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Vascular Effects of Diet Supplementation With Plant Sterols

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Objectives The purpose of this study was to evaluate vascular effects of diet supplementation with plant sterol esters (PSE).

Background Plant sterol esters are used as food supplements to reduce cholesterol levels. Their effects on endothelial function, stroke, or atherogenesis are not known.

Methods In mice, plasma sterol concentrations were correlated with endothelial function, cerebral lesion size, and atherosclerosis. Plasma and tissue sterol concentrations were measured by gas-liquid chromatography-mass spectrometry in 82 consecutive patients with aortic stenosis.

Results Compared with those fed with normal chow (NC), wild-type mice fed with NC supplemented with 2% PSE showed increased plant sterol but equal cholesterol plasma concentrations. The PSE supplementation impaired endothelium-dependent vasorelaxation and increased cerebral lesion size after middle cerebral artery occlusion. To test the effects of cholesterol-lowering by PSE, apolipoprotein E (ApoE)−/− mice were randomized to Western-type diet (WTD) with the addition of PSE or ezetimibe (EZE). Compared with WTD, both interventions reduced plaque sizes; however, WTD + PSE showed larger plaques compared with WTD + EZE (20.4 ± 2.1% vs. 10.0 ± 1.5%). Plant sterol plasma concentration strongly correlated with increased atherosclerotic lesion formation ($r = 0.50$). Furthermore, we examined plasma and aortic valve concentrations of plant sterol in 82 consecutive patients with aortic stenosis. Patients eating PSE-supplemented margarine ($n = 10$) showed increased plasma concentrations and 5-fold higher sterol concentrations in aortic valve tissue.

Conclusions Food supplementation with PSE impairs endothelial function, aggravates ischemic brain injury, effects atherogenesis in mice, and leads to increased tissue sterol concentrations in humans. Therefore, prospective studies are warranted that evaluate not only effects on cholesterol reduction, but also on clinical endpoints. (Concentration of Plant Sterols in Serum and Aortic Valve Cusps; NCT00222950) (J Am Coll Cardiol 2008;51:1553–61)

"Functional foods" supplemented with plant sterol esters (PSE) (e.g., margarines) are advertised for the prevention of cardiovascular diseases and have become a widely used nonprescription approach to lower plasma cholesterol levels. Phytosterols are naturally occurring non-nutritive plant derivatives whose chemical structure differs from that of cholesterol by the presence of modified side chains at carbon C-24 (1). Mammals do not synthesize phytosterols. Sitosterol and campesterol are the most abundant plant sterols. Their primary dietary sources are fat-rich vegetables, including vegetable oils, fruits, and nuts. Their primary mechanism of lowering blood cholesterol levels is the competitive replacement of cholesterol in bile salt micelles, resulting in reduced absorption of unesterified cholesterol from the small intestine (1,2). In humans, doses of PSE ranging from 0.8 to 4.0 g daily have been shown to reduce low-density lipoprotein cholesterol concentrations by 10% to 15% (2). The precise molecular mechanisms for sterol absorption are not well defined, but cholesterol and plant sterol absorption both require the NPC1L1 protein (3). The majority of absorbed plant sterols are excreted by the liver, but small amounts are retained.

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Abbreviations and Acronyms

ApoE = apolipoprotein E
EZE = ezetimibe
LDLR = low-density lipoprotein receptor
NC = normal chow
PSE = plant sterol esters
WTD = Western-type diet

In animal models of atherosclerosis induced by hypercholesterolemia, lowering of cholesterol concentrations with plant sterol–enriched chow reduces plaque volume (4,5). However, 3 lines of evidence raise the question of the net effect of plant sterols on vascular disease. First, individuals with the rare autosomal-recessive disease phytosterolemia have increased plasma concentrations of plant sterols as a result of hyperabsorption and diminished biliary elimination, which is caused by a defect in either the ABCG5 or ABCG8 transporter genes. These patients suffer from premature atherosclerosis and increased risk of coronary heart and aortic valve disease (6,7). Second, plant sterols have been detected in atherosclerotic lesions from individuals with apparently normal cholesterol absorption (8). And third, epidemiological studies have suggested that elevated plasma concentrations of plant sterols are associated with increased risk of vascular disease (9–11). It is not known whether the consumption of plant sterol–enriched food leads to increased concentrations of sterols in vascular tissue, and despite the widespread use of sterol-enriched margarine, the effects of plant sterols on vascular disease remain largely unknown. Therefore we studied the effects of PSE on endothelial function and ischemic brain injury in wild-type mice and characterized the effect of PSE on lipid-driven atherogenesis of apolipoprotein E (ApoE)−/− mice compared with equal lowering of serum cholesterol with ezetimibe (EZE), an inhibitor of cholesterol absorption (3). Finally, to evaluate the effects of the consumption of sterol-enriched margarine in humans, sterol concentrations in plasma and aortic valve tissue were investigated.

Methods

Animals, diets, and sterol analysis. Experiments using male C57/Bl6 (wild-type), 129/SV, and ApoE−/− (C57/Bl6 genetic background) mice, 8 to 12 weeks of age, 20 to 25 g (Charles River, Sulzfeld, Germany) were performed in accordance with the German animal protection law. Sterol supplementation was designed to reflect the composition of commercially available spreads as available at the beginning of the study in 2004 (Table 1). The C57/BL6J mice were fed normal chow (NC) (14 kcal% vegetable oil) with and without 2% PSE supplementation for 4 weeks (n = 10/group). In the second series of experiments, 80 ApoE−/− mice were randomized to 8 treatment groups (n = 10/group) treated for 6 months. Four groups were fed a "Western-type" diet (WTD) (40 kcal% butterfat, 0.15% [w/w] cholesterol). The ApoE−/− on WTD were compared with ApoE−/− on WTD supplemented with 2% PSE (w/w), 0.005% EZE (w/w), and the combination of 2% PSE and 0.005% EZE. Four additional groups were fed an NC cholesterol-free diet (14 kcal% vegetable oil). Normal chow was compared with groups supplemented with 2% PSE (w/w), 0.005% EZE (w/w), and the combination of 2% PSE and 0.005% EZE. To maintain comparable fatty acid profiles and energy contents, 1.4% rapeseed oil was added to all nonsterol ester-supplemented diets. Diets were prepared by ssniff (Soest, Germany) with PSE from RAISIO (Turku, Finland) and EZE from Schering-Plough Research Institute (Kenilworth, New Jersey). All sterol analyses were performed by gas liquid chromatography (12).

