Phytosterols and blood lipid risk factors for cardiovascular disease

Rouyanne Ras

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Rouyanne T. Ras

Thesis committee

Promotor
Prof. Dr F.J. Kok
Professor of Nutrition and Health
Wageningen University

Co-promotors
Dr J.M. Geleijnse
Associate professor, Division of Human Nutrition
Wageningen University

Dr P.L. Zock
Science leader Cardiovascular Health
Unilever Research and Development Vlaardingen

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Rouyanne Thirza Ras

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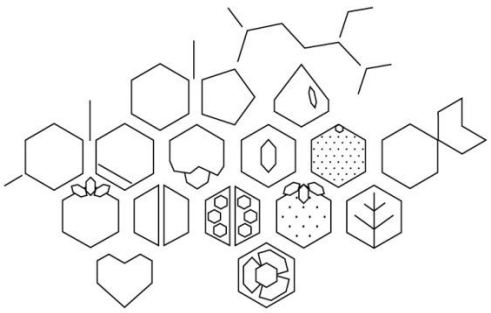

Chapter 1

General Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in many countries around the world[1]. CVD is caused by disorders of the heart and the blood vessels. The most common types of CVD include coronary heart disease (CHD; affecting the heart), cerebrovascular disease (affecting the brain) and peripheral artery disease (affecting the limbs). Many CVD cases can be prevented by addressing unhealthy diets and lifestyles thereby managing cardiovascular risk factors such as raised blood lipids, blood pressure and blood glucose. Efforts should be undertaken to manage and reduce the risk of CVD. This thesis aims to advance insights in the role of phytosterols, lipid-like compounds found in foods of plant origin, in the management of blood lipid risk factors for CVD.

Phytosterols

Chemical structure and function

Phytosterols have a chemical structure comparable to that of cholesterol[2,3]. Both compounds are characterized by having a steroid nucleus containing four cycloalkane rings, a 3β-hydroxyl group and an alkyl side chain (**Figure 1**). The difference in chemical structure between cholesterol and phytosterols is mainly due to the presence of a methyl or ethyl group at carbon atom 24. Small variations in the chemical structure (e.g. in the alkyl side chain and/or in the saturation rate) have led to the existence of more than 200 different phytosterols. The term phytosterols in fact encompasses both plant sterols and plant stanols. Plant stanols are the saturated forms of plant sterols, i.e., lacking a double bond in the steroid nucleus (**Figure 1**). The most abundant phytosterol is sitosterol[4]; other phytosterols include among others campesterol, stigmasterol, sitostanol and campestanol. Phytosterols esterified to fatty acids or other organic acids are called phytosterol esters.

Phytosterols and cholesterol have several biological functions in common[5] although in different hosts, i.e., phytosterols in plants and cholesterol in humans. Both compounds are important building blocks of cell membranes where they regulate membrane fluidity and permeability. Furthermore, they both play a role in cellular differentiation and proliferation and serve as precursors of hormones[2].

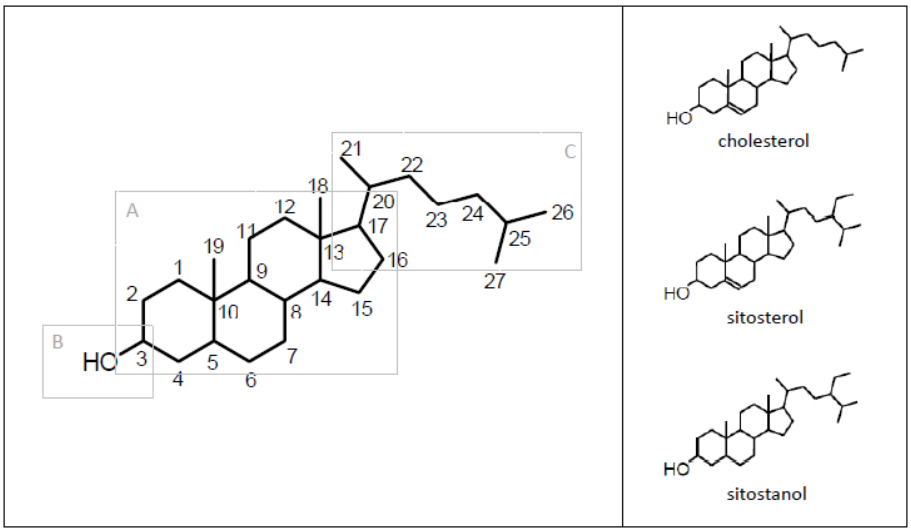

Figure 1. *Chemical structure of sterols. At the left, box A visualizes the steroid nucleus, box B the hydroxyl group at carbon 3 in the β-position, and box C the alkyl side chain. At the right, chemical structures of cholesterol, sitosterol and sitostanol are shown.*

Metabolism

In the intestinal lumen, esterified forms of phytosterols and cholesterol are hydrolyzed into free sterols. These are then dissolved in mixed micelles before entering the intestinal cells via various mechanisms such as transportation by Niemann-Pick C1 like 1 (NPC1L1) proteins[6]. Once in the intestinal cells, phytosterols are actively excreted back into the intestinal lumen by the heterodimer ATP-binding cassette (ABC) transporters ABCG5/8[7], whereas this happens to a lesser degree with cholesterol. ABCG5/8 transporters located in the liver also excrete phytosterols, i.e., from the liver into the bile. Furthermore, phytosterols are a poor substrate for Acetyl-CoA acetyltransferase-2 (ACAT-2)[8]. ACAT-2 esterifies cholesterol and phytosterols in the intestinal cells before they are taken up by chylomicrons for distribution via the lymphatic system to the liver and into the blood. Particularly due to active excretion of phytosterols by the ABCG5/8 transporters, absorption of phytosterols is much lower (<5% for plant sterols and <0.5% for plant stanols[9-11]) compared to absorption of cholesterol (30-80%)[10,12]. Moreover, in contrast to cholesterol, phytosterols cannot be synthesized in the human body. As such, circulating plant sterol concentrations in humans are ~250 times lower compared to cholesterol concentrations[13]. Blood plant stanol concentrations are even lower, i.e., 10-50 times lower compared to plant sterol

concentrations[14]. Patients with homozygeous phytosterolemia, a rare genetic disorder, are an exception. In these patients, mutations in *ABCG5/8* genes[7,15] hamper excretion of phytosterols from the body, resulting in severely elevated phytosterol concentrations in the blood[16].

Phytosterols are known to inhibit intestinal cholesterol absorption by 30-40%[17], thereby reducing blood cholesterol concentrations. Cholesterol inhibition occurs through several hypothesized mechanisms such as competition with cholesterol for solubilization in dietary mixed micelles, interference with transport-mediated processes of cholesterol uptake, and stimulation of cholesterol excretion via the intestine[18-20].

Sources

Phytosterols originate from the diet, i.e., from the lipid- and fiber-rich fractions of plant-based foods such as nuts, seeds, grains, fruits and vegetables[21-23](**Figure 2**). Especially vegetable oils (e.g. corn oil) are rich sources of phytosterols[5]. Plant sterol intakes in the population generally range between 200 and 400 mg/d[24-27]. Only people with specific dietary habits such as vegetarians can reach higher plant sterol intakes of 500-1000 mg/d[28,29], but such high intakes are exceptional. Intakes of naturally occurring plant stanols are much lower, i.e., 10-35 mg/d[24,30,31]. Phytosterols occur in the diet in both free and esterified forms. The composition of phytosterols varies among different sources[5].

Since the cholesterol-lowering properties of phytosterols were discovered in the 1950s[32], large doses of phytosterols in crystalline/powder form, particularly sitosterol, were used to treat hypercholesterolemic patients. Once it was discovered how phytosterols could be esterified with dietary fatty acids to enhance their lipid solubility, research was undertaken to investigate the cholesterol-lowering effect of phytosterols incorporated in fat-based foods like mayonnaise[33] and margarine[34]. To date, a large range of phytosterol-enriched foods with established cholesterol-lowering properties are available on the market[35]. These enriched products contain considerably more phytosterols than natural food sources (**Figure 2**).

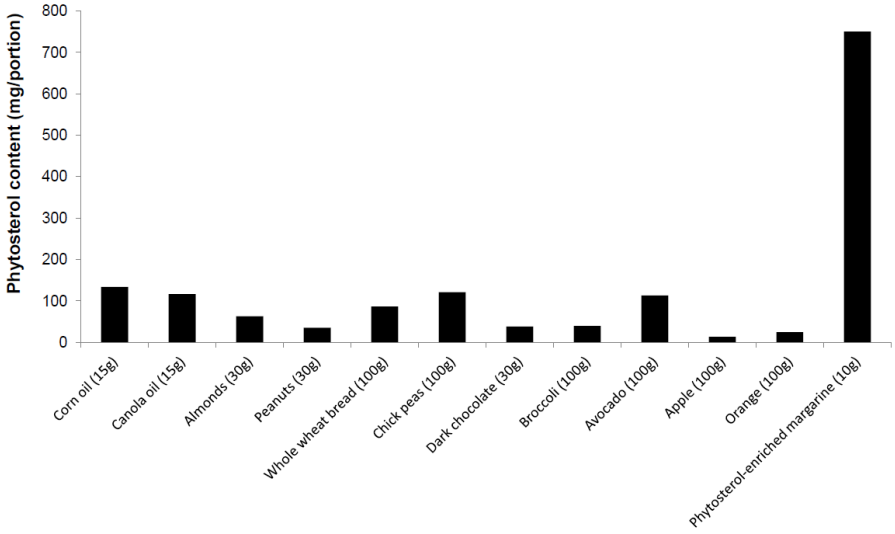

Figure 2. *Phytosterol content of different dietary sources*[21-23].

Summary

Phytosterols encompass both plant sterols and plant stanols. They resemble cholesterol in molecular structure and they have similar biological functions in plants as cholesterol has in humans. Absorption and excretion processes of phytosterols are tightly regulated, resulting in very low phytosterol concentrations in the blood. Phytosterols lower blood cholesterol concentrations through inhibition of cholesterol absorption. Phytosterols originate from plant-based foods and from foods enriched with phytosterols. They cannot be produced by the human body.

Phytosterols and blood lipids

Cholesterol and triglycerides (TGs) are the main lipids circulating in the blood. They are carried by lipoproteins. Lipoproteins are biochemical structures that contain both proteins and lipids and that allow fats to move through water. Low-density lipoproteins (LDL) are lipoproteins that can deposit their fat content into artery walls, attract macrophages, and, consequently, promote the development of arterial plaques, a process leading to atherosclerosis.

Low-density lipoprotein cholesterol

Elevated total cholesterol (TC), and especially LDL-cholesterol (LDL-C), is a major risk factor for CVD, in particular for CHD[36-38]. It has been estimated that elevated cholesterol resulted in 2.6 million deaths and 29.7 million disability-adjusted life years (DALYs), globally, in 2004[39]. According to the World Health Organization (WHO), around 40% of adults (>25 years) worldwide have raised TC concentrations (>5 mmol/L) ranging from ~20-30% in African and Asian countries up to ~50-60% in European and North- and South-American countries[1]. Future economic development, urbanization and nutritional transition will likely lead to further increases in cholesterol concentrations, particularly in developing countries[40].

Foods with added phytosterols lower TC and LDL-C concentrations in the blood[41-45]. The LDL-C-lowering effect of phytosterols appears to be dose-dependent; higher phytosterol doses result in larger reductions in LDL-C[46]. Initially, the dose-response effect for the LDL-C-lowering effect of phytosterols was investigated by calculating average changes in LDL-C for different categories of phytosterol doses[46], so using a categorical and not a continuous approach. Because continuous analysis has the advantage that it allows predicting the LDL-C-lowering effect for any given dose of phytosterols within the range of doses investigated, we determined this continuous relationship by performing a meta-analysis including data from published intervention studies (Chapter 2).

Both plant sterols and plant stanols lower LDL-C concentrations. Some data suggest that the maximal LDL-C-lowering effect of plant sterols (~8%) is reached already at doses of 1.0-1.5 g/d[47], whereas plant stanols continue to reduce LDL-C up to 17% for doses as high as 9 g/d[48]. Evidence for this discrepancy is mainly based on data from continuous dose-response analysis[47] that in some instances over- or underestimates the true effect at certain doses. In fact, a systematic review including studies that investigated the cholesterol-lowering efficacy of plant sterols and plant stanols under the same study conditions showed that plant sterols and plant stanols are equally efficacious in lowering LDL-C[49]. To further elucidate potential differences between plant sterols and plant stanols in their LDL-C-lowering effect, we performed a meta-analysis and compared the LDL-C-lowering efficacy of plant sterols vs. plant stanols within different dose ranges (Chapter 3).

Triglycerides

Elevated TG concentrations are also being considered to play a role in the onset of CVD[50,51]. According to the National Health and Nutrition Examination Survey (NHANES), around 30% of adults (>18 years) in the United States have above desirable (>1.7 mmol/L) TG concentrations[52,53]. This prevalence is expected to increase in the near future due to the increasing prevalence of physical inactivity and obesity. The relationship between elevated TG concentrations and occurrence of future CVD events is not as established as for LDL-C, and its independency of other risk factors (e.g. high-density lipoprotein (HDL)-cholesterol) remains controversial. Nevertheless, several health authorities such as the American Heart Association (AHA)[51] and the European Atherosclerosis Society (EAS)[50] do emphasize the importance of targeting elevated TGs. Especially for subjects at high risk of CVD, such as metabolic syndrome patients, it is recommended to lower TG concentrations in addition to lowering LDL-C.

Reductions in fasting TG concentrations have incidentally been observed in studies with phytosterol-enriched foods[42,54]. These reductions are in general rather modest and variable, and therefore difficult to detect in studies that are primarily set up and statistically powered to investigate effects on LDL-C. For plant stanols, it has been reported that the baseline TG concentration determines the magnitude of their modest TG-lowering effect[55]. As there were no such data available for plant sterols, we conducted a pooled analysis to investigate the TG-lowering effect of plant sterols and the influence of baseline TG concentration on this effect (Chapter 4).

The omega-3 fish fatty acids eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) can substantially lower TG concentrations[56-58]. Most of the evidence for this effect is based on studies that used fish oil supplements with EPA+DHA doses >2 g/d and showed reductions in TG concentrations of around 25-35%[56,58]. Less is known about the TG-lowering effect of lower doses of EPA+DHA (<2 g/d), especially when these low doses of EPA+DHA are combined with plant sterols in a low-fat spread. In such spreads, the maximum amount of fish oil (as the source of EPA+DHA) that can be added is limited. The combination of EPA+DHA and plant sterols is expected to beneficially affect both LDL-C and TG concentrations. We performed a randomized controlled trial to investigate the dose-response relationship between low doses of EPA+DHA (<2 g/d)

and fasting TG concentrations when incorporated in a low-fat spread with added plant sterols while still finding a meaningful reduction in LDL-C (Chapter 5).

Summary

Foods with added phytosterols lower LDL-C, an important risk factor for CVD. So far, a continuous dose-response curve for this effect has not been established. Potential differences between plant sterols and plant stanols in their LDL-C-lowering effect have been suggested; this requires further research. It is not well known whether plant sterols can, like plant stanols, modestly lower fasting TG concentrations, another risk factor for CVD. High doses of the omega-3 fish fatty acids EPA and DHA substantially lower TG concentrations. Whether plant sterols together with EPA+DHA would lower both LDL-C and TG concentrations when low doses of fish oil (as the source of EPA+DHA) are incorporated in a low-fat, plant sterol-enriched spread, requires further investigation.

Phytosterols and CVD risk

Circulating phytosterol concentrations

Phytosterols, so both plant sterols and plant stanols, are poorly absorbed after dietary intake[9-11] mainly due to the activity of ABCG5/8 transporters that excrete phytosterols from the enterocytes into the intestinal lumen and from the liver into the bile. Nevertheless, when phytosterol intakes are increased (e.g. when consuming enriched foods), this is reflected in higher blood concentrations of these phytosterols. Concerns have been raised about increases in circulating phytosterols, particularly plant sterols. Homozygous phytosterolemic patients are characterized by extremely high plant sterol concentrations in their blood and often, but not always[59], experience early onset of atherosclerosis[60,61]. Furthermore, some observational evidence suggests that modestly elevated blood plant sterol concentrations are associated with an increased CVD risk[62,63], but data are conflicting[64]. The magnitude of the increase in circulating plant sterols after plant sterol-enriched food intake has so far not been systematically investigated. Therefore, we performed a meta-analysis to estimate the change in plant sterol concentrations after consumption of plant sterol-enriched foods, and to explore factors that influence this change (Chapter 6).

Cardiovascular endpoints

To date, no randomized controlled studies have been performed on phytosterol intake and incidence of CVD. This type of research would provide the strongest evidence to substantiate a cardiovascular health benefit of phytosterols. However, an adequately powered endpoint study would require 36,000-76,000 hypercholesterolemic individuals with an expected annual CVD risk level of 3% and long-term follow-up (6-10 years)[65]. Performing such an endpoint study is hardly feasible. Observational studies may help clarifying whether intake of phytosterols is related to incidence of CVD, and specifically CHD, at population level. Only a few observational studies have so far been performed and provide data on intake of phytosterols from natural sources, i.e., not on intake of phytosterol-enriched foods. Overall, these studies show that people with higher dietary phytosterol intakes have lower concentrations of LDL-C[30,66,67], and tend to have a lower risk of myocardial infarction (MI)[68]. More prospective research is required to further elucidate the relation between phytosterol intakes and (cholesterol-mediated) reductions in cardiovascular risk. We, therefore, performed a prospective analysis to investigate the association between intake of naturally occurring phytosterols, blood lipids, and incidence of fatal and non-fatal cardiovascular events in a large cohort of Dutch adults (Chapter 7).

Summary

Intake of foods with added phytosterols increases blood phytosterol concentrations. Some observational evidence suggests that circulating plant sterols might be atherogenic. The magnitude of the increase in blood plant sterol concentrations after intake of foods with added plant sterols has so far not been systematically investigated. Clinical trials on phytosterol intake and incidence of CVD are lacking. More observational research is required to investigate relations between dietary phytosterols and cardiovascular risk in the population.

Overall aim of the thesis

This thesis aims to advance insights in the role of phytosterols in the management of blood lipid risk factors for CVD. The studies described here address the effects of phytosterols from enriched foods on LDL-C, TGs and blood plant sterol concentrations (**Figure 3**). In addition, the effect of different low doses of omega-3 fish fatty acids incorporated in a plant sterol-enriched spread on blood lipids was

investigated (**Figure 3**). The association between intake of naturally occurring phytosterols and CVD risk was also investigated (**Figure 3**). This thesis includes data from four meta-analyses (Chapters 2, 3, 4 and 6), a randomized controlled intervention study (Chapter 5) and an epidemiological study (Chapter 7).

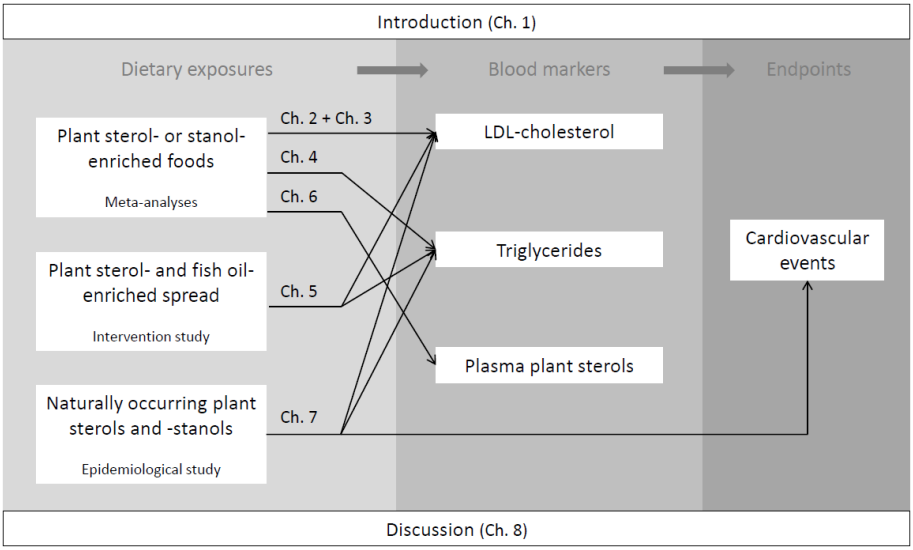

Figure 3. *Schematic overview of the relations between phytosterols, blood lipid risk factors and cardiovascular risk, addressed in this thesis.*

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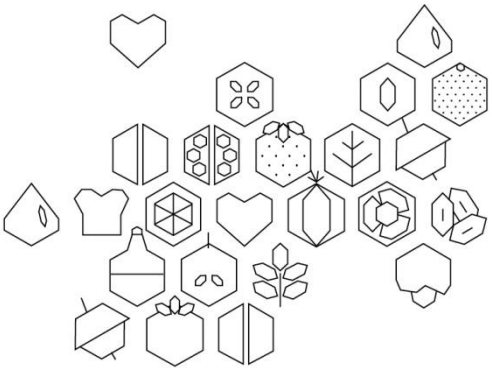

Chapter 2

Continuous dose-response relationship of the LDL-cholesterol-lowering effect of phytosterol intake

Isabelle Demonty
Rouyanne T. Ras
Henk C. M. van der Knaap
Guus S. M. J. E. Duchateau
Linsie Meijer
Peter L. Zock
Johanna M. Geleijnse
Elke A. Trautwein

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Abstract

Phytosterols (plant sterols and stanols) are well known for their low-density lipoprotein cholesterol (LDL-C)-lowering effect. A meta-analysis of randomized controlled trials in adults was performed to establish a continuous dose-response relationship that would allow predicting the LDL-C-lowering efficacy of different phytosterol doses. Eighty-four trials including 141 trial arms were included. A nonlinear equation comprising 2 parameters (the maximal LDL-C lowering and an incremental dose step) was used to describe the dose-response curve. The overall pooled absolute (mmol/L) and relative (%) LDL-C-lowering effects of phytosterols were also assessed with a random effects model. The pooled LDL-C reduction was 0.34 mmol/L (95% CI:-0.36; -0.31) or 8.8% (95% CI: -9.4; -8.3) for a mean daily dose of 2.15 g phytosterols. The impacts of subject baseline characteristics, food formats, type of phytosterols, and study quality on the continuous dose-response curve were determined by regression or subgroup analyses. Higher baseline LDL-C concentrations resulted in greater absolute LDL-C reductions. No significant differences were found between dose-response curves established for plant sterols vs. stanols, fat-based vs. non fat-based food formats and dairy vs. nondairy foods. A larger effect was observed with solid foods than with liquid foods only at high phytosterol doses (>2 g/d). There was a strong tendency (P = 0.054) towards a slightly lower efficacy of single vs. multiple daily intakes of phytosterols. In conclusion, the dose-dependent LDL-C-lowering efficacy of phytosterols incorporated in various food formats was confirmed and equations of the continuous relationship were established to predict the effect of a given phytosterol dose. Further investigations are warranted to investigate the impact of solid vs. liquid food formats and frequency of intake on phytosterol efficacy.

Introduction

Elevated plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are a major risk factor for coronary heart disease (CHD). Phytosterols (plant sterols and stanols) are among the dietary options available to lower elevated plasma TC and LDL-C concentrations. The cholesterol-lowering properties of phytosterols were observed in humans already in the early 1950s[1]. Since then, a vast number of human trials have shown that phytosterols, mainly in the form of plant sterols or stanols esterified to vegetable oil fatty acids (mainly C18),

significantly lower TC and LDL-C when incorporated into various food products[2,3]. The most recent meta-analysis including 41 trials with mainly fat-based foods like spreads, margarine, mayonnaise, or salad dressings enriched with phytosterol esters has shown a nonlinear dose-response relationship between the daily dose of phytosterols consumed and their cholesterol-lowering efficacy[3]. On average, 2 g/d phytosterols (the equivalent dose expressed as free sterols based on 3.3 g/d phytosterol esters) lowered LDL-C concentrations by ~10%[3]. The effect appeared to taper off at intakes of ~2 g/d or more, with little additional benefit at intakes higher than 2.5 g/d. As a consequence, several dietary recommendations now include the daily consumption of 2 g of phytosterols as an additional dietary option to lower elevated LDL-C concentrations[4-7]. The main mechanism of action responsible for the cholesterol-lowering effect of phytosterols is the inhibition of intestinal cholesterol absorption[8]. The recommended daily intake of 2 g of phytosterols reduces cholesterol absorption by 30-40%[3,9].

To date, additional evidence for the cholesterol-lowering efficacy of esterified or free phytosterols incorporated in a wide variety of food formats, including low-fat or fat-free foods such as milk[10-12], yogurt[10,11,13-16], fruit or vegetable juices[17-19], and single daily dose food formats such as yogurt drinks[13,16,20-24], has become available. Although some of these trials suggested that phytosterols incorporated in these food formats lower LDL-C to an extent similar to that observed with fat-based food formats, the impact of food format on the LDL-C-lowering efficacy had not been systematically evaluated. In addition, the most recent meta-analysis[3] pooled together trials in which different phytosterol doses were used and the cholesterol-lowering efficacy was reported for ranges of doses (0.7-1.1, 1.5-1.9, 2.0-2.4, ≥2.5 g/d). Using this approach, it was not possible to predict the cholesterol-lowering effect for a given dose of phytosterols.

The main objective of the present systematic review with meta-analysis was to establish a continuous dose-response relationship that would allow predicting the LDL-C-lowering efficacy of different phytosterol doses using an equation that would take into account the saturable nature of the cholesterol absorption process[25]. Another objective was to evaluate the impact of different treatment characteristics such as phytosterol type (plant sterols vs. stanols) and the impact of food format (fat-based vs. non fat-based, dairy vs. non-dairy, and liquid vs. solid food formats) on the dose-response curve. As part of the investigation of heterogeneity between

trials, the effect of subject characteristics (age, BMI, gender, baseline LDL-C concentrations) and study quality was also evaluated. Finally, because the total-over high-density lipoprotein cholesterol (TC:HDL-C) ratio is a strong predictor of CHD mortality[26] and is affected, but not solely, by changes in LDL-C concentrations, we attempted to determine the dose-response effect of phytosterol intake on this ratio.

Methods

Search strategy

Five databases (MEDLINE, Cab Abstracts, Biological Abstracts, Web of Science, and the Cochrane Library) were searched in July 2007 for articles on phytosterols, with no specification for date of publication. The Medical Subject Headings (terms) phytosterols, lipids, and cholesterol were used, as well as the following search terms: (plant sterol* or plant stanol* or phytosterol* or phytostanol* or sitosterol* or sitostanol* or campesterol* or campestanol* or stigmasterol* or brassicasterol*) and (cholesterol* or blood lipid* or LDL cholesterol* or HDL cholesterol* or triglyceride*), limited to human and clinical trials whenever possible. There was no language restriction.

Inclusion and exclusion criteria

A first selection was made by screening the title and abstract of the publications based on the inclusion criteria (**Table 1**). Because the cholesterol-lowering effect of phytosterols is additive to that of statins[27,28] or ‘‘heart healthy’’ diets (low in total, saturated fat, and cholesterol content)[29-32], they were not considered as a co-intervention as long as they were present in both the control and the treatment groups/phases. The use of a vegetable oil-rich diet as background diet was not considered as co-intervention as long as the background diet was the same in all treatment groups/phases. Because most phytosterol esters result from the esterification of phytosterols to vegetable oil fatty acids, the use of vegetable oil fatty acid esters of phytosterols was not considered as a co-intervention. However, the use of novel, non-vegetable esters of phytosterols such as fish oil fatty acid esters was considered as a co-intervention, because fish oil fatty acids may have a moderate impact on LDL-C[33-35]. This could not be distinguished from the usual phytosterol or phytosterol ester effect and it was not known whether this effect

was additive to that of phytosterols or whether some interactions could exist between fish oil fatty acids and phytosterols.

After the full publications were read, trials were excluded based on the exclusion criteria (**Table 1**). Ferulated phytosterols were excluded, because these phytosterols are not commonly used for food/supplement enrichment and there is no consensus on whether they have a cholesterol-lowering effect[36,37]. Although phytosterols are thought to exert their mechanism of action in the upper gastrointestinal tract[8], colectomized patients were excluded, because the possibility that colectomy could have consequences in the upper tract could not be completely discarded.

Table 1. *Inclusion and exclusion criteria used to select the clinical trials.*

Inclusion criteria used when screening titles and abstracts
1) Randomized controlled trial within human adults (parallel-arm or cross-over trials)
2) Treatment with "usual" phytosterols, where "usual phytosterols" was defined as 4-desmethylsterols and/or 4-desmethylstanols extracted from vegetable or plant oils such as soybean oil, rapeseed oil and tall oil
3) Blood lipids as primary or secondary outcomes
4) Absence of a co-intervention from which consumption of phytosterol-enriched foods or supplements could not be isolated
Exclusion criteria used when reading the full publications
1) Not a randomized controlled trial
2) Relevant blood lipid data missing
3) Phytosterols consumed for less than 2 weeks
4) Phytosterol dose higher than 10 g/d
5) Control group did not receive a placebo
6) Ferulated phytosterols such as rice bran oil and shea nut oil sterols were used
7) Colectomized patients were part of the study

Data extraction

The data were independently extracted by 2 investigators (R.R. and L.M.) using a custom-made database. Codings were defined for the descriptive variables to ensure consistency in recording. In case of discrepancy or indecisiveness, consensus was reached by verbal discussion among the authors. We collected the following data: 1) study identification (author, publication year, country); 2) study design (parallel-arm or cross-over); 3) subject characteristics (number of subjects, gender, age, BMI, body weight, health status, ethnicity); 4) background diet (free living conditions or diet provided by the investigators, typical or "healthy" diet); 5) treatment characteristics [phytosterol dose, phytosterol type (plant sterols or

stanols), phytosterol esterification (in free form or esterified), source of phytosterols, source of fatty acids used for esterification, food format, intake occasion (with or without a meal), frequency of intake (number of portions during the day), and treatment duration]; 6) blood lipid outcomes (LDL-C, HDL-C, and TC); 7) variance measures for these outcomes; and 8) study quality. When required, the original authors were contacted to obtain missing information.

Quality assessment

Trial quality was assessed using a custom-designed tool (**Supplemental Appendix 1**) adapted from the Delphi Consensus[38] and the method by Chalmers *et al.*[39]. Consensus was reached among the authors for the inclusion of the following criteria in the tool due to their high potential to affect the estimate of the treatment effect: random sequence generation, blinding of the subjects, blinding of the investigators, eligibility criteria specified, compliance, and carryover effects taken care of in case of cross-over trials. For each study or trial arm, the overall quality score was calculated by adding the individual criteria scores. The maximal quality score that could be ascribed to a parallel trial was 7. Parallel trials deserving less than 5.5 points were classified as low quality trials, while trials given 5.5 points or more were judged to be of good quality. In case of cross-over trials, the maximal quality score was 8; trials given 6.0 points or less were considered of low quality, and those provided more than 6.0 points were classified as being of good quality.

The quality scores were not used to exclude lower quality trials from the meta-analysis or to weigh the trials, because there is no consensus on which scoring system is the best and hence the use of such a system, which is intrinsically subjective, could have biased the outcome of the meta-analysis[40]. The quality scores were used only for performing subgroup analyses to determine whether the overall quality as well as 2 major quality criteria (randomization and compliance) considered separately could affect the dose-response curves.

Statistical analysis

The main outcome variable was the absolute net change (mmol/L) in LDL-C due to the phytosterol treatment. When the outcome variable was measured at various time points during the intervention, the value corresponding to or closest to the 4-week time point was taken for the analysis. The absolute net change in LDL-C was calculated according to the formulas described in **Supplemental Appendix 2**. When

only relative outcomes were provided in the publications, they were first converted to absolute outcomes using, as the 100% value, the baseline lipid value of the corresponding group for parallel trials and the endpoint lipid value of the control phase for cross-over trials. Absolute changes in the TC:HDL-C ratio were also estimated. Because not all publications reported the ratio, it was calculated from the reported means of TC and HDL-C.

The results of the meta-analysis were also expressed in terms of relative (%) change in LDL-C. When relative net changes were reported, these values were collected. For trials in which relative net changes were not reported, the relative changes were calculated as described in **Supplemental Appendix 2**.

The within-trial variance measures for the absolute net changes in LDL-C were obtained as standard errors (SE) or derived from SD or 100(1-α) % CI. To derive SE from SD and CI, we used the equations described (**Supplemental Appendix 2**). If not provided, the within-trial variance measures of the absolute net changes were estimated according to the equations provided in **Supplemental Appendix 2**.

Pooled estimates of the absolute LDL-C-lowering effect of phytosterols and of the LDL-C concentration at baseline were calculated using a random-effects model according to the method described by DerSimonian and Laird[41] using the inverse of the variance ($1/SE^2$) as weighing factor. A similar weighing factor was used for calculating the pooled estimate of the relative LDL-C-lowering effect. Heterogeneity between studies was assessed by calculating the DerSimonian and Laird Q statistic[41,42] and by looking at the funnel plot in which weights ($1/SE^2$) had been plotted against the absolute net changes in LDL-C[43]. The funnel plot symmetry was examined as an indicator for absence of potential publication bias. The absence of publication bias was also verified with a probability plot of the ranked changes in LDL-C plotted against the normal deviates.

The dose-response curve was determined using the PROC NLIN function of the SAS System (SAS version 8.2, SAS Institute). As a model for the dose-response curve, we used a first-order elimination curve frequently used in pharmacokinetics[44]. The choice of this equation was based on the assumption that the cholesterol-lowering effect of phytosterols would reach a plateau with increasing doses due to the

saturable nature of the processes involved in cholesterol transport and absorption[25]:

$$Change = D\ (1 - \exp[-Kdose]),$$

where D = maximal reduction in LDL-C concentration and K = LDL-C reduction rate. We re-parameterized this equation into:

$$Predicted\ LDL - C\ change = a(1 - \exp\ [\frac{-dose}{\frac{b}{\ln(2)}}])$$

in order to obtain the maximal LDL-C reduction that can be achieved at high phytosterol doses (parameter a) and the incremental dose step needed to achieve an additional effect, which is one-half the size of the previous dose effect (parameter b). Both parameters were estimated using a non-linear, unweighted regression analysis.

When using data from studies in which different phytosterol treatments were administered, we conducted comparisons with a single placebo. Some correlations existed between strata belonging to the same study, but these correlations were not taken into account, because they should not have affected the overall (pooled) reduction in LDL-C but only the error variation of the pooled estimate. In addition, the potential effect of inter-trial correlations on the dose-response curve was expected to be minimal due to the large number of trials included in the meta-regression. To verify whether the nonlinear regression fitted better with the observed relative LDL-C changes than a simple linear relationship (without a maximal reduction estimate), we performed a post hoc analysis to compare the sum of the residuals between the observed and predicted LDL-C changes obtained with the curve vs. a linear fit crossing the y axis at 0.

To explore possible causes of heterogeneity between trials, predefined covariate analyses were performed with the dose-response curve. The predefined continuous covariates were baseline age, BMI, LDL-C concentrations and gender, and the categorical covariates were phytosterol type (plant sterols vs. stanols), food format (fat-based vs. non fat-based foods, dairy vs. non-dairy foods, solid vs. liquid foods), and study quality (low vs. good study quality, well vs. poorly randomized strata, and high vs. low compliance strata). We performed post hoc analyses to evaluate the impact of study design (cross-over vs. parallel) on the dose-response curve as well as the impact of the inclusion of trials in which phytosterol doses >5 g/d were used. The criteria used for classification of the strata

within different categories of treatment or study characteristics are provided (**Supplemental Appendix 3**). For the continuous covariates, residuals (differences between predicted LDL-C changes and observed LDL-C changes) were plotted against the covariates and PROC GLM was used to examine the correlation between the covariates and the residuals. For the categorical covariates, dose-response curves were established for the different subgroups and the differences in the parameters describing the curves were evaluated. $P \leq 0.05$ was considered significant. All analyses were performed with the SAS System.

Results

Overview of trials

A total of 601 articles were identified from the search strategy. Of these, only 165 met the inclusion criteria based on title and abstract content. After full papers were read for the 2nd selection step, 71 articles were excluded based on the exclusion criteria. Ten other articles were excluded because only abstracts could be obtained (n = 2) or the data presented were the same as in previous publications (n = 8), resulting in the inclusion in the meta-analysis of 84 trials/publications comprising 141 strata (phytosterol treatment vs. control) (**Figure 1**); 73 strata were from parallel design studies (**Table 2**) and 68 were from cross-over design studies (**Table 3**).

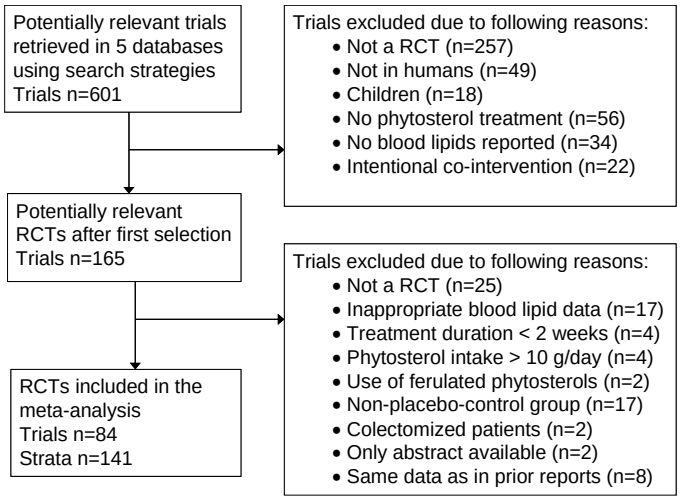

Figure 1. *Flow diagram of the trial selection procedure starting with 601 trials and ending with 84 randomized controlled trials (RCT), including 141 strata with a phytosterol treatment.*

A total of 6805 participants were included in the trials. Most of the strata included European and North American participants who were apparently healthy regardless of baseline lipid levels. Mean age ranged from 22.7 to 66.0 y and mean BMI and body weight at baseline ranged from 22.0 to 31.0 kg/m^2 and 63.0 to 88.3 kg, respectively. Body weight did not change significantly during the intervention except in 9 strata, which reported small (<2 kg) but significant body weight changes. Baseline LDL-C concentrations were reported in 123 strata, with a pooled overall LDL-C concentration at baseline of 3.86 mmol/L (95% CI: 3.77; 3.98). Most strata included both men and women (**Supplemental Appendix 4**).

The mean phytosterol dose given to the study participants was 2.15 g/d (range 0.45-9.00 g/d), for a duration ranging from 21 to 182 d (**Supplemental Appendix 4**). Plant sterols were used in 74 strata and plant stanols in 53 strata; in 14 cases, a combination of plant sterols and stanols was used. Plant sterols and stanols were provided in their esterified form in most cases, except in 39 strata in which free plant sterols or stanols were directly dispersed or mixed in the food products. Phytosterols were incorporated in fat-based foods in ~65% of the strata (n = 91) and in foods with a lower fat content in ~35% of the strata (n = 50). In 26 strata, phytosterols were provided in dairy food formats. Liquid food formats were used in 23 strata. In most strata, phytosterols were consumed in multiple daily intakes (n = 87), at all 3 meals (n = 37), or at various combinations of 2 meals (n = 20). When consumed once a day (n = 14 strata), phytosterols were ingested at breakfast (n = 7 strata), lunch (n = 5 strata), or dinner (n = 2 strata). Subjects were allowed to maintain their usual dietary pattern in the majority (n = 98) of strata. Overall study quality was good for 68 of 141 strata and low for the remaining 73 strata (**Supplemental Appendix 4**).

Between-trial heterogeneity as assessed by the Q-statistic was significant (351.1, P <0.001 and 242583.1, P <0.001 for the absolute and relative changes in LDL-C, respectively). Visual inspection of the funnel plots (**Figure 2**) as well as the probability plot of the ranked changes in LDL-C (not shown) suggested the absence of publication bias.

Table 2. *Overview of the parallel-arm design strata included in the meta-analysis*

Author, publication year, and reference	Subject characteristics					Treatment characteristics					Blood lipid outcomes			Overall quality score[c]
	Sample size		Mean age	Mean BMI	Male	Food format	Sterols or stanols	Ester or free	Duration	Dose phytosterols[a]	LDL-C			
	Control	Treatment									Mean baseline	Net change[b]	95% CI	
			y	*kg/m²*	*%*				*d*	*g/d*	*mmol/L*			
Algorta Pineda et al. 2005[21]	15	17	42.0	26.0	59.4	yogurt drink	stanols	ester	21	1.2	4.06	-0.34	(-0.66 -0.01)	-[d]
Alhassan et al. 2006[45]	9	17	52.7	26.6	53.8	margarine	stanols	ester	28	3.0	3.31	-0.65	(-1.03 -0.27)	6.5 (good)
Andersson et al. 1999[46]	21	19	55.1	25.1	45.9	margarine	stanols	ester	56	1.9	4.68	-0.29	(-0.61 0.03)	6.5 (good)
Ayesh et al. 1999[47]	11	10	36.0	24.0	50.0	margarine	sterols	ester	24	8.6	3.18	-0.58	(-0.77 -0.39)	5.0 (low)
Beer et al. 2000[48] stratum 1[e]	33	33	54.8	27.5	-	low-fat milk	mix	free	26	0.9	4.13	-0.33	(-0.57 -0.08)	5.0 (low)
Beer et al. 2000[48] stratum 2	33	33	56.4	27.3	-	low-fat milk	mix	free	26	1.8	4.16	-0.38	(-0.61 -0.14)	5.0 (low)
Beer et al. 2000[48] stratum 3	33	33	53.3	27.6	-	low-fat milk	mix	free	26	3.6	4.14	-0.57	(-0.82 -0.33)	5.0 (low)
Blair et al. 2000[49]	84	83	56.0	28.5	59.9	margarine	stanols	ester	28	2.9	3.80	-0.36	(-0.52 -0.21)	6.5 (good))
Blomqvist et al. 1993[50]	33	34	45.5	25.5	70.1	mayonnaise	stanols	ester	42	3.4	3.35	-0.33	(-0.54 -0.12)	6.5 (good)
Clifton et al. 2008[51] stratum 1	39	37	55.2	26.8	56.6	margarine	sterols	ester	21	1.6	4.35	-0.39	(-0.63 -0.15)	5.5 (good)
Clifton et al. 2008[51] stratum 2	39	39	54.5	26.9	48.7	margarine	sterols	ester	21	1.6	4.20	-0.45	(-0.67 -0.23)	5.5 (good)
Clifton et al. 2008[51] stratum 3	39	36	54.2	26.7	56.0	margarine	sterols	ester	21	1.6	4.39	-0.33	(-0.55 -0.11)	5.5 (good)
Davidson et al. 2001[52] stratum 1	21	21	45.1	-	52.4	spread / salad dressing	sterols	ester	28	3.0	3.40	-0.14	(-0.47 0.18)	5.0 (low)
Davidson et al. 2001[52] stratum 2	21	19	47.3	-	50.0	spread / salad dressing	sterols	ester	28	6.0	3.36	-0.13	(-0.42 0.16)	5.0 (low)
Davidson et al. 2001[52] stratum 3	21	23	46.1	-	54.5	spread / salad dressing	sterols	ester	28	9.0	3.37	-0.41	(-0.73 -0.09)	5.0 (low)
de Graaf et al. 2002[53]	31	31	57.0	25.4	48.4	chocolate	mix	free	28	1.8	4.70	-0.61	(-0.81 -0.41)	6.5 (good)
de Jong et al. 2008[54] stratum 1	11	15	58.1	26.8	46.2	margarine	sterols	ester	112	2.5	3.57	-0.28	(-0.57 0.01)	6.5 (good)
de Jong et al. 2008[54] stratum 2	11	15	58.3	27.0	46.2	margarine	stanols	ester	112	2.5	3.44	-0.43	(-0.78 -0.08)	6.5 (good)

Table 2. *Continued*

Author, publication year, and reference	Subject characteristics					Treatment characteristics					Blood lipid outcomes			Overall quality score[c]
	Sample size		Mean age	Mean BMI	Male	Food format	Sterols or stanols	Ester or free	Duration	Dose phytosterols[a]	LDL-C			
	Control	Treatment									Mean baseline	Net change[b]	95% CI	
			y	*kg/m²*	*%*				*d*	*g/d*	*mmol/L*			
Devaraj et al. 2004[17]	36	36	42.5	25.5	40.3	orange juice	sterols	free	56	2.0	3.72	-0.44	(-0.62 -0.26)	5.5 (good)
Deveraj et al. 2006[18]	36	36	46.0	24.5	43.1	orange juice	sterols	free	28	2.0	3.72	0.08	(-0.19 0.35)	5.5 (good)
Doornbos et al. 2006[20]	33	38	56.8	25.2	44.0	low-fat yogurt drink	sterols	ester	28	2.8	3.90	-0.37	(-0.49 -0.25)	5.5 (good)
Earnest et al. 2007[55]	29	25	50.5	26.3	53.7	capsules	sterols	ester	84	1.56	4.27	-0.41	(-0.69 -0.13)	6.5 (good)
Goldberg et al. 2006[56]	13	13	59.5	27.2	34.6	tablets	stanols	free	42	1.8	2.89	-0.32	(-0.58 -0.05)	6.5 (good)
Hallikainen & Uusitupa 1999[32] stratum 1	17	18	44.6	25.6	40.0	margarine	stanols	ester	56	2.31	4.54	-0.61	(-0.96 -0.26)	6.5 (good)
Hallikainen & Uusitupa 1999[32] stratum 2	17	20	43.2	24.9	32.4	margarine	stanols	ester	56	2.16	4.25	-0.35	(-0.71 0.01)	6.5 (good)
Hansel et al. 2007[24]	99	95	48.9	23.6	67.0	low-fat fermented milk	sterols	ester	42	1.6	4.09	-0.32	(-0.45 -0.19)	6.5 (good)
Hironaka et al. 2006[19] stratum 1	51	50	43.7	23.9	50.5	vegetable juice	sterols	free	28	0.8	3.68	-0.25	(-0.41 -0.08)	-[d]
Hironaka et al. 2006[19] stratum 2	51	54	43.2	24.0	47.6	vegetable juice	sterols	free	28	1.6	3.70	-0.32	(-0.46 -0.18)	-[d]
Homma et al. 2003[57] stratum 1	34	33	46.5	23.5	33.3	margarine	stanols	ester	28	2.0	3.96	-0.34	(-0.49 -0.19)	3.0 (low)
Homma et al. 2003[57] stratum 2	34	34	47.5	24.0	38.0	margarine	stanols	ester	28	3.0	3.96	-0.26	(-0.44 -0.08)	3.0 (low)
Hyun et al. 2005[13]	28	23	28.7	22.6	51.0	yogurt	stanols	ester	28	2.0	3.08	-0.24	(-0.43 -0.06)	5.0 (low)
Ishiwata et al. 2002[58] study 1[f] stratum 1	19	21	48.2	23.5	22.5	margarine	stanols	ester	28	2.0	4.01	-0.37	(-0.54 -0.20)	2.0 (low)
Ishiwata et al. 2002[58] study 1 stratum 2	19	25	47.8	24.0	29.5	margarine	stanols	ester	28	3.0	4.01	-0.35	(-0.57 -0.13)	2.0 (low)
Ishiwata et al. 2002[58] study 2 stratum 1	11	10	42.6	23.0	61.9	margarine	stanols	ester	28	2.0	3.83	-0.42	(-0.69 -0.16)	2.0 (low)
Ishiwata et al. 2002[58] study 2 stratum 2	11	6	44.6	23.4	58.8	margarine	stanols	ester	28	3.0	3.90	-0.25	(-0.53 0.03)	2.0 (low)

Table 2. *Continued*

Author, publication year, and reference	Subject characteristics					Treatment characteristics					Blood lipid outcomes			Overall quality score[c]
	Sample size		Mean age	Mean BMI	Male	Food format	Sterols or stanols	Ester or free	Duration	Dose phytosterols[a]	LDL-C			
	Control	Treatment									Mean baseline	Net change[b]	95% CI	
			y	*kg/m²*	*%*				*d*	*g/d*	*mmol/L*			
Jauhiainen et al. 2006[59]	34	33	43.3	-	35.8	hard cheese	stanols	ester	35	2.0	3.58	-0.36	(-0.53 -0.18)	6.5 (good)
Jones et al. 1999[30]	16	16	-	-	100.0	margarine	mix	free	30	1.7	4.45	-0.64	(-1.22 -0.06)	7.0 (good)
Korpela et al. 2006[14] study 1	25	25	57.3	27.0	21.3	yogurt	mix	free	42	1.65	4.10	-0.32	(-0.58 -0.06)	5.0 (low)
Korpela et al. 2006[14] study 2	29	33	57.3	27.0	21.3	hard cheese	mix	free	42	2.0	4.10	-0.46	(-0.71 -0.21)	5.0 (low)
Korpela et al. 2006[14] study 3	28	24	57.3	27.0	21.3	fresh cheese	mix	free	42	2.0	4.10	-0.56	(-0.80 -0.32)	5.0 (low)
Lagström et al. 2006[60]	20	22	40.1	25.0	-	capsules	stanols	ester	21	2.0	3.40	-0.2	(-0.46 0.06)	6.5 (high)
Lee et al. 2003[61]	40	41	61.0	29.1	44.4	margarine	sterols	ester	28	1.6	4.33	-0.36	(-0.61 -0.11)	5.0 (low)
Li et al. 2007[62] stratum 1	99	102	44.5	26.0	35.3	milk tea	sterols	ester	35	1.5	3.22	-0.15	(-0.32 0.02)	6.5 (good)
Li et al. 2007[62] stratum 2	99	100	44.5	26.0	37.2	milk tea	sterols	ester	35	2.3	3.08	-0.17	(-0.35 0.00)	6.5 (good)
Maki et al. 2001[63] stratum 1	83	75	58.1	27.5	44.0	margarine	sterols	ester	30	1.1	4.08	-0.31	(-0.45 -0.17)	4.5 (low)
Maki et al. 2001[63] stratum 2	83	35	58.4	27.3	42.4	margarine	sterols	ester	30	2.2	4.03	-0.33	(-0.48 -0.17)	4.5 (low)
Matsuoka et al. 2004[64]	23	23	48.0	25.8	-	mayonnaise	sterols	free	28	0.8	3.78	0.01	(-0.23 0.25)	-[d]
Matvienko et al. 2002[65]	17	17	22.9	26.4	100.0	beef	sterols	ester	28	2.7	4.10	-0.55	(-0.84 -0.26)	7.0 (good)
McPherson et al. 2005[66] study 1	12	13	46.5	26.0	44.0	tablets	stanols	free	42	1.26	3.03	-0.32	(-0.55 -0.08)	5.0 (low)
McPherson et al. 2005[66] study 2[g]	13.5	13.5	50.7	27.8	33.3	capsules	stanols	free	42	1.01	3.50	-0.13	(-0.39 0.12)	5.0 (low)
Mensink et al. 2002[15]	30	30	36.0	23.3	26.7	yogurt	stanols	ester	28	3.0	2.92	-0.4	(-0.53 -0.26)	5.5 (good)
Miettinen & Vanhanen 1994[67] stratum 1	8	9	45.0	25.2	-	mayonnaise	sterols	free	63	1.0	4.09	-0.26	(-0.56 0.04)	6.5 (good)
Miettinen & Vanhanen 1994[67] stratum 2	8	7	45.0	25.2	-	mayonnaise	stanols	free	63	1.04	3.73	-0.11	(-0.43 0.21)	6.5 (good)
Miettinen & Vanhanen 1994[67] stratum 3	8	7	45.0	25.2	-	mayonnaise	stanols	ester	63	1.22	3.39	-0.28	(-0.55 -0.01)	6.5 (good)
Miettinen et al. 1995[68] stratum 1	51	51	50.0	-	-	margarine	stanols	ester	182	2.6.0	3.96	-0.44	(-0.62 -0.26)	5.5 (good)
Miettinen et al. 1995[68] stratum 2	51	51	51.0	-	-	margarine	stanols	ester	182	2.6.0	4.14	-0.52	(-0.70 -0.34)	5.5 (good)

Table 2. *Continued*

Author, publication year, and reference	Subject characteristics					Treatment characteristics					Blood lipid outcomes				Overall quality score[c]
	Sample size		Mean age	Mean BMI	Male	Food format	Sterols or stanols	Ester or free	Duration	Dose phytosterols[a]	LDL-C				
	Control	Treatment									Mean baseline	Net change[b]	95% CI		
			y	*kg/m²*	*%*				*d*	*g/d*	*mmol/L*				
Neil et al. 2001[27]	31	31	50.3	26.0	41.9	margarine	sterols	ester	28	2.5	5.08	-0.72	(-1.21	-0.23)	6.5 (good)
Niittynen et al. 2008[69] study 2	14	12	47.1	25.6	57.7	low-fat yogurt drink	sterols	free	56	2.0	4.73	-0.28	(-0.88	0.29)	6.5 (good)
Plat et al. 2000b[70] stratum 1	42	36	33.0	22.6	37.2	margarine / shortening	stanols	ester	56	3.79	2.94	-0.37	(-0.51	-0.22)	6.5 (good)
Plat et al. 2000b[70] stratum 2	42	34	33.0	23.2	36.8	margarine / shortening	stanols	ester	56	4.03	2.94	-0.34	(-0.51	-0.18)	6.5 (good)
Quilez et al. 2003[71]	29	28	30.9	23.3	43.9	muffin / croissant	sterols	ester	56	3.2	2.50	-0.36	(-0.56	-0.16)	5.0 (low)
Seki et al. 2003a[72]	28	32	39.1	24.2	100.0	bread	sterols	ester	28	0.45	3.01	-0.07	(-0.21	0.07)	5.0 (low)
Seki et al. 2003b[73]	11	11	41.2	24.2	100.0	bread	sterols	ester	28	1.34	2.58	-0.32	(-0.52	-0.12)	5.0 (low)
Seppo et al. 2007[16] study 1	29	31	46.7	25.2	36.1	yogurt	stanols	ester	35	2.0	3.40	-0.10	(-0.31	0.12)	5.0 (low)
Seppo et al. 2007[16] study 2	32	29	46.7	25.2	36.1	low-fat yogurt drink	stanols	ester	35	2.0	3.40	-0.11	(-0.31	0.09)	5.0 (low)
Seppo et al. 2007[16] study 3	9	10	46.7	25.2	36.1	low-fat yogurt drink	stanols	ester	35	2.0	3.40	-0.40	(-0.84	0.03)	7.0 (good)
Seppo et al. 2007[16] study 4	27	32	46.7	25.2	36.1	low-fat milk	stanols	ester	35	2.0	3.40	-0.21	(-0.38	-0.05)	5.0 (low)
Spilburg et al. 2003[74]	13	11	50.6	26.1	33.3	lemonade	stanols	free	28	1.9	3.83	-0.56	(-0.85	-0.27)	4.0 (low)
Taichi et al. 2003[75]	29	26	46.8	25.3	-	mayonnaise	sterols	ester	28	0.88	3.81	-0.31	(-0.50	-0.13)	2.0 (low)
Vanhanen et al. 1994a[76]	8	7	47.3	26.5	73.3	mayonnaise	stanols	ester	63	0.8	3.39	-0.28	(-0.56	0.00)	4.5 (low)
Vanhanen 1994b[77]	7	7	55.0	25.5	35.7	mayonnaise	stanols	ester	42	1.5	3.70	-0.07	(-0.44	0.30)	4.0 (low)
Varady et al. 2004[78]	20	18	56.6	26.3	31.6	margarine	sterols	ester	56	1.8	3.55	-0.39	(-0.56	-0.22)	5.5 (good)
Woodgate et al. 2006[79]	15	14	53.7	27.5	69.0	capsules	stanols	ester	28	1.6	5.35	-0.39	(-0.78	0.00)	6.5 (good)

LDL-C, low-density lipoprotein cholesterol.

