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Effects of plant sterol- or stanol-enriched margarine on fasting plasma oxyphytosterol concentrations in healthy subjects

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A B S T R A C T

Background: Consumption of plant sterols and plant stanols reduces low-density lipoprotein cholesterol (LDL-C) concentrations. At the same time, plasma plant sterol concentrations will increase after plant sterol consumption, but decrease after plant stanol consumption. In contrast to plant stanols, plant sterols can undergo oxidation and form oxyphytosterols. Findings from *in vitro* and animal studies suggest that oxyphytosterols might be atherogenic.

Objective: The objective was to examine whether plant sterol and stanol consumption changes fasting plasma oxyphytosterol concentrations.

Design: A randomized, double blind, cross-over study was performed in which 43 healthy subjects (18–70 years) consumed for 4 weeks a plant sterol-enriched (3.0 g/d of plant sterols), a plant stanol-enriched (3.0 g/d of plant stanols), and a control margarine separated by wash-out periods of 4 weeks. Oxyphytosterol concentrations were determined in BHT-enriched plasma via GC–MS.

Results: Compared to control, serum LDL-C concentrations were reduced after plant sterol (−8.1%; $p < 0.001$) and plant stanol consumption (−7.8%; $p < 0.001$). Plant sterol consumption did not change plasma oxyphytosterol concentrations. On the other hand, intake of the plant stanol margarine reduced 7β-OH-campesterol by 0.07 ng/mL (~14%; $p < 0.01$) and by 0.07 ng/mL (~15%; $p < 0.01$) compared with the control and sterol margarines, respectively. When standardized for serum cholesterol, effects on these oxyphytosterols were comparable. In addition, plant stanol intake reduced cholesterol-standardized 7-keto-campesterol levels compared with plant sterol intake ($p < 0.05$).

Conclusions: Daily consumption of a plant sterol-enriched margarine does not increase oxyphytosterol concentrations, while plant stanol consumption may reduce the concentrations of the oxidative plant sterol metabolites 7β-OH-campesterol and 7-keto-campesterol. This trial is registered at clinicaltrials.gov as NCT01559428.

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1. Introduction

Plant sterols are structurally related to cholesterol and only the side-chain configuration at the C24 position differs. Thus, both components consist of a steroid nucleus with a double bond present at C5–C6. This double bond is missing in plant stanols, the hydrogenated form of plant sterols. Humans are unable to synthesize plant sterols and stanols, which means that serum plant sterols are by definition diet-derived. On average, serum sitosterol concentrations range from 0.12 to 0.62 mg/dL and campesterol concentrations from 0.27 to 1.08 mg/dL [1]. Serum plant stanol concentrations are approximately 100 times lower and vary between 2 and 10 μg/dL in the normal population [2].

Although plant sterol and plant stanol ester-enriched products lower serum low-density lipoprotein cholesterol (LDL-C) concentrations to the same extent at the recommended intake of approximately 2.5 g/day, they have a different effect on serum plant sterol concentrations; i.e. increased plant sterol consumption will elevate serum plant sterol concentrations, while an increased plant

