Plant Sterol Feeding Induces Tumor Formation and Alters Sterol Metabolism in the Intestine of Apc$^{\text{Min}}$ Mice

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PLEASE SCROLL DOWN FOR ARTICLE
Plant Sterol Feeding Induces Tumor Formation and Alters Sterol Metabolism in the Intestine of Apc\textsuperscript{Min} Mice

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Dietary plant sterols reduce the absorption of cholesterol and therefore increase intraluminal cholesterol concentration. We examined how plant sterol esters from functional foods affect intestinal tumorigenesis in tumor-prone adenomatous polyposis coli (Apc)\textsuperscript{Min} mice. Feeding plant sterols at 0.8% increased the number of intestinal adenomas, and the effect was significant in female mice. The concentration of mucosal free sitosterol increased by eightfold in plant sterol males and by threefold in plant sterol females when compared with respective controls. The concentration of mucosal free cholesterol was significantly lower in plant sterol males than in control males, and the decrease in free cholesterol was accompanied with a significant increase in nuclear sterol regulatory element binding protein-2. No difference was found in the levels of β-catenin, cyclin D1, epidermal growth factor receptor, extracellular signal-regulated kinase 1/2, or caveolin-1 in either gender after plant sterol feeding. Among all measured parameters, higher levels of estrogen receptor β and free cholesterol in the mucosa were among the strongest predictors of increased intestinal tumorigenesis. In addition, gene expression data showed significant enrichment of up-regulated genes of cell cycle control and cholesterol biosynthesis in plant sterol females. The results indicate that high intake of plant sterols accelerates intestinal tumorigenesis in female Apc\textsuperscript{Min} mice; however, the mechanism behind the adverse effect remains to be discovered.

INTRODUCTION

Plant derived plant sterols (phytosterols) are dietary compounds that are structurally similar to mammalian cholesterol. Good natural sources of plant sterols are vegetable oils, vegetables, nuts, cereal, and fruit. The intake of plant sterols varies from 200 to 400 mg/day (1–4) but it may notably increase when plant sterol-enriched functional foods are consumed (5,6). For
almost 2 decades, plant sterol and stanol (saturated plant sterol) ester-enriched functional foods have been marketed for their beneficial effect on serum LDL cholesterol. Plant sterols reduce the absorption of dietary and biliary cholesterol from the gut and thereby lower serum LDL cholesterol. An intake of 2 g/day of plant sterols is efficient to achieve a 10% reduction in serum LDL cholesterol (7).

Epidemiological studies have demonstrated that increased intake of plant sterols is associated with reduced risk for several types of cancer [e.g., cancer of stomach (8), breast (9), and lung (10,11)]. The association between plant sterol intake and colorectal cancer is less consistent. The Netherlands Cohort Study on Diet and Cancer did not find an association between high intake of plant sterols and reduced risk for colorectal cancer (1). On the contrary, the authors reported a positive association between some individual plant sterols and rectal cancer in men. Plant sterol supplementation has been shown to inhibit colon tumorigenesis in some studies with carcinogen-treated rats (12,13), but also no effects on colon cell proliferation (14) or colon tumor formation (15) have been reported. We have previously shown that a 0.8% plant stanol-enriched diet accelerated intestinal adenoma formation in adenomatous polyposis coli (Apc)\textsuperscript{Min} mice by upregulating Wnt and epidermal growth factor receptor (Egfr) signaling (16). Dietary plant sterols and stanols are poorly absorbed from the intestine and their serum levels remain low; however, concentrations of plant sterols and cholesterol in the feces increase after plant sterol supplementation. High intraluminal cholesterol concentration has been associated with increased carcinogenesis in the murine colon (17,18).

We conducted the present study to investigate the effect of a 0.8% (w/w) plant sterol-enriched diet in Apc\textsuperscript{Min} mice. The Apc\textsuperscript{Min} mouse is a widely used model to study dietary factors and intestinal carcinogenesis. The mouse carries a germline mutation in the Apc gene that leads to the development of spontaneous adenomas/tumors in the small intestine, although adenomas in the colon are rare (19). In humans, the Apc mutation is involved in the hereditary colon cancer syndrome, FAP (familial adenomatous polyposis), and mutated APC can be detected in a majority of sporadic colon cancer tumors (20). Disturbed function of APC protein results in nuclear accumulation of $\beta$-catenin and activation of Wnt signaling (21). In the nucleus, $\beta$-catenin activates gene expression of, e.g., cyclin D1 and c-Myc that mediate oncogenic transformation in colorectal cancer (22,23). In addition to Wnt, the Egfr pathway plays a pivotal role in colon tumorigenesis (21,24) and its activation results in uncontrolled cell growth. Egfr is a plasma membrane receptor that upon activation phosphorylates downstream targets such as extracellular signal-regulated kinases (ERK1/2).

