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Phytosterols inhibit the tumor growth and lipoprotein oxidizability induced by a high-fat diet in mice with inherited breast cancer

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Abstract

Dietary phytosterol supplements are readily available to consumers since they effectively reduce plasma low-density lipoprotein cholesterol. Several studies on cell cultures and xenograft mouse models suggest that dietary phytosterols may also exert protective effects against common cancers. We examined the effects of a dietary phytosterol supplement on tumor onset and progression using the well-characterized mouse mammary tumor virus polyoma virus middle T antigen transgenic mouse model of inherited breast cancer. Both the development of mammary hyperplastic lesions (at age 4 weeks) and total tumor burden (at age 13 weeks) were reduced after dietary phytosterol supplementation in female mice fed a high-fat, high-cholesterol diet. A blind, detailed histopathologic examination of the mammary glands (at age 8 weeks) also revealed the presence of less-advanced lesions in phytosterol-fed mice. This protective effect was not observed when the mice were fed a low-fat, low-cholesterol diet. Phytosterol supplementation was effective in preventing lipoprotein oxidation in mice fed the high-fat diet, a property that may explain — at least in part — their anticancer effects since lipoprotein oxidation/inflammation has been shown to be critical for tumor growth. In summary, our study provides preclinical proof of the concept that dietary phytosterols could prevent the tumor growth associated with fat-rich diet consumption.

Keywords: Phytosterols; Breast cancer; Cholesterol; Lipoprotein; Oxidation; MMTV-PyMT mice

1. Introduction

Plant sterols or phytosterols are molecules that resemble cholesterol but are found exclusively in plants. The most common phytosterols in the human diet are β-sitosterol, campesterol and stigmasterol [1]. Phytosterols are known to lower serum low-density lipoprotein (LDL) cholesterol levels by reducing intestinal cholesterol absorption [2]. Therefore, readily available food products have been engineered to be enriched in phytosterols and marketed to help lower serum cholesterol and reduce cardiovascular risk. Further, some epidemiologic studies have suggested that dietary phytosterols may exert a protective role not only against cardiovascular diseases but also against cancer [3,4].

According to the cancer statistics published by the American Cancer Society, breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among women in the United States (American Cancer Society, Cancer Facts and Figures 2010, http://www.cancer.org/Research/cancer-facts-and-figures-2010, last accessed May 5, 2011). Interestingly, the incidence of common cancers, such as breast, colon and prostate cancer, is relatively low in Asian countries where people consume predominantly plant-based (and, thus, phytosterol-enriched) diets. Moreover, when Asians emigrate to Western countries and consume more animal-based diets, the incidence of these cancers rises [5]. While

Abbreviations: Abc, ATP-binding cassette transporter; Fasn, fatty acid synthase; GDI, GDP dissociation inhibitor; GP, generalized polarization; HDL, high-density lipoprotein; HFHC, high-fat, high-cholesterol; Hmgcr, 3-hydroxy-3-methyl-glutaryl-CoA reductase; LDL, low-density lipoprotein; Ldlr, LDL receptor; LFLC, low fat, low cholesterol; LXR, liver X receptor; MMTV, mouse mammary tumor virus; oxLDL, oxidized LDL; PAF-AH, platelet-activated factor acetyl-hydrolase; PON1, arylesterase or paraoxonase; PyMT, polyoma virus middle T antigen; REM, relative electrophoretic mobility; Scarb1 (or SR-BI), scavenger receptor class B type I; Srebf, sterol response element binding protein; Tg, transgenic.

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these studies show a potential association between phytosterol consumption and cancer activity prevention [4], they are not demonstrative of a cause–effect relationship. However, support for such a relationship was provided by several experimental studies (reviewed in Ref. [6]) in which β-sitosterol was shown to inhibit the cell growth of several breast, colon and prostate human cancer cell lines. The mechanisms by which phytosterols offered this protection are not fully understood. Some putative mechanisms include inhibition of cancer cell growth, apoptosis promotion, decreased angiogenesis, alteration in sterol metabolism and liver X receptor (LXR) agonism [6–8]. Recently, the existence of common gene networks among cancer, lipid metabolism and inflammation has been reported [9]. Interestingly, both oxidized LDL (oxLDL) and its receptor (OLR-1) were found to promote cellular transformation in a mechanism that may involve nuclear factor (NF) κB activation. Other authors have also related lipoprotein oxidation to the development of several types of cancer [10,11].

In vivo studies on the effect of phytosterol consumption on breast cancer progression have been limited, to date, to xenograft models. The consumption of phytosterol-enriched diets has been shown to reduce tumor growth after fully established human breast cancer cells were injected into rodent hosts [12,13]. Although these cancer models are useful, they do not reflect the complex multistage nature of human tumorigenesis. Therefore, we employed the well-established mouse mammary tumor virus (MMTV) polyoma middle T antigen (PyMT) transgenic (Tg) mouse model to analyze the role of dietary phytosterols in tumor initiation and progression. Further, considering the promoting effect of high-fat diets on cancer development, we studied the effect of a phytosterol supplement on PyMT Tg mice fed both a regular chow diet (which is low fat, low cholesterol; LFLC) and a high-fat, high-cholesterol (HCHF) diet.

2. Materials and methods

2.1. Mice and diets

MMTV-PyMT Tg mice with an FVB/N background were obtained from The Mouse Models of Human Cancers Consortium Repository (National Cancer Institute, Frederick, MD, USA). MMTV-PyMT Tg mice express high levels of the transforming oncogene polyoma virus middle T (PyMT) antigen under the control of the MMTV long terminal repeat promoter, which specifically directs expression to the mammary epithelium [14]. All female PyMT Tg mice spontaneously develop widespread multifocal adenocarcinomas in the mammary gland, with hyperplastic foci occurring as early as 3 weeks after birth [15]. The similitude between the PyMT Tg model and human breast cancer has been validated by histologic studies, which demonstrated a striking similarity between PyMT Tg tumorigenesis and various stages of human ductal adenocarcinoma progression [16,17].

All animals were kept on a 12-h light/dark cycle with access to food and water *ad libitum*. Breeding was carried out with male mice hemizygotes for the PyMT transgene and non-Tg females with the same genetic background. Genotyping was performed as indicated on The Jackson Laboratory's Web site (http://jaxmice.jax.org/). Animal protocols used in this study were approved by the Institutional Animal Care and Use Committee.

