Plant sterol or stanol esters retard lesion formation in LDL receptor-deficient mice independent of changes in serum plant sterols

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Abstract **Statins do not always decrease coronary heart disease mortality, which was speculated based on increased serum plant sterols observed during statin treatment. To evaluate plant sterol atherogenicity, we fed low density lipoprotein-receptor deficient (LDLr$^{+/-}$) mice for 35 weeks with Western diets (control) alone or enriched with atorvastatin or atorvastatin plus plant sterols or stanols. Atorvastatin decreased serum cholesterol by 22% and lesion area by 57%. Adding plant sterols or stanols to atorvastatin decreased serum cholesterol by 39% and 41%. Cholesterol-standardized serum plant sterol concentrations increased by 4- to 11-fold during sterol plus atorvastatin treatment versus stanol plus atorvastatin treatment. However, lesion size decreased similarly in the sterol plus atorvastatin (−99% vs. control) and the stanol plus atorvastatin (−98%) groups, with comparable serum cholesterol levels, suggesting that increased plant sterol concentrations are not atherogenic. Our second study confirms this conclusion. Compared with lesions after a 33 week atherogenic period, lesion size further increased in controls (+97%) during 12 more weeks on the diet, whereas 12 weeks with the addition of plant sterols or stanols decreased lesion size (66% and 64%). These findings indicate that in LDLr$^{+/-}$ mice *1*) increased cholesterol-standardized serum plant sterol concentrations are not atherogenic, *2*) adding plant sterols/stanols to atorvastatin further inhibits lesion formation, and *3*) plant sterols/stanols inhibit the progression or even induce the regression of existing lesions.—**Plat, J., I. Beugels, M. J. J. Gijbels, M. P. J. de Winther, and R. P. Mensink. **Plant sterol or stanol esters retard lesion formation in LDL receptor-deficient mice independent of changes in serum plant sterols. *J. Lipid Res.* 2006.** 47: **2762–2771.**

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Numerous double-blind, placebo-controlled, intervention trials have consistently shown that plant stanol and sterol esters decrease serum LDL cholesterol concentrations in various population and patient groups in a dose-dependent manner (1). Therefore, functional foods enriched with plant stanol or sterol esters have gained a prominent position in strategies to decrease cardiovascular risk (2). Consumption of plant sterol esters, however, increases serum plant sterol concentrations (1), and increased serum plant sterol concentrations may be atherogenic (3). Sitosterolemic patients, for example, who are characterized by ~50- to 60-fold increased serum plant sterol concentrations, often develop coronary heart disease (CHD) at a very young age (4, 5). However, a causal relationship between the severely increased plant sterol concentrations in this rare inheritable disease and CHD risk has never been proven. In addition, epidemiological studies with nonsitosterolemic subjects have suggested that slightly increased cholesterol-standardized serum plant sterol concentrations also are atherogenic (3, 6–8). In a recent study by Willund and coworkers (9), however, these observations could not be confirmed.

Other suggestions that plant sterols are atherogenic come from studies with patients treated with the serum cholesterol-lowering statins (HMG-CoA reductase inhibitors). These drugs increase cholesterol-standardized serum plant sterol concentrations (7, 10, 11). Miettinen and colleagues (7) have now suggested that this is a potential reason why statins did not decrease CHD mortality in a subgroup of patients from the Scandinavian statin survival study (4S study). In that study, patients who did not benefit from simvastatin treatment in terms of mortality had the strongest increases in cholesterol-standardized serum campesterol concentrations, despite comparable decreases in LDL cholesterol compared with other patients (7).

Although suggestive, these epidemiological studies do not prove causality (i.e., the associations can be attribut-

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able to some unknown confounding factor and not to plant sterols per se). However, it is of utmost importance to evaluate these potential unfavorable effects in detail because not only statin treatment but also consumption of functional foods enriched with plant sterols have been shown consistently to increase cholesterol-standardized serum plant sterol concentrations (12, 13). Moreover, the number of subjects taking statins with or without plant sterol-enriched foods is increasing. To study causality, animal studies are helpful. Therefore, we evaluated the potential atherogenicity of plant sterols and stanols in heterozygous low density lipoprotein-receptor deficient ($LDLr^{+/-}$) mice. These animals were fed a Western-type diet (control) or the same diet enriched with atorvastatin plus plant sterols or atorvastatin plus plant stanols. It was expected that both interventions would decrease serum cholesterol concentrations to the same extent but would lead to different serum plant sterol concentrations. In addition, the effects of feeding plant sterols or stanols alone were also evaluated. In a second study, $LDLr^{+/-}$ mice were fed a Western-type diet for 33 weeks, during which lesions were formed. For the next 12 weeks, plant sterols, plant stanols, or atorvastatin were added to the diets to evaluate the effects of these compounds on further lesion development. Both studies showed that plant sterols or stanols, alone, but also in combination with atorvastatin, retarded lesion formation independent of serum plant sterol concentrations. This finding contradicts the suggested atherogenicity of plant sterols in serum, at least in this animal model.

EXPERIMENTAL PROCEDURES

Animals, housing, and diets

Eight week old female and male heterozygous $LDLr^{+/-}$ mice were used for the experiments. These heterozygous $LDLr^{+/-}$ mice were obtained by breeding homozygous LDL receptor-deficient ($LDLr^{-/-}$) male mice (14) with C57BL/6 (Charles River) female mice. Mice had ad libitum access to the semi-synthetic diets. Diets contained 37 percent of energy (en%) fat with a Western-like fatty acid profile (**Table 1**), 0.25% cholesterol, 0.25% cholate, 45 en% carbohydrates (36 en% sucrose and 9 en% corn starch), and 19 en% protein (casein). All diets were prepared by Arie Blok Diervoeding (Woerden, The Netherlands). Atorvastatin-40 was obtained from Parke Davis (Morris Plains, NJ) and pulverized before incorporation into the diets. Plant sterol and stanol esters were prepared by the RAISIO Group (Raisio, Finland). Plant stanols were obtained by saturation of plant sterols from the same batch. For the synthesis of plant sterol and stanol esters, sterols and stanols were esterified with rapeseed oil fatty acids. Plant sterols were wood-based and contained 73% sitosterol, 8% campesterol, and 11% other sterols plus stanols, whereas plant stanols contained 87% sitostanol, 10% campestanol, and 4% other sterols plus stanols. These compositions (i.e., the sitosterol-campesterol ratio or the sitostanol-campestanol ratio) are slightly different from those in plant sterol- or stanol-enriched products currently available on the market. However, we earlier showed that the sitostanol-campestanol ratio of a mixture does not affect its cholesterol-lowering efficacy (15); in addition, at present there are no indications that sitosterol and campesterol or sitostanol and campestanol possess different atherogenic potentials.