In this study, the effect of dietary plant sterols on intestinal tumor formation was studied in both male and female mice. Concentrations of cholesterol, plant sterols and stanols were analyzed from feces and intestinal mucosa. Levels of $\beta$-catenin, cyclin D1, Egfr, ERK1/2, and estrogen receptor (ER) $\alpha$ and $\beta$ subtypes were measured from the histologically normal-appearing intestinal mucosa. Furthermore, RNA was isolated from intestinal mucosa and analyzed for changes in the global gene expression.

**MATERIALS AND METHODS**

**Animals**

The study protocol was approved by the Laboratory Animal Ethics Committee, University of Helsinki, Finland. Male and female C57BL/6J Apc\textsuperscript{Min} mice were bred at the Laboratory Animal Centre in Viikki, Helsinki, from inbred mice originally obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were screened for the Min genotype using a PCR assay described by Dietrich et al. (25). The 5-wk-old Apc\textsuperscript{Min} mice were assigned into the experimental groups by their weight and litter background. The mice were housed in plastic cages in humidity- and temperature-controlled facilities with 12-h light-dark cycle, and food and water was given ad libitum. During the experiment, the mice were weighed and monitored weekly. One male mouse from both experimental groups was sacrificed before the end of experiment due to a considerable weight-loss within 1 wk. These mice were excluded from the data. Furthermore 1 female mouse from the plant sterol group was excluded from the data for having over 170 adenomas in the small intestine. Altogether 6 control male and 7 control female mice, and 6 plant sterol male and 5 plant sterol female mice were included in the study.

**Diets**

The control diet was a modified low-fiber, high-fat (40% of energy from fat) AIN93-G diet (26,27) (Supplemental Table 1). Plant sterols were added to the diet in plant sterol ester-enriched margarine (Becel pro.activ\textsuperscript{R}, Unilever), whereas margarine without plant sterol esters was added to the control diet (Becel\textsuperscript{R}, Unilever). In both diets, AIN93-G diet was modified by adding freeze-dried food products that were used in the control diet in our previous study with plant stanols (16). This was done to make these 2 studies as similar as possible in the diet composition. The plant sterol diet contained 0.8% (w/w) free plant sterols, which corresponds to 1.4% as esterified plant sterols. The approximate daily intake of plant sterols was 20 mg per mouse in the plant sterol group. The protein, carbohydrate, fat, fatty acid, cholesterol, plant sterol, vitamin, and mineral content of both diets were otherwise similar (Supplemental Table 1).

**Evaluation of Adenoma Development and Collection of Samples**

After the 9-wk feeding period, the mice were killed by CO$_2$ asphyxiation. The small intestine, caecum, and colon were removed and opened along the longitudinal axis, rinsed with ice-cold saline, and kept on ice throughout sample collection. The adenomas were counted and their diameter was measured under dissecting microscope by observers unaware of the treatment. The intestinal adenomas were excised from the tissue, and the histologically normal-appearing mucosa was scraped off from
lamina propria and used for protein and RNA analyses. The tissue samples for protein analyses were snap frozen in liquid nitrogen and stored at −70 °C. Samples for RNA analysis were stored in stabilization solution (RNAlater, Qiagen, Valencia, CA) in −20 °C. Fecal samples were collected from the caecum of each mouse and were stored at −20 °C.

