0 (STA) ^k	8	4.9/2.9	-8 (calc.) -8 (calc.)	-13(calc.) -11(calc.)
Vuorio (75)	FH-NK	4(2/2)	41(33-49)	Parallel	<7	<200	2.24 (STA)	12	9.0/7.5 ^l	-10 (no stat.)	-11(no stat.)
	Healthy family members of FH-NK	16(3/13)	32(8-49)	Parallel	<7 or 8-10 ^l	<200 or <300 ^l	2.24 (STA)	12	4.9/3.2 ^l	-7	-12
Relas (79)	NC	11(11/-)	58	Parallel	P/S 0.4	281	3 (STA)	2	4.6/.. ^f	P=NS	..
Jones (5)	HC	15(15/-)	(37-61)	Cross-over	10	..	1.96 (STE) 1.57 (STA) ^m	3	6.0/4.2	-9	-13
Miettinen (78)	Colectomized	11	45(29-64)	Parallel	2 (STA) ⁿ	1 ⁿ	5.3/2.5 ^f	-16	-14 (calc.)
Plat (77)	NC,HC	39(11/28)	31(18-65)	Cross-over	13	231	2.47 (STA) ^o 2.46 (STA)	4	5.0/3.0	-6 -7	-9 -10
Children											
Gylling (66)	FH	14(7/7) heteroz. (1/-) homoz.	9(2-15)	Cross-over	14	114 (3.2 mg/ body WT)	2.8 (STA)	6	7.6/5.5 20.9/17.7	-11 -3 (no stat.)	-15 -9 (no stat.)
Williams (74)	NC,HC	19(8/11)	4(2-5)	Cross-over ^p	11	172	2.9 (STA)	4	4.2/2.4 ^f	-12	-16
Vuorio (75)	FH-NK	24(8/16)	9(3-13)	Parallel	8-10 ^l	<300 ^l	2.24 (STA)	12	7.4/6.0 ^l	-14	-18
Tammi (76)	NH,HC	72(40/32)	6	Cross-over	11	152	1.5 (STA)	12	4.2/2.6	-5 (calc.)	-7 (calc.)

HC=hypercholesterolemia, DM=diabetes mellitus, NC=normocholesterolemia, CAD=coronary artery disease, FH-NK=Familial hypercholesterolemia - North Karelia mutation of low density lipoprotein receptor gene, FH=familial hypercholesterolemia

heteroz.=heterozygous, homoz.=homozygous

SAFA=saturated fatty acids, E%=percent of energy, P/S=polyunsaturated to saturated fatty acids, Chol.=dietary cholesterol, Body WT=body weight
STA=plant stanols; Plant stanols have been added in esterified form to butter (33), vegetable oil-based mayonnaise (47, 54, 64), margarine and
shortening (34, 77) or margarine (in all other studies). STE=plant sterols; Plant sterols have been added in esterified form to vegetable oil-based
margarine. camp:sito=campestanol:sitostanol

calc.=calculated from mean values, no stat.= not statistically analyzed

.. =not reported

^a Actual daily dose of added plant stanols or sterols as informed by investigators or as calculated from actual mean intake of test products (5, 47, 54, 64, 71, 72).

^b Plant stanol ester group (N=7) and control group (N=8)

^c Control group consisted of 8 men.

^d non-HDL-C

^e Total amount of sterol was 2.7 g/d.

^f baseline habitual diet

^g Stanol ester mixture was added to butter. A control group consumed butter without added plant stanols.

^h low-fat diet+low-fat stanol ester margarine group (N=19), low-fat diet+low-fat margarine (control) group (N=21), usual diet+low-fat stanol ester margarine group (N=21)

ⁱ EU 2.2= European-formula vegetable oil-based spread (3.0:6.2:2.6 g/actual daily dose, saturated:monounsaturated:polyunsaturated fatty acids); US 2.7 =US-reformulated vegetable oil-based spread (2.0:7.9:4.6) and US 1.6= US-reformulated vegetable oil-based spread (2.1:7.7:4.3)

^j For men the study period lasted 3 weeks and for women it was 4 weeks.

^k 3.8 g/d of vegetable oil based plant stanols and 4.0 g/d of wood based plant stanols

^l Adults followed the step 2 diet of the National Cholesterol Education Program (81), whereas children followed a step 1 diet. The comparisons have been made to baseline, step 2 or step 1 diet, respectively.

^m Total amount of sterol was 1.76 g/d.

ⁿ Patients consumed light Benecol®. A significant reduction in TC was -3 % (calc.) after 1 day and -11% (calc.) after 3 days.

^o Dose of plant stanol ester has been consumed once per day at lunch.

^p The other test period was a period of high-fiber diet. The comparison was made to the baseline value.

only a few studies. Furthermore, some studies have been conducted in adults with familial hypercholesterolemia - North Karelia mutation of low density lipoprotein receptor gene (FH-NK), women with coronary artery disease (CAD), men with type 2 diabetes mellitus (DM), or colectomized patients. In addition, there have been a few studies of normocholesterolemic, mildly to moderately hypercholesterolemic children, children with familial hypercholesterolemia (FH) or FH-NK. The age of men and women participating in these studies has varied between 18 and 65 years, a great part of them being over 40 years. The age of boys and girls has varied between 2 and 15 years.

In studies with normocholesterolemic (4, 31, 34, 69, 77) or mildly to moderately hypercholesterolemic subjects (4, 5, 31, 33, 34, 47, 64, 68, 71, 72, 77) a net reduction of 5-10% in TC and of 5-15% in LDL-C with the doses of 2-4 g/d of plant stanols or sterols has been observed compared with the control period or control group. In a landmark study (68) in subjects with mild to moderate hypercholesterolemia, the consumption of margarine enriched with plant stanol esters (2.6 g/d of stanols) for a year decreased serum TC and LDL-C concentrations by 10% and 14%, respectively, from the baseline values of the subjects and by 10% and 13%, respectively, from the values of the control group, which consumed margarine without added stanol esters.

In studies with normocholesterolemic or mildly to moderately hypercholesterolemic children aged 2-6 years, the consumption of plant stanol esters (1.5-2.9 g/d of stanols) for 4 or 12 weeks reduced serum TC by 5-12% and LDL-C by 7-16% (74, 76).

The role of plant sterol treatment in different types of lipid disorder is discussed later in the section on factors influencing on the cholesterol-lowering ability of plant sterols.

2.2.1.4 Comparison studies of plant sterols and plant stanols

Some studies have compared the cholesterol-lowering efficacy of plant sterols and plant stanols in free or in esterified form with inconsistent results.

In two comparison studies in hypercholesterolemic men and women (54, 55) with small doses of free plant sterols (0.7 g/d) and free plant stanols (0.6-0.7 g/d), no differences in cholesterol-lowering activity were found. However, in a comparison study in children with severe heterozygous FH, Becker et al. (80) found that 1.5 g/d of free plant stanols given as pastilles reduced serum LDL-C concentrations significantly more than 6 g/d of free plant sterols. The reductions were 33% after 3 months and 29% after 7 months vs. 20% after 3 months, respectively.

In the comparison study of Weststrate and Meijer (4), a soybean sterol ester margarine and a stanol ester margarine (Benecol®) were found to reduce plasma TC and LDL-C concentrations equally effectively, despite the fact that these two margarines differed in the amount of total sterols (3.25 g/d vs. 2.74 g/d, sterol ester margarine vs. Benecol®), in the degree of esterification of sterols and stanols (65% vs. >98.5%) and in fatty acid composition [less saturated (SAFA) and monounsaturated (MUFA) fatty acids and more linoleic acid in sterol ester margarine than in Benecol®]. However, recently,

Jones et al. (5) demonstrated that plant sterol esters reduced plasma LDL-C concentrations more efficiently than stanol esters. In that cross-over study, however, the amount of total sterols was different (1.96 g/d vs. 1.76 g/d, sterol ester vs. stanol ester margarine). Furthermore, the number of subjects was small; only 15 subjects completed the study. In addition, the subjects were randomly assigned to one of six predetermined Latin squares, where each square included three sequenced periods and three subjects. Therefore, the findings in that study might be due to different amounts of total sterols, the methods of randomization and/or the small number of subjects.

2.2.1.5 Dose-response effect of plant sterols

In general, information on the dose-response effect of plant sterols for lowering serum TC and LDL-C is scarce, since in most studies only one dose has been used to evaluate the cholesterol-lowering efficacy of plant sterols. Some studies, however, have evaluated directly the dose-response relationship.

Minimum daily dose

In earlier studies it has been suggested that at least 1 g/d of plant sterols or stanols should be consumed before a clinical response in serum cholesterol is reached (54, 55, 64). This suggestion was based on findings from studies (54, 55, 64) that mainly included a small number of subjects. In those studies, the consumption of 0.6-0.8 g/d of plant sterols, stanols or stanol esters (calculated as free stanols) added to 50 g/d of rapeseed oil-based mayonnaises or spreads resulted in a significant or non-significant reduction of 2-8% (calculated from mean values) in TC and LDL-C. The typical feature of those studies was that serum LDL-C decreased significantly (up to 15%) during the rapeseed oil run-in period. In later studies, more consistent findings have been found with small doses of plant sterols. In two studies (31, 56) a daily intake of 0.80-0.83 g of soybean- or other edible oil-derived plant sterols added as free or in esterified form to margarine reduced TC and LDL-C by 4-7% compared with the control. In addition, in one study 0.74 g/d of free soybean sterols added to butter reduced TC and LDL-C by 10-15% (57).

In conclusion, according to the above-mentioned studies, it seems that a daily dose as little as 0.7-0.8 g of plant sterols or stanols can reduce serum cholesterol concentrations significantly, though of that dose the cholesterol-lowering effect remains below 10%.

Maximum daily dose

In the 1960s Beveridge et al. (82) reported that supplementing 0.3 g/950 kcal (0.87 g/d) of free plant sterols to a diet significantly decreased (-12.72 mg/dl, -0.30 mmol/l) plasma TC. With the greatest supplementation tested (6.4 g/950 kcal, 20.4 g/d), the decrease was 35.43 mg/dl (-0.90 mmol/l, -20%) suggesting that the sterol increments progressively reduced the concentrations of plasma TC (82). In later studies, however, it

has been suggested that the relationship between the intake of plant sterols and serum TC and LDL-C concentration is curvilinear rather than linear. In a series of four clinical trials with type 2 hyperlipoproteinemic adults, Lees and Lees (53) found that an intake of 3 g/d of tall oil sterol suspension or powder reduced plasma TC by 9-12%, but increasing the dose of tall oil sterol suspension from 3 to 6 g/d did not further lower plasma TC. Lees and Lees (53) also confirmed those findings in a cholesterol-balance study. In that study, 3 g/d of tall oil sterols reduced cholesterol absorption markedly, and increasing the dose from 3 to 9 g/d did not achieve any further gains (53). In children, however, the same researchers observed that an intake of 3 g/d of tall oil sterol resulted in only a 3% reduction in TC ($P=0.03$), whereas an intake of 6 g/d ($P=0.06$) achieved a 10% reduction; therefore they suggested that in children, in contrast to adults, 6 g/d of free plant sterols may be more effective than 3 g/d (53).

The optimal dose of plant stanol esters and sterol esters has also been evaluated. Miettinen et al. (68) found that a dose of 2.6 g/d of plant stanols as stanol esters reduced serum TC and LDL-C concentrations slightly, but significantly more (about 0.2 mmol/l) than a dose of 1.8 g/d of plant stanols. They concluded, however, that for practical purposes both doses possessed similar cholesterol-lowering effects. Similarly, Nguyen et al. (72) found that a US-reformulated vegetable oil-based spread containing 2.7 g/d of plant stanols as stanol esters reduced serum LDL-C significantly more than a similar spread containing 1.6 g/d of plant stanols (difference 0.24 mmol/l). On the contrary, Hendriks et al. (31) found no significant differences in cholesterol-lowering effects between the doses of 0.83, 1.61 and 3.24 g/d of plant sterols as sterol esters. However, 95% confidence intervals (compared with the control) suggested that the higher the sterol ester dose, the greater the reduction in plasma cholesterol (4.9% to 6.8% for TC and 6.7% to 9.9% for LDL-C, respectively) (31).

The optimal dose has also been assessed by Wester in his review (83) in which he compared TC and LDL-C responses for different stanol ester doses in different studies. He concluded that the curvilinear dose-response curve plateaus at an intake equivalent to about 2.2 g/d of stanols and that optimal cholesterol-lowering effect is obtained with daily intake of plant stanol esters corresponding to 2-3 g stanols. Furthermore, it has been suggested that increasing the dose above 3 g/d may not lead to any further reductions in serum TC and LDL-C (83, 84). The narrow range of dose responsiveness has been proposed to be a consequence of the compensatory increase in cholesterol synthesis that can be observed after consumption of high doses of plant sterols or stanols (84). This suggestion is based on the findings of Vanhanen et al. (64), in which the intake of about 2 g/d of plant stanols, but not a dose of about 0.8 g/d, was considered to increase cholesterol synthesis by 2 mg/body weight/d. However, the rate of synthesis does not replenish the lost cholesterol, leading to a net reduction of serum cholesterol.

Time needed to response

Plant sterols have been found to reduce serum cholesterol concentrations within 2-3 weeks of the initiation of treatment (62, 72, 74, 85). However, those studies have not actually assessed the minimum time needed to observe an effect of plant sterols on serum cholesterol concentrations. In a one year-long study Miettinen et al. (68), reported that although the reduction in serum TC and LDL-C concentrations had occurred mainly during the first three months, the concentrations tended to continue to fall throughout the study. Lees and Lees (53) reported that a reduction in plasma TC reached during the first 10 months due to the consumption of soy sterols did not diminish during 3 years with their continued consumption. With cessation of ingestion of plant sterols, the serum cholesterol concentrations have been found to return to the initial value within 2-3 weeks (62, 68, 72, 74, 85).

In one specific group, colectomized patients, a significant reduction in serum TC was found already after one day of the consumption of plant stanol esters, and the steady state was reached within just one week (78).

2.2.1.6 Factors influencing on the cholesterol-lowering ability of plant sterols

Large between-subjects variation in cholesterol responses to intake of plant sterols has been reported in many studies (2, 5, 53, 63, 86). Several factors such as gender, age, body weight, initial value of serum cholesterol, type of lipid disorder and genetics as well as experimental diets and plant sterol products can influence the cholesterol-lowering ability of plant sterols (2, 15, 50). However, only a few studies have focused systematically on these issues. Therefore, conclusions have mainly been drawn by comparing findings of different studies, which might have been performed very different study designs.

Gender, age, body weight and initial value of serum cholesterol

No differences in cholesterol-lowering response to plant sterol administration between **genders** have been reported (4, 47, 51, 54, 56, 64, 77).

Serum cholesterol concentration varies with **age** (87). Findings of the effects of age on lipid responses induced by plant sterols have been conflicting in those few studies in which children, adolescents and adults or at least two of these age groups have participated (2, 53, 75, 88). According to the meta-analysis of 14 intervention trials with different groups of adult subjects by Law (89), the reduction in the concentration of LDL-C at each stanol or sterol dose is significantly greater in older people than in younger people. Plant stanol or sterol doses ≥ 2 g/d have been found to reduce serum LDL-C significantly by an average of 0.54 mmol/l (14%) in people aged 50-59 years, by 0.43 mmol/l (9%) in those aged 40-49 years and by 0.33 mmol/l (11%) in those aged 30-39 years (89).

In the few studies in which effects of **body weight** on lipid responses to plant sterols

have been examined, no differences in cholesterol-lowering efficacy between subjects with normal weight and those who are overweight have been observed (53, 72).

The higher the **initial concentration of serum TC**, the greater the reduction in TC which has been observed in several (51, 68, 70, 76, 78), but not in all plant sterol studies (4).

Type of lipid disorder

The type of lipid disorder seems to affect outcomes of plant sterol treatment. In several studies the consumption of plant sterols has been shown to reduce serum TC and LDL-C significantly in subjects with primary moderate hypercholesterolemia (2, 5, 33, 47, 64, 68, 71, 72). Subjects with clinical evidence of atherosclerosis or documented coronary heart disease have been found to respond fairly well to plant sterol treatment (2, 62, 70). Recently, in women with **CAD** (70) the consumption of plant stanol esters (3 g/d of stanols) added to rapeseed oil-based margarine reduced serum TC and LDL-C by 8-15% compared with the control period and by up to 13-20% compared with the baseline diet values. In addition, the use of stanol ester margarine was found to normalize serum LDL-C (<2.6 mmol/l) in about every three women with CAD, especially those with high baseline absorption and low synthesis of cholesterol.

Type 2 DM is associated with accelerated atherosclerosis. In a small number of mildly hypercholesterolemic men with type 2 DM (65, 67) stanol esters (3 g/d of stanols) reduced serum TC and LDL-C concentrations by 6-11% and 9-14%, respectively, compared with the control period. In addition, serum LDL-C was found to be reduced, in particular, in the dense fraction, which is considered to be the most atherogenic LDL particle (90, 91).

Genetics

The finding of the effects of **apolipoprotein (apo) E** genotype or phenotype on lipid responses for an intake of plant sterols have been controversial: in earlier studies it has been suggested that reduction of LDL-C would be more consistent in subjects with the $\epsilon 4$ allele than in those with homozygous $\epsilon 3$ alleles (47, 54), but in later studies no differences have been found among genotype or phenotype groups (34, 66).

Heterozygous FH subjects, especially children, seem to benefit from plant sterol treatment. In FH children, the consumption of free plant sterols (6 g/d) (80, 92) or free plant stanols (1.5 g/d) (80) as pastilles or the consumption of plant stanol esters (2.8 g/d of stanols) added to rapeseed oil-based margarine (66) has been reported to cause a 11-26% reduction in serum TC and a 15-33% reduction in serum LDL-C compared with the control. However, in one homozygous FH boy (66), the reductions were only 3% and 9%, respectively, being in line with the earlier suggestion that monogenic hypercholesterolemia of the homozygous type will not respond or respond only poorly to these compounds (2). In a genetically homogenous FH population containing both

children aged 3-13 years and adults all carrying the FH-NK deletion (75) the consumption of stanol ester margarine (2.24 g/d of stanols) as part of a National Cholesterol Education Program (NCEP) (81) step 1 (children) or step 2 (adults) diet, reduced serum TC and LDL-C by 14-18% in children and by 10-11% in adults compared with the cholesterol-lowering diet the subjects had followed for at least a year before the study. However, in one heterozygous FH-NK child, the serum LDL-C concentration was reported to slightly increase during the trial (75).

Experimental diets

The composition of the diet may have an effect on cholesterol-lowering efficacy of plant sterol treatment. In most studies, the comparisons have been made with a **control or run-in diet** being similar to the study diet except added plant sterols. In some studies (55, 64, 70) replacing the usual dietary fats with rapeseed oil-based products containing substantial amounts of unsaturated fats and thus natural plant sterols, has reduced serum LDL-C significantly, up to 15%, already during the run-in or control diet period alone. Therefore, when a small dose of plant sterol has been added to that diet, no additional cholesterol-lowering effect has been achieved (55, 64). In some studies the comparisons have been made with the **habitual (baseline) diet** of the subjects that might have been varied greatly between subjects (33, 62, 68, 70, 71, 74, 78). Naturally, the reduction in LDL-C is numerically slightly greater when the comparison has been made against the habitual diet than to the control diet (68, 70, 71).

