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Research Article

Dietary sources of lignans and isoflavones modulate responses to estradiol in estrogen reporter mice

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Dietary phytoestrogens, such as the lignan metabolite enterolactone (ENL) and the isoflavone genistein (GEN), are suggested to modulate the risk of estrogen-dependent disease (*e.g.*, breast cancer) through regulation of estrogen signaling. However, the effects of complex food items containing lignans or isoflavones on estrogen receptor (ER) transactivation have not been assessed so far. In this study, the modulation of ER-mediated signaling by dietary sources of lignans (cereals and flaxseed) and isoflavones (soy) was studied *in vivo*. Adult ovariectomized 3 × ERE-luciferase (luc) reporter mice received isocaloric diets supplemented with flaxseed, rye, wheat, or soy for 40 h or two weeks, and an additional group of mice was challenged with 17β-estradiol (E_2) following the two-week dietary intervention. In non-E_2-treated mice, soy diet induced luc expression in liver, mammary gland, and pituitary gland while the other diets had no effects. Interestingly, all diets modulated the E_2-induced luc expression. In particular rye diet efficiently reduced E_2-induced luc expression as well as uterine growth, the hallmark of estrogen action *in vivo*. It is concluded that dietary sources of lignans and isoflavones can modulate estrogen signaling *in vivo*. The results suggest intriguing possibilities for the modulation of the risk of estrogen-dependent diseases by dietary means.

Keywords: Diet / Estrogen reporter mouse / Estrogen response / Isoflavones / Lignans

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1 Introduction

Lignans are phenolic compounds present in foods of plant origin, such as cereals, berries, and vegetables [1 – 3]. The main sources of lignans in Western diets include cereals and cereal products [4, 5]. Plant lignans, such as matairesinol (MR), secoisolariciresinol (SECO), pinoresinol (PINO), and lariciresinol (LAR), are converted to enterolignans enterodiol (END) and ENL by gut microbes [6]. ENL, as well as intake of lignan-rich diets, is suggested to lower the risk of breast cancer through regulation of estrogen signaling [7, 8]. *In vitro*, ENL transactivates estrogen receptors ERα and ERβ with preference for ERα, stimulates proliferation of estrogen-dependent cells, and induces the expression of endogenous estrogen target genes [8 – 10]. We have shown previously that ENL is also estrogenic *in vivo* by inducing luciferase (luc) expression in the uterus and vagina of ovariectomized (OVX) 3 × ERE-luc reporter mice [10]. However, despite the estrogenic activity in several different models, purified ENL and its precursors (plant lignans), given as pure compounds or in their natural plant matrix (flaxseed), reduce the growth of estrogen-dependent tumors in experimental mammary carcinoma models [8, 11, 12], and long-term administration of ENL reduces uterine weight in adult intact female rats [13].

In addition to lignans, there are several other phenolic compounds in the human diet with suggested estrogenic effects. GEN is an isoflavone mainly present in soy [4], and

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Abbreviations: AIN, AIN93M rodent diet; **CPS,** counts per second; **Ctrl,** control; **DAID,** daidzein; **E_2,** 17-estradiol; **END,** enterodiol; **ENL,** enterolactone; **ERα/ERβ**, estrogen receptor α/β; **GEN,** genistein; **GLY,** glycitein; **LAR,** lariciresinol; **luc,** luciferase; **MR,** matairesinol; O**-DMA,** *O*-desmethylangolensin; **OVX,** ovariectomized; **PINO,** pinoresinol; **RLU,** relative light units; **SECO,** secoisolariciresinol; **SYR,** syringaresinol

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intake of isoflavones is suggested to account for the low incidence of breast cancer in Asian populations where consumption of soy is high [14]. *In vitro*, GEN transactivates both ERs with preference for ERβ, induces expression of estrogen-responsive genes, and promotes proliferation of estrogen-dependent cell lines [15 – 17]. Accordingly, GEN induces estrogen reporter gene expression in various tissues in reporter mice *in vivo* [18]. However, in contrast to ENL and flaxseed, GEN and soy promote uterine weight in rodents [19 – 21] and support the growth of estrogen-dependent tumors in experimental mammary carcinoma models [17, 22 – 24].