### Analysis of Fecal and Mucosal Sterols

Free and esterified sterols were analyzed from the caecum content and the mucosa of the proximal small intestine of each mouse. Dihydrocholesterol (95%, Sigma, St. Louis, MO) was added to each sample as an internal standard and 5α-cholestane (Sigma, St. Louis, MO) was added to control the separation of esterified sterols from free sterols by solid phase extraction (SPE). The sample was first homogenized in a mixture of 0.5 M natrium acetate, methanol, and chloroform (1.6:4:2, vol:vol:vol) and incubated in room temperature for half an hour. Next, chloroform was added and incubation continued for another half an hour, and then, Milli-Q-water was added and the mixture was again incubated for half an hour. The chloroform phase was transferred to a round bottom flask and the water phase was washed with chloroform. The chloroform phases were combined, chloroform was evaporated at 50 °C, and the lipids were collected from the flask with heptane-diethylether (9:1, vol:vol:vol). This extract was used to fractionate free and esterified sterols with SiOH-column (Strata SI-1 Silica 500 mg; Phenomenex, Torrance, CA) in a SPE vacuum manifold (Supelco Visiprep DL, Sigma-Aldrich Co.). Silylation was carried out to obtain trimethylsilyl derivatives of sterols, using pyridine and bis(trimethylsilyl) trifluoroacetamide: trimethylchlorosilane (99:1, vol:vol) as silylation agents (1:1, vol:vol) (16). The sample was injected into a RTX-5 w/Integra Guard (crossbond 5% diphenyl-95% dimethyl polysiloxane) capillary column (60 m × 0.32 mm i.d., 0.10 μm film; Restek, Bellefonte, PA) by automated injector of a gas chromatograph equipped with a flame ionization detector (Agilent 6890N Network GC System, Agilent Technologies, Santa Clara, CA). Sterols were quantified using an internal standard method. Identification was based on retention times and GC-mass spectrometric analysis. A sample of rapeseed oil was used to control interassay variation.

### Western Blotting Analysis

Proteins were isolated from histologically normal-appearing mucosa of the distal small intestine, which represents approximately 40% of total small intestine. Sample preparation and Western blot analysis has been previously described by Marttinen et al. (16). The protein lysis buffer was supplemented with proteinase and phosphatase inhibitors (0.4 mM leupeptin, 3.0 μM pepstatin, 1.0 mM PMSF in DMSO, 10 mM sodium fluoride, and 0.1 mM sodium orthovanadate). Antibodies towards β-catenin (sc-7199), lamin B (sc-6216), Egfr (sc-03), ERK1 (sc-94), caveolin-1 (sc-894), ERα (sc-542) and sterol regulatory element binding protein-2 (SREBP-2) (sc-5603) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies were anti-cyclin D1 (SP4, NeoMarkers), anti-β-actin (A541, Sigma), phospho-p44/42 (9101, Cell Signaling Technology), and ERβ (PA1–310B, Thermo Scientific). Blots of β-catenin, cyclin D1, ERK1/2, and ERα were transferred to X-ray film (Amersham), and scanned and analyzed by GSA-800 Calibrated Imaging Densitometer and Quantity One Program (BioRad Laboratories, Hercules, CA). Egfr, caveolin-1, ERα, and SREBP-2 were detected and quantified with Odyssey infrared imager (LI-COR, Inc., Lincoln, NE). β-Actin and lamin B were used to control equal loading of protein samples.

### RNA Isolation and Microarray

Total RNA was extracted from histologically normal-appearing mucosa of the distal small intestine using the RNaseasy Mini kit (Qiagen, Valencia, CA). RNA was quantified using the Nanodrop 1000 spectrophotometer (Thermo Scientific, ABBOTT, Wiesbaden, Germany) and purified using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the RNA integrity number (RIN) was calculated. RNA was reverse transcribed to cDNA using the SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

### Table 1: Fecal sterols by diet and gender

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Control males, M (SD) (μg/100 mg)</th>
<th>Plant sterol males, M (SD) (μg/100 mg)</th>
<th>Control females, M (SD) (μg/100 mg)</th>
<th>Plant sterol females, M (SD) (μg/100 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>81 (32)</td>
<td>265 (94)</td>
<td>83 (17)</td>
<td>307 (112)</td>
</tr>
<tr>
<td>Plant sterols</td>
<td>97 (48)</td>
<td>1442 (564)</td>
<td>209 (57)</td>
<td>2235 (947)</td>
</tr>
<tr>
<td>Avenasterol</td>
<td>8.6 (3.9)</td>
<td>27 (10)</td>
<td>16 (6.6)</td>
<td>43 (23)</td>
</tr>
<tr>
<td>Brassicasterol</td>
<td>8.1 (3.8)</td>
<td>55 (19)</td>
<td>17 (5.6)</td>
<td>81 (32)</td>
</tr>
<tr>
<td>Campesterol</td>
<td>31 (15)</td>
<td>273 (108)</td>
<td>68 (19)</td>
<td>421 (176)</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>49 (25)</td>
<td>1075 (423)</td>
<td>106 (28)</td>
<td>1671 (707)</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>1.5 (0.6)</td>
<td>13 (5.0)</td>
<td>2.8 (1.0)</td>
<td>20 (8.8)</td>
</tr>
<tr>
<td>Plant stanols</td>
<td>7.9 (2.2)</td>
<td>233 (72)</td>
<td>19 (14)</td>
<td>343 (138)</td>
</tr>
<tr>
<td>Campestanol</td>
<td>3.2 (0.5)</td>
<td>28 (7.0)</td>
<td>8.5 (9.1)</td>
<td>38 (15)</td>
</tr>
<tr>
<td>Sitostanol</td>
<td>4.7 (1.7)</td>
<td>206 (65)</td>
<td>10 (5.5)</td>
<td>304 (123)</td>
</tr>
</tbody>
</table>