Table 1 Sterol Composition of Plant Sterol Ester Supplements Compared With Sterol Composition of Commercially Available Spread

Plant Sterol	Experimental Diet	Becel Pro-Activ
Sitosterol	46.2	46.7
Sitostanol*	2.3	2.2
Campesterol	25.3	26.2
Campestanol	0.6	0.6
Stigmasterol	19.1	19.4
Brassicasterol	1.2	2.4
Other plant sterols	4.9	2.5

Percent of total sterols. *Sitostanol + Delta-5-avanasterol.

Endothelial function. Two-millimeter rings of the descending aorta were mounted in organ baths to record isometric tension (13). Drugs were added in increasing concentrations to obtain cumulative concentration-response curves: potassium chloride 20 and 40 mmol/l, phenylephrine 1 nmol to 10 μmol/l, carbachol 10 nmol to 100 μmol/l, and nitroglycerin 1 nmol/l to 10 μmol/l. Endothelial function was expressed as vasorelaxation with increasing concentrations of carbachol in relation to maximal vasoconstriction induced by phenylephrine.

Cerebral ischemia. The 129/SV male wild-type mice on NC and on NC with a 2% plant sterol supplementation (age 6 to 8 weeks, 20 to 24 g) after a feeding period of 4 weeks were subjected to 30 min of left filamentous middle cerebral artery (MCA) occlusion and 72 h of reperfusion (14,15). Regional cerebral blood flow measured with laser-Doppler flowmetry decreased to <20% during ischemia and returned to approximately 100% within 5 min after reperfusion in all experimental groups ($p > 0.05$). Mean arterial blood pressure, pH, Pa_{O_2}, Pa_{CO_2} were measured both before and during cerebral ischemia. Core temperature was controlled and kept constant. Cerebral lesions volumes were determined on 20-μm cryostat sections by computed assisted volumetry (14,15). Indirect lesion areas were calculated by the following formula: contralateral hemisphere (mm^3) − undamaged ipsilateral hemisphere (mm^3).

Analysis of atherosclerotic lesions. At least 25 consecutive cryostat sections (10 μm)/animal were analyzed by oil red O, hematoxylin, and picrosirius red staining (13). Macrophages were detected by immunostaining with MOMA-2, 1:50 (Serotec MCA519G, Oxford, United Kingdom), followed by Alexa Fluor, 1:200 (546 Invitrogen, Carlsbad, California). For smooth muscle cells (SMC)

alpha-actin staining, monoclonal anti–alpha-smooth muscle actin, 1:500 (Sigma, St. Louis, Missouri) was applied. A Nikon E600 microscope and Lucia Measurement Version 4.6 software (Nikon, Tokyo, Japan) was used; 2 observers blindly performed all quantifications (13,16).

Human subjects. The protocol was registered at ClinicalTrials.gov (Identifier NCT00222950) and approved by the ethics committee of Saarland, Germany (number 159/04). We included 82 consecutive patients between 18 and 90 years of age who were admitted to our hospital for elective aortic valve replacement owing to severe aortic stenosis. During a structured interview, study participants were assessed for established cardiovascular risk factors, concomitant statin treatment, family history for cardiovascular diseases, and dietary habits with special attention to the consumption of PSE-supplemented margarine. Venous blood samples were drawn on the day before the scheduled valve replacement. Aortic cusps were removed from aortic rings in the operation room. For sterol quantifications, valve cusps were frozen to dryness in vacuum and extracted with chloroform/methanol for 2 days, followed by alkaline hydrolysis, extraction of the free sterols by cyclohexane, derivatization to the corresponding trimethylsilyl ethers in decane, and analysis by gas chromatography-flame ionization (cholesterol) or mass spectrometry (lathosterol and plant sterols) (12).

Statistics. Data are reported as mean ± SEM. Differences between experimental groups were tested by 2-tailed Student *t* tests or by analysis of variance (ANOVA) followed by the application of Bonferroni test. Statistical calculations of the clinical data were performed with 1-way ANOVA and 2-sided post hoc tests according to the Fisher protected least significant difference method, applied to log-transformed values. Analysis was performed on the log scale to stabilize variances and to satisfy the assumption of normality. Adjusted analyses were performed similarly by use of analysis of covariance. Resulting effects are presented as geometric means and 95% confidence intervals. The p values <0.05 were considered statistically significant. For quantification of correlations, Spearman's correlation coefficient was used. All statistical tests were performed with SPSS (Chicago, Illinois) software.

Results

Effects of plant sterols on endothelial function in wild-type mice. The sterol composition of the plant sterol–supplemented chow and commercially available spreads as quantitated by gas liquid chromatography are depicted in Table 1. Four-week diet supplementation of NC with 2% PSE did not alter cholesterol levels in wild-type mice but increased plasma sitosterol concentrations (1.89 ± 0.12 mg/dl vs. 0.88 ± 0.14 mg/dl) and their ratios to cholesterol (17.77 ± 0.72 μg/mg vs. 8.63 ± 1.01 μg/mg) as well as campesterol concentrations (6.84 ± 0.47 mg/dl vs. 2.65 ± 0.42 mg/dl) and their ratios to cholesterol (64.58 ± 2.49 μg/mg vs. 24.12 ± 3.20 μg/mg) (n = 10/group, $p < 0.05$) (Figs. 1A to 1C). Analysis of aortic rings showed that mice fed NC + PSE developed impaired endothelial-dependent vasorelaxation compared with mice on NC ($p < 0.05$) (Figs. 1D and 1E). Endothelium-independent vasodilation to nitroglycerin was equal between groups.