In most studies a **study diet** has contained a moderate or substantial amount of dietary fat and SAFA and in some cases also large amounts of cholesterol. In those studies, serum TC and LDL-C have been reduced by an average of 5-15% with plant sterols compared with the control (4, 31, 33, 34, 47, 64, 68, 77). Despite the opposite finding of Denke (63) with free plant stanols (discussed more in section 2.2.1.2), plant stanol esters have been found to be effective also as part of a low-fat, low-cholesterol diet (71, 72, 75). Recently, Andersson et al. (71) showed that the cholesterol-lowering effect of low-fat stanol ester margarine was additive, when consumed as part of a cholesterol-lowering diet. The reductions in serum TC and LDL-C were -15% and -19%, respectively, with combination of the low-fat stanol ester margarine (2 g/d of stanols) and the low-fat diet. The respective reductions were -8% and -12% with the low-fat control margarine and low-fat diet, and -9% and -12% with the low-fat stanol ester margarine and usual diet.

Plant sterol products

Serum cholesterol-lowering efficacy of plant sterols may also vary as a result of the composition, form and dose of plant sterols as well as the physical state and consumption frequency of the plant sterol products. The influence of plant sterol dose on outcomes has been discussed in earlier in this thesis.

It has generally been suggested that the greater the sitosterol or sitostanol content in a preparation, the greater will be its hypocholesterolemic efficacy. However, findings on the importance of the **composition** of plant sterols have been inconsistent. Lees and Lees (52, 53) observed that a smaller amount of tall oil sterols (93% sitosterol, with the remainder mostly consisting of campesterol) than soybean sterols (60-65% sitosterol and 35-40% mostly campesterol) was needed to achieve a similar cholesterol-lowering effect. However, recently, wood-based (about 90% sitostanol and 10% campestanol) and vegetable oil-based (about 70% and 30%, respectively) stanol esters dissolved into margarines have been found to reduce serum TC and LDL-C equally effectively (33, 34). In addition, a mixture of sitostanol containing tall oil plant sterols (about 62% sitosterol, 21% sitostanol, 16% campesterol and 1% campestanol) blended into dietary fats has been reported to reduce TC and LDL-C significantly (36, 86). In some earlier studies, stigmasterol, but not γ -sitosterol, has been reported to reduce serum TC concentration (2). Furthermore, rice bran or sheanut oils containing 4,4'-dimethyl sterols (1.7-3.2 g/d) esterified mainly with ferulic (rice bran) and cinnamic and acetic acids (sheanut oil) have not been found to lower serum TC and LDL-C significantly (4, 56).

There are no comparison studies between free and esterified **forms** of plant sterol in contrast to comparisons between the chemical forms of plant sterols. Free plant stanols have been proposed to reduce serum LDL-C more than free plant sterols with large doses (80), but not with small doses (54, 55). Findings of the comparisons between the esterified forms of plant sterols and stanols have been inconsistent (4, 5).

The **physical state of preparation** (vehicle of plant sterols) seems to have a crucial role in determining the cholesterol-lowering efficacy of plant sterols. Powdered plant sterols as such or used in capsules, tablets or granules have been found to be more effective than when they are in suspension (2, 52, 53). Furthermore, dissolving of plant sterols free or in esterified form into dietary fats seems to increase their cholesterol-lowering efficacy when a significant reduction in LDL-C can be reached already with small doses of plant sterols. Findings of two studies in which plant stanol ester-enriched spreads with different fatty acid compositions have been compared, have been contradictory. In one study (33), no differences between rapeseed oil- (monoene-rich) based margarine (3.16-3.18 g/d of stanols) and butter (2.43 g/d of stanols) have been found. However, in another study (72) US-reformulated vegetable oil-based spread (2.7 g/d of stanols) reduced serum LDL-C more than European-formula vegetable oil-based spread (2.2 g/d of stanols). The difference has been suggested to partly be due to the fact that the former spread contained less SAFA and more MUFA and polyunsaturated fatty acids (PUFA) than the latter spread (see Table 2). However, the difference might also partly be due to the different daily intakes of plant stanol esters.

In earlier studies, the consumption of plant sterols with meals, **consumption frequency** (2-3 times/d) and amount of consumption in relation to dietary cholesterol have been suggested to be critical in their cholesterol-lowering abilities (2, 82, 93, 94),

however, these proposals have recently been questioned, at least with respect to the plant stanol esters (77).

2.2.2 Effects of plant sterols on other serum lipids and lipoproteins, and apolipoproteins

Effects on high-density lipoprotein cholesterol

In general, in most studies the consumption of plant sterols has not been found to affect the concentration of serum high-density lipoprotein cholesterol (HDL-C) compared with the control (4, 5, 31, 34, 36, 47, 54, 56, 57, 62-64, 66-71, 74-78, 80, 84, 86, 92). However, in some studies, serum HDL-C concentration has increased (33, 65), while two studies reported decreases in HDL-C (80, 88). The effects of plant stanol esters on subclasses HDL₂-C and HDL₃-C have not been different from that of the control group or the control period (66, 95).

Effects on intermediate and very low-density lipoprotein cholesterol

Effects on serum intermediate density lipoprotein cholesterol (IDL-C) or very low-density lipoprotein cholesterol (VLDL-C) have been examined in a few studies. In some studies, plant stanols or plant stanol esters have not been found to affect IDL-C (54, 65, 67, 71) or VLDL-C concentrations significantly (54, 63, 66, 70, 71, 95), whereas in others, the reduction in serum IDL-C (66, 70) or VLDL-C (65, 67) has been significant compared with the control.

Effects on total and lipoprotein triglycerides

In general, persons who have had serum triglyceride (TG) concentration greater than 3 mmol/l were excluded from intervention studies. In most studies, plant sterols have not been reported to have any significant effect on serum TG concentration (5, 31, 33, 34, 36, 47, 54, 56, 62-68, 70-72, 74-78, 80, 86, 88, 92). However, in some studies or subtrials, serum TG concentration has increased moderately (52, 53, 80, 96) while in others it has decreased (52, 53).

Compared with the control, plant stanol esters have not been found to change concentrations of serum very low-density lipoprotein triglycerides (VLDL-TG) or intermediate density lipoprotein triglycerides (IDL-TG) significantly (66, 70, 71, 95). In one study, serum low-density lipoprotein triglycerides (LDL-TG) decreased slightly, but significantly, compared with the control (70); but in other studies these effects have not been significant (66, 71, 95). Furthermore, no significant changes have been observed in serum high-density lipoprotein triglycerides (HDL-TG) (66, 70, 71) or in subclasses HDL₂-TG and HDL₃-TG (66, 95).

Effects on apolipoproteins

Apo AI and apo B are the major structural components in HDL-C and LDL-C

particles, respectively (97). There are only a few studies in which the effects of plant sterols on apolipoproteins have been investigated. In most of these studies, no significant changes in apo AI concentrations have been found (67, 77, 80, 92, 95, 98). On the other hand, in one study, the consumption of plant stanol esters resulted in a slight, but significant, increase in serum apo AI concentration in parallel to the increase in HDL-C concentration (65). However, in that study, the fractional catabolic rate (FCR) and transport rate (TR) for apo AI were unchanged by plant stanol esters (65). No significant changes in apo AII have been found (65, 67).

Similarly to LDL-C, the consumption of plant sterols has significantly reduced apo B concentrations (65, 67, 80, 92, 95). However, this is not without exception (98). The significant reduction in apo B has been found to result from a significantly diminished TR for LDL apo B (65).

2.3 Absorption and metabolism of plant sterols

2.3.1 Absorption

Absorption under normal conditions

In humans, cholesterol is absorbed in the duodenum and proximal jejunum (99). However, in rat studies plant sterols appear to be absorbed in a somewhat wider region than cholesterol (100-102). Under normal conditions, the concentrations of plant sterols in serum are very low, on average 0.3-1.0 mg/dl (11, 103), and the concentrations of plant stanols are even lower, on average 0-0.03 mg/dl (36, 104). Plant sterol concentrations have been reported to be greater in women than in men (105), and in hypercholesterolemic than in normocholesterolemic subjects (2, 106). Low serum and tissue concentrations have been suggested to be a consequence of poor absorption rate of plant sterols (107-110) and their rapid biliary elimination (11). The poor absorption of plant sterols has been thought to be due to their poor micellar solubility (46, 111, 112), their slow transport rate through the outer surface of mucosal cell to an intracellular site (101, 113) and/or their inadequate esterification rate (112-116). The extent and rates of absorption vary among the different plant sterols; intestinal absorption of plant sterols has been observed to decrease as the number of carbon atoms at the C-24 side chain increases (46, 100, 109, 117) and with saturation of the nucleus double bond of the sterol (118). However, the latter finding is not without exception (109). In humans, sitosterol has been found to be absorbed less (#5%) than campesterol (9.6-16%) and both are absorbed less than cholesterol (30-50%) (11, 109, 119). Sitostanol is virtually non-absorbable, whereas campestanol has been found to be absorbed 5.5-12.5% (109, 119, 120). Consistent with these findings, the fecal recovery of ingested sitostanol in humans has been found to be over 95% (65, 70, 78, 80).

Effects of enhanced intake of plant sterols on their serum concentrations

The effects of the intake of natural plant sterol containing foodstuffs, spreads enriched with plant sterol esters or with stanol esters on serum plant sterols are presented in Table 3.

Serum plant sterol concentrations have been found to reflect intestinal absorption of cholesterol (103, 105), but also to reflect the intake of plant sterols in diet (121). In hypercholesterolemic subjects, the consumption of naturally plant sterols containing rapeseed oil or foodstuffs based on that (mean daily intake about 30-120 mg of campesterol and 40-220 mg of sitosterol) has been found to increase serum campesterol concentrations or the ratio to TC significantly by on average 9-65% from baseline value (47, 54, 55, 64, 70, 71, 104, 121, 122). In contrast, the consumption of spreads enriched with sterol esters (497-810 mg/d of campesterol and 883-1509 mg/d of sitosterol) has been found to increase serum campesterol significantly on average by up to 93% compared with control (4, 5). The effects of the intake of plant sterols on serum sitosterol have generally been smaller than their effects on campesterol. Sitosterol can inhibit absorption of campesterol and vice versa (54, 55). In contrast to the above-mentioned findings, the consumption of commercially prepared infant formulas enriched with vegetable oils (300-400 mg/d of plant sterols), and low-cholesterol, plant sterol rich diets (924-943 mg/d of plant sterols) have been reported to increase serum plant sterol concentrations by three- to fivefold in infants and hypercholesterolemic children or adolescents, respectively, compared with infants receiving breast or cow's milk and children or adolescents consuming self-chosen diets (123). The researchers speculated that the result might be due to that before adulthood, the ability to reject the absorption of plant sterols is not sufficiently matured (123).

The effects of free plant sterol supplementation on serum plant sterol concentrations have been investigated in some studies. Lees and Lees (52, 53) reported in subjects with type 2 hyperlipoproteinemia that the consumption of soy sterol suspension containing 18 g/d of plant sterols (about 6.3 g/d of campesterol and 10.8-11.7 g/d of sitosterol) caused high plasma campesterol concentrations (range 4-21 mg/dl), while sitosterol concentrations remained quite low (range 0.73-0.75 mg/dl). Furthermore, in the same study, the consumption of tall oil sterols of 3 g/d (about 2.85 g/d sitosterol) did not increase plasma sitosterol concentration over 2.5 mg/dl in any of subjects. These trials did not report any baseline values. On the other hand, in children or adolescents with type 2 hyperlipoproteinemia, the supplementation of plant sterols of 12 g/d (about 11.2 g/d sitosterol) increased plasma sitosterol concentrations by about 68% (no statistical significance reported) compared with placebo (88).

Table 3. Effects of the intake of natural plant sterol containing foodstuffs, spreads enriched with plant sterol esters or with plant stanol esters on serum plant sterols in some studies.

Source/Reference	Campesterol	Sitosterol	Campestanol	Sitostanol
Natural plant sterol containing foodstuffs^a				
Vanhanen and Miettinen (55)	á	↑	..	(8)
Vanhanen et al. (47)	á	↑	..	(8)
Miettinen and Vanhanen (122)	á	↑
Vanhanen et al. (64)	á	↑	..	(8)
Gylling et al. (70)	↑	(8)
Sarkkinen et al. (121)	↑	(8)
Gylling et al. (104)	↑	(8)	(8)	(8)
Spreads enriched with plant sterol esters				
Weststrate and Meijer (4)	é	á	ND	ND
Jones et al. (5)	é	á
Spreads enriched with plant stanol esters				
Vanhanen et al. (47)	â	↓	..	(8)
Weststrate and Meijer (4)	â	↓	ND	ND
Gylling and Miettinen (33)	â	↓	8	8
Gylling et al. (104)	â	↓	8	8
Nguyen et al. (72)	â	↓	8	8
Tammi et al. (76)	â	↓	..	8
Jones et al. (5)	â	↓

^a Rapeseed oil, rapeseed oil-based mayonnaises, rapeseed oil-based margarines

↑ increased serum plant sterols; ↓ decreased serum plant sterols; The thickness of arrow reflects the magnitude of change. () nonsignificant change, .. not reported, ND not detectable

The consumption of plant stanols or stanol esters has been found to decrease serum campesterol and sitosterol concentrations or ratios to TC significantly, by on average 10-50% compared with control or baseline (4, 5, 33, 47, 54, 55, 64-68, 70-72, 75, 76, 78-80, 104), but also non-significant changes in sitosterol have been found (71, 79). Furthermore, stanol esters have not been found to affect serum avenasterol significantly (71, 78). The greater the ratio of campesterol or sitosterol to TC in baseline or during the run-in period, the greater the decreases induced by the plant stanol esters (47, 66, 68, 70, 75, 78). Although the consumption of a sitostanol-containing (about 0.3 g/d) plant sterol mixture has not been found to affect serum campesterol and sitosterol concentrations (36, 86), daily doses as low as 0.6-0.8 g of stanols in free or in esterified form have been found to effectively reduce serum campesterol and sitosterol (54, 55, 64). Furthermore, so far only in colectomized patients has the rapidity with which plant stanol esters can lower serum plant sterols been examined (78). A significant reduction in serum campesterol and sitosterol has been observed already on the first day of stanol ester margarine consumption and the reduction has been observed to plateau during the first week (78).

Although in previous studies (4, 54, 62, 71) plant stanols, and especially sitostanol, have been suggested to be virtually nonabsorbable, in recent studies (33, 72, 76, 78, 104), it has been shown that the consumption of mixture of stanol esters (about 0.26-0.95 g/d of campestanol and 2.21-2.91 g/d of sitostanol) can increase serum plant stanol concentrations or the ratio to TC by two- to fivefold (about 5-12 $\mu\text{g}/\text{dl}$ and 12-26 $\mu\text{g}/\text{dl}$, respectively). The relative increase in campestanol appears to be greater than the increase in sitostanol. However, the values of campestanol and sitostanol have still only been 3-15% and 10-19% of the values of campesterol and sitosterol, respectively (78, 104). The different findings between the previous and the recent studies might be due to the preparation used or improved analytical methods. Large amounts of sitostanol in diet have been found to effectively inhibit absorption of campestanol (33, 124). In colectomized patients, the increase of plant stanols in serum has been observed to plateau within 7-18 days after the initiation of stanol ester margarine consumption which has been proposed to be due to their increased biliary secretion (78).

Absorption in subjects with phytosterolemia

In phytosterolemia (sitosterolemia), which is a very rare autosomal recessively inherited lipid storage disease described first in 1974 (125), absorption of plant sterols is high, varying between 16 and 63% (119, 125-127). The characteristics for this disease are xanthomas, early developed of coronary atherosclerosis and hemolysis (128, 129). Increased amounts of plant sterols (sitosterol, campesterol, stigmasterol and avenasterol) and 5α -stanols (cholestanol, 5α -sitostanol and 5α -campestanol) have been found in blood (119, 125, 127, 130, 131) and virtually all tissues except brain (125, 132). The vast majority of plant sterols must be of dietary origin, since they cannot be synthesized endogenously (11, 131). However, 5α -stanols are probably produced endogenously from the corresponding unsaturated sterols, because diet naturally contains some cholestanol, but virtually no plant stanols (130, 133). In phytosterolemic individuals, serum cholesterol concentrations may be normal or elevated (119, 130, 131, 134). In addition to enhanced absorption of plant sterols, their reduced biliary removal by the liver and decreased cholesterol synthesis has been suggested to contribute to this disease (119, 126, 127, 131, 135, 136). In phytosterolemic heterozygotes, serum plant stanol and sterol concentrations are generally not elevated, since these individuals have an almost normal absorption rate and rapid biliary sterol elimination (120, 137). However, moderately increased serum plant sterol concentrations have also been reported (138). Recently, enhanced intake of plant stanols as stanol esters (120) and plant sterols as sterol esters (139) has been reported to increase serum plant stanol and plant sterol concentrations, respectively, to similar levels as found in healthy subjects.

2.3.2 Metabolism

In general, the turnover and biliary excretion of plant sterols are more rapid than that

of cholesterol (11, 140), but slower than that of plant stanols (59). In humans, plant sterols have been found to circulate mainly in LDL, but also in HDL and to lesser extent in VLDL particles (105), either in esterified or unesterified forms (11, 127, 141). In rats, when sterols were intravenously injected, more plant stanols than plant sterols have been found in the esterified form in serum (59). In humans, the stanol metabolism is still incompletely known.

Very little is known about the distribution of plant sterols in tissues of the body. The findings seem to differ somewhat among studies depending on the tissues examined and the administration routes of plant sterols (oral or intravenous). Campesterol has been reported to be the dominant plant sterol in the tissues of rabbits (142). In animal studies, plant sterols have been observed to be deposited mainly in the liver, small intestine, kidney, adipose tissue, adrenal gland and ovaries (59, 140, 142-144). Incorporation of plant stanols into plasma, liver and other tissues has been found to be negligible (61). In humans, Gould et al. (145) reported that small amounts of ingested plant sterols are absorbed and distributed throughout the body. The greatest amounts of plant sterols have been observed in liver, spleen, kidney, lungs, plasma and red blood cells with the lowest amounts in aorta and blood vessels (145).

Under normal conditions, Δ^5 -plant sterols have not been found to be converted enzymatically to stanols in the liver (128). In addition, plant sterols are not converted to bile acids in humans (146), although in previous studies some conversion was claimed to occur (11). Unabsorbed plant sterols have been found to be converted to 24-methyl- and 24-ethyl-coprostanol and coprostanone by intestinal bacteria (147), but no similar conversion has been found with plant stanols (148). Small amounts of plant sterols may also be excreted into skin surface lipids (149).

2.4 Effects of plant sterols on serum cholesterol precursors and cholestanol

Squalene and cholesterol precursor sterols

Cholesterol synthesis has been shown to be stimulated compensatorily in response to cholesterol malabsorption and to depletion of the hepatic cholesterol pool by plant sterols (64, 65, 67, 70, 78). However, in some studies no consistent changes in cholesterol synthesis, as measured by using deuterated water, have been found (5, 36, 86).

