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RESEARCH PAPER

Investigation into the cancer protective effect of flaxseed in Tg.NK (MMTV/*c-neu*) mice, a murine mammary tumor model

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Abstract The aim of the present study was to investigate whether low flaxseed doses relevant to human dietary exposure can prevent mammary tumors in transgenic Tg.NK mice, a model of breast cancer. Animals were exposed to flaxseed through the diet at human relevant levels. Tumor-related parameters and tumor development were evaluated. Hepatic cytochrome P450 and glutathione S-transferase activities were significantly reduced in animals receiving low flaxseed doses. An incidence of palpable tumors before sacrifice, a number of tumors per mouse, and a number of large tumors (>6 mm diameter) at necropsy were statistically significantly lower in the high flaxseed group compared to controls, suggesting a beneficial effect on tumor progression of small dietary doses of flaxseed. However, the number of tumor-bearing mice and multiplicity of tumors at necropsy were not statistically significantly lower compared to the controls. Thus, the effect of small dietary doses of flaxseed on mammary tumor development in Tg.NK mice remains to be established.

Keywords Lignans · Flaxseed · Mammary tumorigenesis · Tg.NK mice · Hepatic enzymes

Abbreviations

End	Enterodiol
Enl	Enterolactone
IsoL	Isolariciresinol
Lar	Lariciresinol
Mat	Matairesinol
Pin	Pinoresinol
Seco	Secoisolariciresinol

Introduction

Breast cancer is the most common form of cancer among the women in the Western world [27]. Epidemiological studies indicate that phytochemicals like flaxseed lignans can protect against mammary cancer [1]. High plasma enterolactone (Enl)[1], a biomarker of lignan exposure, has been correlated with reduced breast cancer risk [2, 3, 32, 33]. Women with breast cancer have been found to have significantly lower urinary excretion of Enl, which indicates lower intake of dietary lignans compared to healthy women [3, 33]. Enl and enterodiol (End) are produced by the gut flora from plant lignan precursors such as secoisolaricesinol (Seco) primarily found in flaxseed and to a much lesser degree in whole grain cereals, berries, nuts, and fruits [42]. The average daily intake of lignans in Western Europe has been estimated to be between 0.4 and 1 mg per person [2, 3, 5, 13, 41] The potential breast cancer protective effect of lignans could be due to their

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weak estrogenic activity [2, 20, 31, 37] and antioxidant properties [18]. Several animal studies indicate that lignans and/or flaxseed can enhance mammary gland differentiation. [38, 40]. Furthermore, flaxseed has been reported to enhance the inhibitory effect of tamoxifen on the growth of human estrogen-dependent mammary cancer [9, 10].

The present study evaluates the effect of flaxseed on mammary gland morphogenesis and tumor development in the Tg.NK (mouse mammary tumor virus [MMTV]/*c-neu*) mice. This animal model carries an activated *c-neu* oncogene, a homologue of the human erbB2 oncogene under the control of mouse mammary tumor virus (MMTV) [34]. ErbB2 oncogene is overexpressed in 25–30% of all human breast cancers [36]. Tg.NK mice develop palpable mammary tumors beginning at the age of 22 weeks without developing tumors in other tissues [34]. This model has been reported as relevant to study dietary prevention of breast cancer and to investigate dietary effects on tumor development. The animals used in the present study were homozygous Tg.NK transgene carriers. The dietary concentrations of flaxseed were aimed to provide intake of lignans comparable to human dietary exposure. The differences in metabolic rates between the mouse and the human were taken into consideration while choosing the dietary concentrations of flaxseed in the experimental diet. In addition, the potential influence of flaxseed on the rate of apoptosis in the developing mammary gland and on the activity of selected drug-metabolizing enzymes was studied.

Materials and methods

Chemicals

Flaxseed was kindly supplied from Kampffmeyermühlen AG, Germany. Carmine, NADPH, glutathione, dicoumarol, cytochrome C, menadione, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 3-amino-9-ethylcarbazole, and HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) were from Sigma Chemical Co. (St. Louis, MO, USA). Beta-glucuronidase/sulfatase (*Helix* pomatia, 5.5 and 2.6 U/ml, respectively) was purchased from Boehringer Mannheim (Mannheim, Germany). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemical Company (IL, USA). 1-Chloro-2,4-dinitrobenzene was from Riedel-de-Häen (Seeize, Germany). Pentoxyresorufin, ethoxyresorufin, and resorufin were from Molecular Probes Europe B. V. (Leiden, The Netherlands), Meyer's hematoxylin and Putts eosin from Prohosp (Denmark) and Eukitt from Bie and Berntsen (Denmark). LM-PCR assay for apoptosis was purchased from Clontech Kit # K 905-1.