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stanol consumption will lower serum plant sterol concentrations [3]. The relevance of increased serum plant sterol concentrations relates in great detail to the ongoing debate whether plant sterol concentrations are positively associated with cardiovascular disease (CVD) risk or not. The atherogenicity of increased plant sterol concentrations was first suggested after the identification of the rare autosomal-recessively inheritable sterol storage disease phytosterolemia. These patients are characterized by extremely high plant sterol concentrations ranging from 10 to 65 mg/dL and a strong predisposition for premature coronary atherosclerosis [4,5]. Moreover, some epidemiological studies have suggested that also in the general population plasma plant sterol concentrations are positively associated with an increased risk to develop CVD [6–8]. Results are however far from conclusive, since there are also numerous studies that did not show this association [9]. How these inconsistencies between study outcomes can be explained is not known. One possibility is that the potential association between circulating plant sterols and CVD risk may not relate to plant sterols per se, but to the fact that serum plant sterols are a marker for intestinal cholesterol absorption [10]. In other words, it is possible that an increased absorption of cholesterol associates with CVD risk [11]. Alternatively, it is possible that oxidation of plant sterols – i.e. the formation of oxyphytosterols – is important to explain the potential association between plant sterol concentrations and CVD risk. Like cholesterol, plant sterols can undergo oxidation, as they possess a double bond between C5–C6 in the steroid nucleus (Fig. 1). Ring-oxidation products of cholesterol may be atherogenic [12–14], while not much is known about the biological effects of ring-oxyphytosterols. Oxyphytosterols first received attention as potential mediators of CVD risk after it was shown that about 1.4% of circulating sitosterol in serum of phytosterolemic patients was present in its oxidized form [15]. Plant stanols do not have a double bond in the steroid nucleus and can therefore not oxidize [16]. In light of the widespread use of plant sterol-enriched products, it is important to identify whether an increased intake of plant sterols will result in increased plasma oxyphytosterol concentrations. Another interesting question is whether an increased plant stanol intake will not only decrease serum plant sterol concentrations, but will also change those of the oxyphytosterols. Therefore, the objective of this study was to examine the effects of consuming plant sterol or stanol ester-enriched margarines on fasting serum oxyphytosterol concentrations in healthy volunteers.

2. Subjects and methods

2.1. Subjects

Subjects were recruited through announcements in local newspapers and university buildings in Maastricht, the Netherlands, between February and April 2010. They were invited for two screening visits if they met the following criteria: aged between 18 and 70 years, BMI between 20 and 30 kg/m^2, no active cardiovascular disease or severe medical condition that might interfere with the study, no use of lipid-lowering medication or a medically prescribed diet, stable body weight during the last three months, and no consumption of plant sterol- or plant stanol-enriched products in the previous month. During the screening visits, weight and height were measured and two fasting blood samples were drawn to determine plasma glucose and serum lipid and lipoprotein concentrations. Forty-seven subjects (18 male and 29 female) were enrolled in the study. Two subjects did not complete the study due to time constraints and difficulties performing the venipuncture. In addition, two subjects were excluded before the analyses of the results: one male subject was not fasted during one blood sampling day and one female subject did not comply with margarine consumption. Baseline characteristics are shown in Table 1. All participants gave written informed consent before entering the study. The protocol was approved by the medical ethical committee of the Maastricht University Medical Centre+ (MUMC+). This trial is registered at clinicaltrials.gov as NCT01559428.

2.2. Diet and design

Subjects participated in a randomized placebo-controlled crossover trial, which consisted of three intervention periods of 4 weeks, separated by wash-out periods of 4 weeks. They were allocated to the intervention periods in a randomized order, based upon a computer-generated table with random numbers. During each period, subjects were asked to replace their own spread with the

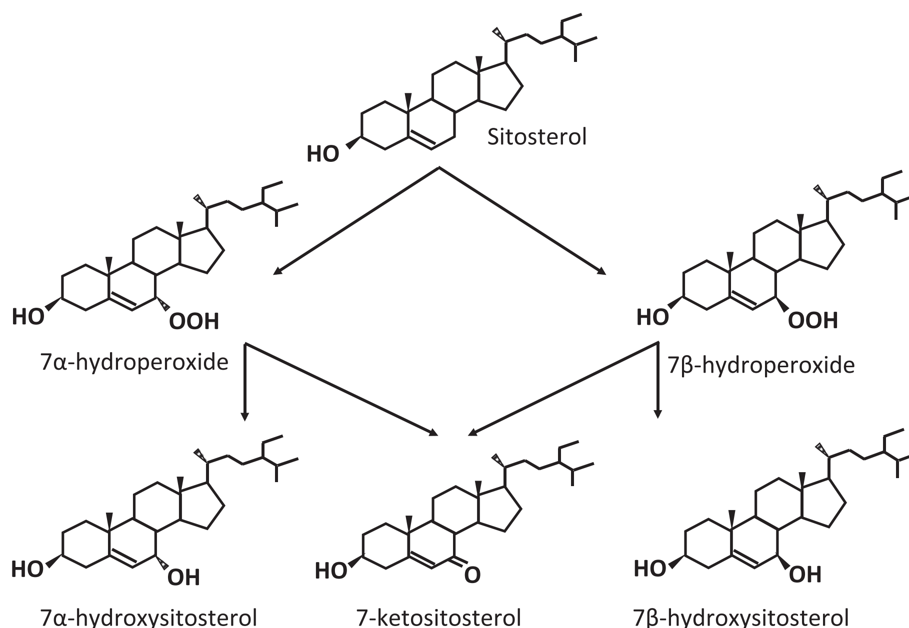

Fig. 1. Non-enzymatically formed oxysitosterol structures. Similar structures are also formed for campesterol and other plant sterols.