For the production of the diets, the plant sterol and stanol esters were mildly heated to 37°C and then gently mixed with the fat phase of the diets. Atorvastatin powder was mixed with the nonfat phase. Next, the different fat phases were mixed with the nonfat phases with or without atorvastatin.

Designs

In a pilot experiment (**Fig. 1**), the effects of increasing doses of plant stanol esters or atorvastatin on serum total cholesterol concentrations were evaluated in 40 heterozygous $LDLr^{+/-}$ mice (20 females and 20 males) to titrate the optimal amounts of these components for studies 1 and 2. In this pilot study, we found that both plant stanol esters (period I) and atorvastatin (period II) decreased serum total cholesterol concentration in a dose-dependent manner. Although mean serum total cholesterol

TABLE 1. Effects of the different diets on absolute and cholesterol-standardized serum noncholesterol sterol concentrations in heterozygous LDL receptor-deficient mice

Treatment	Campesterol	Sitosterol	Campestanol	Sitostanol
		μmol/l		
Absolute concentrations				
Placebo	0.12 ± 0.03	0.03 ± 0.01	0.0029 ± 0.0016	0.0013 ± 0.0007
Stanol	0.08 ± 0.01	0.01 ± 0.00	0.0049 ± 0.0025	0.0026 ± 0.0012
Sterol	0.27 ± 0.04[a]	0.15 ± 0.05[a]	0.0033 ± 0.0011	0.0017 ± 0.0005
Atorvastatin	0.13 ± 0.05	0.04 ± 0.04	0.0021 ± 0.0008	0.0011 ± 0.0004
Stanol plus atorvastatin	0.10 ± 0.12	0.01 ± 0.00	0.0048 ± 0.0020	0.0034 ± 0.0012[c]
Sterol plus atorvastatin	0.32 ± 0.12[a]	0.16 ± 0.01[a]	0.0022 ± 0.0009	0.0013 ± 0.0007
		10^2 ×μmol/mmol cholesterol		
Cholesterol-standardized concentrations				
Placebo	1.17 ± 0.25	0.24 ± 0.08	0.027 ± 0.014	0.013 ± 0.006
Stanol	1.09 ± 0.27	0.10 ± 0.01	0.062 ± 0.034	0.033 ± 0.017
Sterol	3.95 ± 0.80[a]	2.13 ± 0.27[a]	0.046 ± 0.012	0.024 ± 0.009
Atorvastatin	1.50 ± 0.51	0.49 ± 0.40	0.024 ± 0.008	0.013 ± 0.004
Stanol plus atorvastatin	1.61 ± 0.29	0.21 ± 0.06	0.081 ± 0.054	0.050 ± 0.009
Sterol plus atorvastatin	5.64 ± 2.25[a,b]	2.92 ± 0.22[a,b]	0.040 ± 0.018	0.023 ± 0.015

All values are means ± SD and were analyzed after the mice had consumed the different diets for a 35 week period.
[a] $P < 0.001$ versus placebo, stanol, atorvastatin, and stanol plus atorvastatin.
[b] $P < 0.001$ versus sterol.
[c] $P < 0.01$ versus placebo, atorvastatin, and sterol plus atorvastatin.