Except for the whole-mount studies, 4-week-old female PyMT Tg mice were randomized into 2 groups: those consuming and those not consuming a 2% phytosterol supplement added to the powdered food on either an LFLC diet (6.2% fat, no cholesterol, energy density 3.1 kcal/g, calories from protein, fat and carbohydrate, 24%, 18% and 58%, respectively; diet #2018; Teklad diets, Madison, WI, USA) or an HFHC diet (21.2% fat, 0.2% cholesterol, energy density 4.5 kcal/g, calories from protein, fat and carbohydrate, 15.2%, 42% and 42.7%, respectively; diet #TD.88137; Teklad diets). Composition of the diets is detailed in Table 1. Phytosterols were composed of 20% campesterol, 22% stigmasterol and 41% β-sitosterol (Lipofoods S.L., Gavà, Barcelona, Spain). Diets were prepared and mixed with phytosterols by Mucedola srl (Settimo Milanese, Milan, Italy).

Whole mounts of mammary glands were obtained from 4-week-old females whose mothers were fed the different diets during pregnancy and lactation. In all cases, mice were maintained on the diets until euthanized (at 4, 8 or 13 weeks of age). A graphic of the experimental design is shown in Supplementary Figure 1. It is important to note that in this study, experiments were planned to compare mice consuming and mice not consuming the phytosterol supplement rather than to compare the effects of an HFHC diet vs. an LFLC diet. Therefore, some experiments on LFLC and HCHF diets were performed at different timings, and thus, direct comparison of the results would not be appropriate.

2.2. Whole-mount analysis of mammary glands

Right inguinal mammary glands of 4-week-old females were excised, spread onto glass slides, fixed in ethanol/acetic acid for 2 h and stained overnight with carmine alum as previously described [18]. Whole mounts were digitally photographed beside a ruler, and total area measurements for the hyperplastic lesions were quantified using Image J software (available at http://rsbweb.nih.gov/ij/).

2.3. Total tumor burden determination

Total tumor burden was determined in 13-week-old female mice by excising and weighing all the tumor masses formed in each of the 10 mammary glands. Portions of the tumors were then frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin.

2.4. Histologic analysis of mammary glands

Right inguinal mammary glands of 8-week-old female mice were excised, fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin after dehydration. Sections were cut at 5 μm, stained with hematoxylin and eosin and evaluated blindly by an experienced histopathologist (E.L.). Each section was graded as normal (N), hyperplasia (HP), adenoma (A) or carcinoma (C) as defined by the maximum lesion grade developed and in accordance

Table 1
LFLC (Teklad Global diet #2018) and HFHC (Teklad diet #TD.88137) diet composition

	LFLC diet	HFHC diet
Macronutrient composition (%)		
Protein	18.6	17.3
Fat	6.2	21.2
Carbohydrate	44.2	48.5
Cholesterol		0.2
Fatty acid (% of total fat)		
C4:0		1.2
C6:0		1.2
C8:0		0.9
C10:0		2.3
C12:0		3.0
C14:0		10.3
C14:1		0.8
C15:0		1.2
C16:0	12.5	29.4
C16:1		1.7
C17:0		0.8
C18:0	3.6	12.6
C18:1 (oleic)	21.4	20.7
C18:1 isomers		4.7
C18:2 (linoleic)	55.4	2.3
C18:2 isomers		1.0
C18:3 (linolenic)	5.4	0.6

with previous guidelines [19]. The relative abundance of hyperproliferative parenchyma replacing normal fibroadipose stroma was also estimated for each mouse.

2.5. Immunoblot analysis

Left inguinal mammary glands of 8-week-old female mice were homogenized in 50 mM Tris; 150 mM NaCl; 1% Triton X-100; 5 mM EDTA, containing protease (Roche Diagnostics Corp., Indianapolis, IN, USA) and phosphatase (Sigma-Aldrich, Inc., St. Louis, MO) inhibitor cocktails. Tissue lysates were then centrifuged at 12,000*g* for 10 min at 4°C to remove insoluble debris. Protein concentrations were quantified using the bicinchoninic acid protein kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein were loaded, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% acrylamide) and transferred to nitrocellulose. Immunoblot analyses were performed using a mouse monoclonal antibody to cyclin D1 and a rabbit polyclonal antibody to Rho GDIα (Santa Cruz Biotechnology, Inc., Palo Alto, CA, USA) and a mouse monoclonal antibody to IκBα (Cell Signaling Technology, Inc., Danvers, MA, USA).

2.6. Microvessel density quantification

Paraffin-embedded mammary glands underwent immunohistochemical analysis for CD31, a specific marker of endothelial cells, using a rabbit polyclonal antibody against CD31 (Abcam, Inc., Cambridge, MA, USA). Microvessel quantification was made by counting the number of CD31-positive vessels in three representative fields of each different area (normal, hyperplasia, adenoma and carcinoma) in each section. Quantification was performed at a high magnification (×400) field by an experienced histopathologist (E.L.) who was unaware of the group assignment of the samples.

2.7. Plasma cholesterol determination

Fasting plasma samples were collected by intracardiac puncture after each euthanization (4, 8 and 13 weeks). Total cholesterol was determined enzymatically using a commercial kit adapted to a HITACHI 917 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany).

2.8. Sterol content in high-density lipoprotein and mammary gland tumors

Mammary gland lipids were extracted with isopropyl alcohol–hexane (2:3 vol/vol) from 0.1 g of 13-week-old female mammary tumor tissue, dried under nitrogen and reconstituted with 0.5% sodium cholate. After 10-min sonication, mammary gland total cholesterol was determined using a commercial kit adapted to a HITACHI 917 autoanalyzer (Roche Diagnostics).

Phytosterol levels were analyzed by gas–liquid chromatography after lipid extraction using a modification of a previously published method [20]. Epicoprostanol (2 μg) was added to isolated high-density lipoprotein (HDL) or mammary gland tissue (0.2 ml) as internal standard. After alkaline hydrolysis, extraction and derivatization to trimethylsilyl ethers, sterols were quantified on a 30-m nonpolar capillary column (TRB-Esterol; Teknokroma, Barcelona, Spain) equipped with flame ionization detection in an Agilent 7890A GC Autosystem (Agilent Technologies, Wilmington, DE, USA) apparatus. Each run quantified campesterol, stigmasterol and β-sitosterol. Interassay and intra-assay coefficient variations were 1.9% and 1.6% for campesterol, 6.7% and 4.6% for stigmasterol and 2.0% and 1.8% for β-sitosterol, respectively.

2.9. Mammary gland cholesterol uptake

After 4 weeks on an HFHC diet with or without phytosterol enrichment, 8-week-old female PyMT Tg mice received an intragastric load of 4 μCi [1α,2α(*n*)-^{3}H]-cholesterol (GE Healthcare, Buckinghamshire, UK) dissolved in 0.1 ml of olive oil. After 24 h, the mice were euthanized and the two inguinal mammary glands were collected. [^{3}H]-cholesterol was extracted with isopropyl alcohol–hexane, dried under nitrogen, resuspended in scintillation liquid and counted.