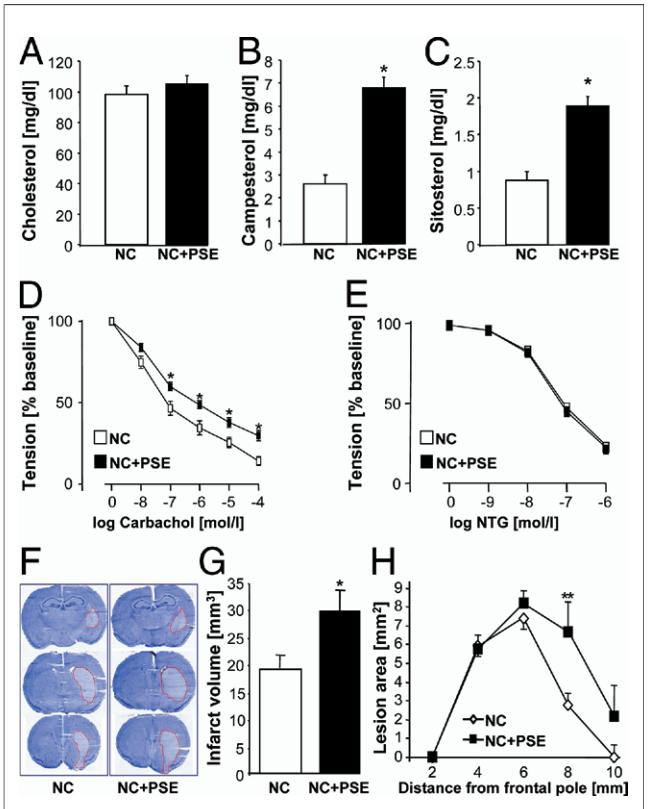

Figure 1 **Effects of Plant Sterols on Endothelial Function and Cerebral Ischemia in Wild-Type Mice**

Effect of a 4-week treatment of wild-type mice with normal chow (NC) and NC supplemented with plant sterol ester (PSE). **(A)** Plasma cholesterol, **(B)** campesterol, and **(C)** sitosterol concentrations determined by gas liquid chromatography-mass spectrometry; n = 10/group. Aortic segments were isolated, and their functional performance was assessed in organ chamber experiments. **(D)** Endothelium-dependent vasorelaxation induced by carbachol and **(E)** endothelium-independent vasodilation induced by nitroglycerin (NTG), expressed in percent of maximal phenylephrine-induced vasoconstriction; n = 10/group. Effects of NC + PSE compared with NC on ischemic stroke. Mice were subjected to 30-min filamentous middle cerebral artery occlusion and 72 h reperfusion. **(F)** Representative examples of 20-μm coronal brain cryostat sections, **(G)** indirect cerebral lesion volumes, and **(H)** direct cerebral lesion areas determined on 5 coronal brain sections (2 mm distance from frontal pole) by computer-assisted volumetry; n = 8/group. Values are mean ± SEM; *$p < 0.05$, **$p < 0.01$.

Effects of plant sterols on ischemic brain injury in wild-type mice. Wild-type SV/129 mice treated with NC or NC + PSE for 4 weeks were subjected to 30 min of left filamentous MCA occlusion and 72 h of reperfusion. Figures 1F and 1G demonstrate that cerebral lesion size determined by an indirect method that corrects for brain swelling was significantly larger in the NC + PSE group compared with NC-fed mice ($p < 0.05$). In addition, greater lesion areas (direct measurement) were evident in

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standardized coronal brain sections (8 mm from frontal pole, $p < 0.01$) (Fig. 1H). Mean arterial blood pressure, blood gas analysis (pH, Pa_{O_2}, Pa_{CO_2}) and rectal core temperature were not different between mice treated with NC or NC + PSE before or during cerebral ischemia (Table 2). The C57/Bl6 mice were subjected to ischemia/reperfusion with very similar results.

Effects of plant sterols on plasma levels and atherosclerosis in ApoE−/− mice. The ApoE−/− mice were treated with EZE and PSE in the presence and absence of a WTD for 6 months. The average plasma cholesterol level in WTD was 1,193 ± 78 mg/dl (Fig. 2). Plant sterol ester supplementation and EZE treatment reduced serum cholesterol concentrations to a comparable extent (388 ± 41 mg/dl vs. 454 ± 43 mg/dl, p = NS between WTD + PSE and WTD + EZE). Ezetimibe but not PSE reduced plasma plant sterol concentrations (0.23 ± 0.02 mg/dl vs. 15.2 ± 1.50 mg/dl campesterol, $p < 0.01$) and their ratios to cholesterol (0.55 ± 0.08 μg/mg vs. 39.78 ± 1.88 μg/mg, $p < 0.01$). Combining PSE and EZE further reduced plasma cholesterol concentrations compared with EZE treatment alone (251 ± 25 mg/dl vs. 454 ± 43 mg/dl; $p < 0.05$) but did not significantly alter plant sterol concentrations (0.23 ± 0.02 mg/dl vs. 0.79 ± 0.15 mg/dl campesterol) or their ratios (0.55 ± 0.08 μg/mg vs. 3.64 ± 0.91 μg/mg). The average plasma cholesterol level in mice fed NC was 460 ± 36 mg/dl. The PSE supplementation and EZE treatment reduced serum cholesterol concentrations in NC to an extent similar to the WTD groups (206 ± 21 mg/dl vs. 206 ± 14 mg/dl). Ezetimibe potently reduced plasma plant sterol concentrations (0.15 ± 0.01 mg/dl vs. 12.3 ± 1.20 mg/dl in NC + PSE for campesterol, $p < 0.05$) and their ratios (0.75 ± 0.04 μg/mg vs. 60.53 ± 2.31 μg/mg, $p < 0.01$). The combination of plant sterols and EZE reduced plasma cholesterol levels to 190 ± 17 mg/dl, and plant sterol concentrations (0.18 ± 0.02 mg/dl vs. 0.15 ± 0.01 mg/dl campesterol) and their ratios (1.00 ± 0.14 μg/mg vs. 0.75 ± 0.04 μg/mg) were not significantly increased, similar to the WTD groups.