Table 1
Baseline characteristics of the subjects ($n = 43$).

Age (years)	41 ± 18
Male/Female (n)	17/26
BMI (kg/m^2)	24.8 ± 2.8
Weight (kg)	73.2 ± 11.0
Height (m)	1.72 ± 0.1
Glucose (mmol/L)	5.25 ± 0.54
Total cholesterol (mmol/L)	5.72 ± 1.12
LDL cholesterol (mmol/L)	3.53 ± 1.06
HDL cholesterol (mmol/L)	1.69 ± 0.37
Triacylglycerol (mmol/L)	1.14 ± 0.46

Values are means ± SD.

test margarine (70% fat) of which 20 g had to be consumed on a daily basis. The daily intake of 20 g of margarine provided no, or 3.0 g of plant sterols or stanols per day, referred to as the control, sterol or stanol condition. The margarines were packed in tubs of 140 g each, providing margarine for 7 days. The margarines were similar in color and taste and the tubs were color coded to blind both subjects and investigators. All volunteers received instructions to consume the margarines divided over two meals, i.e. at lunch and diner. The margarines were all rapeseed oil based with (saturated) soysterols as the source of the plant sterols/stanols. Plant sterols and stanols were provided as fatty acid esters by trans-esterification of free plant sterols and stanols with rapeseed oil fatty acids. The plant sterol ester mixtures contained mainly sitosterol ester (43%), campesterol ester (25%) and stigmasterol ester (20%). Plant stanol mixtures were obtained by saturation of these sterols, resulting in sitostanol ester (76%) and campestanol ester (22%) (Raisio group, Raisio, Finland). Plant sterol, plant stanol and oxyphytosterol content of the margarines are shown in Table 2.

Subjects visited the university at the start of each period, and after 3 and 4 weeks of intervention. They received 4 tubs of margarine at the start of each period, the amount needed for 4 weeks. At the end of each week, the used margarine tubs had to be set aside and returned to the department to be weighed to determine compliance. Subjects completed a validated food frequency questionnaire (FFQ) at the end of all three intervention periods [17], and a dietician checked these questionnaires to calculate energy and nutrient intakes using the Dutch food composition table. Subjects were asked not to change their habitual diet, level of physical exercise or use of alcohol throughout the three intervention periods in the study.

2.3. Blood sampling

Blood was sampled after an overnight fast. The same person performed all venipuncture at approximately the same time of the day. Blood was sampled at the start of each period and after 3 and 4 weeks of intervention. A clotting tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) was sampled at each occasion and serum was obtained by low-speed centrifugation at 1300 × g for 15 min at room temperature, at least half an hour after venipuncture. Serum was stored at −80 °C and used for analysis of lipid and (apo)lipoprotein concentrations, and for plant sterol and plant stanol concentrations. An EDTA tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) was sampled at the start of each period and after 4 weeks and plasma was obtained by low-speed centrifugation at 1300 × g for 15 min at 4 °C, and then stored at −80 °C. To avoid auto-oxidation, oxyphytosterol concentrations were determined in butylated hydroxytoluene (BHT)-enriched plasma; for this 10 μl BHT (25 mg/mL ethanol) was added per 1 mL of EDTA plasma, immediately after centrifugation.

Table 2
Plant sterol, plant stanol and oxyphytosterol content of the margarines[a].