2.10. Lipoprotein isolation

Frozen plasma samples of 8-week-old female PyMT Tg mice fed an HFHC diet with or without a 2% phytosterol supplement were pooled, and HDL (d=1.064–1.21 g/ml) was isolated by sequential ultracentrifugation. LDL from pooled plasma of healthy human donors was also isolated by sequential ultracentrifugation (d=1.019–1.063 g/ml). All lipoproteins were dialyzed against phosphate-buffered saline by gel filtration in PD-10 columns (GE Healthcare) immediately prior to oxidation assays.

2.11. Susceptibility to lipoprotein oxidation

Both mouse HDL susceptibility to copper-induced lipid oxidation and mouse HDL ability to inhibit the oxidative modification of human LDL were assessed [21]. Briefly, oxidation was started by adding 2.5 μmol/l $CuSO_4$ in cuvettes containing HDL alone or HDL in the presence of human LDL (0.2 mmol/l cholesterol in both cases). Conjugated diene formation was measured by continuous monitoring of absorbance at 234 nm in a Synergy HT spectrophotometer (BioTek) at 37°C for 5 h. Reaction was stopped by adding 1 mmol/l EDTA and 2 μmol/l butylated hydroxytoluene, and immediately, 15 μl of lipoproteins was mixed with 5 μl of sucrose 50% and 5 μl of Sudan black at 10 g/l. Ten microliters of this mixture was loaded onto an 0.5% agarose gel and electrophoresed at 90 V for 90 min in a cold room. Relative lipoprotein mobility was evaluated with a Chemi Doc 2000 densitometer using the Quantity One software (Bio-Rad). Results were expressed as relative LDL mobility, considering the mobility of oxLDL as 100% and that of native LDL as 0% [21].

2.12. Laurdan fluorescence

2-Dimethylamino-6-lauroylnaphthalene (Laurdan, Molecular Probes Inc., Eugene, OR, USA) was incorporated into pooled samples of mouse HDL as previously described [22]. Briefly, mouse HDL (50 μg of protein per milliliter) were labeled with Laurdan using a final probe concentration of 0.375 μM. Fluorescence emission spectra of Laurdan were obtained in a Synergy HT spectrofluorometer (BioTek) using an excitation wavelength of 340 nm. After subtraction of the blank, the value of generalized polarization (GP) of Laurdan was calculated using the formula: $GP=(I_{440}-I_{485})/(I_{440}+I_{485})$, where I_{440} and I_{485} are the emission intensities at 440 and 485 nm, respectively [23].

2.13. NF-κB/p65 enzyme-linked immunosorbent assay

Nuclear extracts were prepared from both inguinal mammary glands of 8-week-old female PyMT Tg mice, and nuclear p65 levels were measured using the NF-κB/p65 ActivELISA Kit (Imgenex Corp., San Diego, CA, USA) according to the manufacturer's instructions.

2.14. Statistical analysis

Data are expressed as mean±S.E.M.. Statistical significance between groups was analyzed using the Mann–Whitney *U* test.

Statistical analyses were performed using GraphPad Instat (GraphPad Software, San Diego, CA, USA). P values ≤.05 were considered statistically significant.

3. Results

3.1. A phytosterol supplement delayed the development of hyperplastic mammary lesions in female PyMT Tg mice fed an HFHC diet

One of the earliest premalignant lesions is hyperplasia. Hyperplastic lesions are present in the majority of mammary glands in 3-week-old female PyMT Tg mice and are thought to be lesions that develop into tumors [15]. To determine whether dietary phytosterols affect the development of multifocal hyperplastic lesions, we performed whole-mount mammary gland analyses in 4-week-old female PyMT Tg mice fed an LFLC diet or an HFHC diet, with or without a 2% phytosterol supplement. Fig. 1A and B show representative images of mammary whole-mount preparations obtained from mice on both diets. Quantification of the total area occupied by hyperplastic lesions was performed for each mammary gland. No differences were observed in the size of lesions in mice receiving a phytosterol supplement compared to those that did not (2.12±0.50 mm^2 vs. 2.36±0.34 mm^2) (Fig. 1C) during an LFLC diet. However, consumption of a phytosterol supplement was associated with a 40% reduction in the area occupied by hyperplastic mammary lesions (2.04±0.19 mm^2 vs. 3.37 ±0.50 mm^2, P=.012) when mice were fed an HFHC diet (Fig. 1D).

3.2. Phytosterols reduced the total tumor burden in PyMT Tg mice fed an HFHC diet

To assess the role of dietary phytosterols in mammary tumor formation, we fed 4-week-old female PyMT Tg mice on either an LFLC diet or an HFHC diet, with or without a 2% phytosterol mixture. The mice were kept on the diets for 9 weeks and then euthanized at 13 weeks of age. All tumors were carefully excised and weighed. Consumption of a phytosterol-enriched diet did not affect the total tumor burden (total tumor weight per mouse) when mice were fed an LFLC diet (Fig. 2A). In contrast, phytosterol supplementation when mice were fed an HFHC diet resulted in a significant 28% reduction in the total tumor burden (P=.0050) (Fig. 2B).

3.3. Phytosterols reduced the histologic grade of mammary lesions

We also aimed to ascertain whether dietary phytosterol supplements affected the histologic grade of the lesions. These studies were conducted in 8-week-old female PyMT Tg mice receiving an HFHC diet for 4 weeks to observe tumor growth before large and poorly differentiated tumors developed. Longitudinal sections of the right inguinal mammary gland were prepared for each animal, and the