In brief, ENL and GEN are phenolic compounds derived from human diet with suggested estrogen-like effects. However, neither of these compounds is naturally consumed as a single compound, but as a part of a complex food matrix. Intake of diets rich in lignans or isoflavones will result in exposure to a mixture of potentially bioactive compounds (*e.g.*, GEN, daidzein (DAID), glycitein (GLY), equol, and *O*-desmethylangolensin (*O*-DMA) in the case of soy, and ENL, END, and SECO in the case of flaxseed). Hence, it is difficult to predict the net effect on ER-mediated signaling of dietary intake of diets rich in lignan or isoflavones based on studies conducted with single pure compounds. In the present study, we applied a transgenic estrogen reporter mouse model that has proven to be a sensitive indicator of estrogen activity [10, 25, 26] to assess the effects of dietary sources of lignans (rye, wheat, flaxseed) and isoflavones (soy) on estrogen reporter gene activity, and on the morphology of the mammary gland and uterus *in vivo*. In addition, the possible antiestrogenic effects of the dietary sources of lignans and isoflavones were tested by exposing mice to a single dose of E_2 after a sub-chronic maintenance on the experimental diets.

2 Materials and methods

2.1 Diets

The diets were based on the semisynthetic AIN93M (AIN) rodent diet [27] with a slight modification: soy oil was replaced with rape seed oil in order to eliminate all soy-derived materials. Rye bran, wheat bran, crushed flaxseed, and soy granules were purchased from a local supermarket in Turku, Finland, and analyzed for their nutritional content (crude fat, total protein, carbohydrates, simple sugars, and dietary fiber) at MTT Agrifood Finland (Jokioinen, Finland). The experimental diets were designed by Special Diet Services (SDS, Whitham, Essex, UK) so that the fiber in rye, wheat, and flax diets was derived from the added rye bran, wheat bran, and flaxseed, and the protein in soy diet from the added soy granules. With this definition the experimental diets contained 9.9% wheat bran, 11% rye bran, 14% flaxseed, and 22% soy granules (Table S1 of Supporting Information). The calculated content of macronutrients, fiber, and energy in the diets remained comparable to the basal AIN (Table S2 of Supporting Information).

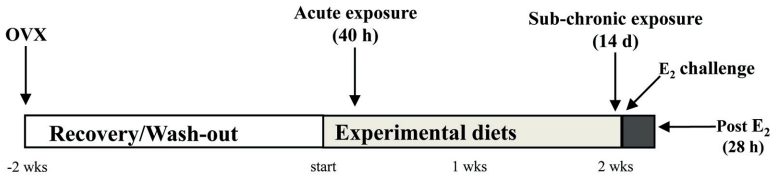
The concentrations of isoflavones (GEN, DAID, GLY, genistin, daidzin) and plant lignans (SECO, MR, LAR, PINO, syringaresinol (SYR)) were determined in all diets. Isoflavones were analyzed as described earlier [28] and plant lignans with a modification of the method previously published by Peñalvo *et al*. [29]. Briefly, homogenized samples (approximately 50 mg) were hydrolyzed as described before, but instead of SPE, samples were extracted twice with diethyl ether. Combined extracts were evaporated and samples were purified with QAE-Sephadex A-25 in acetate form. Instead of GC-MS applied in the original method, samples were analyzed with HPLC-coulometric electrode array detector (CEAD) using the chromatographic conditions described earlier for urinary lignans [30]. The isoflavone analysis was performed in duplicates and the coefficients of variation were less than 5% for all the analytes. The lignan analysis was conducted with single samples. Recovery rates of the analytes were determined with a standard mixture treated like the samples. The recovery rates ranged from 57% (MR) to 100% (PINO) and were comparable to earlier published values [29]. Purity of all reference compounds was 95% or more according to the product information. DAID was synthesized by Professor Hase's group (Laboratory of Organic Chemistry, University of Helsinki, Helsinki, Finland) and GEN and GLY were obtained from Nutrilab (Giessen, the Netherlands). Plant lignans were purchased from ArboNova (Turku, Finland).