	Control margarine	Plant sterol margarine	Plant stanol margarine
Sitosterol	0	64.8	1.4
Campesterol	0	38.1	2.4
Stigmasterol	0	29.6	0
Sitostanol	0	3.8	114.3
Campestanol	0	1.7	33.0
7α-OH-sitosterol	0.35	6.15	0.19
7α-OH-campesterol	0.09	2.48	0.08
7β-OH-sitosterol	0.80	11.39	0.51
7β-OH-campesterol	0.30	2.77	0.09
7-keto-sitosterol	1.67	5.41	2.31
7-keto-campesterol	1.67	5.54	2.23

[a] Plant sterol and stanol content in μg/mg and oxyphytosterol content in ng/mg.

2.4. Analyses

Total cholesterol (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany), high-density lipoprotein cholesterol (HDL-C) (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany) after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium ions, and triacylglycerol (TAG) concentrations, with correction for free glycerol, were analyzed in serum enzymatically (GPO-Trinder; Sigma–Aldrich Corp., St. Louis, MO, USA). All samples from one subject were analyzed within one run at the end of the study. LDL-C concentrations were calculated according to the Friedewald equation [18]. Apolipoprotein concentrations (apoA-1 and apoB100) were analyzed using highly sensitive immuno-turbidimetric assays (Horiba ABX, Montpellier, France). Lipid and (apo)lipoprotein concentrations from weeks 3 and 4 were averaged for data analysis. Plant sterol (sitosterol, campesterol), plant stanol (sitostanol, campestanol), cholesterol precursor (lathosterol), cholestanol and oxyphytosterol concentrations from week 4 were analyzed by gas–liquid chromatography–mass spectroscopy (GC–MS) as described previously [19,20]. Plant sterol, plant stanol and lathosterol concentrations were expressed as $10^2 \times$ μmol/mmol total cholesterol. The measured oxyphytosterols were 7α-hydroxy(OH)-sitosterol, 7α-OH-campesterol, 7β-OH-sitosterol, 7β-OH-campesterol, 7-keto-sitosterol, and 7-keto-campesterol according the procedure as described by Husche et al. (20) and expressed as ng/mL. Oxyphytosterol concentrations were standardized for cholesterol concentrations and expressed as nmol/mmol cholesterol.

2.5. Statistics

To detect a change of 17 ng/mL in plasma oxyphytosterol concentrations with a power of 80% and a known within-subject variation on the response of 34 ng/mL (20), a sample size of 47 subjects was necessary, given an anticipated dropout rate of 10%. All data are presented as means ± standard deviations (SD). Effects of the experimental products were evaluated by a univariate analysis of variance (ANOVA) followed by Bonferroni's correction for multiple comparisons. Results were considered to be statistically significant if $p < 0.05$. Results were analyzed to assess whether gender effects or carryover effects existed, and both effects were not present in the data. All statistical analyses were performed using SPSS 18.0 for Mac Os X (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Dietary intakes

As shown in Table 3, the average daily intakes of total energy (MJ/day), protein (energy%), carbohydrates (CHO, energy%), total fat

Table 3
Daily dietary intake during the study.

	Control condition	Plant sterol condition	Plant stanol condition
Energy (MJ)	10.6 ± 3.4	10.6 ± 3.2	10.5 ± 3.2
Fat (energy%)	37.5 ± 7.2	37.4 ± 6.5	37.4 ± 6.6
SAFA[a]	12.1 ± 3.1	12.2 ± 3.0	11.9 ± 3.0
MUFA	14.4 ± 3.2	14.6 ± 2.7	14.6 ± 3.0
PUFA	7.9 ± 2.4	8.2 ± 2.0	8.0 ± 1.8
Protein (energy%)	15.5 ± 3.6	15.4 ± 3.4	15.4 ± 3.2
CHO (energy%)	45.3 ± 9.5	44.4 ± 7.5	44.6 ± 8.1
Alcohol (energy%)	2.9 ± 2.9	2.6 ± 2.6	2.8 ± 3.2
Fiber (g/day)	23.5 ± 7.8	24.0 ± 7.5	23.3 ± 7.6
Cholesterol (mg/day)	221 ± 111	209 ± 92	205 ± 77
Vitamin E (mg/day)	13.1 ± 5.3	13.6 ± 5.8	13.3 ± 5.7

Values are means ± SD. All subjects ($n = 43$) received the three dietary conditions in random order.
[a] SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, CHO: carbohydrates.