Animal, housing, and clinical observations

Tg.NK female mice approximately 3 weeks of age and weighing 12.6 g ± 1.6 (mean ± SD) were obtained from Taconic Farms, Inc., Germantown, NY, USA. All Tg.NK mice were housed 2/cage. Upon the arrival until the start of the experiment, the Tg.NK mice were fed a control semi-synthetic diet (Table 1). In order to prevent the occurrence of anoestrus, four male NMRI mice approximately 4 weeks old were placed in the same room as Tg.NK female mice. The males were obtained from Bommice Bonholtgaard Breeding and Research Center LTD, Rye, Denmark, housed 1/cage, and fed Altromin 1324 diet (Altromin International, Lage, Germany). All mice were kept under controlled environmental conditions (temperature 21 ± 1°C, the relative humidity 55 ± 5%, 12/12 h light/dark cycle, air changed 10 times/h) and had free access to feed and water acidified to pH 3.0 by citric acid (to prevent growth of microorganisms). Body weight and feed intake were recorded weekly once for female Tg.NK mice. All mice were observed at least twice a day for any abnormalities in clinical appearance.

The animal study was performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee.

Tumor study

The animals were randomized into four groups ($N = 21$/group) based on body weight and fed ad libitum the experimental diets from the 25th day of life for 23 consecutive weeks: group 1 (control) a semi-synthetic diet (Table 1), groups 2–4 this semi-synthetic diet added either 60, 180, or 540 mg flaxseed/kg diet. Starting at the 15th week of life, the animals were palpated twice a week for the presence of subcutaneous tumors. At termination, all the remaining Tg.NK mice were anaesthetized by intraperitoneal injection of a pentobarbital solution (60 mg/kg bw). From an uncovered thoracic cavity, blood samples

Table 1 Composition of the control semi-synthetic diet

Component	g/kg
Carbohydrates	680
Casein	180
Cellulose	60
Soy bean oil[a]	40
Mineral mixture	33
Vitamin mixture	9

[a] The major fatty acids in the oil were C 18:2 linoleic and 18:3 linolenic (51.05 and 8.0% of all fatty acids, respectively; ratio n-6/n-3 of 6.4)

were collected by heart puncture and stabilized in heparin. After bleeding to death, the ventral skin was reflected and visible subcutaneous tumor masses were counted, measured, removed, and fixed in 4% neutral buffered formaldehyde. The thoracic and abdominal cavities were examined for gross lesions. Liver was removed and weighed. All the subcutaneous tumor masses were processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for light microscopic examination. Tumor burden was reported as the number of tumors per mouse and multiplicity (number of tumors per tumor-bearing mouse).

Study of mammary gland development

Additional 10 animals per group fed the respective diets for 2 or 6 weeks were sacrificed by barbiturate intraperitoneal injection (60 mg/kg bw) followed by heart blood extraction. The age at the sacrifices was approx. 6 or 10 weeks. All the 10-week-old animals were sacrificed at estrus stated by examination of vaginal smear. Blood samples were separated into plasma and red blood cells (RBC) and stored at −80°C for further analysis. Liver was excised, rinsed in saline phosphate buffer (PBS), weighed, snap-frozen in liquid N_2, and stored at −80°C. Fourth abdominal gland was excised, placed on an object glass, dried for 5 min, and fixed in Carnoy's fixative (75% ethanol, 25% glacial acid) for 2 h followed by staining with carmine alumina according to Thomsen et al. [39].

The differentiation pattern and development of the 4th abdominal mammary gland were analyzed by quantifying the different structures in the mammary gland in ten representative areas of the gland of animals older than 6 weeks and the whole gland in 6-week-old animals, by measuring the longitudinal growth of the gland and by quantifying the degree of branching using a light microscope equipped with ocular micrometer. Structures were defined based on the classification of Brown and Lamartiniere [7]: terminal end buds (TEB) were recognized by having 3–6 epithelial layers and a diameter of more than 100 μm, terminal ducts (TD) were less than 100 μm in diameter and consisted of a single cell layer, alveolar buds (AB) were identified as terminal or lateral buds further differentiated into 2 or more smaller buds and lobuli as structures consisting of more than 5 AB.