2.2 Animals and study design

The animal experiment was performed at the animal department of the University of Turku (ethical license 1492/05) and the mice were housed under the standard conditions. One hundred adult female 3 × ERE-luc mice [25], heterozygous for the transgene, were OVX and allowed to recover for two weeks, during which they consumed basal AIN diet. After the 2-week recovery/wash-out period, mice were randomized into five treatment groups: AIN ($n = 23$), soy ($n = 19$), flax ($n = 19$), rye ($n = 19$), and wheat ($n = 20$) (Fig. 1A).

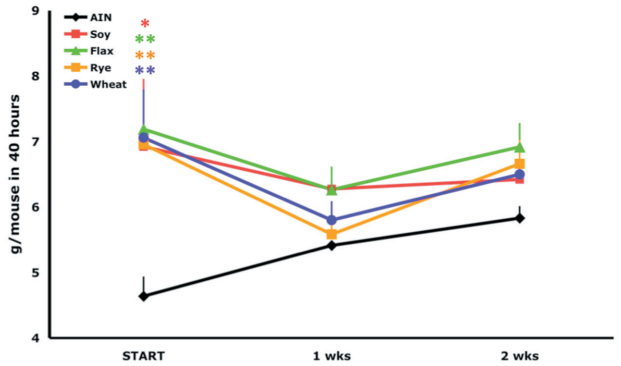
The effects of the diets on the reporter gene expression and uterine morphology were evaluated in three different settings: in acute (40 h), sub-chronic (14 days), and E_2 exposure (Fig. 1A). Mammary gland morphology was assessed after sub-chronic exposure only. For the E_2 challenge, mice kept 15 days on the experimental diets were injected intra-peritoneally with a single dose of E_2 (50 μg/kg) (Sigma, St. Louis, MO, USA). Four animals on AIN received vehicle only (10% DMSO in rape seed oil) and served as negative controls (Ctrls), while the rest ($n = 7$) received E_2 and served as positive Crtls. Food consumption and body weight gain were recorded once a week throughout the experiment.

A



N	AIN	SOY	FLAX	RYE	WHEAT	TOT
Acute	6	6	6	6	7	31
Sub-chronic	6	6	6	6	6	30
Post E_2	7 (+E_2), 4 (-E_2)	7	7	7	7	39
TOT	23	19	19	19	20	100

B



C

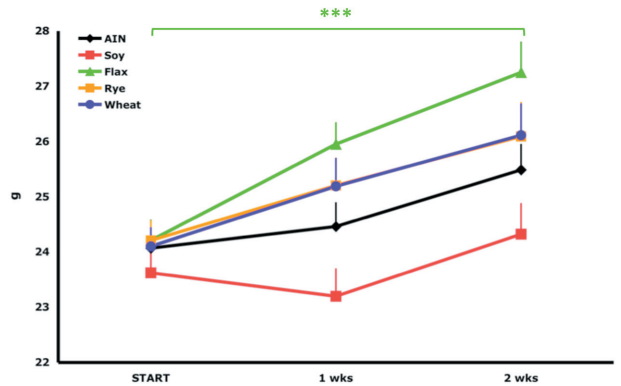


Figure 1. (A) Experimental setup and number of animals in the different dietary groups. Food consumption (B) and body weight gain (C) were measured once a week during the experimental period. The color-coded asterisks indicate statistically significant difference to AIN, unless indicated otherwise, evaluated with two-way ANOVA and Tukey's *post hoc* test at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

In each setting, tissues (brain, pituitary gland, thoracic mammary glands (nos. 2 and 3), bone (tibia), muscle (quadriceps), liver, uterus, and vagina) were collected for *ex vivo* luc analysis. In addition, the weight of the uterus, liver, and kidneys were recorded at sacrifice, and serum was drawn for lignan and isoflavone analysis. One uterine horn and an abdominal mammary gland (no. 4) were collected for histology.