[a] Dose given as free equivalents in g/d.

[b] Net change was calculated by subtracting the mean change in the control group from the mean change in the treatment group (where mean change = LDL-C at the end-of-intervention - LDL-C at baseline).

[c] The maximum overall quality score was 7 for parallel trials. When a trial was given <5.5 points, it was judged to be of low quality and a trial that was given ≥5.5 points was judged to be of high quality.

[d] Publications written in Portuguese or Japanese, so not all descriptive variables could be extracted.

[e] Multiple strata in 1 study corrected for the same single control group: indicated with stratum 1, stratum 2, stratum 3, etc.

[f] Multiple strata in 1 study, each corrected for a respective control group: indicated with study 1, study 2, study 3, etc.

[g] Number of subjects not reported in the publication. Therefore, it was assumed that the 27 subjects were distributed evenly across the groups.

Table 3. *Overview of the cross-over strata included in the meta-analysis.*

Author, publication year, and reference	Subject characteristics				Treatment characteristics					Blood lipid outcomes			Overall quality score[c]
										LDL-C			
	Sample size	Mean age	Mean BMI	Male	Food format	Sterols or stanols	Ester or free	Dura-tion	Dose phyto-sterols[a]	Mean baseline	Net change[b]	95% CI	
		y	*kg/m²*	*%*				*d*	*g/d*		*mmol/L*		
AbuMweis et al. 2006[80] stratum 1[d]	30	59.0	28.0	-	margarine	sterols	free	29	1.72	3.80	-0.05	(-0.27 0.17)	7.0 (good)
AbuMweis et al. 2006[80] stratum 2	30	59.0	28.0	-	margarine	sterols	ester	29	1.72	3.80	-0.05	(-0.28 0.18)	7.0 (good)
Cater et al. 2005[81] study 1[e] stratum 1	8	58.0	28.0	75.0	margarine	stanols	ester	42	2.0	-	-0.52	(-0.79 -0.25)	5.0 (low)
Cater et al. 2005[81] study 1 stratum 2	8	58.0	28.0	75.0	margarine	stanols	ester	42	3.0	-	-0.54	(-0.77 -0.32)	5.0 (low)
Cater et al. 2005[81] study 1 stratum 3	8	58.0	28.0	75.0	margarine	stanols	ester	42	4.0	-	-0.57	(-0.79 -0.35)	5.0 (low)
Cater et al. 2005[81] study 2	13	57.0	27.5	0.0	margarine	stanols	ester	42	3.0	-	-0.54	(-0.67 -0.42)	5.0 (low)
Cater et al. 2005[81] study 3	10	66.0	29.5	100.0	margarine	stanols	ester	60	3.0	-	-0.44	(-0.62 -0.26)	6.0 (low)
Chan et al. 2007[82] stratum 1	21	54.2	25.9	52.4	vegetable oil	sterols	ester	28	1.7	3.91	-0.24	(-0.45 -0.03)	6.0 (low)
Chan et al. 2007[82] stratum 2	21	54.2	25.9	52.4	vegetable oil	sterols	ester	28	1.7	3.91	-0.35	(-0.58 -0.12)	6.0 (low)
Cleghorn et al. 2003[31]	50	46.7	26.0	38.0	margarine	sterols	ester	28	2.1	3.98	-0.27	(-0.40 -0.14)	7.5 (good)
Clifton et al. 2004[10] stratum 1	58/36[f]	54.0	26.2	39.7	bread	sterols	ester	21	1.6	4.03	-0.42	(-0.57 -0.27)	4.5 (low)
Clifton et al. 2004[10] stratum 2	58/40	54.0	26.2	39.7	milk	sterols	ester	21	1.6	4.03	-0.72	(-0.85 -0.58)	4.5 (low)
Clifton et al. 2004[10] stratum 3	58/58	54.0	26.2	39.7	cereals	sterols	ester	21	1.6	4.03	-0.24	(-0.35 -0.13)	4.5 (low)
Clifton et al. 2004[10] stratum 4	58/40	54.0	26.2	39.7	yogurt	sterols	ester	21	1.6	4.03	-0.36	(-0.50 -0.22)	4.5 (low)
Colgan et al. 2004[83]	48	46.0	26.1	56.3	margarine	sterols	ester	21	1.3	3.94	-0.11	(-0.29 0.07)	6.0 (low)
Geelen et al. 2002[84] study 1	31	26.0	23.0	51.6	margarine	sterols	ester	21	3.0	-	-0.31	(-0.48 -0.14)	7.5 (good)
Geelen et al. 2002[84] study 2	57	25.0	23.0	40.4	margarine	sterols	ester	21	3.0	-	-0.34	(-0.47 -0.21)	7.5 (good)
Gylling & Miettinen 1994[85]	11	57.8	-	100.0	margarine	stanols	ester	42	3.0	-	-0.36	(-0.57 -0.15)	4.5 (low)
Gylling & Miettinen 1999[86]	21	52.7	25.7	0.0	butter	stanols	ester	35	2.43	3.98	-0.45	(-0.66 -0.24)	6.0 (low)
Gylling et al. 1997[87]	22	51.0	26.0	0.0	margarine	stanols	ester	49	3.0	3.85	-0.53	(-0.76 -0.30)	5.0 (low)
Hallikainen et al. 2000[88] stratum 1	34	48.8	24.9	-	margarine	stanols	ester	28	2.01	4.43	-0.53	(-0.71 -0.35)	7.5 (good)
Hallikainen et al. 2000[88] stratum 2	34	48.8	24.9	-	margarine	sterols	ester	28	2.04	4.43	-0.44	(-0.59 -0.28)	7.5 (good)
Hayes et al. 2004[89]	7	48.0		66.7	tortilla chips	sterols	free	24	1.5	4.19	-0.62	(-1.10 -0.14)	3.0 (low)
Hendriks et al. 1999[90] stratum 1	80	37.0	22.8	42.0	margarine	sterols	ester	24	0.83	2.97	-0.20	(-0.31 -0.10)	5.5 (low)

Table 3. *Continued*

Author, publication year, and reference	Subject characteristics				Treatment characteristics					Blood lipid outcomes				Overall quality score[c]
	Sample size	Mean age	Mean BMI	Male	Food format	Sterols or stanols	Ester or free	Dura-tion	Dose phyto-sterols[a]	LDL-C				
										Mean baseline	Net change[b]	95% CI		
		y	*kg/m²*	*%*				*d*	*g/d*	*mmol/L*				
Hendriks et al. 1999[90] stratum 2	80	37.0	22.8	42.0	margarine	sterols	ester	24	1.61	2.97	-0.26	(-0.36	-0.15)	5.5 (low)
Hendriks et al. 1999[90] stratum 3	80	37.0	22.8	42.0	margarine	sterols	ester	24	3.24	2.97	-0.30	(-0.41	-0.20)	5.5 (low)
Jakulj et al. 2005[91]	39	55.5	25.9	87.5	margarine	sterols	free	28	2.0	4.50	-0.35	(-0.58	-0.13)	7.5 (good)
Jones et al. 2000[29] stratum 1	15	-	-	100.0	margarine	sterols	ester	21	1.84	4.29	-0.56	(-0.77	-0.35)	8.0 (good)
Jones et al. 2000[29] stratum 2	25	-	-	100.0	margarine	stanols	ester	21	1.84	4.35	-0.27	(-0.50	-0.04)	8.0 (good)
Jones et al. 2003[92]	25	-	-	60.0	non-fat beverage	mix	free	21	1.8	4.15	-0.08	(-0.41	0.25)	8.0 (good)
Judd et al. 2002[93]	53	47.1	26.3	49.1	salad dressing	sterols	ester	21	2.2	3.62	-0.34	(-0.38	-0.29)	6.0 (low)
Kratz et al. 2007[94] stratum 1	17	32.0	22.0	-	margarine	sterols	ester	42	2.03	2.77	-0.10	(-0.30	0.10)	7.5 (good)
Kratz et al. 2007[94] stratum 2	17	32.0	22.0	-	margarine	stanols	ester	42	1.96	2.77	-0.23	(-0.38	-0.08)	7.5 (good)
Lau et al. 2005[95] study 1	14	54.5	30.2	35.7	margarine	sterols	free	21	1.8	3.24	-0.19	(-0.61	-0.23)	8.0 (good)
Lau et al. 2005[95] study 2	15	55.1	26.9	40.0	margarine	sterols	free	21	1.8	3.92	-0.30	(-0.57	-0.03)	8.0 (good)
Lottenberg et al. 2003[96]	60	55.8	26.4	16.7	margarine	sterols	ester	28	1.68	5.00	-0.30	(-0.41	-0.19)	6.0 (low)
Madsen et al. 2007[97]	46	50.6	25.0	25.0	margarine	sterols	ester	28	2.3	3.50	-0.29	(-0.45	-0.14)	6.0 (low)
Mussner et al. 2002[98]	62	42.0	24.0	38.7	margarine	sterols	ester	21	1.82	3.93	-0.26	(-0.37	-0.15)	5.5 (low)
Naumann et al. 2003[99] stratum 1	42	33.8	23.4	35.7	margarine	mix	ester	21	1.96	-	-0.17	(-0.37	-0.02)	4.0 (low)
Naumann et al. 2003[99] stratum 2	42	33.8	23.4	35.7	margarine	mix	ester	21	1.99	-	-0.19	(-0.40	-0.05)	4.0 (low)
Nestel et al. 2001[100] study 1 stratum 1	22	60.0	24.0	81.8	bread / cereal / margarine	sterols	ester	28	2.4	-	-0.45	(-0.76	-0.14)	2.0 (low)
Nestel et al. 2001[100] study 1 stratum 2	22	60.0	24.0	81.8	bread / cereal / margarine	stanols	free	28	2.4	-	-0.30	(-0.60	0.00)	2.0 (low)
Nestel et al. 2001[100] study 2	15	43.7	-	-	margarine	sterols	ester	28	2.4	-	-0.37	(-0.61	-0.13)	4.0 (low)
Niittynen et al. 2008[69] study 1	15	41.0	25.6	100.0	low-fat yogurt drink	sterols	free	28	1.0	3.90	-0.19	(-0.41	0.03)	7.5 (good)
Noakes et al. 2002[101] study 1 stratum 1	46	56.7	26.2	43.5	margarine	sterols	ester	21	2.3	4.38	-0.33	(-0.44	-0.22)	7.5 (good)
Noakes et al. 2002[101] study 1 stratum 2	46	56.7	26.2	43.5	margarine	stanols	ester	21	2.5	4.38	-0.41	(-0.51	-0.31)	7.5 (good)
Noakes et al. 2002[101] study 2	35	57.3	26.0	57.1	margarine	sterols	ester	21	2.0	4.20	-0.40	(-0.52	-0.28)	7.5 (good)
Noakes et al. 2005[11] study 1 stratum 1	39	51.5	25.9	53.8	margarine	sterols	ester	21	2.0	4.83	-0.49	(-0.66	-0.32)	4.5 (low)
Noakes et al. 2005[11] study 1 stratum 2	39	51.5	25.9	53.8	milk	sterols	ester	21	2.0	4.83	-0.38	(-0.50	-0.26)	4.5 (low)

Table 3. *Continued*

Author, publication year, and reference	Subject characteristics				Treatment characteristics					Blood lipid outcomes				Overall quality score[c]
	Sample size	Mean age	Mean BMI	Male	Food format	Sterols or stanols	Ester or free	Dura-tion	Dose phyto-sterols[a]	LDL-C				
										Mean baseline	Net change[b]	95% CI		
		y	*kg/m²*	*%*				*d*	*g/d*	*mmol/L*				
Noakes et al. 2005[11] study 1 stratum 3	39	51.5	25.9	53.8	margarine / milk	sterols	ester	21	4.0	4.83	-0.55	(-0.69	-0.41)	4.5 (low)
Noakes et al. 2005[11] study 2 stratum 1	40	60.4	26.5	42.5	yogurt	stanols	ester	21	1.8	4.48	-0.23	(-0.33	-0.13)	7.5 (good)
Noakes et al. 2005[11] study 2 stratum 2	40	60.4	26.5	42.5	yogurt	sterols	ester	21	1.7	4.48	-0.27	(-0.37	-0.17)	7.5 (good)
Ntanios et al. 2002[102]	53	45.1	23.7	49.1	margarine	sterols	free	21	1.8	-	-0.28	(-0.39	-0.17)	5.5 (low)
Pelletier et al. 1995[103]	12	22.7	22.3	100.0	butter	sterols	free	28	0.74	-	-0.41	(-0.58	-0.24)	5.0 (low)
Plat et al. 2000a[104] stratum 1	39	31.0	22.7	28.2	margarine	stanols	ester	28	2.47	-	-0.29	(-0.39	-0.19)	7.5 (good)
Plat et al. 2000a[104] stratum 2	39	31.0	22.7	28.2	margarine / shortening	stanols	ester	28	2.46	-	-0.31	(-0.41	-0.20)	7.5 (good)
Sierksma et al. 1999[36]	75	44.0	24.4	51.3	margarine	sterols	free	21	0.8	-	-0.19	(-0.23	-0.15)	6.5 (good)
Temme et al. 2002[105]	42	55.0	25.0	52.4	margarine	sterols	ester	28	2.1	4.29	-0.47	(-0.62	-0.31)	7.5 (good)
Thomsen et al. 2004[12] stratum 1	69	60.0	25.9	26.1	milk	sterols	free	28	1.17	4.37	-0.30	(-0.42	-0.18)	4.0 (low)
Thomsen et al. 2004[12] stratum 2	69	60.0	25.9	26.1	milk	sterols	free	28	1.6	4.37	-0.40	(-0.53	-0.28)	4.0 (low)
Vanstone et al. 2002[106] stratum 1	15	47.8	30.8	60.0	butter	sterols	free	21	1.8	4.00	-0.41	(-0.65	-0.17)	8.0 (good)
Vanstone et al. 2002[106] stratum 2	15	47.8	30.8	60.0	butter	stanols	free	21	1.8	4.11	-0.42	(-0.66	-0.18)	8.0 (good)
Vanstone et al. 2002[106] stratum 3	15	47.8	30.8	60.0	butter	mix	free	21	1.8	4.18	-0.46	(-0.70	-0.22)	8.0 (good)
Volpe et al. 2001[107]	30	-	-	70.0	low-fat yogurt drink	sterols	free	28	1.08	4.67	-0.34	(-0.51	-0.17)	6.5 (good)
Weststrate & Meijer 1998[108] stratum 1	76/76[g]	45.0	24.2	50.0	margarine	sterols	ester	21	3.2	3.54	-0.44	(-0.48	-0.40)	5.5 (low)
Weststrate & Meijer 1998[108] stratum 2	76/77	45.0	24.2	50.0	margarine	stanols	ester	21	2.7	3.54	-0.40	(-0.44	-0.36)	5.5 (low)
Yoshida et al. 2006[109] study 1	16	55.2	27.7	43.8	cereal bar	mix	free	21	1.8	4.18	-0.24	(-0.48	0.00)	7.5 (good)
Yoshida et al. 2006[109] study 2	13	56.8	31.0	30.8	cereal bar	mix	free	21	1.8	3.60	-0.09	(-0.33	0.15)	7.5 (good)

LDL-C, low-density lipoprotein cholesterol.

[a] Dose given as free equivalents in g/d.

[b] The net change in LDL-C was calculated as the mean LDL-C concentration at the end of the phytosterol treatment period minus the mean LDL-C concentrations at the end of the control period.

[c] The maximum overall quality score was 8 for cross-over trials. When a trial was given ≤6.0 points, it was judged to be of low quality and a trial that was given >6.0 points was judged to be of high quality.

[d] Multiple strata in 1 study corrected for the same single control group: indicated with stratum 1, stratum 2, stratum 3, etc.

[e] Multiple strata in 1 study, each corrected for a respective control group: indicated with study 1, study 2, study 3, etc.

[f] Clifton et al. (2004)[10] used an incomplete cross-over design with 4 phytosterol treatments and 1 control treatment; all subjects followed a period with control treatment and only 3 periods with phytosterol treatment. Thus, the total number of subjects per treatment was not the same: 58 for the control treatment and 36, 40, 58, and 40 for the phytosterol treatments, respectively.

[g] Weststrate and Meijer (1998)[108] used an incomplete cross-over design with 5 phytosterol treatments (2 of which are included in this meta-analysis) and 1 control treatment; due to 4 drop-outs in the first period and 1 drop-out in the 3rd period, the total number of subjects per phytosterol treatment varied: 76 and 77 during the phytosterol treatments, respectively.

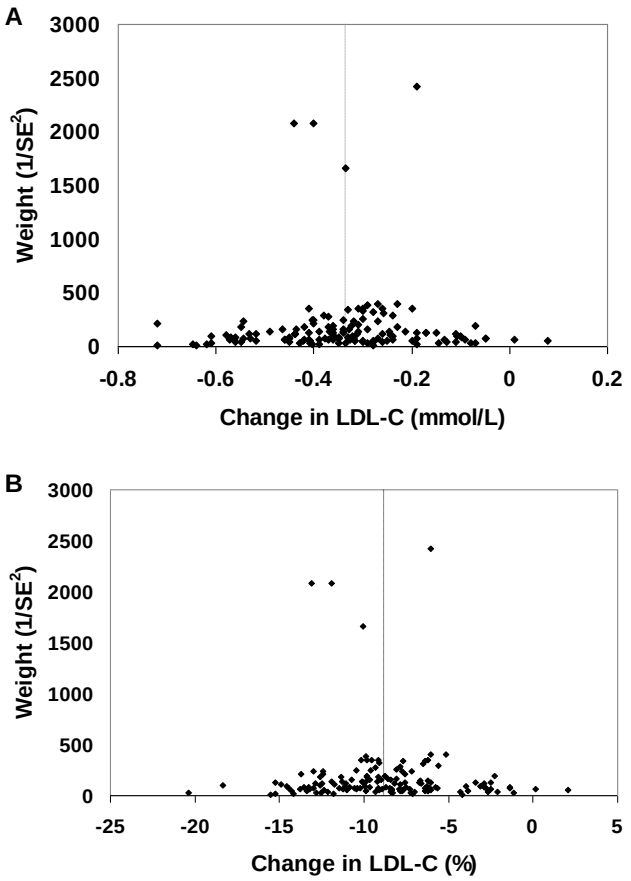

Figure 2. *Funnel plots of the weights ($1/SE^2$) against the absolute changes in LDL-C (A) and the relative changes in LDL-C (B) in 141 strata from 81 randomized controlled trials investigating the cholesterol-lowering effect of phytosterols. The LDL-C changes are scattered around the pooled overall estimate of -0.34 mmol/l (A) and -8.83% (B).*

Effect of phytosterol intake on LDL-C and TC:HDL-C

On average, phytosterols lowered LDL-C by 0.34 mmol/L (95% CI: -0.36; -0.31), which corresponds to a relative decrease of 8.8% (95% CI: -9.4; -8.3). There was an absolute reduction in LDL-C concentrations in 139 of 141 strata (**Tables 2 and 3**) and the reduction was significant in 109 strata. In only 2 strata[18,64], LDL-C concentrations were not decreased at 4 week (time point used for the meta-analysis). Data reported for these strata after 8 or 12 week showed a significant reduction in LDL-C.

The dose-response curve for the relationship between phytosterol dose and LDL-C-lowering was described by the equation, where the best parameters to fit the observed data were: a = -0.43 mmol/L (95% CI: -0.51; -0.35) and b = 0.83 g/d (95% CI: 0.42; 1.23) for the predicted absolute LDL-C change (mmol/L) (P <0.001) and a = -12.68% (95% CI: -15.38; -9.99) and b = 1.12 g/d (95% CI: 0.62; 1.63) for the relative (%) LDL-C change (P <0.001), respectively (**Figure 3**). According to the dose-response relationship, the predicted LDL-C-lowering effect of the recommended daily dose of phytosterols (2 g) would be -0.35 mmol/L or -9%.

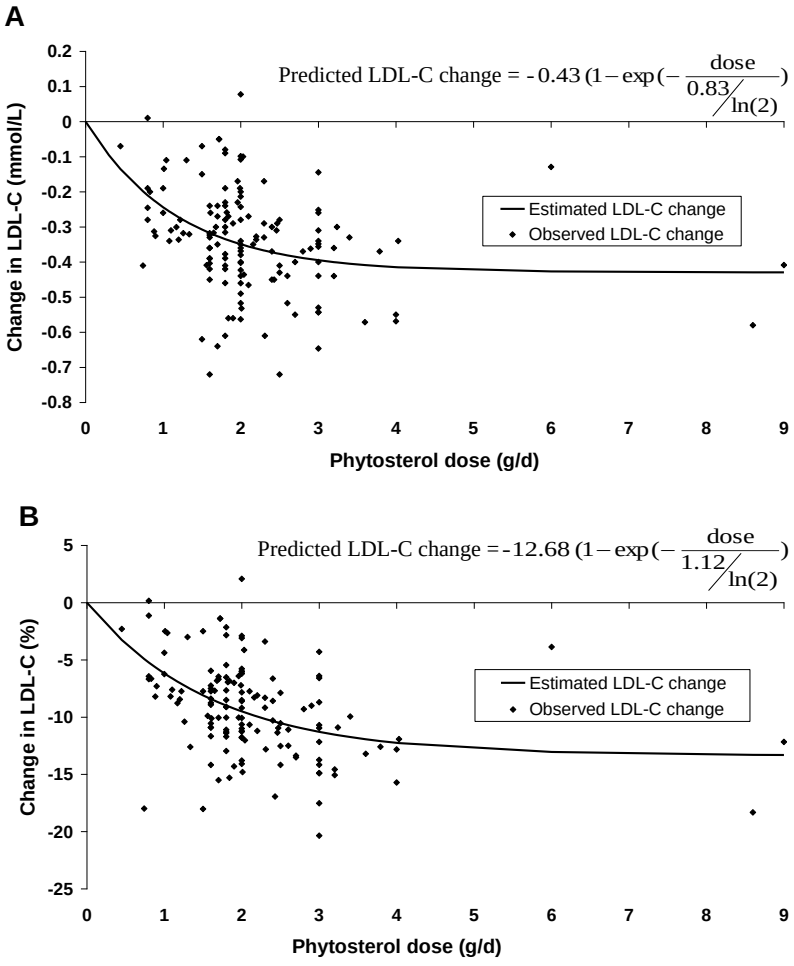

Figure 3. *Dose-response relationship for the absolute (A) and relative (B) LDL-C-lowering effect of phytosterols.*

The equation was also used to describe the relationship between phytosterol dose and absolute changes in TC:HDL-C ratios. The values of parameters a and b obtained for the equation describing the absolute changes in TC:HDL-C were -0.42 (95% CI: -0.57; -0.27) and 1.06 g/d (95% CI: 0.23; 1.90), respectively (P <0.001). For the recommended dose of 2 g/d phytosterols, the equation predicts a 0.31 decrease in the TC:HDL-C ratio. To verify whether this estimate was reliable, the deviations between the mean ratio calculated from individual ratios available from 8 of our previous studies and the ratios calculated from the mean TC and HDL-C concentrations (as was done in the present meta-analysis) were determined. The mean deviation, weighted by the number of subjects, was -6.45% (range: -3.99% to -8.78%), suggesting that TC:HDL-C ratios calculated from the reported means were underestimated.

Impact of subject baseline characteristics on the LDL-C-lowering effect of phytosterols

Residuals (differences between the absolute LDL-C changes predicted with the dose-response curve and the observed LDL-C changes) were most strongly correlated with baseline LDL-C concentrations (r = -0.4; P <0.0001), with 16% of the variation in residuals explained by this variable. Age was also correlated with the residuals (r = -0.17; P = 0.045) but explained only 3% of the variation. BMI was not significantly correlated with gender (r = -0.17; P = 0.051) or residuals (r = -0.18; P = 0.052). When all 4 covariates were simultaneously included in the model, the effect of age on the residuals was no longer significant (P = 0.45), whereas the impact of baseline LDL-C concentrations remained significant (P = 0.001), suggesting co linearity between age and baseline LDL-C concentrations. Given the substantial impact of baseline LDL-C concentrations on the absolute LDL-C reductions due to phytosterol intake, with the larger reductions in populations with higher baseline LDL-C concentrations, comparisons between subgroups of categorical covariates were made by comparing not only the absolute but also the relative curves. Indeed, the use of the relative (%) changes resulted in less variation in residuals (only 0.05% of the variation was due to baseline LDL-C) than the use of the absolute values and the relative curve was more precise (F = 477.1) than the absolute curve (F = 425.9).

Impact of food format and other treatment characteristics on the LDL-C-lowering effect of phytosterols

The impact of the categorical covariates was evaluated by comparing the dose-response curves obtained for the respective subgroups (**Table 4**). The fat content of the food format (fat-based vs. non fat-based) and the type of phytosterols (plant sterols vs. stanols) did not significantly affect the absolute and relative dose-response curves (**Table 4**; **Figure 4**). The dairy or non-dairy nature of the foods also did not significantly affect the absolute dose-response curve (not shown). A relative curve for the dairy food formats could not be calculated due to the small number of strata and the narrow distribution of the net changes in LDL-C. Therefore, the mean relative LDL-C changes were calculated separately for strata in which dairy and non-dairy foods were used and for a narrow range (1.6-2.0 g/d) of doses. The mean LDL-C-lowering effect of dairy and non-dairy food formats was -8.53% (95% CI: -9.71; -7.34) for a mean phytosterol intake of 1.85 g/d and -7.97% (95% CI: -8.79; -7.15) for a mean dose of 1.81 g/d, respectively, indicating no significant difference between dairy and non-dairy food formats. The only significant effect was the effect of solid compared to liquid food format on the relative curve. At high doses, the maximal estimated LDL-C-lowering effect of solid foods was 5.2% larger than that of liquid foods (parameter a), and at low doses, the curve was steeper for liquid than for solid foods (parameter b) (**Table 4**). However, the curves obtained for solid vs. liquid foods crossed at phytosterol intakes of ~1.5 g/d, and at ~2 g/d, the difference between the 2 curves was small (data not shown).

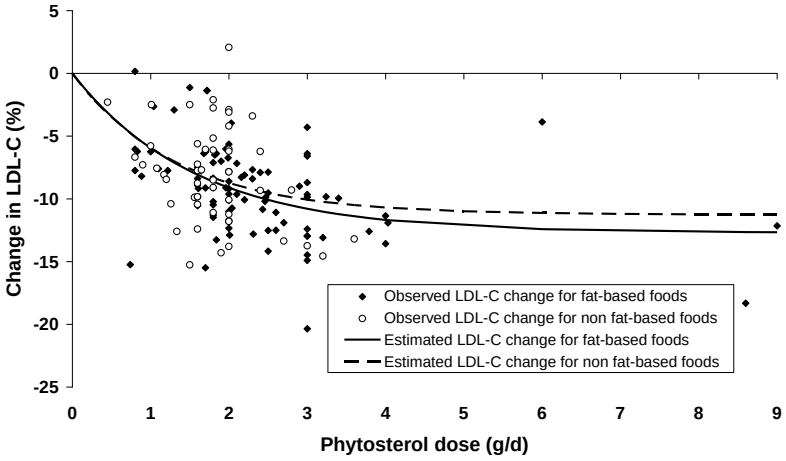

Figure 4. *Relative dose-response curves of the LDL-C-lowering effect of phytosterols incorporated in fat-based compared to non fat-based food formats.*

Post hoc analyses were performed to evaluate the impact of phytosterol esterification and frequency of intake on the dose-response curve. Free phytosterols and phytosterol esters did not differ in the maximal LDL-C reduction or in the incremental dose-step (**Table 4**). Due to the small number of strata (n = 14) in the single daily intake subgroup and the narrow distribution of net LDL-C changes in this subgroup, a dose-response curve could not be established for the once-a-day intakes. Therefore, to evaluate the effect of frequency of intake, the mean relative LDL-C change for a narrow range of doses (1.6-2.0 g/d) was calculated for strata in which phytosterols were consumed once per day compared to ≥2 times/d. The relative LDL-C-lowering effect was more pronounced when phytosterols were consumed in multiple daily intakes (-8.91%; 95% CI: -9.75; -8.07, for a mean phytosterol dose of 1.81 g/d) than in single daily intakes (-6.14%; 95%CI: -8.19; -4.10, for a mean dose of 1.76 g/d). Because the mean dose was slightly higher for the multiple daily intakes, regression analyses were performed to determine the respective impact of dose and frequency of consumption. When included separately in the model, the dose contributed to 14% of the variation in LDL-C changes ($P < 0.0001$) and the frequency of intake contributed to 5% of the variation ($P = 0.0054$). An increase in the number of daily intakes was associated with a larger decrease in LDL-C concentrations. However, when dose and frequency of intake were simultaneously included in the model ($r^2 = 0.26$), the effect of dose on LDL-C changes remained significant ($P <0.0001$), whereas frequency of intake only tended ($P = 0.054$) to affect the relative decreases in LDL-C concentrations. These data suggest that the effect of frequency of intake was partly confounded by the influence of dose.

Impact of study quality and study design on the LDL-C–lowering effect of phytosterols

The overall quality of the trials, the compliance, and the randomization method did not significantly affect either the absolute or the relative dose-response curve. We performed a post hoc analysis to evaluate the effect of study design (cross-over vs. parallel) on the dose-response curves. Study design did not have an impact on the curves (**Table 4**).

Table 4. *Impact of categorical covariates related to the type of phytosterols, food format, study quality and study design on the absolute and relative dose-response curve.*

Treatment or study characteristic	Categories compared (number of strata)	Difference in parameter a[a,b] (mmol/L or %)	95% CI	Difference in parameter b[a,b] (g/d)	95% CI
Absolute curve[c]					
Type of phytosterols	Plant stanols (n = 53) vs. plant sterols (n = 74)	-0.13	(-0.38, 0.12)	0.65	(-0.63, 1.93)
Food format	Non fat-based (n = 50) vs. fat-based (n = 88)	0.05	(-0.12, 0.21)	-0.24	(-1.08, 0.60)
	Non-dairy (n = 114) vs. dairy (n = 26)	-0.02	(-0.18, 0.14)	0.36	(-0.45, 1.16)
	Solid (n = 116) vs. liquid (n = 24)	-0.11	(-0.24, 0.02)	0.51	(-0.27, 1.29)
Quality	High (n = 85) vs. low (n = 52) compliance	-0.01	(-0.17, 0.16)	-0.09	(-0.95, 0.76)
	Well (n = 110) vs. poorly (n = 27) randomized	-0.04	(-0.20, 0.11)	0.15	(-0.46, 1.14)
	High (n = 68) vs. low (n = 69) quality	-0.04	(-0.25, 0.17)	0.29	(-0.76, 1.33)
Design	Cross-over (n = 68) vs. parallel (n = 73)	1.96	(-5.90, 9.81)	-0.38	(-1.79, 1.03)
Relative curve[c]					
Type of phytosterols	Plant stanols (n = 53) vs. plant sterols (n = 74)	-6.66	(-18.33, 5.02)	1.13	(-0.98, 3.23)
Food format	Non fat-based (n = 50) vs. fat-based (n = 88)	1.45	(-4.83, 7.72)	-0.17	(-1.31, 0.97)
	Non-dairy (n = 114) vs. dairy (n = 26)	-	-	-	-
	Solid (n = 116) vs. liquid (n = 24)	-5.23	(-8.63, -1.83)*	0.86	(0.02, 1.71)*
Quality	High (n = 85) vs. low (n = 52) compliance	-0.93	(-7.07, 5.20)	0.09	(-1.05, 1.23)
	Well (n = 110) vs. poorly (n = 27) randomized	-3.36	(-7.73, 1.02)	0.75	(-0.11, 1.61)
	High (n = 68) vs. low (n = 69) quality	-8.66	(-27.49, 10.17)	1.66	(-1.59, 4.90)
Design	Cross-over (n = 68) vs. parallel (n = 73)	0.12	(-0.16, 0.39)	-0.60	(-1.89, 0.69)

[a] The differences in parameters a and b of the curves obtained for subcategories of a covariable were calculated by re-parameterizing the equation with terms for differences between categories.

[b] Parameter a is the maximal LDL-C-lowering effect and parameter b is the dose step needed to achieve an additional effect, which is one half the size of the previous dose effect.

[c] For the absolute curve, the change in parameter a is expressed in mmol/L, and for the relative curve, it is expressed in % from baseline/control.

* $P < 0.05$.

Discussion

The key outcome of this review and meta-analysis is the generation of a physiologically relevant, continuous dose-response relationship for the LDL-C-lowering effect of phytosterols. By including not only fat-based foods consumed multiple times per day but also low-fat or fat-free foods and food formats intended for once-a-day use, this approach provides an updated estimation of the LDL-C-lowering efficacy of phytosterols in the variety of available food formats. The dose-response equation predicts an LDL-C-lowering effect of 29% for the recommended 2 g/d dose of phytosterols, which is consistent with our pooled estimate showing an 8.8% decrease in LDL-C for a mean dose of 2.15 g/d and with the mean 8.9% reduction reported by Katan *et al.*[3] for phytosterol doses of 2.0-2.4 g/d provided mainly in fat-based food formats.

We attempted to estimate as well the dose-response relationship for the effect of phytosterols on the TC:HDL-C ratio, but firm conclusions could not be drawn because the ratio calculated from the reported means of TC and HDL-C was underestimated. Results from a recent meta-analysis of individual subject data[110] provide more insights into this question. Phytosterols (in this case, plant stanols) were shown to significantly lower TC:HDL-C ratios and decreases were more pronounced in subjects with higher baseline values. In subjects with low baseline HDL-C concentrations, HDL-C was slightly increased, while in subjects with high baseline concentrations, it was marginally lowered[110]. According to the authors, this slight reduction in HDL-C in subjects with high baseline values would not increase cardiovascular risk, because at the same time, LDL-C would be decreased substantially.

The LDL-C-lowering dose-response curve obtained from the present meta-analysis had a plateau at phytosterol intakes of ~3 g/d, corresponding to an LDL-C-lowering effect of -10.7%, which is consistent with the estimation by Katan *et al*.[3], according to which doses >2.5 g/d provided only little additional benefit. The present meta-analysis indicated that most phytosterol treatment characteristics (fat-based vs. non fat-based formats, dairy vs. non-dairy formats, free phytosterols vs. phytosterol esters, and plant sterols vs. stanols) had no noticeable impact on the LDL-C-lowering efficacy. The LDL-C-lowering effect of free phytosterols and phytosterol esters has so far not been directly compared in single trials, but cholesterol absorption inhibition was shown to be similar[111] or even larger[112] with

free plant sterols than with the esters. In short (3-4-week)[29,88,101,108] and longer term (up to 85-week)[113] trials where stanols and sterols were compared side by side, no difference was observed between sterols and stanols, which is consistent with the present results.

Results from the present meta-analysis suggest that solid food formats may result in a larger LDL-C-lowering effect than liquid foods when the phytosterol dose is high (>2 g/d). In a previous study, a yogurt drink enriched with ~3 g/d plant sterols had a greater efficacy when consumed with a lunch meal than after an overnight fast[20]. These data could provide support to the hypothesis of a beneficial impact of the simultaneous presence of a solid meal on the cholesterol-lowering efficacy of liquid food formats, perhaps by a longer transit time in the gastrointestinal tract. However, in most studies included in this meta-analysis, the phytosterol-enriched liquid foods were consumed at meal time. Proper side-by-side comparisons in the same trial and using the same daily dose would be needed to confirm a difference in efficacy between solid and liquid food formats. One previous study comparing the efficacy of plant sterol-enriched (1.6 g/d) milk, yogurt, cereal, and bread consumed at meal time showed the greatest efficacy with the milk format[10]. In addition, the greater efficacy of solid food formats was observed in this meta-analysis only at high intakes, for which few strata were available, suggesting that this finding may have little practical relevance for phytosterol doses close to the recommended intake of 2 g/d.

Another factor that may affect the LDL-C–lowering efficacy of phytosterols is the number of portions consumed over the day. So far, only one trial has directly compared the effects of once per day compared to a 3 times/d intake of phytosterols provided in a fat-based spread consumed at meal time and showed no significant difference between the two frequencies of intakes[104]. Other studies in which once-per-day intake of phytosterols was assessed had significant reductions in LDL-C[13,16,21-24]. Nevertheless, the tendency towards a larger effect of multiple daily intakes than single intakes in the present meta-analysis may suggest that a modest effect of frequency of intake may exist but was not detected previously due to a lack of statistical power. Based on the main mechanism of action of phytosterols, which is considered to be the competition with cholesterol for micellar incorporation[8], it could be hypothesized that multiple daily intakes, by favoring the simultaneous presence in the gut of phytosterols, cholesterol, and bile

acids in repeated occasions during the day, would lead to a greater efficacy than a single intake. In fact, the mechanisms by which once-a-day intake of phytosterols would substantially lower LDL-C are not fully understood and warrant further investigations.

The present meta-analysis shows a clear impact of baseline LDL-C concentrations on the magnitude of the absolute decreases in LDL-C concentrations resulting from phytosterol consumption. The previous meta-analyses by Law *et al*.[2] and Katan *et al.*[3] had shown larger reductions in older subjects and it was hypothesized that this effect was due mainly to the higher baseline LDL-C concentrations with increasing age. The regression analysis performed in the present work, with no significant effect of age when baseline LDL-C concentrations were included in the model, confirmed this hypothesis. A recent meta-analysis of individual subject data[110] also showed larger absolute LDL-C reductions with plant stanol consumption when baseline concentrations were higher. The relative dose-response curves obtained from the present meta-analysis therefore present an advantage over the absolute curves by taking into account the baseline LDL-C levels.

The equations describing the continuous dose-response relationship offer a novel approach to predict the LDL-C-lowering effect of a given dose of phytosterols in populations, which could not be derived from previous data[3]. However, due to the large variability between studies in which the same dose of phytosterols was tested, the predicted effect should be used as an indication only. It could be argued that with such variability around the dose-response curve, a linear fit would have performed as well as the nonlinear relationship. To verify this hypothesis, the sum of the residuals between the observed LDL-C changes and the predicted changes obtained with the curve or with a linear fit crossing the y axis at zero (without a maximal reduction estimate) were compared. The sum of the residuals was considerably lower with the curve (370%) than with the linear relationship (475%), indicating that the nonlinear, physiologically relevant model is more appropriate.

The dose-response curves reported here were established by deliberately including studies in which phytosterol intakes could be as high as 10 g/d, because data obtained with such intakes could provide useful information regarding the expected plateau while still being realistically achievable through the consumption of phytosterol-enriched foods or supplements. A post hoc analysis showed that the

dose-response curve was not significantly influenced by the inclusion of studies with doses of 5-10 g/d. Indeed, the maximal LDL-C reduction (parameter a) and the incremental dose step (parameter b) were -13.26 (95% CI: -17.04; -9.48) and 1.22 (95% CI: 0.54; 1.90) for the curve including doses of < 5 g/d compared to -12.68 (95% CI: -15.38; -9.99) and 1.12 (95% CI: 0.62; 1.63) for the curve including doses of up to 10 g/d.

Although various background diets were used in the studies included in the present meta-analysis, comprising usual diets as well as low-fat, low-cholesterol diets consumed both in free-living or more controlled conditions, we did not investigate the potential impact of the background diet on the cholesterol-lowering efficacy of phytosterols. Results from one recent trial suggest that the cholesterol content of the background diet may have no significant effect on plant sterol efficacy[114]. Subject ethnicity is another factor that could potentially affect phytosterol efficacy beyond baseline LDL-C concentrations. Additional investigations to further study this factor, together with the effect of genetic polymorphisms, are warranted.

In summary, the present meta-analysis confirmed the significant LDL-C-lowering effect of phytosterols. Equations based on the underlying mechanism of action of plant sterols and stanols were determined to describe the dose-response relationship and could potentially be used to predict the LDL-C-lowering effect of a given phytosterol dose. However, the use of the curve as a prediction tool should be done cautiously due to the large intertribal variability at fixed doses. For the recommended intake of 2 g/d, the expected LDL-C-lowering effect of phytosterols is 29%. A reduction in LDL-C of ~10% would reduce the incidence of CHD by ~10-20%[2,4]. Although no direct evidence is available yet for the ability of phytosterols to lower CHD incidence, the well-documented cholesterol-lowering effect of phytosterols is the basis for recommendations to include phytosterols into strategies to lower LDL-C concentrations. The present meta-analysis did not show significant differences in efficacy of various food formats providing phytosterol doses around the recommended intake. However, at high phytosterol doses, solid food formats may have a more pronounced LDL-C-lowering effect than liquid food formats. Although not significant (P = 0.054), the possibility of an impact of frequency of intake over the day could not be excluded. Further investigations are warranted to gain more insights into the effect of these factors on the efficacy of phytosterols to lower LDL-C concentrations.

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Supplemental material

Supplemental Appendix 1 - Study quality assessment tool used to evaluate the quality of the studies included in the systematic review with meta-analysis of the LDL-C-lowering effect of phytosterols

Quality item	Coding	Explanation	Quality score
Random sequence generation	Adequate	Sequences obtained are unpredictable: computer generated random-numbers, table of random numbers, coin tossing, throwing dice, drawing lots or opaque, sealed envelopes	2
	Inadequate	Incomplete randomization but treatment sequences obtained seems unpredictable[a]	0
	Not specified	Term randomized or randomly allocated used, but no more explanation, without indication of inadequacy	2
	Not reported	Randomization seems adequate but no term "randomization" in text	0
Blinding of subjects	Adequate	Placebo described as indistinguishable from treatment ("single-blind or double-blind")[b]	1
	Inadequate	Placebo can be distinguished from treatment	0
	Not reported	No details or no term single- or double-blind used in text	0
Blinding of investigators	Adequate	Independent person or panel assessing the outcome or assessment by the investigator but in clear blind conditions ("double-blind")[b]	1
	Inadequate	Clearly reported that the investigator was not blinded	0
	Not specified	No statement on blinding procedures and not deducible	0
Eligibility criteria specified	Adequate	Clear explanation and good follow-up of in- and exclusion criteria	1
	Inadequate	In- and exclusion criteria incomplete or not in accordance to study objectives or not followed-up correctly[c]	0
	Not specified	No statement on procedures and not deducible	0

Quality item	Coding	Explanation	Quality score
Compliance	Adequate with supervision	Phytosterol intake under supervision	2
	Adequate without supervision	Phytosterol intake without supervision: Quantitative description of compliance (>85%) and no difference in compliance between placebo and treatment groups	1.5
	Inadequate	Difference in compliance between placebo and treatment group or very low compliance (<85%) for both groups [d]	0
	Not specified	Only qualitative description of compliance ("good compliance")	0.5
	Not reported	No statement on procedures and not deducible	0
Carry-over effect taken care of for cross-over trials	Adequate	▪ If treatment phase <3 weeks: wash-out period ≥2 weeks included to prevent carry-over effects ▪ If treatment duration ≥3 weeks: no wash-out necessary [e]	1
	Inadequate	Wash-out period insufficient (<2 weeks) or no wash-out period between treatment phases when treatment phases are less than 3 weeks long	0
	Not specified	No statement on procedures and not deducible	0

[a] Inadequate random sequence generation: for example, when the sequence of treatments was not the same for every subject (which is good), but the subjects did not receive all the treatments that were under investigation (e.g. 5 test foods, but the subjects had to consume only 4 out of 5 test foods in 4 different test phases). Another example of inadequate random sequence generation is when the numbers of subjects in the control group and in the test group were noticeably different (e.g. control group with 90 participants and treatment group with 50 participants).

[b] Blinding was scored as follows: double-blind was given 2 points, single-blind was given 1 point and open label was given 0 points.

[c] An example of eligibility criteria not followed-up correctly: the eligibility criteria specified that only subjects with BMI <30 would be selected, but from the reported data it was obvious that subjects with BMI >30 were included.

[d] The threshold of adequate compliance (≥85%) is quite high, but it was considered that high compliance was important, otherwise it could not be assumed that the dose of phytosterols truly consumed by the subjects was as reported in the method section of the article.