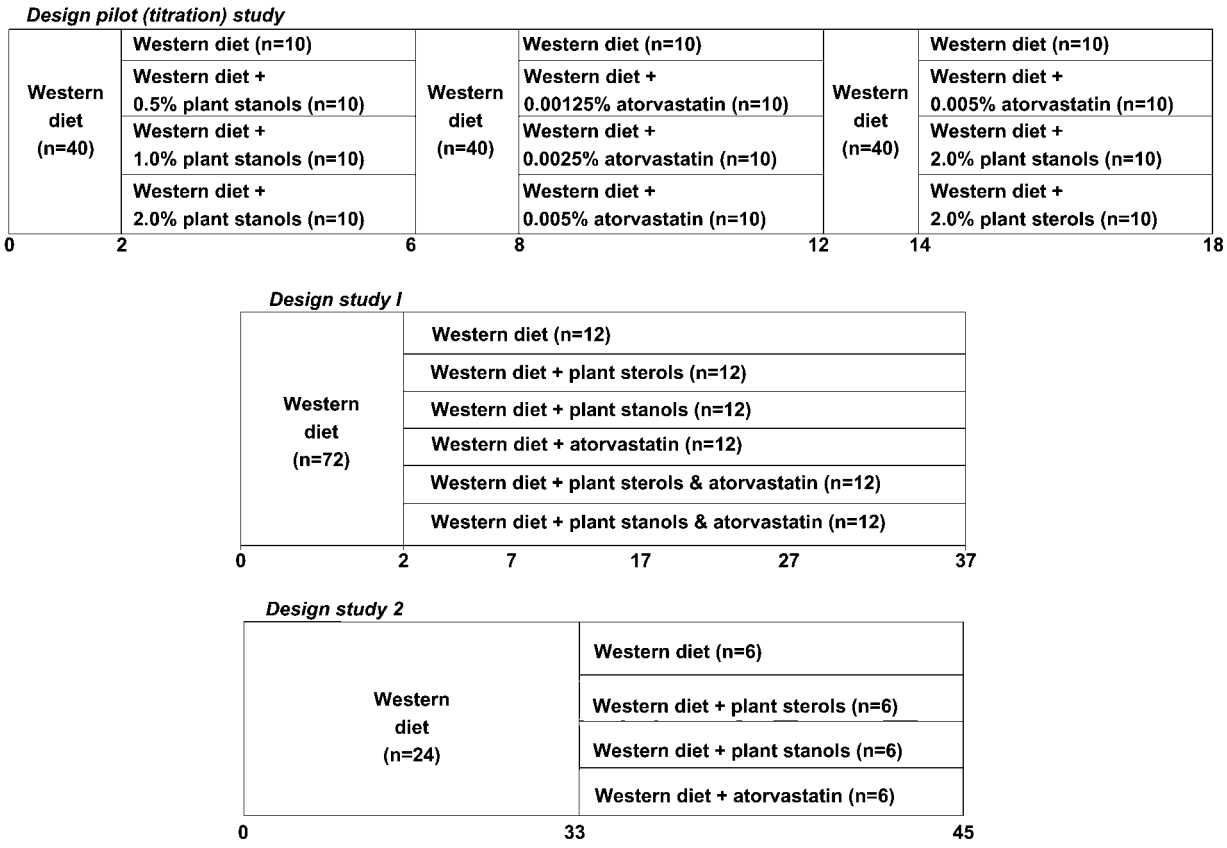

Fig. 1. Design of the studies. In the pilot study with 20 female and 20 male mice, the optimal doses for studies 1 and 2 were titrated. Both studies 1 and 2 had a parallel design. In study 1, 36 male and 36 female mice were used. In study 2, only female mice (n = 36) were used. At the x-axis of each diagram the number of weeks the mice fed on the different diets is indicated.

concentrations were higher in female compared with male mice, the relative reductions in serum total cholesterol concentrations did not depend on gender (data not shown). Moreover, in period III, we showed that diets containing 2% of plant stanol esters or plant sterol esters, or 0.005% of atorvastatin, when compared side-by-side, caused comparable reductions in serum total cholesterol (data not shown). Based on the outcome of this pilot experiment, the amount of plant sterols and stanols (supplied as their fatty acid esters) added to the diets for study 1 was 1% (w/w) and the amount of atorvastatin was 0.0025% (w/w). Expected reductions in serum total cholesterol using these doses were comparable, but not maximal. This made it possible to examine the effects of plant sterol/stanol esters as add-on treatment to statins on serum cholesterol and plant sterol concentrations and on lesion development. As the aim of study 2 was to compare the effects of plant sterol esters, plant stanol esters, and atorvastatin on changes of already existing lesions, the amounts chosen caused the greatest, but still comparable, decreases in serum total cholesterol concentrations [i.e., 2% plant sterols (w/w), 2% plant stanols (w/w), and 0.005% atorvastatin (w/w)].

After the optimal doses of plant sterol esters, plant stanol esters, and atorvastatin for studies 1 and 2 were established, the first intervention study was initiated to evaluate the effects of atorvastatin treatment combined with plant sterol esters or plant stanol esters on serum plant sterol and stanol concentrations and on lesion formation. For this, new mice were bred and fed the control diet for a 2 week run-in period (Fig. 1). Next, cages were randomly allocated to one of the six intervention groups. For the next 35 weeks, the first group (six males, six females) continued to use the Western-type control diet, whereas the other groups (also six males and six females) received the same diets enriched with plant sterols (1%, w/w), plant stanols (1%, w/w), atorvastatin (0.0025%, w/w), plant sterols (1%, w/w) plus atorvastatin (0.0025%, w/w), or plant stanols (1%, w/w) plus atorvastatin (0.0025%, w/w). At the end of the run-in period (week 2) as well as after 5, 15, 25, and 35 weeks on the experimental diets, blood was sampled after 3–4 h of fasting by orbital punctures into plastic tubes (Eppendorf, Hamburg, Germany) filled with glass beads to prepare serum. This serum was used for the analysis of serum concentrations of cholesterol (all time points), plant sterols and stanols (week 35 only), and lipoprotein profiles (week 35 only). After 35 weeks on the experimental diets, all animals were euthanized, and hearts were dissected, directly frozen into Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), and used later for lesion analysis.

The aim of the second intervention study was to compare the effects of plant sterol esters, plant stanol esters, and atorvastatin on the progression or regression of already existing lesions in female heterozygous LDLr$^{+/-}$ mice. Only female mice were used because the first intervention study had shown that, in contrast to female mice, males did not develop atherosclerotic lesions within this time frame. The animals were first fed the western-type control diet for 33 weeks (i.e., the atherogenesis period) (Fig. 1). After 33 weeks, three animals were euthanized to determine the size and severity of the atherosclerotic lesions. These two lesion characteristics of the three mice were averaged and used as a reference point for the remaining animals. For the next 12 weeks, the first group (six female mice) continued to consume the Western-type control diet, and the other three groups (also six females each) consumed the same diets enriched with

plant sterols (2%, w/w), plant stanols (2%, w/w), or atorvastatin (0.005%, w/w). Blood was sampled at the end of the 33 week atherogenic period as well as after 6 and 12 weeks on the experimental diets. Serum was prepared as described for study 1. At the end of the study, all animals were euthanized, and the hearts were dissected, frozen directly into Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), and used later for lesion analysis.

Serum lipoproteins

Concentrations of serum total cholesterol were measured enzymatically with a commercially available kit (CHOD/PAP method; Roche Diagnostics, Basel, Switzerland) directly after sampling. This kit does not differentiate between serum cholesterol and sterol analogues. Serum lipoprotein profiles at the end of the 35 week intervention period were determined by means of fast-protein liquid chromatography (FPLC) as described (16). Briefly, 50 μl of plasma (pooled from two to three mice on the same diet and housed in the same cage) was loaded on a Superose-6PC 3.2/30 column (Amersham Biosciences) in a FPLC system (AKTABasic; Amersham Biosciences) using PBS-EDTA as a mobile phase and automatically fractionated into 36 portions of 50 μl. Total cholesterol concentrations were measured in all fractions as described above.