*a* A significant difference between control and plant sterol males, *P* ≤ 0.01.

*b* A significant difference between control and plant sterol females, *P* ≤ 0.01.

**Note:** The data presented in Table 1 is from a study investigating the effects of plant sterols on fecal and mucosal sterols in a mouse model. The table details the sterol levels (in μg/100 mg) for different groups, including control males, plant sterol males, control females, and plant sterol females. The data is presented in a typical scientific format, with clear headings, column labels, and descriptive text. The table is formatted to align with the expectations for a scientific manuscript, ensuring readability and clarity.
Wilmington, DE), and the integrity and purity of RNA samples were verified using Bioanalyzer 2100 (v2.6, Agilent Technologies, Santa Clara, CA). Equal amounts of total RNA was taken from each mouse to make a pooled sample for control male, control female, plant sterol male and plant sterol female mice. Pooled samples were analyzed by Agilent Whole Mouse Genome 4 × 44 K microarray assay. The quality of the pooled RNA samples was again controlled by Bioanalyzer. All pooled samples had a RIN value >8.90. The labeling, hybridization and scanning were performed at the Biomedicum Functional Genomics Unit, University of Helsinki.

**Statistical Analysis**

Normality of data was tested using the Shapiro-Wilk test, and comparisons within genders were conducted using the independent-samples t-test. The results are expressed as mean ± SD. The Pearson’s correlation was applied to test associations between variables. Statistical analyses were performed using SPSS-PASW 18.0 for Windows software (SPSS). Lasso Poisson regression analysis including all variables simultaneously was conducted to explore which variables had predictive value for the total adenoma number (28,29), and the analysis was performed using glmmnet package in R. The number of predictors with nonzero effects was determined using cross-validation, and the overall significance of the predictive model was assessed with permutation sampling. The lasso regression model was used since there were more variables than observations. For all analyses, a $P < 0.05$ was considered statistically significant.

The microarray data was normalized to medium per chip. To study the effects of sterol treatment, empirical Bayes test with Benjamini-Hochberg correction for multiple testing was performed between the 2 samples of plant sterol and control samples. Genes passing a $P < 0.05$ were considered as candidates for being regulated genes. As there was 1 pooled sample per group, the secondary comparison between the sterol and control groups in male and female mice separately was performed by comparing the difference between the normalized expression values of a gene to the standard deviation of all differences between expression values of all genes, resulting in a z-ratio between the groups for the gene. The genes passing a 2 z-ratio limit between the control and the plant sterol group were considered as candidates for being regulated genes. To diminish the effect of false positives, as only a large body of genes with similar functions may show as significant result in enrichment analysis, statistics were applied to detect enriched functional categories or pathways associated with the genes regulated by the plant sterol feeding by using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (30).

**RESULTS**

**Adenoma Formation**

The mice grew well throughout the experiment and there was no difference in the weight gain between the groups in either gender (Supplemental Fig. 1). Plant sterol feeding increased the total number of intestinal adenomas (control 35.6 ± 8.5, plant sterol 43.7 ± 7.9, $P = 0.025$; Fig. 1A). Adenoma number was significantly increased in plant sterol females but not in males (46.8 ± 7.0 and 35.0 ± 9.1, respectively, $P = 0.036$; Fig. 1B), whereas there was no significant difference between plant sterol and control males (41.2 ± 8.2 and 36.3 ± 8.5, respectively, $P = 0.34$; Fig.1B). There was no difference in the number of colonic adenomas or in the size of adenomas in the small intestine or colon between the experimental groups in either gender (data not shown).

**Fecal Sterols**

Plant sterol feeding increased the concentration of cholesterol, plant sterols, and plant stanols in the feces in both genders when compared with the control diet (all $P < 0.01$; Table 1). Fecal cholesterol concentration was increased by 3.3-fold in plant sterol males and by 3.7-fold in plant sterol females when compared with their respective controls. Fecal total cholesterol correlated positively with intestinal adenoma number (Pearson correlation coefficient $r = 0.417$, $P = 0.047$; Supplemental Fig. 2).