Determination of hepatic phase 1 and 2 enzymes

The microsomal and cytosolic fractions of the liver were prepared as described by Lake [21] and stored at −80°C until use. The microsomes were characterized by the specific activities of major cytochrome P450 (CYP) enzymes as described by Burke et al. [8] with modifications as described by Breinholt et al. [6]. The activities of the CYP isozymes 1A1/2, 3A4, 2B1/2 in the microsomal liver fraction were assayed using ethoxyresorufin, benzyloxyresorufin, methoxyresorufin and pentoxyresorufin (EROD, BROD, MROD and PROD), respectively. Hepatic glutathione S-transferase (GST) was analyzed in hepatic cytosol as described by Ernster [15] and Habig et al. [16]. The protein concentration was determined by either a commercially available kit or the bicinchoninic acid method [35] adapted to a Cobas Mira (Roche) in case of protein concentrations below 10 mg/ml.

Detection of nucleosomal ladders in the fourth abdominal mammary gland by LM-PCR and their densitometric quantitation of DNA from the abdominal mammary gland was isolated according to Ausbel et al. [4]. The detection of the apoptotic nucleosomal DNA ladders in the abdominal mammary gland was made as previously described by Dalgaard et al. [12]. In short, during apoptosis, cellular endonucleases cleave genomic DNA between nucleosomes, producing fragments whose lengths vary by multiples of 180–200 bp. When resolved by agarose/EtBr gel electrophoresis, these DNA fragments appear as a "nucleosomal ladder." The ligation-mediated PCR (LM-PCR assay from Clontech Kit # K 905-1) that uses PCR reaction to specifically amplify the nucleosomal ladder, making it easier to visualize, was used. The densitometric analysis of apoptotic banding pattern obtained by LM-PCR was performed using the densitometer software (Alpha Ease™, Alpha Innotech, San Leandro, CA). Using this densitometer software, outlines with equivalent areas were drawn around the bands and the intensity of the total area was determined.

Analysis of lignans in plasma samples and diets

Metabolic profile of lignans in plasma samples obtained from each animal at the termination of tumor study was carried out after enzymatic hydrolysis by HPLC with coulometric electrode array detector following a previously published method [29, 30]. Enterolignans, Enl and End, formed by gut microflora from dietary precursors such as Seco, Mat, Lar and Pin were used as a biomarker of exposure to dietary lignans. The lignan content of the diets and in flaxseed was determined by isotope dilution GC-MS as reported [29, 30].

Statistics

Data are presented as group mean values plus standard deviation when appropriate. Before subjected to further analysis, the data on body weight, feed intake, liver weight, and tumor size were tested for normal distribution, and the homogeneity of variance among the groups was evaluated

by judgment of standardized residuals plot. The data on body weight and feed intake were analyzed by GLM analysis followed by Dunnett's test. Data on number of tumors per mouse and tumor size were analyzed by Kruskall–Wallis test. Data on tumor incidence were analyzed by Fischer exact test and data on survival using the lifetest procedure. A one-way analysis of variance was used to compare data on mammary gland development and enzyme data. For the statistical analysis of the relative apoptotic density, Mann–Whitney test was employed. All Statistical analyses were performed using SAS v. 6.08 (SAS Institute, Inc., Cary, NC, USA).

Results

Lignan content in diets, flaxseed, and plasma

Seco was the predominant lignan in the flaxseed and the only lignan, in which concentration was increased with increasing concentrations of flaxseed in the diet (Table 2). The concentrations of matairesinol (Mat) and pinoresinol (Pin) were comparable in all flaxseed-added diets. The concentration of larciresinol (Lar) in the flaxseed-added diets was similar to that in the control diet.

