Inhibition of cholesterol absorption by the combination of dietary plant sterols and ezetimibe: effects on plasma lipid levels

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Abstract Consumption of plant sterols and treatment with ezetimibe both reduce cholesterol absorption in the intestine. However, the mechanism of action differs between the two treatments, and the consequences of combination treatment are unknown. Therefore, we performed a double-blind, placebo-controlled, crossover study for the plant sterol component with open-label ezetimibe treatment. Forty mildly hypercholesterolemic subjects were randomized to the following treatments for 4 weeks each: 10 mg/day ezetimibe combined with 25 g/day control spread; 10 mg/day ezetimibe combined with 25 g/day spread containing 2 g of plant sterols; 25 g/day spread containing 2 g of plant sterols; and placebo treatment consisting of 25 g/day control spread. Combination treatment of plant sterols and ezetimibe reduced low density lipoprotein cholesterol (LDL-C) by 1.06 mmol/l (25.2%; P < 0.001) compared with 0.23 mmol/l (4.7%; P = 0.006) with plant sterols and 0.94 mmol/l (22.2%; P < 0.001) with ezetimibe monotherapy. LDL-C reduction conferred by the combination treatment did not differ significantly from ezetimibe monotherapy (−0.12 mmol/l or −3.5%; P = 0.13). Additionally, the plasma lathosterol-to-cholesterol ratio increased with all treatments. Sitosterol and campesterol ratios increased after plant sterol treatment and decreased upon ezetimibe and combination therapy. Our results indicate that the combination of plant sterols and ezetimibe has no therapeutic benefit over ezetimibe monotherapy in subjects with mild hypercholesterolemia.—Jakulj, L., M. D. Trip, T. Sudhop, K. von Bergmann, J. J. P. Kastelein, and M. N. Vissers. Inhibition of cholesterol absorption by the combination of dietary plant sterols and ezetimibe: effects on plasma lipid levels. J. Lipid Res. 2005. 46: 2692–2698.

Supplementary key words low density lipoprotein • humans • sitosterol • hypercholesterolemia

Plasma levels of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) can be substantially reduced by inhibition of cholesterol absorption within the intestine. This reduction in cholesterol absorption can be achieved either by daily consumption of plant sterols or stanols (1) or by treatment with ezetimibe (2). In fact, consumption of 2 g of plant sterols per day decreases plasma LDL-C by ~10% (reviewed in 1, 3, 4), and treatment with 10 mg of ezetimibe once daily reduces plasma LDL-C by 15–20% (5, 6).

The precise mechanism by which free cholesterol is absorbed in the small intestine is not fully understood. Recently, two novel ATP binding cassette transporters, ABCG5 and ABCG8, were identified in this pathway. These proteins are expressed in the intestine as well as in the liver, where they function as heterodimer efflux transporters. Positioned at the apical surface of the intestinal and hepatic cells, they promote intestinal and biliary sterol excretion (7–9). Even more recently, other transporters that may be involved, the Niemann-Pick C1-Like 1 (NPC1L1) and aminopeptidase N proteins, have been identified (10, 11). Both proteins reside on the brush border membrane of enterocytes in the small intestine. They may play a role in cholesterol and plant sterol absorption and may be molecular targets for ezetimibe (11, 12).

Plant sterols and stanols are thought to compete with dietary and biliary cholesterol for incorporation into mixed micelles, thereby reducing the amount of cholesterol available for uptake by the enterocyte (13, 14). Plant sterols themselves are absorbed in exceedingly small amounts, because of the active secretion of those sterols back into the enteric lumen by ABCG5 and ABCG8 (7, 8). Net absorption of plant sterols ranges from 5% to 18% of total sterol mass, depending on the type of sterol (15, 16). In contrast to plant sterols, which increase plasma plant sterol concentrations, plant stanols, the saturated counterparts of plant sterols, reduce the absorption and, conse-

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Ezetimibe, on the other hand, exerts its action in the brush border of the small intestine and reduces cholesterol absorption by inhibiting the uptake of dietary and biliary sterols, most likely through interaction with the NPC1L1 (10, 12) and/or aminopeptidase N (11) protein. Like plant stanols, ezetimibe inhibits both cholesterol and plant sterol absorption (12, 17, 18).