Figure 2 shows a scheme of cholesterol synthesis via certain main cholesterol precursor sterols in humans. Δ^8 -cholestenol, desmosterol and Δ^7 -lathosterol in serum have been found to reflect cholesterol synthesis, while squalene has been found to reflect that less consistently (103, 150-152). A high baseline precursor sterol ratio to TC has been reported to predict a small reduction in serum cholesterol in some stanol ester studies (70, 78, 104), because according to cholesterol homeostasis, the efficacy of cholesterol absorption is low in these subjects. However, in a specific population, FH children, opposite findings have also been presented (66).

In most studies the consumption of plant stanol esters has been found to increase serum $\Delta 8$ -cholestenol (33, 47, 64-66, 70, 75, 76, 78, 79, 104), desmosterol (33, 47, 65-67, 70, 75, 76) and $\Delta 7$ -lathosterol (33, 47, 64-66, 70, 72, 75, 76, 78, 79, 104) concentrations or the ratio to TC significantly, on average by 8-68%, compared with the baseline or control. However, also non-significant changes in serum $\Delta 8$ -cholestenol (64, 67, 71), desmosterol (64, 71, 78, 79) or $\Delta 7$ -lathosterol (67, 71) have been reported. No significant changes in serum squalene have been found (47, 54, 64-67, 78, 79). Furthermore, no congruent effects on serum cholesterol precursors with low daily dose of plant sterols (about 0.7-0.8 g/d of stanols or sterols) have been observed (54, 64). One reason for that might be that the consumption of rapeseed oil has enhanced serum cholesterol precursor concentrations already during the run-in period. In most studies (64, 65, 67, 70), but not in all (54, 78) the results of serum cholesterol precursor sterols have been in accordance with the findings of sterol balance studies.

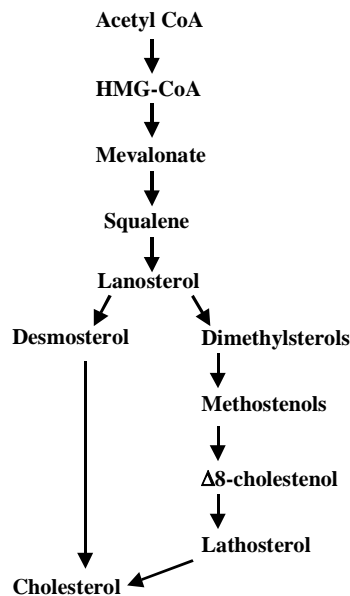


Figure 2. Scheme of cholesterol synthesis via certain main precursor sterols in human serum. HMG-CoA=3-hydroxy-3-methylglutaryl-coenzyme A

Cholestanol

Serum cholestanol, a metabolite of cholesterol, has been reported to reflect the cholesterol absorption efficacy and inversely that of cholesterol synthesis (153). Thus the changes in cholestanol are parallel those seen with plant sterols (background diet non-supplemented with plant sterols) and are opposite to those of cholesterol precursor sterols (153). In most studies, the serum cholestanol concentration or the ratio to TC was significantly lowered on average 6-15% by plant sterols or stanols (33, 54, 65-67, 70, 75, 78, 104), but also non-significant changes have been reported (47, 55, 64, 71, 79).

2.5 Hypocholesterolemic mechanisms of plant sterols

Many theories have been presented about the possible hypocholesterolemic mechanisms of plant sterols. The generally accepted view is that plant sterols inhibit the absorption of dietary and biliary cholesterol from intestine when sufficient amount of plant sterols are present in the intestine. This is supported by many animal and human studies (5, 53, 54, 64, 65, 67, 70, 78, 93, 112, 154), in which even greater than 50% reductions in cholesterol absorption have been reported. Hypocholesterolemic mechanisms of plant sterols have mainly been investigated with rats or other small animals whose lipid metabolism differs from that of humans. In addition to inhibition of cholesterol absorption, other possible hypocholesterolemic mechanisms of plant sterols are reviewed in this section.

Inhibition of absorption of cholesterol

The interference of intestinal absorption of cholesterol by plant sterols is likely related to their close chemical structure to cholesterol. However, the precise mechanisms of action through which plant sterols inhibit the cholesterol absorption and increase its excretion are not totally understood. Among the possible mechanisms are: an inhibition of mixed micelles formation, changes in micellar solubilization, competition with cholesterol for uptake to the brush border membrane, intracellular esterification or/and incorporation into chylomicrons (155). Figure 3 presents a schematic model of the inhibition of cholesterol absorption and lowering of serum LDL-C.

At present, the reduced micellar solubility of cholesterol is thought to be the major mechanism through which plant sterols inhibit cholesterol absorption (111, 157, 158). Solubilization of cholesterol to mixed micelles is essential for intestinal absorption of cholesterol (99). Slota et al. (111) found that increasing the amount of free plant sterols in mixed micellar solution reduced the solubility of cholesterol below that predicted by an equimolar replacement of cholesterol. *In vitro*, free plant sterols, which are more hydrophobic than cholesterol, have been reported to have a lower capacity but higher affinity for binding to cholic acid micelles, and thus to displace cholesterol from micelles with a favorable free energy change (46).

In *in vitro* studies, high amounts of plant sterols in the donor have been reported to be required before they can inhibit uptake of cholesterol to brush border membrane (115, 117, 157, 158). Previously, it was believed that cholesterol crosses the mucosal cell membrane by simple diffusion (99), however, recently, it has been found that uptake of cholesterol to brush border membrane is also energy-independent, protein-mediated process (159-162). Some researchers have suggested that plant sterols might compete with cholesterol for binding to the protein(s) which facilitate sterol uptake in the small-intestinal brush border membrane (16) such as scavenger receptor of class B

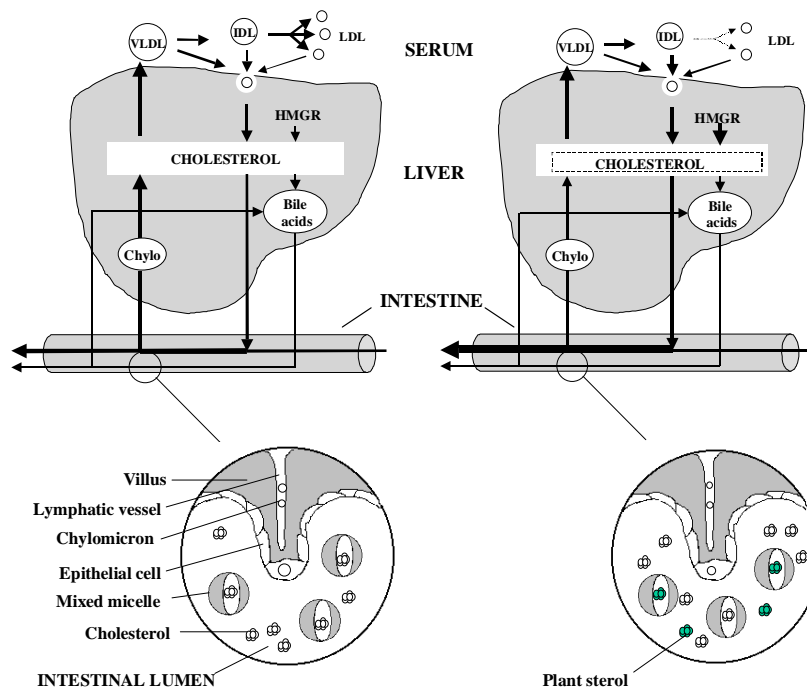


Figure 3. Schematic presentation of inhibition of cholesterol absorption and that of LDL-C lowering by plant sterols adapted by Miettinen and Gylling (156). Left panel: normal situation without plant sterol addition; Right panel: situation with plant sterol addition.

Plant sterols displace cholesterol from mixed micelles when less cholesterol is taken up to epithelial cells (enterocytes). Less cholesterol is packed into nascent chylomicrons, excreted in lymph and transported in chylomicron remnants (Chylo) to liver. As a consequence, the hepatic cholesterol pool is reduced (broken line). This, in turn, stimulates cholesterol synthesis and probably LDL receptor activity. The receptors pick up especially VLDL and IDL particles, precursors of LDL, resulting in reduced production and serum concentration of LDL. Bile acid synthesis is unaffected. HMGR= 3-hydroxy-3-methylglutaryl-coenzyme A reductase

type I (163) or for protein(s) which facilitate intracellular transfer (115).

With respect to intracellular steps, the quantity of plant sterols taken up by intestinal cells may be insufficient to inhibit cholesterol processing, i.e. esterification or incorporation into chylomicrons (157). It seems that plant sterols do not interfere with nor compete with cholesterol for acyl-CoA:cholesterol acyltransferase (ACAT)- or cholesterol esterase-catalyzed esterification in intestinal mucosa (2, 114, 157, 164) and therefore, these enzymes cannot account for plant sterol inhibition of cholesterol absorption. However, there are also some exceptions as presented by Pollak and Kritchevsky (2).

In general, only a minimal amount of the administered plant sterols has been found to be recovered in the lymph compared with cholesterol (112). However, there is little information available about the competition of plant sterols with cholesterol for incorporation into chylomicrons. It has been suggested that transport of cholesterol is

preferential relative to plant sterols during intracellular transport of sterols from plasma membrane to microsomal membranes and to the chylomicrons (165).

Effects of composition or physical state of plant sterols on inhibition of cholesterol absorption

A great part of published studies has been made with plant sterol mixtures containing mainly sitosterol. The effects of different plant sterols on inhibition of cholesterol absorption have been compared only in *in vitro* or animal studies. In most of these studies, the effects of stigmasterol (110, 164) have appeared to be similar to sitosterol, the effects of campesterol (164) and fucosterol (110, 157) have been weaker.

In animal and human studies, free plant stanols have been found to inhibit the cholesterol absorption more efficiently than free plant sterols (58, 61, 166, 167). They appear to be better as reducing micellar solubility of cholesterol (167) and increasing excretion of cholesterol (60, 61, 80, 166). When Heinemann et al. (166) compared effects of free plant stanols and sterols on cholesterol absorption in normo- or hypercholesterolemic volunteers directly by using an intestinal perfusion technique, they found that the cholesterol absorption declined during plant sterol and stanol infusion (3.6 $\mu\text{mol}/\text{min}$ for both) by almost 50% and 85%, respectively.

Esterification, however, seems to make unsaturated plant sterols comparable to the saturated counterparts in inhibition of cholesterol absorption. Normén et al. (168) used a continuous isotope feeding method and demonstrated that unsaturated soy sterol esters could inhibit cholesterol absorption as efficiently as stanol esters (cholesterol absorption 38% vs. 39%) when those had been consumed in the same way in small buns spread with butter. Jones et al. (5) utilized a dual stable isotope ratio technique and demonstrated that plant sterol esters and stanol esters dissolved into margarines reduced cholesterol absorption on average by 36% and by 26%, respectively.

In addition to esterification, solubility of plant sterols and thus their efficacy to reduce cholesterol absorption can be increased by using phospholipids (lecithin) or dietary fats as a vehicle to deliver plant sterols into the small intestine. When plant sterols are offered in a soluble form into the intestine, they might replace and precipitate cholesterol from the absorbable micelles more effectively, and with smaller doses, than might be possible with a crystalline form (94, 169, 170). Recently, Ostlund et al. (170) found that 0.3 g and 0.7 g of plant stanols in lecithin micelles reduced cholesterol absorption significantly by 34% and 37%, respectively. Instead, 1 g of plant stanol powder reduced that only by 11%. The former finding is consistent with the study of Vanhanen (64), in which 0.7-0.8 g/d of stanols as stanol esters dissolved in mayonnaise was reported to reduce efficiently cholesterol absorption. On the other hand, Mattson et al. (94) observed that free plant sterols reduced cholesterol absorption more effectively than oleate esters of plant sterols (42% vs. 33% reduction in cholesterol absorption). However, in that study, free and esterified plant sterols were added to food in different ways: the former was mixed with

omelet and the latter was dissolved in frying fat. It has generally been suggested that before plant sterol esters or stanol esters can inhibit cholesterol absorption, they have to be hydrolyzed to free plant sterols or stanols, respectively, in the intestine (83, 94). Hydrolysis is normally rapid (148), with about 50% of administered plant stanol esters being hydrolyzed in a 50-cm segment of duodenum (148). Since only the sterol monohydrate can affect micellar binding, the reason for different results in the study of Mattson et al. (94) might be incomplete hydrolyzation of sterol esters in the intestine lumen.

In rats, the ability of free plant sterols to alter cholesterol absorption has been compared with that of plant sterols esterified with fatty acids with various chain-lengths or with various degrees of saturation. In those studies both free and esterified plant sterols with acetate (93, 171), decanoate (93) or oleate (93, 171), but not with propionate or palmitate (171) have been observed to cause a similar decrease in cholesterol absorption.

Although in earlier studies it has been proposed that both plant sterols and cholesterol have to be present in diet simultaneously to achieve optimal efficacy in inhibition of cholesterol absorption (2, 94), in a recent study (77) that suggestion has been challenged. Since the researchers (77) did not detect any difference in cholesterol-lowering efficacy between the consumption frequency of plant stanol esters, they hypothesized that plant stanols or stanol esters remain in the intestinal lumen or possibly in or associated with the enterocytes and thus affect micellar solubility of intestinal cholesterol and ultimately cholesterol absorption.

Other hypocholesterolemic mechanisms

In experimental animals, administration of free plant sterols intraperitoneally or subcutaneously has also been reported to cause reduction in serum TC concentrations (155, 172). Thus, it has been proposed that plant sterols may have intrinsic hypocholesterolemic effects via mechanisms other than those involving cholesterol absorption (155). On the other hand, some researchers have suggested that part of the infused plant sterols may be secreted by the liver into the bile and may then impair cholesterol absorption (173). This has been challenged by others claiming that the amount of plant sterols secreted into bile is too low to inhibit cholesterol absorption (174). Only infusion of a large amount (100 mg) of free plant sterols has been observed to achieve even a partial inhibition in cholesterol synthesis (175). An increase in the tissue plant sterol pool has not been observed to reduce 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity, the rate-limiting enzyme of cholesterol synthesis when free plant sterols have been fed (176, 177) or infused intravenously to rats (173, 174). In fact, the enzyme activity has been found to increase twofold in plant sterol-fed rats (176).

In animals, the effects of plant sterols on cholesterol 7 α -hydroxylase activity, the rate-limiting enzyme of conversion of cholesterol to bile acids, have been conflicting (140,

155, 173, 174, 176). In humans, no changes in bile acid synthesis (65, 67, 70) or bile acid composition (78) have been found. In addition, the consumption of plant sterols has not been found to increase bile acid excretion in feces in most studies (54, 64, 65, 67, 70, 78, 168), though one study with plant stanols did report opposite findings (80).

Plant sterols have been observed to be able to inhibit esterification of exogenous cholesterol by ACAT in rat liver (116), and thus to increase cholesterol excretion in bile.

Depletion of intracellular cholesterol in the liver induced by plant stanol esters could be hypothesized to upregulate LDL receptor activity (Figure 3). The receptor may effectively pick up VLDL-C and IDL-C particles resulting in their reduced conversion to LDL and in decreased LDL-C concentrations (65, 67). This hypothesis is based on findings that FCR of LDL apo B remained unchanged and that serum VLDL-C and IDL-C concentrations decreased significantly with the consumption of stanol esters (65, 67).

2.6 Side effects of plant sterols

2.6.1 Effects of plant sterols on serum fat-soluble vitamins and carotenoids

As mentioned earlier, plant sterols inhibit cholesterol absorption from intestine. Therefore, it has been thought that plant sterols might also interfere with the absorption of carotenoids and fat-soluble vitamins. To date, the effects of plant sterols on the absorption of carotenoids and fat-soluble vitamins have been evaluated only by measuring their serum concentrations. In the circulation, tocopherols and carotenoids are transported in lipid particles, therefore changes in these carrier particles may also alter the concentrations of tocopherols and carotenoids. Thus, in the published studies, changes in serum tocopherols or carotenoids have been standardized against simultaneous changes in TC (33, 72, 75, 178), LDL-C (76, 77), total glycerol+TC (4) or TG+TC (31, 71).

Fat-soluble vitamins

Plant sterols have not been found to have significant effects on serum concentrations of retinol (33, 71, 72, 75-77, 178), 25-hydroxyvitamin D₃ (31, 33, 71, 72, 76, 178) or vitamin K (31, 179). Serum α -tocopherol concentrations have reduced significantly, but after lipid standardization, the α -tocopherol concentrations have remained almost unchanged (31, 33, 71, 76, 77, 178). Only the effects of plant stanol esters on serum δ -, γ - or β -+ γ -tocopherol have been studied. No significant changes in δ -tocopherol concentration (77) or in γ - (71) or β -+ γ -tocopherols (77) after lipid standardization have been detected.

Carotenoids

There have been significant reductions in serum concentrations of α -carotene, β -carotene or α -+ β -carotene, even after lipid standardization, after ingestion of plant sterols reported in several (4, 31, 33, 72, 75-77, 80, 178), but not in all (56, 71) studies. The effects on serum lycopene concentrations have been inconsistent. In one study, plant stanol esters have been found to affect serum lycopene also after lipid standardization (4), but not in the others (71, 77). Moreover, free plant sterols (56), but not sterol esters (4, 31), have been found to affect plasma lycopene after lipid standardization. In one study, stanol esters have not been reported to cause any significant reduction in serum phytofluene, lutein/zeaxanthin and β -cryptoxanthin after lipid standardization (77). However, in that study, serum carotenoid as well as tocopherol concentrations have been reported to be slightly lower when stanol esters had been consumed three times per day than consumed once per day (77).

2.6.2 Hormonal effects of plant sterols

Earlier findings in rodents and fish have suggested that plant sterols have effects on the reproductive system, and in particular that they possess estrogenic activity (180-182). However, in recent studies no evidences of estrogenic activity or effects on the reproductive system have been found. Turnbull et al. (183) stated that vegetable oil-based stanols did not increase the proliferation of estrogen-responsive MCF-7 human breast cancer cells at the doses tested (up to 10^{-4} M). Baker et al. (184) observed that plant sterols did not bind to the immature rat uterine estrogen receptor at doses up to 10^{-4} M or to stimulate the transcriptional activity of the human estrogen receptor in a recombinant yeast strain at doses up to 2×10^{-4} M. In addition, vegetable oil- or wood-based stanol esters (183) or vegetable oil-based plant sterols or sterol esters (184) had no estrogenic potential in an *in vivo* rat uterotrophic assay. In two-generation reproductive toxicity studies, no adverse effects on the reproduction or development of male and female rats over two generations were found when the rats were fed a diet containing plant stanol esters in concentration of 8.76% (equivalent to 5% total stanols) (185) or sterol esters in concentration of 8.1% (equivalent to 5% total sterols) (186). In addition, no embryotoxic, fetotoxic, or teratogenic effects were found when rats were fed diet containing stanol esters in a concentration of 8.76% (equivalent to 5% of total stanols) (187).