Plasma Enl concentrations were near detection limits [29, 30]. Although the dietary exposure to Seco was different between groups as confirmed by the diet analysis (Table 2), plasma Enl concentrations were similar in animals receiving control and flaxseed-added diets: 3.22 nM ± 0.28 (SD) in the control, 3.55 nM ± 0.23 in the low-dose group, 2.87 nM ± 0.21 in the middle-dose group, and 2.81 nM ± 0.06 in the high-dose group. Since plasma Enl concentration did not reflect the differences in Seco intake, the plasma concentration of End, the precursor of Enl, was measured: 42.03 nM ± 2.68 (SD) in the control, 95.64 nM ± 0.12 in the low-dose group, 57.59 nM ± 0.28 in the middle group, and 83.80 nM ± 0.22 in the high-dose group. The plasma concentration of enterolignans (Enl and End) was 45 nM in the control group. The concentrations of enterolignans in all the flaxseed groups were comparable: 99, 61 and 87 nM in the low, middle and high flaxseed diet groups, respectively. The plasma levels of enterolignans in the flaxseed-exposed groups did not reflect the difference in lignan content of the flaxseed-added diets.

Table 2 Measured concentrations of lignans in the experimental diets and in the flaxseed (μg/100 g)

	Seco	Mat	Lar[a]	Pin
Control diet	4.98	0.25	21.37	1.90
Low flaxseed diet	5.41	1.13	21.43	5.45
Middle flaxseed diet	20.38	1.00	20.04	4.98
High flaxseed diet	87.02	1.08	22.72	5.30
Flaxseed	609,346	3,565	26,148	12,134

[a] The sum of Lar and Lar decomposition product isolariciresinol (*IsoL*) content

Clinical appearance, body weight, and survival

The clinical appearance of Tg.NK mice did not differ among the groups. With regard to behavior, the Tg.NK mice appeared very active and tended periodically to run in circles. Body weight of mice on diets added low, middle or high concentration of flaxseed were comparable to those of mice fed a control diet from the start of the experimental feeding until the termination (Table 3). The feed intake was comparable between all groups.

In the control group, four mice died prior to the terminal sacrifice: on days 108, 115, 122, and 128 of treatment (Fig. 1). All the mice were tumor-negative. On day 139, one control tumor-positive mouse was euthanized due to ethical reasons. In the low flaxseed group, four mice died prior to the terminal sacrifice: on days 101,119, 120, and 147. All the mice were tumor-negative. In the middle flaxseed group, two mice died prior to the terminal sacrifice: on days 106 and 112. The mice were tumor-negative. In the high flaxseed group, six mice died prior to the terminal sacrifice: on days 95, 96, 103, 120, 122, and 123. All the mice were tumor-negative. The number of mice surviving to the terminal sacrifice was 16, 17, 19 and 15 in the control, low, middle and high flaxseed groups, respectively.

Tumor development

The pattern of palpable tumor incidence is presented in Fig. 2.

The first palpable tumors were recorded from day 134 (week 19) of treatment. The last examination of tumor development by palpation was on day 157 (week 22) of treatment. The incidence of palpable tumors on that day was significantly lower in the high flaxseed group compared to the control group (1 animal vs. 10, $P < 0.01$) (Fig. 2).

Macroscopic examination did not reveal tumors in other tissues than mammary glands. The incidence of mammary tumors recorded at necropsy was not statistically significantly different between the groups (Table 4). The number of tumors per mouse was statistically significantly lower in the high flaxseed group, but multiplicity of tumors (number of tumors per tumor-bearing mice) was not significantly different between the groups. In addition, mice from the high flaxseed group receiving the highest dose of flaxseed had a statistically significantly lower number of big tumors i.e., tumors bigger than 6 mm in diameter compared to control animals ($P < 0.01$) (Fig. 3).

Table 3 Body weight, feed intake, and liver weight of animals in the tumor study

Group/dose of flaxseed	Number of animals		Body weight (g)		Feed intake (g/animal/week)	Liver weight	
	Start	Scheduled termination	Initial	Scheduled termination		Absolute (g)	Relative
Control	21	16	17 ± 1.3	27 ± 2.6	23 ± 1.2	1.1 ± 0.08	4.0 ± 0.29
Low	21	17	17 ± 1.3	27 ± 1.9	22 ± 0.4	1.0 ± 0.08	3.8 ± 0.23
Middle	21	19	17 ± 1.5	28 ± 3.2	24 ± 1.1	1.1 ± 0.12	3.8 ± 0.63
High	21	15	17 ± 1.5	27 ± 2.5	25 ± .1.2	1.1 ± 0.10	4.1 ± 0.48