Prescription of ezetimibe to hypercholesterolemic patients is rapidly increasing, but these patients also often use food products that are enriched with plant sterols or stanols. Hence, it seems clinically relevant to investigate whether these two different modalities exhibit any interaction in terms of the modification of plasma lipid levels. Also, the safety of such a combination should be determined. Therefore, we designed a study to assess the efficacy of ezetimibe and plant sterols alone as well as their combination toward cholesterol and noncholesterol sterol plasma levels.

SUBJECTS AND METHODS

Subjects

Subjects were recruited via advertisements in local newspapers. Each subject gave written informed consent. The study was approved by the institutional review board. Subjects were included in the study if they were 18 years or older and had a plasma LDL-C concentration between 3.5 and 5.0 mmol/l. Exclusion criteria were a history of arterial disease, including unstable angina, myocardial infarction, transient ischemic attack, or a cerebrovascular accident; diabetes mellitus; uncontrolled hypertension; familial hypercholesterolemia; plasma triglyceride (TG) concentration > 4.0 mmol/l at baseline; or excessive alcohol consumption (>3 units per day). During the study, subjects were not allowed to use any other lipid-lowering medication or food products.

Study design

The study was a double-blind, placebo-controlled, crossover study for the plant sterol component with open-label ezetimibe treatment. Subjects started with a 6-week run-in period, in which they were not allowed to consume any plant sterol- or stanol-enriched foods or dietary vitamin supplements. Subjects who regularly consumed plant sterol or stanol products or who used cholesterol-lowering medication started with a 6-week run-in period. After the run-in period, subjects were randomly assigned to one of the following four treatment arms: a) 10 mg/day ezetimibe in combination with 25 g/day control spread; b) 10 mg/day ezetimibe in combination with 25 g/day spread containing 2.0 g of plant sterols; c) 25 g/day spread containing 2.0 g of plant sterols; or d) placebo treatment that consisted of 25 g/day control spread. After 4 weeks of treatment, subjects crossed over to the next study treatment, until they had completed the four treatments. Subjects were requested to maintain their usual pattern of food, drink, and smoking habits as well as physical activity during the whole study.

Plasma levels of TC, LDL-C, and TG were measured after an overnight fast of at least 12 h at baseline and at the end of each treatment period, as were liver and muscle enzymes. Plasma concentrations of lathosterol, cholestanol, sitosterol, and campesterol were measured at the end of each treatment period, because these noncholesterol sterols reflect cholesterol synthesis and absorption, respectively (19). Blood pressure and weight were assessed at each study visit. Physical examination was performed at baseline and at the end of the study.

Spread composition and administration

The placebo and the plant sterol-enriched spreads were produced and blinded by Unilever Research (Vlaardingen, The Netherlands). The placebo spread contained 8.8 g of fat, composed of 25% saturated fatty acids, 30% MUFAs, and 45% PUFAs per 25 g of spread. The plant sterol-enriched spread contained 8.8 g of fat, with a fatty acid composition of 23.5% saturated fatty acids, 25.6% MUFAs, and 49.9% PUFAs, and 2.0 g of plant sterols, composed of 46.5% sitosterol, 29.0% campesterol, 14.5% stigmasterol, and 10% other sterols, per 25 g of spread. The spreads were distributed in identical-looking 25 g tubs, labeled with different colors for each treatment arm. Subjects were instructed to keep the spreads in the refrigerator and to use one tub per day as a spread on sandwiches or as part of a hot meal, by mixing the spread with the food on the plate. The tubs were distributed at the start of each treatment period. Compliance was measured at each study visit by collection and calculation of the empty and full tubs of spread.

Ezetimibe

During two treatment periods, subjects were treated with 10 mg of ezetimibe (Ezetrol®) per day, in addition to their daily consumption of spread. The ezetimibe treatment was not placebo-controlled; therefore, subjects only received tablets during the two ezetimibe periods, one with plant sterol spread, and one with control spread. Subjects were instructed to take one tablet of ezetimibe per day and to return the empty and full blister packs to evaluate compliance.