Histological assessment of lesion size and severity

At the end of the intervention periods (week 35 for study 1 and week 12 for study 2), entire hearts of each mouse were dissected, sectioned perpendicularly to the heart axis just below the atrial tips, and frozen directly into Tissue-Tek. Thereafter, the hearts were sectioned toward the aortic axis. Upon reaching the aortic root, serial 7 μm sections were taken until the valves had disappeared. These sections were mounted on slides and air-dried for 24 h on silica. Next, atherosclerotic lesions were visualized to quantify size, as described (17) (i.e., four serial sections with a 42 μm interval were used per animal). All analyses were performed by the same technician, who was blinded with respect to the design of the study. Characterization of lesion severity was carried out as described previously (18): type I lesion, up to 10 foam cells in the intima (early fatty streaks); type 2 lesion, >10 foam cells in the intima (regular fatty streak); type 3 lesion, presence of a fibrous cap and foam cells located in the media (mild plaque); type 4 lesion, progressed lesions with affected media without the loss of media architecture (moderate plaque); type 5 lesion, severely affected media, cholesterol clefts, calcification, and necrosis (severe plaque). After scoring each mouse individually, lesions were clustered as type 1–2 or type 3–5. Because male mice in study 1 had not developed any lesions, we decided to evaluate the effects of the different interventions on the numbers of monocytes adhering to the endothelium. Monocyte adhesion to the endothelium was determined by counting adherent monocytes in four serial sections after staining with an FA11 antibody (a kind gift from S. Gordon) and expressed as the average number per diet group.

Serum plant sterol and stanol concentrations

Serum plant sterol and stanol concentrations were analyzed by gas chromatography as described previously (19). Briefly, 0.2 ml of serum was saponified for 1.5 h at 70°C with 2 ml of a mixture consisting of a 10 M KOH solution in water and ethanol (1:9%, v/v). After cooling, sterols and stanols were extracted twice with hexane. Next, the hexane extracts were evaporated to dryness at 37°C under a moderate nitrogen flow. The nonsaponifiable lipids were silylated for 15 min after the addition of 200 μl of bis-(trimethylsilyl)-trifluoracetamid containing 1% trimethylchlorosilane and 100 μl of pyridine. After evaporation under a moderate nitrogen flow at 50°C, the samples were dissolved in 500 μl of pentane. Samples were then analyzed with a GC8000 Top gas chromatograph (Carlo Erba, Milan, Italy) fitted with a 25 m AT1701 capillary column with an inner diameter of 0.32 μm and 0.30 μm film thickness (Alltech, Breda, The Netherlands) using cold on-column injection. One hundred micrograms of 5α-cholestane and 2 μg of 5β-cholestan-3α-ol were added to all samples before extraction and used as internal standards for cholesterol and plant sterols/stanols, respectively.

With respect to serum plant sterol concentrations, it is important to stress that in the earlier epidemiological studies (7, 11), not the absolute serum plant sterol concentration (μmol/l) in the circulation but the plant sterol concentration corrected for the number of lipoprotein particles (the plant sterol carriers) in serum (μmol/mmol cholesterol) was important. Statin treatment, for example, decreases serum cholesterol concentrations, whereas serum plant sterol concentrations are decreased, or even increased, depending on the type of statin used. However, cholesterol-standardized plant sterol concentrations (μmol/mmol cholesterol) are increased during statin treatment in general (7, 10, 11), suggesting that lipoprotein particles become enriched in plant sterols, which might be the reason for the observed effects on CHD risk. Therefore, when serum plant sterol concentrations are mentioned in this paper, we always mean cholesterol-standardized concentrations, unless explicitly mentioned otherwise.

Statistics

Data are presented as means ± SEM. Differences between mean values were tested for statistical significance by ANOVA. If a significant treatment effect was found ($P < 0.05$), the treatments were compared pair-wise and corrected for multiple comparisons using the Bonferroni posthoc test.

RESULTS

Study 1: effects of plant sterol/stanol esters plus atorvastatin on serum cholesterol and plant sterol concentrations in relation to atherosclerotic lesions

The aim of study 1 was to evaluate the effects of atorvastatin-induced increases in serum plant sterol concentrations on atherosclerotic lesion formation. During the course of the experiment, body weights of the mice were not differently affected by the treatments ($P = 0.137$; data not shown). As shown in **Fig. 2** (upper panel), 35 weeks of consumption of only plant stanol esters (1%, w/w), plant sterol esters (1%, w/w), or atorvastatin (0.0025%, w/w) significantly decreased serum total cholesterol concentrations by 20%, 26%, and 22%, respectively ($P < 0.01$ vs. the control group). Changes were not significantly different between the three groups. Adding plant sterol or plant stanol esters to the diets that already contained atorvastatin further decreased serum total cholesterol concentrations, resulting in total reductions of 39% and 41%, respectively ($P < 0.001$ vs. the control group). These changes were also significantly different from the change in the atorvastatin group ($P = 0.025$ and $P = 0.044$, respectively, for the plant stanol esters plus atorvastatin and plant sterol esters plus atorvastatin groups) (Fig. 2, upper panel). As shown in Fig. 2 (lower panel), the effects of the different diets on serum total cholesterol concentrations were present over the entire time period. Lipo-