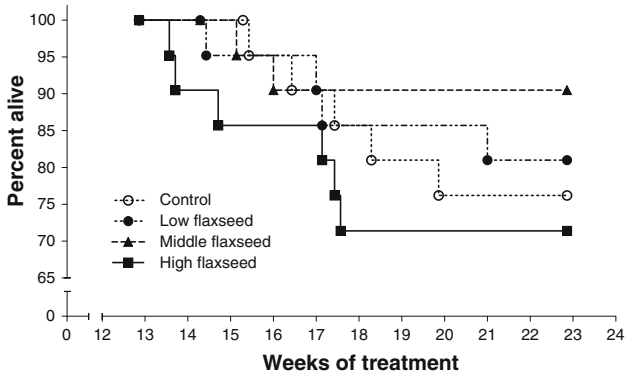

Fig. 1 Survival of Tg.NK mice. ($P > 0.5$, lifetest procedure)

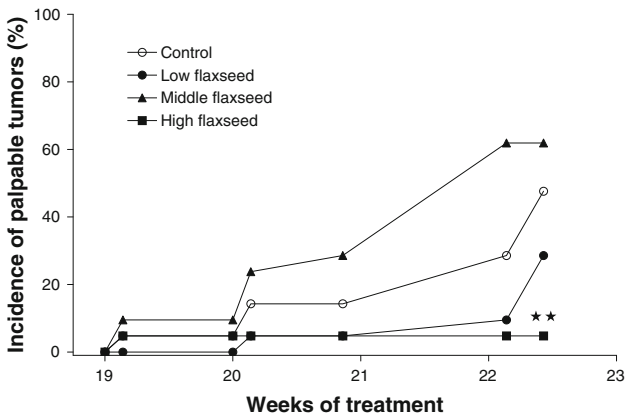

Fig. 2 The percent incidence of palpable mammary tumors per week and treatment group is shown. $^{**}P < 0.01$ (Fischer exact test)

Histopathological examination of mammary tumors indicated that all mammary tumors could be classified as adenocarcinoma. Tumors usually consisted of solid sheets with cystic spaces filled with tissue fluid, blood, or debris.

Effects on mammary gland development

No differences in mammary gland development as assessed by branching, longitudinal growth, and differentiation were recorded between the flaxseed diet groups compared to the control group (Fig. 4a, b). Within the same group, a statistically significant increase in the size of the mammary gland from week 6 to week 10 of age was recorded (Fig. 5): the gland size was more than double in 10-week-old animals reflecting a physiological development of the gland from weaning until adulthood.

Effects on apoptosis within the mammary gland

Apoptosis in the mammary gland was found to decrease with increasing age. Variation in apoptotic levels between animals was high, and no differences between exposure groups were detected (data not shown).

Effects on activity of hepatic phase 1 and 2 enzymes

A statistically significant increase in cytochrome P450 isozymes activities CYP1A1 and 1A2 (EROD and MROD) was recorded in 10-week-old mice exposed to the low dietary dose of flaxseed (Fig. 6).

Hepatic GST activity was statistically significantly decreased in animals receiving the middle and high doses of flaxseed compared to the controls ($P < 0.05$) (Fig. 7).

Discussion

Several epidemiological studies indicated that a high lignan intake had a protective effect against breast cancer. As 20–40% of all human mammary tumors overexpress erbB-2 [36], the Tg.NK mouse model that carries an activated analogue, the *c-neu* oncogene, driven by the mouse mammary tumor virus (MMTV) promoter [25] was chosen as a relevant model for studying in vivo effects of flaxseed on the development of *c-neu*/ErbB-2/HER-2-positive tumors. A semi-synthetic diet was used in the present study to prevent that phytochemicals like phytoestrogens, isoflavones, and lignans commonly present in standard fixed open formula laboratory chows based on plant ingredients could influence the outcome of the experiment in which low

Table 4 Mammary tumor incidence and number

Group/dose of flaxseed	Number of mice			Tumor-bearing mice		Number of tumors per mouse	
	Start	Termination	No.	% of all mice at risk	% of surviving to the termination	All mice at risk	Mice with tumors
Control	21	16	16	76	100	4.24 ± 3.67[a]	5.56 ± 3.20[a]
Low	21	17	15	71	88	3.33 ± 3.35[a]	4.67 ± 3.06[a]
Middle	21	19	18	86	95	4.90 ± 4.56[a]	5.72 ± 4.42[a]
High	21	15	12	57	80	1.81 ± 2.36[b]	3.17 ± 2.33[a]