[e] Carry-over taken care of: Cross-over trials have more potential to be given the highest quality score on this criterion ("adequate" = 1 point) than to be given the lowest score ("inadequate" or "not specified" = 0 points) for the following reasons:

- Duration of the treatment phase was included in the exclusion criteria (exclude when duration is <2 weeks).
- When there was an active treatment period of 3 weeks before crossing over, it was justified to have no wash out period at all, and therefore such a study would still be judged as "adequate" for this quality criterion.
- When carry-over effects were explicitly reported in the articles, only data of the first phase were extracted for the meta-analysis, and therefore the carry-over effect was automatically taken care of and would have no practical influence on the outcome of the meta-analysis. Nevertheless, this criterion was kept in the quality assessment tool, because it was necessary to evaluate whether carry-over was taken care of for studies with treatment durations between 2-3 weeks. Indeed, the absence of an appropriate wash-out period could have biased the outcome of such studies.

Classification of the studies according the quality assessment

For each study or strata, the overall quality score was calculated by adding the individual criteria scores. Adequate random sequence generation, overall blinding and compliance were given 2 points because they were judged to potentially have the greatest impact on the outcome of the meta-analysis. The maximal quality score that could be ascribed to a study was 7 in case of a parallel trial and 8 in case

of a cross-over trial. The cut-off point used to distinguish low and good quality trials was the mean quality score calculated from all trials with the corresponding design. For parallel trials, the mean quality score was 5.4, and a rounded cut-off point of 5.5 was used. Therefore, trials deserving less than 5.5 points were classified as low quality trials, while trials given 5.5 points or more were judged to be of good quality. For cross-over trials, the mean quality score was 6.1 and a cut-off point of 6.0 was used. Trials were of low quality when given 6.0 points or less, or of good quality when given more than 6.0 points.

Supplemental Appendix 2 - Equations used to calculate the absolute and relative net changes in LDL-C as well as the variance measures for the meta-analysis of the LDL-C-lowering effect of phytosterols

A. Calculation of the absolute net changes

Parallel trials:

$$\text{Absolute net change in LDL} - \text{C} = (\text{LDL}_{\text{Tendpoint}} - (\text{LDL}_{\text{Tbaseline}}) - (\text{LDL}_{\text{Cendpoint}} - \text{LDL}_{\text{Cbaseline}}) \quad (1)$$

where $\text{LDL}_{\text{Tendpoint}}$ = mean LDL-C at end-of-intervention in the treatment group
$\text{LDL}_{\text{Tbaseline}}$ = mean LDL-C at baseline in the treatment group
$\text{LDL}_{\text{Cendpoint}}$ = mean LDL-C at end-of-intervention in the control group
$\text{LDL}_{\text{Cbaseline}}$ = mean LDL-C at baseline in the control group

Cross-over trials:

$$\text{Absolute net change in LDL} - \text{C} = \text{LDL}_{\text{Tendpoint}} - \text{LDL}_{\text{Cendpoint}} \quad (2)$$

where $\text{LDL}_{\text{Tendpoint}}$ = mean LDL-C at the end of the intervention period
$\text{LDL}_{\text{Cendpoint}}$ = mean LDL-C at the end of the control period

B. Calculation of the relative net changes

Parallel trials:

$$\text{Relative net change in LDL} - \text{C} = \%\Delta\text{LDL}_{\text{T}} - \%\Delta\text{LDL}_{\text{C}} \quad (3)$$

where

$$\%\Delta LDL_T = 100 \times \frac{LDL_{Tendpoint} - LDL_{Tbaseline}}{LDL_{Tbaseline}} \quad (4)$$

$$\%\Delta LDL_C = 100 \times \frac{LDL_{Cendpoint} - LDL_{Cbaseline}}{LDL_{Cbaseline}} \quad (5)$$

Cross-over trials:

$$\text{Relative net change in LDL} - C = 100 \times \frac{LDL_{Tendpoint} - LDL_{Cendpoint}}{LDL_{Cendpoint}} \quad (6)$$

C. Calculation of the variance measures

To derive SEs from SDs and CIs the following formulas were used:

$$SE = \frac{SD}{\sqrt{n}} \quad (7)$$

$$SE = \frac{(\text{lower limit} + \text{upper limit})/2 - \text{lower limit}}{Z_{\alpha/2}} \quad (8)$$

where n = number of subjects per group/period

$Z_{\alpha/2}$ = normal deviate for 2-sided 100(1-α)%

If not provided, the within-trial variance measures of the absolute net changes were estimated according to the equations detailed below. For these calculations, the method of Follmann *et al.* (J Clin Epidemiol 1992; 45:769-773) was used, assuming a correlation between baseline and endpoint lipid levels for parallel trials, and between lipid levels at the end of the phytosterol treatment and the control treatment for cross-over trials. The 0.80 within-individual correlation coefficient was estimated based on phytosterol trials for which both the within-trial variance measure for the net change and the SEs at (baseline and) endpoint were available for the control and the treatment groups/periods separately.

Parallel trials:

Variance measures of the relative or absolute changes from baseline for the treatment and control groups were used. Otherwise, the variance measures of baseline and endpoint lipid levels were used to estimate the variance measure of the net change:

$$SE_{\text{net change}} = \sqrt{SE_T^2 + SE_C^2} \quad (9)$$

$$SE_T = \sqrt{(SE_{\text{Tbaseline}}^2 + SE_{\text{Tendpoint}}^2) - 2\,r\,(SE_{\text{Tbaseline}}^2)(SE_{\text{Tendpoint}}^2)} \quad (10)$$

$$SE_C = \sqrt{(SE_{\text{Cbaseline}}^2 + SE_{\text{Cendpoint}}^2) - 2\,r\,(SE_{\text{Cbaseline}}^2)(SE_{\text{Cendpoint}}^2)} \quad (11)$$

where SE_T is the SE of the change within the treatment group
SE_C is the SE of the change within the control group
$SE_{Tbaseline}$ is the SE at baseline in the treatment group
$SE_{Tendpoint}$ is the SE at the end-of-intervention in the treatment group
$SE_{Cbaseline}$ is the SE at baseline in the control group
$SE_{Cendpoint}$ is the SE at the end-of-intervention in the control group
r = 0.80 and is the within-subject correlation between LDL-C measurements made before and after the intervention

Cross-over trials:

The variance measure of the net change was estimated using only the variances of endpoint lipid levels:

$$SE_{\text{net change}} = \sqrt{(SE_T^2 + SE_C^2) - 2\,r\,(SE_T^2)(SE_C^2)} \quad (12)$$

where SE_T is the SE at the end of the intervention period
SE_C is the SE at the end of the control period
r = 0.80 and is the within-subject correlation between LDL-C measurements made after the control and the phytosterol treatment

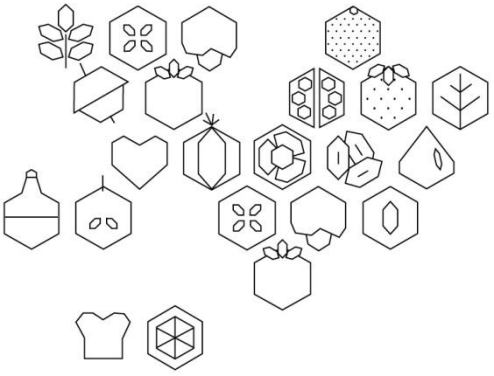
Supplemental Appendix 3 - Definition of the subgroups of treatment or study characteristics used in the meta-analysis of the LDL-C-lowering effect of phytosterols

Treatment or study characteristic	Categories compared	Categories	Definition categories
Saturation of phyosterols	Plant stanols vs. plant sterols	Plant stanols	Strata using plant stanols
		Plant sterols	Strata using plant sterols
Food format	Solid vs. liquid food formats	Solid foods	Strata using margarine, butter, mayonnaise, yoghurt, hard cheese, fresh cheese, beef, cereals, cereal bar, bread, tortilla chips, chocolate, bakery products, salad dressing, shortening, vegetable oil, tablets and capsules
		Liquid foods	Strata using yoghurt-drink, milk, orange juice, lemonade, vegetable juice and milk tea
	Low-fat vs. high-fat food formats	Low-fat foods	Strata using yoghurt, yoghurt-drink, hard cheese, fresh cheese, beef, milk, orange juice, vegetable juice, milk tea, cereals, cereal bar, tortilla chips, chocolate, bakery products, tablets and capsules
		High-fat foods	Strata using margarine, butter, mayonnaise, shortening, salad dressing and vegetable oil
	Non-dairy vs. dairy food formats	Non-dairy foods	Strata using margarine, butter, mayonnaise, beef, orange juice, lemonade, vegetable juice, milk tea, cereals, cereal bar, bread, tortilla chips, chocolate, bakery products, salad dressing, shortening, vegetable oil, tablets, capsules
		Dairy foods	Strata using milk, yoghurt and yoghurt drink, hard cheese, fresh cheese
Quality aspects	Low vs. high compliance strata	Low compliance strata	Strata with compliance score <1
		High compliance strata	Strata with compliance score ≥1
	Bad vs. good randomized strata	Bad randomized strata	Strata with randomization score <1
		Good randomized strata	Strata with randomization score ≥1
	Low quality vs. high quality strata	Low quality strata	Parallel strata with quality score <5.5 and cross-over strata with quality score ≤6
		High quality strata	Parallel strata with quality score ≥5.5 and cross-over strata with quality score >6

Supplemental Appendix 4 - General characteristics of the trials included in the meta-analysis of the LDL-C-lowering effect of phytosterols

	Number of strata
Overall study characteristics	
Total number of trials	84
Total number of strata	141
Parallel design	73
Cross-over design	68
Overall study quality	
Good	68
Low	69
Study participants	
Nationality	
European	63
North-American	42
Australian	19
Asian	16
South-American	1
Health status	
Apparently healthy (regardless of baseline lipid levels)	116
Subjects with specific apoE phenotypes	6
Subjects with health problems	16
Type II diabetics	4
Statin users	9
Previous myocardial infarction	1
Family members of subjects with familial hypercholesterolemia	2
Information could not be recorded (publication could not be translated)	3
Baseline anthropometry	
Mean age: Range 22.7 to 66.0 years	135
Mean baseline BMI: Range: 22.0 to 31.0 kg/m^2	127
Mean baseline body weight Range: 63.0 to 88.3 kg	56
Body weight change mentioned	110
No change	76
Non-significant change	25
Small (<2 kg) body weight change	22
Body weight change >2kg	0
Baseline plasma LDL-C concentrations reported	23
Pooled overall LDL-C concentration at baseline: 3.86 mmol/L (95% CI: 3.77; 3.98)	23
Phytosterol treatment	
Mean phytosterol dose: 2.15 g/d (range: 0.45-9.00 g/d)	141
Duration: 21-182 d	141
Type of phytosterols	
Plant sterols	74
Plant stanols	53
Combination of plant sterols and stanols	14

	Number of strata
Phytosterol source	
Soybean oil	39
Tall oil	31
Undefined vegetable oil or combination of various phytosterol sources	29
Not specified	42
Phytosterol form	
Fatty acid esters	102
Fatty acids from rapeseed oil	42
Fatty acids from sunflower oil	25
Others or information not available	35
Free form, directly dissolved or mixed in the food products	39
Food formats	
Fat-based foods	88
Mix of fat-based and non fat-based foods	3
Non fat-based foods	50
Dairy food formats	26
Mix of dairy and non-dairy food formats	1
Non-dairy food formats	114
Liquid food formats	24
Mix of liquid and solid food formats	1
Solid food formats	116
Frequency and time of intake	
Multiple daily intakes	87
Single daily intakes	14
Frequency of intake not specified	40
Consumption with or without a meal was reported	106
Consumption without a meal	4
Meal(s) with which phytosterols were consumed was reported	73
Single daily intake	14
Breakfast	7
Lunch	5
Dinner	2
Multiple daily intakes	59
All three meals	35
Various combinations of two meals	22
Before two meals	2
Background diet	
Typical (usual) dietary pattern in a free-living setting	87
Typical diet provided	11
Free-living “heart healthy” diet (low in fat and cholesterol) and/or dietary advice	33
At least 2 meals provided every day to the participants	17



Chapter 3

LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: a meta-analysis of randomised controlled studies

Rouyanne T. Ras
Johanna M. Geleijnse
Elke A. Trautwein

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Abstract

Phytosterols (comprising plant sterols and plant stanols) have been proven to lower low-density lipoprotein cholesterol (LDL-C) concentrations. The dose-response relationship for this effect has been evaluated in several meta-analyses by calculating averages for different dose ranges or by applying continuous dose-response functions. Both approaches have advantages and disadvantages. So far, the calculation of averages for different dose ranges has not been done for plant sterols and stanols separately. The objective of the present meta-analysis was to investigate the combined and separate effects of plant sterols and stanols when classified into different dose ranges. Studies were searched and selected based on predefined criteria. Relevant data were extracted. Average LDL-C effects were calculated when studies were categorized by dose, according to random-effects models while using the variance as weighing factor. This was done for plant sterols and stanols combined and separately. In total, 124 studies (201 strata) were included. Plant sterols and stanols were administered in 129 and 59 strata, respectively; the remaining used a mix of both. The average phytosterol dose was 2.1 (range 0.2-9.0) g/d. Phytosterol intakes of 0.6-3.3 g/d were found to gradually reduce LDL-C concentrations by, on average, 6-12%. When plant sterols and stanols were analyzed separately, clear and comparable dose-response relationships were observed. Studies carried out with phytosterol doses exceeding 4 g/d were not pooled, as these were scarce and scattered across a wide range of doses. In conclusion, the LDL-C-lowering effect of both plant sterols and stanols continues to increase up to intakes of approximately 3 g/d to an average effect of 12 %.

Introduction

Phytosterols, comprising both plant sterols and plant stanols, are compounds that naturally occur in all foods of plant origin such as vegetable oils, nuts, seeds, grain products, fruits and vegetables. The intake of naturally occurring phytosterols from the general diet is about 200-400 mg/d[1-3]. Higher phytosterol intakes can be achieved by consuming vegetable-based diets such as vegetarian diets for which phytosterol intakes are almost doubled[4,5] or by consuming food products enriched with phytosterols. Phytosterol-enriched foods are well known for their total cholesterol and especially low-density lipoprotein cholesterol (LDL-C)-lowering properties[6]. Having elevated LDL-C concentrations is one of the most important risk

factors for CVD. Phytosterol-enriched foods are considered a valuable option as part of healthy diet and lifestyle changes in the management of hypercholesterolaemia[7,8].

Since the 1950's, abundant research into the LDL-C-lowering effect of phytosterols has been carried out and this wealth of evidence has been summarized in several meta-analyses[6,9-12]. In these meta-analyses, the dose-response relationship for the LDL-C-lowering efficacy of phytosterols has been investigated. The meta-analyses carried out by Law[9], Katan *et al*.[6] and Abumweis *et al.*[10] described a dose-response relationship based on the calculation of average LDL-C-lowering effects for different categories of phytosterol doses. More recently, Demonty *et al.*[11] have investigated a continuous dose-response relationship, as determined by a first-order elimination function based on the assumption that processes involved in cholesterol transport and absorption are saturable. Musa-Veloso *et al.*[12] subsequently established similar continuous dose-response curves, but this time for plant sterols and stanols separately. Overall, these analyses concluded that with an increasing dose of phytosterols, the LDL-C-lowering effect increases, but that this effect tapers off at doses of 2-3 g/d.

The applied approaches used to study the dose-response relationship differ between showing average effects for ranges of doses and establishing continuous dose-response functions. Both approaches have advantages and disadvantages. Establishing a continuous dose-response relationship has the advantage that it allows predicting effects for a given dose of phytosterols. However, the shape of the curve largely depends on the distribution of studies across the entire range of doses; if this distribution is not balanced, this type of analysis may become vulnerable for over- or underestimation of the estimated effects at certain doses. For example, in the meta-analysis carried out by Musa-Veloso *et al.*[12], the depicted plant sterol curve clearly underestimated the effects of plant sterols at doses of 2.7-3.3 g/d. As a result, it was suggested that a larger maximal lowering effect exists for plant stanols than for plant sterols. The calculation of average effects for predefined ranges of phytosterol doses is less sensitive to potential over- or underestimation, but this approach does not allow predicting effects over a continuous range of doses.

So far, the calculation of weighted averages for different dose ranges has not been done for plant sterols and stanols separately. Such an analysis would provide useful insights into the comparison of the LDL-C-lowering efficacy of these two types of phytosterols for which some debate exists[12-15]. Therefore, the main objective of the present analysis was to investigate the combined and separate LDL-C-lowering effects of plant sterols and stanols when classified into different dose ranges. It was hypothesized that plant sterols and stanols would exert a similar LDL-C-lowering effect at least up to intakes of, on average, 3 g/d[16].

Experimental methods

Search strategy and selection of eligible studies

To retrieve potentially relevant human studies eligible for the present analysis, we relied on the systematic searches carried out by the authors of the two most recent meta-analyses[11,12] that used almost identical search strategies. In the meta-analysis carried out by Demonty *et al.*[11], eighty-one studies with 141 study arms were included, whereas in the more recent meta-analysis carried out by Musa-Veloso et al.[12], 114 studies with 182 study arms were included. To retrieve eligible studies that had been published after these two meta-analyses, an additional search was carried out using nine databases (MEDLINE, Embase, BIOSIS, CAB Abstracts, FROSTI, Food Science and Technology Abstracts, Chemical Abstracts, PASCAL and AGRICOLA) from September 2010 to September 2011. Again, identical search terms were used, limited to human studies with no restriction on language.

Based on the criteria described in the two most recent meta-analyses[11,12], we formulated the following criteria for selecting more recently published studies: (1) randomized controlled studies in human adults; (2) treatment with 4-desmethylsterols and/or 4-desmethylstanols extracted from vegetable oils such as soyabean oil, rapeseed oil and tall oil (so no ferulated phytosterols such as those from rice bran oil or shea nut oil); (3) investigation of blood lipids as primary or secondary outcomes; (4) absence of a co-intervention from which the intake of phytosterol-enriched foods or supplements could not be isolated; (5) availability of relevant LDL-C data; (6) use of proper placebo in the control group/period; (7) consumption of phytosterols for at least 2 weeks; (8) dose of phytosterols not exceeding 10 g/d; (9) no studies including colectomized patients because it cannot be excluded that colectomy does not have an impact on efficacy.

Data extraction and statistical analysis

For the present analysis, the following data were extracted: reference information (first author and year of publication); study design (parallel or cross-over); number of subjects (sample size); test product characteristics (dose, type of phytosterols (plant sterols or plant stanols or mix) and food format); the placebo-adjusted relative (%) change in LDL-C concentration plus accompanying variance measure. In case relative changes were not reported, these were calculated as follows:

For parallel studies,

$$LDL_{change} = \%\Delta LDL_{treatment} - \%\Delta LDL_{control}$$

where

$$\%\Delta LDL_{treatment} = 100 * \frac{LDL_{treatment\_end} - LDL_{treatment\_baseline}}{LDL_{treatment\_baseline}}$$

$$\%\Delta LDL_{control} = 100 * \frac{LDL_{control\_end} - LDL_{control\_baseline}}{LDL_{control\_baseline}}$$

For cross-over studies,

$$LDL - C_{change} = 100 * \frac{LDL_{treatment\_end} - LDL_{control\_end}}{LDL_{control\_end}}$$

When LDL-C concentrations were measured at various time points during the intervention, the concentration corresponding to or closest to the 4-week time point was taken for the analysis. When variance measures of the relative changes were not provided and could not be retrieved based on P values or 95% CI, these were calculated using variance measures at baseline and end of the intervention in active and placebo groups/periods assuming, based on an earlier investigation[17], a within-subject correlation coefficient of 0.8.

Human intervention studies were divided into six categories based on their phytosterol dose: dose <1.0 g/d; 1.0≤ dose <1.5 g/d; 1.5≤ dose <2.0 g/d; 2.0≤ dose <2.5 g/d; 2.5≤ dose <3.0 g/d; 3.0≤ dose ≤4.0 g/d. This approach was chosen so that the incremental dose step was 0.5 g/d except for the lowest and highest categories as the number of studies using doses <0.5 and between 3.5 and 4.0 g/d was rather

limited (n = 6 each). Study arms with doses exceeding 4 g/d were scarce (n = 5) and scattered across a wide range of phytosterol doses (5.8-9.0 g/d); therefore, pooling these studies into a single category was judged to be inappropriate; these studies were solely used for descriptive purposes. For each study, the PS dose was determined by the actual dose administered; when not reported, the intended dose was used. Throughout this article, the doses of plant sterols/stanols are expressed as free (unesterified) plant sterol/stanol equivalents, rounded off at one decimal.

Pooled LDL-C effects were calculated while studies were categorised based on their PS dose (i.e., subgroup analysis with subgroups defined by the PS dose), using random-effects models according to the methods described by DerSimonian & Laird[18]. Random-effects models were used as they take into account the variation in LDL-C-lowering effects observed within and between studies. Studies were weighted by the inverse of their variance ($1/SE^2$). Analyses were carried out for plant sterols and stanols combined and separately. When required, a more in-depth analysis was carried out to investigate the impact of food format on the LDL-C-lowering efficacy of PS. The pooled estimates and accompanying 95% CI were determined using the PROC MIXED function of the SAS System (version 9.2; SAS Institute).

Results

Overview of the included studies

In total, 124 human studies with a total of 201 study arms were included in the present analysis. In 116 study arms, a parallel design was used whereas in 85 study arms, a cross-over design was used. Plant sterols and stanols were administered in 129 and 59 study arms, respectively; in the remaining 13 study arms, a mix of plant sterols and stanols was administered. The number of subjects per study arm was, on average, 48 (range: 7-201). The average phytosterol dose was 2.1 (range: 0.2-9.0) g/d. In most of the studies, (low-fat) margarines/spreads or dairy-type products were used for enrichment with phytosterols; other food formats included, among others, cereals, mayonnaise, salad dressing, soya products, bakery products, orange juice and vegetable oils. An overview of the included studies is given in **Supplemental Appendix 1**.

LDL-cholesterol-lowering effect of plant sterols and stanols combined and separately

The average phytosterol doses and relative effects on LDL-C concentrations for each of the defined dose ranges are summarized in **Table 1**. When plant sterols and stanols were analyzed together, phytosterol intakes were found to reduce LDL-C concentrations in a dose-dependent manner (P <0.001; **Figure 1**). When plant sterols and stanols were analyzed separately, clear and comparable dose-response relationships were observed (**Figure 2**). The impact of dose was significant in both analyses (P <0.001 for plant sterols and P = 0.001 for plant stanols).

Table 1. *Average LDL-cholesterol-lowering effect for different dose ranges of phytosterols combined and separately for plant sterols and stanols (mean values and 95% CI).*

Phytosterol dose category (g/d)[a]	Study arms (n)	Average phytosterol dose (g/d)	Average LDL-C effect (%)					
			Combined		Plant sterols		Plant stanols	
			Mean	95% CI	Mean	95% CI	Mean	95% CI
Dose <1.0	24 (1 mix, 22 sterol, 1 stanol)	0.6	-5.7	-7.1; -4.4	-5.6	-7.1; -4.2	-7.4	-15.2; 0.4
≥1.0 dose <1.5	13 (2 mix, 9 sterol, 2 stanol)	1.1	-6.4	-8.2; -4.6	-6.5	-8.6; -4.4	-6.3	-12.0; -0.6
≥1.5 dose <2.0	55 (7 mix, 39 sterol, 9 stanol)	1.7	-7.6	-8.4; -6.8	-7.6	-8.6; -6.7	-6.7	-8.8; -4.7
≥2.0 dose <2.5	60 (2 mix, 40 sterol, 18 stanol)	2.1	-8.4	-9.2; -7.6	-8.0	-9.0; -7.0	-10.0	-11.3; -8.6
≥2.5 dose <3.0	17 (0 mix, 6 sterol, 11 stanol)	2.6	-10.3	-11.8; -8.9	-10.5	-13.7; -7.3	-10.4	-11.7; -9.1
≥3.0 dose ≤4.0	27 (1 mix, 11 sterol, 15 stanol)	3.3	-12.4	-13.6; -11.2	-12.3	-14.0; -10.6	-12.5	-14.1; -10.8
P (dose effect)			<0.001		<0.001		0.001	

LDL-C, low-density lipoprotein cholesterol

[a] Studies carried out using doses exceeding 4 g/d were not included in the present analysis, as these were scarce and scattered across a wide range of doses; clustering them was judged to be inappropriate.

In the present analysis, in the dose category 2·0≤ dose <2·5 g/d, an apparent difference of 2% in LDL-C-lowering efficacy was observed between plant sterols and stanols. In post hoc analysis that was set up to investigate factors that might explain this finding, it was observed that the consistency of the food format (either solid/edible or liquid/drinkable) may play a role. In fact, within this particular dose category, fifteen of forty plant sterol studies used liquid food formats, whereas only four of eighteen stanol studies used this type of food format. Irrespective of the type of phytosterols used, liquid foods lowered LDL-C concentrations by, on

average, 6.5%, whereas solid foods lowered LDL-C concentrations by, on average, 9.2% (P = 0.003).

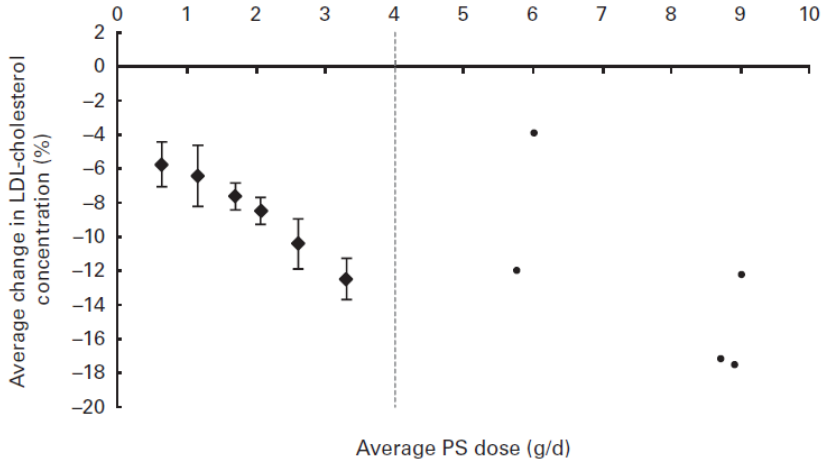

Figure 1. *Average effects on LDL-cholesterol concentration for different dose ranges of phytosterols (PS) up to 4 g/d. The dots represent outcomes of single high-dose studies that were not pooled as these were scarce and scattered across a wide range of doses. Values are means, with 95% CI represented by vertical bars.*

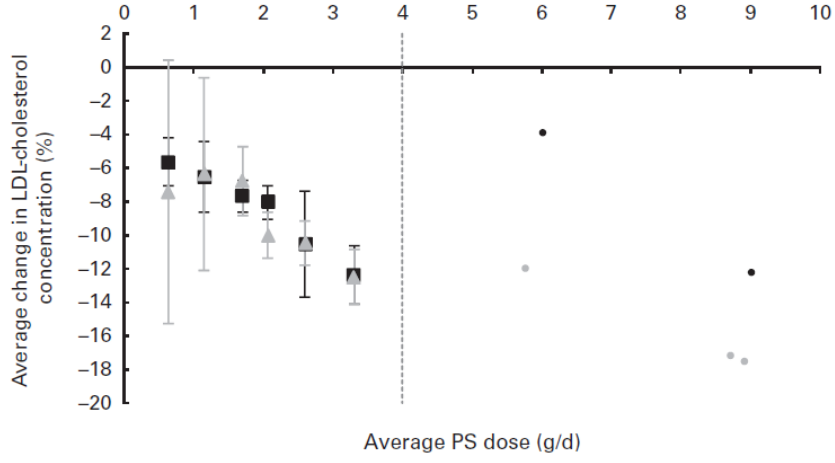

Figure 2. *Average effects on LDL-cholesterol concentration for different dose ranges of phytosterols (PS), separately for plant sterols (black squares) and plant stanols (grey triangles). The dots represent outcomes of single high-dose studies that were not pooled as these were scarce and scattered across a wide range of doses. Values are means, with 95% CI represented by vertical bars.*

Discussion

The present meta-analysis based on dose ranges showed that plant sterols and stanols lower LDL-C concentrations to a similar extent and in a dose-dependent manner, at least up to approximately 3 g/d. The observed comparability between plant sterols and stanols with regard to their cholesterol-lowering potential is in line with the findings of a recent meta-analysis[16]. In this meta-analysis[16], fourteen studies that side by side compared the LDL-C-lowering efficacy of plant sterols with that of plant stanols at doses ranging from 0.6 to 3.3 g/d were included. Of the fifteen study arms reporting usable LDL-C data, seven study arms showed a non-significantly larger LDL-C-lowering effect for plant sterols than for plant stanols, whereas eight study arms showed a relatively larger effect for plant stanols than for plant sterols. Overall, it was concluded that plant sterols and stanols do not have statistically or clinically relevant differing effects on blood lipids. At higher intakes (>4 g/d), some individual studies suggest a larger LDL-C-lowering effect for plant stanols[19,20] than for plant sterols[21]. However, high-dose studies are scarce and scattered across a wide range of phytosterol doses (5.8-9.0 g/d). For proper high-dose equivalence testing, a direct comparison study would be needed with subjects on either high-dose plant sterol or high-dose plant stanol treatment being studied under the same conditions. As such a study has so far not been carried out, drawing conclusions on potential differences in efficacy between plant sterols and stanols at higher doses is not justified, as has been recently discussed by Plat *et al.*[13].

The dose dependency of the LDL-C-lowering effect of phytosterols has previously been demonstrated in several meta-analyses[6,9-12] and in individual dose-response studies[19,22-24]. So far, meta-analyses have suggested that the LDL-C-lowering effect of phytosterols tapers off at intakes of 2-3 g/d with little additional benefit at higher intakes[6,11]. Consequently, several health authorities have included 2 g/d of phytosterols from enriched foods as part of their diet and lifestyle guidelines in the management of hypercholesterolaemia [7,8,25]. From the present analysis, it appears that at least up to approximately 3 g/d of phytosterols, there is a proportional dose-response effect. As the inhibition of cholesterol absorption by phytosterols is probably a saturable process, some tapering-off effect would, however, be expected, but probably at doses slightly higher than 3 g/d. If indeed phytosterol intakes >3 g/d lead to a greater LDL-C benefit, this would be meaningful from a clinical viewpoint as additional LDL-C-lowering could lead to a greater CVD risk

reduction. However, the practical implications of higher phytosterol intakes, such as the technical feasibility of incorporating higher amounts of phytosterols into foods, cost-benefit aspects and, especially, the compliance of consumers, need to be considered. Based on research in populations that actually use foods with added phytosterols, it appears that the intake of phytosterols in real life is far below the recommendation[26,27]; on average, users consume 14 g/d of phytosterol-enriched margarine, which corresponds to a phytosterol intake of approximately 1 g/d. Therefore, encouraging people to consume phytosterols at amounts exceeding approximately 3 g/d seems unrealistic. In addition, because of the observations of premature atherosclerosis in rare homozygous sitosterolaemic patients[28] and due to epidemiological evidence suggesting a positive association between plasma plant sterol concentrations and CVD risk[29], some concerns have been raised related to the increase in plasma plant sterol concentrations following high intakes of plant sterols from enriched foods. However, a recent meta-analysis summarized the totality of observational studies that investigated the association between modestly elevated plasma plant sterol concentrations and CVD risk and concluded that such an association does not exist[30]. Furthermore, plasma plant sterol concentrations after the intake of foods with added plant sterols remain below 1% of total sterol concentrations circulating in the blood[17]. All in all, taking these aspects into account, the current recommendations to consume 2-3 g/d of phytosterols for achieving a significant cholesterol-lowering effect seem to be still valid.

The use of different approaches to investigate dose-response relationships in meta-analyses may sometimes lead to different conclusions being drawn. For instance, Musa-Veloso *et al.*[12] previously concluded that the maximal LDL-C-lowering efficacy was greater for plant stanols (16.4 %) than for plant sterols (8.3 %) when analysing continuous dose-response curves. Also in the meta-analysis carried out by Demonty *et al.*[11], a non-significant 6.7% difference in maximal cholesterol-lowering efficacy was observed between plant stanols and sterols based on continuous analysis. Such an approach offers the opportunity to predict the LDL-C-lowering effect of a given phytosterol dose. However, the applied model seems to underestimate the LDL-C-lowering effect of plant sterols at doses of about 3 g/d. It is likely that this has affected the shape of the overall dose-response curve for plant sterols. This underestimation may have been caused by an unequal distribution of studies across the entire dose range. In fact, the availability of a large number of

low-dose sterol studies with relatively high efficacy probably pulled the plant sterol curve towards a more curvy shape, whereas the stanol curve was mostly influenced by high-dose studies; indeed the number of stanol studies carried out using low doses (<1.5 g/d) was limited. The calculation of average effects for different dose ranges, as has been done in the present analysis, is less influenced by an imbalance of data points across the entire dose range. Moreover, this approach offers the opportunity to better take into account the large between-study variation by means of using random-effects models. On the other hand, one of the limitations of the dose-response approach is that the definition of the dose ranges is rather subjective. Especially between 1·5 and 2·5 g/d, small differences in cut-off values (e.g. <2 or ≤2 g/d) could have a significant impact on the distribution of studies in the adjacent dose ranges and subsequently on the pooled averages for these particular dose ranges. In the present analysis, dose steps of 0·5 g/d were used between adjacent dose ranges, except for the outmost dose ranges, as these ranges would otherwise become too small. Although this approach led to a symmetrical distribution of the number of studies in the different dose ranges (n 24, n 13, n 55, n 60, n 17 and n 27 in ascending ranges), the ratio of plant sterol studies:plant stanol studies was disproportional by this definition (22:1, 9:2, 39:9, 40:18, 6:11 and 11:15, respectively). In any case, one should acknowledge that none of the dose-response approaches is ideal and should consider the pros and cons of the dose range vs. the continuous approach before deciding which approach to choose for the research questions being addressed.

Besides the limitations of the applied dose-response method as discussed above, some other limitations should be mentioned. The present analysis was not set up as a typical meta-analysis, but in fact builds on previous published meta-analyses[11,12] by highlighting the importance of using different analysis techniques. Therefore, heterogeneity tests and publication bias tests were not carried out. However, as between-study variation can never be ruled out, we decided beforehand to use random-effects models that take into account some of this variation. In addition, the baseline cholesterol concentration and the dose of phytosterols have been shown to be important factors affecting the size of the LDL-C-lowering effect of phytosterols[6,10,11]; by looking at relative changes and dose-response relationships, we believe that we have addressed these two important factors. Nevertheless, we cannot exclude that confounding by other factors, such as differences in food formats across the range of phytosterol doses, might have

affected the study outcomes. For example, in the present analysis, we found slightly lower efficacy for plant sterols than for plant stanols in the dose category 2.0≤ dose <2.5 g/d; this was probably due to a larger number of liquid food formats among the plant sterol studies than among the plant stanol studies. Phytosterols in liquid foods vs. solid foods might be less effective at lowering cholesterol concentrations due to a shorter transit time in the gastrointestinal tract. Also, liquid foods (drinks) are not per definition consumed together with a meal; sufficient ingestion of food (i.e., fat) is required to trigger bile release for phytosterols to optimally compete with cholesterol for micellar incorporation and subsequently to optimally inhibit cholesterol absorption[31]. Given the substantial number of studies included, we assume that publication bias had not affected the findings severely. Lastly, the quality of studies was not assessed as we believe that rating study quality is a rather subjective exercise and it has not been shown that excluding low-quality studies leads to different conclusions[10].

In summary, the present analysis showed that the LDL-C-lowering effect of phytosterols continues to increase up to intakes of approximately 3 g/d to an average effect of 12%. This was shown for both plant sterols and stanols. The importance of considering the advantages and disadvantages of different meta-analytical dose-response methods was discussed; future studies should decide on the most suitable dose-response approach depending on the research questions being addressed and the data available.

Acknowledgements

The authors' contributions are as follows: R. T. R. and E. A. T. formulated the research questions and conducted the research. All authors designed the research, interpreted the data and wrote the paper. R. T. R. and E. A. T. are employed by Unilever Research and Development Vlaardingen. Unilever markets food products enriched with plant sterols. J.M.G. has no conflicts of interests to declare.

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Supplemental material

Supplemental Appendix 1 - Overview of the studies

Reference	Design	Sample size	Type of phyto-sterols	Food format	Dose of phytosterols[a]	Relative change in LDL-C	
						Effect (%)	SE
AbuMweis et al. 2006 #1[b]	X	30	sterol	margarine	1.7	-1.4	3.1
AbuMweis et al. 2006 #2	X	30	sterol	margarine	1.7	-1.4	3.2
Algorta-Pineda et al. 2005	P	32	stanol	yoghurt drink	2.0	-8.4	3.9
Alhassan et al. 2006	P	26	stanol	low-fat margarine	3.0	-20.4	6.6
Andersson et al. 1999	P	40	stanol	low-fat margarine	1.9	-7.2	2.9
Athyros et al. 2011	P	100	stanol	margarine	2.0	-13.8	1.3
Banuls et al. 2010	P	40	sterol	low-fat milk	2.0	-8.1	2.8
Banuls et al. 2011	P	75	sterol	low-fat milk	2.0	-9.9	2.8
Beer et al 2000 #1	P	47	mix	low-fat milk	0.9	-7.4	3.5
Beer et al 2000 #2	P	52	mix	low-fat milk	1.8	-8.6	3.3
Beer et al 2000 #3	P	47	mix	low-fat milk	3.6	-13.2	3.4
Blair et al. 2000	P	141	stanol	margarine	2.9	-9.0	1.8
Blomqvist et al. 1993	P	66	stanol	mayonnaise	3.4	-9.9	3.3
Cater et al. 2005 study 1[c] #1	X	8	stanol	margarine	2.0	-12.3	3.3
Cater et al. 2005 study 1 #2	X	8	stanol	margarine	3.0	-13.0	2.6
Cater et al. 2005 study 1 #3	X	8	stanol	margarine	4.0	-13.6	2.3
Cater et al. 2005 study 2	X	13	stanol	margarine	3.0	-13.0	2.3
Cater et al. 2005 study 3	x	10	stanol	margarine	3.0	-14.9	3.3
Chen et al. 2009	x	22	sterol	dressing and margarine	3.3	-12.4	1.9
Christiansen et al. 2001 #1	p	92	sterol	margarine	1.5	-6.4	1.9
Christiansen et al. 2001 #2	p	88	sterol	margarine	3.0	-9.1	2.4
Cleghorn et al. 2003	x	50	sterol	margarine	2.1	-7.2	1.7
Clifton et al. 2004 #1	x	36	sterol	bread	1.6	-9.8	1.4
Clifton et al. 2004 #2	x	40	sterol	milk	1.6	-12.4	1.3
Clifton et al. 2004 #3	x	58	sterol	cereals	1.6	-5.6	1.3
Clifton et al. 2004 #4	x	40	sterol	yoghurt	1.6	-9.8	1.2
Clifton et al. 2008 #1	p	76	sterol	low-fat spread	1.6	-9.1	3.4
Clifton et al. 2008 #2	p	78	sterol	low-fat spread	1.6	-11.4	3.3
Clifton et al. 2008 #3	p	75	sterol	low-fat spread	1.6	-7.3	3.4
Colgan et al. 2004	x	48	sterol	low-fat margarine	1.3	-2.9	2.4
Davidson et al. 2001 #1	p	38	sterol	low-fat spread	3.0	-4.3	5.0
Davidson et al. 2001 #2	p	37	sterol	dressing	6.0	-3.9	4.5
Davidson et al. 2001 #3	p	40	sterol	low-fat spread and dressing	9.0	-12.2	4.9
de Graaf et al. 2002	p	62	mix	chocolate	1.8	-11.1	3.1
de Jong et al. 2008a #1	p	26	sterol	low-fat margarine	2.5	-8.2	5.6
de Jong et al. 2008a #2	p	26	stanol	low-fat margarine	2.5	-12.8	5.1
de Jong et al. 2008b #1	p	35	sterol	low-fat margarine	2.5	-12.4	4.3
de Jong et al. 2008b #2	p	36	stanol	low-fat margarine	2.5	-9.5	4.3
Devaraj et al. 2004	p	72	sterol	orange juice	2.0	-11.8	2.5
Devaraj et al. 2006	p	72	sterol	orange juice	2.0	2.1	3.7
Doornbos et al. 2006 #1	p	71	sterol	low-fat yoghurt drink	3.2	-9.5	2.2
Doornbos et al. 2006 #2	p	71	sterol	low-fat yoghurt drink	2.8	-9.3	2.2
Eady et al. 2011	x	39	sterol	spread	1.6	-5.6	1.7

Reference	Design	Sample size	Type of phyto-sterols	Food format	Dose of phytosterols[a]	Relative change in LDL-C	
						Effect (%)	SE
Earnest et al. 2007	p	54	sterol	capsules	1.6	-9.9	3.4
Escuriol et al. 2010	x	44	sterol	milk	2.0	-4.1	2.0
Fuentes et al. 2008 #1	x	30	sterol	low-fat margarine	2.0	-7.7	2.7
Fuentes et al. 2008 #2	x	30	sterol	low-fat margarine	2.0	-3.3	3.0
Geelen et al. 2002 study 1	x	31	sterol	low-fat margarine	3.2	-9.9	2.6
Geelen et al. 2002 study 2	x	57	sterol	low-fat margarine	3.2	-12.4	2.2
Goldberg et al. 2006	p	26	stanol	tablets	1.8	-9.1	4.5
Goncalves et al. 2007	p	34	sterol	milk	2.0	4.0	4.8
Gylling & Miettinen 1994	x	11	stanol	margarine	3.0	-9.3	2.8
Gylling & Miettinen 1996 #1	x	8	stanol	margarine	3.0	-14.4	2.1
Gylling & Miettinen 1996 #2	x	8	stanol	margarine	3.0	-9.7	3.9
Gylling & Miettinen 1999	x	21	stanol	butter	2.4	-12.0	2.0
Gylling et al. 1997	x	22	stanol	margarine	3.0	-14.5	2.9
Gylling et al. 2010	p	49	stanol	margarine and oat-based drink	8.9	-17.4	2.6
Hallikainen & Uusitupa 1999 #1	p	35	stanol	low-fat margarine	2.3	-14.0	3.0
Hallikainen & Uusitupa 1999 #2	p	37	stanol	low-fat margarine	2.2	-7.8	3.2
Hallikainen et al. 2000 #1	x	34	stanol	margarine	2.0	-12.7	2.2
Hallikainen et al. 2000 #2	x	34	sterol	margarine	2.0	-10.4	1.9
Hallikainen et al. 2008	p	19	stanol	margarine	2.2	-18.6	8.8
Hallikainen et al. 2011	p	24	stanol	margarine	3.2	-13.7	5.2
Hansel et al. 2007	p	194	sterol	low-fat fermented milk	1.6	-9.2	1.0
Hayes et al. 2004	x	7	sterol	tortilla chips	1.5	-15.3	5.9
Heggen et al. 2010 #1	x	59	sterol	low-fat margarine	2.0	-9.0	1.8
Heggen et al. 2010 #2	x	59	sterol	low-fat margarine	2.0	-8.2	1.6
Hendriks et al. 1999 #1	x	80	sterol	margarine	0.8	-6.2	1.8
Hendriks et al. 1999 #2	x	80	sterol	margarine	1.6	-9.2	1.8
Hendriks et al. 1999 #3	x	80	sterol	margarine	3.2	-9.8	1.8
Hendriks et al. 2003	p	185	sterol	low-fat spread	1.6	-4.3	2.4
Hernandez-Mijares et al. 2010	p	55	sterol	low-fat milk	2.0	-10.2	2.3
Hernandez-Mijares et al. 2011 study 1	p	24	sterol	low-fat milk	2.0	-0.5	3.8
Hernandez-Mijares et al. 2011 study 2	p	24	sterol	low-fat milk	2.0	-10.5	3.7
Hironaka et al. 2006 #1	p	101	sterol	vegetable/fruit juice	0.8	-6.7	2.1
Hironaka et al. 2006 #2	p	105	sterol	vegetable/fruit juice	1.6	-8.8	2.0
Homma et al. 2003 #1	p	67	stanol	low-fat spread	2.0	-8.9	1.9
Homma et al. 2003 #2	p	68	stanol	low-fat spread	3.0	-6.6	2.3
Houweling et al. 2009	x	82	sterol	low-fat margarine	2.0	-7.8	2.0
Hyun et al. 2005	p	51	stanol	low-fat yoghurt	2.0	-7.8	3.0
Ishizaki et al. 2003	p	55	sterol	mayonnaise	0.9	-8.2	2.4
Jakulj et al. 2005 #1	x	39	sterol	low-fat spread	2.0	-4.7	1.7
Jakulj et al. 2005 #2	x	39	sterol	low-fat spread	2.0	-3.5	2.3
Jauhiainen et al. 2006	p	67	stanol	low-fat cheese	2.0	-10.3	2.3
Jones et al. 1999	p	32	mix	margarine	1.7	-15.5	5.4
Jones et al. 2000 #1	x	15	sterol	low-fat margarine	1.8	-13.3	2.3
Jones et al. 2000 #2	x	15	stanol	low-fat margarine	1.8	-6.4	2.7
Jones et al. 2003	x	15	mix	non-fat beverage	1.8	-2.1	4.4
Judd et al. 2002	x	53	sterol	salad dressing	2.2	-10.1	0.7

Reference	Design	Sample size	Type of phyto-sterols	Food format	Dose of phytosterols[a]	Relative change in LDL-C	
						Effect (%)	SE
Kassis et al. 2008	x	22	sterol	margarine	1.7	-4.5	1.3
Khandelwal et al. 2009 study 1	p	93	sterol	yoghurt drink	2.0	-3.3	2.9
Khandelwal et al. 2009 study 2	p	85	sterol	yoghurt drink	2.0	-5.0	2.8
Korpela et al. 2006 study 1	p	50	sterol	low-fat yoghurt	1.7	-7.7	3.6
Korpela et al. 2006 study 2	p	62	sterol	low-fat hard cheese	2.0	-11.2	3.4
Korpela et al. 2006 study 3	p	52	sterol	low-fat fresh cheese	2.0	-13.8	3.5
Kratz et al. 2007 #1	x	17	sterol	low-fat margarine	2.0	-4.0	4.2
Kratz et al. 2007#2	x	17	stanol	low-fat margarine	2.0	-9.1	2.7
Kurokawa et al. 2008a #1	p	35	sterol	dressing	0.5	-2.2	5.2
Kurokawa et al. 2008a #2	p	35	sterol	dressing	0.9	-6.7	5.2
Kurokawa et al. 2008a #3	p	34	sterol	dressing	1.3	-7.3	4.8
Kurokawa et al. 2008b	p	59	sterol	dressing	0.8	-5.2	1.7
Lagstrom et al. 2006	p	42	stanol	capsules	2.0	-7.0	3.2
Lau et al. 2005 study 1	x	14	sterol	margarine	1.8	-7.1	7.8
Lau et al. 2005 study 2	x	15	sterol	margarine	1.8	-8.4	3.8
Lee et al. 2003	p	81	sterol	low-fat spread	1.6	-8.1	2.4
Li et al. 2007 #1	p	201	sterol	milk tea powder	1.5	-2.5	1.6
Li et al. 2007 #2	p	199	sterol	milk tea powder	2.3	-3.4	1.6
Lin et al. 2011	x	21	sterol	beverage	2.5	-6.5	3.2
Lottenberg et al. 2003	x	60	sterol	margarine	1.7	-6.4	1.1
Madsen et al. 2007	x	46	sterol	low-fat margarine and low-fat milk	2.3	-7.7	2.2
Maki et al. 2001 #1	p	158	sterol	low-fat spread	1.1	-7.6	1.7
Maki et al. 2001 #2	p	118	sterol	low-fat spread	2.2	-8.1	1.9
Mannarino et al. 2009	p	116	sterol	low-fat fermented milk	1.6	-8.3	1.6
Matsuoka et al. 2004a	p	46	sterol	mayonnaise	0.8	0.2	3.1
Matsuoka et al. 2004b study 1 #1	p	16	sterol	mayonnaise	0.2	-5.3	4.9
Matsuoka et al. 2004b study 1 #2	p	19	sterol	mayonnaise	0.4	3.1	5.0
Matsuoka et al. 2004b study 1 #3	p	16	sterol	mayonnaise	0.6	-0.8	4.9
Matsuoka et al. 2004b study 1 #4	p	16	sterol	mayonnaise	0.8	-7.1	3.9
Matsuoka et al. 2004b study 2 #1	p	15	sterol	mayonnaise	0.8	-12.3	6.3
Matsuoka et al. 2004b study 2 #2	p	17	sterol	mayonnaise	1.6	-8.6	4.0
Matsuoka et al. 2004b study 2 #3	p	15	sterol	mayonnaise	2.4	-11.0	4.8
Matvienko et al. 2002	p	34	sterol	beef	2.7	-13.4	3.6
McPherson et al. 2005 study 1	p	25	stanol	tablets	1.3	-10.4	4.0
McPherson et al. 2005 study 2	p	27	stanol	capsules	1.0	-2.5	3.9
Mensink et al. 2002	p	60	stanol	yoghurt	3.0	-10.3	4.7
Mensink et al. 2010 #1	p	46	stanol	margarine	2.8	-7.4	2.9
Mensink et al. 2010 #2	p	44	stanol	soy-based yoghurt	5.8	-11.9	3.2
Mensink et al. 2010 #3	p	47	stanol	margarine and soy-based yoghurt	8.7	-17.1	3.1

Reference	Design	Sample size	Type of phyto-sterols	Food format	Dose of phytosterols[a]	Relative change in LDL-C	
						Effect (%)	SE
Miettinen and Vanhanen 1994 #1	p	17	sterol	mayonnaise	1.0	-6.2	3.8
Miettinen and Vanhanen 1994 #2	p	15	mix	mayonnaise	1.0	-2.6	4.1
Miettinen and Vanhanen 1994 #3	p	15	mix	mayonnaise	1.2	-7.7	3.7
Miettinen et al. 1995 #1	p	102	stanol	margarine	2.6	-11.1	2.1
Miettinen et al. 1995 #2	p	102	stanol	margarine	2.6	-12.5	2.2
Mussner et al. 2002	x	62	sterol	margarine	1.8	-6.5	1.4
Naumann et al. 2003 #1	x	42	mix	low-fat margarine	2.0	-6.0	3.1
Naumann et al. 2003 #2	x	42	mix	low-fat margarine	2.0	-6.7	3.0
Neil et al. 2001	p	62	sterol	spread	2.5	-14.2	3.3
Nestel et al. 2001	x	15	sterol	dairy spread	2.4	-7.9	2.5
Nigon et al. 2001	x	53	sterol	low-fat spread	1.6	-5.3	1.6
Niittynen et al. 2007 study 1	x	15	sterol	low-fat yoghurt drink	1.0	-4.2	3.3
Niittynen et al. 2007 study 2	p	26	sterol	low-fat yoghurt drink	2.0	-6.0	4.3
Noakes et al. 2002 study 1 #1	x	46	sterol	low-fat spread	2.3	-7.7	1.2
Noakes et al. 2002 study 1 #2	x	46	stanol	low-fat spread	2.5	-9.5	1.2
Noakes et al. 2002 study 2	x	35	sterol	spread	2.0	-9.6	1.5
Noakes et al. 2005 study 1 #1	x	39	sterol	margarine	2.0	-10.1	1.6
Noakes et al. 2005 study 1 #2	x	39	sterol	low-fat milk	2.0	-7.9	1.6
Noakes et al. 2005 study 1 #3	x	39	sterol	low-fat milk and margarine	4.0	-11.4	1.5
Noakes et al. 2005 study 2 #1	x	40	sterol	low-fat yoghurt	1.8	-6.1	1.6
Noakes et al. 2005 study 2 #2	x	40	stanol	low-fat yoghurt	1.7	-5.2	1.7
Ntanios et al. 2002	x	53	sterol	margarine	1.8	-9.1	1.6
Ooi et al. 2007	x	9	sterol	cereal and margarine	2.0	-6.4	7.8
Pelletier et al. 1995	x	12	sterol	butter	0.7	-15.2	3.1
Plana et al. 2008	p	83	sterol	low-fat fermented milk	1.6	-12.2	3.1
Plat and Mensink 2000 #1	p	78	stanol	margarine and shortening	3.8	-12.6	3.3
Plat and Mensink 2000 #2	p	76	stanol	margarine and shortening	4.0	-11.6	3.7
Plat et al. 2000 #1	x	39	stanol	margarine	2.5	-9.4	1.5
Plat et al. 2000 #2	x	39	stanol	margarine and cake/cookie	2.5	-10.4	1.9
Quilez et al. 2003	p	57	sterol	muffin and croissant	3.2	-14.7	4.5
Racette et al. 2010 #1	x	18	sterol	beverage	0.4	-5.0	2.1
Racette et al. 2010 #2	x	18	sterol	beverage	2.0	-8.9	2.3
Raitakari et al. 2008	p	190	stanol	low-fat margarine	2.0	-9.3	3.1
Rudkowska et al. 2008 #1	x	26	sterol	low-fat yoghurt	1.6	-2.3	1.8
Rudkowska et al. 2008 #2	x	26	sterol	low-fat yoghurt	1.6	-5.1	1.8
Ruiu et al. 2009	x	15	sterol	yoghurt drink	1.0	-4.6	2.2
Saito et al. 2006 #1	p	33	sterol	DAG-containing mayonnaise	0.3	-7.1	2.7
Saito et al. 2006 #2	p	33	sterol	DAG-containing mayonnaise	0.4	-5.9	3.2
Saito et al. 2006 #3	p	34	sterol	DAG-containing mayonnaise	0.5	-9.3	2.7

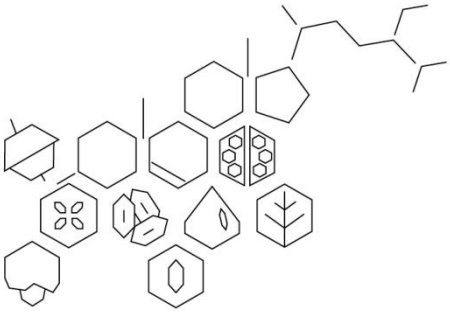
Reference	Design	Sample size	Type of phyto-sterols	Food format	Dose of phytosterols[a]	Relative change in LDL-C	
						Effect (%)	SE
Seki et al. 2003a	p	60	sterol	vegetable oil in bread	0.5	-2.3	2.4
Seki et al. 2003b	p	22	sterol	vegetable oil in bread	1.3	-12.6	3.9
Seki et al. 2003c #1	p	45	sterol	vegetable oil in bread	0.3	0.8	1.8
Seki et al. 2003c #2	p	44	sterol	vegetable oil in bread	0.5	-7.7	1.9
Seppo et al. 2007 study 1	p	60	stanol	low-fat yoghurt	2.0	-2.9	3.2
Seppo et al. 2007 study 2	p	61	stanol	low-fat yoghurt drink	2.0	-3.2	3.0
Seppo et al. 2007 study 3	p	19	stanol	low-fat yoghurt drink	2.0	-11.8	7.0
Seppo et al. 2007 study 4	p	59	stanol	low-fat milk	2.0	-6.2	2.4
Sialvera et al. 2011	p	108	sterol	yoghurt drink	4.0	-19.7	1.7
Sierksma et al. 1999	x	75	sterol	margarine	0.8	-6.1	0.6
Simons 2002 study 1	p	77	sterol	margarine	2.0	-10.2	2.8
Simons 2002 study 2	p	75	sterol	margarine	2.0	-6.1	3.6
Soderholm et al. 2011	p	63	sterol	rye bread	2.0	-8.1	3.5
Spilburg et al. 2003	p	24	stanol	lemonade	1.9	-14.3	4.5
Takeshita et al. 2008	p	29	sterol	DAG-containing cooking oil	0.5	-6.0	4.4
Temme et al. 2002	x	42	sterol	low-fat margarine	2.1	-9.6	1.4
Theuwissen & Mensink 2007	x	40	stanol	cereal	1.5	-4.4	2.1
Theuwissen et al. 2009	p	28	stanol	margarine	2.5	-9.5	4.6
Thomsen et al. 2004 #1	x	69	sterol	low-fat milk	1.2	-7.1	1.5
Thomsen et al. 2004 #2	x	69	sterol	low-fat milk	1.6	-9.6	1.5
Vanhanen 1994	p	14	stanol	mayonnaise	1.5	-2.0	7.6
Vanhanen et al. 1994	p	15	stanol	mayonnaise	0.8	-7.7	3.8
Vanstone et al. 2002 #1	x	15	sterol	butter	1.8	-10.2	2.8
Vanstone et al. 2002 #2	x	15	stanol	butter	1.8	-10.5	2.8
Vanstone et al. 2002 #3	x	15	mix	butter	1.8	-11.5	2.7
Varady et al. 2004 study 1	p	38	sterol	low-fat margarine	1.8	-11.3	2.4
Varady et al. 2004 study 2	p	36	sterol	low-fat margarine	1.8	-12.8	3.8
Volpe et al. 2001	x	30	sterol	low-fat yoghurt drink	1.1	-7.6	1.9
Weidner et al. 2008	p	50	sterol	soy drink	1.6	-5.2	2.8
Weststrate & Meijer 1998 #1	x	76	sterol	margarine	3.2	-13.1	0.6
Weststrate & Meijer 1998 #2	x	77	stanol	margarine	2.7	-11.9	0.6
Woodgate et al. 2006	p	29	stanol	softgel capsules	1.6	-7.2	3.7
Yoshida et al. 2006 study 1	x	16	mix	cereal bar	1.8	-6.1	2.5
Yoshida et al. 2006 study 2	x	13	mix	cereal bar	1.8	-2.8	3.7

DAG, diacylglycerol; LDL-C, low-density lipoprotein cholesterol.