**Mucosal Plant Sterols**

Plant sterol feeding increased concentrations of free sitosterol and campesterol in the intestinal mucosa in both genders (males $P ≤ 0.001$ for both, and females $P = 0.004$ and $P = 0.028$, respectively; Table 2). In plant sterol males the concentration of mucosal free sitosterol was eightfold greater than
TABLE 2

Mucosal plant sterols by diet and gender

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Control males, M (SD) (μg/100 mg)</th>
<th>Plant sterol males, M (SD) (μg/100 mg)</th>
<th>Control females, M (SD) (μg/100 mg)</th>
<th>Plant sterol females, M (SD) (μg/100 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant sterols, free</td>
<td>9.6 (1.5)</td>
<td>37.6 (14.4)a</td>
<td>15.0 (2.9)</td>
<td>25.4 (5.3)b</td>
</tr>
<tr>
<td>Campesterol</td>
<td>6.6 (1.1)</td>
<td>12.8 (2.4)a</td>
<td>10.2 (1.6)</td>
<td>12.9 (2.3)b</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>3.0 (0.5)</td>
<td>24.8 (12.1)a</td>
<td>4.4 (1.7)</td>
<td>12.5 (3.7)b</td>
</tr>
<tr>
<td>Plant stanols, free</td>
<td>1.2 (0.3)</td>
<td>5.4 (3.2)a</td>
<td>1.3 (0.3)</td>
<td>2.1 (0.7)b</td>
</tr>
<tr>
<td>Campestanol</td>
<td>0.52 (0.1)</td>
<td>0.98 (0.5)a</td>
<td>0.58 (0.1)</td>
<td>0.65 (0.1)</td>
</tr>
<tr>
<td>Sitostanol</td>
<td>0.66 (0.1)</td>
<td>4.4 (2.8)a</td>
<td>0.70 (0.2)</td>
<td>1.5 (0.6)b</td>
</tr>
<tr>
<td>Plant sterols, esterified</td>
<td>0.40 (0.2)</td>
<td>2.8 (2.4)a</td>
<td>0.89 (0.5)</td>
<td>0.96 (0.6)</td>
</tr>
<tr>
<td>Campesterol</td>
<td>0.23 (0.1)</td>
<td>0.60 (0.4)</td>
<td>0.44 (0.1)</td>
<td>0.37 (0.2)</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>0.18 (0.1)</td>
<td>2.2 (1.9)a</td>
<td>0.45 (0.4)</td>
<td>0.59 (0.5)</td>
</tr>
<tr>
<td>Plant stanols, esterified</td>
<td>0.33 (0.3)</td>
<td>0.74 (0.5)</td>
<td>0.35 (0.2)</td>
<td>0.29 (0.1)</td>
</tr>
<tr>
<td>Campestanol</td>
<td>0.15 (0.1)</td>
<td>0.17 (0.1)</td>
<td>0.14 (0.02)</td>
<td>0.11 (0.01)</td>
</tr>
<tr>
<td>Sitostanol</td>
<td>0.18 (0.2)</td>
<td>0.57 (0.4)</td>
<td>0.21 (0.2)</td>
<td>0.17 (0.1)</td>
</tr>
</tbody>
</table>

*aA significant difference between control and plant sterol males, P < 0.05.
*bA significant difference between control and plant sterol females, P < 0.05.

in control males, but only 2.8-fold greater in plant sterol females than in control females. Whereas campesterol was the predominant plant sterol in the mucosa of control male and female mice, the concentration of free sitosterol exceeded the concentration of free campesterol in plant sterol males (24.8 ± 12.1 μg/100 mg and 12.8 ± 2.4 μg/100 mg), and in plant sterol females the concentration of free sitosterol was equal to that of campesterol (12.5 ± 3.7 μg/100 mg and 12.9 ± 2.3 μg/100 mg, respectively). In addition, plant sterol feeding resulted in increased concentrations of esterified sitosterol and campesterol in males (P = 0.027 and P = 0.058, respectively; Table 2), but not in females. The total concentration of free plant stanols was increased in plant sterol males and females (P = 0.009 and P = 0.011, respectively), but no difference was observed in the concentration of esterified plant stanols in either gender.