(energy%), fatty acids (saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), energy%), fiber (g/day), cholesterol (mg/day) and vitamin E (mg/day) did not differ between the three intervention periods.

3.2. Serum lipids and lipoproteins

Serum lipid and lipoprotein concentrations are shown in Table 4. Compared with the control condition, serum total cholesterol concentrations decreased by 0.30 ± 0.51 mmol/L (−5.3%; $p < 0.001$) in the sterol condition and by 0.29 ± 0.42 mmol/L (−5.3%; $p < 0.001$) in the stanol condition. These reductions could almost completely be ascribed to decreases in serum LDL-C concentrations. In the sterol condition, serum LDL-C concentrations decreased by 0.29 ± 0.43 mmol/L (−8.1%) and in the stanol condition by 0.26 ± 0.41 mmol/L (−7.8%; $p < 0.001$ for both conditions). Serum HDL-C concentrations were not different between the three conditions. This shift towards a more beneficial partitioning of cholesterol was also reflected in the total cholesterol/HDL cholesterol ratio, which decreased in the sterol condition by 0.14 ± 0.36 (−3.1%; $p < 0.05$) and in the stanol condition by 0.18 ± 0.35 (−4.9%; $p < 0.01$). Serum apolipoprotein B100 concentrations were reduced in the sterol condition by 0.05 ± 0.09 g/L (−4.4%) and in the stanol condition by 0.05 ± 0.08 g/L (−5.1%; $p < 0.01$ for both conditions).

Table 4
Effect of consumption of plant sterol and plant stanol-enriched margarines on serum lipid and lipoprotein concentrations, and on serum plant sterol, plant stanol and lathosterol levels.

	Control condition	Plant sterol condition	Plant stanol condition
Total cholesterol (mmol/L)	5.56 ± 1.07	5.26 ± 1.08[1]	5.27 ± 1.11[1]
LDL cholesterol (mmol/L)	3.36 ± 1.06	3.08 ± 1.00[1]	3.10 ± 1.05[1]
HDL cholesterol (mmol/L)	1.69 ± 0.39	1.65 ± 0.39	1.68 ± 0.39
Total cholesterol/HDL	3.46 ± 1.00	3.32 ± 0.93[2]	3.28 ± 0.96[3]
Triacylglycerol (mmol/L)	1.14 ± 0.40	1.18 ± 0.44	1.08 ± 0.41
ApoB100 (g/L)	0.97 ± 0.25	0.93 ± 0.24[3]	0.92 ± 0.26[3]
ApoA1 (g/L)	1.57 ± 0.31	1.55 ± 0.31	1.55 ± 0.27
Sitosterol*	140 ± 69	226 ± 255[3]	88 ± 35[4]
Campesterol	214 ± 83	346 ± 172[1]	131 ± 59[1,4]
Sitostanol	4.3 ± 3.3	5.5 ± 5.7	22.4 ± 11.7[1,4]
Campestanol	2.8 ± 1.6	3.5 ± 2.0	13.1 ± 7.7[1,4]
Lathosterol	115 ± 57	134 ± 56[3]	130 ± 55[2]
Cholestanol	165 ± 0.33	153 ± 32[2]	155 ± 32[2]

Values are means ± SD and *sterols are expressed as $10^2\times$ μmol/mmol cholesterol. All subjects ($n = 43$) received the three dietary conditions in random order.
Significantly different compared with control condition: [1]($p < 0.001$), [2]($p < 0.05$), [3]($p < 0.01$).
Significantly different compared with sterol condition [4]($p < 0.001$).

No differences were found in serum TAG and apolipoprotein A1 concentrations between the three conditions.

