Dietary Plant Stanol Esters Reduce VLDL Cholesterol Secretion and Bile Saturation in Apolipoprotein E*3-Leiden Transgenic Mice

Oscar L. Volger, Hans van der Boom, Elly C.M. de Wit, Wim van Duyvenvoorde, Gerard Hornstra, Jogchum Plat, Louis M. Havekes, Ronald P. Mensink, Hans M.G. Princen

Abstract—Dietary plant stanols lower serum cholesterol levels in humans and in hyperlipidemic rodents, mainly by inhibition of the intestinal cholesterol absorption. We used female apolipoprotein E*3-Leiden transgenic mice to investigate the consequences of this effect on serum lipid levels and hepatic lipid metabolism. Five groups of 6 or 7 mice received for 9 weeks a diet containing 0.25% cholesterol and 0.0%, 0.25%, 0.5%, 0.75%, or 1.0% (wt/wt) plant stanols (sitostanol 88% [wt/wt], campestanol 10% [wt/wt]) esterified to fatty acids. Compared with the control diet, plant stanol ester treatment dose-dependently reduced serum cholesterol levels by 10% to 33% (P<0.05), mainly in very low density lipoproteins (VLDLs), intermediate density lipoproteins, and low density lipoproteins. Furthermore, 1.0% of the dietary plant stanols significantly decreased the liver contents of cholesteryl esters (−62%), free cholesterol (−31%), and triglycerides (−38%) but did not change the hepatic VLDL-triglyceride and VLDL–apolipoprotein B production rates. However, plant stanol ester feeding significantly decreased the amounts of cholesteryl esters and free cholesterol incorporated in nascent VLDLs by 72% and 30%, respectively, resulting in a net 2-fold decreased VLDL cholesterol output. Liver mRNA levels of low density lipoprotein receptors, 3-hydroxy-3-methylglutaryl coenzyme A synthase, cholesteryl 7α-hydroxylase, and sterol 27-hydroxylase were not changed by plant stanol ester feeding. Nevertheless, the serum lathosterol-to-cholesterol ratio was significantly increased by 23%, indicating that dietary plant stanol esters increased whole-body cholesterol synthesis. Plant stanol esters also significantly decreased the cholesterol saturation index in bile by 55%. In conclusion, in apolipoprotein E*3-Leiden transgenic mice, plant stanol ester feeding dose-dependently lowered serum cholesterol levels as a result of a reduced secretion of VLDL cholesterol. This was caused by a decreased hepatic cholesterol content that also resulted in a lowered biliary cholesterol output, indicative of a reduced lithogenicity of bile in these mice. (Arterioscler Thromb Vasc Biol. 2001;21:1046-1052.)

Key Words: plant sterols ■ sitostanol ■ apolipoprotein E*3-Leiden transgenic mice ■ lipoproteins ■ liver lipid metabolism

Plant sterols are structurally closely related to cholesterol. In contrast to cholesterol, they are not synthesized by animals but by plant cells, in which they are abundantly present. Plant stanols, like sitostanol and campestanol, are saturated forms of the plant sterols β-sitosterol and campesterol, respectively, and occur in nature only in trace amounts. However, by saturation of plant sterols, large amounts of plant stanols can be obtained. Simply, through trans-esterification with rapeseed oil fatty acids, plant stanols and sterols can be converted into more fat-soluble stanol and sterol esters; this conversion makes them suitable for the addition to fat-containing foods, such as margarine and spreads. In several mammalian animal models, the hypocholesterolemic and antiatherosclerotic properties of plant sterols have been shown.1–4 Plant sterols and stanols lower serum cholesterol through inhibition of the intestinal cholesterol absorption of dietary and biliary cholesterol, leading to an increased fecal cholesterol excretion.2,5–9 In hypercholesterolemic children, unesterified sitostanol, compared with unesterified sitosterol, appears to be more effective in lowering serum cholesterol.10 However, fatty acid chain–esterified plant stanols (plant stanol esters) and plant sterols (plant sterol esters) are equally potent in lowering serum cholesterol levels.11,12