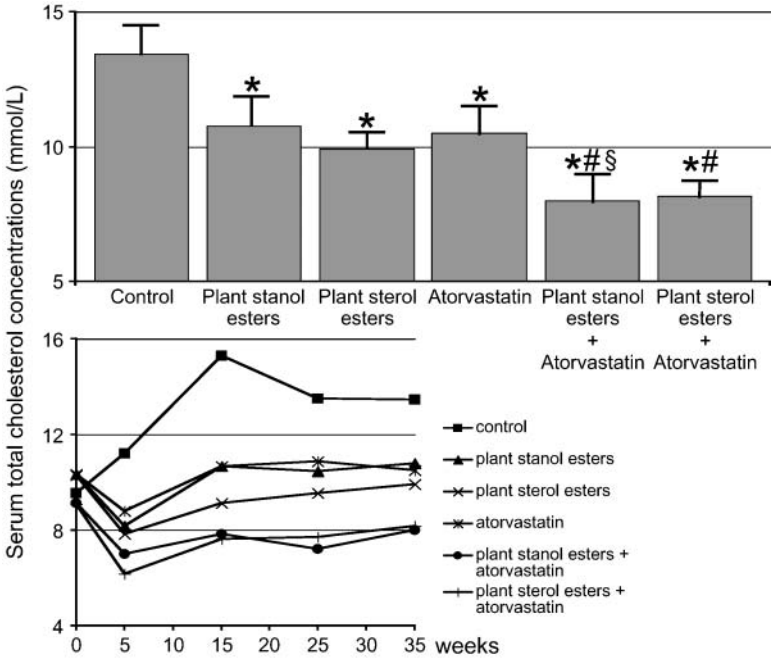

Fig. 2. Serum total cholesterol concentrations in heterozygous LDL receptor-deficient ($LDLr^{+/-}$) mice fed plant stanols (1%, w/w), plant sterols (1%, w/w), atorvastatin (0.0025%, w/w), plant stanols (1%, w/w) plus atorvastatin (0.0025%, w/w), or plant sterol ester (1%, w/w) plus atorvastatin (0.0025%, w/w) for 35 weeks. Plant sterols and stanols were fed as fatty acid esters. The upper panel shows serum total cholesterol concentrations at the end of the 35 week period, and the lower panel shows the development of serum cholesterol concentrations over time. Data are presented as means ± SEM. * $P < 0.01$ versus control; # $P < 0.05$ versus atorvastatin; § $P < 0.05$ versus stanol ester.

protein profiles as determined by FPLC indicated a reduction in the VLDL and intermediate density lipoprotein, but not in the LDL, fractions for all treatments (data not shown).

As expected, the plant sterol ester-enriched diet significantly increased serum sitosterol ($P < 0.001$) and campesterol ($P < 0.001$) concentrations (both absolute and cholesterol-standardized) compared with the control group (Table 1, **Fig. 3**). Serum cholesterol-standardized plant sterol concentrations increased even further, by up to 11-fold for sitosterol ($P < 0.001$ vs. the control group) and up to 4-fold for campesterol ($P < 0.001$ vs. the control group), when the diets contained plant sterol esters plus atorvastatin (Fig. 3). The increases in both cholesterol-standardized serum sitosterol and campesterol concentrations in the plant sterol ester plus atorvastatin group were significantly greater compared with those in the plant sterol group ($P < 0.001$ for sitosterol and $P = 0.001$ for campesterol). Absolute concentrations were not significantly higher in the plant sterol ester plus atorvastatin group compared with the plant sterol group (Table 1). However, because the cholesterol-standardized concentrations were increased, this indicates that the lipoprotein particle became enriched in plant sterols. Plant sterol ester consumption did not affect serum plant stanol (sitostanol and campestanol) concentrations. As for humans, serum plant stanol concentrations (both absolute and cholesterol-standardized) were much lower than serum plant sterol concentrations. Although serum cholesterol-standardized plant stanol concentrations increased in the plant stanol ester group, by up to 3-fold ($P = 0.607$ vs. the control group) and up to 2-fold ($P = 0.143$ vs. the control group) for sitostanol and campestanol, respectively, and particularly in the group that consumed plant stanol esters plus atorvastatin, by up to 5-fold for sitostanol ($P = 0.033$ vs. the control group) and up to 3-fold for campestanol ($P = 0.188$ vs. the control group), these increases were less pronounced compared with changes in cholesterol-standardized serum plant sterol concentrations (Fig. 3).

As shown in **Fig. 4**, all interventions significantly decreased lesion areas ($P < 0.001$ vs. control) in female $LDLr^{+/-}$ mice. The effects of the two combination treatments were most pronounced and even significantly larger than treatment by atorvastatin only ($P = 0.032$ and $P = 0.024$ for plant stanol esters plus atorvastatin and plant sterol esters plus atorvastatin, respectively, vs. atorvastastin alone). Effects on lesion severity showed the same pattern. All treatments shifted lesion severity from predominantly type 3–5 lesions, as seen in the control group, toward mainly type 1–2 lesions (**Fig. 5**). However, the effects were less pronounced in the atorvastatin group ($P = 0.022$ vs. control) and most pronounced in the two combination treatment groups (both $P < 0.001$ vs. control).

In male mice, 35 weeks of consumption of the same Western-type diet did not induce any detectable lesion formation, despite ~2 mmol/l higher serum total cholesterol concentrations compared with female mice. Therefore, as an alternative for lesion size and severity, the total number of monocytes attached to the endothelium was quantified in these animals. All interventions decreased adhering monocyte numbers from 3.1 ± 1.2 in control males to hardly any detectable numbers in the plant stanol ester plus atorvastatin (0.5 ± 0.4) and plant sterol ester plus atorvastatin (0.3 ± 0.4) groups ($P < 0.001$ and $P < 0.001$, respectively, vs. the control group).