As expected, atherosclerotic lesion formation was most pronounced in mice on WTD (44 ± 2.3% of luminal area), and diet supplementation with plant sterols, EZE, and the combination significantly reduced atherosclerotic lesion formation compared with WTD control animals (Figs. 3A to 3D). However, the extent of atherosclerotic lesion reduction was greater in WTD mice treated with EZE (10 ± 1.5%) compared with WTD + PSE (20.4 ± 2.1%; $p < 0.05$). The WTD + EZE + PSE showed a trend toward more pronounced atherosclerotic lesion formation compared with WTD + EZE, but this difference was not significant (13.2 ± 3.8% vs. 10.0 ± 1.5%).

Table 2 Physiologic Parameters During Cerebral Ischemia

Parameter	NC	NC+PSE
MABP (mm Hg)		
Before	86 ± 3	89 ± 7
During	103 ± 7	108 ± 9
pH		
Before	7.36 ± 0.03	7.33 ± 0.05
During	7.29 ± 0.06	7.28 ± 0.03
$PaCO_2$ (mm Hg)		
Before	46 ± 5	46 ± 8
During	46 ± 2	47 ± 7
PaO_2 (mm Hg)		
Before	99 ± 7	103 ± 8
During	93 ± 10	95 ± 3

129/SV male wildtype mice (ages 6 to 8 weeks, 20 to 24 g) were fed with normal chow (NC) or with NC supplemented with 2% plant sterol esters (PSE). Animals were then subjected to 30 min middle cerebral artery occlusion followed by reperfusion. Mean arterial blood pressure (MABP) was measured both before and during cerebral ischemia. Fifty microliters of blood were withdrawn twice before and during cerebral ischemia, respectively, for blood gas determination (pH, PaO_2, $PaCO_2$). Rectal (core) temperature was controlled and kept constant by means of a feedback temperature-control unit, n = 5 animals/group. Values are mean ± SD.

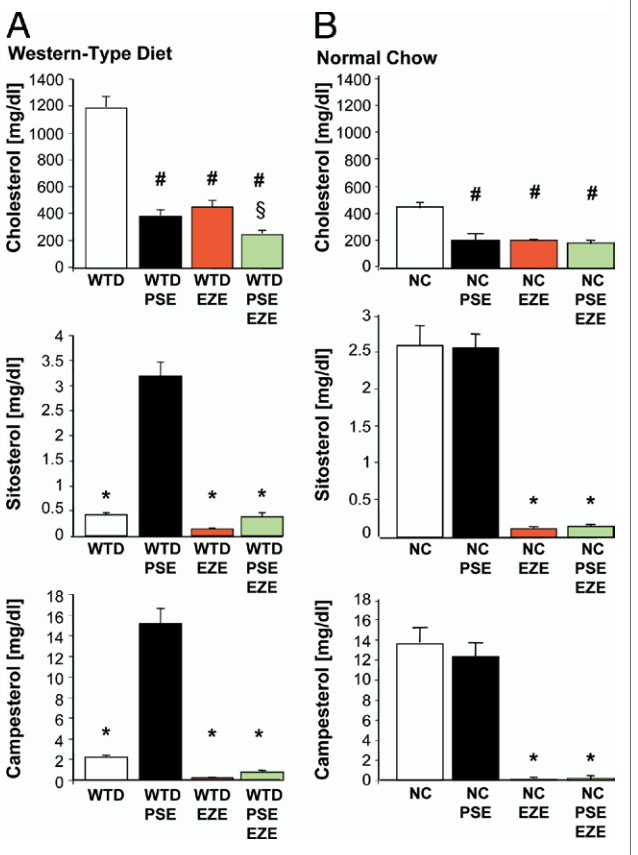

Figure 2 Effects of Plant Sterols on Plasma Cholesterol, Sitosterol, and Campesterol in ApoE−/− Mice

Effect of 6-month treatment of apolipoprotein E (ApoE)−/− mice with a high-cholesterol Western-type diet (WTD) or NC supplemented with PSE, ezetimibe (EZE), and their combination (PSE + EZE) on plasma cholesterol, sitosterol, and campesterol concentrations determined by gas liquid chromatography-mass spectrometry. Values are mean ± SEM (n = 10/group). **(A)** #$p < 0.05$ for WTD versus WTD + PSE, WTD + EZE, and WTD + PSE + EZE. §$p < 0.05$ for WTD + EZE versus WTD + PSE + EZE. *$p < 0.05$ for WTD + PSE versus WTD, WTD + EZE, and WTD + PSE + EZE. **(B)** #$p < 0.05$ for NC versus NC + PSE, NC + EZE, and NC + PSE + EZE. *$p < 0.05$ for NC, NC + PSE versus NC + EZE, and NC + PSE + EZE. Abbreviations as in Figure 1.

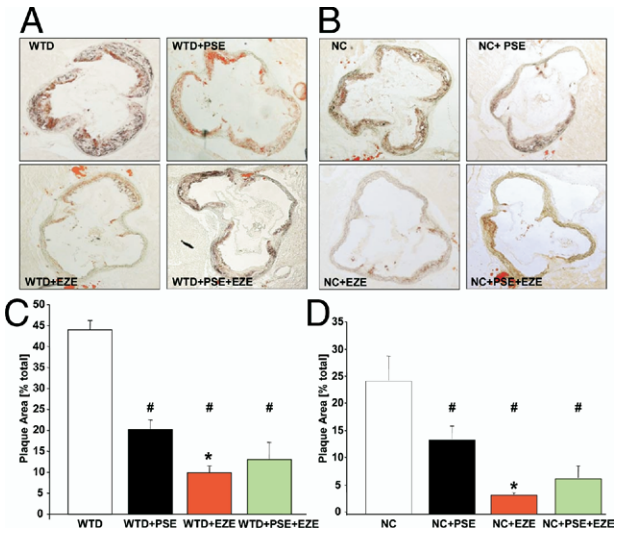

Figure 3 **Effects of Plant Sterols on Atherosclerotic Lesions in ApoE−/− Mice**

Atherosclerotic lesion formation was quantitated in the aortic sinus of ApoE−/− mice treated with high-cholesterol WTD or NC supplemented with PSE, EZE, and their combination (PSE + EZE). **(A and B)** Representative examples, and **(C and D)** histomorphologic analysis. #$p < 0.05$ compared with WTD or NC; *$p < 0.05$ for WTD + EZE versus WTD + PSE or NC + EZE versus NC + PSE; values are mean ± SEM (n = 10/group). Abbreviations as in Figures 1 and 2.