The present study uses apoE*3-Leiden transgenic mice and was conducted to investigate the physiological mechanism underlying the reducing effects of dietary plant stanol esters on serum cholesterol levels. We chose apoE*3-Leiden mice because these mice, in contrast to nontransgenic rodents, display a human-like lipoprotein profile with abundant VLDL/LDL-sized lipoproteins.13,14 When given semisinthetic sucrose–based diets, the serum lipid levels of these
mice can easily be varied by changing the amount of cholesterol and fat supplied via the diets.\textsuperscript{13} Furthermore, the serum cholesterol and triglyceride levels are highly responsive to small changes in chylomicron and VLDL metabolism.\textsuperscript{13,15} ApoE*3-Leiden transgenic mice respond well to hypolipidemic drugs and dietary interventions, such as fish oil and cafestol, the cholesterol-raising factor in boiled coffee.\textsuperscript{14,16,17}

In the present study, we observed that in apoE*3-Leiden mice, the feeding of plant stanol esters dose-dependently lowered serum cholesterol levels by reducing the hepatic availability of cholesterol for incorporation into nascent VLDLs. This also resulted in a reduced biliary cholesterol output.

**Methods**

**Animals, Housing, and Diets**

Three-month-old female apoE*3-Leiden transgenic mice were used for the experiments.\textsuperscript{18} Transgenic mice were identified with an ELISA for human apoE\textsuperscript{13} and were the F13 generation produced by breeding male apoE*3-Leiden transgenic mice with female C57BL/6J mice (The Broeckman Institute bv, Someren, the Netherlands). All animals were housed in wire-topped cages with sawdust as bedding, and all diets and water were given ad libitum. During the study period, animals were fed semisynthetic diets (see Table 1 for composition). During the complete study period, food disappearance (expressed as gram per mouse per day) was determined by periodically weighing of the diets. Dietary plant stanol esters were obtained by saturation of corresponding plant sterols and subsequent esterification with fatty acids from low erucic rapeseed oil (Raisio Group).

**Experimental Design**

At the start of a 4-week run-in period, diets were changed from a chow diet (SRM-A, Hope Farms) to a semisynthetic diet (diet group 1, Table 1). After this run-in period, the animals were separated into 5 different dietary groups in a first experiment and into 2 different groups in a second experiment; the animals were matched for age and serum cholesterol levels. In the first experiment, the respective dietary plant stanol contents were 0.0% (control group), 0.25%, 0.50%, 0.75%, and 1.0% (wt/wt) (Table 1), and in the second experiment, the plant stanol contents were 0.0% (control) and 1.0% (wt/wt) in the first and second groups, respectively. In experiment 1, the control group was composed of 6 mice, and the dietary stanol ester groups consisted of 7 mice. In the second experiment, the dietary groups each consisted of 12 mice. Blood was collected from the tail vein at week 0 (run-in end point), weeks 4 and 8 (first experiment), and week 9 (second experiment), after 4 hours of fasting.

**Serum Lipids, Lipoproteins, and Noncholesterol Sterols**

Levels of total serum cholesterol and triglycerides were measured enzymatically by using commercially available kits (236691, Boehringer-Mannheim, and 337-B, GPO-Trinder kit, Sigma Chemical Co). For determination of serum lipoprotein profiles, pooled serum was subjected to density gradient ultracentrifugation in an SW41 rotor (Beckman Instruments, Inc). After fraction collection, density (assessed by a DNA 602 mol/L densitometer, Paar) and total cholesterol and triglyceride levels were measured by using commercial enzymatic kits (cholesterol was measured as described above; triglycerides were measured by use of a GPO-PAP kit, Sigma). Serum noncholesterol sterols were determined as described by Kempen et al.\textsuperscript{19} 5α-Cholestane was used as an internal standard. The different sterols were separated on a 25-m×0.35-mm capillary GLC column (CP Sil 5CB, Chrompack Int) in a Varian 3800 gas chromatograph equipped with a flame ionization detector. The injector temperature was raised from 240°C to 280°C in 10 minutes, and the flame ionization detector temperature was kept at 300°C. Quantification was based on the area ratio of the individual sterol to 5α-cholestane.