3.3. Serum plant sterols, plant stanols, lathosterol and cholestanol

Cholesterol-standardized serum plant sterol, plant stanol, lathosterol and cholestanol levels are shown in Table 4. Sitosterol levels increased in the sterol condition by 86.6 ± 199 $10^2\times$ μmol/mmol cholesterol (49%; $p < 0.01$) compared with the control condition and by 138 ± 248 $10^2\times$ μmol/mmol cholesterol (167%; $p < 0.001$) compared with the stanol condition. For campesterol, these values were 132 ± 137 $10^2\times$ μmol/mmol cholesterol (66%; $p < 0.001$) and 214 ± 138 $10^2\times$ μmol/mmol cholesterol (179%; $p < 0.001$). In addition, campesterol levels decreased in the stanol condition by 82.3 ± 53.3 $10^2\times$ μmol/mmol cholesterol (37.3%) compared with the control condition ($p < 0.001$).

Sitostanol levels increased in the stanol condition by 182 ± 114 $10^2\times$ μmol/mmol cholesterol (511%; $p < 0.001$) compared with the control condition and by 170 ± 117 $10^2\times$ μmol/mmol cholesterol (404%; $p < 0.001$) compared with the sterol condition. For campestanol, these values were 103 ± 70 $10^2\times$ μmol/mmol cholesterol (398%; $p < 0.001$) and 96 ± 66 $10^2\times$ μmol/mmol cholesterol (295%; $p < 0.001$). Lathosterol levels, a marker for cholesterol synthesis, increased in the sterol condition by 189 ± 454 $10^2\times$ μmol/mmol cholesterol (24.1%; $p < 0.01$) and in the stanol condition by 147 ± 322 10^2x μmol/mmol cholesterol (17.5%; $p < 0.05$) compared with the control condition. Cholestanol levels, a marker for cholesterol absorption, decreased in the sterol condition by 112 ± 292 $10^2\times$ μmol/mmol cholesterol (5.2%; $p < 0.05$) and in the stanol condition by 96 ± 205 $10^2\times$ μmol/mmol cholesterol (4.9%; $p < 0.05$) compared with the control condition.

3.4. Plasma oxyphytosterols

Absolute oxyphytosterol concentrations (ng/mL) in BHT-enriched EDTA plasma are shown in Table 5. 7β-OH-campesterol concentrations were decreased in the stanol condition by 0.07 ± 0.17 ng/mL (13.6%) compared with the control condition ($p < 0.01$) and by 0.07 ± 0.14 ng/mL (15.0%) compared with the sterol condition ($p < 0.01$). Concentrations of the other oxyphytosterol were comparable between the three conditions.

Cholesterol-standardized oxyphytosterol levels (nmol/mmol cholesterol) showed comparable results, i.e. a decreased 7β-OH-campesterol level in the stanol condition compared with the

Table 5
Effect of consumption of plant sterol and plant stanol-enriched margarines on plasma oxyphytosterol concentrations.

		Control condition	Plant sterol condition	Plant stanol condition
A	7α-OH-sitosterol	0.21 ± 0.09	0.22 ± 0.11	0.26 ± 0.35
	7α-OH-campesterol	0.09 ± 0.05	0.09 ± 0.06	0.08 ± 0.05
	7β-OH-sitosterol	1.17 ± 0.35	1.09 ± 0.52	1.09 ± 0.58
	7β-OH-campesterol	0.32 ± 0.16	0.32 ± 0.15	0.25 ± 0.11[1,2]
	7-keto-sitosterol	2.49 ± 0.58	2.35 ± 0.78	2.35 ± 0.60
	7-keto-campesterol	0.48 ± 0.19	0.49 ± 0.28	0.44 ± 0.22
B	7α-OH-sitosterol	0.10 ± 0.05	0.11 ± 0.06	0.12 ± 0.16
	7α-OH-campesterol	0.04 ± 0.03	0.05 ± 0.03	0.04 ± 0.02
	7β-OH-sitosterol	0.54 ± 0.17	0.56 ± 0.26	0.50 ± 0.26
	7β-OH-campesterol	0.15 ± 0.08	0.17 ± 0.08	0.12 ± 0.05[1,3]
	7-keto-sitosterol	1.18 ± 0.34	1.22 ± 0.45	1.10 ± 0.30
	7-keto-campesterol	0.23 ± 0.10	0.26 ± 0.15	0.21 ± 0.10[4]

Panel A: Values are means ± SD and are expressed as ng/mL. Panel B: Values are means ± SD and are expressed as nmol/mmol cholesterol. All subjects ($n = 43$) received the three dietary conditions in random order.
Significantly different compared with sterol condition [1]($p < 0.01$), [4]($p < 0.05$).
Significantly different compared with control condition [2]($p < 0.01$), [3]($p < 0.05$).

control and sterol condition ($p < 0.01$ and $p < 0.05$, respectively). In addition, the 7-keto-campesterol level was reduced in the stanol condition compared with the sterol condition ($p < 0.05$).