Atherosclerotic lesions in the NC group were reduced compared with WTD mice (24.5 ± 4.5%, $p < 0.05$). The NC with plant sterol supplementation (13.7 ± 2.4%) and NC with the combination therapy (6.6 ± 2.1%) reduced atherosclerotic lesions compared with NC alone, similar to the results of the WTD groups. Again, the effect of atherosclerotic lesion reduction was most pronounced in mice treated with NC + EZE (3.5 ± 0.4%) and significantly greater than in mice fed NC + PSE; $p < 0.05$.

Within the 3 treatment groups with comparable plasma cholesterol concentrations (NC: 460 ± 36 mg/dl, WTD + PSE: 388 ± 41 mg/dl, WTD + EZE: 454 ± 43 mg/dl cholesterol), both plasma sitosterol ($r = 0.50$; $p < 0.008$) and campesterol ($r = 0.49$; $p < 0.01$) correlated with atherosclerotic lesion size. This positive correlation of plaque size and sterol concentrations was independent of plasma cholesterol levels.

Histological characterization of atherosclerotic lesions in ApoE−/− mice. The EZE-treated animals showed reduced lipid accumulation compared with animals on a dietary supplementation with PSE (Fig. 4A). The localization of the lipid droplets within the plaques did not differ between groups. Levels of interstitial collagen were increased in WTD animals with cholesterol-lowering treatment (Fig. 4B), but there was no difference between PSE- and EZE-treated animals. In all groups macrophages localized mostly in the shoulder region of plaques and in the area surrounding the lipid core. Cholesterol-lowering reduced macrophage infiltration (Fig. 4C). Intimal SMC content was less pronounced in WTD animals compared with animals on cholesterol-lowering therapy (Fig. 4D).

Effects of diet supplementation on plant sterol concentrations in plasma and aortic valves in humans. Plasma levels and aortic valve cusp concentrations of cholesterol, the cholesterol precursor lathosterol, and the plant sterols sitosterol and campesterol were measured by gas liquid chromatography-mass spectrometry in 82 consecutive patients who were admitted to our hospital for aortic valve replacement (Table 3). Ten patients had consumed a sterol ester–supplemented margarine (Becel pro-activ [Unilever Deutschland GmbH, Werk Pratau, Germany], a brand popular in Germany) for more than 2 years before aortic valve replacement. Four patients reported an irregular consumption, averaging 1 serving/day. Six patients stated that sterol ester–supplemented margarine had been part of their regular diet with at least 2 servings/day for up to 4 years. Ten grams (1 serving) of Becel pro-activ margarine contains 0.75 g of PSE (manufacturer's information confirmed by own gas liquid chromatography). Seventy-two patients re-

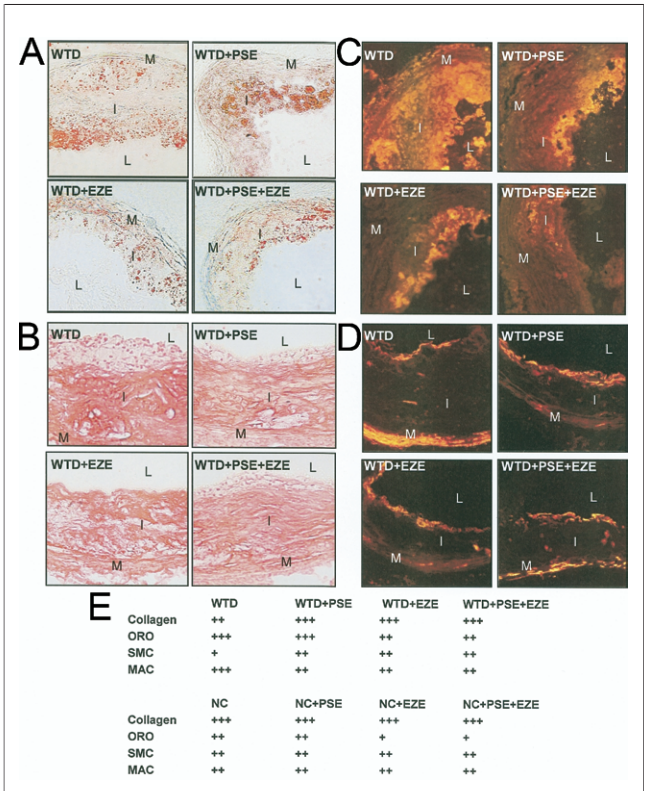

Figure 4 **Histological Characterization of Atherosclerotic Lesions in ApoE−/− Mice**

Representative atherosclerotic lesions from ApoE−/− mice treated with high-cholesterol WTD or NC supplemented with PSE, EZE, and their combination (PSE + EZE) stained for **(A)** oil red O (ORO), **(B)** collagen (picrosirius red), **(C)** macrophages (MAC, MOMO-2), and **(D)** vascular smooth muscle cells (SMC alpha-actin). **(E)** Semiquantitative grading of stainings: (+) weak staining, (++) moderate staining, (+++) intense staining. I = intima; L = lumen; M = media; other abbreviations as in Figures 1 and 2.