[a] Dose given as free equivalents in g/d, rounded off at 1 decimal.

[b] Multiple study arms in 1 study corrected for the same single control group/period: indicated with # 1, # 2, # 3, etc. For some cross-over studies, different active treatments were compared with a separate corresponding placebo treatment; however, as the same subjects were included in those periods, these are indicated with #1, #2, #3 etc.

[c] Multiple study arms in 1 study, each corrected for a respective control group (i.e., different set of subjects): indicated with study 1, study 2, study 3, etc.



Chapter 4

The effect of plant sterols on serum triglyceride concentrations is dependent on baseline concentrations: a pooled analysis of 12 randomised controlled trials

Isabelle Demonty
Rouyanne T. Ras
Henk C.M. van der Knaap
Linsie Meijer
Peter L. Zock
Johanna M. Geleijnse
Elke A. Trautwein

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Abstract

Purpose - Plant sterols (PS) are well-known for their low-density lipoprotein cholesterol-lowering effect. Until recently, they were believed to have little or no impact on blood triglycerides (TG). However, studies taken individually were possibly lacking statistical power to detect modest TG decreases. This study was performed to quantify the TG-lowering effect of PS by pooling individual subject data from 12 randomized controlled trials that investigated the effects of PS on blood lipids.

Methods - The main outcome variable was the control-adjusted PS effect on relative (%) and absolute (mmol/L) changes in TG. The relative and absolute changes in high-density lipoprotein cholesterol (HDL-C) were also assessed. Differences in changes of serum lipid concentrations between PS and control treatments were estimated by an ANCOVA using a random effect model which included PS intake (active or control), study, and pre-defined subject characteristics.

Results - The twelve randomized controlled trials included in total 935 hypercholesterolemic subjects not preselected based on their baseline TG concentrations. In most studies, the PS dose ranged between 1.6 and 2.5 g/d. PS intake significantly lowered serum TG by 6.0% (95% CI: -10.7; -1.2) or 0.12 mmol/L (95% CI: -0.20; -0.04). No significant interaction was observed between PS intake and baseline TG concentrations on relative changes, but, on absolute changes, interaction was significant with larger TG decreases observed with higher TG concentrations at baseline. No effects were observed on HDL-C concentrations.

Conclusions - These results show that PS exert a modest TG-lowering effect which is dependent on baseline concentrations.

Introduction

Plant sterols (PS) and stanols, their saturated counterparts, are well known for their total and low-density lipoprotein cholesterol (LDL-C)-lowering effect. To date, several meta-analyses have summarized and quantified the LDL-C-lowering effect of PS/stanol-enriched foods and their dose-response relationship[1-4]. Possibly due to the fact that the large number of human intervention studies with PS/stanols were designed and powered to detect a significant effect on LDL-C, in most studies taken individually the effect of PS/stanols on serum triglycerides (TG) was not estimated

or not detected. However, significant reductions in TG concentrations after PS intervention have incidentally been observed[5-8]. Furthermore, a recent meta-analysis of individual subject data from five studies, which aimed at studying the relationship between subjects' baseline characteristics and the effects of plant stanol-enriched spreads on serum lipid concentrations, indicated that plant stanols not only lower serum concentrations of LDL-C, but also TG concentrations[9]. More recently, large TG reductions were observed in metabolic syndrome patients consuming PS/stanol-enriched foods[10,11].

Elevated TG concentrations are increasingly being recognized as a possible independent risk factor for coronary heart disease (CHD), and TG-lowering therapy next to lowering LDL-C may be considered relevant especially in high risk populations such as e.g. subjects with dyslipidemia as characterized in the metabolic syndrome[12-14].

In the recent meta-analysis that indicated a TG-lowering effect of plant stanols[9], significant interaction was observed between baseline TG concentrations and plant stanol intake, resulting in larger TG reductions (expressed in mmol/L) with higher baseline TG concentrations. Even when expressed in terms of relative (expressed in %) changes from baseline, TG reductions were more pronounced when baseline TG concentrations were higher. For investigating the TG-lowering effect of PS, having individual subject data would thus allow making better adjustments for baseline TG concentrations resulting in more precise estimations. As such, the aim of the present study was to quantitatively evaluate the TG-lowering effect of PS by pooling individual subject data from randomized controlled trials that were made available by investigators from independent research groups.

In order to specifically take into account the baseline TG concentrations in the estimation of the TG-lowering effect, the main outcome was expressed as the relative change in TG from baseline values. In addition, and for better understanding the impact of baseline concentrations on the observed reductions in TG, the absolute changes were calculated. As high-density lipoprotein cholesterol (HDL-C) metabolism is closely related to that of TG via the action of the cholesterol-ester transfer protein (CETP)[15], the effect of PS-enriched food consumption on HDL-C concentrations was also evaluated.

Methods

Selection of the studies

Data sets of 14 Unilever-sponsored PS intervention studies published in 12 publications were made available by different independent research groups[5,16-26] that published their findings in peer-reviewed journals. Studies were eligible for the current pooled analysis if they were randomized placebo-controlled trials with human adults not preselected based on their baseline TG concentrations, had used the 'usual' plant sterols (4-desmethylsterols), had disposal of TG data at baseline and at end-of-intervention as well as relevant co-variable data, and had no co-intervention from which the effect of PS could not be isolated.

Ferulated PS as found e.g. in rice bran oil were excluded because these are not commonly used for food/supplement enrichment. In addition, there is no consensus on their cholesterol-lowering effect[17,27], thus their potential impact on serum TG and/or HDL-C may also be different from that of other PS. Because the cholesterol-lowering effect of PS is additive to that of statins[18,28] and dietary fat modifications (diets low in total, saturated fat, and cholesterol content or high in vegetable oil)[29-31], we assumed that a similar additive effect could be expected in case of an impact on serum TG and HDL-C. Therefore, studies that prescribed statins or dietary fat modifications in both the control and the treatment group/phase within each study were included in the present analysis.

Eligibility for inclusion in the pooled analysis was judged by evaluating the full publication, the study protocol and the data set. Out of the 14 studies, one study was excluded because it did not measure TG concentrations[19] and another because initial lipid values were not readily available[20]. One study[24] consisted of two parallel arms with a randomized controlled cross-over design within each arm; these parallel arms were considered as two separate cross-over studies. In another study[25], 2 separate cross-over trials were described. Thus, individual subject data from a total of 12 studies from 10 publications that met the selection criteria were available for inclusion in the current pooled analysis[5,16-18,21-26].

Data extraction and quality assessment

For each subject, the following data was extracted from the different data sets: study identification, gender, BMI, age, treatment (active or control), and TG and

HDL-C data at baseline and at end-of-intervention. When the lipids were measured at various time points during the intervention, the values corresponding to or closest to the 4-week time point were taken for the analysis. If measurements were done on two different days at the end of the intervention, the mean value of those two measurements was taken.

Study quality was assessed as previously reported[3] using a custom-designed tool adapted from the Delphi Consensus[32] and the method by Chalmers *et al.*[33]. However, due to a lack of consensus on which scoring system is the best and hence scoring is intrinsically subjective[34], quality scores were not used to exclude lower quality trials or to weigh the data accordingly.

Statistical analysis

The primary outcome variables were the control-adjusted relative (%) and absolute (mmol/L) changes from baseline in TG due to the PS treatment. The secondary outcome variables were defined as the control-adjusted relative and absolute changes from baseline in HDL-C. The relative changes in serum TG and HDL-C were calculated as follows for each subject:

$$Relative\ change = 100 * \frac{Lipid_{end} - Lipid_{baseline}}{Lipid_{baseline}}$$

Baseline lipid concentrations were defined as the lipid concentrations at the start of the intervention phase (end of run-in when a run-in phase was present). For cross-over trials in which start-of-intervention measurements were not available (n = 1), the lipid concentrations at screening were used as baseline concentrations.

In order to standardize the variability structure of all data in the overall pooled analysis, we only used the data from the first study phase of cross-over studies, so that all studies were treated as parallel studies.

For the absolute changes, analysis was done on end-of-intervention serum lipid concentrations while adjusting for baseline concentrations. Differences in mean relative changes and absolute serum TG and HDL-C concentrations between the PS group and the control group were determined by an ANCOVA using a model which initially included plant sterol intake (active or control), study and the predefined

subject characteristics age, gender, BMI and baseline lipid concentrations and their interactions with PS intake. Because age and gender did not significantly ($P > 0.1$) contribute to the model, the subject characteristics kept in the final model were the respective baseline lipid concentrations and BMI (and the interaction between baseline TG concentrations and PS intake in the case of absolute changes). The statistical analysis was performed for the quasi intention-to-treat population[35], i.e., using all subjects for whom end-of-intervention TG or HDL-C values were available, and according to a random effect model.

Sensitivity analysis was performed to determine whether the presence of one study with patients on statins[18] influenced the outcome. The effect of PS on TG and HDL-C (expressed as relative change) were thus also determined when using only the eleven studies with healthy subjects. In order to verify that the use of only the first phase of cross-over trials in the overall analysis did not affect the outcome, a separate analysis was performed by using all phases of the cross-over trials.

Heterogeneity between studies was assessed by calculating the Q statistic as described by DerSimonian and Laird[36].

All analyses were performed with the statistical software program The SAS System (SAS Version 9.2, SAS Institute, Inc.,Cary, NC, USA). ProcMixed was used to perform the analyses.

Results

Overview of included studies and subjects

In total, 12 studies from 10 publications were available for the current pooled analysis[5,16-18,21-26]. The study by Noakes *et al.*[25] included PS and plant stanol treatments; only the data from the PS arm were used. When parallel design studies included different PS treatments (e.g. PS from different sources) provided in the same food format, these strata were combined[5,23]. In all studies, blood lipid concentrations were measured after an overnight fast. TG concentrations were included in the eligibility criteria of 9 out of 12 studies and were defined as less than 3.4-4.5 mmol/L in most (n = 8) studies. **Table 1** shows the characteristics of the studies included. The majority of studies was judged as of good quality (data not shown).

PS were esterified to vegetable oil fatty acids in all studies except one[17] which used free PS. The food format was margarine or spread in the majority of studies (n = 9). In one study, a combination of spread and milk (n = 1) was used[26], and in two studies, the vehicle for PS was a salad dressing[24]. The PS dose varied between 0.8 and 4 g/d, with the majority of studies (n = 9) testing doses ranging between 1.6 and 2.5 g/d. Doses of 0.8, 1.3 and 4 g/d were used in the other studies[16,17,26]. In most cases, PS-enriched foods were consumed for a period of 3 weeks; in three studies, the treatment duration was longer than 4 weeks, namely 5, 8 or 52 weeks[18,21,23]. In these cases, data obtained at 3 or 4 weeks were used in order to standardize the data from all studies to a similar point in time after the start of the intervention. Frequency of test product intake was not reported in three studies[16,17,26], whereas PS were consumed 2-3 times/d with meals in the other studies. Subjects were allowed to keep their usual, self-selected diet during the intervention in half of the studies[5,17,18,21,22,26]. In the other studies, the subjects were either provided a typical North-American diet[24], or were advised to follow the NCEP Step 1 diet[16,23] or to consume a diet rich in carotenoid-rich fruits and vegetables[25].

A total of 935 participants were included in the current pooled analysis. In 11 of the 12 studies, the subjects were overall healthy and were not taking any lipid-lowering medication. The only exception was the study by Neil *et al.*[18] in which subjects received statins and half of them had familial hypercholesterolemia. In all studies, subjects were Caucasian. The mean age of the study populations varied between 44 $\pm$ 12 and 58 $\pm$ 11 years. On average, the subjects were slightly overweight (mean BMI ranging between 24.0 $\pm$ 2.9 and 27.3 $\pm$ 3.7 kg/m^2). Mean baseline TG concentrations were on average normal to borderline high (ranging from 1.37 $\pm$ 0.52 to 1.93 $\pm$ 1.08 mmol/L) according to the NCEP classification[14], whereas LDL-C concentrations were on average above optimal to very high (ranging from 3.15 $\pm$ 0.86 to 5.11 $\pm$ 1.07 mmol/L). The baseline characteristics of the subjects in each of the studies are presented in **Table 1**.

Heterogeneity analysis

For the relative changes in TG, there was no significant heterogeneity between the studies as assessed by the Q statistic (Q = 0.22, 11 degrees of freedom, P >0.95). For HDL-C, no significant heterogeneity was observed either (Q = 2.18, 11 degrees of freedom, P >0.95).

***Table 1**. Overview of the studies included in the current study*

Author and reference	Original design	Sample size		Subject characteristics[a]							PS treatment characteristics		
		Con-trol	PS	Mean age	Mean BMI	Male	TG eligibility criteria	Mean baseline TG	Mean baseline TC	Mean baseline LDL-C	Mean baseline HDL-C	Food format	Dose	Duration
		n	*n*	*y*	*kg/m²*	*%*	*mmol/L*	*mmol/L*	*mmol/L*	*mmol/L*	*mmol/L*		*g/d*	*weeks*[b]
Sierksma et al. 1999[17]	Cross-over[c]	26	24	44.3 ± 11.6	24.9 ± 2.3	52.0	None	1.37 ± 0.52	5.09 ± 1.07	3.15 ± 0.86	1.33 ± 0.44	margarine	0.8[d]	3
Maki et al. 2001[23]	Parallel	89	131	58.0 ± 10.7	27.3 ± 3.7	44.8	<4.0	1.59 ± 0.73	6.17 ± 0.76	4.13 ± 0.67	1.32 ± 0.36	margarine	1.1 or 2.2	4
Neil et al. 2001[18]	Parallel	29	29	50.2 ± 12.7	26.1 ± 4.2	43.1	≤3.5	1.48 ± 0.76	7.29 ± 1.09	5.11 ± 1.07	1.45 ± 0.32	margarine	2.5	3.5
Judd et al. 2002 study 1[24]	Cross-over	12	15	47.9 ± 12.5	27.0 ± 2.8	51.9	<3.4	1.46 ± 0.73	5.76 ± 0.75	3.68 ± 0.57	1.42 ± 0.44	salad dressing (Ranch)	2.2	3
Judd et al. 2002 study 2[24]	Cross-over	14	12	46.4 ± 9.9	27.0 ± 3.0	46.2	<3.4	1.49 ± 0.69	5.48 ± 0.64	3.57 ± 0.53	1.24 ± 0.32	salad dressing (Italian)	2.2	3
Mussner et al. 2002[22]	Cross-over	31	31	-	24.0 ± 2.9	-	<1.8	1.19 ± 0.37	6.16 ± 0.71	4.11 ± 0.62	1.51 ± 0.35	margarine	1.8	3
Noakes et al. 2002 study 1[25]	Cross-over	14	18	57.5 ± 8.3	25.8 ± 2.9	40.6	<4.5	1.55 ± 0.54	6.31 ± 0.67	4.44 ± 0.57	1.17 ± 0.33	margarine	2.3	3
Noakes et al. 2002 study 2[25]	Cross-over	16	19	57.3 ± 9.7	26.0 ± 2.4	57.2	<4.5	1.73 ± 0.93	6.06 ± 0.71	4.20 ± 0.58	1.12 ± 0.24	margarine	2.0	3
Hendriks et al. 2003[21]	Parallel	97	91	48.5 ± 7.7	24.8 ± 3.2	48.4	None	1.35 ± 0.71	6.01 ± 1.01	3.79 ± 0.97	1.65 ± 0.41	margarine	1.6[d]	3
Colgan et al. 2004[16]	Cross-over	17	30	46.0 ± 8.8	26.2 ± 3.2	55.3	None	1.44 ± 0.65	6.23 ± 0.66	4.01 ± 0.79	1.18 ± 0.35	margarine	1.3[d]	3
Noakes et al. 2005[26]	Cross-over	21	18	51.5 ± 11.2	26.0 ± 2.1	53.9	≤4.5	1.67 ± 0.71	6.83 ± 0.82	4.83 ± 0.79	1.26 ± 0.39	spread and milk	4.0	3
Clifton et al. 2008[5]	Parallel	39	112	54.0 ± 8.7	26.5 ± 3.4	56.6	<4.5	1.93 ± 1.08	6.48 ± 0.77	4.34 ± 0.80	1.45 ± 0.48	margarine	1.6	3

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PS, plant sterols; TC, total cholesterol; TG, triglycerides

[a] Data are mean ± SD unless mentioned differently

[b] Data at the time point closest to 4 weeks were taken

[c] For cross-over studies, only the 1st phase was used in the current study and treated as a parallel design

[d] Compliance data were available to determine the dose truly consumed

TG outcomes

When combining the individual subject data from all studies, PS significantly lowered serum TG by 6.0% (95% CI: -10.7; -1.2, P = 0.02) (**Figure 1**). No significant interaction was observed between TG effects of PS intake and baseline TG concentrations (P = 0.38).

When the study with statin users[18] was removed from the analysis, the pooled estimate was a 6.3% reduction in TG (95% CI: -11.3; -1.3, P = 0.02). An analysis of only cross-over studies including all treatment phases showed a similar effect, namely a 5.6% reduction in TG (95% CI: -9.3; -2.0). The ANCOVA performed for each study separately showed non-significant TG reductions in 8 out of 12 studies (**Figure 1**).

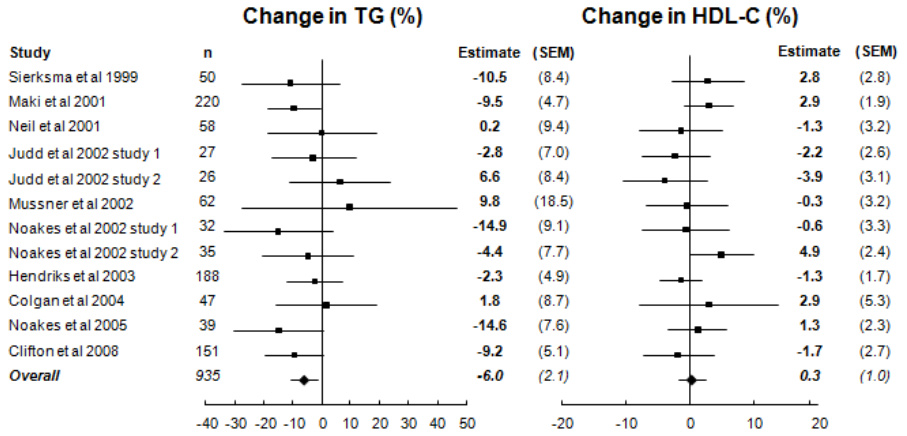

Figure 1. *Forest plots. Forest plots showing the effect of PS on TG and HDL-C estimated for each of the studies included in the overall analysis using individual subject data. The squares represent the averages for each of the individual studies. Error bars represent 95% CI. The diamonds represent the pooled results. The solid vertical line extending upward from zero is the null value. In both the overall and individual study analyses, only the first phase of cross-over trials was used. Both types of analyses were performed using individual subject data. The overall estimate was obtained by pooling together the individual subject data from all studies. The same statistical model was used for the individual studies and the overall analysis; the model included PS intake, study, age, gender, BMI, and the respective baseline concentrations and their interactions with PS intake.*

When the effects were expressed in absolute values, PS intake modestly but significantly lowered TG by 0.12 mmol/L (95% CI: -0.20; -0.04, P = 0.01). In contrast

with the results obtained when the effects were expressed relatively, a significant (P <0.01) interaction between PS intake and baseline TG concentrations was observed on absolute end-of-intervention concentrations. In line with this finding, larger reductions vs. control were observed in subjects with higher baseline TG concentrations (**Figure 2**).

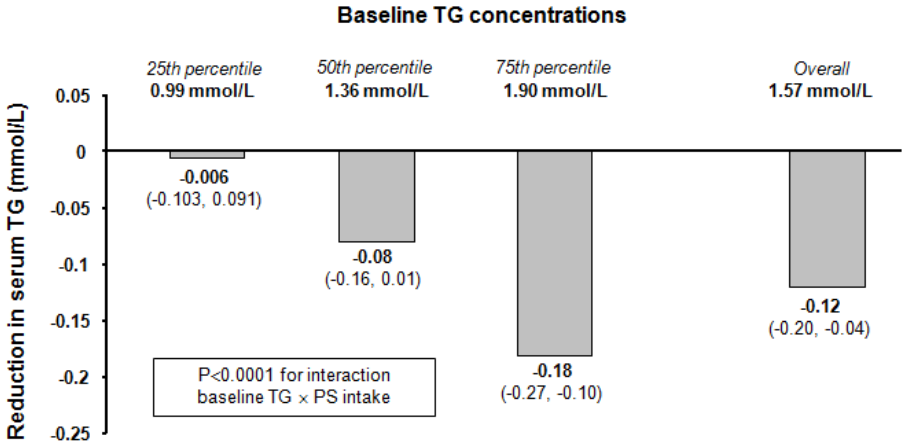

Figure 2. *Impact of baseline TG concentrations. Impact of baseline TG concentrations on the absolute (expressed in mmol/L) TG reductions achieved with PS consumption in twelve randomized controlled trials. In the majority of studies (n = 9), doses of 1.6-2.5 g/d were tested (range: 0.8-4.0 g/d).*

HDL-C outcomes

No significant effect of PS was observed on HDL-C; the relative change from baseline was +0.3% (95% CI: -1.8; +2.5, P = 0.73) (**Figure 1**). There was no interaction between PS intake and baseline HDL-C concentrations (P = 0.75). The removal of the study with statin users[18] did also not have an impact (HDL-C change: +0.5%, 95% CI: -1.8; +2.8, P = 0.66).

When the analysis was performed on the absolute HDL-C concentrations, also no significant effect of PS intake was observed (+0.01 mmol/L; 95% CI: -0.02, +0.04, P = 0.54) and there was no PS intake × baseline HDL-C interaction (P = 0.44).

Discussion

The present pooled analysis including individual subject data from 12 randomized controlled trials shows that PS intakes of around 2 g/d exert a modest TG-lowering effect of about 6% or 0.12 mmol/L in hypercholesterolemic subjects not preselected based on their baseline TG concentrations. Given the high inter-individual variation in TG concentrations, and the fact that the individual PS studies were primarily powered to assess the effect of PS on LDL-C concentrations, it is likely that the absence of statistically significant TG-lowering effects in these studies was due to insufficient statistical power. For example, a recent study by Mensink *et al*.[37] studied the serum lipid effects of doses of plant stanols up to 9 g/d but failed to show a significant TG reduction (e.g. ~8% for 9 g/d; P = 0.187) with only a limited number of subjects in each of the treatment groups (~22 to 25 subjects).

The 6% TG-lowering effect observed here with PS use is consistent with the outcome of a previous meta-analysis of individual subject data from five studies[9] which showed a 4% reduction in TG after 2 g/d plant stanol intake in subjects with baseline concentrations of ~2 mmol/L. These data thus show that both PS and stanols exert a comparable TG-lowering effect. Other recently published studies using similar doses of PS (~2 g/d) also support the findings of our pooled analysis; TG concentrations were significantly lowered by 9-19% after 4-6 weeks of intervention with PS-enriched (soy)milk or spread in subjects with baseline TG concentrations >1.5 mmol/L[5-8]. For plant stanols as well, significant decreases in TG concentrations were shown in subjects with overt hypertriglyceridemia[38].

The TG-lowering effect observed in our pooled analysis seems robust. Heterogeneity analysis did not reveal significant variability between studies. In addition, the sensitivity analysis showed that removing the study with statin users did not affect the outcome. Also, the use of only the first phase of cross-over trials in the overall analysis did not change the results. At last, the majority of studies included in the pooled analysis were of good quality, and most individual studies showed a tendency towards the same direction in the form of non-significant TG reductions.

Our results indicate that the absolute (mmol/L) reductions in TG achieved with PS intake are dependent of baseline TG concentrations. A significant interaction on

relative (%) TG changes was not present. However, it cannot be fully excluded that the current analysis may have been underpowered to detect such an effect. Nevertheless, the present results suggest that the impact of baseline TG is more pronounced on absolute changes in TG concentrations than on relative changes from baseline. By expressing TG changes as % change from baseline, at least part of the variability in PS effects due to inter-individual variations in baseline TG is taken into account. Therefore, it appears preferable to express the TG changes in relative terms when referring to the mean effect in a population.

Our data fit well with the findings of two studies reporting large control-adjusted TG reductions of 19-28% (corresponding to 0.23 to ~0.4 mmol/L) following the consumption of 2-4 g/d PS/stanols in metabolic syndrome subjects with baseline TG concentrations of 2.2-2.4 mmol/L[10,11]. We estimated, for our study population, a reduction of 0.18 mmol/L in subjects with baseline TG concentrations at the 75th percentile (1.9 mmol/L). If our pooled analysis had comprised a larger proportion of subjects with higher baseline TG concentrations and/or subjects with the metabolic syndrome, it is likely that even larger TG reductions would have been observed. Taken together, these data suggest that PS/stanols would be particularly useful for a dual benefit on both LDL-C and TG in subjects with both lipid abnormalities.

Based on the significant reductions in large and medium size VLDL particles observed in subjects with the metabolic syndrome, Plat *et al*.[39] suggested that a reduced hepatic VLDL1 secretion could be a mechanism involved in the TG-lowering effect of plant stanols. The unaltered CETP mass observed in their subjects coupled with unchanged HDL-C concentrations[39] are consistent with the absence of effect of PS on HDL-C observed in the present study. Overall, these data suggest that the reduced TG concentrations attributable to either PS or stanol consumption may not be ascribed to a remodeling of TG-rich lipoproteins via CETP activity.

The findings of the current pooled analysis are limited by the fact that the randomized controlled trials included in the analysis present only a selection of studies available in the literature. Also because the included studies were all industry-sponsored, selection bias might possibly be present. However, all studies were planned and executed by independent research groups and published in peer-reviewed journals. Because we re-analyzed individual subject data of a large

number of subjects (935 in total), we believe that there was sufficient power to substantiate the conclusions drawn, and that adding more subject data from other studies would not have changed the outcomes. In addition, because most studies used PS doses within a narrow range (between 1.6 and 2.5 g/d), this does not allow drawing any conclusion on a possible dose-response relationship for the TG-lowering effect of PS.

In the absence of intervention studies that directly quantified the CHD risk reduction resulting from lowering TG only, it is difficult to determine whether the additional effect that a modest 6% TG reduction may have on CHD risk is clinically relevant next to the average 10% LDL-C reduction achievable with an intake of 2 g/d of PS. Nevertheless, although not as strong as LDL-C, elevated TG is increasingly being recognized as a possible risk factor for CHD[12-14]. Additional research into the relevance of TG-lowering for CHD risk reduction, and into interventions (e.g. diet and lifestyle interventions) that beneficially impact TG, is therefore warranted.

In conclusion, foods enriched with PS modestly lower TG concentrations, especially in those with high TG concentrations at baseline. This effect may add to the overall benefit of using PS-enriched foods as part of therapeutic lifestyle and diet changes for improving blood lipid profiles.

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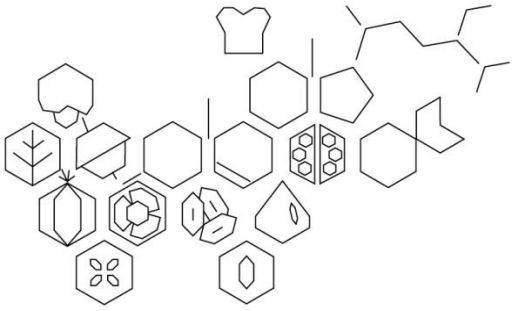

Chapter 5

Low doses of eicosapentaenoic acid and docosahexaenoic acid from fish oil dose-dependently decrease serum triglyceride concentrations in the presence of plant sterols in hypercholesterolemic men and women

Rouyanne T. Ras
Isabelle Demonty
Yvonne E.M.P. Zebregs
Johan F.A. Quadt
Johan Olsson
Elke A. Trautwein

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Abstract

Plant sterols (PS) lower low-density lipoprotein cholesterol (LDL-C) concentrations, whereas the n–3 (ω-3) fish fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) lower triglyceride (TG) concentrations. Incorporating both PS and EPA+DHA from fish oil (FO) in a single food format was expected to beneficially affect two blood lipid risk factors. The aim of this study was to investigate the dose-response relation between low doses (<2 g/d) of EPA+DHA from FO, incorporated in a low-fat PS-enriched spread, and TG concentrations. In addition, effects on LDL-C were investigated. The study was designed as a randomized, double-blind, placebo-controlled parallel study. After a 4-week run-in period, subjects were randomly assigned to consume either a control (C) spread (no PS, no FO) or 1 of 4 intervention spreads containing a fixed amount of PS (2.5 g/d) and varying amounts of FO (0.0, 0.9, 1.3, and 1.8 g/d of EPA+DHA) for 4 weeks. Before and after the intervention, fasting blood samples were drawn for measuring serum lipids and EPA and DHA in erythrocyte membranes. In total, 85 hypercholesterolemic men and 247 women with a mean age of 57.9 y (range: 25-74 y) were included. Eighteen subjects dropped out during the study. At baseline, mean TG and LDL-C concentrations were 1.09 and 4.00 mmol/L, respectively. After the intervention, a significant dose-response relation for the TG-lowering effect of EPA+DHA ($\beta_{Ln(TG)}$ = -0.07mmol/L per gram of EPA+DHA; P <0.01) was found. Compared with the C group, TG concentrations were 9.3-16.2% lower in the different FO groups (P <0.05 for all groups). LDL-C concentrations were 11.5-14.7% lower in the different PS groups than in the C group (P <0.01 for all groups). EPA and DHA in erythrocyte membranes were dose-dependently higher after FO intake than after the C spread, indicating good compliance. Consumption of a low-fat spread enriched with PS and different low doses of n-3 fatty acids from FO decreased TG concentrations in a dose-dependent manner and decreased LDL-C concentrations. This trial was registered at clinicaltrials.gov as NCT01313988.

Introduction

Elevated low-density lipoprotein cholesterol (LDL-C) is an established risk factor for coronary heart disease (CHD)[1]. Phytosterols, including both plant sterols (PS) and their saturated counterparts, plant stanols, are proven to lower LDL-C. To date, several meta-analyses have been published that quantified the LDL-C-lowering

effect of phytosterols when incorporated into various foods; a mean phytosterol intake of 2 g/d decreases LDL-C by 0.31-0.34 mmol/L or 8-10%[2-5]. Although not as strong and established as LDL-C, elevated triglyceride (TG) concentrations also represent an emerging blood lipid risk factor for CHD. A recent Mendelian randomization study even suggested a causal role of TG-rich lipoproteins in the development of CHD[6]. Especially in individuals at high risk of CHD, such as individuals with diabetes, attempts to decrease elevated TG concentrations are recommended in addition to treating elevated LDL-C[7,8]. The very long-chain n-3 fatty acids (FAs) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were shown to decrease fasting TG concentrations[9-13]. Evidence for this is mainly based on studies that used EPA+DHA in the form of fish oil (FO) capsules at doses >2 g/d and showed reductions in TG concentrations of 25-35%[9,11].

The consumption of a combination of PS and EPA+DHA from FO would address two blood lipid risk factors simultaneously. Some studies have investigated the lipid-modifying effects of this combination, with both ingredients being provided in separate formats or being esterified with each other. Overall, these studies showed decreasing effects on both LDL-C and TGs[14-19]. Whether the combination of EPA+DHA from FO and PS is also efficacious when both are incorporated into a single food format, i.e., a spread with a reduced-fat content, is unknown. Spreads are rich sources of unsaturated FAs and would therefore fit well within dietary approaches for improving blood lipid profiles. However, in low-fat spreads (~35% fat), the maximum amount of FO that can be added is limited. Furthermore, FO contains relatively large amounts of saturated FAs (SFAs), which are known to increase LDL-C concentrations[20]. Adding large amounts of FO into PS-enriched spreads could thus potentially lessen the LDL-C-lowering effect of PS. Recent dose-response investigation revealed that intakes of EPA+DHA as low as 0.2-0.5 g/d decrease TG concentrations by ~3-7%[10].

The aim of the present study was to investigate the dose-response relation between low doses of EPA+DHA from FO, incorporated in a low-fat spread enriched with PS, and TG concentrations. Also, we investigated the effect of PS and FO on LDL-C concentrations. Furthermore, the effects of PS and FO on total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and, as a compliance marker, on EPA and DHA in erythrocyte membranes were investigated.

Materials and Methods

This study was conducted according to the ethical principles laid down in the Declaration of Helsinki, as adopted in 1964 with later revisions. The protocol, informed consent and other subject information were approved by the ethical committee of Uppsala, Sweden (Regionala etikprövningsnämnden i Uppsala). The study took place from March 2011 to November 2011 at the clinical research organization Food Files, formerly known as Good Food Practice, in Uppsala, Sweden. Written informed consent was obtained from all subjects. The study was registered at clinicaltrials.gov (NCT01313988).

Study population

Subjects were recruited among inhabitants of Uppsala and surroundings. Interested subjects (n = 704) were referred to the study web site where they were requested to fill out a short questionnaire. Subjects whose eligibility was indicated by the results of the questionnaire (n = 562) were invited to join the screening procedure. Subjects were eligible if they met the following main selection criteria: apparently healthy; aged 25-75 y; fasting TC concentration between 5 and 8 mmol/L [i.e., borderline-high or high TC concentrations[8] as usually used in studies investigating the effects of PS on blood lipids]; BMI between 18 and 30 kg/m^2; systolic blood pressure ≤160 mm Hg, diastolic blood pressure ≤90 mm Hg and heart rate between 50 and 100 beats/min; no use of medication that could influence the study outcomes (e.g., lipid-lowering drugs or antibiotics); no use of nicotine-containing products; 10-y cardiovascular disease risk ≤10 according to the Systematic Coronary Risk Evaluation (SCORE); willing to comply with the study protocol (e.g., consume test products and follow several dietary and lifestyle restrictions); and having signed the informed and biobank consents. In total, 332 men and women fulfilled all inclusion and exclusion criteria and were enrolled into the study (**Figure 1**).

Study design

This study was designed as a randomized, double-blind, placebo-controlled, parallel efficacy study. Subjects followed a 4-week run-in period during which they consumed the control (C) spread to stabilize blood lipids and to get familiarized with the study regimen. After the run-in phase, subjects were randomly allocated, without further stratification, to consume either the C spread or 1 of 4 intervention

spreads containing a fixed amount of PS (2.5 g/d) and varying amounts of EPA+DHA (0-1.8 g/d) for 4 weeks. At the end of the run-in and intervention phases, fasted blood samples were drawn on two consecutive days for measuring serum lipids (on the basis of double blood sampling) and percentage of EPA and DHA of total erythrocyte membrane FAs (on the basis of single blood sampling). Body weight was also measured. Breakfast was served on all four test days. Health and well-being, compliance with test product intake and dietary restrictions, use of concomitant medication, and adverse events (AEs) were monitored online throughout the study.

Test products and dietary and lifestyle instructions

During the intervention phase, subjects were provided with 1 of the following test spreads: 30 g/d low-fat spread (C), 30 g/d low-fat spread with 12.5% PS esters (PS), 30 g/d low-fat spread with 12.5% PS esters and 11% (i.e., low-dose) FO (PS+FOL), 30 g/d low-fat spread with 12.5% PS esters and 16.5% (i.e., medium-dose) FO (PS+FOM), or 30 g/d low-fat spread with 12.5% PS esters and 22% (i.e., high-dose) FO (PS+FOH). The PS esters consisted of 60% PS and 40% FA esters (BASF Corporation). The FO consisted of 27% EPA+DHA as TG molecules (Ocean Nutrition). The ratio of EPA to DHA was 2:1. The FA composition of the FO is provided in **Supplemental Appendix 1**. The formulations of the 5 test spreads were similar (same base composition) except for FO, which replaced sunflower oil, and PS esters, which replaced water (PS) and sunflower oil (FA esters). In the PS+FOH spread, as much sunflower oil as possible was replaced by FO (i.e., 1.8 g EPA+DHA per daily serving); the PS+FOL spread consisted of half this maximal FO dose (i.e., 0.9 g EPA+DHA per daily serving). The nutritional compositions of the test spreads are shown in **Table 1**. All test spreads were produced in 3 production batches at the pilot plant of Unilever Research and Development Vlaardingen. Content analysis was performed after the production of the test spreads; the mean amounts of EPA+DHA were 0.9, 1.3, and 1.8 g per daily serving of the different FO spreads, whereas the mean amount of PS was 2.5 g (as free equivalents) per daily serving of the spreads containing PS. This amount is at the upper end of the recommended PS intake of 1.5-2.4 g/d[21,22]. All test products used in this study underwent standard microbiologic clearance and safety testing. The test spreads were provided in 10-g tubs packed in carton boxes and were stored under cooled (4-8°C) conditions. Subjects were instructed to consume 3 tubs daily (i.e., 1 tub with each main meal). Subjects were requested to consume the spread on bread or on other foods at

room temperature; the use of the spread on top of hot meals could have released a fishy smell and was therefore not allowed to avoid making them aware of their treatment group. The subjects and all staff involved in the conduct of the study were unaware of the treatment groups; the different test products were as similar as possible with respect to taste and appearance.

Table 1. *Nutritional composition of the control spread, the spread with plant sterols alone and the spreads with plant sterols and various amounts of fish oil.*

	Test spread				
	C	**PS**	**PS+FOL**	**PS+FOM**	**PS+FOH**
			unit/30 g spread		
Energy, *kJ*	388.9	388.9	388.2	388.2	388.2
Energy, *kcal*	94.6	94.6	94.4	94.4	94.4
Total protein, *g*	0.0	0.0	0.0	0.0	0.0
Total carbohydrates, *g*	0.0	0.0	0.0	0.0	0.0
Sugar, *g*	0.0	0.0	0.0	0.0	0.0
Total fat, *g*	10.5	10.5	10.5	10.5	10.5
SFAs, *g*	2.4	2.4	3.0	3.3	3.7
MUFAs, *g*	2.5	2.5	2.5	2.4	2.4
PUFAs, *g*	5.5	5.5	4.8	4.5	4.1
Total n-3 PUFAs, *g*	0.0	0.0	0.9	1.4	1.8
ALA, *g*	0.0	0.0	0.0	0.0	0.1
EPA, *mg*	0.0	0.0	594	891	1188
DHA, *mg*	0.0	0.0	297	446	594
Total n-6 PUFAs, *g*	5.5	5.5	3.4	2.4	1.4
TFA, *g*	0.1	0.1	0.2	0.2	0.2
Cholesterol, *mg*	0.2	0.2	33.1	49.6	66.1
PS ester[a], *g*	0.00	3.75	3.75	3.75	3.75
Sodium, *mg*	3.5	3.2	3.2	3.2	3.2
Potassium, *mg*	7.8	7.8	7.8	7.8	7.8
Vitamin A, *µg*	30.0	30.0	30.0	30.0	30.0
Vitamin E, *mg*	4.8	4.2	2.4	1.5	0.6
Water, *g*	19.4	17.2	17.2	17.2	17.2

ALA, a-linolenic acid; C, control; MUFA, monounsaturated fatty acids; PS, plant sterols; PS+FOH, plant sterols + high-dose fish oil; PS+FOL, plant sterols + low-dose fish oil; PS+FOM, plant sterols + medium-dose fish oil; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TFA, trans fatty acids.

[a] 3.75 g of PS esters contains 2.5 g free PS.

Subjects were asked to maintain their normal diet and lifestyle during the entire study period but to refrain from consuming foods or supplements enriched with FO, EPA+DHA, or PS/stanols and to restrict the intake of fish to a maximum of 3 portions/week. Furthermore, the intake of aspirin or other anticoagulants on a daily basis was discouraged. Strenuous exercise was not allowed during the 24 h

preceding each blood sampling. Test product intake and deviations from the protocol were recorded daily by the subjects in an online diary. If subjects did not enter any data in their diary for 3 days or if they showed noncompliance on a regular basis, they were contacted. Compliance with test product intake was determined by counting test product intake as reported in the online diaries and by measuring EPA and DHA in erythrocyte membranes.

Blood sampling and assays

Venous blood was collected from all subjects after an overnight fast (of at least 12 h) on 2 consecutive days pre- and post intervention. Blood samples for the serum lipid analysis were prepared at the test facility by centrifuging at 850 x g for 5 min; serum was then separated into aliquots and stored at -80°C until analysis after the study was completed. The EDTA samples for the FA analysis in erythrocytes were prepared at the test facility by centrifuging at 1400 x g for 10 min at 4°C. Two aliquots of erythrocytes were washed twice with HEPES buffered saline and stored at -80°C until further preparation. Erythrocyte membranes were isolated through several centrifuge and wash steps with decreasing concentrations of phosphate buffered saline (PBS). The final membrane pellets were suspended in isotonic stock PBS, and the tubes were dipped in dry ice/ethanol before being placed in an -80°C freezer until analysis after completion of the study. Serum concentrations of TC, LDL-C, HDL-C, and TGs were analyzed directly by photometry on an Abbot Architect ci8200 auto-analyzer. FAs were analyzed with the FAME-N3 method as previously reported[23,24]. All samples obtained from 1 subject were analyzed within the same assay.

Statistical analyses

The study was powered to find a significant slope of the dose-response relation between low doses of EPA+DHA and serum TG concentrations. Assuming a mean baseline TG concentration of 1.3 mmol/L and an SD of 0.5 mmol/L, a total of at least 222 subjects divided across the 4 PS groups were required for reaching a power of 0.8 (α = 0.05, 2-sided) when aiming for a 10% reduction in TG concentration. The study was also powered to find a significant LDL-C-lowering effect of 8% in each of the PS groups vs. the C group. Assuming a mean baseline LDL-C concentration of 4.0 mmol/L and an SD of 0.5 mmol/L, 60 subjects per treatment group were required (i.e., 300 in total) to arrive at a power of 0.8 (α = 0.05, 2-sided). This number covered the 222 subjects required to power the study

to find a significant TG slope. To account for possible dropouts (10%), a total of 330 subjects were included, 66 per treatment group. Two additional subjects acted as reserves and replaced dropouts during the run-in period.

Data were analyzed according to the intention-to-treat principle and the per-protocol principle, i.e., excluding data from subjects who had been noncompliant with the protocol (i.e., low test product compliance, not being weight stable, or use of prohibited drugs). Here, we only report the results based on the intention-to-treat analysis; the per-protocol analysis yielded similar results. For each subject, serum lipid concentrations as determined on the 2 consecutive days pre- and post intervention were averaged. In case of not normally distributed variables (i.e., for TGs), natural log transformation was applied and statistical analysis was performed on the basis of the log transformed data [ln(TG)]. Statistical analysis was performed on end-of intervention concentrations with corrections for baseline.