Plasma analyses

Plasma TC, HDL-C, and TG levels as well as the hepatic transaminases, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), and creatine phosphokinase (CPK) were measured with standard (automated) methods. Plasma LDL-C levels were calculated using the Friedewald equation (20).

Plasma concentrations of lathosterol, cholestanol, sitosterol, and campesterol were analyzed as trimethylsilylates by gas-liquid chromatography (Hewlett-Packard 5890) using an automatic injection system (Automatic Sampler; Hewlett-Packard 7673A) with 5α-cholestanol as the internal standard (15). Because noncholesterol sterols are transported in serum by lipoproteins, changes in lipoprotein concentrations also affect concentrations of noncholesterol sterols (21). Therefore, noncholesterol sterols are ex-
pressed in concentrations (µg/dl) as well as in ratios to cholesterol (µg/mg).

Statistical analysis
Based on 40 subjects, our study had a statistical power of 80% to detect a difference of 0.15 mmol/l in LDL-C. In other words, we were able to detect an effect of 3.8% with a baseline level of 4 mmol/l and of 5% with a baseline level of 3 mmol/l.

Data were analyzed by ANOVA using the general linear model (GLM) of SAS (SAS Institute, Inc., Cary, NC). Tukey’s procedure was used for pairwise comparisons and for the calculation of 95% confidence intervals between active treatments and placebo. The data were analyzed by ANOVA using the general linear model (GLM) of SAS. Tukey’s procedure was used for pairwise comparisons.

RESULTS

General
All 40 subjects completed the trial. The clinical characteristics of these subjects are shown in Table 1. Five of the subjects were females, aged 53–63 years, and none of them was on hormone replacement therapy. Based on the number of returned empty tubs of spread, the mean compliance for both types of spread was >94%. Based on the number of empty packs of ezetimibe, the mean compliance for ezetimibe treatment was >91%. One subject was excluded from statistical analysis because of high TG concentrations and possible noncompliance with the study protocol. The data were analyzed by ANOVA using the general linear model (GLM) of SAS. Tukey’s procedure was used for pairwise comparisons.

Table 2. Plasma lipid and lipoprotein concentrations after 4 weeks of treatment with placebo, plant sterols, ezetimibe, and the combination of plant sterols with ezetimibe (n = 39)

| Lipid/Lipoprotein | Placebo | Plant Sterol Spread | Ezetimibe | Plant Sterol Spread + Ezetimibe |
|-------------------|---------|---------------------|-----------|---------------------------------|
| TC                | 6.44 ± 0.90a | 6.09 ± 0.85b | 5.38 ± 0.69c | 5.28 ± 0.67c |
| LDL-C             | 4.07 ± 0.76a | 3.84 ± 0.69b | 3.13 ± 0.57c | 3.01 ± 0.62c |
| HDL-C             | 1.75 ± 0.44 | 1.68 ± 0.47 | 1.71 ± 0.50 | 1.74 ± 0.53 |
| TG                | 1.36 ± 0.75 | 1.25 ± 0.62 | 1.19 ± 0.61 | 1.20 ± 0.68 |

All values are means ± SD. Values in the same row with different letters are significantly different (P < 0.05). One subject was excluded from statistical analyses because of high TG concentrations and possible noncompliance with the study protocol. The data were analyzed by ANOVA using the general linear model (GLM) of SAS. Tukey’s procedure was used for pairwise comparisons.

Fig. 1. Percentage changes (±SD) of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglyceride (TG) after treatment with plant sterols, ezetimibe, and the combination of plant sterols and ezetimibe. * Significant difference between treatment and placebo (P < 0.05).
ezetimibe reduced LDL-C by 0.12 mmol/l (−3.5%) compared with ezetimibe treatment alone, which was not statistically significant (95% CI: −0.54 to 0.10 mmol/l; \( P = 0.13 \)). Of the 39 subjects, 26 showed reduced LDL-C levels with plant sterol treatment, 37 with ezetimibe treatment, and 38 with the combination of plant sterols and ezetimibe, compared with placebo (Fig. 2). The absence of significant treatment-by-period interactions with respect to TC and LDL-C levels indicated that there were no important carry-over effects (data not shown). HDL-C and TG levels were not affected by any treatment.