**Liver Lipids, mRNA Analyses, and Cholesterol 7α-Hydroxylase Activity**

Lipids were extracted from livers and separated by high-performance thin-layer chromatography.\textsuperscript{20} Quantification was performed after charring. Total RNA was isolated from the liver by using a single-step method.\textsuperscript{21} Northern blotting and hybridization techniques were performed as described by Post et al.\textsuperscript{22} Blots were subsequently hybridized with \textsuperscript{32}P-labeled cDNA probes of LDL receptor, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase,

**TABLE 1. Composition of Experimental Diets**

<table>
<thead>
<tr>
<th>Dietary Components, g/100-g Diet</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western fat</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Shortening</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Experimental</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Sucrose</td>
<td>38.5</td>
<td>38.5</td>
<td>38.5</td>
<td>38.5</td>
<td>38.5</td>
</tr>
<tr>
<td>Corn starch</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cellulose</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Cellulose*</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
</tr>
<tr>
<td>Free stanols*</td>
<td>0.0</td>
<td>0.25</td>
<td>0.50</td>
<td>0.75</td>
<td>1.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Western fat contained 40.7% monounsaturated fatty acid, 17.4% polyunsaturated fatty acid, and 41.9% saturated fatty acids. Total fat provides 37.0% energy; sucrose, 35.0% energy; corn starch, 9.2% energy; and casein, 18.8% energy.

*Percentages (wt/wt) were calculated for the amount of free stanols but were provided as stanol esters, which contained 87.6% sitostanol, 9.5% campestanol, 1.1% β-sitosterol, 0.8% campesterol, 0.5% sitostane, and 0.1% campestanol in the first experiment. In the second experiment, the stanol esters consisted of 73.0% sitostanol, 24.7% campestanol, 1.1% β-sitosterol, 0.9% campesterol, 0.3% sitostane, and 0.1% campestanol. All diets contained 1% choline chloride (50% [wt/vol]), 0.2% methionine, 4.85% mineral mixture, and 0.25% vitamin mixture (all wt/vol).
cholesterol 7α-hydroxylase, and sterol 27-hydroxylase. SB34 and GAPDH probes were used as internal standards to correct for differences in the amount of RNA applied to the gel or filter. The intensity of the hybridization signal was quantified with a PhosphorImager (Molecular Dynamics). Microsomal cholesterol 7α-hydroxylase activity of freshly isolated liver microsomes was determined as described by Post et al.17

Bile Analyses
After the animals had fasted for 4 hours and were anesthetized as described below, gallbladders were cannulated (littermates of the study mice were used for measuring VLDL production and nascent VLDL lipid composition), and bile was immediately collected as described by Kuipers et al.21 Biliary bile acid concentration was determined by an enzymatic kit (Nycomed Pharma AS). After gall bladder bile was collected, newly produced bile was collected for 90 minutes in intervals of 15 minutes. Hepatic bile acid synthesis rates were determined at the nadir of individual bile acid output versus time curves (average of 60- to 75-minute and 75- to 90-minute intervals).23 The biliary bile acid pool size was determined by calculating the area under the curve of individual bile acid output versus time plots, after subtractions of the hepatic bile acid synthesis rates. Biliary cholesterol and phospholipids were measured enzymatically by using kits as described above. The cholesterol saturation index of bile was determined by using calculations described by Carey.24