To investigate the dose-response effect of EPA+DHA on TG concentrations, regression analysis was performed including only the 4 groups who consumed PS. To investigate between-group differences vs. the C group, a mixed-model ANCOVA was carried out followed by post hoc multiple comparisons of the least square means (LSMeans) by using a Dunnett-Hsu adjustment. Between-group differences vs. the PS group were also investigated but were only reported for TGs. Full models included treatment, baseline, treatment x baseline, gender, age, BMI, change in body weight, period (before or after summer), and cohort (time of study start per subject). Reduced models included treatment and baseline and a selection of the other covariates in case these contributed significantly to the model (if $P < 0.10$). Results obtained with the reduced models are reported here. Relative differences in LSMeans were calculated with the LSMean of the C group (or the PS group) as the reference. A P value <0.05 was considered significant. All analyses were performed with the statistical software package SAS version 9.2 (SAS Institute).

Results

Subject characteristics and compliance

A total of 247 women (74.4%) and 85 men (25.6%) were included in the study. Eighteen subjects (5.4%) dropped out during the study (**Figure 1**); 8 subjects chose to discontinue for personal reasons, 6 experienced an AE (e.g., fever, diarrhea,

upset stomach), 3 were lost to follow-up, and 1 subject was excluded by the study physician for medical reasons (low hemoglobin). An overview of subjects' characteristics at baseline is provided in **Table 2**. Compliance with test product intake on the basis of the diaries was excellent (98.2%), with no difference between the run-in period (98.2%) and the intervention period (98.2%). Compliance with dietary and lifestyle restrictions was also high. Body weights after intervention did not differ between the groups (P = 0.75).

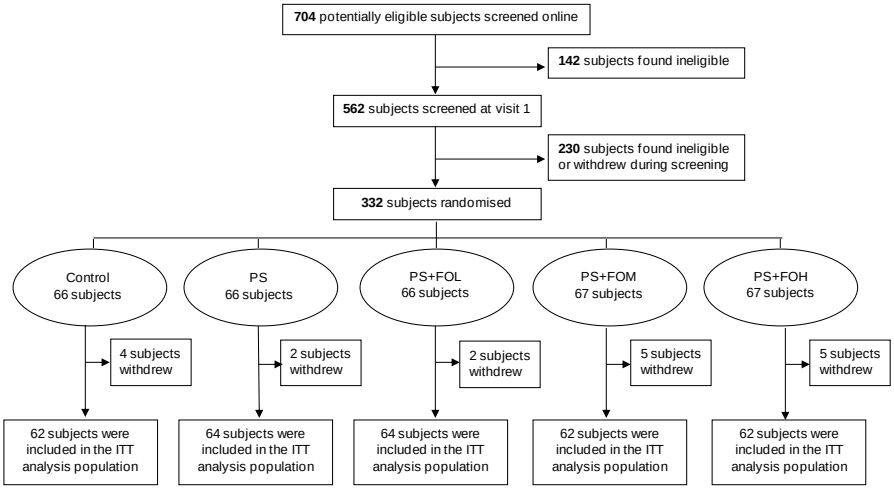

Figure 1. *Subject flow throughout the study. Hypercholesterolemic men and women were randomly assigned across 5 different treatment groups consuming a control spread (C), a spread with plant sterols (PS) or one of the spreads with PS and a low dose of fish oil (PS+FOL), a medium dose of fish oil (PS+FOM) or a high dose of fish oil (PS+FOH). ITT, intention-to-treat.*

Serum lipids

The analysis of the ln(TG) data revealed a clear dose-response relation (β = -0.07, P <0.01) for the TG-lowering effect of EPA+DHA (**Figure 2**). After 4 weeks, serum TGs were significantly lower in all FO groups than in the C group (ranging from -9.3% to -16.2%; P <0.05 for all; **Table 3**). The effect was not present after treatment with PS only (-5.3%; P = 0.36). Compared with the PS group, TGs were significantly lower in the PS+FOM and PS+FOH groups [-9.4% (P = 0.02) and -11.5% (P <0.01), respectively], whereas no effect was observed in the PS+FOL group (-4.3%; P = 0.47).

Table 2. *Overview of the subject characteristics at baseline.*

		Treatment group				
	All	**C**	**PS**	**PS+FOL**	**PS+FOM**	**PS+FOH**
Gender (F/M), *n/n*	230/84	42/20	45/19	45/19	48/14	50/12
Age, *y*	57.9 ± 0.6	56.2 ± 1.5	58.3 ± 1.5	55.8 ± 1.4	59.9 ± 1.2	59.4 ± 1.3
Weight, *kg*	72.3 ± 0.6	73.5 ± 1.2	71.1 ± 1.4	73.3 ± 1.4	73.8 ± 1.5	69.7 ± 1.4
BMI, kg/m^2	25.0 ± 0.1	25.0 ± 0.3	24.6 ± 0.3	25.1 ± 0.3	25.7 ± 0.3	24.3 ± 0.4
SBP, *mmHg*	128.2 ± 0.8	127.1 ± 1.5	127.7 ± 1.9	130.0 ± 1.9	129.6 ± 1.8	126.7 ± 1.8
DBP, *mmHg*	77.7 ± 0.4	77.6 ± 0.9	77.5 ± 1.0	77.6 ± 0.9	79.0 ± 0.8	76.8 ± 1.0
Heart rate, *beats/min*	65.7 ± 0.5	64.4 ± 1.2	65.5 ± 1.4	65.5 ± 1.1	66.0 ± 1.1	67.1 ± 1.3
SCORE[a]	2.5 ± 0.1	2.5 ± 0.3	2.7 ± 0.3	2.6 ± 0.3	2.6 ± 0.3	2.3 ± 0.3
Serum TC, *mmol/L*	6.45 ± 0.05	6.39 ± 0.11	6.39 ± 0.10	6.49 ± 0.10	6.60 ± 0.12	6.36 ± 0.10
Serum LDL-C, *mmol/L*	4.00 ± 0.04	4.01 ± 0.09	3.91 ± 0.09	4.10 ± 0.09	4.06 ± 0.10	3.89 ± 0.10
Serum HDL-C, *mmol/L*	1.63 ± 0.02	1.59 ± 0.04	1.67 ± 0.06	1.57 ± 0.05	1.68 ± 0.05	1.64 ± 0.04
Serum TG, *mmol/L*	1.09 ± 0.03	1.09 ± 0.06	1.13 ± 0.07	1.11 ± 0.08	1.09 ± 0.06	1.02 ± 0.05

C, control; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PS, plant sterols; PS+FOH, plant sterols + high-dose fish oil; PS+FOL, plant sterols + low-dose fish oil; PS+FOM, plant sterols + medium-dose fish oil; SBP, systolic blood pressure; SCORE, Systematic Coronary Risk Evaluation; TC, total cholesterol; TG, triglycerides. Values are means ± SEs.

[a] Ten-year cardiovascular disease risk according to the SCORE.

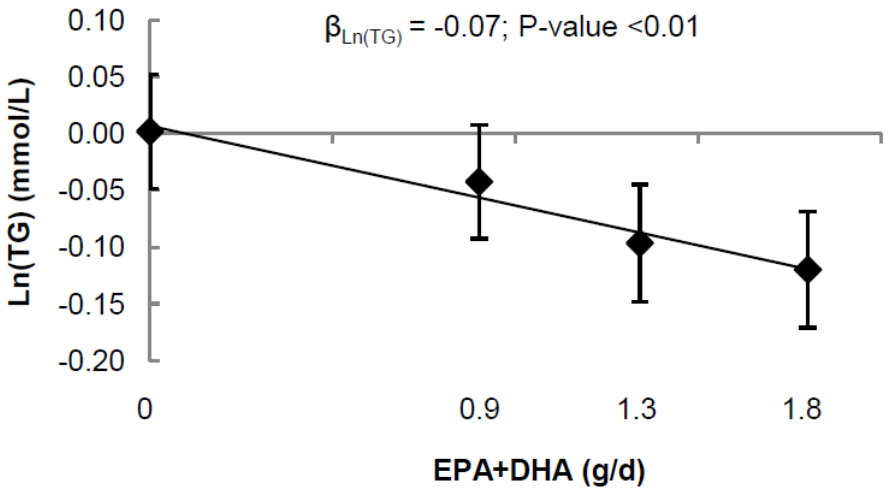

Figure 2. *Dose-response effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil incorporated in a plant sterol-enriched spread on serum triglyceride (TG) concentrations in hypercholesterolemic men and women. Least square means and 95% CIs of log-transformed TG [ln(TG)] concentrations are shown, n = 252. The relation was linear.*

LDL-C concentrations were significantly lower after the intervention with all spreads containing PS vs. the C spread; the average effects ranged from -11.5% to -

14.7% (P <0.01 for all) (**Table 3**). TC concentrations were also significantly lower vs. the C group (-5.6% to -9.0%; P <0.01 for all). HDL-C concentrations did not differ except for a 4.7% higher concentration in the PS+FOH group vs. the C group (P = 0.03).

Table 3. *Serum lipid concentrations in hypercholesterolemic men and women supplemented with plant sterols or with plant sterols and various amounts of fish oil for 4 weeks.*

Outcome and treatment group	Baseline	End-of-intervention	Absolute difference (95% CI) in LSMeans[a] vs. C	*P*	Relative difference in LSMeans vs. C
	mmol/L	*mmol/L*	*mmol/L*		*%*
Ln(TG)[b]					
C	0.02	0.04	-	-	-
PS	0.06	0.00	-0.05 (-0.13; 0.02)	0.36	-5.3
PS+FOL	0.05	-0.04	-0.10 (-0.17; -0.03)	0.03	-9.3
PS+FOM	0.03	-0.09	-0.15 (-0.22; -0.08)	<0.01	-13.9
PS+FOH	0.06	-0.13	-0.18 (-0.25; -0.11)	<0.01	-16.2
LDL-C					
C	3.80	3.85	-	-	-
PS	3.77	3.34	-0.45 (-0.59; -0.32)	<0.01	-11.7
PS+FOL	3.98	3.50	-0.45 (-0.58; -0.31)	<0.01	-11.5
PS+FOM	3.93	3.46	-0.49 (-0.63; -0.36)	<0.01	-12.7
PS+FOH	3.77	3.26	-0.57 (-0.70; -0.43)	<0.01	-14.7
TC					
C	6.16	6.23	-	-	-
PS	6.25	5.72	-0.57 (-0.74; -0.40)	<0.01	-9.0
PS+FOL	6.34	5.91	-0.44 (-0.61; -0.27)	<0.01	-6.9
PS+FOM	6.40	6.09	-0.36 (-0.53; -0.19)	<0.01	-5.6
PS+FOH	6.26	5.93	-0.39 (-0.56; -0.22)	<0.01	-6.2
HDL-C					
C	1.58	1.59	-	-	-
PS	1.68	1.66	-0.03 (-0.09; 0.02)	0.64	-1.9
PS+FOL	1.58	1.58	-0.01 (-0.06; 0.05)	1.00	-0.4
PS+FOM	1.65	1.72	0.06 (0.00; 0.11)	0.16	3.4
PS+FOH	1.64	1.72	0.08 (0.02; 0.13)	0.03	4.7

C, control; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LSMean, least square mean; PS, plant sterols; PS+FOH, plant sterols + high-dose fish oil; PS+FOL, plant sterols + low-dose fish oil; PS+FOM, plant sterols + medium-dose fish oil; TC, total cholesterol; TG, triglycerides.

[a] LSMeans were corrected for baseline and, if significantly contributing to the model (P <0.10), for treatment x baseline, gender, age, BMI, change in body weight, period (before or after summer), and cohort (time of study start per subject).

[b] Statistical analysis was conducted by using log-transformed TG concentrations [ln(TG)] because these were not normally distributed. Negative end-of-intervention values indicate that the values are <1 mmol/L on the normal scale. Relative differences in LSMeans vs. C are based on back-transformed LSMeans.

EPA and DHA in erythrocyte membranes

After intervention, the percentages of EPA and DHA in total erythrocyte membrane FAs were not different in the PS group compared with the C group. In the different FO groups, the erythrocyte contents of EPA were 61.1%, 87.5%, and 120.8% higher, respectively, vs. the C group (P <0.01 for all). The erythrocyte contents of DHA were also significantly higher in the different FO groups vs. the C group (ranging from 7.1% to 9.4%; P <0.01 for all), although the relative effect sizes were smaller compared with EPA (**Table 4**).

Table 4. *EPA and DHA of total erythrocyte membrane fatty acids in hypercholesterolemic men and women supplemented with plant sterols or with plant sterols and various amounts of fish oil for 4 weeks.*

Outcome and treatment group	Baseline	End-of-intervention	Absolute difference (95% CI) in LSMeans[a] vs. C	*P*	Relative difference in LSMeans vs. C
	% total FAs	*% total FAs*	*% total FAs*		*%*
EPA					
C	1.15	1.09	-		-
PS	1.14	1.10	0.02 (-0.13; 0.17)	1.00	1.7
PS+FOL	1.17	1.76	0.68 (0.53; 0.83)	<0.01	61.1
PS+FOM	1.15	2.16	0.97 (0.82; 1.12)	<0.01	87.5
PS+FOH	1.26	2.56	1.34 (1.19; 1.49)	<0.01	120.8
DHA					
C	4.93	4.86	-	-	-
PS	4.98	4.91	-0.01 (-0.19; 0.16)	1.00	-0.3
PS+FOL	4.96	5.23	0.37 (0.19; 0.54)	<0.01	7.4
PS+FOM	4.95	5.24	0.35 (0.17; 0.52)	<0.01	7.1
PS+FOH	5.13	5.53	0.46 (0.29; 0.63)	<0.01	9.4

C, control; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FA, fatty acid; LSMean, least square mean; PS, plant sterols; PS+FOH, plant sterols + high-dose fish oil; PS+FOL, plant sterols + low-dose fish oil; PS+FOM, plant sterols + medium-dose fish oil.

[a] LSMeans were corrected for baseline and, if significantly contributing to the model (P <0.10), for treatment x baseline, gender, age, BMI, change in body weight, period (before or after summer), and cohort (time of study start per subject).

Adverse events

A total of 126 subjects experienced 214 AEs during the intervention period. Overall, AEs were mild, with major complaints being headache (n = 43), acute nasopharyngitis (n = 35), abdominal pain (n = 11), and nausea (n = 10) and were unlikely to be or not related to the study procedures. In total, 3 subjects experienced a serious AE (concussion, hospitalized due to chest pain, or diagnosed with an ileal diverticulum); none were related to the study procedures and all were resolved. There was no remarkable difference in the number of subjects

experiencing AEs or in the nature and frequency of AEs between the 5 treatment groups.

Discussion

This randomized controlled intervention study showed that the combination of EPA+DHA from FO (with doses ranging from 0.9 to 1.8 g/d) and PS (at a dose of 2.5 g/d) decreases TG concentrations in a dose-dependent manner (9-16%) while also decreasing LDL-C concentrations (~13%) in a population with elevated cholesterol but normal TG concentrations. On the basis of data from statin trials, a 0.45-0.57 mmol/L (~13%) reduction in LDL-C could potentially reduce the risk of CHD by ~12-14%[25]. On the basis of currently available Mendelian randomization data[6], it can be estimated that a 10% decrease in TG could lower CHD risk by 4-5% independently of changes in LDL-C. Whether these estimated CHD risk reductions, if present at all, would be additive when consuming a combination of PS and FO incorporated in a single food format remains unclear and requires further investigation.

The dose-response relation for the TG-lowering effect of EPA+DHA was previously shown in 2 meta-analyses[10,12]. In the meta-analysis by Eslick *et al.*[12], 47 studies were selected, all including adults at risk of cardiovascular disease. Mean EPA+DHA intakes ranged between 0.9 and 6.8 g/d. On the basis of linear meta-regression analysis, it was shown that the decrease in TGs was significantly related to the dose of EPA+DHA ($P < 0.01$). The meta-analysis by Musa-Veloso *et al.*[10] included 15 studies and established a continuous dose-response curve from which the TG-lowering effect for a given dose of EPA+DHA could be calculated. Mean intakes of EPA+DHA ranged between 0.2 and 5.6 g/d. On the basis of this dose-response curve, EPA+DHA doses of 0.9, 1.3, and 1.8 g/d were predicted to decrease TGs by 11.7%, 15.4%, and 18.9%, respectively. These outcomes are in accordance with the findings of our study that investigated different doses of FO side-by-side; compared with the C group, mean changes in TG concentrations were -9.3%, -13.9%, and -16.2%, respectively. On the basis of the established dose-response curve (i.e., correcting for the PS group), TG-lowering effects of 6.3%, 8.9%, and 12.2%, respectively, were found. Hence, this suggests that part of the TG-lowering effect of the combination of PS and EPA+DHA from FO might be explained by the presence of PS that seem to exert a modest ~6% TG-lowering effect[26].

So far, 6 studies have investigated the effects on blood lipids of PS in combination with EPA+DHA[14-19]. In these studies, PS were either esterified to fish FAs and provided in supplements[16,18] or oil or margarine[15,19], or were provided separately from the FO, i.e., applying FO capsules next to PS-enriched yogurt drinks[17] or spreads[14]. The current study is the first to our knowledge that used a single food format (i.e., low-fat spread) that was enriched with both PS, in their ester form, and FO. Overall and in accordance with our study findings, the previous studies showed that the combination of PS and EPA+DHA from FO decreased both TG and LDL-C concentrations, although the decrease in LDL-C seemed somewhat diminished with high intakes of EPA+DHA (≥5 g/d), possibly due to the relatively high SFA content of FO.

Strengths of this randomized study include the large number of subjects (n = 332) who were followed up under well-controlled, double-blind conditions. Furthermore, this study was designed as a parallel study, minimizing the risk of carryover effects. Last, self-reported compliance with test product intake was excellent; this was further reflected in a dose-dependent higher content of EPA and DHA in erythrocyte membranes. DHA in erythrocyte membranes is known to be higher and tends to increase less upon intervention than does EPA[27,28]. In the study by Katan *et al*.[27], for example, 1 g/d of EPA resulted in an increase in erythrocyte EPA of 2% of total FAs, whereas a similar intake of DHA resulted in an increase in erythrocyte DHA of 1% of total FAs after 12 months of intervention. The average increases observed in the current 4-week study are somewhat lower (~1.3% for EPA and ~0.5% for DHA); reaching a new steady state probably requires more time than 1 month of intervention. Nevertheless, it is clear from these analyses that, overall, subjects used different, i.e., increasing, doses of EPA+DHA in the different treatment groups.

Some limitations of this study should be mentioned as well. First, in the current study, FO was used as a rich source of EPA+DHA. Although the TG-lowering effect of FO is known to be attributable to its EPA+DHA content, we cannot exclude that other ingredients in FO (e.g., trans fat and SFAs) may have affected the blood lipid concentrations to some extent (e.g., smaller PS-induced reductions in LDL-C with increasing doses of SFA-containing FO). In the current study, however, we did not observe such effects. It is likely that the amount of SFAs in the FO used was too small to partly counteract the LDL-C-lowering effect of PS. Second, it is known that

the magnitude of the TG-lowering effect is influenced by the initial TG concentration[10,12]. In the current study, subjects were selected on the basis of elevated cholesterol concentrations, whereas elevated TG concentrations were not a requirement for inclusion. Indeed, the mean TG concentration at baseline was within normal ranges (~1.12 mmol/L). We cannot rule out that larger effects would have been found if subjects with higher initial TG concentrations had been included. Nevertheless, it is remarkable that significant reductions in TGs of 9-16% were observed in our study population with normal TG concentrations.

According to the World Health Organization, ~40% of adults (>25 y) worldwide have elevated TC (>5 mmol/L) concentrations[29]. Future economic development, urbanization, and nutritional transition might lead to further increases in cholesterol concentrations, particularly in developing countries[30]. Furthermore, ~30% of adults (>18 y) in the United States have above desirable (>1.7 mmol/L) TG concentrations[31,32], and this proportion is expected to increase in the near future due to the increasing prevalence of obesity and type 2 diabetes. Interestingly, lifelong lower exposures to risk factors (e.g., LDL-C) seem to be associated with a greater reduction in CHD risk per unit of cholesterol-lowering than that observed with a statin treatment later in life[25,33]. Thus, from a preventative point of view, there is an increasing need to manage blood lipid risk factors to prevent future CHD events.

In summary, the consumption of a low-fat spread enriched with PS and different low (<2 g/d) doses of EPA+DHA from FO lowers TG concentrations in a dose-dependent manner in addition to significantly decreasing LDL-C concentrations. The use of low-fat spreads enriched with both PS and FO may thus offer an interesting opportunity for a combined blood lipid benefit that would fit in diet and lifestyle changes for improving blood lipid profiles.

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JFAQ and JO have no conflicts of interest. R.T.R., I.D., Y.E.M.P.Z., and E.A.T. designed the research; Y.E.M.P.Z. and J.O. conducted the research; J.F.A.Q. performed the statistical analysis; R.T.R. and E.A.T. wrote the manuscript; and R.T.R. had primary responsibility for final content. All authors read and approved the final manuscript.

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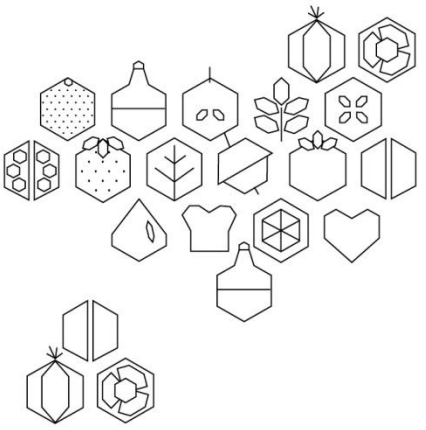
Supplemental material

Supplemental Appendix 1 – Fatty acid composition of the fish oil

	Composition, g/100g
SFAs	
14:0	7.4
15:0	0.5
16:0	16.9
17:0	0.4
18:0	3.4
MUFAs	
16:1	9.0
17:1	0.3
18:1n-9	9.3
18:1n-7	3.1
20:1n-9	1.0
22:1n-11	1.0
24:1n-9	0.4
PUFAs	
16:2n-6	1.4
18:2n-6	1.2
18:3n-3	0.7
18:4n-3	2.3
20:4n-6	1.1
20:4n-3	0.8
20:5n-3 (EPA)	19.8
21:5n-3	0.9
22:5n-6	0.4
22:5n-3	2.5
22:6n-3 (DHA)	10.4
TFAs	<5
Cholesterol	<1.5

EPA, eicosapentaenoic fatty acid; DHA; docosahexaenoic fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TFA, trans fatty acid



Chapter 6

Consumption of plant sterol-enriched foods and effects on plasma plant sterol concentrations - a meta-analysis of randomized controlled studies

Rouyanne T. Ras
Harry Hiemstra
Yuguang Lin
Mario A. Vermeer
Guus S.M.J.E. Duchateau
Elke A. Trautwein

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Abstract

Objective - Intake of plant sterol (PS)-enriched foods effectively lowers plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations while increasing plasma PS concentrations. The magnitude of this increase has not been systematically assessed. This study aimed to investigate the effect of PS-enriched foods on plasma PS concentrations by performing a meta-analysis of randomized controlled studies.

Methods - Published PS intervention studies reporting plasma PS concentrations were searched through June 2012. Studies were selected that fulfilled predefined in- and exclusion criteria. Data were extracted, particularly on campesterol, sitosterol, TC and LDL-C. Random-effects models were used to calculate net effects while weighing each study by the inverse of its variance. Potential sources of heterogeneity were investigated.

Results - The meta-analysis included data from 41 studies (55 strata) with in total 2084 subjects. The average dose of PS from enriched foods was 1.6 g/d (range: 0.3-3.2 g/d). Plasma sitosterol and campesterol concentrations were increased by on average 2.24 µmol/L (31%) and 5.00 µmol/L (37%), respectively, compared to control. TC and LDL-C were reduced by on average 0.36 mmol/L (5.9%) and 0.33 mmol/L (8.5%), respectively. The increase in sitosterol and campesterol was impacted by the dose of PS, the baseline PS concentration and the PS composition of the test products. In the highest PS dose category (2.0-3.2 g/d), increases in sitosterol and campesterol were on average 3.56 and 7.64 µmol/L, respectively.

Conclusion - Intake of PS-enriched foods increases plasma sitosterol and campesterol concentrations. However, total PS remain below 1% of total sterols circulating in the blood.

Introduction

Plant sterols (PS) are found in all foods of plant origin and are structurally similar to cholesterol except for a slight difference in their side chain, i.e., an additional ethyl or methyl group at C24. The two major PS are sitosterol (24α-ethylcholesterol) and campesterol (24α-methylcholesterol). Intake of PS-enriched foods or supplements has been shown to effectively lower total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations[1,2]. Based on recent meta-analyses, a PS intake of 2 g/d lowers LDL-C by on average 0.31-0.34 mmol/L or 8-10%[3-5].

Elevated TC, and especially LDL-C, is an established risk factor for cardiovascular disease (CVD) and reducing cholesterol by dietary or drug interventions is known to reduce the risk of CVD[6,7]. Hence, the cholesterol-lowering properties of PS have been acknowledged by health associations such as the National Cholesterol Education Program Adult Treatment Panel III[8], the American Heart Association[9], the European Society of Cardiology and the European Atherosclerosis Society[10].

PS lower plasma cholesterol by partly inhibiting cholesterol absorption in the gut, mainly through competition with cholesterol for micellar incorporation[11]. In contrast to cholesterol, PS themselves are not bioavailable in significant quantities as they are excreted back from the intestinal mucosa into the intestinal lumen by the heterodimer ATP-binding cassette (ABC) transporters ABCG5/8[12]. Only a small amount of dietary PS can be absorbed and reaches the systemic circulation[13]. Furthermore, PS are not synthesized in the human body. As such, circulating PS concentrations are ~200 times lower compared to cholesterol concentrations in subjects consuming habitual diets[14].

When people consume the recommended dose of 2 g/d PS for cholesterol-lowering purposes, they ingest 7-10 times more PS than what is normally reached when consuming typical Western diets which contain natural sources of PS such as vegetable oils, cereals, vegetables, fruits, nuts and seeds. In these Western-type diets, PS intakes range between 200 and 300 mg/d[15-18] whilst vegetarians can consume up to 500-1000 mg/d of PS[19,20]. Despite the low bioavailability of PS, higher intakes of PS, especially with enriched foods, do eventually result in increased plasma/serum PS concentrations.

Recently, potential health concerns have been voiced related to elevated PS concentrations following the intake of PS-enriched foods mainly because of two reasons. First, patients with homozygous sitosterolemia, a rare genetic disorder with mutations in *ABCG5/8* genes, have extremely elevated PS concentrations (~500-1200 µmol/L) and often experience early onset of atherosclerosis independent of circulating cholesterol[21,22]. Second, some, but not all, observational studies suggest a positive association between modestly elevated PS concentrations and CVD risk although the overall evidence, as summarized in a recent meta-analysis, does not support such an association[23].

Until now, the effect of PS-enriched food intake on plasma PS concentrations has not yet been systematically investigated and the size of the increase in circulating PS seems often overestimated by referring to single studies. Therefore, the objective of this study was to perform a meta-analysis of randomized controlled studies to estimate the absolute and relative change in plasma concentrations of the main PS (i.e., sitosterol and campesterol) with and without correction for TC concentrations after consumption of PS-enriched food. Additionally, we estimated the change in plasma LDL-cholesterol and TC concentrations in the selected PS intervention studies. Furthermore, sources that could possibly explain some of the between-study heterogeneity in changes in plasma PS and cholesterol concentrations were investigated.

Methods

Search strategy

To retrieve as many potentially relevant studies as possible, six databases (Medline, Embase, Cab Abstracts, Food Science & Technology Abstracts, HCA Plus and Biosis) were systematically searched through June 2012. For this, a search strategy was developed including the Medical Subject Heading 'phytosterols' and the search terms 'plant sterol* or phytosterol* or sitosterol* or campesterol* or stigmasterol* or brassicasterol*' and 'blood* or plasma or serum', limited to humans and intervention studies were possible. There was no restriction on language. For simplicity, throughout this paper, the term "plasma" is used when referring to plasma or serum depending on what has been used in the different studies.

Selection of studies

The following criteria for selecting eligible studies were pre-defined: (a) randomized placebo-controlled study in humans (studies with (familial hypercholesterolemic) children were allowed); (b) oral intake of PS-enriched foods or supplements as active treatment (throughout this paper, the term "enriched foods" encompasses also supplements which were used in only a few studies); (c) absence of co-intervention from which consumption of PS-enriched foods could not be isolated; (d) no studies with colectomized patients or patients with hetero- or homozygous sitosterolemia; (e) duration of treatment of at least two weeks; (f) reporting of treatment effects on plasma sitosterol and campesterol concentrations; (g) treatment with "common" plant sterols defined as 4-

desmethylsterols extracted from common vegetable oils and no ferulated PS such as from rice bran oil and/or sheanut oil; (h) dose of PS <10 g/d; (i) composition of the phytosterol mixture containing at least 80% PS (max 20% plant stanols); (j) no treatment with ezetimibe; and (k) no conference proceedings or duplicates.

Selection of studies was done in two rounds. In the first selection round, titles and abstracts were screened and those studies that were obviously not fulfilling the predefined selection criteria were excluded, e.g. reviews, studies testing other ingredients than PS or acute-effect studies. Because investigating effects on plasma PS is usually not the primary objective in PS intervention studies, we did not limit our search by only selecting studies that reported results on plasma PS concentrations in their abstracts. In the second selection round, full publications were read to judge eligibility of the studies. A co-intervention was defined as any additional test ingredient next to PS which was not added to the placebo intervention (e.g. the portfolio diet containing soluble fiber, nuts, PS and soy protein vs. a placebo diet). The source of fatty acid esters used to esterify PS into PS esters was not considered as a co-intervention. The PS mixtures used in the studies were not allowed to contain more than 20% plant stanols[24] as stanols are known to reduce plasma PS concentrations[25]. Studies including ezetimibe treatment were not selected because ezetimibe is known to directly impact plasma PS concentrations via mechanisms in the gut. In case of indecisiveness, eligibility was discussed amongst authors until consensus was reached.

Data extraction and transformation

Data were collected on (a) publication characteristics (reference details and year of publication); (b) study characteristics (parallel or cross-over, sample size and study duration); (c) subject characteristics (health status of subjects, mean age, mean BMI and gender distribution); (d) treatment characteristics (PS dose, form of PS (free or esterified PS), food format, PS source, etc); (e) measurement characteristics (methodology used and serum or plasma); and (f) outcome variables (plasma concentrations of sitosterol, campesterol (including those standardized for TC, e.g. expressed in µmol/mmol TC), LDL-C, TC and high-density lipoprotein cholesterol (HDL-C)). We have not assessed the quality of the individual studies because scoring of quality is rather subjective and excluding studies based on this subjective scoring was judged not appropriate.

For each of the outcome variables, data (mean absolute concentration and accompanying variance measure) were extracted at baseline and at end-of-intervention. When outcome variables were measured at different time points, the data closest to 4 weeks of intervention were selected in order to standardize the intervention duration amongst the studies. Original authors were contacted in case the sitosterol and campesterol data were solely expressed as concentrations corrected for TC[26-28].

In case concentrations of cholesterol were expressed in mg/dL, data were transformed to derive concentrations in mmol/L by using the molecular weight of cholesterol (386.65 g/mol). In case concentrations of sitosterol and campesterol were expressed in mg/L, mg/dL, µg/dL, ng/dL, µg/mL or ng/mL, data were transformed based on the molecular weights of sitosterol (414.71 g/mol) or campesterol (400.68 g/mol) to derive concentrations in µmol/L. These transformations were done both for means and SEs or SDs.

Control-adjusted absolute (µmol/L or mmol/L) and relative (%) changes plus accompanying within-study SEs for sitosterol, campesterol, LDL-C, TC and HDL-C were calculated for each study. For parallel studies, the absolute and relative changes plus accompanying SEs were calculated based on the average concentrations and variance measures at baseline and at end-of-intervention of treatment and control groups. For cross-over studies that reported baseline data, the absolute and relative changes were calculated similarly as for the parallel studies. Otherwise, these were calculated based on the data at the end of the treatment and control periods. In **Supplemental Appendix 1**, a complete overview is provided of the formulas that were used to transform the data.

Statistical analysis

For each of the main outcome variables, a net effect was calculated according to a random-effects model while weighing the studies by the inverse of their within-study variance ($1/SE^2$)[29]. This was done for baseline concentrations, end-of-intervention concentrations, absolute changes and relative changes. In contrast to fixed-effects models, random-effects models take into account both the within-study variation as well as the large variation between studies and assume that the treatment effects of the individual studies vary around some overall average treatment effect.

Funnel plots were developed with the effect sizes of all individual studies expressed against their precisions (1/SE). These plots visualize the likeliness of heterogeneity (when effect sizes fall outside the confidence limits) as well as the likeliness of publication bias (when clear holes in the funnel (i.e., asymmetry) are detected). Heterogeneity was furthermore assessed by calculating Q-statistics and I^2-statistics[29] whereas publication bias was analyzed according to Egger tests[30]. Forest plots were developed for the absolute (µmol/L) and relative changes (%) in sitosterol and campesterol.

Covariate analysis was performed to investigate the impact of pre-specified covariates on the absolute and relative changes in plasma PS and cholesterol concentrations after consumption of PS-enriched foods. These covariates were dose of PS tested, baseline PS or cholesterol concentration and PS composition (i.e., amount of sitosterol or campesterol in the PS mixture of the test products). Subgroup analysis was performed for determining differences between subgroups after stratification based on the above mentioned covariates. Also meta-regression analysis was used for assessing their correlations with the effect sizes found.

P values below 0.05 were considered statistically significant based on two-sided hypothesis testing. All analyses were performed with the statistical software package SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA). The PRISMA statement guidelines for reporting in systematic reviews and meta-analyses were followed.

Results

Overview of included studies

With the systematic search, 1034 papers were identified. After two selection rounds, 41 human intervention studies including 55 strata were judged eligible for inclusion in the current meta-analysis (**Figure 1**). Most of the studies were excluded because they were no randomized controlled studies with human subjects, investigated a different active ingredient or did not report plasma or serum PS concentrations.

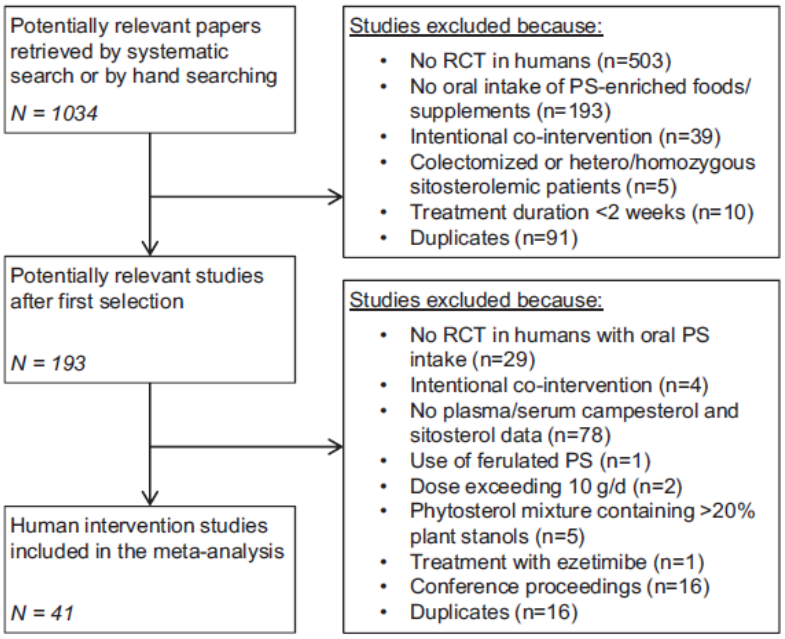

Figure 1. *Flow-chart of the study selection process.*

Of the 41 studies, 21 studies were parallel studies[26-28,31-48], 19 were cross-over studies[49-67] and 1 paper described a parallel and a cross-over study[68]. Non-standardized concentrations of sitosterol and campesterol were available for all studies (55 strata). For 12 studies (14 strata), PS concentrations corrected for TC concentrations (e.g. expressed in µmol/mmol TC) were available.

In total, 2084 subjects were included in the studies. The average age of the subjects was 49.3 years (range: 10.5-60.1 years) and the average BMI was 26.0 kg/m^2 (range: 19.0-35.2 kg/m^2). Six strata included healthy or normocholesterolemic subjects, 39 strata included hypercholesterolemic but otherwise healthy subjects and 10 strata included (hypercholesterolemic) subjects characterized as metabolic syndrome patients, diabetics or statin users.

The median duration of studies was 28 days (range: 21-315 days). The PS dose tested was an average 1.6 g/d (median: 1.7 g/d; range: 0.3-3.2 g/d). The majority of studies used (low-fat) spreads (n = 28) whilst others used dairy products like yoghurt, milk etc (n = 12) or other formats like dressing, mayonnaise, bread or supplements (n = 15). PS were in most cases esterified to different fatty acids (n =

43); only 12 strata used free PS as active ingredient. Depending on the source of PS, PS mixtures typically contained 20-30% campesterol, 45-50% sitosterol and some other minor sterols/stanols for soybean PS or 5-10% campesterol, 75-80% sitosterol and some other minor sterols/stanols for tall oil PS[33]. **Tables 1 and 2** show overviews of the parallel and cross-over studies, respectively, including sitosterol and campesterol data. In **Supplemental Appendix 2**, an overview is provided summarizing the blood cholesterol data.

Plasma PS outcomes

At baseline, plasma sitosterol and campesterol concentrations were on average 6.92 and 13.07 µmol/L, respectively. After intake of on average 1.6 g/d PS from enriched foods, plasma PS concentrations were significantly increased by on average 2.24 µmol/L for sitosterol and 5.00 µmol/L for campesterol, compared to control. Corresponding relative increases were 31.3% and 37.3%, respectively. Total PS remained below 1% of total sterols circulating in the blood. When corrected for TC, sitosterol concentrations significantly increased by on average 0.59 µmol/mmolTC (41.7%) and campesterol by on average 1.34 µmol/mmolTC (60.8%). **Table 3** gives an overview of the weighted net effects. Forest plots of the absolute changes in sitosterol and campesterol are shown in **Figure 2**. Forest plots of the relative changes are provided in **Supplemental Appendix 3**.

For both absolute and relative changes in sitosterol and campesterol concentrations, there was significant heterogeneity between the studies as assessed by inspecting funnel plots (a considerable number of studies reported effects outside the confidence limits) and calculating I^2-statistics ($P < 0.05$) (**Supplemental Appendix 4**). Furthermore, regression analysis of the standard normal deviate as a function of the precision and the asymmetrical shape of the funnel plots indicated that publication bias was likely present in all sitosterol and campesterol analyses (Egger test: P (intercept) <0.05; studies reporting relatively small increases in plasma PS concentrations at the bottom of the funnel seemed lacking).

Table 1. *Overview of parallel studies*

Reference information	Subject characteristics					Treatment characteristics				Plasma PS			
	Sample size		Gender (% male)	Age (y)	BMI (kg/m^2)	Free or ester	Dose (g/d)[a]	Food format	Duration (days)	Sitosterol		Campesterol	
	Con-trol	Active								Absolute change (µmol/L)	Relative change (%)	Absolute change (µmol/L)	Relative change (%)
Carr et al. 2009	16	16	50.0	37.1	25.5	ester	1.8[a]	capsules	28	0.97	16.3	3.69	42.5
Christiansen et al. 2001 stratum 1[b]	46	46	35.5	50.7	25.7	free	1.5	rapeseed oil margarine	90	0.97	33	-1.09	-12.3
Christiansen et al. 2001 stratum 2[b]	46	42	35.5	50.7	25.9	free	3.0	rapeseed oil margarine	90	1.02	36.6	-1.52	-21.2
Clifton et al. 2008 stratum 1[b]	39	37	56.6	55.2	26.8	ester	1.6	low-fat margarine	21	1.70	23.7	6.63	67.6
Clifton et al. 2008 stratum 2[b]	39	39	48.7	54.5	26.9	ester	1.6	low-fat margarine	21	6.36	78.9	1.37	11.4
Clifton et al. 2008 stratum 3[b]	39	36	56.0	54.2	26.7	ester	1.6	low-fat margarine	21	3.41	46	5.87	49.3
Hansel et al. 2007	99	95	67.0	48.9	23.6	ester	1.6	low-fat fermented milk	42	1.81	27.3	-0.75	-14.1
Hendriks et al. 2003[bc]	96	89	48.6	48.0	24.9	ester	1.6	low-fat margarine	182	2.67	23.7	13.38	76.2
Hernandez-Mijares et al. 2010[bd]	24	31	27.3	49.5	28.2	ester	2.0	low-fat milk	90	8.53	93.4	6.64	105.3
De Jong et al. 2006[e]	11	15	46.2	58.1	26.8	ester	2.5	low-fat margarine	112	4.10	36.4	25.50	95.3
De Jong et al. 2008[e]	17	18	60.0	59.5	25.7	ester	2.5	low-fat margarine	315	3.84	35.2	16.52	96.4
Korpela et al. 2006	82	82	21.3	57.3	27.0	free	1.9[f]	low-fat milk products	42	3.62	51.7	-1.25	-6.5
Kurokawa et al. 2008	27	32	69.9	44.7	23.2	ester	0.8	dressing	84	2.41	50	4.99	40.0
Maki et al. 2001 stratum 1[bc]	92	92	44.0	58.1	27.5	ester	1.1	low-fat margarine	35	1.11	19.8	4.49	24.1
Maki et al. 2001 stratum 2[bc]	92	40	42.4	58.4	27.3	ester	2.2	low-fat margarine	35	2.36	34.4	9.53	43.6
Mannarino et al. 2008	56	60	44.0	50.1	25.0	ester	1.6	low-fat fermented milk	42	0.58	3.9	-0.5	-3.5
Masuda et al. 2007	49	48	59.8	46.7	23.1	ester	0.8	drink	84	0.30	5	-0.07	-1.7
Neil et al. 2001[bcd]	29	29	41.9	51.6	26.0	ester	2.5	margarine	56	1.17	20.4	7.21	71.5
Nittynen et al. 2007 study 2	14	12	57.7	45.9	26.0	free	2.0	low-fat yogurt drink	56	9.65	56.9	6.99	29.6
Plana et al. 2008[ce]	40	43	41.0	51.4	26.7	Ester	1.6	low-fat fermented milk	42	2.52	33.5	0.79	16.7

Table 1. *Continued*

Reference information	Subject characteristics					Treatment characteristics				Plasma PS			
	Sample size		Gender (% male)	Age (y)	BMI (kg/m^2)	Free or ester	Dose (g/d)[a]	Food format	Duration (days)	Sitosterol		Campesterol	
	Con-trol	Active								Absolute change (µmol/L)	Relative change (%)	Absolute change (µmol/L)	Relative change (%)
Saito et al. 2006 stratum 1	17	16	100.0	38.4	23.6	ester	0.3	DAG-containing mayonnaise	28	0.42	5.5	1.60	9.4
Saito et al. 2006 stratum 2	17	16	100.0	39.2	23.6	ester	0.4	DAG-containing mayonnaise	28	1.79	23.9	4.60	27.1
Saito et al. 2006 stratum 3	17	17	100.0	38.7	23.9	ester	0.5	DAG-containing mayonnaise	28	0.93	10.5	3.80	19.8
Seki et al. 2003a	28	32	100.0	39.1	24.2	ester	0.5	vegetable oil-based bread	28	0.72	16.1	2.25	39.5
Seki et al. 2003b	11	11	100.0	41.2	24.2	ester	1.3	vegetable oil-based bread	28	0.96	23.2	3.74	78.4
Takeshita et al. 2007a	21	18	100.0	37.0	24.4	ester[g]	0.4	DAG-containing mayonnaise	28	1.01	17.4	3.62	27.1
Takeshita et al. 2008	15	14	20.7	59.4	21.9	free	0.5	DAG-containing cooking oil	84	-0.46	-5.0	1.85	10.2
Varady et al. 2004 study 1	20	18	31.6	56.6	26.3	ester	1.8	low-fat margarine	56	0.95	28.9	2.94	38.4
Varady et al. 2004 study 2	18	18	22.2	52.1	29.9	ester	1.8	low-fat margarine	56	1.04	24.0	3.99	48.9

DAG, diacylglycerol; PS, plant sterols.

[a] PS dose expressed as free equivalents. Carr et al. 2009 reported the PS dose as esters; the amount of free equivalents was calculated assuming that 40% of PS esters are fatty acids and 60% are PS.

[b] The following papers reported serum/plasma PS concentrations in a subset of the total number of subjects included in the study: Christiansen et al. (n = 52), Clifton et al. (n = 25), Hendriks et al. (n = 83), Hernandez-Mijares et al. (n = 34), Neil et al. (n = 54) and Maki et al. (n = 71).

[c] Cholesterol and plasma PS data are based on raw data.

[d] No average age was reported; thus, the average of the medians per group was used.

[e] Non-standardized plasma PS data were obtained from original authors.

[f] The dose of PS was calculated by taking the average of 2, 2 and 1.65 g/d which were the doses used in the different foods.

[g] PS were mostly esterified PS.

Table 2. *Overview of cross-over studies*

Reference information	Subject characteristics				Treatment characteristics				Plasma PS			
									Sitosterol		Campesterol	
	Sample size	Gender (% male)	Age (y)	BMI (kg/m^2)	Free or ester	Dose (g/d)[a]	Food format	Duration (days)	Absolute change (μmol/L)	Relative change (%)	Absolute change (μmol/L)	Relative change (%)
AbumWeis et al. 2006 stratum 1	30	-	59.0	28.0	free	1.7	margarine	29	2.90	41.4	0.80	4.3
AbumWeis et al. 2006 stratum 2	30	-	59.0	28.0	ester	1.7	margarine	29	1.70	24.3	5.50	29.6
Amundsen et al. 2004[b]	41	46.3	10.5	19.0	ester	1.6	low-fat margarine	56	4.00	33.4	10.37	76.3
Casas-Agustench et al. 2012	43	51.2	49.0	26.6	ester	2.0	skimmed milk	28	12.01	90.6	9.22	62.8
Clifton et al. 2004 stratum 1[c]	58	39.7	54.0	26.2	ester	1.6	bread	21	2.70	31.6	4.09	44.1
Clifton et al. 2004 stratum 2[c]	58	39.7	54.0	26.2	ester	1.6	milk	21	2.34	27.4	4.89	52.7
Demonty et al. 2006	21	52.4	54.2	25.9	ester	1.7	orange juice	29	1.38	19.9	8.19	67.9
Hallikainen et al. 2000	34	47.6[d]	48.8	24.9	ester	2.0	margarine	28	2.00	24.4	6.89	35.8
Heggen et al. 2010 stratum 1	59	72.9	52.0	24.8	ester	2.0	low-fat margarine	28	5.73	83.1	1.10	4.8
Heggen et al. 2010 stratum 2	59	72.9	52.0	24.8	ester	2.0	low-fat margarine	28	5.97	86.5	15.05	65.6
Houweling et al. 2009 study 1	41	100.0	50.4	27.8	ester	2.0	low-fat margarine	28	2.77	23.1	4.25	21.0
Houweling et al. 2009 study 2	41	100.0	52.1	29.0	ester	2.0	low-fat margarine	28	2.71	34.7	3.23	27.0
Jakulj et al. 2005	39	87.5	55.5	25.9	ester	2.0	low-fat margarine	28	1.91	20.7	10.96	78.7
Jones et al. 2000	15	100.0	-	-	ester	1.8	low-fat margarine	21	2..50	29.1	8.60	67.3
Kratz et al. 2007	10	-	30.0	21.9	ester	2.0	low-fat margarine	42	0.90	12.5	4.00	24.0
Lau et al. 2005 study 1[b]	14	35.7	54.5	30.2	free	1.8	margarine	21	0.84	34.1	0.31	8.6
Lau et al. 2005 study 2[b]	15	40.0	55.1	26.9	free	1.8	margarine	21	0.66	20.3	0.72	9.3
Mussner et al. 2002	62	38.7	42.0	24.0	ester	1.8	margarine	21	1.57	35.3	6.96	78.2
Myrie et al. 2012	15	53.3	33.8	30.4	free	1.6	capsules	29	0.21	3.0	2.84	22.8
Nittynen et al. 2007 study 1	15	100.0	41.0	26.0	free	1.0	low-fat yogurt drink	28	2.41	38.5	5.99	34.3
Ooi et al. 2007	9	100.0	60.1	35.2	ester	2.0	breakfast cereal and	28	1.95	44.3	5.19	80.6
Rudkowska et al. 2008 stratum 1	26	-	59.6	26.4	ester	1.6	low-fat yogurt	30	0.85	42.7	2.74	27.8
Rudkowska et al. 2008 stratum 2	26	-	59.6	26.4	ester	1.6	low-fat yogurt	30	1.02	55.5	3.87	39.0
Takeshita et al. 2007b	14	0.0	52.2	23.0	Free	0.6	DAG-containing cooking oil	28	1.44	22.2	4.38	33.3
Vanstone et al. 2002	15	60.0	47.8	30.8	free	1.8	Butter	21	2.40	28.3	13.1	90.9
Weststrate et al. 1998[e]	40	50.0	46.2	24.2	ester[f]	3.2	margarine	21	2.89	37.5	12.23	71.0

DAG, diacylglycerol; PS, plant sterols.

[a] PS dose expressed as free equivalents.

[b] The following papers reported serum PS concentrations in a subset of the total number of subjects included in the study: Amundsen et al. (n = 29) and Lau et al. (n = 27).

[c] Incomplete cross-over design: not all subjects received all treatments ($n_{control}$ = 58, n_{bread} = 36, n_{milk} = 40).

[d] Gender distribution is based on 42 subjects (8 dropped out).

[e] Age, gender, cholesterol and plasma PS data are based on raw data of n = 40. BMI is based on total n in the study (n = 100).

[f] PS were mostly esterified PS.