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Table 3 Patient Characteristics

N	82
Age (yrs)	71 ± 8
Male/female	58/24
Coronary heart disease	45
Risk factors	
Hypertension	61
Hyperlipidemia	44
Statin treatment	37
Diabetes mellitus	16
Current or ex-smoker	35
Body mass index (kg/m^2)	26.9 ± 3.9
Total cholesterol (mg/dl)	173 ± 33
Creatinine (mg/dl)	1.1 ± 0.41
Aortic valve area (cm^2)	0.78 ± 0.34

Values are mean ± SD.

ported a regular Western diet without deliberate consumption of PSE-enriched food. Of these 72 patients, 40 were not treated with a statin. The concentrations of the 2 most common plant sterols, sitosterol and campesterol, strongly correlated in plasma and aortic valve cusps ($r = 0.98$, $p < 0.0001$) (Fig. 5A for aortic valve cusps). Importantly, there was a significant correlation of plasma plant sterol levels to tissue concentrations. Figure 5B shows a strong correlation of the ratio of campesterol to cholesterol in plasma with aortic valve cusps ($r = 0.76$; $p < 0.0001$).

Patients who consumed margarine supplemented with PSE showed significantly higher plasma concentrations of sitosterol ($p < 0.003$; ANOVA comparing no, irregular, and regular intake of margarine, applied on log scale) and campesterol ($p < 0.001$) compared with patients on a nonsupplemented diet. Figure 5C shows the effect of sterol ester–supplemented margarine on plasma campesterol concentrations. Plasma campesterol concentrations of patients with irregular intake of sterol ester–supplemented margarine showed a nonsignificant trend toward increase (0.439 ± 0.10 mg/dl vs. 0.303 ± 0.02 mg/dl; $p = 0.13$). Patients consuming sterol ester–supplemented margarine on a regular basis were characterized by higher campesterol concentrations compared with patients on a regular Western diet (0.838 ± 0.14 mg/dl; $p < 0.0001$). Figure 5D shows that both irregular and regular consumption of sterol ester–supplemented margarine increased campesterol deposition in aortic valve cusps (164.4 ± 41.0 ng/mg and 299.0 ± 27.4 ng/mg vs. 61.6 ± 5.0 ng/mg; $p < 0.005$). The effect seemed to be dose-dependent. Similar results were observed for the ratios of campesterol to cholesterol in serum and in plasma (Figs. 5E and 5F) as well as sitosterol (data not shown).

Statin treatment decreased concentrations of the cholesterol precursor lathosterol in plasma (0.5 ± 0.10 μg/mg vs. 0.9 ± 0.06 μg/mg lathosterol/cholesterol; $p < 0.005$) and in aortic valve tissue (0.5 ± 0.04 μg/mg vs. 0.8 ± 0.06 μg/mg; $p < 0.005$). As expected (8,17), statin treatment increased plant sterol concentrations in plasma (2.1 ± 0.10 μg/mg vs. 1.3 ± 0.10 μg/mg campesterol/cholesterol; $p < 0.005$) and in aortic valve cusps (2.0 ± 0.20 μg/mg vs. 1.4 ± 0.10 μg/mg; $p < 0.001$). Figure 6A shows the effects of statin treatment ($n = 32$) in patients without PSE-supplemented margarine consumption ($n = 72$) on campesterol and lathosterol ratios to cholesterol. The positive correlations of PSE-enriched food consumption with plasma and tissue concentrations of plant sterols were independent of both statin treatment and cholesterol concentrations (Table 4).

A subgroup analysis of patients with neither prior statin treatment nor functional food consumption ($n = 40$) demonstrated that patients with a positive family history of

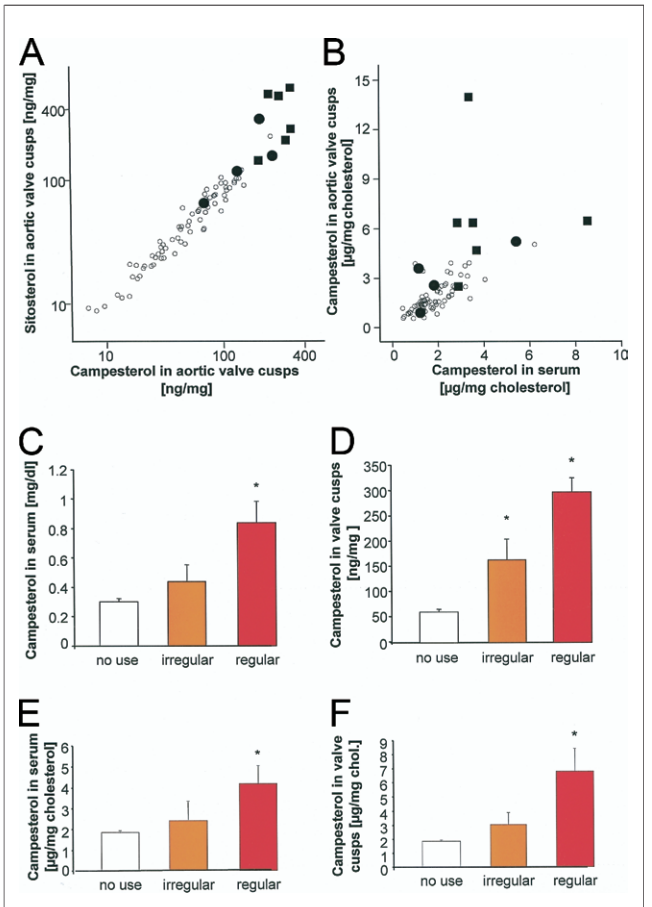

Figure 5 Effect of Diet Supplementation on Plant Sterol Concentrations in Plasma and Aortic Valves in Humans

Plasma and tissue analysis of cholesterol and plant sterol concentrations in 82 consecutive patients undergoing elective aortic valve surgery determined by gas liquid chromatography-mass spectrometry. **(A)** Correlation of sitosterol to campesterol in aortic valve cusps ($r = 0.98$; $p < 0.0001$). **(B)** Correlation of campesterol to cholesterol ratios between plasma and aortic valves ($r = 0.76$; $p < 0.0001$). Concentrations of sitosterol and campesterol in aortic valve cusps of patients using sterol ester–enriched margarine: **open circles** = no intake of sterol ester–enriched margarine ($n = 72$); **solid circles** = irregular intake of sterol ester–enriched margarine ($n = 4$); **solid squares** = regular intake of sterol ester–enriched margarine ($n = 6$). Effect of frequency of sterol-enriched margarine consumption on **(C)** plasma and **(D)** aortic valve tissue campesterol concentrations and campesterol to cholesterol ratios in plasma and tissue **(E and F)**. *$p < 0.01$ compared with "no use." Values are mean ± SEM.