Study 2: effects of plant sterol esters, plant stanol esters, or atorvastatin on the progression of already existing lesions

The aim of study 2 was to compare the effects of plant sterol esters or plant stanol esters with those of atorvastatin on the progression or regression of already existing ath-

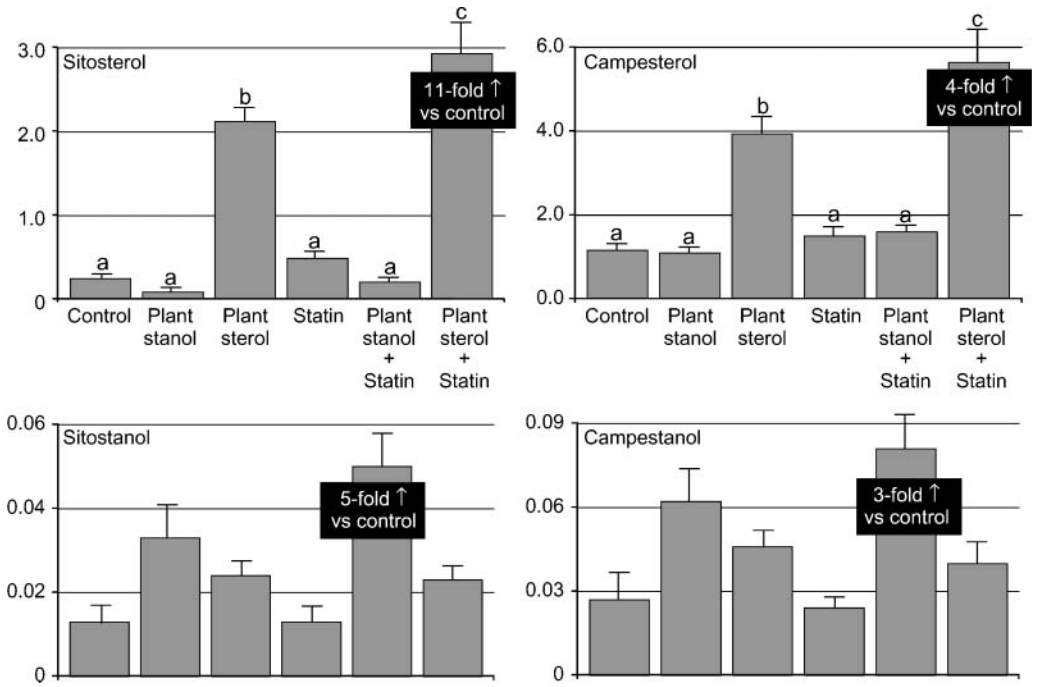

Fig. 3. Cholesterol-standardized serum plant sterol and stanol concentrations ($10^2 \times$ μmol/mmol cholesterol) in heterozygous LDLr$^{+/-}$ mice fed plant stanols (1%, w/w), plant sterols (1%, w/w), atorvastatin (0.0025%, w/w), plant stanols (1%, w/w) plus atorvastatin (0.0025%, w/w), or plant sterols (1%, w/w) plus atorvastatin (0.0025%, w/w) for 35 weeks. Plant sterols and stanols were fed as fatty acid esters. Data are presented as means ± SEM. Different letters (a, b, c) indicate statistically significant differences ($P < 0.001$).

erosclerotic lesions. As in study 1, body weights of the mice were not affected by the treatments ($P = 0.120$; data not shown). At the end of the 33 week atherogenic period, in which all animals consumed the Western-type diet, both type 1–2 and type 3–5 lesions were present in approximately equal numbers, and mean lesion area was 70,000 μm^2. As shown in **Fig. 6**, at the end of the 12 week intervention period, both lesion area (137,000 μm^2) and lesion severity progressed further (25% type 1–2 and 75% type 3–5 lesions) in the control group. In contrast, consuming the same Western-type diets enriched with plant stanol esters, plant sterol esters, or atorvastatin decreased lesion area and severity. In both the plant stanol and sterol ester groups, lesion sizes were ~25,000 μm^2, and that in the atorvastatin group was 55,000 μm^2. Only for the plant stanol ester group ($P = 0.016$ vs. the control group) and the plant sterol ester group ($P = 0.026$ vs. the control group) did the effects reach statistical significance. For the atorvastatin group, a trend was observed ($P = 0.065$). In the plant stanol and sterol ester groups, the lesions found were mainly (95% and 90%, respectively) type 1–2 lesions, whereas hardly any type 3–5 lesions were detected ($P < 0.001$ vs. the control group). The effects of atorvastatin were less pronounced, as shown by 60% type 1–2 lesions and 40% type 3–5 lesions ($P = 0.023$ vs. the control group).

DISCUSSION

Recent findings from epidemiological surveys have suggested that increased serum plant sterol concentrations are an independent risk marker for CHD (3, 6–8). In particular the observation by Miettinen and coworkers seems relevant that simvastatin treatment did not decrease mortality in a subgroup of patients characterized by the greatest increases in cholesterol-standardized serum campesterol concentrations. LDL cholesterol reductions between the subgroups were comparable (7). Although suggestive, these studies do not prove causality. Therefore, we evaluated in the first study the effects of atorvastatin-induced increases in serum plant sterol and stanol concentrations on lesion size and severity in heterozygous LDLr$^{+/-}$ mice. In the second study, we analyzed the effects of plant sterol or stanol ester consumption or atorvastatin treatment on already existing lesions that were formed during a 33 week atherogenic period.

Because in study 1, reductions in serum total cholesterol concentrations in the plant sterol ester plus atorvastatin group and the plant stanol ester plus atorvastatin group were comparable, whereas both absolute and serum cholesterol-standardized plant sterol concentrations increased only in the plant sterol ester plus atorvastatin group, we were able to evaluate the effects of increased plant sterol concentrations on atherosclerotic lesion formation at the same serum cholesterol background. Our results clearly indicate that even 11-fold or 4-fold differences in cholesterol-standardized serum sitosterol or campesterol concentrations (or 16-fold or 3-fold differences in absolute plant sterol concentrations) do not affect atherosclerotic lesion area and lesion type. As expected, cholesterol-standardized serum plant stanol concentrations were highest in the plant stanol ester plus