FIG. 2. The effect of plant sterol (PS) feeding on free cholesterol (μg/100 mg; A), esterified cholesterol (μg/100 mg; B), and nuclear SREBP-2 (arbitrary units; C) in the mucosa of small intestine in male and female ApcMin mice. The concentration of esterified cholesterol was significantly decreased in plant sterol fed mice. The decrease in the mucosal free cholesterol concentration and the increase in nuclear sterol regulatory element binding protein-2 (SREBP-2) level were significant in plant sterol males when compared to control males (P < 0.05). Representative bands of SREBP-2 from Western blot are shown below the box plot for SREBP-2 results. The results are presented as box-whisker plots and the median is indicated by a line across the box. C = control. Control males, n = 6; plant sterol males, n = 6; control females, n = 7; and plant sterol females, n = 5.
**Mucosal Cholesterol and SREBP-2**

The concentration of mucosal free cholesterol was significantly lower in plant sterol male mice when compared with control males (−17%; 139 ± 16.9 μg/100 mg vs. 167 ± 12.5 μg/100 mg; P = 0.008; Fig. 2A). There was no difference in the free cholesterol between females (control 162 ± 16.2 μg/100 mg, plant sterol 159 ± 7.2 μg/100 mg; P = 0.24). The concentration of esterified cholesterol in the intestinal mucosa was decreased both in plant sterol males (−56%; control 4.8 ± 2.0 μg/100 mg, plant sterol 2.1 ± 0.7 μg/100 mg; P = 0.012; Fig. 2B) and in plant sterol females (−38%; control 6.0 ± 1.5 μg/100 mg, plant sterol 3.7 ± 1.5 μg/100 mg; P = 0.024). We measured the level of sterol regulatory element binding protein SREBP-2 in the intestinal mucosa to confirm the changes in the mucosal free cholesterol concentration. The SREBP-2 acts as transcription factors for genes that upregulate cholesterol biosynthesis, and when cellular free cholesterol concentration decreases, the level of nuclear SREBP-2 increases (31). In plant sterol males, the level of nuclear SREBP-2 (68 kDa) was increased when compared with control males (P = 0.048; Fig. 2C). The level of nuclear SREBP-2 did not differ between females (P = 0.823). There was no difference in the level of precursor SREBP-2 (125 kDa) or in the ratio of precursor to mature SREBP-2 (68 kDa) between the experimental groups in either gender.

**Levels of β-catenin, Egfr, ERK1/2, Caveolin-1, and Estrogen Receptors in the Intestinal Mucosa**

We determined the levels of proteins related to Wnt and Egfr signaling because in our previous work on plant stanols, the increased adenoma formation was accompanied by upregulation of Wnt and Egfr signaling (16). There was no difference in the level of membranous, cytosolic or nuclear β-catenin between the groups in either gender (Table 3). The level of nuclear cyclin D1, a β-catenin target, was not altered after plant sterol feeding. Plant sterol feeding did not alter levels of Egfr and ERK1/2 either. Plant sterols have been reported to regulate caveolin-1 levels (32), hence we measured the level of membranous caveolin-1 from the intestinal mucosa, but detected no difference between the groups in either gender. Because plant sterol feeding induced tumor formation more strongly in female than in male ApcMin mice, we evaluated the level of ERα and ERβ subtypes from the intestinal mucosa. There was no difference in the level of ERα between the groups in either gender (Table 3), however a tendency toward an elevated level of ERβ was observed in plant sterol females when compared with control females (P = 0.188, Fig. 3A). There was a significant positive correlation between the total number of adenomas and ERβ level among females (r = 0.83, P = 0.001; Fig. 3B), but not among males (r = 0.347, P = 0.269; data not shown).

A lasso regression analysis was performed to find those variables that would best predict increased tumorigenesis in this study. All measured parameters including fecal and mucosal sterols, and signaling proteins were included in the model. Those variables that received a coefficient more than 0.1 or less than −0.1 were considered strong predictors in the model. Higher levels of ERβ, mucosal sitosterol esters, mucosal free cholesterol, and a lower level of caveolin-1, in the aforementioned order, were the strongest predictors, and in combination predicted increased tumorigenesis significantly (P = 0.027).