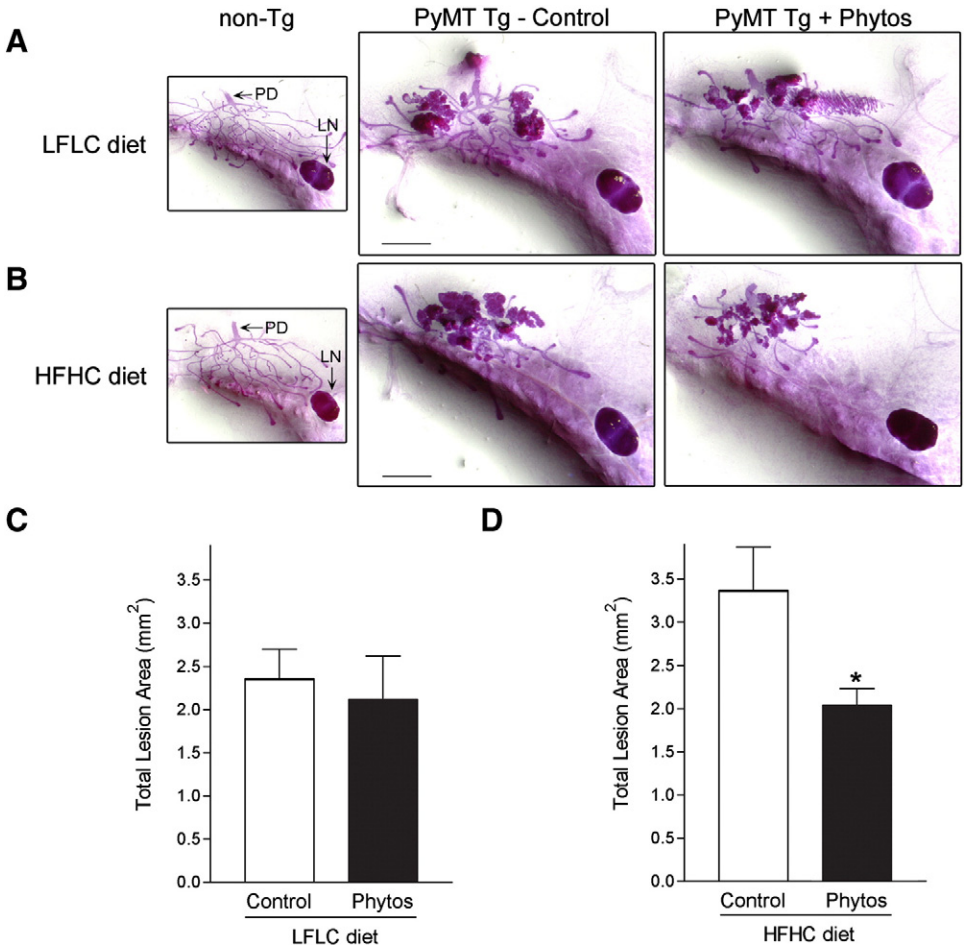

Fig. 1. Phytosterol effect on the development of multifocal hyperplastic mammary lesions. Right inguinal mammary glands were harvested from 4-week-old PyMT Tg or non-Tg (control) female mice, fixed and stained with carmine dye. Representative, equally magnified images are shown for animals consuming and mice not consuming the phytosterol supplement in an LFLC diet (A) or an HFHC diet (B). The primary duct (PD) originates from the nipple area and is visible in the top left corner of all images. The subiliac lymph node (LN) is visible on the right of all images. Scale bars=1 mm. Quantification of the total area occupied by multifocal hyperplastic lesions in 4-week-old female PyMT Tg mice fed an LFLC diet (C) or (D) an HFHC diet. Data are expressed as mm^2 of lesion per gland±S.E.M. (n=8–11 in all experimental groups; *P<.05).

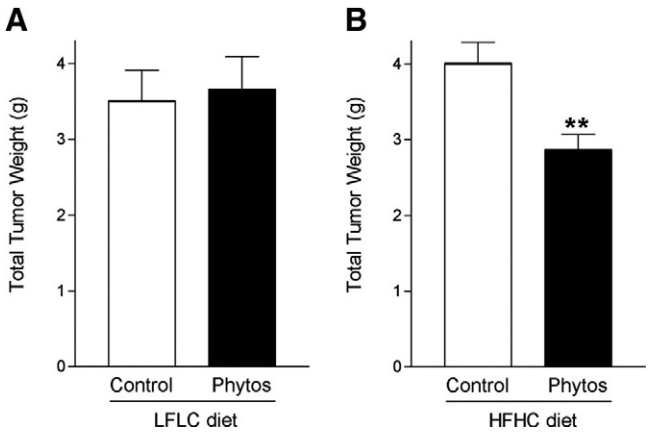

Fig. 2. Phytosterol effect on total tumor burden. Total tumor burden expresses the mean±S.E.M. of the grams of tumor weighed after careful dissection of each of the 10 mammary glands of each 13-week-old female PyMT Tg mouse fed an LFLC diet (A) with or without phytosterol supplementation (n =13 in each group) or an HFHC diet (B) (n=16 and 17, respectively; **P<.01).

relative abundance of hyperproliferative parenchyma replacing normal fibroadipose stroma was estimated for each section. Although not significant, the percentage of mammary tissue occupied by hyperproliferative areas was shown to be 38% lower in female PyMT Tg mice that received the phytosterol supplement (Fig. 3A). After histopathologic examination, each section was classified as being normal (N) or at one of the following stages: hyperplasia (HP), adenoma (A) or carcinoma (C). Each animal was defined by the maximum lesion grade reached. The percentage of mice reaching a maximum of hyperplasia and adenoma was 29% and 43%, respectively, in mice not consuming the phytosterol supplement. In contrast, these percentages were 57% and 14% in phytosterol-consuming mice, thus showing a trend toward a less-advanced histopathologic grade (Fig. 3B). Representative images for each histologic grade are shown in Fig. 3C. The expression level of cyclin D1, a marker of tumor progression, in left inguinal mammary glands was examined by Western blot (a representative result is shown in Fig. 3D). Cyclin D1 protein expression was shown to be significantly decreased in PyMT Tg mice consuming the phytosterol supplement compared to those that did not. As it has been suggested that phytosterols may promote the expression or activity of certain endogenous angiogenesis inhibitors [24], we determined the formation of new blood vessels in the mammary gland through CD31 immunostaining. As shown in Fig. 3E, no differences in the number of CD31-positive vessels were found between PyMT Tg mice consuming the phytosterol supplement and those not consuming the phytosterol supplement in the areas of normal mammary stroma, hyperplasia, adenoma or carcinoma.

3.4. Phytosterols did not affect weight

Phytosterol treatment did not change the weight of mice fed either an LFLC diet (not shown) or an HFHC diet. Mice fed an HFHC diet containing the phytosterol supplement had weights (in grams) of 18.6±1.4, 22.4±2.5 and 28.4±2.2 at 4, 8 and 13 weeks, respectively, and these did not differ from those in mice fed an HFHC without added phytosterols (18.8±1.0, 23.8±2.8 and 30.1±3.5).

3.5. Hypocholesterolemic effect of phytosterols

Fasting plasma samples were collected from female PyMT Tg mice at 4, 8 and 13 weeks of age. Total plasma cholesterol levels were determined after 4 weeks (4- and 8-week-old female mice) and 9 weeks (13-week-old female mice) of HFHC diet. Mice presented significantly reduced plasma cholesterol levels after 4 weeks of phytosterol supplementation (P=.0055 and P=.05 in 4- and 8-week-old mice, respectively) (Fig. 4A). However, plasma cholesterol levels were not affected by phytosterol supplementation in 13-week-old female PyMT Tg mice fed an HFHC diet. This may be explained by the significant reduction in plasma cholesterol levels observed in PyMT Tg mice at this age compared to their non-Tg counterparts (data not shown), which suggests that tumor progression was responsible for a cholesterol reduction that masked the hypocholesterolemic effect of phytosterols.