Means within the same column bearing different superscripts are significantly different ($P < 0.05$, analysis of variance with Duncan's multiple-range test)

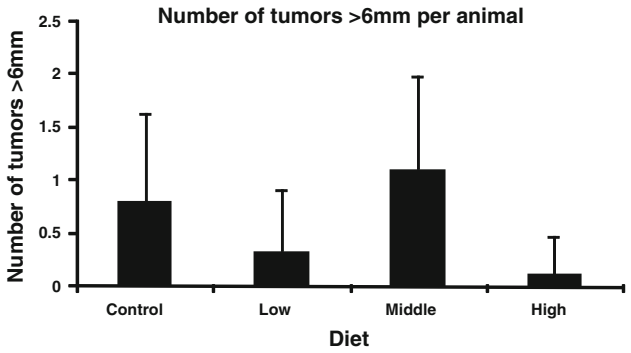

Fig. 3 Number of tumors >6 mm found per animal after dietary exposure to the different flaxseed doses (60, 180, or 540 mg flaxseed per kg diet referred to as low, middle, and high diet, respectively). *Bars* indicate the mean ± SD ($n = 10$). $^{**}P < 0.01$ (Kruskal–Wallis)

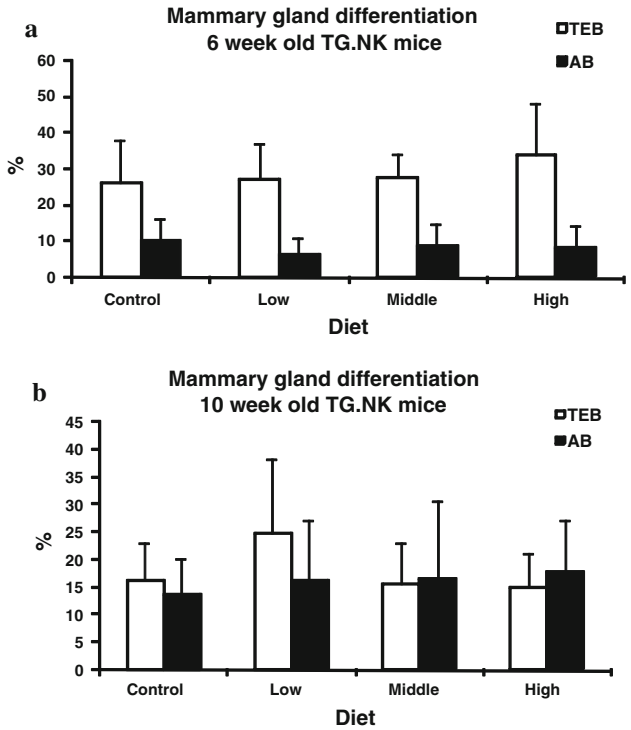

Fig. 4 **a** Mammary gland differentiation in 6-week-old animals as assessed by the percent of the mammary gland structure representing terminal end buds (*TEB*) and alveolar buds (*AB*) in the 4th abdominal gland for the different treatment groups (60, 180, or 540 mg flaxseed per kg diet referred to as low, middle, and high diet, respectively). *Bars* indicate the mean ± SD ($n = 10$). $P > 0.05$ (Dunnetts test). **b** Mammary gland differentiation in 10-week-old animals assessed by the percent of the mammary gland structure representing terminal end buds (*TEB*) and alveolar buds (*AB*) in the 4th abdominal gland for the different treatment groups (60, 180, or 540 mg flaxseed per kg diet referred to as low, middle, and high diet, respectively). *Bars* indicate the mean ± SD ($n = 10$). $P > 0.05$ (Dunnetts test)