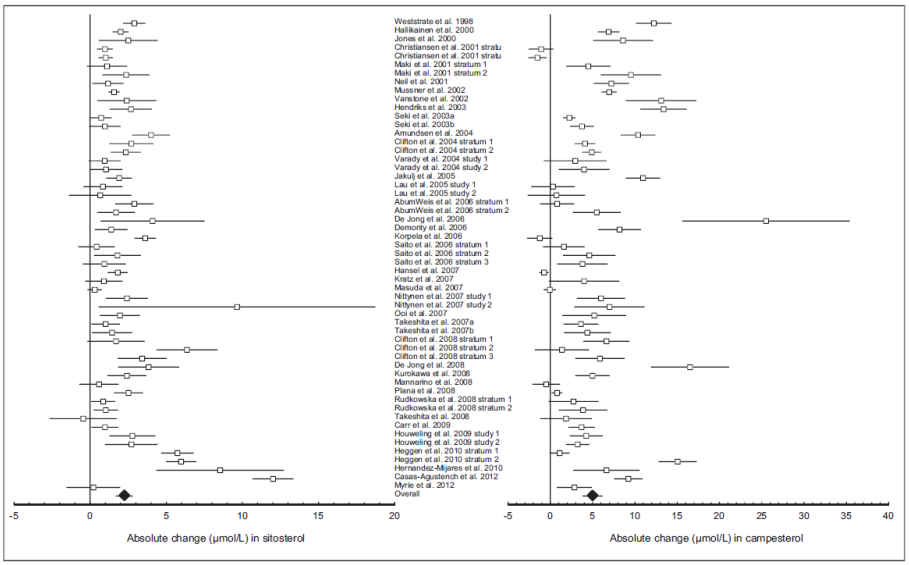

Figure 2. *Forest plots of the absolute (µmol/L) changes in plasma sitosterol and campesterol concentrations.*

Plasma cholesterol outcomes

LDL-C and TC concentrations at baseline were on average 3.90 and 6.04 mmol/L, respectively. LDL-C was reduced by on average 0.33 mmol/L (8.5%) and TC by 0.36 mmol/L (5.9%) with an average PS intake of 1.6 g/d, compared to control (**Table 3**). Heterogeneity was statistically significant for absolute changes in TC (P = 0.029) whereas it was not significant for absolute and relative changes in LDL-C and for relative changes in TC (P >0.05). The heterogeneity in cholesterol changes was clearly less obvious as compared to the heterogeneity in plasma PS changes. Visual inspection of symmetrical funnel plots as well as the outcomes of the Egger tests (P of intercept ranging between 0.397 and 0.613) suggested absence of publication bias for LDL-C and TC (**Supplemental Appendix 5**). HDL-C did not change upon PS intervention (-0.00 mmol/L or -0.1%; **Table 3**).

Table 3. *Weighted net effects (baseline, end-of-intervention, absolute change and relative change) of plasma sitosterol, campesterol, LDL-cholesterol, total cholesterol and HDL-cholesterol, based on random effects models.*

Parameter	Unit	Baseline concentration[a]	Concentration after PS intervention[b]	Absolute change vs. placebo	Relative change vs. placebo (%)
Sitosterol	µmol/L	6.92 (6.23; 7.61)	9.29 (8.20; 10.38)	2.24 (1.71; 2.77)	31.3 (26.0; 36.7)
	µmol/mmolTC[c]	1.22 (0.88; 1.56)	1.77 (1.14; 2.41)	0.59 (0.25; 0.92)	41.7 (31.0; 52.5)
Campesterol	µmol/L	13.07 (11.65; 14.48)	18.18 (15.99; 20.38)	5.00 (3.86; 6.14)	37.3 (29.3; 45.3)
	µmol/mmolTC[c]	2.10 (1.63; 2.56)	3.39 (2.43; 4.34)	1.34 (0.83; 1.85)	60.8 (44.7; 76.9)
LDL-C	mmol/L	3.90 (3.76; 4.03)	3.59 (3.47; 3.72)	-0.33 (-0.37; -0.30)	-8.5 (-9.2; -7.7)
TC	mmol/L	6.04 (5.90; 6.18)	5.69 (5.56; 5.82)	-0.36 (-0.40; -0.32)	-5.9 (-6.5; -5.3)
HDL-C	mmol/L	1.42 (1.37; 1.47)	1.41 (1.36; 1.47)	-0.00 (-0.02; 0.01)	-0.1 (-1.1; 0.9)

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PS, plant sterols; TC, total cholesterol; TG, triglycerides.

[a] The weighted average baseline concentration was calculated based on the baseline concentrations in the active and placebo groups for parallel studies. For cross-over studies, the baseline concentrations were used when reported; otherwise the end-of-intervention concentrations of the placebo periods were used.

[b] The weighted average concentration after PS intervention was calculated based on the concentrations after PS intervention in the active treatment groups for parallel studies, and based on the end-of-intervention concentrations of the active periods in case of cross-over studies.

[c] The weighted net effects of the PS to cholesterol ratios were based on only 12 studies (14 strata) that reported plasma PS concentrations corrected for total cholesterol concentrations. The non-standardized weighted net effects are based on 41 studies (55 strata).

Expressed as means (95% CI)

Covariate analyses

Meta-regression analyses revealed that dose of PS, baseline PS concentration and PS composition significantly impacted the absolute changes in plasma PS concentrations. Absolute increases in sitosterol and campesterol were larger in studies with higher doses of PS (β = 1.02, P = 0.014 and β = 2.37, P = 0.009, respectively), with higher average baseline concentrations (β = 0.39, P <0.001 and β = 0.35, P <0.001, respectively), and with higher amount of either sitosterol or campesterol in the PS mixture (β = 0.06, P = 0.004 and β = 0.27, P <0.001, respectively). When looking at the relative changes, the impact of baseline concentrations was, as expected, not present anymore. Subgroup analyses showed comparable results except for a weaker (non-significant) impact of PS composition on absolute and relative changes in plasma sitosterol and a weaker impact of PS dose on relative changes in plasma campesterol. In the subgroup with the highest dose studies (2.0-3.2 g/d PS), increases in sitosterol and campesterol were on average 3.56 µmol/L (42.2%) and 7.64 µmol/L (47.9%), respectively. The results of the covariate analyses are shown in **Table 4**.

Table 4. *Results of the covariate analysis for absolute and relative changes in plasma sitosterol, campesterol, LDL-cholesterol and total cholesterol concentrations.*

Trial characteristic	Stratification variable	No of study arms	Subgroup analysis			Meta-regression analysis		Subgroup analysis			Meta-regression analysis	
			Change vs. placebo	95% CI	P between subgroups[a]	β	P meta-regression[b]	Change vs. placebo	95% CI	P between subgroups[a]	β	P meta-regression[b]
			Absolute change (µmol/L) in sitosterol					*Relative change (%) in sitosterol*				
Baseline concentration	below median (≤6.9 µmol/L)	26	1.38	(0.71; 2.05)	<0.001	0.39	<0.001	28.1	(20.3; 36.0)	0.284	0.4	0.677
	above median (>6.9 µmol/L)	29	3.08	(2.41; 3.76)				33.9	(26.8; 41.1)			
Dose of PS	≥0.3 g/d and ≤1.5 g/d	13	1.08	(0.13; 2.04)	0.001	1.02	0.014	18.7	(9.0; 28.5)	0.002	12.0	0.002
	>1.5 g/d and <2.0 g/d	25	2.00	(1.31; 2.69)				30.0	(22.9; 37.2)			
	≥2.0 g/d and ≤3.2 g/d	17	3.56	(2.68; 4.44)				42.2	(33.6; 50.9)			
PS composition (% sitosterol)	below median (<50%)	27	1.81	(1.06; 2.56)	0.128	0.06	0.004	26.8	(19.1; 34.4)	0.107	0.8	<0.001
	above median (≥50%)	28	2.62	(1.90; 3.34)				35.4	(28.2; 42.6)			
			Absolute change (µmol/L) in campesterol					*Relative change (%) in campesterol*				
Baseline concentration	below median (≤12.6 µmol/L)	28	3.44	(1.97; 4.91)	0.003	0.35	<0.001	35.8	(24.3; 47.4)	0.724	0.4	0.572
	above median (>12.6 µmol/L)	27	6.64	(5.10; 8.17)				38.7	(27.6; 49.8)			
Dose of PS	≥0.3 g/d and ≤1.5 g/d	13	3.03	(0.92; 5.15)	0.003	2.37	0.009	24.1	(8.7; 39.5)	0.084	12.8	0.042
	>1.5 g/d and <2.0 g/d	25	4.26	(2.72; 5.79)				37.4	(26.0; 48.9)			
	≥2.0 g/d and ≤3.2 g/d	17	7.64	(5.72; 9.55)				47.9	(33.8; 62.0)			
PS composition (% campesterol)	below median (<25%)	27	3.11	(1.67; 4.54)	<0.001	0.27	<0.001	26.4	(15.9; 37.0)	0.005	1.6	0.001
	above median (≥25%)	28	6.85	(5.39; 8.31)				47.5	(37.1; 58.0)			
			Absolute change (mmol/L) in LDL-C					*Relative change (%) in LDL-C*				
Baseline concentration	below median (≤3.9 mmol/L)	26	-0.26	(-0.31; -0.21)	0.001	-0.10	0.011	-7.4	(-8.7; -6.0)	0.057	-0.6	0.559
	above median (>3.9 mmol/L)	29	-0.37	(-0.41; -0.34)				-8.9	(-9.8; -8.1)			
Dose of PS	≥0.3 g/d and ≤1.5 g/d	13	-0.25	(-0.32; -0.18)	0.038	-0.07	0.017	-6.6	(-8.3; -4.8)	0.052	-1.7	0.015
	>1.5 g/d and <2.0 g/d	25	-0.35	(-0.40; -0.30)				-8.8	(-9.8; -7.7)			
	≥2.0 g/d and ≤3.2 g/d	17	-0.35	(-0.40; -0.31)				-9.1	(-10.2; -7.9)			
			Absolute change (mmol/L) in TC					*Relative change (%) in TC*				
Baseline concentration	below median (≤6.0 mmol/L)	26	-0.26	(-0.32; -0.20)	<0.001	-0.14	<0.001	-4.6	(-5.6; -3.6)	0.004	-1.3	0.064
	above median (>6.0 mmol/L)	29	-0.41	(-0.45; -0.37)				-6.4	(-7.0; -5.8)			
Dose of PS	≥0.3 g/d and ≤1.5 g/d	13	-0.28	(-0.36; -0.20)	0.039	-0.07	0.034	-4.6	(-5.8; -3.3)	0.01	-1.2	0.011
	>1.5 g/d and <2.0 g/d	25	-0.35	(-0.41; -0.30)				-5.7	(-6.5; -4.9)			
	≥2.0 g/d and ≤3.2 g/d	17	-0.4	(-0.46; -0.35)				-6.8	(-7.6; -6.0)			

β, regression coefficient; LDL-C, low-density lipoprotein cholesterol; PS; plant sterols; TC, total cholesterol.

[a] P between subgroups <0.05 indicates a significant difference in pooled effect sizes between subgroups.

[b] P meta-regression <0.05 indicates a significant correlation between the variable under investigation and the effect sizes.

Post hoc analyses

To investigate the shape of the dose-response relationship between PS doses and changes in plasma PS, we established continuous dose-response curves based on first-order elimination functions. A slight tapering-off effect seemed present for changes in both plasma sitosterol (**Figure 3, Panel A**) and campesterol concentrations (**Figure 3, Panel B**). It should however be noted that these curves are severely limited by the heterogeneous distribution of the observed changes across the range of doses included.

In addition, we investigated the impact of food format (dairy-type foods vs. (low-fat) margarine), blood matrix (serum vs. plasma), subjects' health status (diabetics/metabolic syndrome patients vs. hypercholesteromic subjects vs. normocholesterolemic/healthy subjects) and study duration (≤4 weeks vs. >4 weeks) on the changes in plasma PS concentrations. No significant impact of these potential covariates on the absolute and relative changes in plasma PS concentrations could be detected ($P > 0.05$). Regarding duration, we additionally analyzed whether there was a statistically significant difference between halfway and end-of-intervention plasma PS changes in studies that reported plasma PS concentrations at several time points[27,32,44,47]; again, no significant impact of duration was detected ($P > 0.05$).

Covariate analyses furthermore revealed that absolute and relative reductions in LDL-C and TC were larger with higher doses of PS and that absolute reductions in LDL-C and TC were larger with higher respective baseline concentrations. The impact of baseline cholesterol concentrations on the relative changes was weaker, especially for LDL-C (see **Table 4**).

At last, we analyzed whether the relative changes in LDL-C were related to the relative changes in plasma PS (**Supplemental Appendix 6**). In fact, no such correlation was found. Perhaps differences in metabolic fates between cholesterol and PS, e.g. circulating cholesterol is derived from synthesis and absorption whereas circulating PS can only be obtained through absorption, provide an explanation for this finding. Also, the considerable heterogeneity in plasma PS changes might have blurred the association with LDL-C.

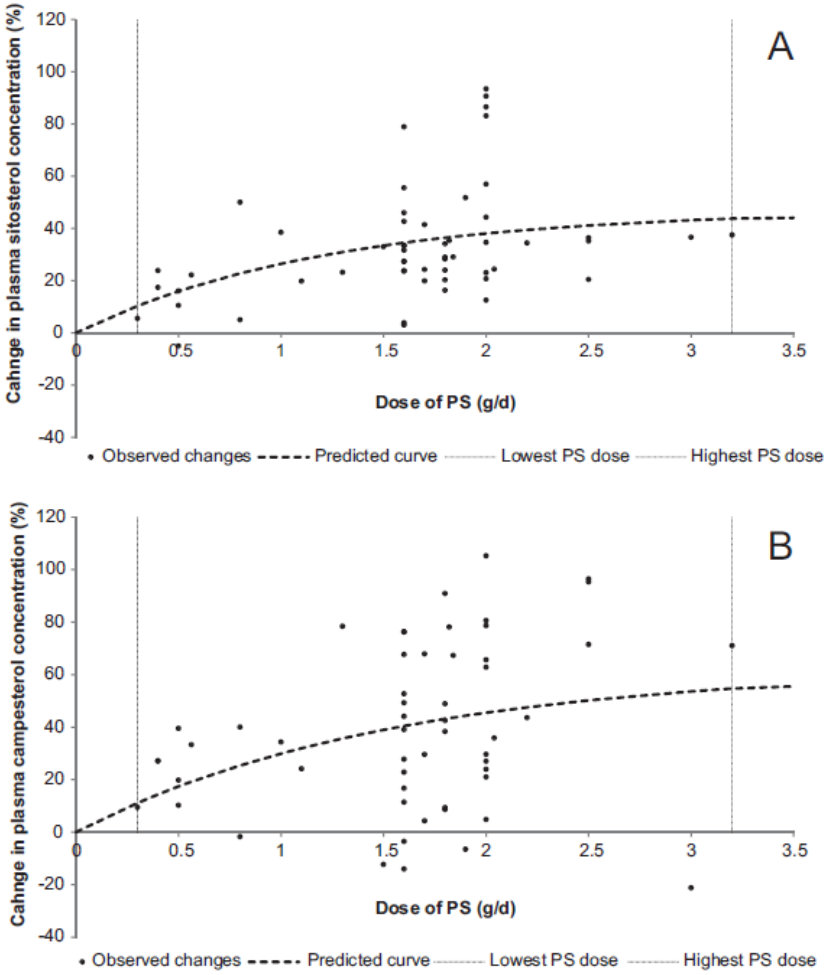

Figure 3. *Dose-response relationship between doses of plant sterols (PS) and relative (%) changes in plasma sitosterol (Panel A) and campesterol (Panel B) concentrations. A first-order elimination curve was plotted through the observed changes.*

Discussion

For the first time, the effect of PS-enriched food intake on plasma PS concentrations was systematically investigated by reviewing available data from published PS intervention studies. We observed significant increases in plasma sitosterol and campesterol concentrations after intake of PS-enriched foods, as was expected. The average increases in plasma sitosterol and campesterol concentrations were 2.24 µmol/L (31%) and 5.00 µmol/L (37%), respectively, with

an average intake of 1.6 g/d PS. In the highest PS dose category (2.0-3.2 g/d), the average absolute increases were 3.56 µmol/L (42%) and 7.64 µmol/L (48%), respectively. The baseline plasma sitosterol and campesterol concentrations that we observed in the current meta-analysis (6.9 and 13.1 µmol/L, respectively) were comparable to what has previously been reported by Chan *et al*.[14]. In this review including data of 45 studies, average baseline concentrations for sitosterol and campesterol in the general population were 7.9 and 14.2 µmol/L, respectively. We furthermore observed an average reduction in LDL-C of 0.33 mmol/L (8.5%) which is similar to the pooled LDL-C-lowering effect expected for 1.6 g/d of PS based on several recent meta-analyses[3-5]. So, despite the smaller number of studies (reporting plasma PS concentrations) included in the current meta-analysis compared to the more extensive cholesterol-lowering efficacy meta-analyses, our selection of studies seems representative for a broader range of studies. Also, with no less than 41 studies, a robust overview of the available literature has been developed.

In the current meta-analysis, the change in plasma PS concentrations was related to the dose of PS consumed per day, i.e., the higher the dose, the larger the increase in both sitosterol and campesterol concentrations. For PS-induced cholesterol-lowering, it is known that the decrease in plasma cholesterol concentrations would reach a plateau with increasing dose of PS due to saturable processes in cholesterol uptake and transport and subsequent feedback on cholesterol synthesis. Whether such tapering-off effect exists for plasma PS concentrations is yet unclear. In an attempt to investigate this, we established continuous dose-response curves for the relationship between PS doses and changes in plasma PS concentrations. These curves suggest that some tapering-off might exist although the maximal increase in plasma PS will likely be reached at doses higher than 3.2 g/d which was the highest dose tested in our meta-analysis. Studies investigating higher PS doses are scarce. Only two studies tested PS doses exceeding 3 g/d and reported serum PS concentrations. The study by Davidson *et al*.[69] tested PS intakes of 3, 6 and 9 g/d from enriched foods, but only reported medians and ranges of plasma PS concentrations. Based on their analysis, the increase in serum PS did not significantly differ between the three PS doses, except for the TC-standardized increase in campesterol. Noteworthy, even with the highest dose of PS (9 g/d), overall absolute PS concentrations remained below 2 mg/dL (~50 µmol/L). Another study by Tuomilehto *et al*.[70] investigated increasing

intakes of PS (1.25, 2.5 and 5 g/d) together with a mix of minerals during three consecutive 5-week periods. Serum sitosterol concentrations increased in a dose-dependent manner whereas no dose-dependent increase was observed in serum campesterol concentrations. From these data, together with the findings of the current meta-analysis which included studies investigating PS doses in the range of 0.3 to 3.2 g/d, no firm conclusions can be drawn on the dose-response behaviour for plasma PS concentrations at higher PS doses (>3 g/d). The composition of the PS mixture, and related to this the PS source, also influenced the magnitude of the increase in plasma sitosterol and campesterol concentrations. For instance, studies that used PS derived from tall oil which contains less campesterol (~5-10%) and more sitosterol (75-80%) compared to e.g. soybean oil (20-30% campesterol and 45-50% sitosterol), showed smaller increases in plasma campesterol concentrations whereas increases in sitosterol concentrations were larger.

The concentrations of PS at baseline also seemed to explain part of the heterogeneity observed between different study results; in studies with higher average baseline PS concentrations, the absolute increase in plasma sitosterol and campesterol concentrations was larger compared to studies with lower average baseline concentrations. It could be that subjects with higher cholesterol/PS absorption efficiency (as indicated by higher baseline PS concentrations) are likely to absorb more PS when on PS intervention. Alternatively, the use of different analytical techniques to measure plasma PS concentrations could potentially have caused differences (systematic errors) in baseline concentrations and thus in changes upon intervention. This latter hypothesis is supported by the observation that baseline concentrations had no impact on relative changes in plasma PS concentrations which are less affected by systematic errors. Interestingly, Hendriks *et al*.[35] found that in subjects with the highest baseline PS concentrations, the average relative increase after one year consumption of PS-enriched margarine was even smaller as compared to subjects with lower baseline PS concentrations. This might suggest that some kind of feedback mechanism arises (e.g. upregulation of ABCG5/8) when PS are consumed for a longer period of time. Indeed, based on studies that reported plasma PS concentrations at different time points[27,32,44,47], the increase in plasma sitosterol and campesterol seemed to stabilize over time, which we confirmed in post hoc analyses. For example, in the study by de Jong *et al*.[27], plasma sitosterol and campesterol concentrations were similar after 45 weeks and 85 weeks of PS intervention.

The plasma PS concentrations that we observed in our meta-analysis are much lower than those reported in patients with homozygous sitosterolemia. Patients with this disease display plasma PS concentrations in the range of ~500-1200 µmol/L (~20 to 50 mg/dL)[22]. This is 20-45 times higher than the average total plasma PS concentration after intake of PS-enriched foods observed in the current meta-analysis. In this respect, the PS-induced increase in plasma PS can be considered modest and is not reaching the levels seen in homozygous sitosterolemics. Subjects with heterozygous sitosterolemia do not have such elevated plasma PS concentrations although their plasma PS concentrations are somewhat elevated (35-37%) compared to healthy controls[71]. These elevated concentrations are of the same order of magnitude as the increases in plasma sitosterol and campesterol concentrations after PS-enriched food intake seen in our study. Several studies[60,63,72,73] investigated what would happen if subjects with heterozygous sitosterolemia would regularly consume PS-enriched foods and found that these subjects showed similar plasma PS responses as compared to control subjects.

Recent evidence suggests that moderate, lifelong elevations in plasma PS concentrations in heterozygous sitosterolemics being carriers of the *ABCG8-G574R* variant are not associated with increased intima-media thickness (IMT). These subjects even showed lower IMT compared to controls[71]. IMT is a commonly used predictor for CVD, although evidence does so far not convincingly support that progression of IMT is associated with CVD risk[74]. In contrast to the findings by Horenstein *et al.*[71], in a genome wide association study, gene variants in *ABCG8* were found to be significantly associated with increased serum PS concentrations and increased CVD risk[75]. However, as stated by Plat *et al.*[76], it cannot be ruled out that this association may be an epiphenomenon because plasma PS concentrations also reflect cholesterol absorption and, therefore, the association with CVD risk may be explained by increased absorption of cholesterol. Genser *et al.*[23] recently published a meta-analysis of observational studies that aimed to investigate the association between serum sitosterol and campesterol concentrations and CVD risk. The individual studies included in this meta-analysis showed conflicting evidence. However, based on seventeen studies reporting either plasma PS concentrations in CVD cases vs. controls or relative risks for CVD, it was concluded that, overall, no association between circulating PS (sitosterol and campesterol) and risk of CVD exists. In our meta-analysis, the observed control-adjusted average

changes in plasma sitosterol and campesterol concentrations (2 and 5 µmol/L, respectively) were at least smaller than the difference between the upper and the lower tertiles of the sitosterol and campesterol distributions (6 and 10 µmol/L, respectively) reported in the Genser meta-analysis. Evidence from endpoint studies demonstrating a reduced risk of CVD has so far not been generated with intake of PS.

This meta-analysis has some limitations that need to be addressed. A considerable amount of heterogeneity was observed among the studies, more for circulating sitosterol and campesterol than for LDL-C and TC concentrations. Some of this heterogeneity could be explained by differences in PS dose, baseline PS concentrations and in PS composition. However, many other factors could have induced variability between studies such as differences in study designs, test products and study populations. In particular, between-study differences in plasma PS concentrations may have been induced by differences in analytical methods used to measure plasma PS (i.e., differences in PS separation and detections methods). For better comparison between studies, there is a clear need for standardization of methods to measure plasma PS concentrations. Furthermore, the quality of the meta-analysis depends on the quality of the studies that have been included. As such, we had pre-defined rigorous selection criteria in order to exclusively retrieve studies that were suitable to answer our study objectives. We have not considered the quality of each individual study due to the rather subjective nature of such quality scoring. Finally, there is considerable indication that publication bias was present; the funnel plots suggested that studies reporting relatively small increases in plasma PS concentrations with low precision were lacking. In PS studies, determining changes in plasma PS concentrations is usually not the primary aim, and thus, it may well be that in some studies, blood samples were drawn to measure circulating PS but were eventually not analyzed or reported due to unknown reasons. In any case, given the observation that studies reporting relatively small increases in plasma PS seemed lacking, our findings are likely not underestimated. Despite these limitations, the current meta-analysis provides a good overview of all evidence available on this topic.

In summary, our meta-analysis including data from 41 randomized controlled studies showed that intake of PS-enriched foods (average PS dose was ~1.6 g/d) increases circulating sitosterol and campesterol concentrations (2.24 and 5.00

µmol/L, respectively) whilst reducing TC and LDL-C concentrations (0.36 and 0.33 mmol/L, respectively). Overall, total PS remained below 1% of total sterols circulating in the blood which is far below levels seen in homozygous sitosterolemics. Since a considerable amount of heterogeneity was observed in plasma PS concentrations amongst the included studies, attempts to harmonize methods for measuring plasma PS concentrations should be undertaken.

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Supplemental material

Supplemental Appendix 1 - Overview of data transformation steps

1. General remarks

In parallel studies, the mean age and BMI of the study population was usually reported per study group (treatment or control). To derive one mean for the whole group, the following formulas were used:

- $N_{tot} = N_C + N_T$
- $X_{tot} = \frac{(N_C * X_C)+(N_T * X_T)}{N_C+N_T}$

where

- N_{tot} is the number of subjects of the whole group
- X_{tot} is the mean X of the whole group
- N_C is the number of subjects in the control group
- N_T is the number of subjects in the treatment group
- X_C is the mean X in the control group
- X_T is the mean X in the treatment group

For the current meta-analysis, the main outcome variables were the following: sitosterol, campesterol, LDL-cholesterol, total cholesterol and HDL-cholesterol. For each of these variables, the following information was extracted from the papers:

- N (number of subjects per group/period)
- At baseline: mean and SE (standard error) or SD (standard deviation)
- At end of intervention: mean and SE or SD

To derive SEs from SDs or vice versa, the following formulas were used:

- $SD = SE * \sqrt{n}$
- $SE = \frac{SD}{\sqrt{n}}$

2. Baseline data

To calculate the baseline means plus accompanying variance measures for each of the main variables in each of the studies, the following formulas were used.

2.1. Calculation of the baseline mean

Parallel studies:

- $N_{tot} = N_C + N_T$
- $X_{totbase} = \frac{(N_C * X_{Cbase}) + (N_T * X_{Tbase})}{N_C + N_T}$

where

- N_{tot} is the number of subjects of the whole group
- N_C is the number of subjects in the control group
- N_T is the number of subjects in the treatment group
- $X_{totbase}$ is the mean X at baseline of the whole group
- X_{Cbase} is the mean X at baseline in the control group
- X_{Tbase} is the mean X at baseline in the treatment group

Cross-over studies:

- $N_{tot} = N_C = N_T$
- $X_{totbase} = \frac{(N_C * X_{Cbase}) + (N_T * X_{Tbase})}{N_C + N_T}$

where

- N_{tot} is the number of subjects of the whole group
- N_C is the number of subjects at the start of the control period
- N_T is the number of subjects at the start of the treatment period
- $X_{totbase}$ is the mean X at baseline of the whole group
- X_{Cbase} is the mean X at the start of the control period
- X_{Tbase} is the mean X at the start of the treatment period

2.2. Variance measure of baseline mean

To estimate the within-trial variance measures of the baseline means, approximately the same formula was used for parallel and cross-over studies.

Parallel studies:

- $SE_{totbase} \approx \sqrt{\frac{N_{Tbase}}{N_{totbase}} SE_{Tbase}^2 + \frac{N_{Cbase}}{N_{totbase}} SE_{Cbase}^2}$

Cross-over studies:

- $SE_{totbase} \approx \sqrt{\frac{1}{2}SE_{Tbase}^2 + \frac{1}{2}SE_{Cbase}^2}$

where

- $SE_{totbase}$ is the SE at baseline of the whole group
- N_{Tbase} is the number of subjects at baseline in the treatment group
- N_{Cbase} is the number of subjects at baseline in the control group
- $N_{totbase}$ is the number of subjects of the whole group at the start of the intervention
- SE_{Tbase} is the SE at baseline in the treatment group/period
- SE_{Cbase} is the SE at baseline in the control group/period

3. Absolute changes

To calculate the absolute changes plus accompanying variance measures for each of the main variables in each of the studies, the following formulas were used.

3.1. Calculation of the absolute change

Parallel studies:

- $X_{abschange} \approx (X_{Tend} - X_{Tbase}) - (X_{Cend} - X_{Cbase})$

where

- $X_{abschange}$ is the absolute change in X of the whole group
- X_{Tend} is the mean X at the end-of-intervention in the treatment group
- X_{Tbase} is the mean X at baseline in the treatment group
- X_{Cend} is the mean X at the end-of-intervention in the control group
- X_{Cbase} is the mean X at baseline in the control group

Cross-over studies:

In case baseline data of both intervention periods were reported, absolute changes were calculated based on the formula mentioned above (same as for parallel studies). Otherwise, absolute changes were calculated using the following formula.

- $X_{abschange} \approx X_{Tend} - X_{Cend}$

where

- $X_{abschange}$ is the absolute change in X of the whole group

- X_{Tend} is the mean X at the end of the treatment period
- X_{Cend} is the mean X at the end of the control period

3.2. Variance measure of absolute change

To estimate the within-trial variance measures of the absolute changes, a correlation was assumed between baseline and endpoint values of X for parallel studies, and between values of X at the end of the phytosterol period and the control period for cross-over studies. This within-subject correlation coefficient was estimated based on studies for which both the SE of the net change and the SEs at baseline and end-of-intervention for parallel studies, and SEs at both endpoints for cross-over studies, were available. It was estimated that for all main outcome variables, a correlation coefficient of 0.8 should be used.

Parallel studies:

- $SE_{abschange} \approx \sqrt{SE_T^2 + SE_C^2}$
- $SE_T \approx \sqrt{(SE_{Tbase}^2 + SE_{Tend}^2) - 2r * SE_{Tbase} * SE_{Tend}}$
- $SE_C \approx \sqrt{(SE_{Cbase}^2 + SE_{Cend}^2) - 2r * SE_{Cbase} * SE_{Cend}}$

where

- $SE_{abschange}$ is the SE of the absolute change of the whole group
- SE_T is the SE of the absolute change in the treatment group
- SE_C is the SE of the absolute change in the control group
- SE_{Tbase} is the SE at baseline in the treatment group
- SE_{Tend} is the SE at the end-of-intervention in the treatment group
- SE_{Cbase} is the SE at baseline in the control group
- SE_{Cend} is the SE at the end-of-intervention in the control group
- r is the within-subject correlation between repeated measurements of X (i.e., 0.8 for cholesterol and plasma/serum PS)

Cross-over studies:

In case baseline data of both intervention periods were reported, variance measures of the absolute changes were calculated based on the formulas mentioned above (same as for parallel studies). Otherwise, variance measures of the absolute changes were calculated using the following formula.

- $SE_{abschange} \approx \sqrt{SE_{Tend}^2 + SE_{Cend}^2 - 2r * SE_{Tend} * SE_{Cend}}$

where

- $SE_{abschange}$ is the SE of the absolute change of the whole group
- SE_{Tend} is the SE at the end of the treatment period
- SE_{Cend} is the SE at the end of the control period
- r is the within-subject correlation between repeated measurements of X (i.e., 0.8 for cholesterol and plasma/serum PS)

4. Relative changes

To calculate the relative changes plus accompanying variance measures for each of the main variables in each of the studies, the following formulas were used.

4.1. Calculation of the relative change

Parallel studies:

- $X_{relchange} \approx \%\Delta X_T - \%\Delta X_C$

where

- $\%\Delta X_T \approx 100 * \frac{X_{Tend} - X_{Tbase}}{X_{Tbase}}$
- $\%\Delta X_C \approx 100 * \frac{X_{Cend} - X_{Cbase}}{X_{Cbase}}$

where

- $X_{relchange}$ is the relative change in X of the whole group
- $\%\Delta X_T$ is the relative change in X of the treatment group
- $\%\Delta X_C$ is the relative change in X of the control group
- X_{Tend} is the mean X at the end-of-intervention in the treatment group
- X_{Tbase} is the mean X at baseline in the treatment group
- X_{Cend} is the mean X at the end-of-intervention in the control group
- X_{Cbase} is the mean X at baseline in the control group

Cross-over studies:

In case baseline data of both intervention periods were reported, relative changes were calculated based on the formulas mentioned above (same as for parallel studies). Otherwise, relative changes were calculated using the following formulas.

- $X_{relchange} \approx 100 * \frac{X_{Tend} - X_{Cend}}{X_{Cend}}$

where

- $X_{relchange}$ is the relative change in X of the whole group
- X_{Tend} is the mean X at the end of the treatment period
- X_{Cend} is the mean X at the end of the control period

4.2. Variance measure of relative change

It was estimated that for all main outcome variables, a within-subject correlation coefficient of 0.8 should be used.

Parallel studies:

- $SE_{relchange} \approx \sqrt{SE_{Tratio}^2 + SE_{Cratio}^2}$

where

- $SE_{Tratio} \approx \sqrt{\frac{Var_{Tratio}}{N_T}}$
- $SE_{Cratio} \approx \sqrt{\frac{Var_{Cratio}}{N_C}}$

where

- $Var_{Tratio} \approx \left(\frac{X_{Tend}}{X_{Tbase}} * 100\right)^2 * \left(\frac{SD_{Tend}^2}{X_{Tend}^2} + \frac{SD_{Tbase}^2}{X_{Tbase}^2} - \frac{2r * SD_{Tend} * SD_{Tbase}}{X_{Tend} * X_{Tbase}}\right)$
- $Var_{Cratio} \approx \left(\frac{X_{Cend}}{X_{Cbase}} * 100\right)^2 * \left(\frac{SD_{Cend}^2}{X_{Cend}^2} + \frac{SD_{Cbase}^2}{X_{Cbase}^2} - \frac{2r * SD_{Cend} * SD_{Cbase}}{X_{Cend} * X_{Cbase}}\right)$

where

- $SE_{relchange}$ is the SE of the relative change of the whole group
- SE_{Tratio} is the SE of the relative change in the treatment group
- SE_{Cratio} is the SE at of the relative change in the control group
- Var_{Tratio} is the variance of the relative change in the treatment group
- Var_{Cratio} is the variance of the relative change in the control group
- N_T is the number of subjects in the treatment group
- N_C is the number of subjects in the control group
- X_{Tend} is the mean X at the end-of-intervention in the treatment group
- X_{Tbase} is the mean X at baseline in the treatment group
- X_{Cend} is the mean X at the end-of-intervention in the control group
- X_{Cbase} is the mean X at baseline in the control group
- SD_{Tend} is the SD at the end-of-intervention in the treatment group
- SD_{Tbase} is the SD at baseline in the treatment group
- SD_{Cbase} is the SD at baseline in the control group
- SD_{Cend} is the SD at the end-of-intervention in the control group

- r is the within-subject correlation between repeated measurements of X (i.e., 0.8 for cholesterol and plasma/serum PS)

Cross-over studies:

In case baseline data of both intervention periods were reported, variance measures of the relative changes were calculated based on the formulas mentioned above (same as for parallel studies). Otherwise, variance measures of the relative changes were calculated using the following formulas.

- $SE_{relchange} \approx \sqrt{\frac{Var_{ratio}}{N_{tot}}}$
- $Var_{ratio} \approx \left(\frac{X_{Tend}}{X_{Cend}} * 100\right)^2 * \left(\frac{SD_{Tend}^2}{X_{Tend}^2} + \frac{SD_{Cend}^2}{X_{Cend}^2} - \frac{2r * SD_{Tend} * SD_{Cend}}{X_{Tend} * X_{Cend}}\right)$
- $N_{tot} = N_C = N_T$

where

- $SE_{relchange}$ is the SE of the relative change of the whole group
- Var_{ratio} is the variance of the relative change of the whole group
- X_{Tend} is the mean X at the end of the treatment period
- X_{Cend} is the mean X at the end of the control period
- SD_{Tend} is the SD at the end of the treatment period
- SD_{Cend} is the SD at the end of the control period

Supplemental Appendix 2 - Study overview with blood cholesterol data

Reference information	LDL-C		TC		HDL-C	
	Absolute change (mmol/L)	Relative change (%)	Absolute change (mmol/L)	Relative change (%)	Absolute change (mmol/L)	Relative change (%)
Parallel studies[a]						
Carr et al. 2009	-0.29	-6.7			0.11	6.8
Christiansen et al. 2001 stratum 1	-0.27	-6.4	-0.34	-5.1	0.00	0.0
Christiansen et al. 2001 stratum 2	-0.39	-9.1	-0.40	-5.9	-0.01	-0.6
Clifton et al. 2008 stratum 1	-0.38	-8.7	-0.43	-6.6	-0.02	-1.5
Clifton et al. 2008 stratum 2	-0.45	-10.5	-0.58	-9.0	-0.03	-2.2
Clifton et al. 2008 stratum 3	-0.33	-7.5	-0.46	-7.1	-0.02	-1.5
De Jong et al. 2006	-0.29	-8.2	-0.34	-6.0	0.05	3.8
De Jong et al. 2008	-0.40	-12.4	-0.48	-9.1	0.00	0.0
Hansel et al. 2007	-0.32	-7.8	-0.30	-4.8	0.02	1.1
Hendriks et al. 2003	-0.24	-6.6	-0.31	-5.3	-0.02	-1.3
Hernandez-Mijares et al. 2010	-0.43	-10.2	-0.44	-7.2	0.07	5.3
Korpela et al. 2006	-0.45	-11.0	-0.42	-6.5		
Kurokawa et al. 2008	-0.23	-5.9	-0.23	-3.9	0.08	5.4
Maki et al. 2001 stratum 1	-0.37	-9.0	-0.45	-7.3	-0.03	-2.3
Maki et al. 2001 stratum 2	-0.55	-13.0	-0.57	-9.0	0.03	2.2
Mannarino et al. 2008	-0.40	-9.4	-0.40	-5.8	-0.04	-3.4
Masuda et al. 2007	-0.21	-5.7	-0.14	-2.4	0.02	1.2
Neil et al. 2001	-0.51	-10.0	-0.57	-7.8	0.04	2.7
Nittynen et al. 2007 study 2	-0.30	-6.0	-0.41	-6.0	-0.14	-9.5
Plana et al. 2008	-0.36	-9.5	-0.36	-6.1	-0.01	-0.9
Saito et al. 2006 stratum 1	-0.27	-7.1	-0.24	-4.0	-0.05	-3.4
Saito et al. 2006 stratum 2	-0.24	-5.9	-0.35	-5.6	-0.05	-3.0
Saito et al. 2006 stratum 3	-0.31	-9.3	-0.38	-6.6	-0.04	-2.6
Seki et al. 2003a	-0.07	-2.3	-0.13	-2.5	0.02	1.6
Seki et al. 2003b	-0.32	-12.6	-0.43	-9.5	-0.01	-0.5
Takeshita et al. 2007a	-0.10	-3.0	-0.29	-5.5	-0.10	-7.4
Takeshita et al. 2008	-0.24	-6.0	-0.26	-4.2	0.02	0.8
Varady et al. 2004 study 1	-0.40	-11.6	-0.42	-6.9	0.01	0.8
Varady et al. 2004 study 2	-0.46	-12.8	-0.41	-7.5	-0.03	-1.8
Cross-over studies[b]						
AbumWeis et al. 2006 stratum 1	-0.05	-1.4	-0.07	-1.2	0.10	7.9
AbumWeis et al. 2006 stratum 2	-0.05	-1.4	-0.04	-0.7	0.08	6.3
Amundsen et al. 2004	-0.59	-10.2	-0.53	-7.2	0.03	2.4
Casas-Agustench et al. 2012	-0.36	-8.0	-0.52	-7.8	-0.03	-1.9
Clifton et al. 2004 stratum 1	-0.42	-9.8	-0.35	-5.4	0.04	2.7
Clifton et al. 2004 stratum 2	-0.53	-12.4	-0.53	-8.2	-0.03	-2.1
Demonty et al. 2006	-0.22	-5.6	-0.21	-3.7	0.05	4.0
Hallikainen et al. 2000	-0.45	-10.7	-0.46	-7.5	0.05	3.3
Heggen et al. 2010 stratum 1	-0.39	-9.8	-0.40	-6.6	-0.03	-1.9

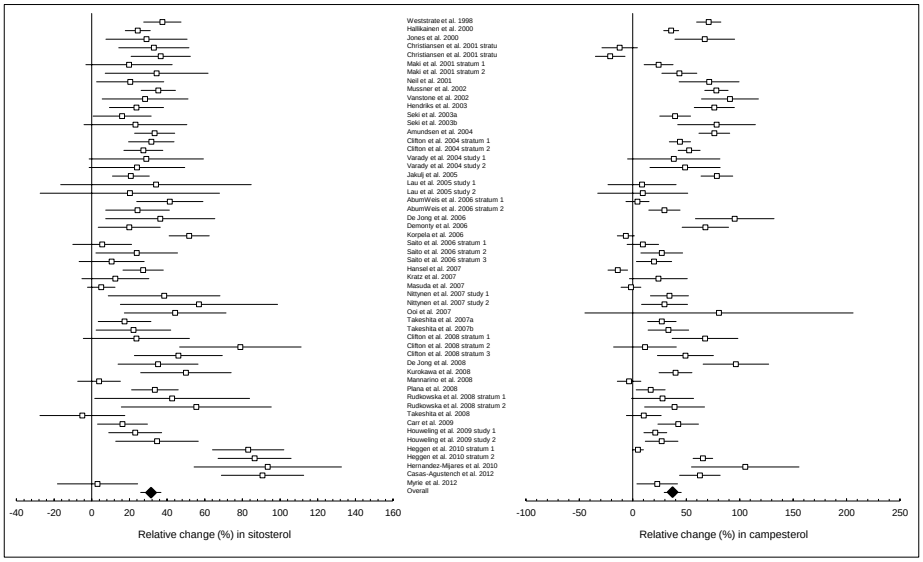
Reference information	LDL-C		TC		HDL-C	
	Absolute change (mmol/L)	Relative change (%)	Absolute change (mmol/L)	Relative change (%)	Absolute change (mmol/L)	Relative change (%)
Heggen et al. 2010 stratum 2	-0.35	-8.8	-0.40	-6.6	-0.05	-3.1
Houweling et al. 2009 study 1	-0.30	-8.2	-0.34	-6.1	-0.01	-0.9
Houweling et al. 2009 study 2	-0.25	-7.8	-0.26	-5.3	-0.02	-2.0
Jakulj et al. 2005	-0.23	-5.7	-0.35	-5.4	-0.07	-4.0
Jones et al. 2000	-0.39	-9.3	-0.47	-7.4	-0.01	-1.0
Kratz et al. 2007	-0.13	-5.3	-0.15	-3.0	0.02	1.1
Lau et al. 2005 study 1	-0.84	-26.5	-0.34	-5.3	0.10	8.5
Lau et al. 2005 study 2	-0.38	-9.6	0.05	0.5	0.03	2.1
Mussner et al. 2002	-0.26	-6.5	-0.23	-3.8	0.05	3.5
Myrie et al. 2012	-0.62	-18.7	-0.43	-7.5	-0.13	-10.9
Nittynen et al. 2007 study 1	-0.17	-4.2	-0.13	-2.1	0.02	1.4
Ooi et al. 2007	-0.22	-6.4	-0.15	-2.8	0.03	2.6
Rudkowska et al. 2008 stratum 1	-0.14	-3.8	-0.24	-4.1	-0.08	-5.5
Rudkowska et al. 2008 stratum 2	-0.29	-7.7	-0.42	-7.0	-0.11	-7.5
Takeshita et al. 2007b	-0.41	-11.6	-0.60	-10.0	-0.09	-5.3
Vanstone et al. 2002	-0.35	-8.8	-0.44	-7.4	-0.01	-1.0
Weststrate et al. 1998	-0.40	-12.2	-0.41	-8.0	0.00	0.3

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol.

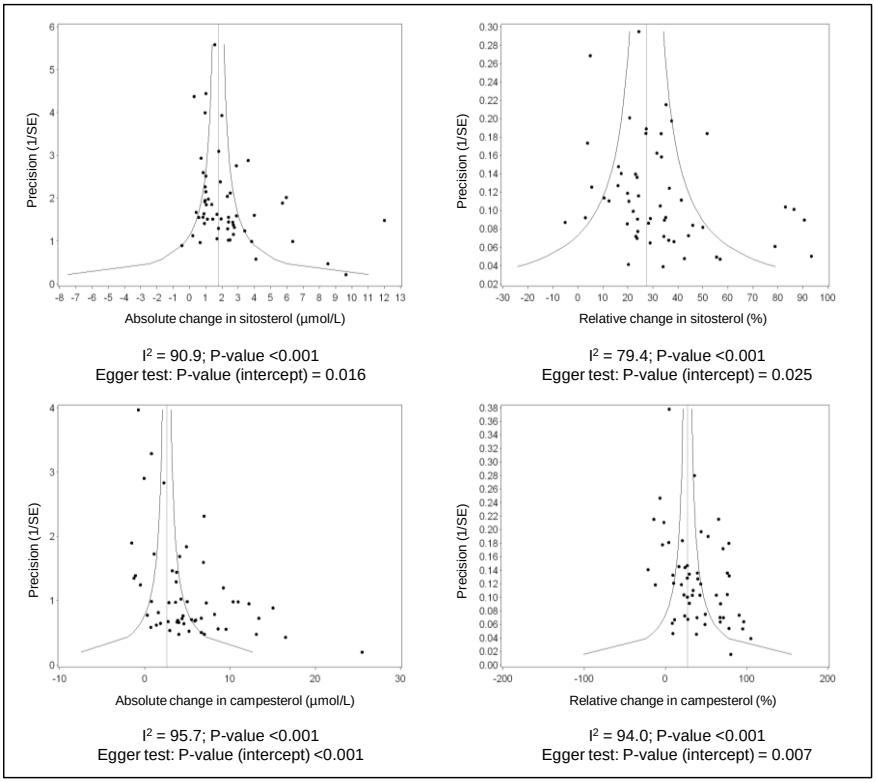
[a] For parallel studies, the absolute and relative changes were calculated based on the average concentrations at baseline and at end-of-intervention of treatment and control groups.

[b] For cross-over studies that reported baseline data, the absolute and relative changes were calculated similarly as for the parallel studies. Otherwise, these were calculated based on the data at the end of the treatment and control periods.