3.6. Phytosterols did not affect mammary gland cholesterol availability

We sought to determine whether phytosterol supplementation could affect mammary gland cholesterol availability in PyMT Tg mice fed an HFHC diet. No differences in mammary cholesterol content were detected (Fig. 4B), whereas a nonsignificant trend toward a reduced labeled cholesterol mammary uptake was found in animals fed a phytosterol-supplemented diet (Fig. 4C). Mammary gland messenger RNA (mRNA) levels of scavenger receptor class B type I (*Scarb1*) and LDL receptor (*Ldlr*) (Supplementary Figure 2A), which regulate cholesterol uptake in the mammary gland, as well as protein levels of SR-BI (Supplementary Figure 2B), were unaffected by phytosterol supplementation. Transcription of both 3-hydroxy-3-methyl-glutaryl-CoA reductase (*Hmgcr*) and sterol response element binding protein (*Srebf*) 2 genes, which control cholesterol synthesis by the cell, was activated in the liver of PyMT Tg mice consuming the phytosterol supplement (P=.0012) (Supplementary Figure 2C); however, this was not observed in mammary glands (Supplementary Figure 2D). Further, mammary gland gene expression levels of LXRα (*Nr1h3*), a major regulator of cholesterol exit from cells and of reverse cholesterol transport within the whole body, and several well-known LXR-target genes such as ATP-binding cassette transporters (*Abc*) *a1* and *g1*, and fatty acid synthase (*Fasn*) were not modified either by phytosterol supplementation (Supplementary Figure 2E).

3.7. Phytosterols reduced lipoprotein susceptibility to oxidation and NF-κB

The extent of copper-induced lipid HDL oxidation was evaluated by measuring the conjugated diene formation in pooled HDL obtained from 8-week-old PyMT Tg mice fed a control or a phytosterol-enriched HFHC diet. A significant decrease in the amount of conjugated dienes was observed in mice consuming the phytosterol supplement (P=.05; Fig. 5A). The kinetics of HDL of mice consuming a phytosterol supplement rapidly reached a plateau, indicating that extensive oxidation of HDL was prevented in these samples (Fig. 5B). The ability of HDL to inhibit LDL oxidation was also tested by examining the relative electrophoretic mobility (REM) of human LDL exposed to oxidative modification in the presence of HDL from PyMT Tg mice fed a control or a phytosterol-enriched HFHC diet. As shown in Fig. 5C, HDLs from PyMT Tg mice fed a phytosterol-enriched diet were able to better inhibit (68% vs. 28%) the increase in electrophoretic mobility of LDL due to oxidation (REM=72%±1.9% and 32%±8.2%) than HDL from mice fed with no added phytosterols (P=.0286). As shown in Fig. 5D, the value of Laurdan GP was found to be significantly decreased in HDL obtained from mice consuming added phytosterols compared to those who did not (P=.0286). Decreased Laurdan GP values are associated with increased packing of the surface lipids [22], a phenomenon that promotes lipoprotein resistance to oxidative processes [25]. By contrast, these phytosterol-induced changes were not associated with changes in apolipoprotein (apo)A-I or apoA-IV concentrations nor in paraoxonase (PON1) and platelet-activated factor acetyl-hydrolase activities, which are