concentrations of flaxseed were used. Flaxseed was used as a dietary lignan source. In addition, flaxseed also contains n-3 polyunsaturated fatty acid (n-3 PUFA) and has been shown to be inversely associated with breast cancer risk [19]. Tg.NK mice fed diets high in n-3 PUFA developed mammary tumors later than when fed n-6 PUFA diets [23]. It has been suggested that the ratio of n-6/n-3 fatty acids may be important in mammary cancer development in Tg.NK mice [23]. However, it is believed that the cancer protective effect of n-3 PUFA can be attributed to a general cancer protective effect and is not specific to mammary cancer in contrast to lignans which is considered due to their estrogenic properties. The dietary exposure of mice to flaxseed was aimed to mimic a one-third, the mean, or three times the mean human dietary exposure to lignans from the Western diet [13]. Thus, the concentrations of flaxseed in the experimental diets were calculated based on the average feed intake by laboratory mouse, the flaxseed content of lignans according to information received from the supplier, and on the difference in metabolic rate between human and mouse applying mouse *versus* human compensation factor [24]. Analysis of the diets revealed lower content of lignans to what was expected based on the information from the supplier. The analysis further demonstrated that the flaxseed-added diets primarily contained two lignans Seco and Lar and only minor amounts of Mat and Pin. However, only the concentration of Seco, a predominant lignan in the flaxseed used, was increased with increasing concentrations of flaxseed in the diet. The measured plasma concentrations of enterolignans indicated an enhanced systemic exposure to lignans in flaxseed groups compared to the control group, but it demonstrated

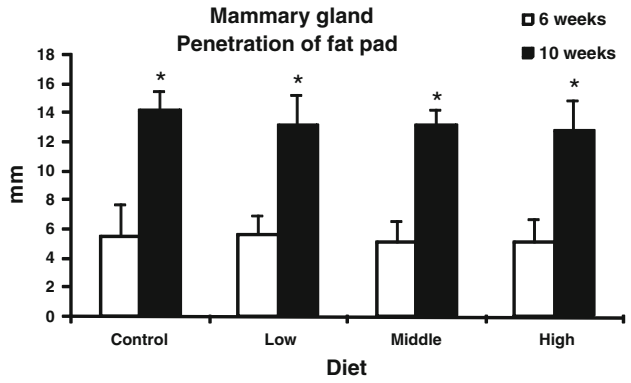

Fig. 5 Penetration of the fat pad by the mammary gland in 6-week-old and 10-week-old animals for the different treatment groups (60, 180, or 540 mg flaxseed per kg diet referred to as low, middle, and high diet, respectively). The penetration into the fat pad was measured by a *straight line* from the outer lymph node of the 4th abdominal gland into the fat pad. *Bars* indicate the mean ± SD ($n = 10$). *$P < 0.05$ (Dunnetts test)

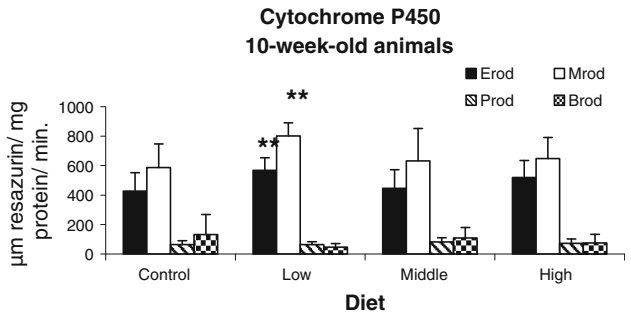

Fig. 6 Cytochrome P450 activities in Tg.NK mice liver microsomes after dietary exposure to the flaxseed doses (60, 180, or 540 mg flaxseed per kg diet referred to as low, middle, and high diet, respectively) for 6 weeks (i.e., 10-week-old animals). Cytochrome P450 activities are expressed in µmol resorufin/mg protein/minute. *Bars* indicate the mean ± SD ($n = 10$). **$P < 0.01$ (Dunnetts test)

Fig. 7 GST activities in the cytosolic liver fraction of Tg.NK mice after dietary exposure to three flaxseed doses (60, 180, or 540 mg flaxseed per kg diet referred to as low, middle, and high diet, respectively)for 6 weeks (i.e., 10-week-old animals). GST activity is expressed in Units GST (µmol glutathione-conjugate/minute)/g protein. *Bars* indicate the mean ± SD ($n = 10$). *$P < 0.05$ (Dunnetts test)

lack of the difference in the systemic exposure between the three flaxseed groups probably because of the small differences in the doses. The latter was considered a confounding factor in the interpretation of the results. It is noteworthy, however, that the plasma levels of enterolignans in the mice from flaxseed diet groups were comparable to plasma Enl levels found in humans [32, 44].