In humans, effects of plant stanol esters or sterol esters on serum female sex hormone concentrations have been investigated in few short-term studies. Gylling et al. (70, 188) did not find changes in serum estradiol concentrations in postmenopausal women with CAD when the women had consumed stanol esters (3 g/d of stanols) for seven weeks. Furthermore, Ayesh et al. (73) did not observe any biologically relevant effects on serum female sex hormone concentrations in normocholesterolemic or hypercholesterolemic women when they had consumed sterol esters (8.6 g/d of sterols) for four weeks.

Plant sterols have been used in the treatment of benign prostatic hyperplasia, because they have been found to ease urologic symptoms and improve measures of flow (189).

2.6.3 Other adverse effects of plant sterols in humans

In general, in the vast majority of clinical trials, oral administration of plant sterols has been well tolerated and without any side effects. However, some adverse effects have been reported. In one study, some subjects described mild constipation when they had consumed 3-6 g/d of tall oil sterols (53). Diarrhea has also been reported to occur occasionally in some studies (50). One subject reported a skin reaction when he had used sterol ester margarine (8.6 g/d of sterol) (73). In addition, in two children, appetite was depressed during the first two weeks of plant sterol treatment (92).

Minor changes in routine hematology and clinical chemistry parameters have been reported. However, the values have remained within the normal ranges (4, 31, 71-73). No significant effects on coagulation or fibrinolytic parameters have been observed (34) when subjects have consumed stanol ester margarine (3.8-4 g/d of stanols) for eight weeks. Furthermore, no significant changes in the formation of bile acids or neutral sterol metabolites (190) or the bacterial profile of the gut microflora (73) or urine parameters (5, 73) have been observed when subjects had consumed sterol ester margarine containing up to 8.6 g/d of total sterols.

3 AIMS OF THE STUDY

The general aim of these studies was to examine the role of stanol ester- and sterol ester-enriched margarines in lowering elevated serum cholesterol concentrations with different study designs. In addition, the safety of plant stanol esters and sterol esters was evaluated by measuring the serum concentrations of carotenoids and fat-soluble vitamins as well as concentrations of plant stanols and plant sterols during the intervention studies.

Specific questions in the separate studies were as follows:

1. Do the low-fat margarines enriched with plant stanol esters offer an additional cholesterol-lowering effect to a cholesterol-lowering diet alone (**I**).
2. Do the margarines enriched with plant stanol esters or sterol esters reduce serum TC and LDL-C concentrations as part of a low-fat, low-cholesterol diet (**I, V**).
3. Do the low-fat margarines enriched with wood- or vegetable oil-derived plant stanol esters differ in their abilities to lower serum cholesterol concentrations (**I**).
4. Do the margarines enriched with plant stanol esters or sterol esters differ in their abilities to lower serum TC and LDL-C concentrations (**V**).
5. Do plant stanol esters reduce serum TC and LDL-C concentrations in a dose-dependent manner and what is the optimal dose of plant stanol esters (**III**).
6. Do plant stanol ester- or sterol ester-enriched margarines affect serum fat-soluble vitamin or carotenoid concentrations as part of a cholesterol-lowering diet or a standardized habitual diet (**I, II, III, V**).
7. How do different doses of plant stanol esters affect cholesterol metabolism using serum cholesterol precursors as biomarkers and how do the stanol esters affect serum plant sterol and stanol concentrations (**IV**).

4 SUBJECTS AND METHODS

Detailed descriptions of subjects and methods are presented in the original publications (I-V).

4.1 Subjects

A total of 128 mildly to moderately hypercholesterolemic men and women with normal or slightly elevated body weight [body mass index (BMI) 19-30 kg/m²] were recruited to the studies from the occupational health care system (I/II, V), from former studies carried out in the Department of Clinical Nutrition, University of Kuopio (I-V) and from the local society of the Finnish Heart Association (III/IV). In addition, employees of the city of Kuopio were recruited to study V. To be included in the studies the subject had to have normal liver, kidney and thyroid function. They were not allowed to have DM, gastrointestinal diseases, lipid-lowering drug treatment, alcohol abuse (>45 g ethanol/day) or irregular eating habits. The subjects were requested to maintain any medication, and to keep their weight, alcohol consumption, smoking habits and physical activity constant during the studies. All study protocols were approved by the Ethics Committee of the University of Kuopio, and all subjects gave their informed consent.

Study I/II

A total of 60 hypercholesterolemic subjects met the inclusion criteria, which were for serum TC 5.4-7.5 mmol/l, for serum TG <3.0 mmol/l, and for age 20-60 years. Five subjects dropped out at the beginning of the run-in period for personal reasons. Ten of the subjects were smokers.

Study III/IV

A total of 26 subjects were recruited to the dose-response study. To be included to the study, the subjects had to have serum TC of 5.0-8.5 mmol/l and TG <3.5 mmol/l after the pre-trial period, and to be aged 25-65 years. Four subjects dropped out during the study due to personal reasons, prolonged infection (bronchitis, stomatitis), prostatitis or use of a plantago ovata product (Visiblin[®]) for constipation. One of the subjects was a smoker.

Study V

A total of 42 subjects were recruited to the study, in which the main inclusion criteria were as follows: serum TC 4.8-7.0 mmol/l and TG below 2.5 mmol/l at screening, and age 30-65 years. Eight subjects dropped out during the study due to personal reasons or poor commitment. Two subjects were smokers.

Baseline characteristics of the subjects completed studies I-V are shown in Table 4.

Table 4. Baseline characteristics of the subjects in studies I-V.

	Study I/II			Study III/IV	Study V
	Control group	WSEM group	VOSEM group		
N(men/women)	17(6/11)	18(8/10)	20(6/14)	22(8/14)	34(15/19)
Age (years)	46.0 ± 8.2	43.2 ± 8.2	40.8 ± 9.3	50.5 ± 11.7	48.8 ± 8.1
BMI (kg/m ²)	25.7 ± 3.5	25.6 ± 4.0	24.2 ± 3.0	26.3 ± 3.4	24.9 ± 2.4
Serum TC (mmol/l)	5.93 ± 0.64	6.36 ± 0.76	6.15 ± 0.79	6.87 ± 1.28	6.24 ± 0.83
Serum TG (mmol/l)	1.24 ± 0.66	1.42 ± 0.67	1.25 ± 0.39	1.24 ± 0.43	1.11 ± 0.52

Mean ± SD. WSEM=wood stanol ester-enriched margarine, VOSEM=vegetable oil stanol ester-enriched margarine

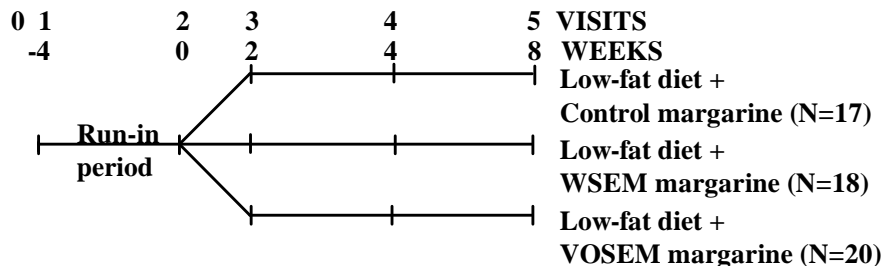
4.2 Study designs

In study I/II, a double blind, parallel study design was used (Figure 4). The subjects were randomly assigned into three low-fat [control, wood stanol ester (WSEM) and vegetable oil stanol ester (VOSEM)] margarine groups taking smoking habits and phase of menstrual cycle into account. All groups followed the experimental, low-fat and low-cholesterol diet for eight weeks preceded by a 4-week run-in, high-fat diet period.

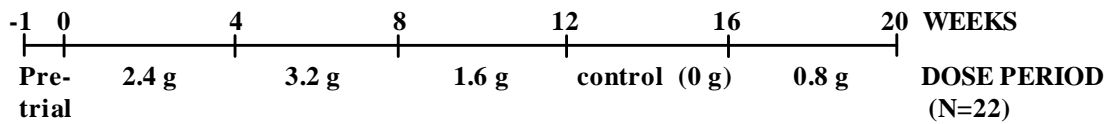
In study III/IV, a randomized single blind repeated measures design was used (Figure 4). After a 1-week pre-trial period, all subjects consumed five different plant stanol doses in the same randomly determined order [2.4 g, 3.2 g, 1.6 g, control (0 g), 0.8 g].

In study V, a double-blind randomized repeated measures design with three test margarines [control, stanol ester (STAEST) and sterol ester (STEEST)] and three periods of 4-week duration was used (Figure 4). The randomization was made according to a Latin square model. The experimental periods were preceded by a 2-week pre-trial period. During the entire study, the subjects followed a low-fat diet.

Study I/II



Study III/IV



Study V

Subjects (groups)

	1	2	3
I	STEEST margarine, N=11 (C)	STAEST margarine, N=12 (A)	Control margarine, N=11 (B)
II	STAEST margarine, N=11 (A)	Control margarine, N=12 (B)	STEEST margarine, N=11 (C)
III	Control margarine, N=11 (B)	STEEST margarine, N=12 (C)	STAEST margarine, N=11 (A)

Figure 4. Study designs.

4.3 Methods

4.3.1 Diets

Margarines

Test margarines were low-erucic acid rapeseed oil-based margarines (Raisio Group Plc, Raisio, Finland) except in study I/II where the spread used in the run-in period was a milk fat-based spread. Plant stanol esters (I-V) were prepared using commercially available plant sterols by recrystallization, hydrogenation to form plant stanols, and esterification with low-erucic acid rapeseed oil-based fatty acids to produce fatty acid esters of plant stanols. The respective plant sterol esters (V) were prepared by recrystallization, and esterification with low-erucic acid rapeseed oil-based fatty acids to produce fatty acid esters of plant sterols. In study I/II, the WSEM margarine contained plant stanol esters were derived from wood sterols (Ultra sitosterol, Kaukas Oy, Finland), and the VOSEM margarine contained plant stanol esters from vegetable oils (Archer Daniels Midland Co, Decatur, IL). In studies III/IV and V, the stanol ester margarines were prepared from wood and vegetable sterols (DRT, Les Derives Resiniques & Terpeniques Granel S.A. Dax Cedex, France and Archer Daniels Midland Co, Decatur, IL, respectively). In study V, the sterol ester margarine was prepared from vegetable oil-based sterols (Archer Daniels Midland Co, Decatur, IL).

The daily dose of the test margarines was 25 g (I/II, III/IV) or 20 g (V) taken in 2 to 3 portions with meals. The fatty acid composition and the amount of plant stanols and sterols in daily dose of the test margarines are presented in Table 5. The low-fat control and test margarines contained absorbable fat 35% and 32%, respectively (I/II). In the other studies, the test margarines contained absorbable fat 68-70% (III/IV) or 70-71% (V). The theoretical (planned intake x actual composition of spread) daily intake of plant stanols was 2.34 g in the WSEM group and 2.20 g in the VOSEM group (I/II). In study III/IV, the amount of plant stanols in the test margarines differed slightly from that planned being 0.81 g (planned 0.8 g), 1.56 g (planned 1.6 g), 2.29 g (planned 2.4 g) and 3.03 g (planned 3.2 g) per daily dose. In addition, the theoretical daily amount of total plant sterols and stanols was 2.02 g in the STAEST margarine and 2.06 g in the STEEST margarine (V). The control margarines did not contain added stanols or sterols, neither did the spreads used during the run-in or pre-trial periods. All test spreads were fortified with vitamin A [550 µg as retinol equivalents (RE), I/II; 445 µg RE, III/IV; 870 µg RE, V] and vitamin D (7 µg, I/II; 6.4 µg, III/IV; 7 µg, V) per 100 g spread. This kind of fortification of margarines is a normal procedure, in fact it is stipulated legally in Finland.

The subjects received the coded tubs of test margarines when visiting the study unit. They were given detailed instructions on how to use of test spreads. Furthermore, the subjects were asked to record the use of test fats daily (I-V) and to return the empty and partly empty tubs and the extra tub of test margarine to the study unit at the end of each

Table 5. The target daily intake of absorbable fat, fatty acids and plant sterols and plant stanols (g) from daily margarine dose (25 g in I/II and III/IV and 20 g in V) /their the actual mean intake.

	Study I/II			Study III/IV					Study V		
	Control	WSEM	VOSEM	Dose 0 g	Dose 0.8 g	Dose 1.6 g	Dose 2.4 g	Dose 3.2 g	Control	STAEST	STEEST
Absorbable fat	8.8/8.6	8.0/7.9	8.0/7.8	17.5/17.8	17.4/17.7	17.2/17.6	17.0/17.3	17.2/17.3	14.2/14.1	14.0/13.9	14.0/13.9
Fatty acids											
PUFA	2.1/2.1	2.1/2.0	2.1/2.1	3.8/3.9	3.9/4.0	3.8/3.9	3.8/3.9	3.9/3.9	3.1/3.1	3.1/3.1	3.2/3.2
MUFA	4.1/4.1	4.2/4.2	4.1/4.0	8.5/8.7	8.9/9.0	8.7/8.9	8.3/8.4	8.7/8.8	7.1/7.1	7.0/7.0	6.9/6.8
SAFA	2.0/2.0	1.1/1.1	1.1/1.1	4.2/4.3	3.8/3.9	3.8/3.9	3.9/4.0	3.7/3.7	3.2/3.2	3.3/3.3	3.3/3.3
Total plant stanols	0/0	2.34/2.31	2.20/2.16	0/0	0.81/0.82	1.56/1.59	2.29/2.33	3.03/3.05	0/0	1.92/1.91	0.09/0.09
Sitostanol	0/0	2.15/2.13	1.50/1.47	0/0	0.62/0.63	1.19/1.22	1.74/1.77	2.30/2.32	0/0	1.43/1.42	0.06/0.06
Campestanol	0/0	0.19/0.19	0.70/0.69	0/0	0.19/0.19	0.37/0.38	0.55/0.56	0.73/0.74	0/0	0.49/0.49	0.02/0.02
Total plant sterols	0.05/0.05	0.10/0.10	0.15/0.15	0.10/0.10	0.13/0.13	0.15/0.15	0.17/0.17	0.21/0.21	0.09/0.09	0.10/0.10	1.98 /1.96
Brassicasterol	0.01/0.01	-	0.01/0.01	0.01/0.01	0.01/0.01	0.01/0.01	0.01/0.01	0.01/0.01	0.01/0.01	-	0.06/0.06
Campesterol	0.02/0.02	0.04/0.04	0.06/0.06	0.04/0.04	0.05/0.05	0.05/0.05	0.06/0.06	0.06/0.06	0.03/0.03	0.04/0.04	0.57/0.56
Sitosterol	0.03/0.03	0.07/0.07	0.08/0.08	0.05/0.05	0.07/0.07	0.09/0.09	0.10/0.10	0.12/0.12	0.04/0.04	0.06/0.06	1.00/0.99
Stigmasterol	-	-	-	-	-	-	-	-	-	-	0.34/0.34
Total plant sterols and stanols	0.03/0.03	2.44/2.41	2.35/2.30	0.10/0.10	0.93/0.94	1.70/1.74	2.46/2.50	3.24/3.27	0.09/0.09	2.02/2.01	2.06/2.04

period (III/IV, V). The packages and the test spread left over were weighed and the result recorded (III/IV, V).

Diets

The goals of fat composition of diets are presented in Table 6. In study I/II, during the run-in period, a diet rich in fat and SAFA was used. During the experimental diet period of study I/II as well as during the entire study V, the subjects followed a diet which resembled the NCEP step 1 diet (81). In study III/IV, subjects followed a standardized background diet, which resembled their habitual diet, throughout the study.

Table 6. Goals for the composition of diets during the studies.

	Study I/II		Study III/IV	Study V
	Run-in	Experimental	Pre-trial and experimental	Pre-trial and experimental
Fat (E%)	36-38	28-30	34	<30
SAFA (E%)	16-18	8-10	<12	8-10
MUFA (E%)	14	12	14	12-14
PUFA (E%)	6	8	8	5-7
Cholesterol (mg/MJ)	35.7	23.8	23.8	23.8

The diets were composed of normal Finnish food items. In studies I/II and V, all subjects received individual oral and written instructions on the isocaloric diet including the precise amounts and quality of foods to be eaten by main food groups: fats, dairy products, meat and meat products, cereals, fruits and berries, and leaf vegetables and roots. In study III/IV, the diet plan included only precise amounts and quality of fat and cheese, and the precise quality of liquid milk and meat products. Depending on the goals for the amount of fat and the fatty acid composition of the different studies, the diets were adjusted with moderate rich fat (I/II, run-in) or low-fat and fat free dairy and meat products (I-V), sunflower (I/II, run-in) or rapeseed (I-V) oil, salad dressing (III/IV, V), milk fat-based spread (I/II, run-in) or vegetable oil-based margarines (I-V). The diet plans were made for several energy levels. The energy requirement of each subject was estimated by a 4-day food record kept before the study (I/II) and by the Harris-Benedict formula (I-V) added with the energy need due to physical activity (191). The body weight was not allowed to change more than ± 1 kg during the studies. Therefore, if necessary the energy intake level was changed in order to ensure stable body weight. The feasibility of diet was improved by providing the test spreads, vegetable oils and liquid milk products (I/II) or test margarines, vegetable oil, salad dressing and low-fat cheese (III/IV, V) to the participants free of charge.

4.3.2 Evaluation of the feasibility of the diets

Food records

Adherence to the background diet was monitored by 3-day (III/IV) or 4-day (I/II, V) food records kept before the end of the run-in period (I/II) or the pre-trial period (III/IV, V) and by 4-day food records kept before the end of each experimental period (III/IV, V) or 3 times during the experimental period (I/II). One of the recording days was a weekend day or the person's day off from work. The subjects recorded their food consumption after consulting a portion size booklet containing photographs of food (192). 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.

The diets were planned and the nutrient intake in 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 mainly on analyses of the Finnish food and international food composition tables (193). In addition, the database was updated for the purposes of each study.

Fatty acid composition of serum cholesteryl esters

Fatty acid composition of serum cholesteryl esters was determined as an objective marker of dietary adherence in studies III/IV and V.

4.3.3 Height, body weight and blood pressure

Height was measured on the first visit of the studies to the closest 0.5 cm. Body weight with light clothing was measured at every visit with a digital scale. Blood pressure (I/II, V) was measured in the sitting position from right arm using a digital blood pressure monitor (Hem-705c, Omron Corporation, Japan) after the subjects had rested for 5-10 min. Two measurements were taken, with the mean being used in the analyses.

4.3.4 Laboratory measurements

Fasting blood samples were taken in study I/II at the beginning of the run-in (-4 wk) and the experimental diet (0 wk) periods and at weeks 2, 4 and 8. In the other studies, fasting blood samples were taken at the beginning of the pre-trial (-1 wk, III/IV or -2 wk, V) period, at the beginning of the first experimental period (0 wk) and at the middle and the end of each experimental period. Serum lipids were determined from blood samples at every visit, but the main comparisons were made between the mean values of the weeks 0 and 8 (I) or among the mean values at the end of each experimental period (III, V). Samples for other variables were taken only at the beginning and the end of the studies (I-V), at the beginning and the end of the experimental diet period (I/II) or at the end of each experimental period (III/IV, V). Since the phase of menstrual cycle may affect cholesterol concentration (194), in study I/II in those women with a menstrual

cycle, the main blood samples were taken at the same time of the cycle, and in studies III/IV and V, the end measurement of each period was performed at days 5-10 of the cycle.