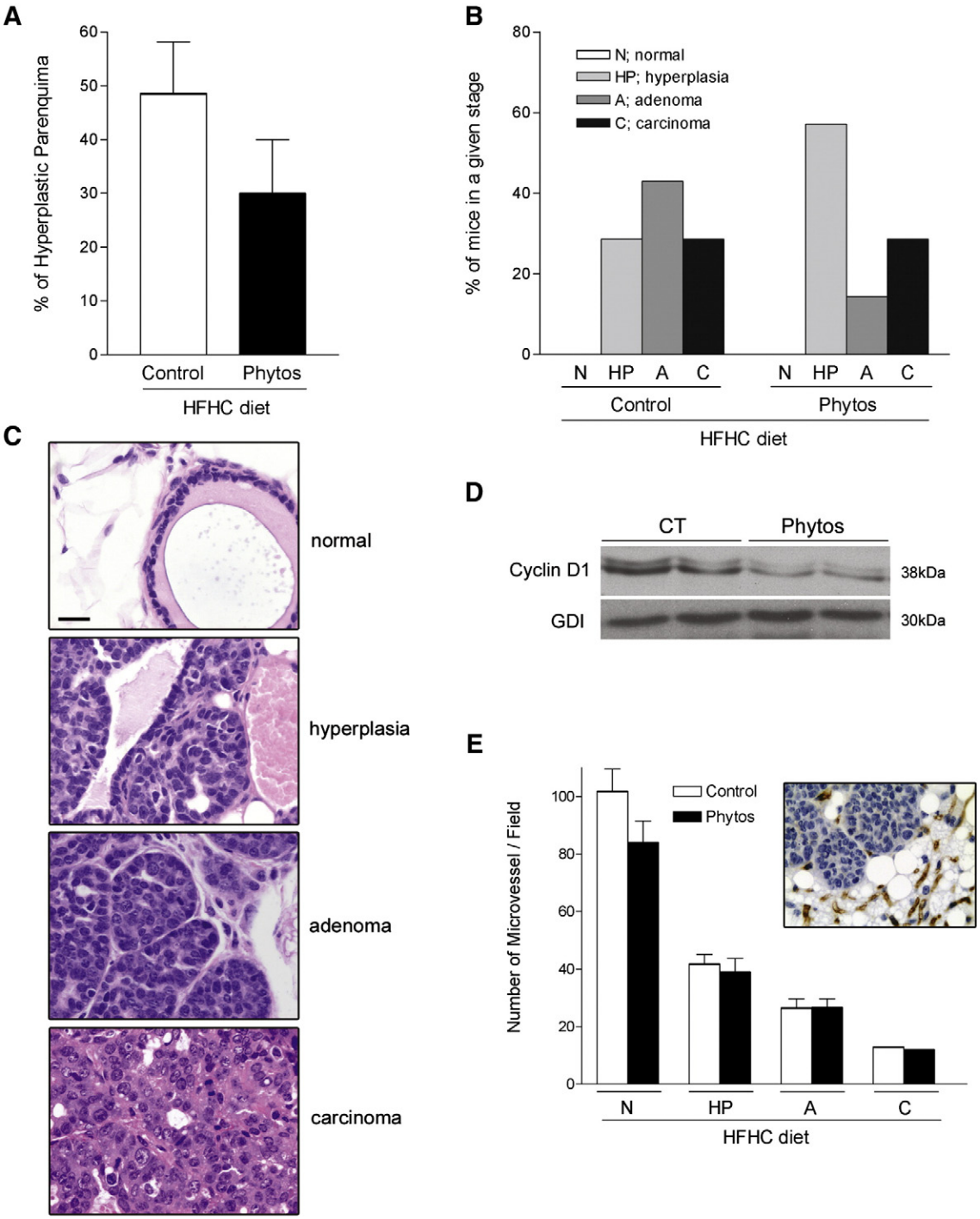

Fig. 3. Phytosterol effect on tumor lesion grade. Eight-week-old female PyMT Tg mice fed an HFHC diet with or without phytosterols were euthanized, and the two inguinal mammary glands were excised. (A) Relative abundance of hyperproliferative parenchyma expressed as averaged percentage±S.E.M. of mammary glands occupied by hyperproliferative parenchyma in right inguinal mammary gland sections (n=7 for each dietary group) stained with hematoxylin and eosin. (B) Each section was graded as the maximum grade of lesion demonstrated in it and then classified as follows: normal (N), hyperplasia (HP), adenoma (A) or carcinoma (C); the percentage of mice in each histopathologic stage is represented. (C) A representative image of each histopathologic stage is shown. Original magnification ×40. Scale bars=20 μm. (D) Left inguinal mammary glands were lysed and subjected to cyclin D1 immunoblot analyses using GDI as a control of equal loading. Two representative images (n=5 for each dietary group) are shown. (E) Mammary gland microvessels were quantified by counting the number of CD31-positive vessels in three representative fields of each different area (normal, hyperplasia, adenoma and carcinoma; note that not all lesion types were found in each mouse) in one section of each mouse (n=7 in each group). Inset, a representative image with CD31-positive microvessels. Results of panels A, B and E were obtained by an experienced pathologist (E.L.) blinded to the group assignment of the samples.

thought to confer part of the antioxidant properties on HDL (data not shown). No evidence was found either of changed liver paraoxonase 1 and 3 mRNA levels due to phytosterol supplementation (data not shown). As lipoprotein oxidation has been linked to inflammation through NF-κB, nuclear p65 levels were studied and found to be decreased (P=.056; Fig. 5E), whereas IκBα protein showed a 1.8-fold significant increase (P=.029) (Fig. 5F) in the mammary gland of PyMT Tg mice consuming the phytosterol supplement compared to

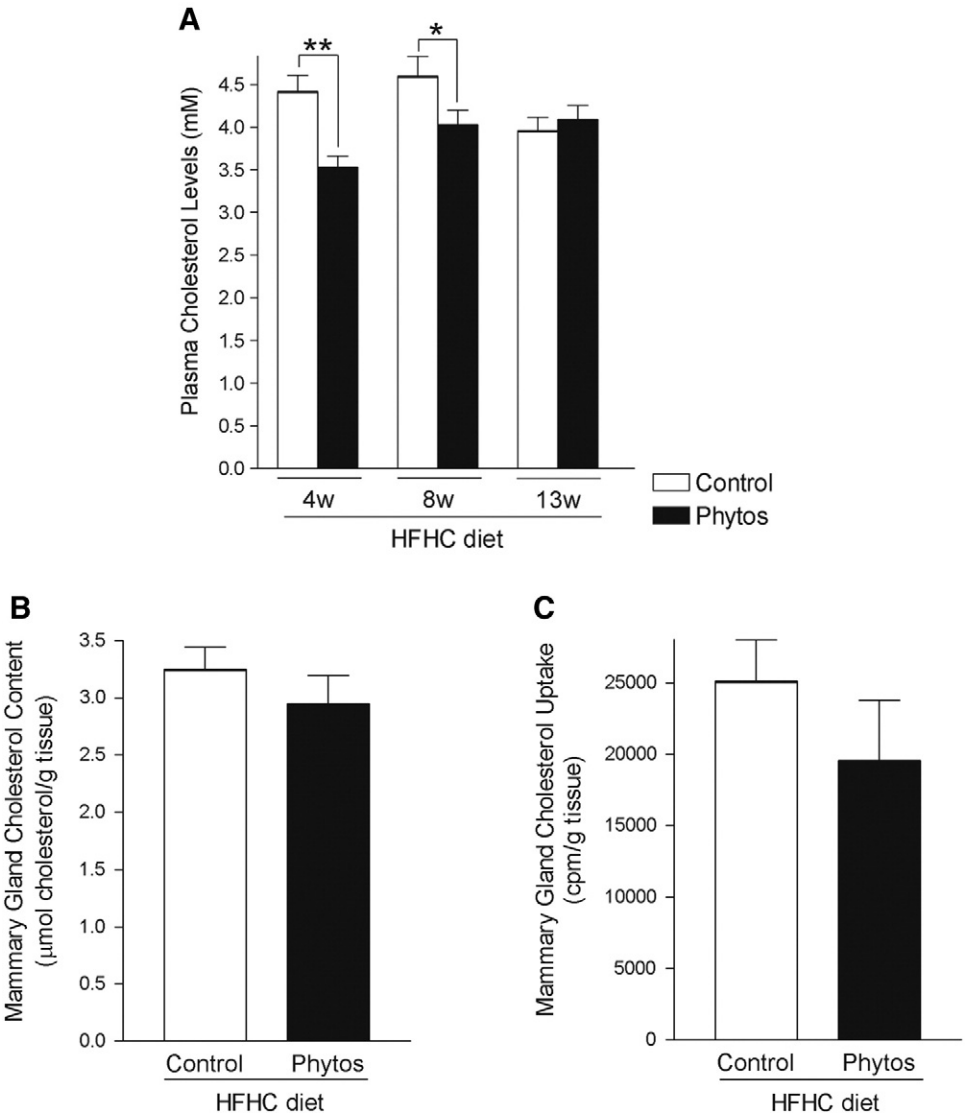

Fig. 4. Phytosterol effect on plasma cholesterol levels and mammary gland cholesterol availability. (A) Total plasma cholesterol content was determined in fasting plasma samples from 4-, 8- and 13-week-old female PyMT Tg mice fed an HFHC diet with or without phytosterol supplements. Data are expressed as mean (in mmol/l)±S.E.M. (n=8–16 in all experimental groups; **P<.01 and *P≤.05 vs. PyMT Tg mice not consuming phytosterol supplements). (B) Cholesterol content of mammary tumors obtained from 13-week-old PyMT Tg mice maintained on an HFHC diet with or without a 2% phytosterol supplement. Results are expressed as μmol of cholesterol per g of tissue±S.E.M. (n=10 tumors analyzed for each dietary group). (C) Inguinal mammary gland cholesterol uptake in 8-week-old PyMT Tg mice fed an HFHC diet with or without phytosterol supplements, 24 h after receiving an intragastric load of [1α,2α(n)-^{3}H]-cholesterol. Data are expressed as cpm per g of tissue (n=8 and 9 animals in each group).

those that did not. However, no significant differences were found in mammary gland monocyte chemotactic protein 1, interleukin 5 and interleukin 1beta mRNA levels of mice fed an HFHC diet with or without added phytosterols (data not shown). This might be due to a compensatory action by other regulators and, further, does not rule out other effects mediated by decreased activation of NF-κB. Indeed, there are several recent reports consistent with an antioxidant and anti-inflammatory activity of phytosterols [26,27].