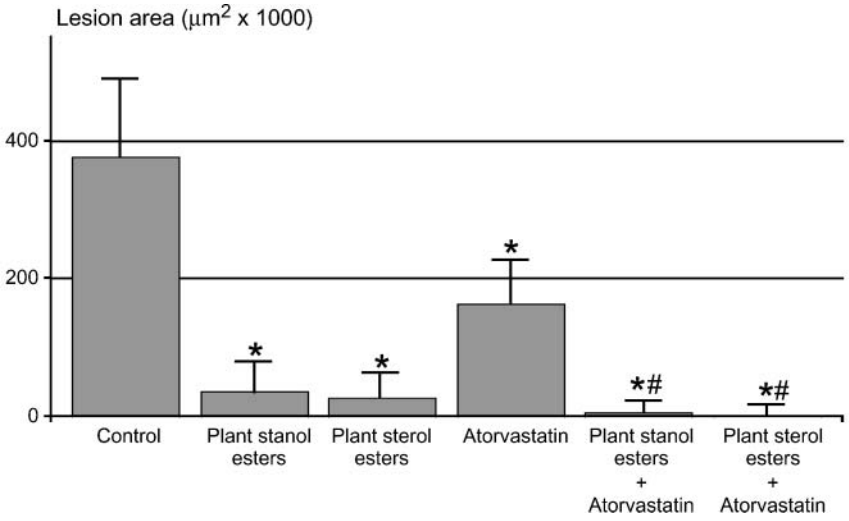

Fig. 4. Lesion areas (μm^2) in female heterozygous $LDLr^{+/-}$ mice fed plant stanols (1%, w/w), plant sterols (1%, w/w), atorvastatin (0.0025%, w/w), plant stanols (1%, w/w) plus atorvastatin (0.0025%, w/w), or plant sterols (1%, w/w) plus atorvastatin (0.0025%, w/w) for 35 weeks. Plant sterols and stanols were fed as fatty acid esters. Data are presented as means ± SEM. * $P < 0.001$ versus control; # $P < 0.05$ versus atorvastatin.

atorvastatin group. However, this increase was only a fraction (100-fold lower) of the increase in serum plant sterols in the plant sterol ester plus atorvastatin group (Fig. 3). Also in a situation of already existing lesions (study 2), consumption of plant sterols or stanols improved, or at least slowed the progression of, lesion area and type compared with the control group. Therefore, suggestions from the epidemiology studies that a 2-fold difference in serum cholesterol-standardized sitosterol concentrations (3) or 4-fold higher serum cholesterol-standardized campesterol concentrations (7) have proatherogenic effects could not be confirmed in this study with heterozygous $LDLr^{+/-}$ mice.

Recently, Willund and coworkers (9) also concluded that there is no evidence that increased serum plant sterol concentrations are atherogenic, as lesion sizes in homozygous $ABCG5/G8^{-/-}$ $LDLr^{-/-}$ double knockout mice fed an atherogenic diet were comparable to those in homozygous $LDLr^{-/-}$ deficient mice. However, in their editorial commenting on that study, Sehayek and Breslow (20) supply several arguments why this study, in their opinion, does not provide the necessary data to conclude that plant sterols are not atherogenic. The study of Willund et al. (9) differed in several important respects (which are in our opinion exactly the shortcomings indicated by

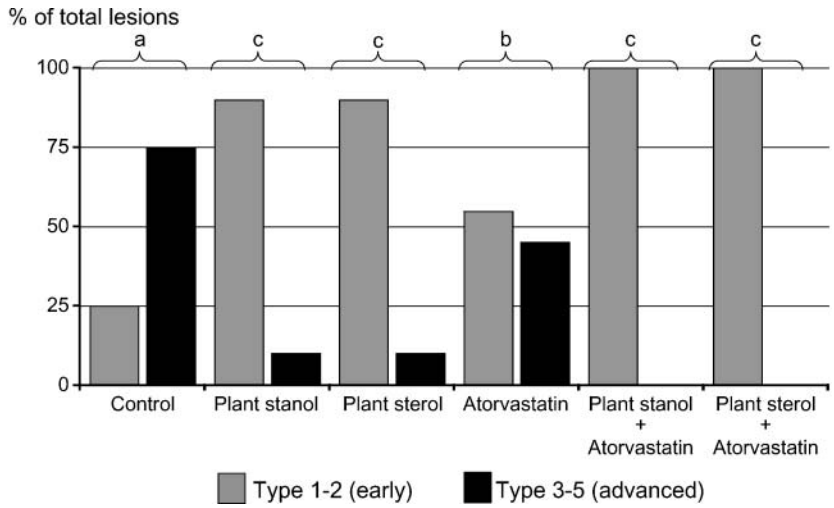

Fig. 5. Lesion types (% of total lesions) in female heterozygous $LDLr^{+/-}$ mice fed plant stanols (1%, w/w), plant sterols (1%, w/w), atorvastatin (0.0025%, w/w), plant stanols (1%, w/w) plus atorvastatin (0.0025%, w/w), or plant sterols (1%, w/w) plus atorvastatin (0.0025%, w/w) for 35 weeks. Plant sterols and stanols were fed as fatty acid esters. Lesions were typed as described in Experimental Procedures and categorized into type 1–2 (early lesions) or type 3–5 (advanced lesions). a versus b, $P = 0.022$; a versus c, $P < 0.001$; b versus c, $P < 0.001$.