4. Discussion

There is an ongoing debate whether there is a relation between circulating serum plant sterol concentrations and cardiovascular risk. Based on findings from animal studies [21–23], we hypothesized that plant sterols themselves are not, but become atherogenic, when oxidized. We here show that in healthy volunteers, despite the increase in non-oxidized plant sterols concentrations, absolute concentrations of oxyphytosterols do not increase after consumption of plant sterol-enriched margarines. However, plant stanol consumption reduced 7β-OH-campesterol concentrations and – when standardized for cholesterol also 7-keto-campesterol levels – compared with the control as well as with the plant sterol-enriched margarine.

Oxyphytosterol concentrations in humans have only been determined in a few – merely cross-sectional – studies. In 2001, Plat et al. identified oxyphytosterols in serum of phytosterolemic patients, but not in serum of healthy subjects [15]. A few years later, Grandgirard et al. used a method with a lower detection limit and detected α- and β-epoxysitosterol, 7-keto-sitosterol and sitostanetriol in plasma of healthy subjects in a range of 5–57 ng/mL [24]. We found oxyphytosterol concentrations in a range of 0.08–2.49 ng/mL, but measured different oxyphytosterols. 7-keto-sitosterol, however, was measured in both studies and while we measured a mean concentration of 2.5 ng/mL, Grandgirard et al. found a mean concentration of 6.1 ng/mL. The amount BHT added to the samples differed between our and Grandgirard's study (1% vs. 0.05%), which might explain the discrepancies in oxyphytosterol concentrations. Menéndez-Carreño measured the same oxyphytosterols as we did, but found somewhat higher oxyphytosterol concentrations in serum of healthy volunteers (0.25–4.48 ng/mL) [25]. This difference between our results with Menéndez-Carreño's data is probably due to the use of different methods, emphasizing the need for standardization of oxyphytosterol analyses. Recently, Husche et al. determined oxyphytosterol concentrations in plasma from 16 volunteers, who were given a plant sterol-enriched margarine for 4 weeks, providing 3.0 g of plant sterols per day. Oxyphytosterol concentrations did not change after plant sterol consumption, except for an 85% increase in 7β-OH-sitosterol concentrations [20]. In our study 7β-OH-sitosterol concentrations did not change after consumption of a plant sterol-enriched margarine, despite the fact that baseline 7β-OH-sitosterol concentrations were comparable between the two studies (1.20 ± 0.54 ng/mL vs. 1.17 ± 0.35 ng/mL). The study of Husche et al., however, was not placebo-controlled, which might have influenced the results. The range of the different oxyphytosterol concentrations in Husche's study and our study was comparable (0.07–3.01 ng/mL vs. 0.08–2.49 ng/mL) and this consistency in baseline oxyphytosterol concentrations may help to define normal serum values.

So far oxyphytosterol concentrations have been reported as absolute values in literature. The question arises whether concentrations, as for non-oxidized plant sterols, should be standardized for cholesterol concentrations or not. Plant sterols concentrations are standardized for cholesterol, as plant sterols are transported by lipoproteins. This may be true for oxyphytosterols as well. When oxyphytosterols are standardized for cholesterol concentrations, conclusions were very comparable to those of the absolute changes, i.e. there was a significant decrease in 7β-OH-campesterol concentration in the stanol condition compared to the sterol and control condition. In addition, 7-keto-campesterol was significantly decreased in the stanol compared with the sterol condition. Alternatively, oxyphytosterols levels can also be expressed as the proportion of circulating plant sterol concentrations, which more or less indicates the percentage of substrate that is oxidized. The percentage oxidized plant sterols of total plant sterols is the highest in the stanol condition and the lowest in the sterol condition, but this is mostly due to a change in plant sterol concentrations and not in oxyphytosterol concentrations. For this reason, it was chosen to only express oxyphytosterols in absolute and cholesterol-standardized levels (Table 5).