**Enrichment of Regulated Genes Among the KEGG Pathways**

Global gene expression was analyzed by microarray assay using RNA isolated from the histologically normal-appearing mucosa of the distal small intestine. Enriched pathways are shown in more detail in Supplemental Tables 2 and 3. The analysis was conducted within genders. Among the KEGG pathways, a significant enrichment of differentially regulated genes associated with the cell cycle control was observed in plant sterol females when compared with control females (12 genes, P = 0.009). Of these 12 genes 10 were upregulated and 2 downregulated (Table 4). No significant enrichment of regulated genes of the cell cycle was observed in plant sterol males. Furthermore, there was significant enrichment of differentially regulated genes in the terpenoid backbone biosynthesis pathway in plant sterol females (P = 0.014, Fig. 4) but not in plant sterol males. In this pathway expression of genes belonging to the mevalonate pathway upstream of cholesterol biosynthesis (mevalonate kinase, phosphomevalonate kinase, isopentenyl-diphosphate delta-isomerase) were upregulated in plant sterol females.

**DISCUSSION**

For the first time, we show that plant sterols derived from plant sterol ester-enriched food products accelerate intestinal
Plant sterols and the ApcMin mouse. After feeding the plant sterol-enriched diet, sitosterol became the predominant mucosal plant sterol in males, whereas concentrations of campesterol and sitosterol were close to equal in females. In addition, plant sterol feeding increased concentrations of esterified plant sterols in the intestinal mucosa, but the effect was significant only in males. Our results support earlier findings that genders diverge in their sterol metabolism (39,41,42). The regulation of plant sterol metabolism may be related to differences in intestinal sterol transporters, because the gene expression of sterol transporters Abcg5 and Abcg8 in the small intestine has been shown to be higher in females than in males (43). As a consequence of high ATP binding cassette subfamily G 5/8 expression, the efflux of sterols from the enterocyte back to the intestinal lumen is increased and cellular plant sterol content is lowered.

In plant sterol males, accumulation of free sitosterol may have caused a reduction in the concentration of free cholesterol in the mucosa, a phenomenon previously observed also in the adrenal gland (44). As a matter of fact, we observed a significant negative correlation between mucosal free cholesterol and free sitosterol (results not shown), suggesting that sitosterol could replace cholesterol in enterocytes. The reduction in mucosal free cholesterol in males was accompanied by an increase in mature nuclear SREBP-2, a transcription factor that induces cholesterol synthesis when cellular cholesterol level is reduced (31). Although the nuclear SREBP-2 level was increased in plant sterol males, genes of the cholesterol biosynthesis were not differentially regulated when compared with control males. We propose that in plant sterol males the high concentration of mucosal free sitosterol may have inhibited the expression of SREBP-2 target genes. Previously, β-sitosterol itself has been reported to reduce gene expression and activity of HMG-CoA reductase, despite a decrease in intracellular free cholesterol content, and this effect of β-sitosterol was independent of SREBP-2 maturation (45).

Plant sterol feeding increased fecal cholesterol excretion in both genders; however the concentration of free cholesterol in

<table>
<thead>
<tr>
<th>β-Catenin</th>
<th>Control males, M (SD)</th>
<th>Plant sterol males, M (SD)</th>
<th>Control females, M (SD)</th>
<th>Plant sterol females, M (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>2.3 (1.8)</td>
<td>2.7 (1.3)</td>
<td>2.7 (2.0)</td>
<td>4.0 (1.1)</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.18 (0.2)</td>
<td>0.16 (0.1)</td>
<td>0.52 (0.4)</td>
<td>0.61 (0.2)</td>
</tr>
<tr>
<td>Nucleus</td>
<td>1.5 (0.7)</td>
<td>1.3 (0.4)</td>
<td>1.9 (1.3)</td>
<td>2.2 (0.8)</td>
</tr>
<tr>
<td>Cyclin D1, nucleus</td>
<td>0.56 (0.2)</td>
<td>0.92 (0.8)</td>
<td>1.8 (1.6)</td>
<td>1.4 (0.8)</td>
</tr>
<tr>
<td>Egfr</td>
<td>2.0 (1.1)</td>
<td>1.9 (1.2)</td>
<td>2.3 (1.8)</td>
<td>3.0 (0.7)</td>
</tr>
<tr>
<td>Phospho-Egfr</td>
<td>0.86 (0.3)</td>
<td>1.0 (0.5)</td>
<td>0.96 (0.4)</td>
<td>0.89 (0.1)</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>5.5 (0.4)</td>
<td>5.7 (0.6)</td>
<td>5.5 (0.5)</td>
<td>5.3 (0.6)</td>
</tr>
<tr>
<td>Phospho-ERK1/2</td>
<td>1.1 (0.9)</td>
<td>1.3 (1.0)</td>
<td>0.93 (0.3)</td>
<td>0.77 (0.4)</td>
</tr>
<tr>
<td>Caveolin-1, membrane</td>
<td>2.7 (2.5)</td>
<td>6.6 (10.9)</td>
<td>2.1 (1.9)</td>
<td>3.7 (3.7)</td>
</tr>
<tr>
<td>ERα</td>
<td>0.82 (0.6)</td>
<td>0.40 (0.2)</td>
<td>0.94 (0.7)</td>
<td>0.91 (0.5)</td>
</tr>
</tbody>
</table>