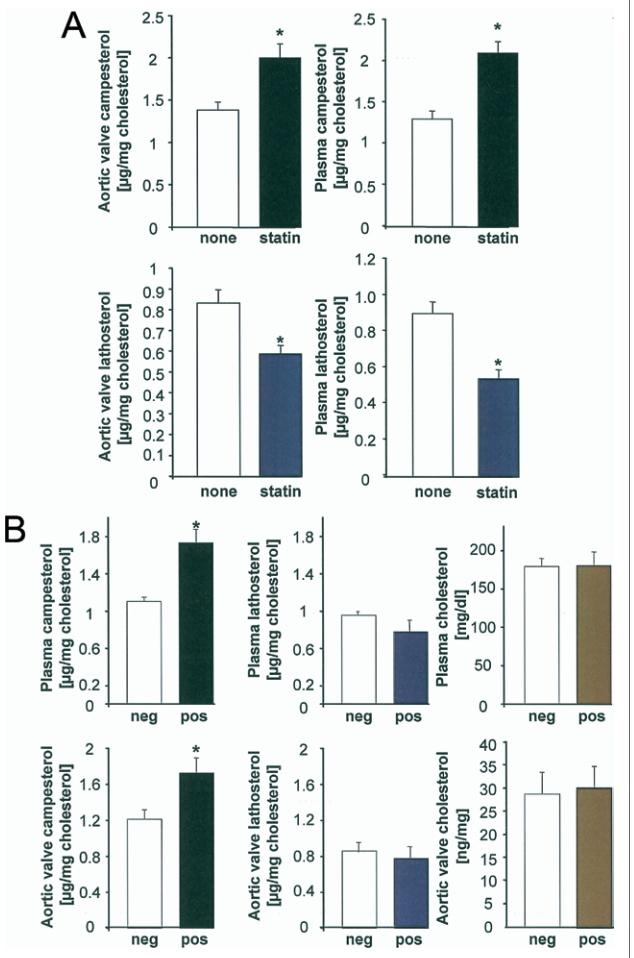

Figure 6 **Effects of Statin Treatment and Family History of Cardiovascular Diseases**

(A) Effect of statin treatment (n = 32/72) on campesterol and the cholesterol precursor lathosterol in relation to cholesterol in plasma and in aortic valve cusps; *p < 0.05. Values are mean ± SEM. **(B)** Effect of a positive (pos) family history of cardiovascular diseases (n = 15) on campesterol and lathosterol in relation to cholesterol in plasma and in aortic valve cusps in patients without statin treatment and consumption of plant sterol ester (PSE)-enriched food (n = 40). Neg = negative.

cardiovascular diseases (n = 15) showed increased campesterol/cholesterol ratios both in plasma (1.7 ± 0.10 μg/mg vs. 1.1 ± 0.05 μg/mg; $p < 0.005$) and aortic valve tissue (1.7 ± 0.17 μg/mg vs. 1.2 ± 0.10 μg/mg; $p < 0.05$). The concentrations of cholesterol and the cholesterol precursor lathosterol/cholesterol were not affected (Fig. 6B).

Discussion

Supplementation of chow with PSE impaired endothelium-dependent vasodilatation in wild-type mice and increased lesion size after cerebral ischemia. Plant sterols reduced cholesterol levels and atherosclerotic plaque formation in a model of lipid-driven atherogenesis; however, equal cholesterol-lowering with EZE resulted in significantly smaller plaques. The clinical study shows that consumption of sterol-enriched margarine correlates with increased plasma concentrations of sterols and, importantly, increased tissue deposition.

The functional integrity of the endothelium exerts potent anti-atherosclerotic effects. Endothelial dysfunction represents 1 of the earliest detectable stages of atherosclerosis and predicts cardiovascular events. Supplementation of NC with vegetable-based plant sterols did not change plasma cholesterol levels in wild-type mice, but increased campesterol and sitosterol by approximately 2-fold, a relative increase that is smaller than the increase observed in the patients of the clinical study consuming sterol-enriched margarine regularly. Unexpectedly, feeding with plant sterol–supplemented chow impaired endothelium-dependent vasodilation, whereas endothelium-independent vasodilator function remained unchanged.

These data suggested a potential negative vascular effect, depending on sterol rather than cholesterol plasma concentrations. Previous studies had shown that endothelial function is the determinator of the survival of the penumbra and development of cerebral lesion in the well-characterized model of transient cerebral ischemia (14,15). Mice fed the sterol-enriched diet showed equal physiological parameters, such as blood pressure or heart rate, but exhibited significantly greater ischemic stroke risk after transient occlusion of the middle cerebral artery.