VLDL Production and Nascent VLDL Lipid Composition
After 4 hours of fasting, the mice were anesthetized (with 2.5 mL/kg Dormicum, Roche, and 2.5 mL/kg Hypnorm, Janssen Pharmaceutica). In vivo hepatic VLDL apoB and VLDL triglyceride production were determined after intravenous [3H]methionine and Triton WR-1339 injection, as described by Post et al.17 Blood samples were taken at 30 seconds and at 30, 60, and 90 minutes after Triton WR 1339 injection, and serum triglycerides were measured. Liver triglyceride production was calculated from the slope of the curve and was expressed as micromoles per hour per kilogram body weight.15 Nascent VLDL was isolated from serum (collected 90 minutes after Triton WR 1339 injection) by density gradient ultracentrifugation. For determination of the lipid composition of nascent VLDL, total cholesterol, free cholesterol, phospholipid, and triglyceride contents were measured enzymatically by using kits (cholesterol and phosphatidylcholine; Boehringer-Mannheim; and triglycerides; Boehringer-Mannheim). Cholesteryl esters were calculated as the difference between total and free cholesterol. For quantification of apoB content and serum cholesterol, apoB was isolated by precipitation of VLDL with isopropanol (VLDL-to-isopropanol ratio was 1:1 by volume) for 1 hour at room temperature, followed by centrifugation (Biofuge A, Heraeus Sepatech GmbH). Supernatants were removed, and pellets were dissolved in 0.1 mol/L NaOH at 95°C. VLDL total protein and apoB contents were determined by using the method of Lowry. Statistical Analyses
For determination of the relationship between the dietary stanol content and serum cholesterol levels, regression analysis (curve estimation) was performed. For comparisons of 2 groups, data were analyzed nonparametrically by using the Mann-Whitney U test.APOB CONTENT AND SERUM CHOLESTEROL LEVELS Table 2

Results

Serum Lipids, Lipoproteins, and Noncholesterol Sterols
Food intake and body weights were not affected by stanol ester feeding (data not shown). Serum cholesterol levels decreased dose-dependently after the feeding of stanol esters (Figure, panel A). Maximal cholesterol lowering had already been obtained after 4 weeks of treatment in the dietary plant stanol groups treated with 0.5%, 0.75%, and 1.0% (wt/wt) plant stanols. Regression analysis revealed a significant negative relationship between dietary stanol ester content and serum cholesterol levels (Figure, panel A, insert). Serum triglyceride levels remained unaffected by dietary stanol esters (data not shown). Separation of lipoproteins showed that stanol ester feeding caused a dose-dependent decrease in VLDL, IDL, and (to a lesser extent) LDL cholesterol (Figure, panel B). Plant stanol ester feeding had no effect on serum HDL cholesterol levels (Figure, panel B).

Because the effects on serum cholesterol levels were most pronounced in the groups treated with 1% (wt/wt) dietary plant stanols, we studied the underlying mechanism of this cholesterol-lowering effect at a 1% (wt/wt) dose of dietary plant stanols. The ratios of serum levels of plant steroles to cholesterol reflect the fractional intestinal absorption of (dietary and biliary) cholesterol.25 Compared with the control diet, the plant stanol ester diet lowered the ratio of serum plant steroles to cholesterol (Table 2), indicating that the intestinal cholesterol absorption was reduced by dietary plant stanol esters. Serum plant stanol levels were also measured and were virtually undetectable in control mice (Table 2). Plant stanol levels were increased in plant stanol ester–treated mice compared with control mice. However, in the plant stanol ester–treated mice, the plant stanol levels were still markedly lower than the levels of the plant sterols.

Lathosterol is a cholesterol precursor sterol, and its serum levels relative to serum cholesterol indicate whole-body cholesterol synthesis.19 Compared with the control diet, the plant stanol ester diet significantly increased serum lathosterol-to-cholesterol ratios (Table 2), indicating that plant stanol ester feeding increased whole-body cholesterol synthesis.

Hepatic Lipid and Bile Metabolism
Livers of stanol ester–treated mice had significantly lower cholesteryl ester, free cholesterol, and triglyceride contents...
TABLE 2. Effect of Dietary Plant Stanol Esters on Serum Levels of Plant Stanols and Noncholesterol Sterols Relative to Serum Cholesterol

<table>
<thead>
<tr>
<th>Plant Stanol</th>
<th>Control Diet</th>
<th>Plant Stanol Ester Diet</th>
<th>μmol/mmol Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campesterol</td>
<td>ND</td>
<td>0.21 ± 0.02* (840)</td>
<td></td>
</tr>
<tr>
<td>Sitostanol</td>
<td>0.08 ± 0.05</td>
<td>0.20 ± 0.03* (253)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD (n = 3 pools of 2 mouse sera each). ND indicates not detectable. Mice were treated with 1% (wt/wt) plant stanols or control diet (respective diet groups 5 and 1; see Table 1) for 8 weeks. Serum plant stanols and noncholesterol sterols were determined by using gas-liquid chromatography as described in Methods and were expressed as μmol/mmol cholesterol, because serum cholesterol levels influence the levels of the measured sterols. Values between parentheses represent the percentage of the value obtained in mice on a control diet.