Supplemental Appendix 3 - Forest plots of the relative (%) changes in plasma sitosterol and campesterol concentrations



Supplemental Appendix 4 - Funnel plots of the absolute (µmol/L) and relative (%) changes in plasma sitosterol and campesterol



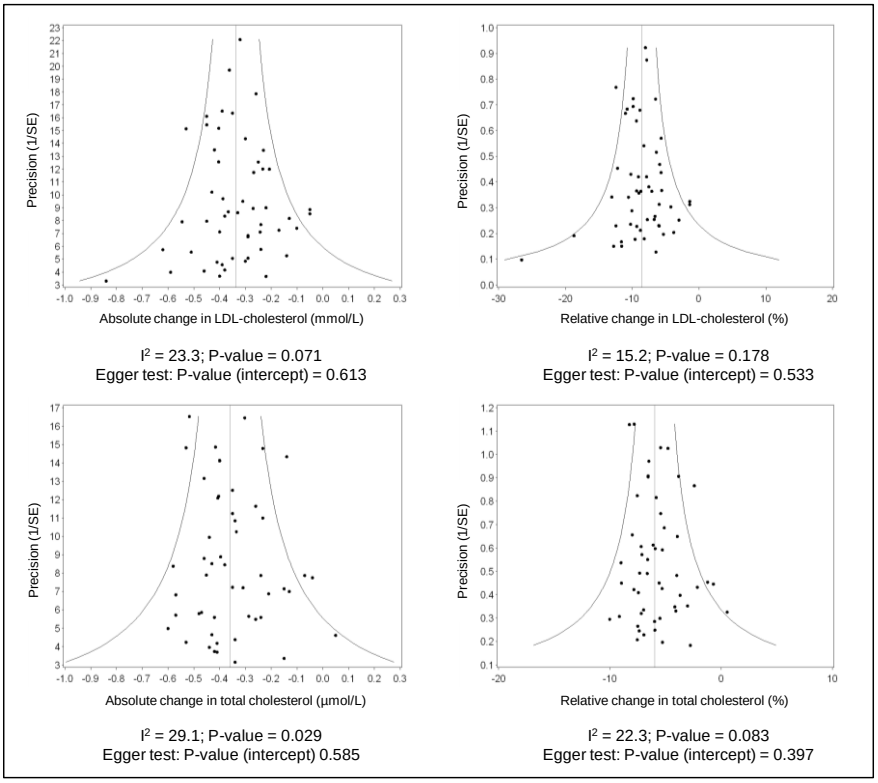
I^2 = 90.9; P-value <0.001
Egger test: P-value (intercept) = 0.016

I^2 = 79.4; P-value <0.001
Egger test: P-value (intercept) = 0.025

I^2 = 95.7; P-value <0.001
Egger test: P-value (intercept) <0.001

I^2 = 94.0; P-value <0.001
Egger test: P-value (intercept) = 0.007

Supplemental Appendix 5 - Funnel plots of the absolute (mmol/L) and relative (%) changes in plasma LDL-cholesterol and total cholesterol



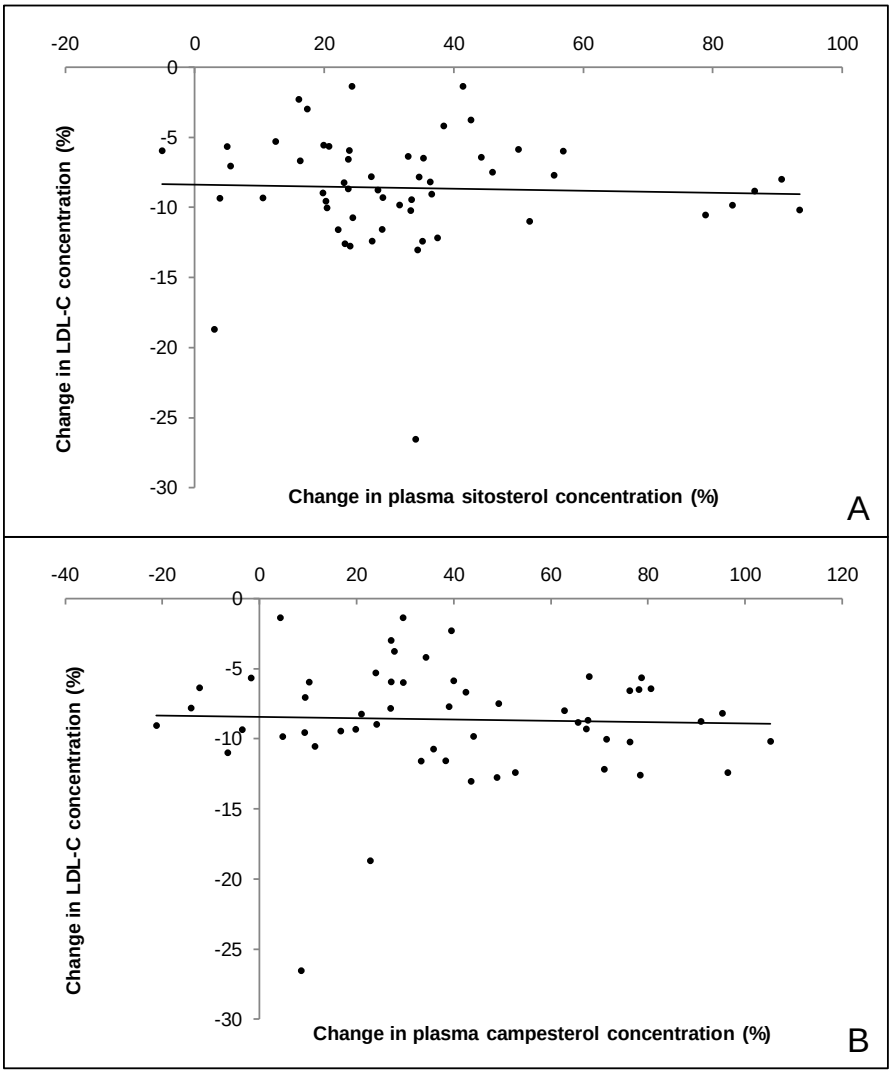
I^2 = 23.3; P-value = 0.071
Egger test: P-value (intercept) = 0.613

I^2 = 15.2; P-value = 0.178
Egger test: P-value (intercept) = 0.533

I^2 = 29.1; P-value = 0.029
Egger test: P-value (intercept) 0.585

I^2 = 22.3; P-value = 0.083
Egger test: P-value (intercept) = 0.397

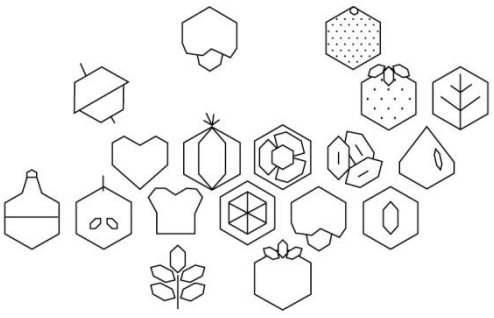
Supplemental Appendix 6 - The changes in LDL-cholesterol concentrations (%) expressed against the changes in plasma sitosterol (%, see Panel A) and plasma campesterol (%, see Panel B)



Chapter 7

Intake of phytosterols from natural sources and risk of cardiovascular disease in the EPIC-NL population

Rouyanne T. Ras
Yvonne T. van der Schouw
Elke A. Trautwein
Isabelle Sioen
Geertje W. Dalmeijer
Peter L. Zock
Joline W.J. Beulens



Submitted

Abstract

Background - Phytosterols are known to lower low-density lipoprotein cholesterol (LDL-C), an established risk factor for cardiovascular disease (CVD). Whether a high intake of phytosterols reduces CVD risk is unknown. This observational study aimed to investigate the associations between intake of naturally occurring phytosterols, blood lipids and CVD risk.

Methods - The study included 35,597 Dutch men and women, participating in the EPIC-NL study. At baseline, intakes of naturally occurring phytosterols were estimated with a validated food frequency questionnaire and non-fasting blood lipids were measured. Occurrence of CVD, coronary heart disease (CHD) and myocardial infarction (MI) was determined through linkage with registries.

Results - The average energy-adjusted phytosterol intake at baseline was 296 mg/d (range: 83-966 mg/d). During 12.2 years of follow-up, 3,047 CVD cases (8.6%) were documented. After adjustment for confounders, phytosterol intake was not associated with risk of CVD, CHD or MI (P trend >0.05); hazard ratios ranged from 0.90 to 0.99 for CVD, from 0.83 to 0.90 for CHD and from 0.80 to 0.95 for MI risk across quintiles of phytosterol intake and were almost all non-significant. Higher phytosterol intake was associated with lower total cholesterol (-0.06 mmol/L per 50 mg/d; P = 0.038) and lower LDL-C (-0.07 mmol/L; P = 0.007), particularly among men. In mediation analysis, LDL-C did not materially affect the association between phytosterol intake and CVD risk.

Conclusions - In this population with relatively narrow range of low naturally occurring phytosterol intakes, intake of phytosterols was not associated with reduced CVD risk despite lower LDL-C concentrations in men.

Introduction

Plant sterols and plant stanols (together they are referred to as phytosterols) are bioactive compounds found in all foods of plant origin. Phytosterols are well-known for their total cholesterol (TC)-lowering, and especially low-density lipoprotein cholesterol (LDL-C)-lowering properties; an average phytosterol intake of 2 g/d lowers LDL-C by on average 8-10%[1,2]. Intakes of around 2 g/d of phytosterols cannot be reached with habitual diets; phytosterol intakes in the general population usually range between 200-400 mg/d[3,4]. With specific dietary habits

such as vegetarians diets, higher phytosterol intakes of 500-1000 mg/d can be reached[5,6].

Elevated LDL-C is an established risk factor for cardiovascular disease (CVD) [7]. As phytosterols lower LDL-C, one could assume that high intakes of phytosterols would reduce CVD risk. Direct evidence supporting such a reduced risk of CVD is however lacking. Given the difficulties in performing fully controlled CVD endpoint trials with phytosterol intervention, observational studies could help to clarify whether intake of naturally occurring phytosterols is associated with blood lipid risk markers and incidence of CVD.

A few observational studies with dietary phytosterol intakes have been performed and showed that people with higher intakes of naturally occurring phytosterols have lower concentrations of LDL-C[8-10] and tend to have a lower carotid intima-media thickness.[9] A recent study showed that a high intake of naturally occurring phytosterols was related to a lower risk of a first myocardial infarction (MI) [11]. However, this association was not apparent when phytosterol intake was corrected for energy intake and no significant associations were observed in women.

We aimed to prospectively investigate the association between intake of phytosterols from natural sources and occurrence of cardiovascular events (total CVD, total coronary heart disease (CHD) and MI. As secondary objectives, we cross-sectionally investigated the association between naturally occurring phytosterol intake and blood lipid concentrations at baseline and whether associations between phytosterol intake and CVD were mediated through effects on LDL-C.

Subjects and Methods

Study population

The EPIC-NL cohort[12] consists of two contributions to the EPIC collaboration; the Monitoring Project on Risk Factors for Chronic Diseases (MORGEN) cohort and the Prospect cohort. The MORGEN cohort consists of 22,654 men and women, aged 20-64 years, recruited through random sampling from the general population between 1993 and 1997. Prospect is a cohort study among 17,357 women, aged 49-70 years, recruited during the same time period (1993-1997) through a breast cancer screening programme. The procedures in both cohorts were set up

simultaneously and using similar methods with the exception of the blood pressure (BP) assessment. The data have been harmonized and merged in one database in 2006. The study complies with the Declaration of Helsinki and was approved by local medical ethical committees. All participants provided informed consent before study inclusion.

For the prospective analysis, the following exclusion criteria were applied: prevalent CVD based on self-report or identified through linkage with the National Medical Registry (1990-1997) (n = 1,264), missing dietary intake data (n = 203), having extremely low or high reported energy intakes (i.e. ratio of energy intake over basal metabolic rate in the lowest or highest 0.5%) (n = 385), and missing follow-up data (n = 2,562). Thus, in total 35,597 participants were included. For the cross-sectional analysis, we only included participants of a random 6.5% sample (n = 2,604) for which we had data on complete blood lipid profile. Similar exclusion criteria were applied as mentioned above, except that participants were excluded when blood lipid data instead of follow-up data on CVD endpoints were missing. The numbers of participants included in the blood lipid analyses were 2,417 for TC, 2,383 for LDL-C, 2,383 for high-density lipoprotein cholesterol (HDL-C) and 2,410 for triglycerides (TGs).

Baseline assessments

At baseline, participants filled out a general questionnaire on demographics, disease history and lifestyle characteristics, a physical activity questionnaire and a validated food-frequency questionnaire (FFQ)[13]. A physical examination was performed as earlier described[12] and non-fasting venous blood samples were drawn at baseline. Physical activity was assessed by calculating the Cambridge Physical Activity Score. Smoking was classified in current, past or non-smoker and education level was categorized based on nine categories ranging from primary education to university completed. Menopausal status was classified as pre-, peri, or (surgical) postmenopausal; men were considered postmenopausal. Diagnosis of hyperlipidemia was determined based on self-report (‘ever diagnosed?’ yes/no), whereas hypertension was determined based on measured BP (>140 mmHg systolic or >90 mmHg diastolic BP), use of BP-lowering medication or self-report.

Assessment of nutrient and phytosterol intake

The self-administered FFQ contained questions on consumption frequency of 79 main food items during the past year[13]. Additional questions were asked about sub-items, preparation methods or additions. Consumption of in total 178 foods when considering the sub-items could be calculated. Portion sizes were estimated using specified units or photographed portions. Energy and nutrient intakes were calculated based on the Dutch food composition table. Because this table does not contain information on phytosterol content of foods, we estimated total phytosterol intake by using a phytosterol database that was developed by Ghent University, Belgium[4], based on the Finnish, United Kingdom and United States food composition tables[14-16], scientific literature[17], Dutch recipes[18], ingredient lists on packaging, and known phytosterol composition of equivalent foods. Intake data of individual phytosterols, such as sitosterol or campesterol, were not available. phytosterol-enriched foods were not available on the market at the time of the dietary intake assessment and, information on consumption of such products during later years was not available.

We used data from a previous validation study[13] among 63 men and 58 women to estimate the relative validity of the phytosterol intake as measured with the FFQ against twelve standardized 24-hour recalls. Reproducibility was tested against two other FFQs taken at 6-month intervals. We observed a reasonable to good relative validity of the estimated phytosterol intake with Pearson correlation coefficients of 0.72 for the crude phytosterol intake and 0.59 for the energy-adjusted phytosterol intake. Reproducibility was good with Pearson correlation coefficients ranging between 0.84-0.87 for the crude phytosterol intake and 0.68-0.69 for the energy-adjusted phytosterol intake.

Assessment of blood lipids

Data on baseline blood lipids were available for a random 6.5% sample of the total study population (n = 2,604) representative of the full cohort[12], and for all CVD cases that occurred until January 2006 (n = 2,068). Non-fasting TC and TG were measured using enzymatic methods. Non-fasting HDL-C and LDL-C were measured using a homogeneous assay with enzymatic endpoint, on an autoanalyser (Beckman Coulter, Mijdrecht, the Netherlands).

Follow-up assessments

Participants were followed for occurrence of chronic diseases and death through linkage with several national registries. Vital status was obtained through linkage with municipal population registries. Causes of death were obtained via 'Statistics Netherlands'. Data on morbidity were obtained from the Dutch Hospital Association and Order of Medical Specialists. Registries were linked to the cohort based on a validated probabilistic method[19]. Follow-up was complete until January 2008. Incidences of fatal and non-fatal events were combined, taking only the first-occuring events into account. The CVD events were coded according to ICD-9. CVD was based on codes 410-414 (ischemic heart disease), 427.5 (cardiac arrest), 428 (heart failure), 415.1 (pulmonary disease), 443.9 (unspecified peripheral vascular disease), 430-438 (cerebrovascular disease), 440-442 (atherosclerosis and aneurysms), 444 (arterial embolism and thrombosis) and 798.1, 798.2 and 798.9 (sudden death), CHD based on codes 410-414, 427.5, 798.1, 798.2 and 798.9 and acute MI based on code 410.

Data analysis

Person-years were calculated from the date of return of the questionnaire until the date of CVD occurrence, date of death or 1 January 2008, whichever came first. Data on physical activity were missing in 14% of all participants. Missing values for physical activity were therefore imputed using the single imputation method (SPSS Missing Value Analysis). For all other variables, the few missing values (<0.5%) were imputed using the mean for continuous variables and a missing indicator for categorical variables. Nutrients were adjusted for energy intake using the regression residual method[20]. Blood lipid variables were log transformed in case of non-normally distributed data.

Cox proportional hazard models were used to prospectively analyze associations between intake of naturally occurring phytosterols and risk of total CVD, total CHD and MI. Associations were analyzed categorically based on quintiles of energy-adjusted phytosterol intake with the lowest quintile as the reference. All analyses were stratified for cohort (i.e. MORGEN or Prospect). Associations were adjusted for confounders. The first model adjusted for age and gender. The second model additionally adjusted for CVD risk factors, i.e. BMI, education, smoking status, physical activity, menopause and total energy intake. The third, fully-adjusted, model additionally adjusted for dietary factors known to affect blood lipids and/or

CVD risk, i.e. energy-adjusted intakes of saturated, polyunsaturated and monounsaturated fat, fiber, dietary cholesterol and alcohol. Two additional models were investigated to explore possible confounding by intake of sodium, retinol, β-carotene, vitamin D and vitamin E (model 4) or by hypertension (model 5). Effect modification by gender, waist circumference and hyperlipidemia was investigated by including interaction terms with phytosterol intake in the third model. Exploratory analyses were performed with stroke as outcome variable. The proportionality assumption was checked in the final models. Sensitivity analyses were performed to ensure robustness of the findings. We checked the impact of censoring at 2000 (i.e. the year that phytosterol-enriched foods were introduced onto the market), exclusion of participants with cancer or diabetes at baseline, exclusion of energy under- and over-reporters as determined by the Goldberg criteria[21], exclusion of participants with a survival time <2 years (i.e. any undiagnosed illness preceding the early censoring may have changed a partipants' diet) and additional adjustment for diabetic status/drug use.

Associations between energy-adjusted phytosterol intake and blood lipids at baseline were analyzed using linear regression analysis based on the same models as defined for the prospective analysis. Effect modification by gender, waist circumference and hyperlipidemia was tested. To investigate whether associations between phytosterol intake and CVD risk were mediated through effects on LDL-C, we applied a case-cohort design including all cases until January 2006 and the random 6.5% sample for which we had LDL-C data. Modified Cox proportional hazard models were used accounting for case-cohort design by Prentice-weighting[22]; LDL-C was included in the third model to assess its mediation effect. Even if associations would be non-significant, mediation analysis could reveal relevant information as long as the HR is not 1.00.

A P-value below 0.05 was considered statistically significant. All statistical analyses were performed using the statistical package SAS (SAS version 9.2, SAS Institute).

Results

Overview of study population

Of the 35,597 participants, 25% were men and 75% were women **(Table 1**). The average age was 49.3 years. After a median of 12.2 years of follow-up, 3,047 cases

of CVD were documented, including 1,807 cases of CHD and 606 cases of MI. The average baseline energy-adjusted phytosterol intake in the whole population was 295.8±49.2 mg/d (mean±SD). Average phytosterol intakes ranged from 231.3±22.0 to 366.0±34.9 mg/d between the lowest and the highest quintiles. The most important dietary sources of phytosterols were fruits and vegetables (25.5%), bread and cereal products (25.1%), and fats, oils and sauces (19.3%). With higher naturally occurring phytosterol intakes, participants were younger, more often female, had higher BMI, were more physically active, were lower educated and smoked less (**Table 1**). Furthermore, intakes of carbohydrates, mono- and polyunsaturated fat, and fiber were higher, whereas intakes of protein, saturated fat, cholesterol and alcohol were lower with higher phytosterol intakes.

Cardiovascular disease risk

In the fully-adjusted model (**Table 2**), no association was observed between energy-adjusted intake of naturally occurring phytosterols and total CVD risk (P_{trend} = 0.94) with non-significant hazard ratios (HRs) ranging between 0.90 and 0.99 across quintiles of phytosterol intake. Phytosterol intake was also not associated with total CHD risk (P_{trend} = 0.17); however, phytosterol intake was significantly associated with a lower risk of CHD in the second (HR=0.83, 95% CI: 0.72; 0.97) and in the third (0.84; 0.72; 0.98) quintiles of phytosterol intake vs. the quintile with the lowest phytosterol intake. In the fourth and fifth quintiles, HRs for CHD were 0.90 (95% CI: 0.76; 1.06) and 0.84 (95% CI: 0.70; 1.01). Phytosterol intake was not associated with MI risk (P_{trend} = 0.19) after adjustment for confounders; non-significant HRs ranged from 0.80 to 0.95 across quintiles of phytosterol intake.

Models 4 and 5 showed essentially similar results indicating that a possible relation was not obscured by confounding of other dietary factors or hypertension. Interactions of phytosterol intake with gender, waist circumference or hyperlipidemia were not statistically significant. No associations were observed between phytosterol intake and occurrence of stroke (**Supplemental Appendix 1**). In sensitivity analyses, censoring the analysis at year 2000, excluding participants with cancer or diabetes at baseline, excluding energy under- and over-reporters, excluding participants with a survival time <2 years and adjusting additionally for diabetic status/drug use did not change our results (data not shown).

Table 1. *Overview of the study population when classified into categories of energy-adjusted phytosterol intake.*

Characteristics	Quintiles based on energy-adjusted phytosterol intake[a]					All participants
	Q1 (<257 mg/d)	Q2 (257-282 mg/d)	Q3 (283-305 mg/d)	Q4 (306-333 mg/d)	Q5 (>333 mg/d)	
Demographics						
Total n	7120	7118	7121	7118	7120	35597
CVD cases	713 (10.0)	588 (8.3)	553 (7.8)	567 (8.0)	626 (8.8)	3047 (8.6)
CHD cases	436 (6.1)	327 (4.6)	333 (4.7)	359 (5.0)	352 (4.9)	1807 (5.1)
MI cases	154 (2.2)	121 (1.7)	115 (1.6)	103 (1.5)	113 (1.6)	606 (1.7)
Stroke cases	124 (1.7)	118 (1.7)	113 (1.6)	93 (1.3)	132 (1.9)	580 (1.6)
Cohort (MORGEN[b])	3830 (53.8)	3962 (55.7)	4046 (56.8)	4076 (57.3)	3839 (53.9)	19753 (55.5)
Age (y)	50.4 ± 11.7	49.2 ± 12.0	48.9 ± 12.0	48.6 ± 11.9	49.3 ± 11.5	49.3 ± 11.9
Gender (male)	1943 (27.3)	1755 (24.7)	1847 (25.9)	1789 (25.1)	1592 (22.4)	8926 (25.1)
BMI (kg/m^2)	25.6 ± 4.0	25.5 ± 13.8	25.6 ± 4.0	25.6 ± 3.8	26.0 ± 4.2	25.6 ± 4.0
Waist circumference (cm)	86.0 ± 11.8	84.9 ± 11.2	85.1 ± 11.2	84.7 ± 11.1	84.9 ± 11.5	85.1 ± 11.4
Smoking status (non-smoker)	2367 (33.2)	2699 (37.9)	2878 (40.4)	2868 (40.3)	2884 (40.5)	13696 (38.5)
Physically active	2710 (38.1)	2960 (41.6)	3089 (43.4)	3105 (43.6)	3100 (43.5)	14964 (42.0)
Education (higher level)	1502 (21.1)	1571 (22.1)	1501 (21.1)	1407 (19.8)	1279 (18.0)	7260 (20.4)
Pre-menopausal status	1336 (18.8)	1687 (23.7)	1735 (24.4)	1788 (25.1)	1781 (25.0)	8327 (23.4)
Hypertension	2641 (37.1)	2567 (36.1)	2613 (36.7)	2585 (36.3)	2719 (38.2)	13125 (36.9)
SBP (mmHg)	127.2 ± 19.2	126.3 ± 19.0	126.3 ± 18.9	125.8 ± 18.3	126.2 ± 19.0	126.4 ± 18.9
DBP (mmHg)	78.1 ±10.7	77.7 ± 10.5	77.9 ± 10.7	77.8 ± 10.5	78.1 ± 10.6	77.9 ± 10.6
Hyperlipidemia	522 (7.3)	502 (7.1)	532 (7.5)	611 (8.6)	607 (8.5)	2774 (7.8)
Diet[c]						
Total energy intake (kcal/d)	2026 ± 595	2046 ± 584	2077 ± 593	2082 ± 609	2024 ± 640	2051 ± 605
Total carbohydrate intake (g/d)	214.2 ± 34.5	221.7 ± 30.3	223.5 ± 28.6	224.5 ± 28.6	224.9 ± 29.6	221.8 ± 30.7
Total protein intake (g/d)	78.0 ± 12.3	77.0 ± 10.9	76.1 ± 10.4	75.0 ± 10.0	73.1 ± 10.4	75.9 ± 11.0
Total fat intake (g/d)	76.1 ± 11.9	76.6 ± 10.9	77.4 ± 10.7	78.3 ± 11.0	80.1 ± 11.6	77.7 ± 11.3
SFA intake (g/d)	34.3 ± 6.6	32.9 ± 5.7	32.4 ± 5.4	32.0 ± 5.4	31.6 ± 5.8	32.6 ± 5.8
MUFA intake (g/d)	28.9 ± 5.2	29.1 ± 4.9	29.4 ± 4.9	29.6 ± 5.1	30.3 ± 5.3	29.5 ± 5.1
PUFA intake (g/d)	12.2 ± 2.9	14.0 ± 3.0	15.0 ± 3.2	16.0 ± 3.6	17.5 ± 4.2	14.9 ± 3.9
Phytosterol intake (mg/d)	231.3 ± 22.0	270.1 ± 7.3	293.4 ± 6.7	318.2 ± 8.1	366.0 ± 34.9	295.8 ± 49.2
Fiber intake (g/d)	19.9 ± 4.1	22.3 ± 3.9	23.7 ± 4.1	24.7 ± 4.2	26.4 ± 4.9	23.4 ± 4.8
Cholesterol intake (mg/d)	237.8 ± 63.8	221.1 ± 55.3	214.0 ± 54.3	210.4 ± 54.9	204.7 ± 59.6	217.6 ± 58.8
Alcohol intake (g/d)	17.0 ± 23.4	11.8 ± 16.7	9.9 ± 15.1	8.8 ± 13.2	7.7 ± 13.2	11.0 ± 17.1

CHD, coronary heart disease; CVD, cardiovascular disease; DBP diastolic blood pressure; MI, myocardial infarction; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SBP, systolic blood pressure.

[a] P for trend was <0.001, except for prevalence of stroke cases, DBP, prevalence of hypertension and total energy intake.

[b] MORGEN is the name of one of the two cohorts that were part of this study

[c] All nutrients, except for total energy intake, were energy-adjusted.

Values are mean ± SD or n (%).

Table 2. *Energy-adjusted phytosterol intake and risk of cardiovascular disease, coronary heart disease and myocardial infarction in the EPIC-NL cohort.*

	Quintiles based on energy-adjusted phytosterol intake					P for trend
	Q1 (<257 mg/d)	Q2 (257-282 mg/d)	Q3 (283-305 mg/d)	Q4 (306-333 mg/d)	Q5 (>333 mg/d)	
n	7120	7118	7121	7118	7120	
HR (Total CVD)						
Model 1[a]	1.0	0.90 (0.80; 1.00)	0.85 (0.76; 0.95)	0.88 (0.79; 0.98)	0.98 (0.88; 1.09)	0.6033
Model 2[b]	1.0	0.96 (0.86; 1.07)	0.92 (0.82; 1.03)	0.95 (0.85; 1.06)	1.03 (0.92; 1.15)	0.7334
Model 3[c]	1.0	0.95 (0.84; 1.06)	0.90 (0.79; 1.02)	0.92 (0.81; 1.05)	0.99 (0.86; 1.14)	0.9401
HR (Total CHD)						
Model 1	1.0	0.82 (0.71; 0.95)	0.84 (0.73; 0.97)	0.92 (0.80; 1.06)	0.91 (0.79; 1.04)	0.4232
Model 2	1.0	0.88 (0.76; 1.01)	0.90 (0.78; 1.04)	0.98 (0.85; 1.13)	0.94 (0.82; 1.08)	0.7476
Model 3	1.0	0.83 (0.72; 0.97)	0.84 (0.72; 0.98)	0.90 (0.76; 1.06)	0.84 (0.70; 1.01)	0.1722
HR (MI)						
Model 1	1.0	0.88 (0.69; 1.11)	0.83 (0.65; 1.06)	0.76 (0.59; 0.97)	0.84 (0.66; 1.07)	0.0763
Model 2	1.0	0.99 (0.78; 1.26)	0.95 (0.74; 1.21)	0.86 (0.66; 1.10)	0.93 (0.72; 1.18)	0.3267
Model 3	1.0	0.95 (0.74; 1.22)	0.90 (0.68; 1.17)	0.80 (0.59; 1.06)	0.84 (0.62; 1.15)	0.1878

CHD, coronary heart disease; CVD, cardiovascular disease; HR, hazard ratio; MI, myocardial infarction.

[a] Model 1: corrected for age, gender and cohort (only for women).

[b] Model 2: corrected for variables in model 1 + BMI, smoking status, education, physical activity level, menopausal status (only for women) and total energy intake.

[c] Model 3: corrected for variables in model 2 + intake of saturated, polyunsaturated and monounsaturated fat, dietary cholesterol, fiber and alcohol.

Values are HR (95% CI).

Blood lipids

In the fully-adjusted model, energy-adjusted intake of naturally occurring phytosterols was significantly, inversely associated with TC, LDL-C and HDL-C (P <0.05); each 50 mg/d incremental phytosterol intake was significantly associated with a 0.06 mmol/L (95% CI: -0.11; -0.00) lower TC, a 0.07 mmol/L (95% CI: -0.11; -0.02) lower LDL-C, and a 0.02 mmol/L (95% CI: -0.04; -0.00) lower HDL-C. Furthermore, a significant association was observed between phytosterol intake and TG concentrations (0.04 mmol/L, 95% CI: 0.01; 0.06). Effect modification by

gender was significant for LDL-C. When stratifying according to gender, phytosterol intake was more strongly inversely associated with LDL-C in men (-0.18 mmol/L, 95% CI: -0.29; -0.08) than in women (-0.03 mmol/L, 95% CI: -0.08; 0.03). Interactions of phytosterol intake with waist circumference or hyperlipidemia were not significant for the lipid parameters. An overview of the associations with blood lipids is provided in **Supplemental Appendix 2**.

In mediation analysis, LDL-C hardly changed the association between phytosterol intake and cardiovascular risk; the mediation effect was low for each quintile and at maximum 5% for total CVD, 3% for total CHD and 6% for MI risk. When analyzing the mediation effect of LDL-C separately for men and women, we observed similar results.

Discussion

In this large cohort of 35,597 Dutch men and women, we observed no association between energy-adjusted intake of phytosterols from natural sources and CVD risk during 12 years of follow-up. However, higher naturally occurring phytosterol intake was significantly associated with lower TC and LDL-C concentrations at baseline, particularly among men.

Intake of two grams per day of phytosterols has been shown to lower LDL-C by on average 10%.[1] Based on data from statin trials[23], such a reduction in LDL-C could potentially reduce the absolute risk of CHD by ~9%. Considering that the intakes of naturally occurring phytosterols are much lower than 2 g/d (i.e. on average 296 mg/d in the current study), only small risk reductions were expected: ~2% lower CHD risk given the predicted LDL-C lowering effect for a difference in phytosterol intake of 150 mg/d between the highest and lowest quintiles of phytosterol intake or ~4% lower CHD risk given the observed ~5% lower LDL-C concentration between the highest and lowest quintiles of phytosterol intake. In the current study, we observed surprisingly strong CHD hazard ratios ranging between 0.83 and 0.90 across quintiles of phytosterol intake, but these were not all statistically significant. Klingberg *et al*. recently showed in a nested case-referent study[11] that a high absolute phytosterol intake was related to a reduced risk of a first MI in men with an odds ratio in the highest vs. the lowest quartile of 0.71. However, when corrected for total energy intake, this association of phytosterol intake with MI was not significant anymore. In women, neither the absolute nor the energy-adjusted

phytosterol intakes were associated with risk of MI[11]. In our opinion, adjustment for total energy intake is required, since associations of phytosterol intake with CVD risk may easily be confounded by energy intake. All in all, the findings of the current study are in line with previous investigation[11].

The significant associations between intake of naturally occurring phytosterols and lower TC and LDL-C concentrations were also found in previous observational studies with similar ranges of naturally occurring phytosterol intakes[8-10,24,25]. It should be noted that in our study, the association with TC and LDL-C was only present in men whereas evidence from randomized controlled trials have shown that TC and LDL-C are lowered in both men and women[1,2]. It is not clear why this discrepancy exists. The association observed between phytosterol intake and lower HDL-C concentrations was also found in other population studies, with some studies showing more pronounced effects in women[10] (similar to our observation) whereas other studies showed more pronounced effects in men.[8,9] As randomized controlled trials clearly show that HDL-C concentrations are not changed upon phytosterol intervention[26], it might be that residual confounding has played a role in this association.

The mechanism by which phytosterols are expected to reduce CVD risk is their LDL-C-lowering effect. However, in our population with relatively narrow range of low naturally occurring phytosterol intakes, mediation analysis did not support that low dietary phytosterol intakes are associated with reduced CVD risk through reductions in LDL-C. Whether higher intakes of phytosterols would eventually be significantly associated with reduced CVD risk through effects on LDL-C has yet to be investigated. This should preferably be done in populations with higher and broader ranges of phytosterol intakes, for example by including people with diets containing predominantly rich sources of phytosterols (e.g. cereal products and vegetable oils) and users of foods enriched with phytosterols. Users of phytosterol-enriched foods consume much higher amounts of phytosterols (~1.0-1.3 g/d) and seem to have lower TC concentrations vs. non-users after 5 years of follow-up[27,28].

Strengths of this study include the large sample size and its continuous, prospective and almost complete follow-up for disease occurrence, but there are also some limitations. First, the intakes of phytosterols from natural sources were low within a relatively narrow range, thereby limiting the capacity to detect an association

between dietary phytosterol intake and CVD risk. Second, dietary intake was assessed only at baseline. It cannot be ruled out that participants changed their dietary behaviours during follow-up thereby influencing the occurrence of disease, and, thus, the findings of this study. However, excluding participants that most likely changed their dietary habits (those with chronic diseases at baseline and cases occurring during the first 2 years) did not alter our findings. Furthermore, assessment of the long-term reproducibility of the FFQ in the EPIC-Heidelberg cohort showed fairly high correlation between dietary assessments at baseline and at follow-up[29]. Related to this, we cannot exclude that our findings may be confounded by a small part of the study population that started using phytosterol-enriched foods or cholesterol-lowering medication during follow-up. Although the proportion of people consuming phytosterol-enriched foods was only ~6% in a subset of our study population[28], these foods contain high concentrations of phytosterols and can therefore contribute considerably to the daily intake of phytosterols. A sensitivity analysis with follow-up until 2000 (i.e. the year that phytosterol-enriched foods were introduced onto the European market) however did not reveal different results. The influence of cholesterol-lowering medication use during follow-up could not be tested in sensitivity analysis and remains a limitation of our study. Third, because a national database with phytosterol composition data did not exist for the Netherlands, a specific database was developed for the analysis[4]. Although this database was developed with utmost care, some misclassification of the level of phytosterol exposure may have occurred due to incomplete information on phytosterol content in foods. At last, food intake was estimated with FFQs that are vulnerable for misreporting. Exclusion of misreporters in sensitivity analysis did however not affect the results. Moreover, the main dietary phytosterol sources (fruits and vegetables, cereal products and vegetable oils) and the average phytosterol intakes in the current study were comparable to those observed in other populations[3,4,30]. Additionally, we could demonstrate good relative validity and reproducibility of the phytosterol intake estimated with our FFQ.

In summary, intake of phytosterols from natural sources was not associated with a reduced CVD risk despite a lower LDL-C concentration particularly in men. Future studies should preferably investigate the association between phytosterol intake and CVD risk in populations with higher and broader ranges of phytosterol intake.

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Supplemental material

Supplementary Appendix 1 - Energy-adjusted phytosterol intake and risk of stroke in the EPIC-NL cohort

	Quintiles based on energy-adjusted intake of phytosterols					P for trend
	Q1 (<257 mg/d)	Q2 (257-282 mg/d)	Q3 (283-305 mg/d)	Q4 (306-333 mg/d)	Q5 (>333 mg/d)	
n	7120	7118	7121	7118	7120	
HR (Stroke)						
Model 1[a]	1.00	1.04 (0.81; 1.34)	1.02 (0.79; 1.32)	0.85 (0.65; 1.11)	1.20 (0.94; 1.54)	0.3771
Model 2[b]	1.00	1.10 (0.86; 1.42)	1.10 (0.85; 1.42)	0.91 (0.69; 1.19)	1.27 (0.99; 1.63)	0.1917
Model 3[c]	1.00	1.12 (0.86; 1.46)	1.12 (0.84; 1.48)	0.93 (0.68; 1.26)	1.30 (0.94; 1.79)	0.2411
HR (Ischemic stroke)						
Model 1	1.00	0.90 (0.64; 1.27)	0.99 (0.71; 1.38)	0.73 (0.51; 1.05)	1.25 (0.91; 1.72)	0.3191
Model 2	1.00	0.97 (0.69; 1.36)	1.08 (0.77; 1.51)	0.80 (0.55; 1.15)	1.33 (0.97; 1.84)	0.1765
Model 3	1.00	0.97 (0.68; 1.39)	1.08 (0.74; 1.57)	0.80 (0.52; 1.22)	1.34 (0.88; 2,04)	0.2475
HR (Hemorrhagic stroke)						
Model 1	1.00	1.15 (0.73; 1.83)	0.84 (0.51; 1.38)	0.99 (0.62; 1.61)	0.95 (0.59; 1.54)	0.6683
Model 2	1.00	1.22 (0.77; 1.93)	0.89 (0.54; 1.48)	1.07 (0.66; 1.73)	1.01 (0.62; 1.65)	0.8793
Model 3	1.00	1.36 (0.83; 2.21)	1.05 (0.60; 1.84)	1.31 (0.75; 2.31)	1.33 (0.71; 2.47)	0.4639

HR, hazard ratio.

[a] Model 1: corrected for age, gender and cohort (only for women).

[b] Model 2: corrected for variables in model 1 + BMI, smoking status, education, physical activity level, menopausal status (only for women) and total energy intake.

[c] Model 3: corrected for variables in model 2 + intake of saturated, polyunsaturated and monounsaturated fatty acids, dietary cholesterol, fiber and alcohol.

Values are HR (95% CI).

Supplementary Appendix 2 - Energy-adjusted phytosterol intake and blood lipid concentrations in the EPIC-NL cohort

	Overall			Men			Women		
	β per 50 mg/d phytosterols	95% CI	P	β per 50 mg/d phytosterols	95% CI	P	β per 50 mg/d phytosterols	95% CI	P
TC (mmol/L)									
n		2417			605			1812	
Average		5.32 ± 1.05			5.59 ± 1.11			5.23 ± 1.02	
Model 1[a]	-0.05	(-0.09; -0.01)	0.0070	-0.07	(-0.16; 0.01)	0.1024	-0.05	(-0.09; -0.00)	0.0324
Model 2[b]	-0.05	(-0.09; -0.01)	0.0093	-0.08	(-0.17; 0.01)	0.0679	-0.04	(-0.09; 0.00)	0.0529
Model 3[c]	-0.06	(-0.11; -0.00)	0.0384	-0.13	(-0.24; -0.01)	0.0307	-0.03	(-0.09; 0.03)	0.2891
LDL-C (mmol/L)									
n		2383			593			1790	
Average		3.09 ± 0.87			3.19 ± 0.98			3.06 ± 0.83	
Model 1	-0.05	(-0.09; -0.02)	0.0029	-0.12	(-0.20; -0.04)	0.0039	-0.03	(-0.07; 0.01)	0.0926
Model 2	-0.05	(-0.09; -0.02)	0.0038	-0.12	(-0.20; -0.04)	0.0023	-0.03	(-0.07; 0.01)	0.1367
Model 3	-0.07	(-0.11; -0.02)	0.0074	-0.18	(-0.29; -0.08)	0.0007	-0.03	(-0.08; 0.03)	0.3294
HDL-C (mmol/L)									
n		2383			593			1790	
Average		1.27 ± 0.35			1.14 ± 0.28			1.32 ± 0.35	
Model 1	-0.02	(-0.03; -0.01)	0.0025	-0.01	(-0.03; 0.01)	0.4088	-0.02	(-0.04; -0.01)	0.0035
Model 2	-0.02	(-0.03; -0.01)	0.0026	-0.01	(-0.03; 0.02)	0.5478	-0.02	(-0.04; -0.01)	0.0027
Model 3	-0.02	(-0.04; -0.00)	0.0211	0.00	(-0.03; 0.03)	0.9442	-0.03	(-0.05; -0.01)	0.0123
ln(TG) (mmol/L)									
n		2410			605			1805	
Average		0.30 ± 0.54			0.54 ± 0.55			0.22 ± 0.51	
Model 1	0.01	(-0.01; 0.03)	0.3450	0.03	(-0.01; 0.08)	0.1350	0.00	(-0.02; 0.03)	0.8493
Model 2	0.01	(-0.01; 0.03)	0.4075	0.02	(-0.02; 0.07)	0.2684	0.00	(-0.02; 0.03)	0.8548
Model 3	0.04	(0.01; 0.06)	0.0142	0.04	(-0.01; 0.10)	0.1494	0.03	(-0.00; 0.07)	0.0516

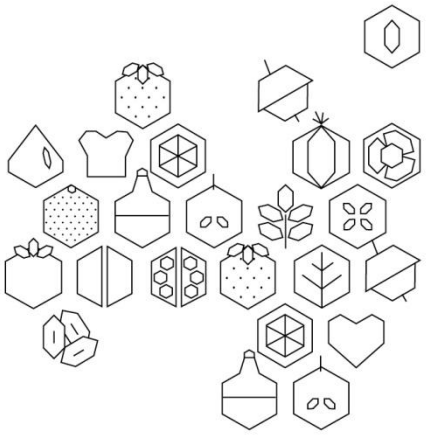
β, regression coefficient; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

[a] Model 1: corrected for age, gender and cohort (only for women).

[b] Model 2: corrected for variables in model 1 + BMI, smoking status, education, physical activity level, menopausal status (only for women) and total energy intake.

[c] Model 3: corrected for variables in model 2 + intake of saturated, polyunsaturated and monounsaturated fat, dietary cholesterol, fiber and alcohol.

Averages are mean ± SD. Effects are β (95% CI).



Chapter 8

General Discussion

The aim of this thesis was to advance insights in the role of phytosterols in the management of blood lipid risk factors for cardiovascular disease (CVD). Phytosterols are lipid-like compounds that occur naturally in small amounts in plant-based foods and in high amounts in specific enriched foods. An overview of the main results of this thesis is presented in **Table 1**.

Table 1. *Overview of the main results of the studies presented in this thesis.*

Chapter	Type of research	Exposure	Main results
2	Meta-analysis of 84 randomized controlled trials	Plant sterol- or stanol-enriched food intake	A non-linear, continuous dose-response relationship was established for the LDL-C-lowering effect of plant sterol/stanol intakes. The pooled LDL-C reduction was 0.34 mmol/L (95% CI: -0.36; -0.31) or 8.8% (95% CI: -9.4; -8.3) for a mean daily dose of 2.15 g plant sterols/stanols.
3	Meta-analysis of 124 randomized controlled trials	Plant sterol- or stanol-enriched food intake	Plant sterol/stanol intakes of 0.6-3.3 g/d gradually reduced LDL-C concentrations by, on average, 6-12%. When plant sterols and stanols were analyzed separately, clear and comparable dose-response relationships were observed.
4	Meta-analysis of 12 randomized controlled trials, using individual subject data	Plant sterol-enriched food intake	Plant sterol intake (~2 g/d) significantly lowered fasting TG concentrations by 6.0% (95% CI: -10.7; -1.2) or 0.12 mmol/L (95% CI: -0.20; -0.04). Larger absolute decreases were observed with higher TG concentrations at baseline.
5	Randomized controlled trial with 332 subjects	Plant sterol- and fish oil-enriched spread intake	Intake of a low-fat spread with added plant sterols (2.5 g/d) and different low doses (<2 g/d) of omega-3 fatty acids from fish oil decreased fasting TG concentrations in a dose-dependent manner (5.3% to 16.2%) while also decreasing LDL-C concentrations (on average 13%).
6	Meta-analysis of 41 randomized controlled trials	Plant sterol-enriched food intake	Intake of foods with added plant sterols (~1.6 g/d) increased blood sitosterol and campesterol concentrations by on average 2.24 µmol/L (31%) and 5.00 µmol/L (37%), respectively, while reducing TC and LDL-C by 0.36 mmol/L (6%) and 0.33 mmol/L (9%), respectively. Overall, total plant sterol concentrations remained below 1% of total sterols circulating in the blood.
7	Epidemiological study with 35,597 Dutch participants followed-up for ~12 years	Intake of naturally occurring phytosterols	In a population with a relatively narrow range of low naturally occurring phytosterol intakes (231-366 mg/d), intake of phytosterols was not associated with reduced CVD, CHD or MI risk. Phytosterol intake was associated with lower LDL-C concentrations at baseline in men (-0.18 mmol/L per 50 mg/d; 95% CI: 0.29; -0.08).

CHD, coronary heart disease; CVD, cardiovascular disease; LDL-C, low-density lipoprotein cholesterol; MI, myocardial infarction; TC, total cholesterol; TG, triglyceride

In this chapter, the methodological aspects of the studies presented in this thesis are considered and the relevance of our findings in relation to CVD risk is discussed. Implications for public health are indicated and recommendations for future research are given.

Methodological considerations

This thesis includes data from four meta-analyses of randomized trials (Chapters 2, 3, 4 and 6), one intervention study (Chapter 5) and one epidemiological study (Chapter 7). In this section, the main strengths and limitations of these studies are highlighted.

Meta-analyses

In meta-analyses, the results from multiple studies are combined to increase the precision of the overall effect estimate and to identify and quantify sources of variation in results across studies. Some limitations of meta-analyses should however be considered.

In meta-analyses, bias in the selection of studies can occur. Especially meta-analyses based on individual subject data (like in Chapter 4) are prone to selection bias when only the original data of part of the studies performed can be accessed. To estimate the effect of plant sterol intake on triglyceride (TG) concentrations, data of twelve industry-sponsored studies were available. These studies also formed part of the meta-analysis on low-density lipoprotein cholesterol (LDL-C) (Chapter 3), which included a total of 124 studies. We assessed whether the twelve selected studies were representative of the total body of evidence by comparing the LDL-C response in that subset with the overall response reported in Chapter 3. The change in LDL-C in the twelve industry-sponsored studies was around 8% for an average plant sterol dose of 1.9 g/d. In Chapter 3, an average phytosterol dose of 1.7 g/d (based on 55 study arms) lowered LDL-C by ~7.6% whereas an average dose of 2.1 g/d (60 study arms) lowered LDL-C by ~8.4%. Based on this observation, it can be assumed that also the effects on TGs in the twelve studies included in Chapter 4 are representative of the total body of evidence.

A concern related to the validity of meta-analyses is publication bias. This type of bias occurs when published studies are systematically unrepresentative of all the

studies that have been done. For example, small studies with unexpected or neutral results are less likely to be published than studies with statistically significant, positive results. In our meta-analyses on phytosterols and LDL-C (Chapters 2 and 6), funnel plots suggested absence of publication bias. On the contrary, for the plasma plant sterol concentrations (Chapter 6), publication bias may have been present; studies reporting no or relatively small increases in plasma plant sterols with low precision were lacking. The estimated increase in plasma plant sterols, for which some concern exists[1], may thus have been slightly overestimated. However, this bias is probably not substantial as studies with low precision would not contribute much in estimating the variance-weighted net effect.

Heterogeneity in meta-analyses refers to the degree of variation in results of individual studies, which may be caused by differences in study design (methodological variation) and/or study populations (biological variation). If the results of individual studies are too heterogeneous, the conclusions of a meta-analysis cannot be generalized but only apply to the average population under the average conditions of the included studies. Conversely, if the presence of heterogeneity can be explained by variables such as the health status of the subjects or the dose of the active ingredient, this provides relevant information that may not have been picked up in single studies. In our meta-analyses, heterogeneity was clearly present and we identified two factors that were repeatedly shown to influence the relationships under investigation. Both the dose of phytosterols (Chapters 2, 3 and 6) and the pre-intervention lipid values (Chapters 2, 4 and 6) clearly explained part of the variation in observed effects on LDL-C, TGs and/or plasma plant sterols. Regarding the plasma plant sterol concentrations (Chapter 6), another source of variation was likely present. Measurements of plasma non-cholesterol sterols, like plant sterols, are not well standardized resulting in considerable variability when measured by different research groups[2]. Differences in internal standards, extraction, derivatization, separation and detection techniques explain about 25% of the variability in reported plasma plant sterol concentrations[2,3]. As relative changes are overall less affected by systematic errors, these are probably more reliable than the absolute changes in plasma plant sterol concentrations observed in our meta-analysis.

Intervention studies

Randomized controlled trials, if correctly designed and executed, provide the strongest evidence for a causal relationship. High quality trials have a randomized, placebo-controlled, double-blind design, a sufficient number of subjects to detect the expected effects and a high degree of compliance with the study protocol. The intervention study described in this thesis (Chapter 5) fulfills these criteria. With 332 subjects, it had ample power to detect effects on LDL-C and TGs. Plant sterols were provided via enriched spreads and compliance with these foods was high (>95%). The number of subjects that dropped out during the study was low (5.4%). The effects on blood lipids in the intervention group were compared to the effects in a placebo control group. The observed effects on LDL-C and TGs can therefore be fully ascribed to the plant sterol and/or fish oil interventions.

Intervention studies, however, also have their limitations. They are often performed in selected populations with relatively high doses of the food or active ingredient and it may be difficult to ensure sufficient compliance. The findings of such studies cannot easily be translated to the general population in free-living settings. In our study, we selected hypercholesterolemic but otherwise healthy subjects and instructed them to consume 30 g/d of the test spread with main meals. In free-living settings, people usually consume less, typically half of this amount of spread[4,5]. The effects on blood lipids in the general population are thus likely lower than in well-controlled studies. Furthermore, it is challenging to conduct large intervention studies under controlled conditions for long periods of time. Nutritional intervention studies in primary prevention settings therefore focus more frequently on metabolic risk factors (e.g. blood lipids or blood pressure) rather than on actual disease outcome (e.g. CVD events)[6]. Also, the intervention is often dietary advice or counseling rather than supply of actual foods/diets. Hence, dietary recommendations rely not only on intervention studies, but also take into account evidence from epidemiological studies on associations of dietary exposure with disease outcome.

Epidemiological studies

Epidemiological studies examine associations between exposures and outcomes at the population level or in a selected subset of the population. While epidemiological studies in general cannot prove causality, they may provide insights that cannot be obtained from short-term intervention studies, like for

example insights in relation to disease outcome. The EPIC-NL cohort that we used for our epidemiological investigations (Chapter 7) included 35,597 participants with extensive information on diet and lifestyle at baseline and almost complete follow-up for disease incidence[7].

Our epidemiological study was however limited by the relatively low and narrow intake of phytosterols from natural sources (231-366 mg/d between the lowest and highest quintiles). The interquintile difference in phytosterol intake of ~150 mg/d is estimated to lower LDL-C by ~0.05 mmol/L[8] as was confirmed in our cross-sectional analysis. Based on this estimate, the expected reduction in CVD risk would be ~2%[9]. We observed hazard ratios between 0.90 and 0.99 across quintiles of phytosterol intake, but these were all not statistically significant (Chapter 7). Apart from no relationship being present, there are alternative explanations why we found no significant association. Phytosterol intake was estimated only once at baseline, using a self-administered food frequency questionnaire. Next to measurement imprecision, people could have over-reported the intake of healthy plant-based foods and (consequently) of phytosterols. Furthermore, the database of phytosterol concentrations in foods that was used for our study (see Chapter 7) may have been incomplete. These measurement errors have likely caused misclassification of individuals for their true phytosterol intake, which could have diluted the association with CVD outcomes.

A general limitation of observational studies is confounding. Confounding occurs when a certain variable correlates with both the exposure and the outcome and is not an intermediate in the causal pathway from exposure to outcome. People who have a high phytosterol intake have also a high intake of plant-based foods and are often more health conscious. In our analyses, we adjusted for many potential confounders that are related to diet and lifestyle, including smoking, education, physical activity, saturated and unsaturated fats and fiber. Nevertheless, some residual confounding from unknown or imprecisely measured variables may have remained. If this is the case, the inverse, though non-significant, associations that we observed may have been overestimated. On the other hand, it is also possible that we have overcorrected for confounders. In that case, significant inverse associations with CVD risk may have been missed. Considering its limitations, our epidemiological study does not provide a definitive answer on the relation between

intake of phytosterols from habitual, non-fortified foods and CVD risk. It at least shows the need for population studies with broader ranges of phytosterol intakes.

Interpretation of findings

The data presented in this thesis showed that foods enriched with phytosterols dose-dependently lower LDL-C concentrations (Chapters 2 and 3), and that plant sterols alone and in combination with omega-3 fatty acids from fish oil lower fasting TG concentrations (Chapters 4 and 5). Blood levels of plant sterols increased significantly during intake of enriched foods, indicating that some of the dietary plant sterols were absorbed (Chapter 6). Whether phytosterols through their effects on blood lipids could lower the risk of CVD has not been established (Chapter 7). To date, CVD endpoint trials with phytosterols have not been performed.