Theoretically, oxyphytosterols can be derived from absorption of oxidized plant sterols present in food, as well as from endogenous synthesis (either from enzymatic or non-enzymatic ring-oxidation processes). Since this study cannot distinguish between endogenously formed and diet-derived oxyphytosterols, we can only speculate about the origin of the measured circulating oxyphytosterols. Although phytosterolemic patients follow a strict plant sterol-poor (and consequently also oxyphytosterol poor) diet, they still have elevated plasma (oxy)phytosterol concentrations [4]. This could indicate that plasma oxyphytosterols originate from *in vivo* oxidation of the high circulating non-oxidized serum plant sterol concentrations. A second potential indication for the *in vivo* formation of oxyphytosterols might be found in the oxyphytosterol content of the margarines. Oxyphytosterols in the diet are mainly derived from plant sterol-enriched products [16]. Our plant sterol-enriched margarine contained ~34 ng oxyphytosterols per milligram margarine, while the oxyphytosterol content of the other two margarines was ~5 ng/mg margarine (Table 2). However, this higher oxyphytosterol content did not translate into increased serum concentrations, which might suggest that serum concentrations are not related to absorption of oxyphytosterols from the diet. On the other hand, while non-oxidized plant sterol concentrations increased, oxyphytosterol concentrations did not increase, which could imply that oxyphytosterols are not formed *in vivo* but origin from intestinal absorption. In this respect, the relative absorption of different oxyphytosterols might give more insight into the origin of circulating oxyphytosterols. The sterol-enriched margarines contained twice as much sitosterol oxidation products as campesterol oxidation products, while concentrations of sitosterol oxidation products in plasma were four to five times as high as campesterol concentration products. This may suggest that sitosterol oxidation products are preferentially absorbed or formed *in vivo*. However, in humans, non-oxidized campesterol is better absorbed than non-oxidized sitosterol and serum concentrations are also higher [26]. Human data is lacking, but animal studies have consistently shown that – analog to the non-oxidized sterols – campesterol oxidation products are better absorbed than sitosterol oxidation products [27,28]. This may suggest that *in vivo* formation of plant sterol oxidation products is more likely than intestinal absorption, or perhaps that excretion of sitosterol oxidation products is low. To definitively answer the question whether plasma oxyphytosterols result from absorption or from *in vivo* formation, future studies should be performed in which for example labeled non-oxidized plant sterols are consumed and subsequent enrichment in labeled plasma oxyphytosterols is measured. In addition, little is known about tissue distribution of oxyphytosterols and whether serum oxyphytosterol concentrations relate to tissue concentrations. These questions should be addressed in future studies to elucidate the physiological role of circulating oxyphytosterols in humans.

We observed a decrease in absolute 7β-OH-campesterol concentrations or cholesterol-standardized 7β-OH-campesterol and 7-keto-campesterol after plant stanol-enriched margarine consumption. Again, it is not known whether this is due to effects on *in vivo* formation or on absorption. Also, it may be a chance finding. However, if true, it is interesting that 7β-OH-cholesterol (cholesterol oxidation product) is regarded as the most atherogenic

oxysterol and has been identified as the strongest predictor of a rapid progression of carotid atherosclerosis in humans [29]. Whether 7β-OH-campesterol might also be regarded as an atherogenic compound remains to be determined.

Potential differences in dietary oxyphytosterol intake between treatments cannot be eliminated as the study is performed in a free-living setting, but due to the cross-over design and low oxyphytosterol content in daily diet this is not very likely.

In conclusion, we have shown that a daily consumption of a plant sterol-enriched margarine for a period of 4 weeks does not increase plasma oxyphytosterol levels. We further found that plant stanol consumption reduced the absolute and cholesterol-standardized 7β-OH-campesterol levels and the cholesterol-standardized 7-keto-campesterol level. The reproducibility and possible clinical implication of these reductions remain to be determined and deserve further investigation.

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