3.8. Phytosterols are found in HDL and mammary gland tumors

Phytosterols were quantified by direct measurement in HDL (one pool of three to seven individual samples per group) and mammary gland tumors (three individual samples per group) in 8-week-old PyMT Tg mice fed an HFHC diet with or without phytosterols. Campesterol, stigmasterol and β-sitosterol concentrations in HDL of mice fed with a phytosterol supplement were 4.9, 0.1 and 5.4 mg/l, respectively, whereas those of HDL of mice fed without phytosterols were 1.3, 0.1 and 1.4 mg/l. Campesterol, stigmasterol and β-sitosterol concentrations in mammary gland tumors of mice fed with a phytosterol supplement were 16.0±4.2, 1.4±1.0 and 11.6±3.0 μg/g of tissue, whereas those in mice fed without added phytosterols were 1.7±0.2, 0.3±0.0 and 0.5±0.1 μg/g of tissue, respectively.

4. Discussion

In the present study, female PyMT Tg mice were fed a 2% phytosterol mixture supplement in either an LFLC or an HFHC diet. Dietary phytosterol supplementation delayed tumor onset and progression only in mice fed an HFHC diet. In particular, the phytosterol supplement consumption was associated with reduced size of hyperplastic lesions (at 4 weeks of age) and total tumor burden (at 13 weeks of age). Histopathologic examination of the mammary glands (at 8 weeks of age) revealed the presence of less-advanced carcinogenic lesions in phytosterol-fed mice.

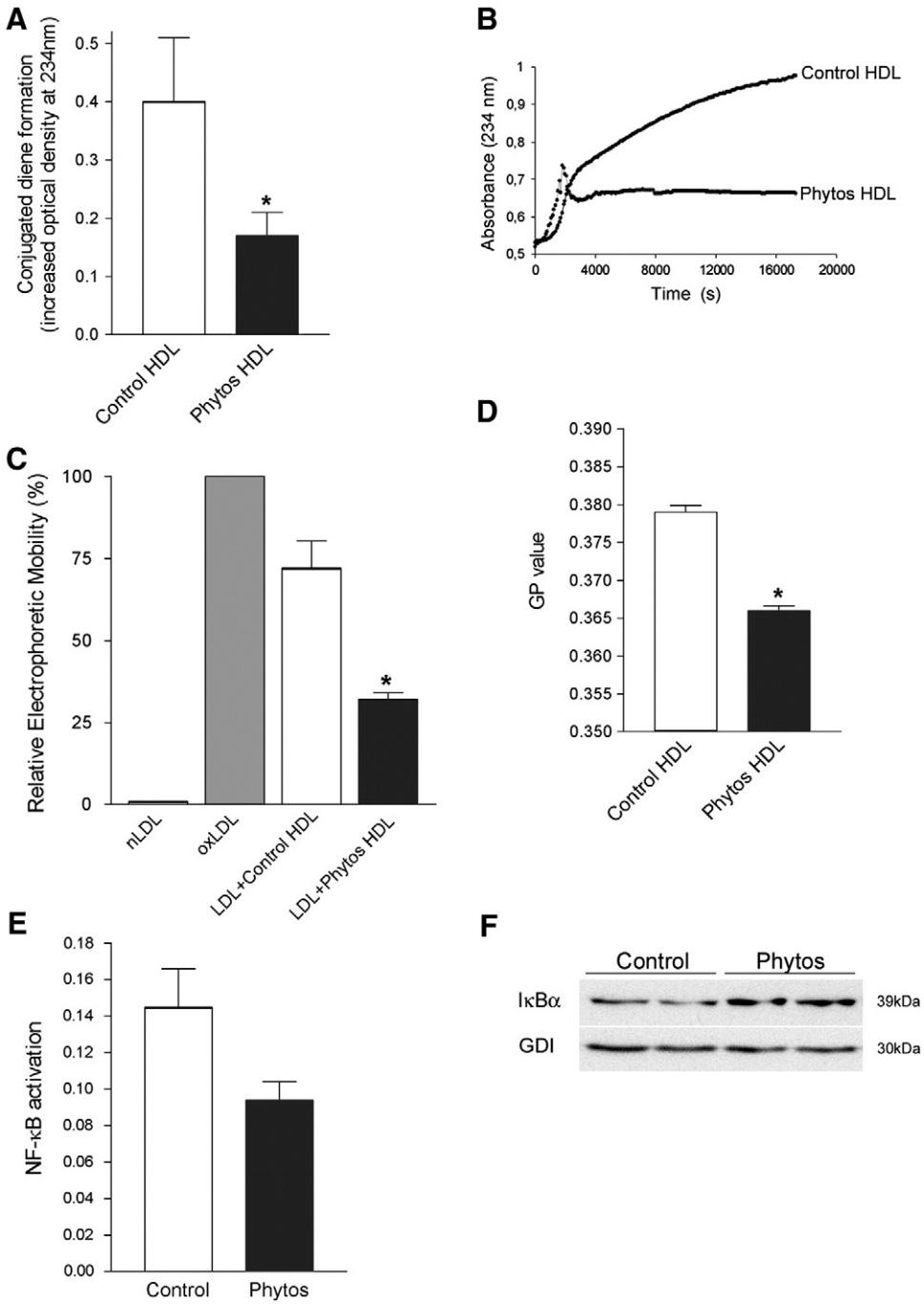

Fig. 5. Phytosterol effect on lipoprotein susceptibility to oxidation and NF-κB in mice fed an HFHC diet with or without phytosterol supplements. HDL were isolated from four pools of five plasma samples for each diet, corresponding to 8-week-old female PyMT Tg mice fed an HFHC diet with or without a 2% phytosterol supplement. (A) Conjugated diene formation of HDL isolated from pooled plasma samples in the presence of copper expressed as mean±S.E.M. (B) Representative diene formation curves. (C) Quantification of human LDL REM analysis after being exposed to oxidation in the presence of HDL. Electrophoretic mobilities of native and oxidized LDL (100% mobility) are shown for comparison (*P<.05). (D) GP values of Laurdan in HDL expressed as mean±S.E.M. (E) NF-κB/p65 in mammary gland nuclear extracts from 8-week-old female PyMT Tg mice fed an HFHC diet with or without phytosterol supplements. Samples were loaded in duplicate, and data are presented as absorbance at 405 nm±S.E.M. (n=8 and 9 animals in each group). (F) Mammary gland total protein immunoblot of IκBα using GDI as a control of equal loading (two representative samples of eight animals examined for each dietary group are shown).

The PyMT Tg model has been extensively validated for the study of breast carcinoma progression [16,17]. Compared to xenograft models, which are generally created by injection of human cancer cell lines into immunodeficient mice, genetically engineered mouse models have many advantages since they better mimic human disease development. In genetically modified mouse models of cancer, normal cells spontaneously evolve to a hyperplastic, dysplastic and, eventually, a more malignant stage. Moreover, tumor cells develop in concert with their native environment as well as with exposure to the natural growth factors, hormonal and angiogenic milieux and in the setting of an intact immune system [28].