Plant sterol–enriched foods reduce plasma cholesterol levels (1,18,19), which results in the reduction of plaque volume in animal models of atherosclerosis induced by severe hypercholesterolemia (4,5,20). The ApoE−/− mice fed with a WTD have plasma cholesterol concentrations well above 1 g/dl; any intervention that reduces this severe hyperlipidemia is likely to slow the progression of this primarily lipid-driven atherogenesis. Therefore the aim of the ApoE−/− study was to compare PSE-supplementation with equal cholesterol-lowering by a second intervention. Ezetimibe was chosen as a comparator, because similar to plant sterols, this 2-azetidione is an inhibitor of intestinal cholesterol absorption and lowers plasma cholesterol. Ezetimibe undergoes glucuronidation in the intestine and liver, and both the parent compound and its glucuronide localize to the brush border of the small intestine, where they block the absorption of dietary and biliary sources of cholesterol including plant sterols without affecting absorption of triglycerides, bile acids, or fat-soluble vitamins. The molecular mechanism relates to the inhibition of NPC1 L1, which is enriched in the brush border membrane of enterocytes in the small intestine (3,21). The novel finding of the experiments is a differential effect of plant sterols and EZE on the development of atherosclerotic lesions. Long-term treatment with plant sterols and EZE conferred equal lowering of plasma cholesterol both in the presence of the high-fat, high-cholesterol WTD and the cholesterol-free NC groups. As expected, the substantial lipid-lowering by both treat-

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Table 4 Effect of Sterol-Enriched Margarine Consumption on Plasma and Aortic Valve Campesterol Concentrations

Sterol Ester	Margarine Consumption	Geometric Mean Ratio	p Value *t* Test [*F* Test]
Aortic valve cusps			
Campesterol	Irregular vs. no	2.77 (1.14–6.73)	0.0249
	Regular vs. no	6.03 (2.49–14.65)	0.0001 [0.0001]
Adjusted for statin treatment	Irregular vs. no	2.53 (1.07–6.02)	0.0355
	Regular vs. no	4.83 (2.00–11.67)	0.0007 [0.0006]
Adjusted for statin treatment and cholesterol	Irregular vs. no	1.40 (0.6–2.43)	0.2279
	Regular vs. no	2.37 (1.34–4.17)	0.0033 [0.009]
Plasma			
Campesterol	Irregular vs. no	1.60 (0.81–3.15)	0.1709
	Regular vs. no	3.44 (1.75–6.78)	0.0005 [0.0012]
Adjusted for statin treatment	Irregular vs. no	1.52 (0.78–2.97)	0.2167
	Regular vs. no	3.02 (1.52–5.98)	0.0019 [0.0049]
Adjusted for statin treatment and cholesterol	Irregular vs. no	1.24 (0.72–2.14)	0.4271
	Regular vs. no	2.10 (1.20–3.68)	0.0102 [0.0306]

ment principles reduced lesion formation. However, despite equal plasma cholesterol concentrations, sterol ester supplementation was associated with twice the amount of plaque formation compared with EZE.

Histological evaluation of atherosclerotic plaque morphology revealed no difference of collagen content, vascular SMC-expression, and macrophage accumulation between PSE- and EZE-treated animals. Atherosclerotic lesions of EZE-treated animals were characterized by reduced lipid accumulation that correlated directly with reduced plasma plant sterol levels and reduced atherosclerotic lesion size. Increased plasma plant sterol concentrations might play a key role in cholesterol tissue deposition in sitosterolemic patients, either by attracting cholesterol from plasma by way of net transfer or by stimulating biosynthesis of cholesterol locally. Sitosterol-containing lipoproteins trigger free sterol-induced caspase-independent death in macrophages (22). Plant sterols are more readily oxidized than cholesterol (23). Oxidized sterols are present in human atherosclerotic plaque; the oxysterol/cholesterol ratio in plaque is higher than in normal tissues. Oxysterols in some animal models exhibit more potent effects than cholesterol per se. In vitro, oxysterols have been shown to perturb cholesterol biosynthesis, esterification, and efflux; impair vascular reactivity; and are cytotoxic and/or induce apoptosis (24). Wilund et al. (25) studied mice lacking 2 adenosine triphosphate–binding cassette half transporters and the low-density lipoprotein receptor (LDLR) (G5−/−; G8−/−; LDLR−/− mice). Exposed to WTD, these mice showed increased sitosterol plasma concentrations but equal plaque formation. One potential explanation of the difference to our results is a role of the LDLR for the sterol-induced vascular effects. Food supplementation with plant sterols in genetic models of lipid metabolism, such as LDLR−/−, will help to further elucidate the effect of sterols in vascular cells.

To assess whether the negative observations of the mouse models might have implications for humans, a prospective study on 82 consecutive patients undergoing aortic valve surgery was performed. Ten individuals (12%) reported consumption of plant sterol–supplemented margarine, which might be an indicator of the wide distribution of functional foods that are specifically advertised by their manufacturers for individuals with cardiovascular risk. In agreement with previous data by Miettinen et al. (8), the study shows a strong correlation between plasma and tissue concentrations. Importantly, the patient history of sterol-enriched margarine consumption predicted plant sterol tissue concentrations. Adjustment to statin treatment and cholesterol concentration showed an effect on top of statin treatment and independent of blood cholesterol concentration. Furthermore, increased plant sterol concentrations in plasma and in aortic valve cusps correlated with a positive history of cardiovascular events in patients without statin treatment and without sterol-supplemented margarine consumption. Interestingly, this correlation was not observed for cholesterol or the cholesterol precursor lathosterol. These data suggest that consumption of PSE-enriched food significantly changes the composition of vascular tissue and requires further and prospective testing in larger populations.

Study limitations. The study has several limitations: aside from a potentially negative effect of plant sterols, our animal data do not exclude additional effects of EZE that could potentially be beneficial during atherogenesis. The amount of PSE calculated as milligrams/day/kilogram of body weight in the animal experiments was approximately 100 times higher than that typically consumed by patients regularly with PSE-supplemented margarines. In addition, the dietary habits in the years before study enrollment were not controlled. Finally, the prospective observational design of the clinical study is a limitation but was needed to set the stage for a prospective randomized interventional trial with cardiovascular end points.

Conclusions

In summary, plant sterol supplementation impairs endothelial function, aggravates ischemic brain injury, effects atherogenesis in mice, and leads to increased tissue sterol concentrations in humans. In the light of the severe premature atherosclerosis in patients with phytosterolemia (6,7) and epidemiological observations suggesting an association of plant sterols with increased vascular risk (9–11), the findings of this study underline the need for prospective clinical studies with cardiovascular end points for functional foods supplemented with PSE that are currently advertised for patients with cardiovascular diseases.

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