2.3 *In vivo* imaging

Luc induction in the E_2-exposed animals was monitored in real time with *in vivo* imaging using Xenogen IVIS50 Imaging System equipped with XGI-8 Gas Anesthesia System (Caliper Life Sciences, Runcorn, UK). Mice were anesthetized with isofluran (Baxter, Helsinki, Finland), administered D-luciferin (25 mg/kg in PBS *s.c.*) (Synchem, Altenburg, Germany), and imaged 4, 8, 12, 16, 22, and 28 h (±10 min) post E_2 (or vehicle) injection. A background (0 h) image was taken prior to the E_2 (or vehicle) injection. An area over the upper abdomen, where the strongest luc activity was observed, was quantified with LivingImage 4.09A Carbon software (Xenogen, Lake Oswego, Oregon, USA). The time point of maximal luc induction was determined for each mouse, the area under the curve calculated with GraphPad Prism, and the CV in the dietary groups calculated for each time point after the E_2-injection.

2.4 *Ex vivo* luc analysis

Luc activity in tissue samples was measured as described earlier [10]. The results were calculated according to Eq. (1), where RLU stands for relative light units, CPS for counts *per* second, and CPS (background) is the average CPS measured from each plate before the luc reaction is started. Protein (sample) refers to protein concentration measured with Bio-Rad's Protein Assay (based on the method of Bradford) using BSA standards (Bio-Rad, Hercules, CA, USA).

$$\mathrm{RLU} = \frac{\mathrm{CPS(sample)} - \mathrm{CPS(background)}}{\mathrm{protein(sample)}} \quad (1)$$

Negative RLU values were considered as no detectable activity and assigned a value of 0. In figures, the activity in the AIN group is set to 1.

2.5 Phytoestrogens in serum

Three serum samples per group from the acute and subchronic time points were selected for serum phytoestrogen analysis. Due to the limited sample volume, serum from the wheat-, rye-, and flax-fed mice was only analyzed for lignans and serum from the soy-fed mice for isoflavones. The samples were prepared for the analyses according to a slight modification of the method previously described by Nurmi and Adlercreutz [31] and Peñalvo *et al*. [32]. Briefly, samples were thawed and 200 μL was hydrolyzed overnight at 37°C with four volumes of 0.2 M sodium acetate buffer pH 5, containing 0.2 Units/mL of β-glucuronidase and 2 Units/mL of sulfatase. In cases where available sample volume was less than 200 μL, the amount of hydrolysis buffer was scaled down. Cool samples were extracted twice with 4 mL of diethyl ether and combined extracts were evaporated under N_2-flow. Isoflavones and their metabolites were measured from samples dissolved in 70% methanol. Samples from the AIN mice were first dissolved in methanol, then half of the volume was taken for further purification carried out prior to lignan analysis, and the other half was evaporated under N_2-flow again and dissolved in half the original volume of 70% methanol for isoflavone analysis. Samples for the lignan analysis were purified with QAE-Sephadex A-25 in acetate form as described earlier [32]. Isoflavones, lignans, and their metabolites were analyzed with an HPLC-CEAD under conditions reported earlier for food isoflavones [28, 30]. The origin and purity of the used reference compounds were the same as described above in the analysis of the diets. Additionally, equol and *O*-DMA were obtained from Professor Hase's group (University of Helsinki, Helsinki, Finland) and END and ENL purchased from VTT Technical Research Center (Espoo, Finland) (purity of all > 95%).

2.6 Morphology of the mammary gland and uterus

Abdominal mammary glands were processed into whole mounts, and paraffin-embedded uterine horns into 5 μm thick hematoxylin & eosin-stained tissue sections. The mammary whole mounts were evaluated under Olympus SZX9 Stereo Microscope, and the uterine sections under Olympus BX51 Microscope equipped with an Olympus DP70 Digital Camera System. Images of the uteri were taken for the measurement of epithelial thickness. The measurement was performed with ImageJ (http://rsb.info.nih.gov/ij/) calibrated against scale bars in the images. The height of the luminal epithelium was evaluated by taking 15 measures per cross-section from the basal lamina to the apical surface of the epithelial cell lining. Two cross-sections *per* mouse, cut from the middle part of the uterine horn, were measured. Average of the two estimations (both being averages of the 15 measures) were considered as the height of luminal epithelium in one sample.