*P < 0.05 for control diet vs stanol ester diet (Mann-Whitney U test).

than did livers of control mice (Table 3). To investigate the consequences of the decreased hepatic cholesterol content, we measured liver mRNA levels of HMG-CoA synthase and LDL receptors.

TABLE 3. Effect of Dietary Plant Stanol Esters on Hepatic Lipids and mRNA Levels

<table>
<thead>
<tr>
<th>Lipids, μg/mg protein</th>
<th>Control Diet</th>
<th>Stanol Ester Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl ester</td>
<td>46.6 ± 1.7</td>
<td>17.6 ± 3.4* (38)</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>17.7 ± 2.1</td>
<td>12.3 ± 0.4* (69)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>106.8 ± 4.3</td>
<td>66.2 ± 10.7* (62)</td>
</tr>
</tbody>
</table>

mRNA levels, % of control diet

<table>
<thead>
<tr>
<th>LDLC receptor</th>
<th>SB34</th>
<th>100 ± 14</th>
<th>102 ± 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>100 ± 25</td>
<td>98 ± 10</td>
<td></td>
</tr>
<tr>
<td>HMG-CoA synthase</td>
<td>SB34</td>
<td>100 ± 19</td>
<td>92 ± 13</td>
</tr>
<tr>
<td>GAPDH</td>
<td>100 ± 29</td>
<td>87 ± 11</td>
<td></td>
</tr>
<tr>
<td>Cholesterol 7α-hydroxylase</td>
<td>SB34</td>
<td>100 ± 19</td>
<td>94 ± 9</td>
</tr>
<tr>
<td>GAPDH</td>
<td>100 ± 28</td>
<td>90 ± 9</td>
<td></td>
</tr>
<tr>
<td>Sterol 27-hydroxylase</td>
<td>SB34</td>
<td>100 ± 7</td>
<td>104 ± 10</td>
</tr>
<tr>
<td>GAPDH</td>
<td>100 ± 12</td>
<td>102 ± 8</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD (n = 3 pools of 2 mouse livers each). Mice were treated with 1% (wt/wt) plant stanols or control diet (respective diet groups 5 and 1; see Table 1) for 8 weeks. Livers were isolated immediately after the VLDL-apoB and VLDL-triglyceride production experiment. In liver homogenates, free cholesteryl, cholesteryl ester, and triglycerides were determined by using high-performance thin-layer chromatography as described in Methods. Values between parentheses represent the percentage of the value obtained in mice on a control diet. mRNA was isolated from liver homogenates as described in Methods.

*P < 0.05 for control diet vs treated mice (Mann-Whitney U test).

Compared with the control diet, the plant stanol ester diet had no effect on liver mRNA levels of HMG-CoA synthase and LDL receptors (Table 3). In addition, dietary plant stanols had no influence on hepatic mRNA levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase (Table 3).

In line with the mRNA measurements, no effect of 8-week plant stanol ester feeding on microsomal cholesterol 7α-hydroxylase activity was found (Table 4). Furthermore, no effects of plant stanol ester treatment on bile flow and the biliary outputs of bile acids and phospholipids were found (Table 4). In addition, control and plant stanol ester–treated mice had similar bile acid pool sizes and hepatic bile acid synthesis rates (Table 4). However, compared with the control diet, the dietary plant stanol ester diet significantly reduced biliary cholesterol output by > 50%. As a consequence, the biliary cholesterol saturation index was significantly decreased by 55% (Table 4).