In the present study, the dietary exposure to flaxseed had no effect on mammary gland differentiation. It could be speculated that the dietary concentrations were too low to affect age-related maturation of mammary gland. However, much higher dietary concentrations of flaxseed (5 or 10%) administered to weaned rats had no effect on mammary gland morphogenesis [40]. Several reports indicate that only gestational and/or lactational exposure to flaxseed can alter mammary gland development, whereas exposure after weaning does not influence mammary gland differentiation in rats [14, 17].

Cytochrome P450 isozymes play an important role in the metabolism of xenobiotics and are known to play an role in the activation of carcinogens and drug metabolism. They are often induced by the compounds they metabolize, and as cytochrome P450 isozymes are involved in the metabolism of mammalian lignans [26], the effect of flaxseed dietary intake on their activity was of interest. A recorded significantly increased activity of CYP1A1 and 1A2 in 10-week-old mice from the low flaxseed group when compared to the control group may indicate that even low dietary exposure to flaxseed can modulate cytochrome P450 isoenzymes. It is, however, noteworthy that the same effect was not detected in the middle- and high-dose groups, and it could speculated if this might have due to with an increasing intake of n-3 PUFA as inhibitory effects of n-3 PUFA toward several CYP isozymes including CYP1A1 and 1A2 have been observed [28, 43]. Thus, an induction of CYP1A1 and 1A2 by lignans might be counteracted by an inhibiting effect of n-3 PUFA in flaxseed, resulting in the observed no effect at the higher dose levels.

Induction of hepatic phase 2 enzymes' activity is considered a cancer protective effect. Although it can be argued whether modulation of xenobiotic metabolism is relevant to tumorigenesis in Tg.NK mice, as tumors in these animals are induced by a genetic alteration rather than a chemical, the inhibition of GST activity in 10-week-old animals exposed to dietary flaxseed concentrations mimicking the average human lignan intake could be of interest. The GST isoenzymes constitute one of the major groups of enzymes involved in the detoxification of both endogenous and exogenous chemicals. A decreased activity of GST in the liver might result in impaired resistance to toxic agents such as oxidants or chemical carcinogens. Information about whether hepatic or red blood cell GST is

also down-regulated in humans receiving lignan-rich and/or supplemented diet is needed.

In the present study, apoptosis was found to decrease with increasing age in accordance with physiological mammary gland development; i.e., high proliferation rate during puberty followed by proliferation decrease with age [39] but the dietary exposure to low flaxseed doses did not influence apoptosis as no difference in apoptotic levels was found between exposure groups.

The only tumors observed in the Tg.NK mice were mammary tumors in agreement with a previous report on tumor development in this animal model [34]. A study in nude mice revealed that flaxseed enhanced the inhibitory effect of tamoxifen on the growth of estrogen-dependent human breast cancer [9]. It has been reported that exposure to flaxseed and its purified lignan Seco during lactation inhibited chemically induced mammary carcinogenesis in rats exposed to flaxseed solely [11]. Exposure of Tg.NK mice during post-weaning life to flaxseed added to standard chow or a high-fat diet did not statistically significantly affect the onset and incidence of mammary tumors [22]. In the present study an incidence of palpable tumors at the end of a clinical phase of the experiment (day 157 of treatment), a number of tumors per mouse, and a number of large tumors (i.e., tumors bigger than 6 mm in diameter) at necropsy were statistically significantly lower in the high flaxseed group compared to the control group. The significantly decreased number of tumors per animal in the high-dose group could indicate a delay in tumor development due to increased exposure to flaxseed. Inhibition of GST at the middle- and high-dose groups could result in increased availability of flaxseed lignans and n-3 PUFA acids resulting from reduced metabolism and excretion of those components. These findings might suggest that dietary exposure to small doses of flaxseed may delay mammary tumor progression. However, in the same flaxseed group, the number of mammary tumor-bearing mice and the multiplicity of tumors recorded at necropsy were not statistically significantly lower compared to the controls. Considering that the plasma levels of enterolignans, though higher than in the control groups, were comparable in all flaxseed groups, the recorded beneficial effects on tumor-related endpoints in the high flaxseed group might be an incidental finding. Therefore, it is concluded that the effect of small dietary doses of flaxseed on mammary tumor development in Tg.NK mice remains to be established.

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