Serum samples for analysis of apo AI and B, carotenoids and fat-soluble vitamins, cholesterol precursors, plant sterols and cholestanol, as well as fatty acid composition were stored at -70°C until analysis.

Routine laboratory measurements

The blood samples for the routine laboratory examinations (B-Hemoglobin, B-Thrombocytes, S-Thyroid stimulating hormone, S-Alanine aminotransferase, S-Gamma glutamyltransferase and S-Creatinine) were drawn at the beginning and the end of each study. The samples were analyzed with standardized methods.

Fatty acid composition of serum cholesteryl esters

In the analysis of fatty acid composition of serum cholesteryl esters, serum samples were extracted with chloroform-methanol (2:1, vol:vol), and lipid fractions (cholesteryl esters, triglycerides and phospholipids) were separated with an aminopropyl column (195). Fatty acids were analyzed in a gas liquid chromatograph (GLC) (Hewlett-Packard 5890 series II, Hewlett-Packard Company, Waldbronn, Germany) equipped with a 25-m long FFAP-column. Fatty acids are presented as molar percentage of total fatty acids.

Serum total and lipoprotein lipids and apolipoproteins

Lipoproteins were separated by ultracentrifugation for 18 h at density 1.006 to remove the VLDL fraction. HDL in the infranatant was separated from LDL by precipitation of LDL with dextran sulfate and magnesium chloride (196). LDL-C was calculated as the difference between the mass of cholesterol in the infranatant and HDL, and VLDL-C was calculated as the difference between the whole serum and the infranatant. Enzymatic photometric methods were used for the determination of cholesterol and TG 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 coefficients of variation between measurements for serum TC were 1.3-2.1% (I), 0.9-1.6% (III) and 1.3-1.4% (V), for TG were 3.0-4.7% (I), 1.6-2.4% (III) and 1.7-1.9% (V), for HDL-C were 1.1-1.4% (I), 1.2-1.9% (III) and 1.1-1.2% (V), and for HDL-TG were 1.6% (I), 1.9% (III) and 1.1% (V).

Analyses of apolipoproteins were based on the measurement of immunoprecipitation enhanced by polyethylene glycol at 340 nm (197). A Kone Specific Clinical Analyser and apo AI and apo B reagents from Kone Corporation (Espoo, Finland) were used. The coefficients of variation within measurements for serum apo AI were 1.5-2.3% (I), 2.8-5.5% (III) and 3.7-4.9% (V), and for apo B were 1.2-1.8% (I), 1.8-2.2% (III) and 1.7-

2.8% (V).

Serum cholesterol precursors, plant sterols and cholestanol

In study I, serum plant sterols were quantified from nonsaponifiable serum materials by GLC (Hewlett-Packard 5890A, Palo Alto, CA) (126) equipped with a 25-m long silica CP-Sil 5-CB capillary column (Chrompack, Raritan, NJ). 5 α -cholestane and 5 β -coprostanol were used as internal standards.

In studies III/IV and V, serum cholesterol precursors, plant sterols, cholesterol and cholestanol were measured by GLC (HP 5890 Series II, Hewlett Packard, Delaware, Little Falls, USA) (198, 199) equipped with a 50-m long Ultra 1 capillary column (methyl-polysiloxane) (Hewlett Packard, Delaware, Little Falls, USA) for cholestanol, squalene, Δ 8-cholestenol, Δ 7-lathosterol, desmosterol, campesterol and sitosterol, and equipped with a 50-m long Ultra 2 capillary column (5% Phenyl-methyl siloxane) (Hewlett Packard, Delaware, Little Falls, USA) for sitostanol and campestanol. 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. 5 α -cholestanol for cholesterol and epi-coprostanol for cholesterol precursors, plant sterols and cholestanol were used as internal standards.

Serum carotenoids and fat-soluble vitamins

Serum carotenoids and fat-soluble vitamins were analyzed with a high performance liquid chromatographic system (Perkin-Elmer, Norwalk, CT) on a C₁₈ column (Waters, Milford, MA) (200-202) except in study V where serum 25-OHD₃ was analyzed with a radioimmunoassay method (25-OHD₃ I125 RIA KIT, DiaSorin, Stillwater, MN).

Apo E genotypes

Apo E genotypes were analyzed with the polymerase chain reaction-restriction fragment length polymorphism method described by Tsukamoto et al. (203) with a slight modification.

4.3.5 Questionnaires

At the beginning of each study, previous and present diseases, current medication, alcohol and tobacco consumption, physical activity, use of vitamins or other nutrient supplements were interviewed using a structured questionnaire. Alcohol and tobacco consumption and physical activity were reviewed also at the end of each study. The possible adverse effects and symptoms were enquired repeatedly based on a structured questionnaire (III/IV,V) except in study I/II where a non-structured interview was used at every study visit.

4.3.6 Statistical methods

Statistical methods are reported in detail in the original publications I-V. The data were analyzed with SPSS for Windows 6.0 (I/II, III/IV) or Windows 7.5 (V) statistics program (SPSS, Chicago, IL, USA) (204-206).

Normal distribution and homogeneity of variance were checked before further analyses. Overall changes in continuous variables were analyzed with analysis of variance for repeated measurements [SPSS procedures MANOVA (I/II, III/IV) or GLM (V)]. In study V, GLM was used to assess the effect of the order of margarine consumption periods, carry-over effect and gender on the main end-point variables among the different experimental margarine periods. In further analyses, Student's test in between-group comparisons (I/II) and paired t-test (I/II, III/IV) or GLM (V) within-group comparisons were used. Statistical significances for the response variables (I/II) were tested with a single measurement simple factorial analysis of variance (ANOVA) followed by Student's t-test. In study I/II if the initial concentration differed significantly among the study groups, the concentrations were adjusted in the between-groups comparisons by dividing the response variable with the initial concentration. Variables which were not normally distributed even after logarithmic transformation or non-continuous variables were tested with the Friedman two-tailed ANOVA, Mann-Whitney test, Kruskal-Wallis test, Chi-square test or Wilcoxon matched-paired signed rank test. The analysis of covariance was used for checking whether some variables had effects on lipid responses. In addition, for some variables of interest, Pearson correlation coefficient were calculated. To control the overall α level, Bonferroni adjustment was used. The results are expressed as means \pm SDs, means \pm SEMs or means.

5 RESULTS

5.1 Baseline characteristics

Body weight and blood pressure

During study I/II body weight decreased slightly and similarly (mean decrease 1.1-1.2 kg, $P < 0.001-0.01$) within the control, WSEM and VOSEM groups. During studies III/IV and V body weight remained unchanged. There were no significant changes in systolic or diastolic blood pressure in studies I/II and V.

Routine laboratory measurements

Blood hemoglobin and thrombocytes, and serum γ -glutamyl and alanine amino transferase and creatinine were all within the normal ranges in all studies, even if there were slight, but significant changes in these variables.

Side effects

No adverse effects related to the use of test margarines were recorded. The symptoms (gastrointestinal or skin) reported by the participants were slight and they occurred occasionally and were not related to any particular experimental period or test product.

5.2 Feasibility of the diets

The compliance with the use of test margarines was good. The average daily consumption of the test margarines was between 96-102% of the target amount in all studies. The actual daily intake of plant stanols and plant sterols from test margarines is shown in Table 5.

The actual intakes of the energy nutrients during the different studies are shown in Figure 5. The goals for the composition of experimental diets were well achieved in all studies. Actually, in study I/II during the low-fat diet the mean intakes of fat, SAFA and cholesterol were lower than the goal (step 1) (81) and the intake of dietary cholesterol (mean 137-161 mg/d, 18-21 mg/MJ/d) achieved the goal of the step 2 diet (< 200 mg/d) and the intake of SAFA (mean 6.8-7.3 E%/d) was near to these goals (7 E%/d) in all study groups. The intake of nutrients did not differ among the study groups.

During study III/IV, the mean intake of SAFA was slightly, but significantly, greater during the control period than during the 2.4 g dose period. The mean intake of alcohol was slightly higher in the control period than during the 1.6 g, 2.4 g and 3.2 g dose periods owing to the fact that the eve of May Day and May Day occurred at the end of the control period. In addition, the mean intake of fiber was slightly lower during the control and 0.8 g dose periods than during the 2.4 g and 1.6 g dose periods. However, according to analyses of covariance, the differences in the intake of these nutrients during the different dose periods did not interfere with the results.

During study V, the mean intake of SAFA was within the goal, the mean intake of fat (30.0-31.1 E%) met almost the goal and the mean intake of cholesterol (166-179 mg/d, 20-22 mg/MJ/d) was even lower than the goal. There were no significant differences in the intake of nutrients among the different test margarine periods.

There were no significant changes in the intake of β -carotene or fat-soluble vitamins during any of the studies. The mean intakes of β -carotene, vitamin A and E were 3056-4726 μ g/d, 812-1337 μ g RE/d and 10.4-16.8 mg/d, respectively, in all studies.

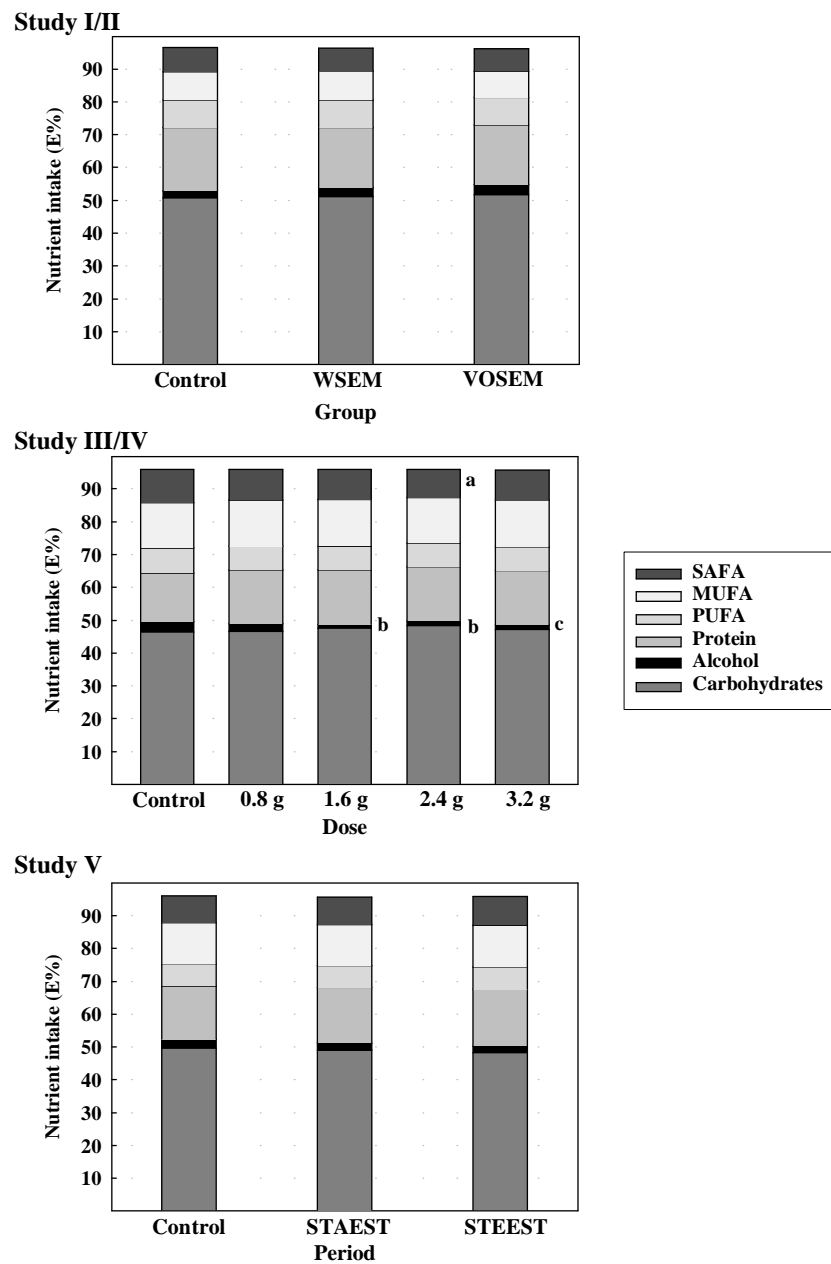


Figure 5. Actual intake of energy nutrients (E%) during studies I/II, III/IV and V. a $P < 0.001$, b $P < 0.05$, c $P < 0.01$ vs. control.

Fatty acid composition of serum cholesteryl esters

There were no major differences in the fatty acid composition of serum cholesteryl esters during the experimental periods (III/IV, V), and the results of this biomarker paralleled the food records (Table 7). Proportions of fatty acids which reflect the intake of SAFA (myristic acid, palmitic acid and stearic acid), the intake of MUFA (oleic acid), and the intake of PUFA (γ -linolenic acid, α -linolenic acid, dihomo- γ -linolenic acid and their metabolites) did not differ among the experimental periods (III/IV, V).

5.3 Serum total lipids, lipoprotein lipids and apolipoproteins

Serum TC and LDL-C

Study I

Serum TC and LDL-C decreased significantly in all study groups during the low-fat, low-cholesterol diet (Figure 6). The reduction in serum TC was significantly greater in both the WSEM and VOSEM groups compared with the control group (mean reduction 18.3% and 15.7% vs. 7.7% from baseline value, respectively). Furthermore, the reduction in LDL-C was significantly greater in the WSEM group compared with the control group (mean 23.6% vs. 9.9%, $P < 0.01$). There were no significant differences in the reduction of TC or LDL-C compared with the baseline between the WSEM and VOSEM groups.

Study III

Serum TC and LDL-C decreased in a dose-dependent manner and significant decreases in serum TC and LDL-C concentrations were reached with the daily stanol dose equal to or greater than 1.6 g (Figure 6). The percentage mean reductions in TC compared with the control were 2.8%, 6.8%, 10.3% and 11.3% with the daily doses of 0.8 g, 1.6 g, 2.4 g and 3.2 g, respectively. The respective reductions for LDL-C were 1.7%, 5.6%, 9.7% and 10.4%. The reduction in serum TC was significantly greater with the doses of the 1.6 g/d, 2.4 g/d and 3.2 g/d than with the dose of the 0.8 g/d, but the reduction in serum LDL-C was significantly greater only with the doses of the 2.4 g/d and 3.2 g/d compared with the 0.8 g/d dose.

Study V

There were no significant differences in cholesterol-lowering efficacy between the STAEST and STEEST margarines (Figure 6). The STAEST and STEEST margarines resulted in significantly lower serum TC (mean 9.2% and 7.3%, respectively) and LDL-C (12.7% and 10.4%, respectively) concentrations compared with the control.

Table 7. Fatty acid composition of serum cholesteryl esters during studies III/IV and V.

Fatty acid, mol%	Study III/IV					Study V		
	Control (0 g)	Dose 0.8 g	Dose 1.6 g	Dose 2.4 g	Dose 3.2 g	Control	STAEST	STEEST
Myristic acid	0.96 ± 0.20	0.94 ± 0.18	0.96 ± 0.20	1.02 ± 0.20	1.02 ± 0.19	1.17 ± 0.33	1.14 ± 0.36	1.22 ± 0.42
Palmitic acid	12.05 ± 0.72	12.06 ± 0.69	12.07 ± 0.59	12.23 ± 0.79	12.03 ± 0.68	12.46 ± 0.87	12.37 ± 0.86	12.45 ± 0.89
Palmitoleic acid	3.11 ± 1.26	3.05 ± 1.05	3.24 ± 1.28	3.38 ± 1.35	3.17 ± 1.30	3.80 ± 0.88	3.68 ± 0.91	3.58 ± 0.80
Stearic acid	0.55 ± 0.12	0.51 ± 0.15	0.49 ± 0.18 ^a	0.50 ± 0.19	0.45 ± 0.13 ^{a,b}	0.78 ± 0.15	0.67 ± 0.13 ^c	0.67 ± 0.13 ^c
Oleic acid	20.29 ± 1.06	20.04 ± 1.64	19.91 ± 1.34	20.51 ± 1.87	19.95 ± 1.45	21.22 ± 1.50	21.07 ± 1.49	20.66 ± 1.52 ^d
Linoleic acid	53.78 ± 3.08	54.04 ± 3.62	53.79 ± 3.17	52.50 ± 4.02 ^b	53.72 ± 3.29	51.45 ± 3.21	52.27 ± 3.27	52.20 ± 3.37
γ-linolenic acid	0.78 ± 0.38	0.69 ± 0.28	0.71 ± 0.28	0.80 ± 0.32	0.74 ± 0.34	0.65 ± 0.29	0.63 ± 0.32	0.59 ± 0.26
α-linolenic acid	1.12 ± 0.17	1.03 ± 0.15	1.06 ± 0.13	1.10 ± 0.18	1.03 ± 0.13 ^c	1.07 ± 0.20	1.00 ± 0.16	0.99 ± 0.23
Dihomo- γ-linolenic acid	0.48 ± 0.10	0.49 ± 0.10	0.50 ± 0.08	0.51 ± 0.10	0.49 ± 0.08	0.52 ± 0.15	0.49 ± 0.10	0.53 ± 0.17
rachidonic acid	4.78 ± 0.83	5.00 ± 0.97	5.02 ± 1.00	4.99 ± 1.02	4.93 ± 0.88	4.58 ± 0.95	4.50 ± 0.89	4.63 ± 0.82
EPA	1.59 ± 0.73	1.59 ± 0.67	1.64 ± 0.61	1.85 ± 0.73	1.87 ± 0.76	1.68 ± 1.00	1.57 ± 0.66	1.86 ± 1.04
DHA	0.54 ± 0.13	0.56 ± 0.16	0.60 ± 0.17 ^c	0.62 ± 0.17 ^a	0.61 ± 0.16 ^c	0.63 ± 0.18	0.60 ± 0.19	0.63 ± 0.20

Mean ± SD. EPA=Eicosapentanoic acid, DHA=Docosahexanoic acid

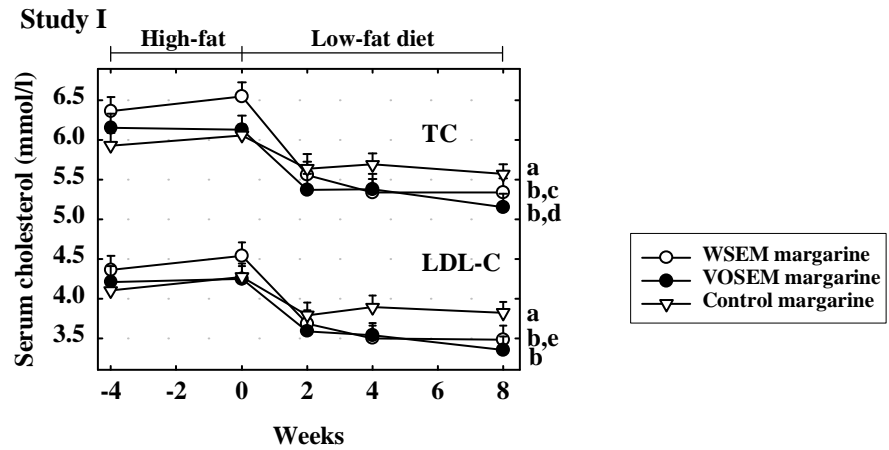
Study III/IV:

^a P<0.01, ^c P<0.05, vs. control

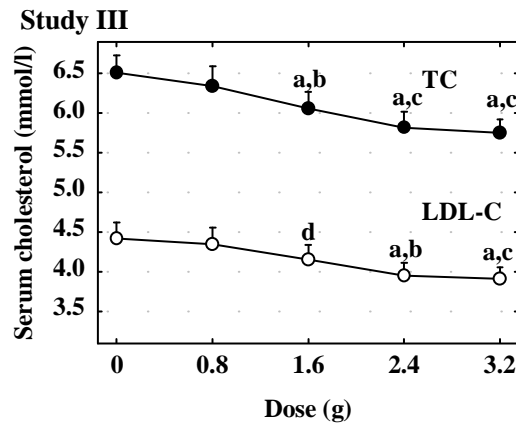
^b P<0.05 vs. 0.8 g dose

Study V:

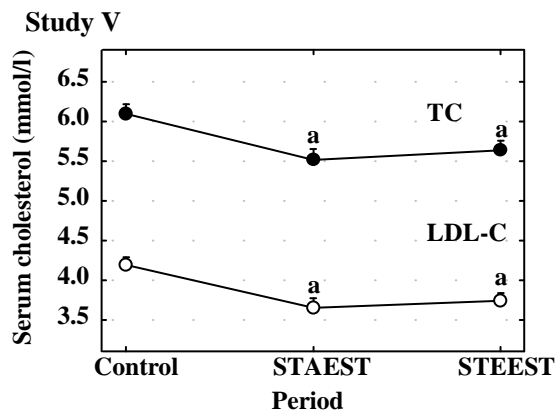
^c P<0.001, ^d P<0.01 vs. control



a $P < 0.01$, b $P < 0.001$ change within-group from 0 to 8 weeks
 c $P < 0.001$, d $P < 0.05$, e $P < 0.01$ vs. control group



a $P < 0.001$, d $P < 0.05$ vs. control dose
 b $P < 0.05$, c $P < 0.001$ vs. 0.8 g dose



a $P < 0.001$ vs. control margarine

Figure 6. Serum TC and LDL-C concentrations (mmol/l) during studies I, III and V. Mean \pm SEM.