Noncholesterol sterol concentrations

The concentrations of the cholesterol precursor lathosterol and the cholesterol absorption markers cholestanol, sitosterol, and campesterol as well as the ratios of the non-cholesterol sterols to cholesterol are presented in Table 3 and Fig. 3. The mean lathosterol concentration and the lathosterol-to-cholesterol ratio increased after inhibition of cholesterol absorption, with the smallest increase after plant sterol treatment and the largest increase after the combination treatment of plant sterols with ezetimibe. The mean cholestanol concentration and the cholestanol-to-cholesterol ratio decreased when cholesterol absorption was inhibited with plant sterols, ezetimibe, or their combination. However, the effect of the combination treatment did not differ significantly from that of ezetimibe monotherapy. As expected, plasma sitosterol and campesterol concentrations and ratios increased after plant sterol treatment and decreased after ezetimibe treatment. However, even though their concentrations and ratios increased when the plant sterol spread was added to the ezetimibe treatment, they remained lower than on placebo treatment.

Adverse events

No serious adverse events were reported. Two subjects experienced mild gastrointestinal complaints during placebo treatment, one subject during plant sterol consumption, four subjects during ezetimibe treatment, and four subjects during the combination treatment period. ASAT, ALAT, and CPK were not relevantly affected by plant sterol consumption, ezetimibe, or their combination as com-

![Plots](image)

**Fig. 2.** Individual differences in plasma low density lipoprotein cholesterol (LDL-C) concentration between the end of the 4-week treatment with plant sterols, ezetimibe, or their combination and the end of the 4-week placebo treatment.

![Plots](image)

**Fig. 3.** The mean lathosterol concentration and the lathosterol-to-cholesterol ratio decreased when cholesterol absorption was inhibited with plant sterols, ezetimibe, or their combination. However, the effect of the combination treatment did not differ significantly from that of ezetimibe monotherapy.

| Variable | Placebo (n = 39) | Plant Sterol Spread (n = 39) | Ezetimibe (n = 39) | Plant Sterol Spread + Ezetimibe (n = 39) |
|----------|-----------------|-----------------------------|-------------------|---------------------------------------|
| Plasma sterol concentrations | | | | |
| Cholesterol (mg/dl) | | | | |
| Lathosterol (\( \mu \)g/dl) | 258 ± 35a | 241 ± 36b | 216 ± 31c | 212 ± 28c |
| Cholestanol (\( \mu \)g/dl) | 318 ± 115a | 353 ± 140a | 418 ± 190b | 467 ± 147c |
| Sitosterol (\( \mu \)g/dl) | 402 ± 111a | 350 ± 89b | 301 ± 70c | 285 ± 59c |
| Campesterol (\( \mu \)g/dl) | 381 ± 160a | 460 ± 179b | 200 ± 74c | 235 ± 89c |
| Plasma sterol-cholesterol ratios | | | | |
| Lathosterol (\( \mu \)g/mg) | 1.26 ± 0.49a | 1.47 ± 0.53b | 1.94 ± 0.57c | 2.20 ± 0.63d |
| Cholestanol (\( \mu \)g/mg) | 1.55 ± 0.30a | 1.46 ± 0.29b | 1.40 ± 0.24c | 1.35 ± 0.22c |
| Sitosterol (\( \mu \)g/mg) | 1.48 ± 0.54a | 1.92 ± 0.68b | 0.93 ± 0.31c | 1.11 ± 0.58d |
| Campesterol (\( \mu \)g/mg) | 2.16 ± 0.83a | 4.15 ± 1.53b | 1.09 ± 0.42c | 1.72 ± 0.65d |

All values are means ± SD. Values in the same row with different letters are significantly different (\( P < 0.05 \)). One subject was excluded from statistical analyses because of high TG concentrations and possible noncompliance with the study protocol. The data were analyzed by ANOVA using the general linear model (GLM) of SAS. Tukey’s procedure was used for pairwise comparisons.