VLDL Production

To assess whether the decreased hepatic cholesterol levels in plant stanol ester–fed mice had an effect on the secretion of cholesterol into the circulation, we measured VLDL-triglyceride and apoB production and the lipid content of nascent VLDL (Table 5). Plant stanol ester feeding had no effect on the secretion by the liver of VLDL-triglyceride. In addition, VLDL-apoB production was also unchanged, indicating that the number of VLDL particles produced by the liver was not affected by plant stanol ester feeding. However, dietary plant stanols significantly reduced the absolute amounts of cholesteryl ester (−70%) and free cholesterol (−28%) per nascent VLDL particle, resulting in a net decreased hepatic cholesterol output (−58%). This is indicative of the secretion of a β-VLDL–like particle less enriched in cholesterol. In contrast, the average phospholipid and triglyceride contents of nascent VLDL were not increased; the latter is in line with the absence of a change in serum triglyceride levels.

Discussion

In the present study with transgenic apoE*3-Leiden mice, plant stanol esters dose-dependently decreased serum cholesterol levels, mainly in the VLDL and IDL fractions and, to a lesser degree, in LDL. No effect on serum triglycerides was found. Dietary plant stanol esters lowered liver lipid levels and reduced hepatic secretion of cholesterol via VLDL and bile. No adverse health effects on plant stanol ester feeding were observed.

Similar to our findings, plant stanols esters lower serum cholesterol without affecting serum triglycerides in mildly hypercholesterolemic nondiabetic humans, although the hypercholesterolemic effect is mainly in the LDL fraction. In addition, in non–insulin-dependent diabetic patients, dietary plant stanol esters reduce serum cholesterol levels in VLDL and LDL without having an effect on serum triglycerides, which is even more comparable to our findings in mice than in nondiabetic humans. The sensitive hypocholesterolemic response to plant stanol ester feeding in apoE*3-Leiden mice is explained by the dominance of the apoE*3-Leiden mutation, defecting the clearance of postprandial lipoproteins. This leads to accumulation of these lipoproteins and results in a human-like lipoprotein profile under dietary challenge with fat and cholesterol. In this phenotype, any change in
TABLE 4. Effect of Plant Stanol Ester Feeding on Flow and Composition of Bile and Cholesterol 7α-Hydroxylase Enzyme Activity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Diet</th>
<th>Stanol Ester Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol 7α-hydroxylase activity, mmol·h⁻¹·mg protein⁻¹</td>
<td>10.7±0.7</td>
<td>11.7±4.0 (110)</td>
</tr>
<tr>
<td>Bile flow, μL·min⁻¹·100 g body wt⁻¹</td>
<td>7.2±1.1</td>
<td>7.1±1.0 (94)</td>
</tr>
<tr>
<td>Output, mmol·min⁻¹·100 g body wt⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile acid output</td>
<td>438.8±299.6</td>
<td>403.8±129.3 (92)</td>
</tr>
<tr>
<td>Cholesterol output</td>
<td>4.3±1.3</td>
<td>2.1±0.1* (49)</td>
</tr>
<tr>
<td>Phospholipid output</td>
<td>70.1±7.5</td>
<td>74.3±9.9 (105)</td>
</tr>
<tr>
<td>Bile cholesterol saturation index</td>
<td>0.98±0.56</td>
<td>0.44±0.055* (45)</td>
</tr>
<tr>
<td>Bile acid pool size, μmol/100 g body wt</td>
<td>11.3±5.0</td>
<td>11.4±2.1 (101)</td>
</tr>
<tr>
<td>Hepatic bile acid synthesis, nmol·min⁻¹·100 g body wt⁻¹</td>
<td>171±25</td>
<td>186±14 (109)</td>
</tr>
</tbody>
</table>

Values are mean±SD of 6 mice per group. Cholesterol 7α-hydroxylase enzyme activity was determined in hepatic microsomes as described in Methods. Bile was collected for 15 minutes immediately after bile flow by creation. Bile flow and bile acid synthesis were determined as described in Methods. Values between parentheses represent the percentage of the value obtained in mice on a control diet.

*P<0.05 for control diet vs stanol ester diets (Mann-Whitney U test).