Variation in LDL-C responses to plant stanol esters or sterols esters

Serum LDL-C increased in one subject in the VOSEM group and in three subjects in the control group (I). The greater the stanol ester dose, the smaller the number of non-responders (III). However, there was no subject who did not respond at least one of the four stanol ester doses (III). In addition, in both STAEST and STEEST margarine periods (V) serum LDL-C increased slightly in five subjects and in two of them with both test margarine periods. When the reduction in LDL-C was examined in thirteen subjects who participated in at least two of three studies, no real non-responders were found.

Serum HDL-C, VLDL-C and TG

Serum HDL-C concentrations remained almost unchanged in all studies.

In study I, serum VLDL-C decreased within all study groups, but only significantly within the VOSEM group (0.47 ± 0.24 mmol/l to 0.34 ± 0.18 mmol/l, 0 to 8 wk). There were no significant differences in the reduction in VLDL-C among the three study groups (I). In study III, the serum VLDL-C was significantly lower with the doses of 1.6 g/d (0.39 ± 0.22 mmol/l), 2.4 g/d (0.38 ± 0.29 mmol/l) and 3.2 g/d (0.34 ± 0.23 mmol/l) compared with control value (0.62 ± 0.25 mmol/l), but there were no significant differences in serum VLDL-C concentrations between any two stanol doses. No significant changes in serum VLDL-C were found during study V.

There were no significant changes in serum total TG (I,III,V), VLDL-TG (I), LDL-TG (I) or HDL-TG (I) concentrations among the study groups (I) or in serum TG among the experimental periods (III, V). However, there was a significant decrease in the serum VLDL-TG concentration (0.96 ± 0.62 mmol/l to 0.77 ± 0.64 mmol/l, 0 to 8 wk) within the WSEM group, and an increase in the serum LDL-TG concentration (0.27 ± 0.06 mmol/l to 0.30 ± 0.08 mmol/l, 0 to 8 wk) within the control group (I).

Serum apo AI and B

Serum apo AI was reduced on average by 9.0%, 8.6%, and 6.1% from the baseline within the WSEM, VOSEM and control groups, respectively, $P < 0.01-0.05$ (I). However, no significant differences in the changes of apo AI were found among the study groups (I) or among the experimental periods (III, V).

Serum apo B was reduced significantly in all studies and the reduction paralleled the reduction in LDL-C. In study I, the mean reduction was 19.2%, 13.7% and 5.2% from the baseline, within the WSEM, VOSEM and control groups, respectively, $P < 0.001-0.05$. In study III, a significant reduction (8.7%) in apo B was reached already with the lowest stanol dose (0.8 g/d). With the other doses (1.6 g/d, 2.4 g/d and 3.2 g/d) the mean reduction of apo B was 9.3%, 10.2% and 13.7%, respectively. In addition, the STAEST margarine resulted in a 10.7% reduction and the STEEST margarine in a 10.4% reduction in serum apo B concentrations compared with the control margarine, $P < 0.001$ for both.

5.3.1 Non-dietary factors affecting serum lipid responses

No differences between **genders** or different **age** groups (Figure 7) were found in serum lipid responses to plant stanol esters or sterol esters in any of studies I, III and V. No differences in the lipid results between normal weight and slightly **overweight** subjects were found (I, III, V). In addition, there were no significant correlations between the **initial value of LDL-C** and the percentage reduction in LDL-C in any of the studies when subjects were divided to tertiles of the initial LDL-C concentrations.

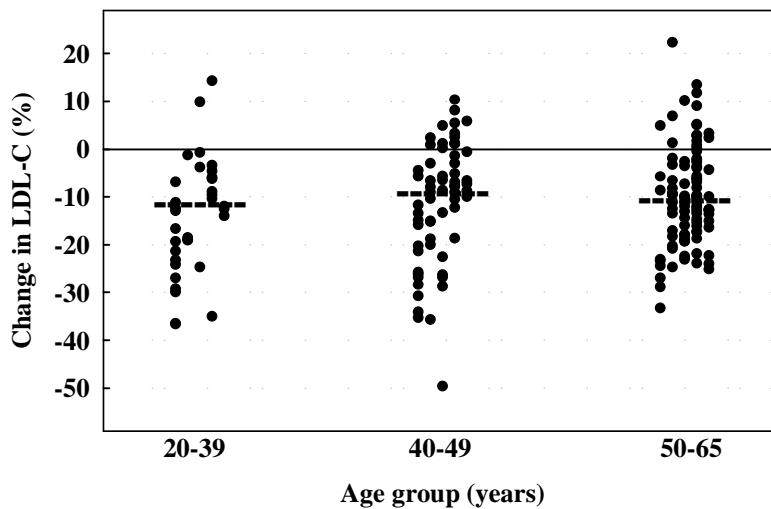


Figure 7. Serum LDL-C response (%) in different age groups during studies I, III and V.

The changes in LDL-C were similar for those subjects with **apo E genotype 3/3** and those with apo E allele 4 (3/4 or 4/4) in both experimental groups during study I (Figure 8). However, in the control group, the reduction in LDL-C seemed to be somewhat greater in those with apo E3/4 than those with apo E3/3. Also in study III, serum LDL-C reduced similarly in two apo E groups with different stanol ester doses. In study V, the subjects with apo E3/4 had a significantly greater reduction in LDL-C during the STAEST margarine period than during the STEEST margarine period. However, there were no significant differences in the reduction in LDL-C in those with apo E3/3 between the two test margarine periods or between the two apo E groups during either experimental period.

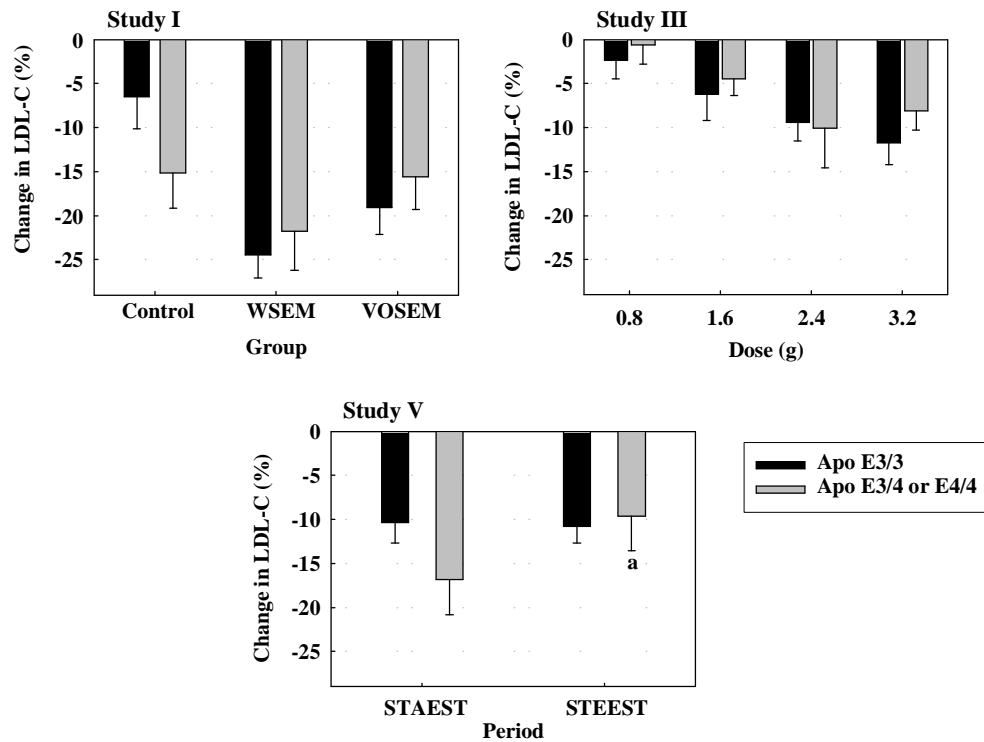


Figure 8. Changes in serum LDL-C (%) in different apo E groups during studies I, III and V. ^a $P < 0.05$ STAEST vs. STEEST in the subjects with apo E3/4. Mean \pm SEM.

5.4 Serum cholesterol precursors and cholestanol

In general, the changes in the concentrations of serum cholesterol precursors were small. The most pronounced changes were found in serum $\Delta 8$ -cholestenol and $\Delta 7$ -lathosterol concentrations, especially in their ratios to serum TC (Figure 9). In study V, also the increase in the ratios of the serum desmosterol and squalene to TC reached statistical significance (Figure 9).

The serum $\Delta 7$ -lathosterol/TC ratio (IV) did not increase in a dose-dependent manner but plateaued with a dose equal to or greater than 1.6 g/d (Figure 9).

Serum cholestanol concentration reduced significantly with daily doses of the 1.6 g, 2.4 g and 3.2 g in comparison with the control (IV; Table 2). However, the serum cholestanol/TC ratio remained almost unchanged during that study. In study V, the serum cholestanol concentration was lower during the STAEST and STEEST margarine periods than during the control (V; Table 5). The ratio was also significantly lower during the STEEST margarine period than during the control or STAEST margarine period.

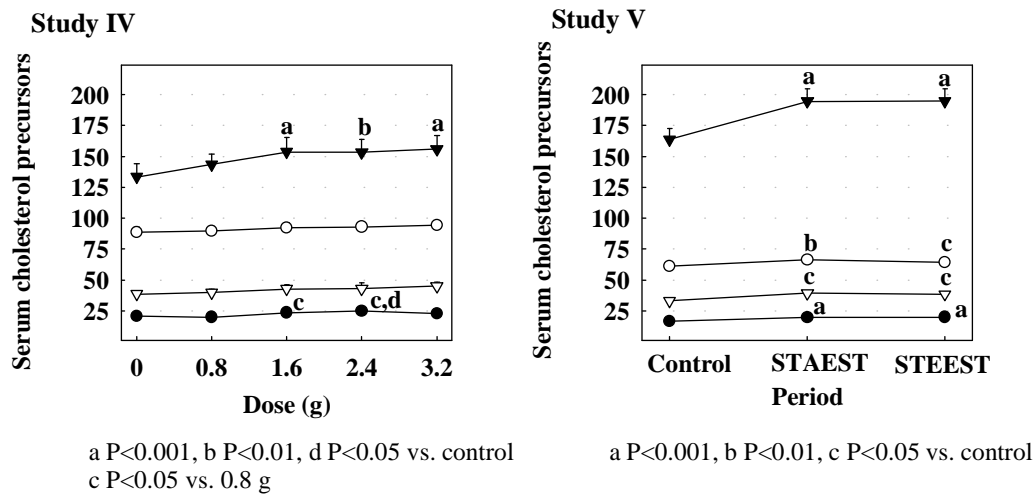


Figure 9. Serum cholesterol precursors Δ 7-lathosterol (Y), desmosterol (TM), squalene (C) and Δ 8-cholestenol (~) (10^{-2} μ g/mg of TC) during studies III/IV and V. Mean \pm SEM.

5.5 Serum plant sterols

The consumption of plant stanol esters containing margarines (I, III/IV, V) resulted in a significant reduction in serum campesterol concentration (Figure 10) and the greater the stanol ester dose, the greater the reduction in serum campesterol (III/IV). The consumption of STEEST margarine increased serum campesterol concentration significantly (V).

In study I, serum sitosterol concentrations did not decrease significantly within the WSEM and VOSEM groups. However, in study III/IV, a significant decrease in sitosterol concentration occurred already with a dose of 0.8 g/d (Figure 10). In study V, serum sitosterol concentration decreased significantly with the STAEST margarine and increased significantly with the STEEST margarine compared with the control (Figure 10).

The changes in serum avenasterol (III/IV, V) were parallel, but somewhat smaller than the changes in serum campesterol and sitosterol (Figure 10). Furthermore, the changes in the ratios of serum campesterol, sitosterol or avenasterol to serum TC paralleled the changes in their serum concentrations.

Serum campestanol and sitostanol concentrations did not change significantly within any of the three study groups during study I (Figure 10). Similarly, during the STEEST margarine period (V) those concentrations remained close to the control values. On the other hand, in studies III/IV and V, significant increases in serum campestanol and sitostanol concentrations were found during the stanol ester margarine periods (Figure 10). Serum campestanol concentration became doubled already with the stanol dose of 0.8 g/d compared with the control, and the concentration increased only slightly further with greater doses (III/IV). The greatest increment in serum sitostanol concentration was

achieved also with the dose of 0.8 g/d, and only a minor further increase was found with the greater stanol ester doses (III/IV). Similarly to serum plant sterol concentrations, the changes in the ratios of serum sitostanol and campestanol to serum TC paralleled the changes in their serum concentrations in studies I, III/IV and V.

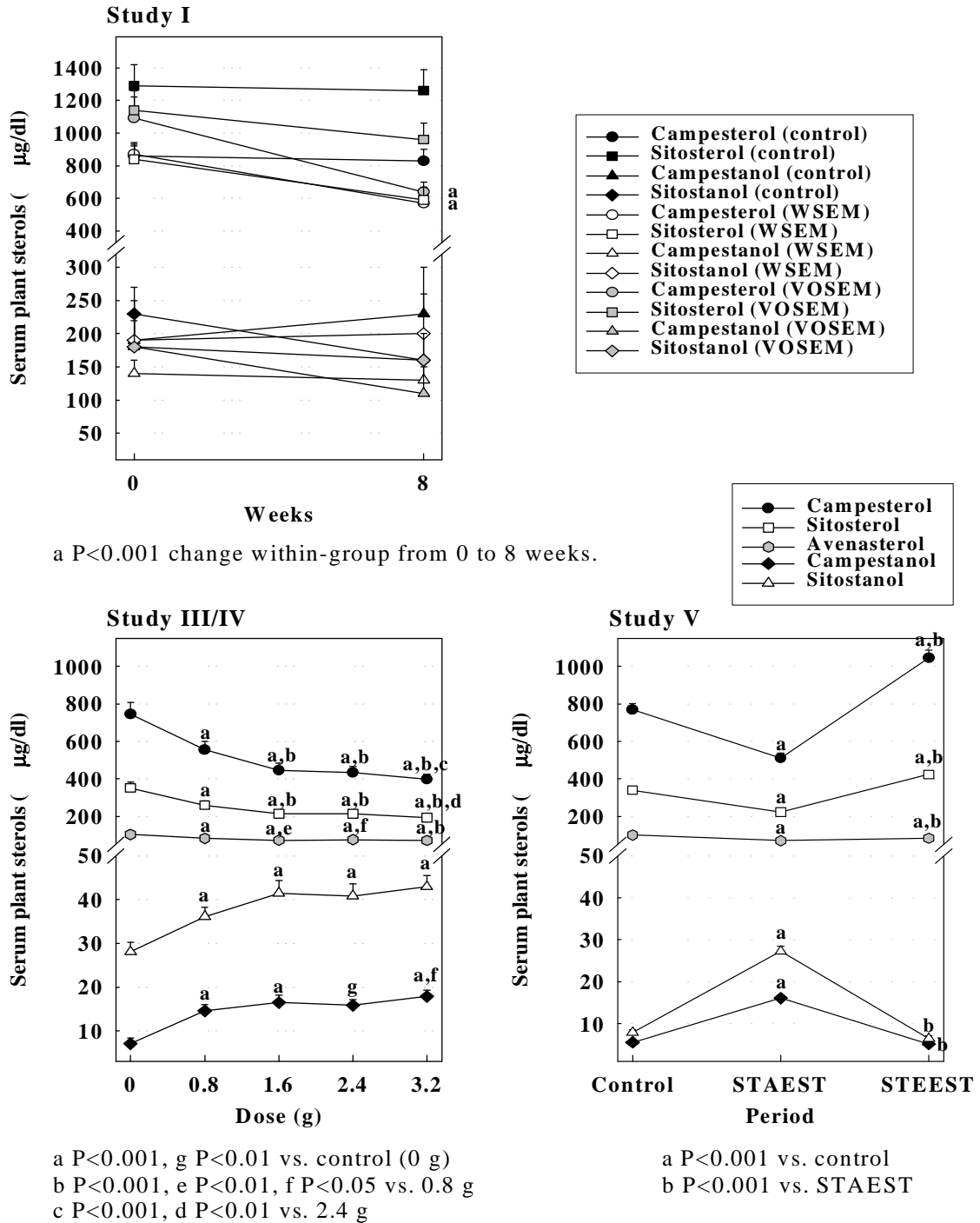


Figure 10. Serum plant sterol and stanol concentrations (µg/dl) during studies I, III/IV and V. Mean ± SEM.