As measured by GC.
pared with placebo treatment, and their levels did not exceed three times the upper limit of normal (Table 4). Blood pressure, weight, and body mass index did not differ between the four treatment periods (data not shown).

DISCUSSION

In this study, we showed that in subjects with mild hypercholesterolemia, combination treatment of ezetimibe and plant sterols reduces LDL-C by 25% compared with 5% by plant sterols alone and 22% by ezetimibe monotherapy. In fact, the LDL-reducing effect of the combination treatment did not significantly differ from that of ezetimibe monotherapy.

The LDL-C lowering effects of plant sterols and ezetimibe monotherapy per se are in agreement with previous results. Although the LDL-C lowering effect of plant sterols in our study was relatively small, it was within the range of previous findings (1, 3, 4). Conversely, the LDL-C lowering effect of 22% with ezetimibe was relatively pronounced, because a mean reduction of ~18% is more frequently reported (5, 6). There were no carry-over effects that could explain these results, and previous findings by others also do not suggest the existence of a carry-over effect in the current study. Mensink et al. (22) showed that the effects of 4-week treatment with 3 g/day plant stanols on TC and LDL-C concentrations were already maximal after 1 week, and values between the active treatment and placebo groups were comparable already 2 weeks after discontinuation of the treatments. Knopp et al. (23) showed that the maximal LDL-C reduction by ezetimibe was evident at 2 weeks. Moreover, Sudhop et al. (17) demonstrated that there was no carry-over effect in a crossover study with ezetimibe if subjects were treated with ezetimibe and placebo for 2 weeks each. Although they included a 2-week washout period between the two treatments, they also measured the effect of the one treatment 4 weeks after discontinuing the other. Therefore, the existence of carry-over effects is not very likely.

To our knowledge, this is the first study to investigate the combined effects of plant sterols and ezetimibe in humans. The combination has previously been studied in wild-type Kyoto rats (24), but their cholesterol levels increased after ezetimibe in combination with or without plant sterol treatment, probably because they possess a homozygous guanidine-to-thymine transversion in exon 12 of the Abcg5 gene (24). In our study, ezetimibe decreased cholesterol levels in correspondence with other human studies (5, 6). However, the combined effect of ezetimibe and plant sterols on lipid levels was unknown, although their mechanism of action is probably different. Ezetimibe reduces the absorption of cholesterol as well as that of plant sterols (17, 18, 25), probably through an interaction with the pathway that also involves NPC1L1 (10, 12). Plant sterols, on the other hand, are hypothesized to compete with cholesterol for incorporation into mixed micelles (26–29). Combining plant sterols and ezetimibe did not result in a significant decrease in LDL-C compared with ezetimibe alone, which may be attributable to a reduced amount of cholesterol that is available for the cholesterol transporter in the case of the combination treatment. As a consequence of the competition between plant sterols and cholesterol, the proportion of plant sterols in the micelles will increase and that of cholesterol will decrease (28). Because the rate of cholesterol and sitosterol absorption is directly proportional to their contents in micelles (30), an increased amount of plant sterols and a reduced amount of cholesterol will be transported through the brush border membrane and consequently be blocked by ezetimibe. In other words, the

Fig. 3. Plasma lathosterol- (A), cholestanol- (B), and campesterol- (C) to-cholesterol ratios at the end of treatment with placebo, plant sterols, ezetimibe, and the combination of plant sterols and ezetimibe. Values with different letters are significantly different from each other (P < 0.05).
The combined effects of plant sterols and ezetimibe

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amount of cholesterol that can be blocked in the brush border may decrease when ezetimibe is combined with plant sterols because of an altered proportion of sterols in the micelles. However, more studies are needed to confirm this hypothesis.