TABLE 5. Effect of Plant Stanol Ester Feeding on Production of VLDL-Triglyceride and VLDL-ApoB and on Lipid Composition of Nascent VLDL

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Diet</th>
<th>Stanol Ester Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL-triglyceride production, μmol·h⁻¹·kg body wt⁻¹</td>
<td>80.8±13.1</td>
<td>98.7±19.5 (122)</td>
</tr>
<tr>
<td>VLDL-apoB production, 10³ dpm·h⁻¹·kg body wt⁻¹</td>
<td>145±45</td>
<td>131±59 (90)</td>
</tr>
<tr>
<td>Nascent VLDL lipid composition, μmol/mg apoB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>13.4±3.0</td>
<td>3.8±1.8* (28)</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>6.3±1.3</td>
<td>4.4±1.2* (70)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>17.4±2.0</td>
<td>20.5±4.9 (117)</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>9.6±1.0</td>
<td>8.0±2.1 (83)</td>
</tr>
</tbody>
</table>

Values are mean±SD of 6 mice per group. Mice were treated with 1% (wt/wt) plant stanols or control diet (respectively diet groups 5 and 1; see Table 1) for 8 weeks. After [35S]methionine and Triton WR-1339 injection, triglyceride and de novo apoB production and lipid composition of VLDL were determined as described in Methods. Values between parentheses represent the percentage of the value obtained in mice on a control diet.

*P<0.05 for control diet vs stanol ester diet (Mann-Whitney U test).

Cholesterol input is reflected by changes in serum cholesterol levels. Plant stanol ester feeding reduced ratios of serum plant sterol to cholesterol, indicating inhibition of the intestinal absorption of plant sterols and cholesterol, which is in line with observations in humans and rodents. As a consequence of the decreased intestinal cholesterol absorption, leading to a reduced flux of esterified and free cholesterol from the intestines to the liver via chylomicrons, we found that stanol ester feeding resulted in a reduction in hepatic cholesterol and free cholesterol content. Theoretically, a decrease in the hepatic pool of free cholesterol would lead to an upregulation of the genes involved in cholesterol synthesis and in the LDL receptor gene. This process is mediated via sterol regulatory element–binding proteins, which act as sensitive sensors of the putative regulatory pool of free cholesterol in the cells. We found that plant stanol ester feeding did not alter mRNA levels of HMG-CoA synthase and the LDL receptor, indicating that the liver was not depleted of cholesterol in these cholesterol-fed mice. The livers of stanol ester–treated mice still contained considerable amounts of cholesterol compared with the amount in the livers of chow-fed apoE*3-Leiden mice (cholesterol ester in the latter is 3 to 5 μg/mg protein). It has been shown that intravenous infusion of a high dose of phytosterols can inhibit hepatic cholesterol synthesis and that HMG-CoA reductase mRNA is reduced in sitosterolemics. Although we cannot fully exclude the possibility that accumulation of plant stanols in the liver may explain the lack of effect on HMG-CoA synthase and LDL receptor mRNAs, we think that this is unlikely. In our experiment, liver levels were not measured, but serum sterol levels were decreased, and although serum plant stanol levels were increased, they remained markedly lower than the plant sterol concentrations. Furthermore, plant sterols and stanols are efficiently removed from the liver into bile by specific transport mechanisms. In addition, at least high tissue β-sitosterol concentrations do not inhibit HMG-CoA reductase activity and mRNA levels.

In line with observations in humans and rodents, plant stanol ester treatment did increase whole-body cholesterol synthesis, which is reflected by an elevated serum ratio of lathosterol to cholesterol in apoE*3-Leiden mice. There may be several other reasons for this apparent discrepancy. The cholesterol synthesis, in which HMG-CoA reductase plays a key role, is regulated (next to the regulation of mRNA levels) at multiple levels, ie, gene transcription, mRNA and protein stability, enzyme activity, and availability of substrate. Furthermore, it should be noted that lathosterol is a marker of whole-body cholesterol synthesis. The liver and intestines are the major organs involved in cholesterol synthesis. However, it is conceivable that a reduced supply of lipoprotein cholesterol in stanol ester–treated mice may also lead to upregulation of synthesis in other tissues. The compensatory upregulation of whole-body cholesterol synthesis, in response to the reduced intestinal cholesterol absorption, is obviously not sufficient to overcome the hypocholesterolemic effect of stanol ester treatment.