5.6 Serum carotenoids and fat-soluble vitamins

Plant sterols and stanols had the greatest effect on serum β -carotene concentration (Figure 11). There was a significant reduction in absolute serum β -carotene concentration within the WSEM and VOSEM groups (I/II) and during the STAEST and STEEST margarine periods (V), but no significant reduction in the β -carotene concentration was found during the different stanol dose periods (III). When the serum β -carotene concentration was related to the serum TC concentration, no significant differences in the changes of the ratio were found in any of the studies. Within the control group, the changes in serum α -carotene were small and only the reduction in the α -carotene/TC ratio was statistically significant (II). The changes in the α -+ β -carotene paralleled the changes in β -carotene. However, in study V, serum α -+ β -carotene concentration did not differ significantly between the STAEST margarine and the control periods as it did between the STEEST margarine and the control periods. Serum lycopene and lycopene/TC did not change significantly within the WSEM and VOSEM groups (II) or during the STAEST and STEEST margarine periods (V). In women, serum lycopene concentration was significantly greater with the control and the dose of 0.8 g/d than with the dose of 2.4 g/d, and the lycopene/TC ratio was significantly greater with the dose of 0.8 g/d than with the dose of 2.4 g/d and control (III). However, no significant differences in the serum lycopene concentrations were found in men during the same study (III).

Serum retinol concentration remained almost unchanged in all studies. There were no major changes in the serum 25-OHD₂ concentration during study I or in serum 25-OHD₃ concentration during studies III and V. The concentration of 25-OHD₃ increased significantly within the WSEM, VOSEM and control groups, and the increase was significantly smaller in the WSEM than in the VOSEM group (I; Table 5). However, there was no significant difference in the percentage increase of 25-OHD₃ among the study groups.

Serum α -tocopherol concentrations were reduced significantly within the WSEM, VOSEM and control groups (I; Table 5). The reduction differed significantly only between the WSEM and control groups. Serum α -tocopherol concentration was significantly lower with all test doses compared with the control, and in addition, the concentration was significantly lower with the dose of 3.2 g/d than with the dose of 0.8 g/d (III; Table 6). Furthermore, the serum α -tocopherol concentration was significantly lower during the STAEST and STEEST margarine periods than during the control period (V; Table 6). However, after relating α -tocopherol to TC there were no significant changes among the study groups (I) or experimental periods (III, V). The changes in serum γ -tocopherol concentrations were smaller than the changes in serum α -tocopherol concentrations (III and V; Table 6). Only in study III was the concentration significantly lower with the stanol doses of 2.4 g/d and 3.2 g/d than with the control. The

γ -tocopherol/TC ratio remained almost unchanged during these two studies (III, V). In addition, the changes in serum α - γ -tocopherol paralleled the changes in serum α -tocopherol (III, V).

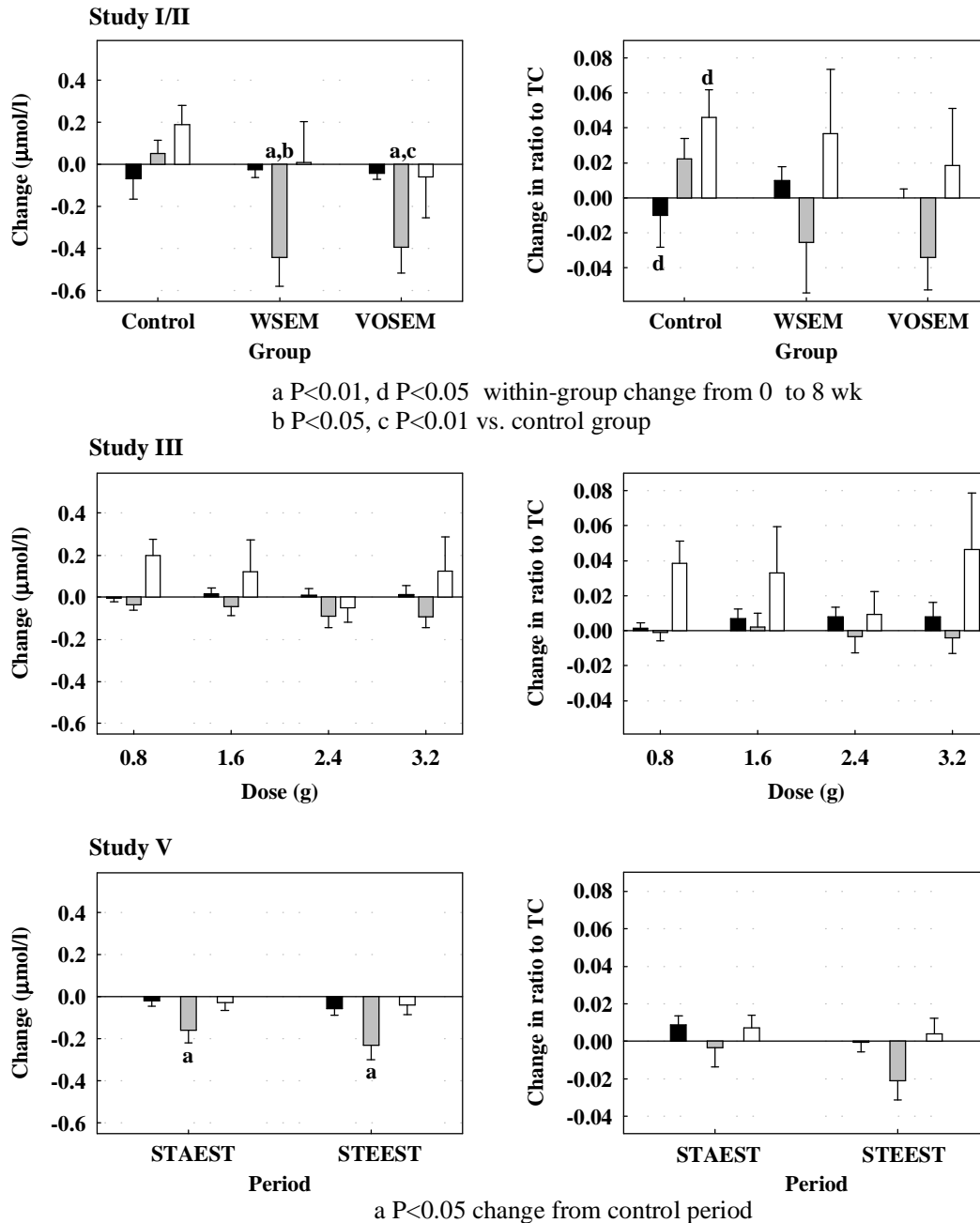


Figure 11. Changes in serum α -carotene (black), β -carotene (gray) and lycopene (white) concentrations ($\mu\text{mol/l}$) and their ratios to TC (mmol/mol of TC) during studies I/II, III and V. Mean \pm SEM.

6 DISCUSSION

6.1 Subjects and study designs

Subjects representing the potential users of plant sterol-enriched products i.e. middle-aged, normal weight or slightly overweight and mildly or moderately hypercholesterolemic individuals were recruited into the studies. The subjects completing the studies did not differ from those who dropped out with respect to background variables. The subjects can be considered to be well representative of the target study population.

In study I/II a parallel **study design** with a 4-week high-fat diet period and a 8-week low-fat diet period was used, because our aim was to examine how much stanol ester-enriched low-fat margarines could potentiate the lipid reduction induced by a low-fat diet alone compared with the typical Western diet. On the other hand, because in studies III/IV and V the main interest was to compare the different stanol ester doses or the different test margarines, the repeated measures design with 4-week experimental periods was chosen as the study design. The length of the experimental period in study I/II was eight weeks instead of four weeks, because the implementation of a low-fat diet takes a longer time than four weeks, and thus study I/II could not be arranged with the cross-over design.

In studies III/IV and V, the subject consumed each test margarine in a randomized order, which was determined as a whole study group (III/IV) or according to the Latin square model (V). The randomization as a group in study III/IV was chosen for practical reasons; there were five test doses and if each subject had been randomized separately or according to the Latin square model, there would have to have been many more subjects in the study. The benefit of the repeated measures design is that it increases the power of the study by eliminating the between-individual variation. In addition, the randomization according to the Latin square model in study V enabled us to control the effect of time. On the contrary, the randomization as a group in study III/IV did not allow to control for the effect of time but did allow to control for systematic bias due to the order of periods. However, should time have affected the results of that study, the greatest reduction in serum cholesterol would have been expected to occur during the first dose period (2.4 g/d), but this was not true the case in that study. Moreover, in study V no effect of time was observed.

The disadvantage in the repeated measures design is a possible carry-over effect. To eliminate the carry-over effect, it is possible to use wash-out periods between test periods or to use a sufficiently long study period. In studies III/V and V, the duration of the experimental period was four weeks and the main comparisons were made among the mean values at the end of each experimental period. It is well-known that effects of plant stanols and plant sterols become apparent within a short time. In earlier studies, it has

been shown that plant sterols reduce cholesterol concentrations within 2-3 weeks of the initiation of treatment (4, 15, 72, 74). Furthermore, in one specific patient group, colectomized patients, a significant reduction in serum cholesterol was found already after one day of the consumption of plant stanol esters and a new steady state was reached within seven days (78). Moreover, the serum cholesterol concentration has been shown to return to its initial value within 2-3 weeks, upon cessation of the ingestion of plant sterols (4, 15, 68, 74). In addition, in dietary intervention studies, a new steady state in the cholesterol concentration has been attained within 2 weeks (207). Altogether, the four-week study period in studies III/IV and V can be considered as being sufficiently long enough to eliminate any carry-over effects of the previous period, and long enough to illustrate the effects of a given test margarine on serum cholesterol concentration.

6.2 Compliance of subjects and feasibility of the diets

All subjects participating in the present studies were free-living. In order to gain the dietary goals and eliminate changes in body weight, the subjects received precise oral and written instructions about isocaloric diets, which were individually tailored to each subject within the set dietary goals. The subjects themselves took care of the practical implementation with normal Finnish foodstuffs and only the test spreads and the other study products (vegetable oils, salad dressings, low-fat cheeses or liquid milk products) were given to them free of charge. In some other plant sterol studies, all meals have been prepared entirely by the research unit (36, 71, 86) to improve the compliance of the subjects and to diminish the variability of the background diets. However, the lipid results of our studies were comparable to the result of those studies. In the present studies, the subjects were well motivated to participate in the studies and only three out of seventeen dropped out due to lack of commitment while other reasons for dropping outs were not connected to motivation. In addition, there were only minor changes in medication and life-style of the subjects during the studies and they did not affect the results. Therefore, our free-living setting was sufficiently strict to allow us to evaluate the test diet at the level demanded by the hypothesis. The advantage of our chosen procedure is that it gives a more realistic view on the effects of the study products and experimental diets on examining variables in free living individuals under normal conditions than providing of prepared meals to subjects or studies in a metabolic unit setting.

In study I/II, test margarines were enriched with stanol esters derived from wood (WSEM) or vegetable oils (VOSEM) and in the other studies (III/IV, V) they were a blend of wood and vegetable oils. The sterol esters were only vegetable oil-based as has been the case in the other published sterol ester studies (4, 5, 31). In most of previous studies, plant stanol esters have been derived from wood (47, 64-68). However, recently published studies, blends of wood and vegetable oils have been used (4, 71, 72, 77, 78).

The amount of absorbable fat, fatty acid composition of the test margarines and the esterification degree of plant stanols or sterols in each study was identical (I-V). However, there were slight differences in the total amount of plant stanols between the WSEM and VOSEM margarines (difference 0.18 g/d; I/II) and between the amount of planned and actual plant stanols in the different stanol ester doses (#0.15 g/d of stanols; III/IV). These differences, however, were minor and therefore, it can be assumed that they have any impact on the results.

The dietary compliance of the subjects was monitored by three to four consecutive days of food records (I-V) and by measuring the fatty acid composition of serum cholesteryl esters (III/IV, V) regularly. The use of these two methods ensured the reliability of the dietary follow-up. The three to four consecutive days food recording has been found to be sufficient to estimate the intake of energy nutrients at a group level (208, 209). However, for the monitoring cholesterol intake more recording days may be needed (208). In study I, the mean of data from the three four days' food records was used as the estimate of nutrient intake. In addition, in all studies the subjects followed a certain dietary regimen. This diminished within-person variation and thus decreased the number of recording days required to obtain reliable information on nutrient intake (210).

The subjects were given oral and written instructions on how to keep the food records. In addition, the nutritionist reviewed the records during the study visits to complement data that were lacking. It is known that subjects attempt to please the nutritionist by manipulating their records to match the dietary goals of the study (211, 212). Therefore, it was emphasized to the subjects that they have to report their true food intake to ensure the reliability of the study. In addition, the results of fatty acid composition of serum cholesteryl esters (III/IV, V), which is an objective marker of dietary adherence in terms of quality of fat, reflecting the fatty acid composition of a diet during the past 3-4 weeks (213, 214), suggests that the subjects were honest in their reporting. The subjects were aware of the use of this objective measurement.

According to the food records (I-V), the experimental diets met well the goals for fatty acid composition and dietary cholesterol. In study I/II, the consumption of the low-fat test margarines combined with the low-fat diet enabled the achievement of the goals of the step 2 diet (81) in the intake of fat and dietary cholesterol. Also the intake of SAFA was close to the goal of the step 2 diet (81) in all three study groups. No significant differences were found in the nutrient intake among the groups of study I/II or among the experimental margarine periods of study V. In addition, the minor differences in the intake of SAFA, alcohol and fiber during the different dose periods (III/IV) had a non-significant effect on the serum lipid responses caused by the stanol esters. The fatty acid composition of serum cholesteryl esters was similar during the different experimental periods indicating that the background diets of these studies did not change. In addition, the similar serum fatty acid composition as the biomarker confirms

good compliance to the use of test margarines and intended fat modification.

6.3 Serum total lipids, lipoprotein lipids and apolipoproteins

Effects of plant stanol esters as part of a cholesterol-lowering diet

The low-fat WSEM and VOSEM margarines (I) combined to the cholesterol-lowering diet reduced serum TC and LDL-C concentrations on average by 16-18% and 18-24%, respectively, from the high-fat baseline diet. The reductions seen here were greater than those achieved (range of mean reduction 4-10% and 5-15%, respectively) in several studies with mildly to moderately hypercholesterolemic subjects in which full-fat stanol ester margarine combined to a habitual moderate rich or rich fat diet has been used (33, 34, 68). In fact, our results (I) indicate that combining plant stanol esters to a low-fat margarine and as part of a strict low-fat, low-cholesterol diet, can reduce serum cholesterol concentration nearly as much as some cholesterol-lowering drugs (215, 216). In addition, our findings show that low-fat stanol ester margarines do provide an additional, about 10% reduction, in serum cholesterol concentrations to that which can be obtained with a strict cholesterol-lowering diet alone as was recently also shown with a similar study design by Anderson et al. (71). This additional benefit is remarkable, because the dietary changes have been reported to obtain only a 3-6% reduction in serum cholesterol at the population level (1).

Our findings that the stanol ester (I, V) and sterol ester (V) margarine reduced serum TC and LDL-C significantly as part of a low-fat, low-cholesterol diet are contrary to the earlier suggestion that plant stanols and sterols are ineffective when the diet is low in cholesterol. This suggestion is based on the findings of Denke (63), in which 3 g/d of plant stanols taken in capsules and as part of a low-fat and low-cholesterol diet reduced serum TC and LDL-C only slightly. However, the most probable reason for Denke's finding was that plant stanols in capsules were suspended, not dissolved, in sunflower oil and were thus in a poorly soluble, less effective, form. The findings of Denke have now also been rejected in several other studies (70-72, 75-77). The finding that plant stanols can reduce serum cholesterol concentrations even when combined with a low-cholesterol intake, indicates that they must inhibit both the absorption of dietary as well as biliary cholesterol.

In the present studies, as in many others (4, 34, 65-72) the daily dose of plant stanol esters was taken in 2-3 portions with meals. However, most recently Plat et al. (77) demonstrated that it is not necessary to eat plant stanol esters simultaneously with dietary cholesterol or with each meal to obtain the optimal cholesterol-lowering effect.

Serum HDL-C and TG concentrations remained almost unchanged, in agreement with previous studies (5, 34, 47, 64, 66-68, 71, 72, 74-78).

Dose-response effect of plant stanol esters

In study III, plant stanol esters reduced the serum cholesterol concentrations in a dose-dependent manner. The significant reduction in serum TC and LDL-C concentrations was reached with a daily stanol dose equal to or greater than 1.6 g compared with the control. Furthermore, increasing the daily dose of plant stanol from 2.4 g to 3.2 g did not provide additional cholesterol-lowering effect. Therefore, based on the present findings and the findings of the other stanol ester or sterol ester studies (31, 68, 72) the optimal daily stanol or sterol dose seems to be about 1.6-2.4 g. These findings indicate that the dose-response of plant stanols or sterols in esterified form on serum cholesterol is curvilinear and that the response plateaus with a dose of equal to or greater than 2.4 g/d. Above that level of plant stanol or sterol, the cholesterol-lowering efficacy increases only marginally. These findings are interpreted to mean that the ability of plant stanols or sterols to disturb the cholesterol absorption from intestine is dependent on their relative amounts in the intestine. Therefore, if there is an excessive amount of plant stanol or sterol in the intestine in relation to cholesterol, no additional benefit can be obtained by increasing the dose of stanol ester or sterol ester. In adults, each day between 1000-1500 mg of cholesterol, either of biliary or dietary origin, enters the lumen of the small intestine (97). This could be one reason why the reduction plateaus with the dose of 2.4 g/d.

In general, the changes in serum apo B paralleled the changes in serum LDL-C. However, a dose as low as of 0.8 g/d resulted in a significant reduction in apo B concentrations compared with the control, although with that dose the reduction in serum LDL-C was small and non-significant. In recent studies, it has been suggested that as little as 0.7-0.8 g/d of plant sterols or stanols are needed to achieve a significant reduction in serum cholesterol (31, 54, 56, 57). In this study, only one blood sample was taken at the end of each dose period. Therefore, the slight, but non-significant, reduction during the 0.8 g dose period in serum cholesterol concentrations might be concealed by within-subject variation in serum cholesterol concentrations. Within-subject variation in serum cholesterol concentration is 5-10% (217, 218). In addition, the power of the study (0.8) was calculated to detect a 0.4-0.5 mmol/l difference in TC response between different doses. Therefore, the number of subjects (N=22) was too small to observe such small reductions in serum cholesterol as being statistically significant.

Variation in serum LDL-C responses to plant stanol esters or sterol esters

In one study about 8% of subjects were reported to be non-responders to the stanol ester treatment (104). However, we did not find any real non-responders when we compared the reduction in LDL-C in thirteen subjects who participated in at least in two of our three studies. Although there were subjects who did not respond to stanol ester or sterol ester treatment in one study, in another study their LDL-C concentrations did decrease in response to the treatment. In addition, it seems that the more strict the

background diet, the fewer non-responders found in the present studies.