ERK1/2 = extracellular signal-regulated kinase 1/2; ER = estrogen receptor.
been associated with increased cell proliferation (48,49) and
thesis intermediates, such as mevalonate and isoprenoids, has
expression of enzymes involved in lipid and cholesterol
Like SREBP-2, SREBP-1 is a transcription factor that regulates
females. The level of nuclear SREBP-2 was, however, not af-
sterol group were considered as being regulated genes.
been shown to accumulate in ovaries and adrenal glands (39), where they could derегulate the synthesis of steroid hormones, including female sex hormones. In fact, plant sterol supplementation has been reported to reduce serum estrogen levels in animals (60,61). In addition, plant sterols may disturb estrogen metabolism by competing of binding to estrogen receptors α and β with estrogens (62). In the colonic epithelium, ERβ is the predominant estrogen receptor (63, 64). Plant sterols themselves bind to ERβ with low affinity, and their estrogenic effect seems to be weaker than estrogens (65). Epidemiological and clinical studies suggest that estrogens could protect against colon cancer (66,67,68). In Ap Min mouse, the loss of endogenous estrogen production was reported to increase the number of small intestinal tumors and the expression of ERβ in the small intestine of ovariectomized females when compared with intact females (69). Likewise, we suggest that a compensatory mechanism due to reduced estrogen signaling may explain the slight increase in the level of ERβ in plant sterol females and the positive correlation between tumor number and ERβ level among females. The regression analysis also proposed a higher ERβ level to be among the predictors.
FIG. 4. Regulated genes of cholesterol biosynthesis after plant sterol feeding. The cholesterol biosynthetic pathway showed significant enrichment of regulated genes only in plant sterol females. Fold-change values represent the change in gene expression in plant sterol females compared to control females (a) and in plant sterol males compared to control males (b). The genes passing a 2 z-ratio limit between the control and the plant sterol group were considered as being regulated genes.

- Hmgcs2 = 3-hydroxy-3-methylglutaryl-CoA synthase
- Hmgcr = 3-hydroxy-3-methylglutaryl-CoA reductase
- Mvk = mevalonate kinase
- Pmvk = phosphomevalonate kinase
- Mvd = mevalonate decarboxylase
- Fdps = farnesyl diphosphate synthetase
- Idi1 = isopentenyl-diphosphate delta-isomerase
- Fdft1 = farnesyl-diphosphate farnesyl-transferase 1
- Cyp51 = cytochrome P450, family 51
- Tm7sf2 = delta-14-sterol reductase

of increased adenoma number. Previously, Bises and coworkers demonstrated that soy feeding upregulated markers of premalignancy in the colon of healthy female mice, but not in male mice (70). The adverse effect was not caused by isoflavone genistein, which was tested separately, but by an unknown compound in soy. In addition to isoflavones, soy contains high concentrations of plant sterols.

In conclusion, we show that feeding plant sterol at 0.8% (w/w) accelerated intestinal tumorigenesis in \( \text{Apc}^{\text{Min}} \) mice, and the effect was stronger in female mice. Furthermore, we demonstrate that there were differences in sterol metabolism between genders after a high intake of plant sterols. Our results suggest that plant sterols may have adverse effects in the intestine; however, the exact mechanism remains unresolved. In the present study, the intake of plant sterols corresponds to an energy-adjusted intake of approximately 5 g/day in humans, which is possible to achieve using enriched products (5,6). Because the use of plant sterol-enriched products becomes more common later in life, when the prevalence of \( \text{APC} \) mutations in the colon increases (71), the \( \text{Apc}^{\text{Min}} \) mouse provides a reasonable approach to study the effect of plant sterols on intestinal carcinogenesis. Our results warrant the need for future studies on the effect of high plant sterol intake on intestinal health.

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SUPPLEMENTAL DATA
Supplemental data for this article can be accessed on the publisher’s website at http://dx.doi.org/10.1080/01635581.2014.865244.

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