An additional reason why the LDL receptor is not regulated is that liver cholesterol homeostasis in mice (in contrast to humans) is mainly regulated via cholesterol synthesis rather than via LDL receptor–mediated uptake of cholesterol. This finding also indicates that the hypocholesterolemic effect of plant stanol esters cannot be explained by an increased LDL receptor–dependent clearance of apoB-containing lipoproteins in apoE*3-Leiden transgenic mice. Plant stanol ester feeding had no effect on hepatic mRNA levels and enzyme activity of bile acid synthetic enzymes or on biliary bile acid output. These data indicate that the decreased hepatic cholesterol content had no effect on the neutral and acidic pathways in bile acid synthesis. This is in line with observations in mildly hypercholesterolemic non–
Non–insulin-dependent diabetic patients, the LDL-apoB pro-
triglycerides in VLDL and vice versa.3 8–4 0 In addition, in
cholesterol pool is required for the secretion and transport of
ester, regulates apoB secretion and that the hepatic metabolic
that hepatic cholesterol, specifically the amount of cholesteryl
atherogenic
the hepatic production of cholesterol-poor and, therefore, less
particles (apoB). Thus, plant stanol ester treatment resulted in
rates of VLDL triglycerides and the amount of VLDL
levels, we found that the decreased liver cholesterol content
lead to a decreased lithogenicity of bile in humans, which is
at variance with findings in apoE*3-Leiden mice.
As an explanation for the decreased serum cholesterol
evels, we found that the decreased liver cholesterol content
led to a reduced hepatic output of cholesteryl ester and free
cholesterol in nascent VLDL, without affecting the output
rates of VLDL triglycerides and the amount of VLDL
particles (apoB). Thus, plant stanol ester treatment resulted in
the hepatic production of cholesterol-poor and, therefore, less
atherogenic β-VLDL particles.

Data from in vivo and in vitro studies in animals indicate
that hepatic cholesterol, specifically the amount of cholesteryl
ester, regulates apoB secretion and that the hepatic metabolic
cholesterol pool is required for the secretion and transport of
triglycerides in VLDL and vice versa.3 8–4 0 In addition, in
non–insulin-dependent diabetic patients, the LDL-apoB pro-
duction rate was lowered by dietary plant stanol esters.3 8 Our
finding that there was no change in VLDL-apoB production with
plant stanol ester treatment indicates that in mildly
cholesterol-fed apoE*3-Leiden mice, the size of the hepatic
cholesteryl ester pool did not become rate limiting for apoB
secretion.
In apoE*3-Leiden mice, plant stanol ester feeding lowered
the liver triglyceride content. The biochemical background of
this finding awaits further investigation. The lowered liver
triglyceride content implicates that during the assembly of
nascent VLDL, the reduced cholesteryl ester incorporation
was not compensated by an increased incorporation of tri-
glycerides, as observed.

In conclusion, in apoE*3-Leiden mice, the reduced hepatic
cholesterol content on plant stanol ester feeding was respon-
sible for the serum cholesterol-lowering effect via a de-
creased incorporation of cholesterol in nascent VLDL. In
addition, the reduced hepatic cholesterol content led to a
reduced biliary cholesterol saturation. Reduction of the litho-
genicity of bile may be an additionally favorable feature of
plant stanol ester consumption, which may only be applicable
to these mice. We have recently shown that feeding plant
stanol esters to these mice also dramatically reduced the
extent and severity of atherosclerosis.4 1

Acknowledgments
We thank Raisio Benecol Ltd, Finland, and the Netherlands Heart
Foundation (grant No. 95.057) for funding and Marjan Bekkers for
technical assistance.

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Dietary Plant Stanol Esters Reduce VLDL Cholesterol Secretion and Bile Saturation in Apolipoprotein E*3-Leiden Transgenic Mice

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doi: 10.1161/01.ATV.21.6.1046

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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