Origin and form of plant stanols or plant sterols

In study I, the wood and vegetable oil-based stanol esters enriched margarines reduced serum TC and LDL-C concentrations equally effectively, as was recently also stated by Gylling and Miettinen (33) and Plat and Mensink (34). In addition, in study V the cholesterol-lowering effect of plant sterol esters and stanol esters (saturated form of plant sterols) did not differ significantly. The findings in two recent comparison studies have been inconsistent (4, 5): in one study soy oil-based sterol ester margarine and stanol ester margarine (Benecol®) reduced serum cholesterol concentrations similarly (4), but in the second study sterol esters reduced serum LDL-C concentrations somewhat more than stanol esters (5). In some earlier studies it has been suggested that free plant stanols reduce serum cholesterol concentrations more effectively than free plant sterols (58-61, 80). Therefore, there might be differences in the cholesterol-lowering efficacy between the large and small doses of plant sterols and stanols. However, with the doses used currently, there does not seem to be differences in the efficacy of plant sterols and stanols. Furthermore, based on recent findings (56, 170) it seems that the vehicle by which plant sterols or stanols are delivered to the small intestine is a more critical factor determining their ability to disturb the cholesterol absorption and thus reduce serum cholesterol concentrations than the degree of saturation of plant sterols.

6.4 Non-dietary factors affecting serum lipid responses

In none of the studies (I, III, V) were there any differences detected between **genders** in serum lipid responses on plant stanol esters or sterol esters in agreement with other studies (4, 47, 54, 64, 77).

Although it has been suggested that the subjects **aged** 30-39 years would have a lower LDL response to plant stanol or plant sterol treatment compared with those aged 40-49 or 50-59 years (89), the findings of the present studies I, III and V do not confirm this suggestion.

No differences in lipid responses between the subjects with normal **weight** and those who were slightly overweight were found in any of the studies (I, III, V) in accordance with the findings of other studies (53, 72). Furthermore, the changes in body weight can not be considered to have any confounding effect on serum lipid results, since in studies III and V the body weight remained unchanged and in study I the significant decrease in body weight was only marginal and similar within the three study groups. In study I, the decrease in body weight was primarily ascribed to the lower intake of energy during the experimental period than during the run-in period (mean 7.1-7.8 MJ/d vs. 8.0-8.7 MJ/d). In turn, this was attributed to the low-fat diet, all of the subjects could not eat the planned amount of food, which would have covered their energy requirements.

The **initial value** of serum LDL-C did not affect the magnitude of response to plant stanol esters or sterol esters in any of the present studies (I, III, V). This is in contrast to the findings of several other studies (68, 70, 76, 78), although also similar findings to ours have been presented (4).

In previous studies, the results of the effects of **apo E genotype** or phenotype on LDL-C responses to plant stanols or plant sterols have been controversial (34, 47, 54, 66). According to secondary analyses performed in studies I, III and V, there were no significant differences in LDL-C response between the apo E3/3 and apo E3/4 genotype groups. Surprisingly, the subjects with apo E3/4 had a greater percentage reduction in serum LDL-C during the STAEST period than during the STEEST period (V). It is difficult to assess the validity of this finding, since there are no previous reports in which the effects of plant stanols on serum cholesterol concentrations in different apo E groups have been compared with that of plant sterols. Therefore, this finding should be verified in prospective studies.

6.5 Serum non-cholesterol sterols as a marker of cholesterol metabolism

The concentrations of serum plant sterols (campesterol and sitosterol; I, III/IV, V) and cholestanol (IV, V) were determined to evaluate cholesterol absorption (103, 105, 153), whereas the concentrations of serum cholesterol precursors ($\Delta 8$ -cholestenol and $\Delta 7$ -lathosterol and desmosterol; IV, V) were measured to estimate cholesterol synthesis (103, 150-152).

Serum plant sterol concentrations, and in particular the serum campesterol concentration, have been found to reflect cholesterol absorption efficacy from intestine in individuals on a normal background diet without plant sterol or stanol supplementation (103, 105). As in previous plant stanol or stanol ester studies (4, 5, 33, 47, 54, 55, 64-68, 70-72, 75, 76, 78, 79, 104) in the present studies, stanol esters reduced serum campesterol concentrations significantly. Compared with the control the significant reduction in serum plant sterol concentration was already attained with a stanol dose of 0.8 g/d (III/IV) indicating that plant stanols can effectively inhibit intestinal absorption of cholesterol even at low doses. This confirms the findings of the previous studies in which 0.6-0.8 g/d of plant stanols in free or esterified form caused marked reductions in serum plant sterol concentrations (54, 55, 64). Our findings are also in agreement with the studies where 0.7-0.8 g/d of plant stanol as stanol esters effectively reduced intestinal absorption of cholesterol as measured by the continuous isotope feeding method (54, 64). The greater the stanol ester dose, the greater the reduction in serum campesterol, indicating an even greater reduction in cholesterol absorption with the higher daily stanol doses of 1.6 g, 2.4 g and 3.2 g (III/IV). On the other hand, the increased serum plant sterol concentrations with the STEEST margarine reflected the absorption of plant sterols from that margarine rather than the increased cholesterol absorption.

In studies IV and V, serum cholestanol, a metabolite of cholesterol, seemed to be a weak marker of cholesterol absorption. However, this is not surprising, in view of the fact that most published reports of the effects of plant stanol esters on serum cholestanol have been inconsistent (33, 47, 54, 55, 64-67, 70, 71, 75, 78, 79, 104).

Cholesterol malabsorption and depletion of hepatic cholesterol pool induced by plant sterols have been suggested to stimulate a compensatory increase in endogenous cholesterol synthesis (64, 65, 67, 70, 78). The marker of that is the enhanced concentrations of serum cholesterol precursors, and particularly the elevated serum Δ^7 -lathosterol/TC ratio (103, 151, 152). In studies IV and V, the enhanced serum Δ^7 -lathosterol/TC ratio indicated that cholesterol synthesis had increased in compensation for the cholesterol malabsorption and the decrease in hepatic cholesterol. The plant stanol dose of 0.8 g/d increased serum Δ^7 -lathosterol/TC only slightly and this agreed with earlier findings (54, 64). Furthermore, serum Δ^7 -lathosterol/TC plateaued with the stanol dose of 1.6 g/d which might indicate that cholesterol synthesis does not increase in a dose-dependent manner but the endogenous cholesterol synthesis reaches its maximum level with a stanol dose of 1.6-2.4 g/d (III/IV). Despite the increase in cholesterol synthesis, plant stanols and sterols induced a marked decrease in serum TC and LDL-C concentrations. This can be ascribed to that the decreased hepatic cholesterol pool in addition to increased cholesterol synthesis enhanced LDL receptor activity resulting in a reduction of serum levels of cholesterol rich particles (32).

6.6 Serum concentrations as a marker of absorption of plant sterols and plant stanols

Absorption of plant sterols and stanols is a key issue for the evaluation of systemic effects possibly caused by the consumption of plant sterol ester or stanol ester. Therefore, concentrations of plant sterols and plant stanols in serum were determined in studies I, III/IV and V.

Under normal conditions, serum plant sterol concentrations are very low, only about 1/1000 of the serum cholesterol concentration, and the concentrations of plant stanols in serum are even lower. During the present studies, both plant sterol and plant stanol concentrations in serum remained very low. Similarly to the other plant sterol studies (4, 5, 53), in study V the consumption of the sterol ester-enriched margarine increased serum campesterol and sitosterol concentrations. Furthermore, in studies III/IV and V, the consumption of the stanol ester-enriched margarines increased serum campestanol and sitostanol concentrations confirming the findings of other recently published studies (33, 72, 76, 78, 104) that also plant stanols are absorbed, but that the absorbed amounts were very small compared with the daily intake of plant stanols from the test margarines. However, the findings of study I differed from the findings of studies III/IV and V and the recent reports (33, 72, 76, 78, 104). In study I, serum campestanol and sitostanol

concentrations did not change significantly within any of the three study groups, and the serum concentrations in that study were higher than in studies III/IV and V. The differences in the higher values of serum stanols might partly be due to the analytical method, especially different column used. In study I, the column probably did not differentiate sitostanol from avenasterol, and therefore, the sitostanol values might represent a mixture of sitostanol and avenasterol rather than sitostanol alone. However, that cannot explain the higher sitostanol values entirely. Therefore, the reason for the higher serum sitostanol and campestanol concentrations remains unresolved. Presumably, the used analytical method is also one reason for the findings of the several earlier intervention studies, in which sitostanol has been suggested to be virtually nonabsorbable (4, 54, 62, 64). In study I, serum plant sterol concentrations were also higher than in studies III/IV and V. Furthermore, in that study serum sitosterol concentration was exceptionally higher than serum campesterol concentration.

The increases in serum campesterol and campestanol concentrations were greater than that in serum sitosterol and sitostanol, respectively, when the consumed amounts were taken into account, reflecting the better absorption rates of campesterol and campestanol compared with sitosterol and sitostanol, respectively. That is in accordance with the findings of absorption studies (109, 119) as well as clinical trials (4, 5, 33, 76, 78, 104). The differences in the absorption rates between the individual plant sterols and plant stanols were attributable to the small differences in their chemical structures (i.e. the extra carbon atom in the side chain of sitosterol and sitostanol) which make campesterol and campestanol more readily absorbable.

According to the findings of study III, the absorption of plant stanols seems to plateau already at a stanol dose of 0.8 g/d (III), since with higher doses the increases of sitostanol and campestanol were only minor. However, in addition to negligible absorption of plant stanols, the low serum concentrations could also be a consequence of fast and effective clearance of absorbed stanols (11). To date, there are no published data on whether the unsaturated plant sterol concentrations increase in a dose-dependent manner or whether they level off with higher plant sterol doses.

6.7 Serum carotenoids and fat-soluble vitamins

Since plant stanol esters and sterols esters inhibit intestinal absorption of cholesterol, they may also affect the absorption of carotenoids and fat-soluble vitamins. Therefore, those concentrations were determined in studies I/II, III and V. Furthermore, the concentrations of serum carotenoids and tocopherols were related to the concentrations of serum TC, since carotenoids and tocopherols are transported in lipoprotein particles, such as LDLs (219-221). Therefore, changes in serum cholesterol concentrations are reflected in serum carotenoid and tocopherol concentrations.

Serum carotenoids (α - and β -carotene and lycopene) and fat-soluble vitamins (retinol,

vitamin D and tocopherols) were all within the normal range in the present studies. Similarly to the results of several other studies (31, 33, 71, 72, 75-77, 178) plant stanol esters and sterol esters had no effect on the concentrations of serum retinol, vitamin D and the concentrations of tocopherols related to serum TC (I, III, V). Furthermore, plant stanol esters and sterol esters had no effect on the concentrations of serum α -carotene or lycopene (II, III, V). Although in women there were differences in serum lycopene between the different dose periods (III), the differences were not related to the stanol ester dose. Women had also lower serum lycopene concentrations than men, which could partly be due to their older age (222). The greatest effects of plant stanol esters and sterol esters focused on serum β -carotene concentrations. However, after relating changes in β -carotene to the simultaneous changes in TC, the changes were minor and non-significant. As in the recent dose-response study of Hendriks et al. (31) with plant sterol esters, in study III the effects of plant stanol esters on carotenoids and fat-soluble vitamins were not dose-dependent but rather dependent on the achieved reduction in serum cholesterol. Therefore, it can be assumed that the reduction in serum carotenoid concentration plateaus with the same dose (2.4 g/d of stanols) as the reduction in serum cholesterol does. In addition, because the effects were not dose-dependent, it can be proposed that there are some other factors e.g. nutrient density of the background diet, which could affect the fluctuations on serum carotenoid concentrations rather than the dose of plant stanol or sterol. In the present studies the background diet was standardized including instructions about the intake of vegetables, but this was probably not the case in most of the other studies (4, 31, 33, 72, 178). Therefore, the variability in composition of background diets might explain why in most of the other plant sterol studies (4, 31, 33, 72, 75, 178) the effects of plant stanols or sterols on serum β - or α - β -carotene concentrations have been greater than those seen here even after relating the changes in β - or α - β -carotene concentrations to the changes in lipid concentrations.

Finally, according to the present studies, the changes in serum carotenoids were minor and possibly clinically unimportant. In addition, the findings of present studies showed that by ensuring the intake of vegetables in the diet, a reduction in carotenoid concentrations induced by plant stanol esters or sterol esters can easily be prevented. It should be noted that although in context of plant sterols much attention has been paid to their effect on serum carotenoids, the clinical importance of β -carotene has diminished due to reports of the harmful effects following β -carotene supplementation (223, 224).

6.8 Adverse effects

According to the laboratory tests no changes in the health status of the subjects in any of the present studies were found. In addition, the recorded gastrointestinal and skin symptoms were slight and occurred occasionally and were not related to any particular experimental or control period or test product. These findings are in accord with the findings of the previous stanol ester or sterol ester studies (4, 5, 31, 34, 71, 72, 76, 225).

Are systemic effects possible? According to the present findings in studies I, III/IV and V and the findings of the other studies in healthy subjects (4, 5, 33, 72, 104) it can be postulated that the absorbed amounts of plant stanols from stanol ester margarines and plant sterols from sterol ester margarines are so small that the systemic effects are most unlikely. In addition, even with the long-term use of stanol ester-enriched margarine, the increase in plant stanol concentrations has been minor (104). There is, however, a specific group, phytosterolemic patients, to whom the consumption of plant sterols and stanols is harmful. In phytosterolemia (119, 125-127) absorption of plant sterols is elevated and thus the serum plant sterol concentrations are very high, about 100 times greater than those detected in study V with sterol ester margarine. In that disease, serum plant stanol concentrations are also enhanced. High concentrations of plant sterol in serum are suggested to have atherogenic potential (134). However, in phytosterolemic heterozygotes, the consumption of plant sterols or stanols in esterified form has recently been reported to increase only slightly serum plant sterol or stanol concentrations, respectively (120, 139). Based on some earlier animal studies (180-182) it has been suggested that plant sterols and stanols may have hormonal effects. However, no relevant effects on serum female sex hormone concentrations in normocholesterolemic or hypercholesterolemic women (73) or women with CAD (70, 188) have been found in clinical studies.

6.9 Clinical implications

Based on the present studies and the other plant stanol ester (4, 5, 34, 47, 64, 68, 71, 72, 77) or sterol ester studies (4, 5, 31, 73) it can be concluded that subjects with mild or moderate hypercholesterolemia and high intestinal absorption rate of cholesterol most likely would benefit from plant stanol ester or sterol ester treatment. However, also subjects with type 2 DM (65, 67) or CAD (70) and children with heterozygous FH (66) have been found to respond favorably to stanol ester treatment. In mildly or moderately hypercholesterolemic subjects serum LDL-C is typically reduced by 10-15%, and a reduction of that magnitude has been proposed to decrease the risk of heart disease by about 25% (89).

Treatment of hypercholesterolemia begins usually with diet therapy. The diet generally recommended for hypercholesterolemic individuals is low in total fat (#30E%), and in SAFA (<10E%) and dietary cholesterol <300 mg/d (81, 226). If serum cholesterol

concentrations are still elevated after dietary changes, individuals could be advised to replace their usual spreads with stanol ester- or sterol ester-containing spreads. Based on the available data, the daily plant stanol or sterol dose should be about 2 g to achieve the optimal cholesterol-lowering benefit. The daily dose can be taken two to three times per day, but also once per day may be equally effective (77). If the above-mentioned dietary modifications do not normalize the elevated serum cholesterol concentrations, the final step is cholesterol-lowering drug therapy possibly combined with the dietary stanol esters. The combining of these two has been found to potentiate the cholesterol-lowering effects of drugs such as statins, neomycin and cholestyramine in mildly dyslipidemic men with type 2 DM (67), in women with CAD (70) or adults with FH-NK (75). Another advantage of combining a cholesterol-lowering drug and stanol esters is that the dose of drug can possibly be reduced and thus the risk of side effects associated with higher drug doses can be diminished (227).

The number of stanol esters or sterol esters containing products on the market continues to expand. This increases the need for knowledge about foodstuffs and nutrition in health care, in the food retail trades and in the food industry. The amount of stanol esters or sterol esters in products should be planned so that the dosage would be easy to assess, even when different stanol ester- or sterol ester-containing food products being consumed during the day. The consumers and patients must be counseled on the effective and safe dose of plant stanols or sterols as well as on how to incorporate these food products into their daily diet. In addition, written instructions should be available for all consumers.

7 SUMMARY AND CONCLUSIONS

The aim of the present studies was to investigate the effects of stanol ester and sterol ester margarines on serum lipids and lipoprotein lipids. Furthermore, the dose-response effect of plant stanol esters was examined. Cholesterol-lowering efficacy of plant stanol esters and sterol esters was also compared. In addition, the safety of plant stanol esters or sterol esters was evaluated by measuring the serum concentrations of carotenoids and fat-soluble vitamins, as well as concentrations of plant sterols and plant stanols. Altogether 111 mildly to moderately hypercholesterolemic subjects participated in the three different studies.

The results of the present studies can be summarized as follows:

1. The low-fat margarines enriched with plant stanol esters offered an **additional**, clinically significant reduction (8-14%) in serum TC and LDL-C to that obtained with a cholesterol-lowering diet alone.
2. The margarines enriched with plant stanol esters or sterol esters reduced serum TC and LDL-C concentrations effectively when used as part of a **low-fat, low-cholesterol** background diet.
3. There was no significant difference in the cholesterol-lowering efficacy of two low-fat margarines enriched with **wood- or vegetable oil**-derived plant stanol esters.
4. The margarines enriched with **stanol esters or sterol esters** did not differ in their ability to reduce serum TC and LDL-C concentrations.
5. Plant stanol esters reduced serum TC and LDL-C concentrations in a **dose-dependent** manner. The significant reduction was achieved with the daily stanol dose of 1.6 g. Increasing the daily dose from 2.4 g to 3.2 g did not provide additional cholesterol-lowering effect.
6. Plant stanol esters or sterol esters did not affect **serum fat-soluble vitamins**. The effects of plant stanol esters or sterol esters on **serum carotenoids** were minor and possibly clinically unimportant when plant stanol esters or sterol esters were consumed as part of a cholesterol-lowering diet or a standardized habitual diet and the intake of vegetables was ensured.
7. Plant stanol esters reduced **serum plant sterol concentrations** significantly already with a dose 0.8 g/d of stanols, indicating that cholesterol absorption was effectively reduced already with the small stanol ester doses. As judged from the serum Δ^7 -lathosterol/TC ratio, it is considered that plant stanol esters stimulated cholesterol synthesis. However, the synthesis did not seem to increase further when the daily stanol doses increased from 1.6 g to 2.4 g or 3.2 g. The consumption of plant stanol esters increased serum sitostanol and campestanol concentrations by about twofold, but the concentrations remained extremely low, and they plateaued with doses equal to or greater than the 0.8 g/d.

In conclusion, plant stanol ester- and sterol ester-enriched margarines reduce serum cholesterol concentrations effectively as part of the diet recommended for subjects with elevated serum cholesterol concentrations. Furthermore, the effects on serum carotenoids are minor and possibly clinically unimportant when stanol ester and sterol ester margarines are consumed as part of a recommended and healthy diet.

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