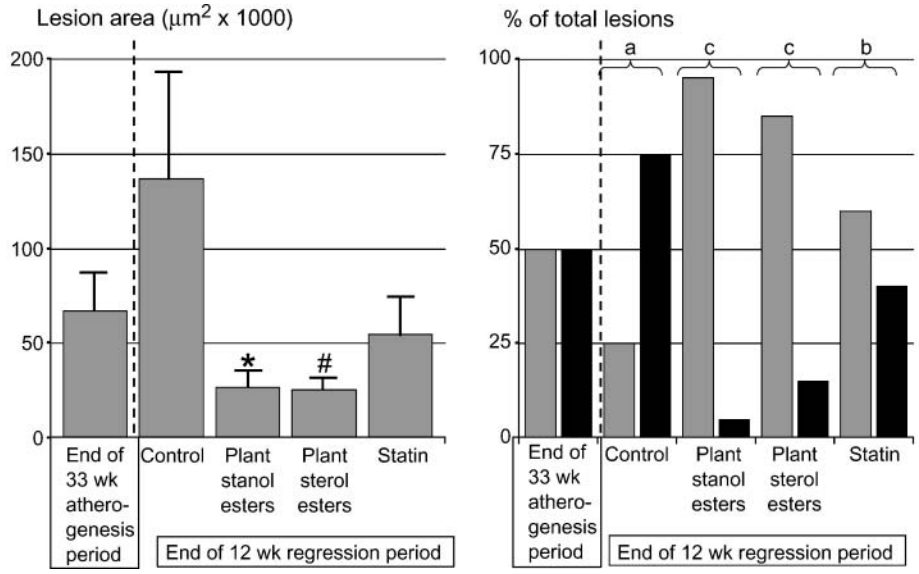

Fig. 6. Lesion areas (left panel; μm^2) and lesion types (right panel; % of total lesions) in female heterozygous $LDLr^{+/-}$ mice fed a Western-type control diet for 33 weeks (atherogenesis period) followed by a 12 week period (regression period) in which animals were fed plant stanols (2%, w/w), plant sterols (2%, w/w), or atorvastatin (0.005%, w/w). Plant sterols and stanols were fed as fatty acid esters. Data are presented as means ± SEM. For effects on lesion size (left panel): * $P = 0.016$, # $P = 0.026$. For lesion type (right panel), lesions were typed as described in Experimental Procedures and categorized into type 1–2 (early lesions) or type 3–5 (advanced lesions). a versus b, $P = 0.023$; a versus c, $P < 0.001$; b versus c, $P < 0.05$.

Sehayek and Breslow) from our study. First, our approach was not to increase serum plant sterol concentrations by overexpressing or knocking out genes but by means of diet or drug treatment, which more mimics the human situation. Second, we also evaluated the effects of plant stanols, because sitosterolemic patients not only have increased serum plant sterol concentrations but also increased serum plant stanol concentrations. As recently indicated by Connor et al. (21), plant stanols also are highly absorbed by sitosterolemic subjects and show a prolonged retention time. However, in epidemiological studies, serum plant stanol concentrations are frequently not reported, because sitostanol and campestanol concentrations are much lower and consequently difficult to measure. Third, Willund et al. (9) evaluated aortic arch en face lesion area, a technique that may be less sensitive than other methods to detect diet-induced atherosclerosis (22). Therefore, we have evaluated the effects of increased serum plant sterol or stanol levels on aortic root lesion area and morphology. The last difference is that serum cholesterol levels in the $ABCG5/G8^{-/-}$ $LDLr^{-/-}$ double knockout mice on a Western-type diet were ~20 mmol/l, which may have masked any atherogenic effect of increased plant sterol levels. In our study, serum total cholesterol concentrations were more moderately increased (~7–8 mmol/l). This is more physiologic and more representative of the human situation, because comparable combination treatments are frequently advised in patients with these serum cholesterol concentrations. Also, the magnitude of plant sterol increases in our study, which was much smaller than the difference between $ABCG5/G8^{-/-}$ $LDLr^{-/-}$ double knockout mice and the $LDLr^{-/-}$ knockout mice used by Willund et al. (9), is more realistic for the human situation (7, 11, 23). However, also under these conditions, increased concentrations of plant sterols were not atherogenic. Our second study even suggested that plant sterols or stanols could induce regression of already existing lesions. Plaque regression is regarded as a new target for antiatherosclerotic therapies (24), and in humans, positive effects have already been reported for statins (25, 26) and apolipoprotein A-I Milano (27). A relevant question is whether the effects of statins on lesion development may be ascribed to lipid lowering alone or whether statins have pleiotropic effects. Our finding that cholesterol reduction by plant sterol or stanol esters retards lesion development suggests that lipid lowering alone, independent of pleiotropic effects, already affects lesion development. However, it cannot be excluded that plant sterols or stanols themselves also have pleiotropic effects.

There has been at least one other study that has examined the effects of plant sterols on lesion regression (28). In that study, male apolipoprotein E-deficient mice were fed a Western-type diet [9% (w/w) fat and 0.15% (w/w) cholesterol] for 18 weeks to induce atherosclerosis. However, feeding plant sterols (2%, w/w) for the subsequent 25 weeks did not significantly change lesion development: in the plant sterol group, lesion size increased by 28% compared with a 40% increase in the control group. Surprisingly, plant sterols also did not decrease serum total cholesterol concentrations, which might explain the absence of a statistically significant "regression" effect. The absence of a cholesterol-lowering and consequent regression effect in this study cannot be

ascribed to the lack of responsiveness toward plant sterols of this model in general. Moreover, both in earlier and in more recent studies, Moghadasian et al. (29–31) have shown that plant sterols are effective at decreasing serum cholesterol concentrations in apolipoprotein E-deficient mice.

Although our studies did not reveal any atherogenic effects of plant sterols or stanols, other systemic effects cannot be excluded. For example, sitosterolemic patients also have anemic episodes, probably related to disturbed red blood cell characteristics. Indeed, in SPHR rats, which are characterized by increased plant sterol concentrations attributable to a defect in ABCG5, high plant sterol concentrations were associated with increased stroke risk, which was ascribed to a reduced erythrocyte deformability (32). However, in humans consuming plant sterol-enriched margarines for 16 weeks, we could not confirm these effects despite an increase in red blood cell plant sterol content (33). In addition, Yang et al. (34) have shown severe accumulation of plant sterols in adrenals of ABCG5/G8$^{-/-}$ mice, which led to depletion of cholesteryl esters. However, acute adrenocorticotropic hormone challenge to mimic a response to stress indicated that corticosterone production was normal in these mice, suggesting normal adrenal functioning.

In conclusion, we have shown that increased serum sitosterol and campesterol concentrations induced by feeding plant sterols to heterozygous LDLr$^{+/-}$ mice with or without atorvastatin treatment did not have any atherogenic effects. These findings do not support suggestions from epidemiological studies that moderately increased serum plant sterol concentrations are an atherogenic factor. However, although our data refute the possibility of a direct atherogenic effect of increased plant sterol concentrations in heterozygous LDLr$^{+/-}$ mice, we cannot exclude the possibility that in free-living humans, increased serum plant sterols are still a marker for CHD risk, for example, as an (indirect) marker for a step in the atherogenesis process.

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