The results of the cholesterol absorption markers support the hypothesis that ezetimibe strongly inhibits the absorption of plant sterols (12, 17, 18). When we combined treatment with plant sterols and ezetimibe, the sitosterol and campesterol levels and their ratios to cholesterol were increased compared with those after ezetimibe monotherapy (Fig. 3, Table 3). However, the increase of plasma sitosterol and campesterol levels and ratios by the addition of plant sterols to ezetimibe treatment was only approximately one-third of the increase after plant sterol consumption alone, which confirms that the absorption of plant sterols was reduced by ezetimibe treatment. Surprisingly, plasma sitosterol and campesterol concentrations and ratios were lower after the combination than after placebo treatment. On the one hand, this finding suggests that ezetimibe decreases the absorption of a larger amount of plant sterols than is additionally available from micelles by the consumption of 2 g/day plant sterols, but it also confirms that ezetimibe strongly inhibits plant sterol absorption.

It has also been suggested that plant sterols may exert a cholesterol-lowering effect within the enterocyte besides the competition with cholesterol for incorporation into the micelles (29, 31). For instance, an increased amount of plant sterols within the enterocyte may upregulate liver ABC transporters or NPC1L1 (33); thus, data with respect to the upregulation of transport proteins by plant sterols or stanols are as yet inconsistent.

Our study had 80% power to detect a difference in LDL-C of 0.15 mmol/l, corresponding with 3.8% at a baseline level of 4 mmol/l or 5% at a baseline level of 3 mmol/l. Consequently, our study may have been underpowered to detect an effect of 0.12 mmol/l or 3.5% by adding plant sterols to ezetimibe. By increasing the number of subjects, the study would have gained more power and a difference of 0.12 mmol/l would have been statistically significant. However, an additional effect of only 0.12 mmol/l does not have much therapeutic advantage. We chose an LDL-lowering effect of >0.15 mmol/l to be clinically relevant, which was realized by plant sterols alone as well as by ezetimibe monotherapy (Table 2), but not by adding plant sterols to ezetimibe treatment. On the other hand, our subjects were mildly hypercholesterolemic, and including subjects with severe hypercholesterolemia may have resulted in a more pronounced difference between the combination therapy and ezetimibe monotherapy. Therefore, our results indicate that there is no therapeutic benefit of the combination of plant sterols and ezetimibe over ezetimibe monotherapy, at least in subjects with mildly increased cholesterol levels.

The lack of a placebo treatment for the ezetimibe arm is a limitation of this study. However, we included a placebo period consisting of a placebo spread to compare the effects of the other treatments. Usually, when a study lacks a placebo treatment, the effect of the intervention is calculated by subtracting baseline levels from levels after treatment, and consequently, a time effect or regression to the mean cannot be excluded. Because we included a placebo period, the effect of ezetimibe is not likely to be attributable to a time effect. Thus, even though the study design would have been more appropriate if a placebo treatment for the ezetimibe arm had been included, we think our data reflect real treatment consequences.

In conclusion, our results demonstrate that combination therapy of plant sterols and ezetimibe is safe. Nevertheless, the LDL-C reducing effect of combination therapy was not significantly greater than that of ezetimibe monotherapy, which indicates that combining the two cholesterol absorption inhibitors has hardly any therapeutic benefit compared with ezetimibe monotherapy in subjects with mild hypercholesterolemia.

### Table 4. Plasma liver and muscle enzyme concentrations after 4 weeks of treatment with placebo, plant sterols, ezetimibe, and the combination of plant sterols with ezetimibe

| Enzyme  | Placebo (n = 39) | Plant Sterol Spread (n = 39) | Ezetimibe (n = 39) | Plant Sterol Spread + Ezetimibe (n = 39) |
|---------|----------------|-----------------------------|-------------------|----------------------------------------|
| ASAT    | 24 [20–52]     | 25 [17–47]                  | 26 [19–92]        | 28 [19–59]                             |
| ALAT    | 25 [11–58]     | 25 [13–64]                  | 26 [11–95]        | 29 [13–72]                             |
| CPK     | 118 [58–534]   | 118 [55–483]                | 124 [54–281]      | 113 [53–419]                           |

All values are median and [range]. ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CPK, creatine phosphokinase.
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