The section below discusses the relevance of changes in LDL-C, TGs and plasma phytosterols with phytosterol intake for future CVD risk (**Figure 1**).

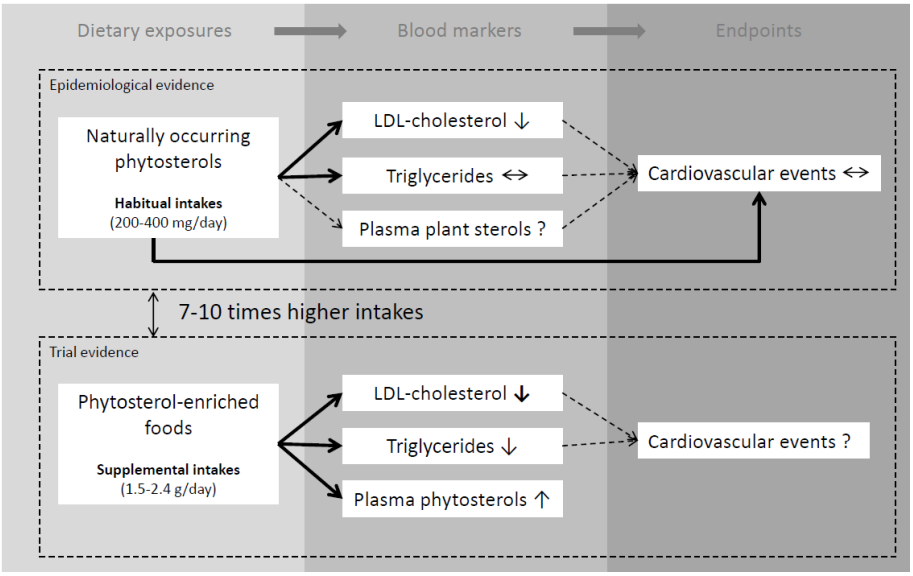


Figure 1. *Overview of the relationships between habitual and supplemental intakes of phytosterols, various blood markers and cardiovascular risk as observed in epidemiological and/or intervention studies. Bold lines indicate relations that were investigated in the current thesis. Dotted lines indicate relations that were investigated in other published studies.*

Relevance of blood low-density lipoprotein cholesterol

LDL-C is an established risk factor for CVD, particularly for CHD. Substantiation for this relationship is based on several types of evidence. Epidemiological studies have shown that people with elevated LDL-C are more likely to suffer from a cardiovascular event[10,11]. In randomized trials, LDL-C lowering by means of drugs (statins)[9,12] or diets[13] has convincingly been shown to reduce the risk of CHD. This effect is irrespective of the type of intervention[14]. Furthermore, the reduction in CHD incidence appears to be related to the magnitude of the decrease in LDL-C with no indication for a threshold level at lower LDL-C concentrations[15]. These reductions in CHD risk were overall caused by relatively short interventions (2-5 years) beginning later in life in adult populations at various degrees of risk. If LDL-C concentrations remain at lower levels for longer periods of time, reductions in CHD risk are expected to be more pronounced[16,17]. Indeed, life-long exposure to lower LDL-C (0.07-0.43 mmol/L) due to the presence or absence of specific variations in DNA sequences (single-nucleotide polymorphisms, SNPs) was associated with a 6-28% lower risk of CHD[16]. When standardized per unit lower LDL-C, these alleles were associated with a 9%, 18%, 33% or 54% lower risk of CHD for each 0.125, 0.25, 0.5 or 1 mmol/L lower LDL-C, respectively[16]. This risk reduction due to prolonged exposure to lower LDL-C early in life is 2- to 3-fold larger compared to the risk reduction from statin treatment started later in life (i.e., 21% per 1 mmol/L LDL-C for CHD or 24% per 1 mmol/L for CVD)[9]. In the current thesis, an average phytosterol intake of 2 g/d was shown to lower LDL-C by on average 0.35 mmol/L or 9%. Such a decrease in LDL-C is predicted to reduce the risk of CHD by ~9% in 5 years at population level[9]. For this estimation, data from statin trials are used; it should be realized that these LDL-C-lowering drugs may impact CHD risk also via other mechanisms, e.g. via decreases in low-grade inflammation. In the case of life-long decreases in LDL-C, the reduction in CHD risk may augment to ~25% [16].

Relevance of blood triglycerides

While the clinical relevance of decreases in LDL-C is well established, this is less so for decreases in TGs. Observational evidence suggests that people with higher TG concentrations have a higher risk of CVD[18] or CHD[19]. However, whether this association is independent of changes in other blood lipids, particularly high-density lipoprotein cholesterol (HDL-C), remains uncertain. For example, in a large meta-analysis with >300,000 people, the hazard ratio for CHD per SD increase in plasma TGs dropped from 1.37 (95% CI: 1.31; 1.42) after adjustment for non-lipid

risk factors to 0.99 (95% CI: 0.94; 1.05) after further adjustment for HDL- and non-HDL-C[20]. Randomized controlled trials with fibrates, an effective group of TG-lowering drugs, have shown that reducing TGs lowers the risk of CVD, particularly in populations with initially high levels of TGs and low levels of HDL-C[21,22]. Fibrates though also reduce, to some extent, LDL-C and increase HDL-C. Based on evidence from genetic studies, SNPs that have strong associations with TGs but minimal associations with other lipids are significantly related to CHD risk[23,24]. Thus, although assessment of TGs appears to have little predictive value for CHD risk on top of HDL-C, data from these genetic studies do suggest some role of TGs in the development of CHD. It might be that blood TGs are a marker of TG-rich lipoprotein remnants, particularly intestinal-derived chylomicron remnants and liver-derived very-low-density lipoprotein (VLDL) remnants. Increasing evidence suggests that these remnants are atherogenic[25,26]. To establish the relevance of TG-lowering effects of phytosterols and/or omega-3 fish fatty acids for CVD or CHD risk, their effects on these atherogenic lipoprotein remnants should be further explored.

Relevance of blood phytosterols

Concerns have been raised about a potential atherogenic effect of increased plasma plant sterol concentrations (Chapter 6) based on several lines of evidence.

First, patients with homozygous phytosterolemia cannot sufficiently excrete phytosterols from the body due to mutations in *ABCG5/8* genes. These patients therefore have extremely high levels of plant sterols (~500-1200 µmol/L) and plant stanols (up to 200 µmol/L) in their blood[27,28]. In the very few patients who suffer from this genetic disorder (approximately 50-80 reported worldwide[29]), sterol-rich fat depositions in tendons and other body parts, so called xanthomas, are formed. In these patients, symptoms of premature atherosclerosis are observed[28,29]. However, in five recently published case studies, no signs of atherosclerosis were reported[30]. Our meta-analysis on plasma plant sterol concentrations (Chapter 6) showed concentrations in plasma plant sterols after intake of plant sterol-enriched foods that were 20-45 times lower than plasma plant sterol concentrations observed in patients with homozygous phytosterolemia. Heterozygous phytosterolemic subjects have moderately higher (35-37%) plasma plant sterol concentrations compared to healthy controls[31]. The relative plasma plant sterol responses in heterozygous phytosterolemic subjects after consumption of plant

sterol-enriched foods are similar to the responses in subjects without phytosterolemia [32,33].

Second, elevated plasma plant sterol concentrations have been associated with increased CVD risk in some[34,35], but not all[36,37], observational studies. In a meta-analysis of 17 observational studies, no overall association between circulating sitosterol and campesterol and CVD risk could be identified[38]. The sizes of the average increases in plasma sitosterol and campesterol (about 2 and 5 µmol/L, respectively) observed in Chapter 6 were covered by the ranges of plasma sitosterol and campesterol investigated in this meta-analysis (about 3-9 µmol/L for sitosterol and about 4-14 µmol/L for campesterol)[38]. In a genome-wide association study with data from 3 studies (4,412 subjects)[39], genetic variants related to plasma plant sterol levels were detected. A meta-analysis of 11 studies (27,394 subjects) presented in the same paper[39] showed that SNPs related to elevated plasma plant sterol levels were associated with increased CHD risk whereas SNPs related to decreased plasma plant sterol levels were associated with reduced risk. Plasma plant sterol concentrations may however reflect cholesterol absorption efficiency[40,41]. In another genetic study[42], the same SNPs were associated with increases in the cholestanol-to-cholesterol ratio, a measure of cholesterol absorption that is independent of plasma plant sterols. This high cholestanol-to-cholesterol ratio was significantly related to increased CVD risk[42]. The association between plasma plant sterols and CVD risk may thus, at least partly, be explained by increased absorption of cholesterol and not by plant sterols *per se*.

Several potential mechanisms have been suggested why circulating plant sterols might be atherogenic. These include: 1) plant sterols are susceptible to oxidation[43] and it can be hypothesized that oxyphytosterols, like oxycholesterol[44] are atherogenic; 2) plant sterols are taken up by human aortic tissue where they may relate to the degree of aortic valve stenosis[45]; and 3) circulating plant sterols have been shown to be correlated with worsening of endothelial function in mice[46]. These observations have so far not been confirmed in individuals that consume plant sterol-enriched foods. Intake of such foods by healthy subjects did not significantly change blood levels of oxidized plant sterols[47]. Also, the ratio of plant sterols over cholesterol in aortic tissue after intake of phytosterol-enriched foods does not exceed this ratio in plasma. This suggests that plant sterols are not

preferentially taken up in these tissues [48]. Furthermore, the intake of plant sterol-enriched foods does not result in a worsening of endothelial function in humans[49].

Not only circulating plant sterols, but also plant stanol concentrations are increased in homozygous phytosterolemic patients[28] and after intake of plant stanol-enriched foods[50,51]. In absolute terms, however, the increases in plant stanols are much smaller than the increases in plant sterols due to a lower absorption rate[52]. Also, plant stanols do not have a double bound in the steroid nucleus and can therefore not be oxidized. Epidemiological studies on blood plant stanol concentrations and CVD risk have so far not been performed.

The effect of phytosterols on experimental atherosclerosis has extensively been studied in different animal models including chickens, rabbits, hamsters and genetically-modified mouse models of atherosclerosis, as recently summarized by Gylling *et al*.[53] These studies with high doses of phytosterols (0.1-2.0% (w/w)) showed overall atheroprotective effects including attenuation of foam cells, inhibition of lesion formation and regression of existing lesions[46,54-56]. Although these observations in animals cannot directly be translated to humans, the findings suggest that phytosterol intake may induce atheroprotective effects despite increases (up to 10-fold) in blood levels of phytosterols.

Phytosterols and CVD risk

Whether dietary phytosterols can impact CVD risk has so far not been investigated in randomized trials. Only a few epidemiological studies with phytosterol intake and occurrence of CVD, including the study described in Chapter 7, have been performed. Our study showed no significant association between intakes of naturally occurring phytosterols and CVD risk. In a recent prospective analysis by Klingberg *et al*.[57], a significant inverse association between intakes of naturally occurring phytosterols and risk of myocardial infarction was shown in men, but not in women. However, when adjusting for energy intake, the association in men was no longer significant, in line with our findings. As intakes of phytosterols from natural sources are low and limited in range (200-400 mg/d; **Figure 2**), the results from these two observational studies cannot be taken as strong evidence for absence of a relation between dietary phytosterol intake and CVD risk, and cannot merely be extrapolated to effects on CVD risk of supplemental phytosterol intake through enriched foods. Such foods contain much higher amounts of phytosterols,

e.g. 0.75 g per 10 g portion of phytosterol-enriched spread. In controlled trial settings, phytosterol intakes from enriched foods ranged overall between 1.5 and 2.4 g/d. In free-living settings, however, users of such foods consume lower amounts of 1.0-1.3 g/d[4,5]. Furthermore, such foods are consumed by only a small part of the population[58] as compared to the widespread intake of naturally occurring phytosterols with habitual diets (**Figure 2**).

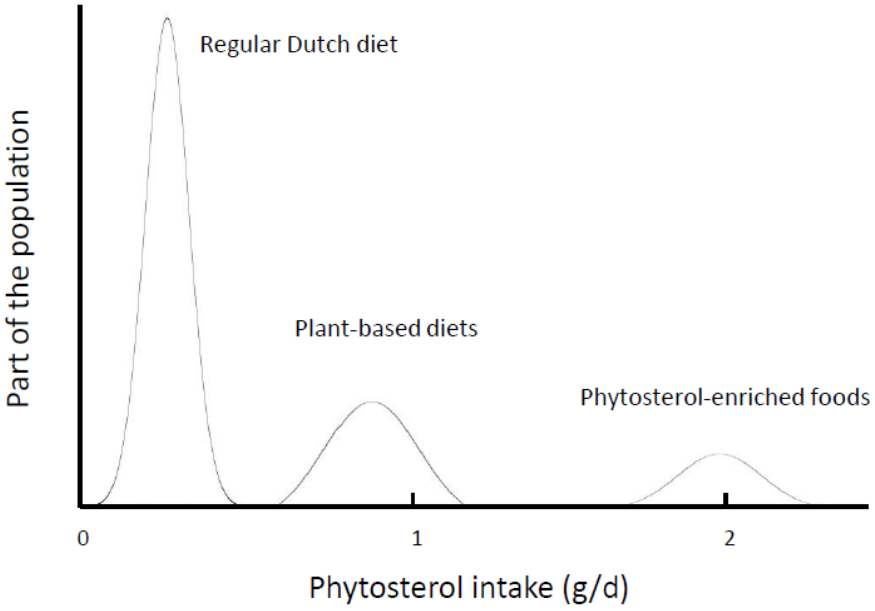


Figure 2. *Intake of phytosterols in the general population and in populations with plant-based diets vs. intake of phytosterols from enriched foods as tested in trials.*

In individuals with diets that emphasize plant-based foods, the intake of phytosterols is higher than in a general Western diet (**Figure 2**). Examples of such diets are the Mediterranean diet[59] and the Dietary Approaches to Stop Hypertension (DASH) diet for lowering blood pressure[60]. With these diets, phytosterol intakes of 500-550 g/d can be achieved (**Table 2**). The Predimed study, a randomized trial with 7,447 persons at high cardiovascular risk[61], showed a 28 to 30% lower incidence of major CVD events for a Mediterranean diet with additional extra-virgin olive oil or nuts. The DASH diet was associated with an 18% lower estimated 10-year CHD risk (based on the Framingham risk equation) in individuals with (pre-)hypertension[62]. A lacto-vegetarian diet that emphasizes phytosterol-rich

foods (**Table 2**) may yield phytosterol intakes up to 1 g/d. Such types of vegetarian diets have been associated with a ~24% reduced risk in CHD mortality, which could partly be mediated through favorable effects on blood cholesterol[63].

Table 2. *Estimated phytosterol intakes with various diets*

	Lacto-vegetarian diet	**Predimed diet**	**DASH diet**
Concept	A diet that excludes meat, fish, poultry and eggs	A Mediterranean diet with addition of 50 mL extra-virgin olive oil or 30 g nuts	A diet low in saturated fat, trans fat and sodium and high in potassium
Hypothetical daily menu	**Breakfast** ¾ cup (50 g) bran flakes 1 cup (200 mL) low-fat milk 1 cup (150 g) fruit salad 1 cup orange juice (200 mL) **Lunch** 2 slices (70 g) whole-wheat bread 2 tsp. (10 g) margarine 1 ½ tbsp. (30g) peanut butter 1 ¼ cup (250 mL) broccoli soup **Dinner** 1 whole-wheat roll (50 g) 2 tbsp. (40 g) hummus 2 tbs. (10 g) canola oil ½ cup (150 g) couscous 1 avocado (150 g) ½ cup (75 g) corn ½ onion (30 g) ½ tomato (50 g) ½ carrot (50 g) 2 tbsp. (30 g) feta cheese 4 tsp. (20 g) corn oil 2 tsp. (10 g) lemon juice 20 g pine nuts ½ cup (100 mL) fruit yogurt **Snacks** ¼ cup (50 g) almonds ¼ cup (50 g) pistachios 1 bar (25 g) dark chocolate 100 g grapes	**Breakfast** 150 g Greek yogurt 75 g strawberries 1 tsp. (5 g) honey 1 slice (35 g) whole-wheat toast ½ avocado (100 g) **Lunch** 1 slice (35 g) whole-wheat toast 2 tbsp. (40 g) hummus 1 cup (20 g) lettuce ½ tomato (50 g) 1 cup (200 mL) minestrone soup 1 medium orange (150 g) **Dinner** 100 g salmon 1 tsp. (5 g) mustard ½ cup (150 g) couscous ½ cup (100 g) egg plant 4 asparagus (100 g) ½ cup (10 g) rucola ½ cup (10 g) spinach 1 tbsp. (15 g) parmesan cheese 1 tbsp. (15 g) vinaigrette 150 mL red wine 100 g grapes ½ cup (100 mL) lemon sorbet **Snacks** 1/8 cup (25 g) almonds 1/8 cup (25 g) peanuts **Additional** 50 mL olive oil or 30 g nuts	**Breakfast** ¾ cup (50 g) bran flakes 1 medium banana (100 g) 1 cup (200 mL) low-fat milk 1 slice (35 g) whole-wheat bread 1 tsp. (5 g) unsalted margarine 1 cup (200 mL) orange juice **Lunch** 2 slices (70 g) whole-wheat bread ¾ cup (50 g) chicken salad 1 tsp. (5 g) mustard ½ cup (50 g) fresh cucumber slices ½ cup (50 g) tomato wedges 1 tbsp. (15 g) sunflower seeds 1 tsp. (5 g) dressing ½ cup (75 g) fruit cocktail **Dinner** 100 g beef 2 tbsp. (30 g) fat-free beef gravy 1 cup (150 g) green beans 1 tsp. (5 g) canola oil 1 small baked potato (75 g) 1 tbsp. (15 g) fat-free sour cream 1 tbsp. (15 g) cheddar cheese 1 tbsp. (15 g) chopped scallions 1 whole-wheat roll (50 g) 1 tsp. (5 g) unsalted margarine 1 small apple (100 g) 1 cup low-fat milk (200 mL) **Snacks** ¼ cup (50 g) almonds, unsalted ¼ cup (50 g) raisins ½ cup (100 mL) fruit yogurt
Phytosterol intake[a] (mg/d)	±1000	500-550	±500

[a] Phytosterol intakes are estimated using the phytosterol database that was developed by Sioen *et al.*[67]

It should be noted that these plant-based diets not only contain phytosterols, but also a wide array of other nutrients (e.g. fiber, B-vitamins and vegetable protein) and bioactive compounds (e.g. flavonoids) that could exert a beneficial effect on cardiovascular health. An additional intake of phytosterols of 250-750 mg/d, attainable with a plant-based diet, is predicted to lower LDL-C by 2-5% based on the dose-response curve presented in Chapter 2. The effect of phytosterols on LDL-C has been shown to be additive to that of a healthy diet[64-66]. To what extent the 2-5% reduction in LDL-C by phytosterols, which is expected to lower CHD risk by ~2-12%[9,16], could contribute to the cardio protective effect of plant-based foods has not yet been investigated.

Public health implications

Opinions of regulatory authorities

The LDL-C-lowering efficacy of phytosterols has been acknowledged by regulatory bodies. The European Food and Safety Authority (EFSA) approved a disease risk reduction health claim (article 14.1a) for phytosterols[68]. This health claim, authorized by the European Union (EU) in 2009, was formulated as follows[69,70]: "Plant sterols and plant stanol esters have been shown to lower/reduce blood cholesterol. High cholesterol is a risk factor in the development of coronary heart disease". This positive opinion from the EFSA panel was based on the established efficacy of phytosterols in lowering LDL-C and the established relation between LDL-C and CHD risk. The conditions of use for the approved health claim specify that intakes of 1.5-2.4 g/d plant sterols or stanols, incorporated in yellow fat spreads, dairy products, mayonnaise or salad dressings, are required to lower LDL-C by 7-10% or intakes of 2.5-3.0 g/d plant sterols or stanols to lower LDL-C by 10-12.5%, within 2-3 weeks[71]. In 2012, the EFSA delivered an opinion stating that plant sterols and stanols have similar cholesterol-lowering efficacy at intakes of 1.5-3.0 g/d[72]. The United States Food and Drug Administration (US FDA)[73] also approved the use of a health claim on phytosterols stating that plant sterol/stanol esters may reduce the risk of CHD provided that at least 1.3 g/d of plant sterol esters or 3.4 g/d of plant stanol esters is consumed as part of a diet low in saturated fat and cholesterol.

The safety of phytosterols was assessed as part of the EU Novel Foods approval process. Overall, no safety issues with prolonged intakes of phytosterols were

noted by the Scientific Committee on Food (SCF); intakes up to 8% phytosterols per 100 g spread were considered safe for human use[74]. Phytosterols may, however, interfere with the absorption of fat-soluble vitamins, particularly β-carotene[75]. This effect on β-carotene, a vitamin A precursor, is not expected by the SCF to have health consequences except in situations where vitamin A requirements are greater than normal such as in pregnancy, lactation or infancy. Phytosterol-enriched foods are therefore not nutritionally appropriate for these groups as clarified in the EU labeling regulation[76]. Although no numerical upper level of total intake could be established, it was concluded by the SCF that a prudent upper level of 3 g/d phytosterols should be considered[75]. In a recent post-launch monitoring study[58] in users of phytosterol-enriched foods, it was shown that the daily upper intake (95th percentile) of phytosterols at the household level ranged from 1.1 g in France up to 3.7 g in the Netherlands. Mean intakes ranged from 0.4 to 0.9 g/d. In total, 75-85% of the volume of phytosterol-enriched products was purchased by 1-2 person households whereas only 1.3-2.5% of the volume was purchased by households with children <5 years[58].

The French Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) recently evaluated the benefits and risks relating to the consumption of foods with added phytosterols. In contrast with reports from other authoritative bodies, ANSES concluded that, based on the available evidence, foods enriched with phytosterols are not appropriate means for preventing heart disease[77]. Arguments that were used by ANSES to support their conclusion were the increase in blood phytosterol concentrations and the reduction in β-carotene concentrations with phytosterol-enriched food intake, the observation that some individuals fail to reduce LDL-C with such foods and the absence of data from endpoint trials[77].

Guidelines and recommendations

The blood cholesterol-lowering properties of phytosterols have been acknowledged in recent guidelines by the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS) for the management of dyslipidemia[78]. These guidelines recognize that there are no clinical trial data showing that cholesterol-lowering through phytosterol intake prevents CVD. The recent Joint British Societies' guidelines[79] mention that it is reasonable to postulate a beneficial effect on CVD outcomes based on the LDL-C-lowering hypothesis. In the United

States, recent guidelines by the American College of Cardiology (ACC) and the American Heart Association (AHA) do not explicitly mention the use of phytosterol-enriched foods for lowering CVD risk[80,81]. These guidelines only considered dietary options that have supporting endpoint evidence with emphasize on dietary patterns rather than on individual dietary components. For example, for adults who would benefit from LDL-C-lowering, a diet that emphasizes vegetables, fruits, whole grains, low-fat dairy, lean meat, fish, legumes, nuts and vegetable oils is advised, as well as reduced saturated fat and trans fat intakes[80].

An EAS panel of experts in the field of cholesterol metabolism, phytosterol biology and CVD recently concluded that phytosterols may be a useful dietary adjunct for people with elevated cholesterol at intermediate or low CVD risk who do not yet qualify for drug treatment, for high risk patients who fail to achieve LDL-C targets while on drug treatment, and for people with familial hypercholesterolemia[53]. Foods with added phytosterols should not be used as a substitute for adopting a healthy dietary pattern. Rather, phytosterols may be incorporated in an overall healthy diet and lifestyle approach to manage hypercholesterolemia.

For the general population, current dietary guidelines do not include specific recommendations on phytosterol intake. Nevertheless, most dietary guidelines include the advice to consume more foods of plant origin[82,83]. For example, in the Netherlands, consumption of ~200 g/d of vegetables, ~200 g (i.e., two pieces) of fruit, and fiber-rich foods are part of the dietary recommendations[84,85]. When adhering to these guidelines, the intake of phytosterols will increase up to 0.5-1.0 g/d.

Recommendations for future research

Intervention studies

High intakes of phytosterols lower LDL-C, an established risk factor for CVD, particularly CHD. The direct relation between increased phytosterol intake and CVD risk has so far not been assessed in randomized controlled trials. Such a trial would require a sample size of 36,000-76,000 hypercholesterolemic individuals in primary and secondary prevention settings with an expected annual CVD risk level of 3%, and follow-up of 6-10 years[53]. Because of challenging practical issues and high

costs, it is highly unlikely that such a trial on hard CVD endpoints will be conducted in the next decades.

Alternatively, future randomized controlled trials may investigate the effect on CVD risk of phytosterol-enriched foods as part of a healthy diet. For example, a randomized trial with 7,447 subjects has recently shown that the Mediterranean diet reduces the risk of CVD by 30%[61]. Phytosterol-enriched foods have been shown to lower LDL-C on top of a healthy diet[65]. A follow-up study may investigate whether addition of phytosterol-enriched foods to this Mediterranean diet may further improve the health outcome of the subjects. Also, a combination of supplemental phytosterols with other LDL-C-lowering foods such as soluble fiber, nuts and soy protein could be tested in CVD endpoint studies. This combination, known as the Portfolio diet, has been shown to lower LDL-C to a similar extent (~30%) as statins[86]. The contribution of phytosterol-enriched foods to this LDL-C-lowering effect is over one third[87]. As the expected effect on LDL-C and subsequently on CVD risk with this diet is ~3 times higher as compared to the effect of phytosterols alone, it is expected that such a study would require less than half of the subjects as estimated for a randomized controlled trial with phytosterols alone[53]. Trials with phytosterols may furthermore focus on markers for CVD risk beyond LDL-C-lowering such as measuring progression of intima-media thickness, using advanced techniques.

Epidemiological studies

Future epidemiological studies of long-term CVD risk may focus on populations with higher levels and a wider distribution of natural phytosterol intake from dietary sources. These studies may include, for example, cohorts with a large number of vegetarians or vegans who consume a predominantly plant-based diet, or individuals consuming Mediterranean diets. To enable these epidemiological studies, food composition tables with extensive information on phytosterol content of foods are needed. Only a few food composition tables, e.g. in Finland[88] and the US[89], contain such detailed information on phytosterol content. To date, the Dutch NEVO table is lacking this information[90].

Other suggestions for epidemiological studies include the prospective investigation of CVD events in regular users of foods with added phytosterols compared to non-users. Such foods have been on the market in Europe and in the US for almost 15

years now. Exposure to phytosterol-enriched foods is around 2-6% in the Netherlands[4,5,91]. To enable this type of investigation, questions that allow accurate assessment of intake of phytosterol-enriched foods need to be incorporated in food frequency questionnaires. In cohorts with dietary assessments performed before the year 2000, including the EPIC-NL cohort (Chapter 7), this information is not available. Investigating phytosterol-enriched food intake in CVD cases vs. controls may also provide useful evidence.

Concluding remarks

In the current thesis, a high intake of phytosterols (i.e., plant sterols and plant stanols) with enriched foods was shown to lower LDL-C in a dose-dependent manner. Furthermore, a high intake of plant sterols with enriched foods modestly lowered TG concentrations and increased plasma plant sterol concentrations. A low intake of naturally occurring phytosterols in the general population did not show a clear association with CVD risk. Based on these findings, we conclude that the intake of phytosterols may be considered in the management of hypercholesterolemia. Whether a high intake of phytosterols can play a role in CVD prevention in the population at large remains to be established.

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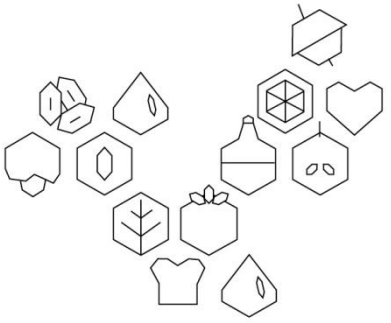
Thesis overview

What was known
• Dietary plant sterols and plant stanols lower blood LDL-C, but the dose-response relationship for this effect has not yet been extensively studied. • The maximal LDL-C-lowering effect may be larger for plant stanols than for plant sterols. • The intake of plant stanols modestly lowers blood TG concentrations; studies with plant sterols were mostly underpowered to detect effects on TGs. • The intake of high doses of omega-3 fish fatty acids (2-4 g/d) lowers TG concentrations. • The intake of plant sterols results in increased blood concentrations of plant sterols; the size of this increase has not yet been systematically investigated. • Observational studies with intake of plant sterols or plant stanols and long-term risk of CVD are lacking.
What this thesis adds
• The LDL-C-lowering effect of plant sterols and stanols is dose-dependent and reaches a plateau at doses around 3 g/d. • Plant sterols and plant stanols at doses up to 3 g/d are equally effective in lowering blood LDL-C. • Not only plant stanols, but also plant sterols modestly lower fasting TG concentrations. • Low doses (<2 g/d) of omega-3 fish fatty acids incorporated in a low-fat plant sterol-enriched spread lower blood TGs and LDL-C. • The intake of foods with added plant sterols increases plasma plant sterol concentrations but these remain below 1% of total sterols circulating in the blood. • The intakes of plant sterols and stanols from a regular Dutch diet is ~300 mg/d; these low intakes are associated with lower LDL-C, but not with a reduced CVD risk.
Recommendations for future research
• Randomized controlled trials of supplemental phytosterol intake and hard CVD endpoints are lacking and it is uncertain whether such trials will be conducted in the near future; trials on surrogate CVD endpoints such as carotid intima-media thickness are warranted. • Randomized controlled trials of phytosterol intake as part of the healthy diet or the Portfolio diet in relation to CVD risk factors and (surrogate) CVD endpoints are needed. • There is a need for long-term epidemiological studies on CVD risk in populations with higher levels and wider distributions of phytosterol intake. • Dietary assessment methods in epidemiological studies should be adapted to enable studies on the association of long-term intake of phytosterol-enriched foods and CVD risk in the general population.

CVD, cardiovascular disease; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride



English summary
Nederlandse samenvatting

English summary

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Lifestyle improvements including dietary changes are important for CVD prevention. This thesis aimed to advance insights in the role of phytosterols, lipid-like compounds present in foods or plant origin, in the management of blood lipid risk factors for CVD. Phytosterols include plant sterols and their saturated form, plant stanols. These compounds resemble cholesterol in both structure and function, but cannot be produced by the human body. The intake of phytosterols occurs through plant-based foods and/or enriched foods like margarine.

Elevated blood low-density lipoprotein cholesterol (LDL-C) is a major risk factor for CVD, especially for coronary heart disease (CHD) resulting from atherosclerosis. We studied the dose-response relationship between dietary phytosterols and blood LDL-C in two meta-analyses (Chapters 2 and 3). A meta-analysis of 81 randomized controlled trials (Chapter 2) demonstrated a non-linear, continuous dose-response relationship for the LDL-C-lowering effect of phytosterols. Based on this dose-response curve, it may be predicted that phytosterols at a dose of 2 g/d lower LDL-C by 0.35 mmol/L or 9%. The dose-response curve reached a plateau at phytosterol doses of ~3 g/d, above which there is limited additional LDL-C-lowering effect. In another meta-analysis of 124 randomized controlled trials (Chapter 3), we showed that plant sterols and plant stanols up to ~3 g/d are equally effective in lowering LDL-C by a maximum of 12%. No conclusions could be drawn for phytosterol doses exceeding 4 g/d because of the limited number of studies.

Elevated blood triglycerides (TGs) may also be involved in the onset of CVD, although its role is less established than for LDL-C. The effect of plant sterols on blood TG concentrations was assessed in a meta-analysis of individual subject data from 12 randomized controlled trials (Chapter 4). We showed that plant sterols, at a dose of ~2 g/d, modestly reduce TG concentrations by on average 0.12 mmol/L or 6%. The TG-lowering effect of plant sterols was larger in subjects with higher initial TG concentrations. Our double-blind, placebo-controlled, randomized trial with 332 subjects (Chapter 5) showed more pronounced TG-lowering effects of 9-16% when plant sterols (2.5 g/d) were combined with low doses of omega-3 fish fatty acids (0.9 to 1.8 g/d).

Dietary phytosterols are, after initial absorption by intestinal cells, actively excreted back into the intestinal lumen. Nevertheless, small amounts reach the circulation.

We assessed the effect of plant sterol intake on blood plant sterol concentrations in a meta-analysis of 41 randomized controlled trials (Chapter 6). The intake of plant sterols, at a dose of ~1.6 g/d, increased blood sitosterol concentrations by on average 2 µmol/L (31%) and campesterol concentrations by 5 µmol/L (37%). At the same time, total cholesterol and LDL-C concentrations were reduced by on average 0.36 mmol/L (6%) and 0.33 mmol/L (9%), respectively. After supplemental intake, plant sterol concentrations remained below 1% of total sterols circulating in the blood.

Whether phytosterols, due to their LDL-C-lowering properties, affect the risk of CVD events is at present unknown. The relation between phytosterol intake from natural sources (e.g. vegetables, cereals, nuts) and CVD risk in the population was examined in a large prospective cohort of 35,597 Dutch men and women with 12 years of follow-up (Chapter 7). The intake of phytosterols from natural sources (~300 mg/d) was not related to risk of CVD (total of 3,047 events) with a relative risk ranging from 0.90 to 0.99 across quintiles of phytosterol intake. Also, no association with incident CHD and myocardial infarction were found. In a cross-sectional analysis using baseline data of this cohort, phytosterol intake was associated with lower blood LDL-C in men (-0.18 mmol/L per 50 mg/d; 95% CI: -0.29; -0.08) but not in women (-0.03 mmol/L; 95% CI: -0.08; 0.03).

Most randomized trials with enriched foods have tested phytosterol doses between 1.5 and 2.4 g/d. In practice, however, users of such foods consume much lower amounts (~1 g/d), which is about 3 times higher than obtained from a regular Western diet. Individuals who consume diets with emphasis on plant-based foods (e.g. vegetarians) may reach phytosterol intakes between 0.5 and 1 g/d. Health authorities recommend various types of diets for CVD prevention, almost all rich in plant-based foods and, consequently, relatively rich in phytosterols.

In conclusion, a high intake of phytosterols with enriched foods was shown to lower LDL-C in a dose-dependent manner. Furthermore, a high intake of plant sterols with enriched foods modestly lowered TG concentrations and increased plasma plant sterol concentrations. A low intake of naturally occurring phytosterols in the general population did not show a clear association with CVD risk. Based on these findings, the intake of phytosterols may be considered in the management of hypercholesterolemia. Whether a high intake of phytosterols can play a role in CVD prevention in the population at large remains to be established.

Nederlandse samenvatting

Hart- en vaatziekten (HVZ) vormen de belangrijkste oorzaak van morbiditeit en mortaliteit wereldwijd. Verbeteringen in de levensstijl waaronder veranderingen in eetgewoonten, zijn belangrijk voor de preventie van HVZ. Het doel van dit proefschrift was om inzicht te verkrijgen in de rol die fytosterolen spelen in het beïnvloeden van bepaalde vetten in het bloed die een risico (kunnen) vormen op het krijgen van HVZ. Fytosterolen zijn vetachtige verbindingen die aanwezig zijn in plantaardig voedsel. Onder de fytosterolen vallen de plantensterolen en hun verzadigde vorm, de plantenstanolen. Deze verbindingen lijken op cholesterol in zowel structuur als functie, maar kunnen niet worden geproduceerd door het menselijk lichaam. Fytosterolen worden geconsumeerd in lage doseringen via plantaardig voedsel en/of in hoge doseringen via verrijkte producten zoals in sommige margarines.

Een verhoogd cholesterol in lage-dichtheids lipoproteÏnes (LDL-C) is een belangrijke risicofactor voor HVZ, in het bijzonder voor coronaire hartziekten, als gevolg van aderverkalking. We bestudeerden de dosis-effectrelatie tussen fytosterolen en LDL-C in het bloed in twee meta-analyses (Hoofdstukken 2 en 3). In een meta-analyse van 81 gerandomiseerde, gecontroleerde studies (Hoofdstuk 2) werd er een niet-lineair, dosisafhankelijk verband gevonden tussen fytosterolinname en LDL-C. Op basis van deze relatie kan worden voorspeld dat 2 g/dag fytosterolen het LDL-C-gehalte met gemiddeld 0,35 mmol/L of 9% verlaagt. Deze dosis-effectrelatie liet verder zien dat een inname van meer dan 3 g/dag weinig extra effect geeft. In een andere meta-analyse van 124 gerandomiseerde, gecontroleerde studies (Hoofdstuk 3) werd aangetoond dat plantensterolen en plantenstanolen tot een inname van ~3 g/dag even effectief zijn in het verlagen van LDL-C. Er konden geen conclusies getrokken worden over innamen boven de 4 g/dag omdat er slechts een beperkt aantal studies is uitgevoerd met dergelijke hoge innamen.

Een verhoogd triglyceriden (TG)-gehalte in het bloed is mogelijk ook een risicofactor voor HVZ. Het effect van plantensterolen op het TG-gehalte in het bloed werd onderzocht in een meta-analyse van 12 gerandomiseerde, gecontroleerde studies waarvan data van individuele proefpersonen beschikbaar waren (Hoofdstuk 4). We toonden aan dat een inname van ~2 g/dag plantensterolen het TG-gehalte met gemiddeld 0,12 mmol/L of 6% verlaagt. Het TG-verlagende effect van plantensterolen bleek groter bij proefpersonen met een

hoger initieel TG-gehalte. In een dubbelblinde, placebogecontroleerde, gerandomiseerde studie met 332 patiënten (Hoofdstuk 5) toonden we aan dat grotere verlagingen in TG (9-16%) bereikt kunnen worden als plantensterolen (2.5 g/dag) worden gecombineerd met omega-3 visvetzuren variërend in doseringen tussen de 0.9 en 1.8 g/dag.

Fytosterolen worden over het algemeen, na opname via de darmwand, weer uitgescheiden in het darmkanaal. Toch komen er kleine hoeveelheden in de bloedsomloop terecht. De mate waarin het gehalte van plantensterolen in het bloed toeneemt na inneming van plantensterolen werd onderzocht in een meta-analyse van 41 gerandomiseerde, gecontroleerde studies (Hoofdstuk 6). De inname van ~1.6 g/dag plantensterolen bleek het sitosterolgehalte in het bloed te verhogen met gemiddeld 2 µmol/L (31%) en het campesterolgehalte met gemiddeld 5 µmol/L (37%). Tegelijkertijd werden de gehaltes van totaal cholesterol en LDL-C verlaagd met respectievelijk 0.36 mmol/L (6%) en 0.33 mmol/L (9%). Na hoge inname van plantensterolen bedroegen de plantensterolgehaltes minder dan 1% van alle sterolen die in het bloed circuleren.

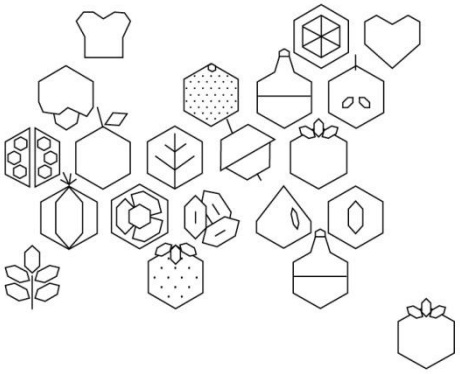
Het is niet zeker of fytosterolen het risico op HVZ kunnen beïnvloeden. De relatie tussen fytosterolinname uit natuurlijke bronnen (zoals groenten, granen en noten) en het risico op HVZ werd onderzocht in een populatie van 35.597 Nederlandse mannen en vrouwen die 12 jaar werden gevolgd (Hoofdstuk 7). In totaal werden er 3.047 nieuwe gevallen van HVZ geconstateerd. De inname van fytosterolen uit natuurlijke bronnen (gemiddeld 300 mg/dag) hield geen verband met het risico op HVZ. Het risico van groepen met toenemende innamen van fytosterolen ten opzichte van de groep met de laagste fytosterolinname varieerde tussen 0.90 en 0.99. Ook werd er geen significant verband gevonden met coronaire hartziekten of acute hartinfarcten. In een cross-sectionele analyse werd een omgekeerd verband waargenomen tussen fytosterolinname en LDL-C voor mannen (-0.18 mmol/L per 50 mg/dag) maar niet voor vrouwen (-0.03 mmol/L per 50 mg/dag).

In de meeste gerandomiseerde studies met fytosterolen van verrijkte voedingsmiddelen zijn doseringen getest tussen de 1.5 en 2.4 g/dag. In de praktijk consumeren de gebruikers van dit soort voedingsmiddelen echter lagere hoeveelheden (~1 g/dag fytosterolen). Dit is ongeveer drie keer de hoeveelheid die van nature in onze dagelijkse voeding aanwezig is. Mensen die voornamelijk

plantaardige voeding eten (bijvoorbeeld vegetariërs) kunnen over het algemeen hogere fytosterolinnamen van 0.5 tot 1.0 g/dag bereiken. Gezondheidsautoriteiten adviseren over het algemeen diëten die rijk zijn aan plantaardig voedsel, waarin ook veel fytosterolen voorkomen, ten behoeve van HVZ preventie.

Samengevat kan gesteld worden dat een hoge inname van fytosterolen via verrijkte voedingsmiddelen het LDL-C-gehalte in het bloed verlaagt. Verder kan een hoge inname van plantensterolen het TG gehalte iets verlagen en het gehalte plantensterolen in het bloed verhogen. Een lage inname van fytosterolen uit natuurlijke bronnen blijkt vooralsnog niet geassocieerd te zijn met het risico op HVZ. Of fytosterolen daadwerkelijk een rol kunnen spelen in de preventie van HVZ in de algemene bevolking moet nog definitief worden vastgesteld.

Acknowledgements
(Dankwoord)



Het werk zit erop, mijn proefschrift is klaar. Het is nu de tijd om mijn dankwoord te schrijven, want zonder de steun van velen was me dit niet gelukt.

Allereerst, Frans Kok, Marianne Geleijnse en Peter Zock, heel erg bedankt dat jullie mij tijdens het gehele promotietraject zo goed hebben begeleid. Peter, vooral jou ben ik dankbaar dat je me hebt gestimuleerd om, al werkende bij Unilever, te gaan promoveren in Wageningen. Marianne en Frans, bedankt voor het mogelijk maken van deze promotieconstructie en voor de fijne en inzichtgevende gesprekken die we in Wageningen met elkaar hebben gevoerd. Jullie hebben me alledrie flink uitgedaagd (vooral bij die discussie!) om tot dit mooie resultaat te komen.

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Bedankt ook (oud)collega's Mario Vermeer, Yuguang Lin, Harry Hiemstra, Guus Duchateau, Henk van der Knaap, Frits Quadt en Yvonne Zebregs voor alle input die jullie mij hebben gegeven en voor de gezellige samenwerking. Statistici, zonder jullie had ik mijn meta-analytische vaardigheden, waarop dit proefschrift voornamelijk is gebaseerd, niet zo goed kunnen ontwikkelen. Ik moet eerlijk toegeven dat ik wel van getalletjes houd... Ook mijn externe coauteurs, bedankt! Joline Beulens, fijn dat jij het mogelijk maakte om samen te werken waardoor ik ook een epidemiologisch hoofdstuk aan dit proefschrift kon toevoegen.

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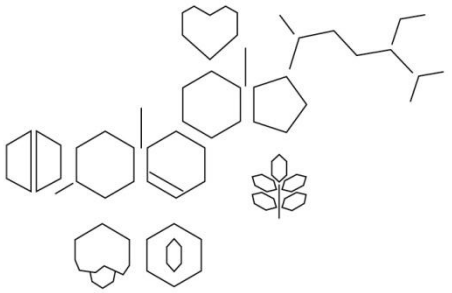

About the author

Curriculum vitae

Rouyanne Ras was born on the 11th of April 1982 in Alphen aan den Rijn, the Netherlands. After finishing high school in 2000, she studied Architecture for 1 ½ years at the Technical University in Delft. From 2002-2006, she studied Human Movement Sciences at the Vrije Universiteit in Amsterdam. As part of her training, she participated in the Women International Space Simulation for Exploration study in Toulouse, France, which was commissioned by the European Space Agency. After graduation in Amsterdam in 2006, Rouyanne continued education at the Wageningen University, to extent her knowledge in nutrition (MSc Nutrition and Health). For her Master's thesis, she was appointed by Unilever Research and Development Vlaardingen to perform a meta-analysis on the effect of phytosterols on blood cholesterol. For her internship, she worked for the University Medical Centre Groningen, within the Lifelines project.

After obtaining her Master of Science degree in 2008, Rouyanne was employed by Unilever Research and Development Vlaardingen as a nutrition scientist. She performed several meta-analyses and co-managed several clinical studies in the field of nutrition and cardiovascular health, some of which are described in this thesis. In 2012, she formally started her PhD research as an external fellow at Wageningen University. She participated and presented at several national and international conferences. After her graduation, she will continue her work on phytosterols and cardiovascular health for Unilever.

Publication list

Ras RT, Demonty I, Zebregs YEMP, Quadt JFA, Olsson J and Trautwein EA. Low doses of EPA and DHA from fish oil dose-dependently decrease serum triglyceride concentrations in the presence of plant sterols in hypercholesterolemic men and women. Journal of Nutrition 2014; doi: 10.3945/jn.114.192229.

Greyling A, **Ras RT**, Zock PL, Lorenz M, Hopman MT, Thijssen DH and Draijer R. The effect of black tea on blood pressure: a systematic review with meta-analysis of randomized controlled trials. PloS One 2014; 9(7): e103247.

Ras RT, Geleijnse JM and Trautwein EA. LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: a meta-analysis of randomised controlled studies. British Journal of Nutrition 2014; 112(2): 214-219.

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Ras RT, Zock PL, Zebregs YE, Johnston NR, Webb DJ and Draijer R. Effect of polyphenol-rich grape seed extract on ambulatory blood pressure in subjects with pre- and stage I hypertension. British Journal of Nutrition 2013; 110(12): 2234-2241.

Ras RT, Streppel MT, Draijer R and Zock PL. Flow-mediated dilation and cardiovascular risk prediction: a systematic review with meta-analysis. International Journal of Cardiology 2013; 168(1): 344-351.

Demonty I, **Ras RT**, Knaap HCM van der, Meijer L, Zock PL, Geleijnse JM and Trautwein EA. The effect of plant sterols on serum triglyceride concentrations is dependent on baseline concentrations: a pooled analysis of 12 randomised controlled trials. European Journal of Nutrition 2013; 52(1): 153-160.

Demonty I, **Ras RT**, Trautwein EA. Comment on: ‘‘A comparison of the LDL-cholesterol lowering efficacy of plant stanols and plant sterols over a continuous dose range: Results of a meta-analysis of randomized, placebo controlled trials’’ by Musa-Veloso K et al., Prostagl. Leucot. Essential Fatty Acids 2011, 85, 9–28. Prostaglandins Leukotrienes & Essential Fatty Acids 2011; 85(1): 7-8

Ras RT, Zock PL and Draijer R. Tea consumption enhances endothelial-dependent vasodilation; a meta-analysis. PLoS ONE 2011; 6(3): e16974

Trautwein EA, Demonty I and **Ras RT**. Plant sterols - A dietary approach for effective blood lipid lowering as part of a heart healthy diet. Current Topics in Neutraceutical Research 2010; 8(4): 137-148

Demonty I, **Ras RT**, Knaap HCM van der, Duchateau GSMJE, Meijer L, Zock PL, Geleijnse JM and Trautwein EA. Continuous dose-response relationship of the LDL-cholesterol-lowering effect of phytosterol intake. Journal of Nutrition 2009; 139(2): 271-284

Overview of completed training activities

Discipline specific activities (symposia, congresses)

- Nederlands Hypertensie Genootschap symposium, Zeist, the Netherlands (2010)
- Unilever symposium 'Dietary fats and health', Vlaardingen, the Netherlands (2010)
- 20th European Society of Hypertension congress, Oslo, Norway (2010)
- NWO Nutrition meeting, Deurne, the Netherlands (2010)
- American Heart Association Epidemiology and Prevention / Nutrition, Physical Activity and Metabolism scientific sessions, Atlanta, United States (2011)
- 79th European Atherosclerosis Society congress, Goteborg, Sweden (2011)
- Erasmus University master class 'Epidemiology', Rotterdam, the Netherlands (2011)
- NWO Nutrition meeting, Deurne, the Netherlands (2011)
- Stresa-2 meeting (phytosterol expert meeting), Maastricht, the Netherlands (2011)
- American Heart Association Epidemiology and Prevention / Nutrition, Physical Activity and Metabolism scientific sessions, San Diego, United States (2012)
- 80th European Atherosclerosis Society congress, Milan, Italy (2012)
- Unilever symposium 'Change behavior', Vlaardingen, the Netherlands (2012)
- NWO Nutrition meeting, Deurne, the Netherlands (2012)
- American Heart Association Epidemiology and Prevention / Nutrition, Physical Activity and Metabolism scientific sessions, New Orleans, United States (2013)
- 81st European Atherosclerosis Society congress, Lyon, France (2013)
- European Society of Cardiology congress, Amsterdam, the Netherlands (2013)
- NWO Nutrition meeting, Deurne, the Netherlands (2013)
- EuroPrevent congress, Amsterdam, the Netherlands (2014)
- 82nd European Atherosclerosis Society congress, Madrid, Spain (2014)

General courses and activities

- Patents course, Vlaardingen, the Netherlands (2008)
- Introduction to Project Management course, Rotterdam, the Netherlands (2009)
- Advances Statistics course, Rotterdam, the Netherlands (2009)
- Presentations Skills course, Rotterdam, the Netherlands (2011)
- Research & Development Foundation course, Chester, United Kingdom (2010-2011)
- Assertiveness & Self-confidence course, Vlaardingen, the Netherlands (2012)
- Central Committee on Research Involving Human Subjects workshop, Vlaardingen, the Netherlands (2012)
- Business Savviness course, Vlaardingen, the Netherlands (2013)

Optional courses and activities

- Preparing PhD research proposal (2011-2012)
- Expertise team meetings, project team meetings, science forums, etc (ongoing)

Colophon

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