In accordance with the above discussion and for the first time to the best of our knowledge, our experiments were performed using the PyMT Tg mouse model. Our study covered different stages in the development of inherited breast cancer, and the results thus clarify and validate the preventive effect of dietary phytosterols previously

examined in xenograft models [12,13]. Further, it provides a proof of concept that could be tested in patients with breast cancer. In this context, it is noteworthy that a 56% reduction in prostate cancer incidence was observed in Transgenic Adenocarcinoma Mouse Prostate mice fed with a *Pygeum africanum* extract (highly enriched in β-sitosterol) for 5 months [29]. Thus, the anticancer effects of phytosterols may be extended to other cancer types beyond that of the breast.

Phytosterols inhibit cancer cell growth, angiogenesis and invasion by promoting apoptosis of cancerous cells or altering sterol metabolism [6,7]. However, little is known about the molecular mechanisms underlying these protective effects. Phytosterols are absorbed from the diet in small but significant amounts [30].

Several recent studies showed that consumption of HCHF diets resulted in accelerated tumor formation in different mouse models of cancer [31–33], including one in PyMT Tg mice [18]. Fatty acids can fuel cell growth and induce migration, invasion and survival, all major cancer characteristics [34]. Elevated plasma cholesterol levels — a direct consequence of the HFHC diet — increased the cholesterol content in lipid rafts and improved survival of cancer cells by reducing apoptosis [32,35].

It has been suggested that phytosterols may promote beneficial changes in signal transduction by lowering the cholesterol content of lipid rafts, by being directly incorporated into these membrane microdomains or by both mechanisms [36,37]. Importantly, we also showed for the first time that phytosterol supplementation delayed mammary gland tumor onset and progression, only when mice were fed an HFHC diet. As only mice fed an HFHC diet showed reductions in plasma cholesterol levels after consuming phytosterols (data on plasma cholesterol in mice fed an LFLC are not shown), our data suggest that the anticarcinogenic properties of phytosterols may somehow be related to their hypocholesterolemic action. Treatment with ezetimibe, a hypocholesterolemic drug that inhibits cholesterol intestinal absorption, also reduced tumor growth in mice bearing human prostate cancer xenografts, only when they were on an HFHC diet [31]. However, no differences were found in our analysis of mammary gland cholesterol content or uptake. Further and in contrast to the liver, which is more exposed to phytosterols, the mammary gland gene expression of several critical regulators of cellular cholesterol metabolism did not change with phytosterol consumption, and this includes LXR signaling [8,38–40]. Taken together, these results indicate that the hypocholesterolemic effect of phytosterols did not change mammary gland cholesterol availability. Thus, other possible mechanisms by which phytosterols may act as cancer chemopreventive agents were studied in our model.

Several studies reported that oxidized lipoproteins stimulate cell proliferation and cell migration and are related to enhanced tumor progression [9,11,41]. Hyperlipidemia in mice is known to promote LDL oxidative modification and also impairs the antioxidant ability of HDL [42]. Several *in vitro* [43–45] and *in vivo* [26] studies suggested that plant sterols may exert a protective effect against oxidative stress, and our results do demonstrate that phytosterol supplementation protects HDL from lipid peroxidation. Analysis of GP of Laurdan indicates that this protection might be due to changes in the physicochemical properties of HDL induced by their increased phytosterol content. This would increase lipid packing at the lipoprotein surface and inhibit propagation of lipoperoxidative reactions. We also showed that HDL from PyMT Tg mice fed a phytosterol-enriched diet protected human LDL from oxidation. Phytosterols have already been shown to exert an inhibitory effect against copper-lipid peroxidation of LDL *in vitro* [46]. Moreover, it has been demonstrated that the significant decrease in plasma LDL-cholesterol levels in subjects whose diet was supplemented with 2 g/d of plant sterols was associated with a decrease in oxLDL [47] and plasma isoprostanes [48], both being markers of free radical-initiated lipid peroxidation. On the other hand, inhibition of OLR-1 was shown to inhibit cellular transformation in a manner associated with reduced NF-κB activity [9], which has, in turn, been associated with delayed onset and a number of new tumors in the adult mammary epithelium of MMTV-ErbB2 mice [49]. Therefore, we suggest that the antioxidant activity exerted by phytosterols may explain, at least in part, their anticancer effect by protecting cells from oxidative damage and inflammation through decreased NF-κB activation. Finally, activation of the nuclear factor E2-related protein 2-antioxidant response element (Nrf2-ARE) signaling pathway has been considered an effective strategy for antioxidant cell defense and cancer chemoprevention [50]. However, phytosterols did not induce this molecular mechanism in our model (data not shown). In this context, and taking into account that human HDL protects LDL from oxidative modification, it is noteworthy that apoA-I, the main HDL protein component, was recently shown to inhibit tumor development in mice [51]. Further, baseline HDL cholesterol has been found to inversely correlate with the risk of cancer in randomized, controlled trials on lipid-altering therapy [52].

Despite their feasibility, the findings regarding lipoprotein oxidizability and NF-κB have not been shown to be causally linked to the anticancer effects of phytosterols. This topic would warrant further studies. It is, however, noteworthy that the molecular mechanisms by which phytosterols produce their different effects, including the lowering of intestinal cholesterol absorption, have remained elusive despite being the object of intense study over decades. Another limitation of the study is the lack of a food intake record. However, as previously mentioned, the promotional effects of HFHC diet on cancer growth are well established, and we consider unlikely a major effect on palatability of high-fat diets (with a 21.2% fat content), depending on the presence or absence of 2% phytosterol. We do not know of reports showing differences in food intake related to phytosterol supplementation, and furthermore, no statistical difference was found between the weights of mice fed an HFHC with or without added phytosterols.

As mentioned in "Methods," some experiments on mice fed LFLC or HCHF diets were performed at different time points, and thus, direct comparison may not be appropriate. However, there are reports using the same murine model that show that an HFHC diet promoted breast cancer development [18], a finding that was extended also to a genetic murine model of prostate cancer [33]. It is unclear whether the phytosterol inhibition of the tumor-promoting effects of an HFHC diet is due to blocking of the effects on cholesterol or saturated fat [9,18,31,35], and this also warrants further study.

In summary, we showed that dietary phytosterol supplementation delayed tumor onset and progression in the setting of a typical Western diet and suggest that phytosterols may exert these anticancer effects by preventing oxidative damage. It is noteworthy that the protection against cancer provided by phytosterols occurred at the same dose that reduced blood cholesterol and cardiovascular risk. Hence, phytosterols could be incorporated as a dietary supplement, not only to lower the risk of cardiovascular disease but also to potentially prevent or delay cancer development. However, significant biological differences exist between murine and human malignancies, and benefits to patients will need to be directly demonstrated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.01.007.

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