2.7 Statistical analysis

Normally distributed data (animal weight gain, food consumption, organ weights) were analyzed with one-way or two-way ANOVA followed by Tukey's *post hoc* test. Unparametrical data (all luc data and serum lignan concentrations) were first log-transformed to achieve normality and then analyzed as above. The time point of maximal luc activity was analyzed by Chi Square test. In tables and figures, results are presented as average + SEM and asterisks indicate statistically significant differences to AIN (or AIN + E_2 where indicated) at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3 Results and discussion

3.1 Food consumption and body weight gain

At the start of the experiment, all mice receiving supplemented diet (soy, rye, wheat, flax) consumed significantly more feed than mice that continued on AIN diet. This difference was transient and lost statistical significance at later points (Fig. 1B). Consumption of the flax diet remained higher (difference not statistically significant) than consumption of the other diets throughout the experiment, and only mice on the flax diet gained weight significantly during the experiment (Fig. 1C). Experimental diets did not affect the relative weights of the liver and kidneys (data not shown).

3.2 Exposure to phytoestrogens

The concentrations of lignans and isoflavones in the diets were determined (Table 1), intake of these compounds cal-

Table 1. Lignans and isoflavones in the diets (μg/100 g)

	AIN diet	WHEAT diet	RYE diet	FLAX diet	SOY diet
DAID[a)]	0	0	0	0	4372
GEN[a)]	0	0	0	0	14003
GLY	0	0	0	0	1990
TOT ISOFLAVONES	*0*	*0*	*0*	*0*	*20365*
SECO	0	20.8	0	51000	80.3
MR	0	0	0	349.5	0
LAR	0	43.6	47.0	538.2	151.9
PINO	0	0	28.3	747.7	42.4
SYR	4.7	135.8	180.3	0	93.8
TOT LIGNANS	*4.7*	*200.2*	*255.6*	*52635*	*368.4*

a) Sum of aglycone and glycoside (*i.e.*, genistin + GEN, daidzin + DAID).

Table 2. Calculated exposure to isoflavones and lignans (mg/kg/40 h ± SEM[a)])

		GEN	DAID	GLY	SECO	MR	LAR	PINO	SYR	*TOT I*[b)]	*TOT L*[c)]
AIN	Start	–	–	–	–	–	–	–	0.0	–	*0.0*
	1 wk	–	–	–	–	–	–	–	0.0	–	*0.0*
	2 wks	–	–	–	–	–	–	–	0.0	–	*0.0*
Wheat	Start	–	–	–	0.1	–	0.1	–	0.4	–	*0.6*
	1 wk	–	–	–	0.0	–	0.1	–	0.3	–	*0.5*
	2 wks	–	–	–	0.0	–	0.1	–	0.3	–	*0.5*
Rye	Start	–	–	–	–	–	0.1	0.1	0.5	–	*0.7*
	1 wk	–	–	–	–	–	0.1	0.1	0.4	–	*0.6*
	2 wks	–	–	–	–	–	0.1	0.1	0.5	–	*0.7*
Flax	Start	–	–	–	150.4 ± 3.0	1.0	1.6	2.2	–	–	*155.2 ± 3.1*
	1 wk	–	–	–	121.1 ± 4.5	0.8	1.3	1.8 ± 0.1	–	–	*125.0 ± 4.7*
	2 wks	–	–	–	128.1 ± 4.6	0.9	1.4	1.9 ± 0.1	–	–	*132.2 ± 4.7*
Soy	Start	38.1 ± 2.2	11.9 ± 0.7	5.4 ± 0.3	0.2	–	0.4	0.1	0.2	*55.3 ± 3.2*	*1.0 ± 0.1*
	1 wk	37.3 ± 0.8	11.6 ± 0.3	5.3 ± 0.1	0.2	–	0.4	0.1	0.2	*54.2 ± 1.2*	*1.0*
	2 wks	37.4 ± 1.1	11.7 ± 0.3	5.3 ± 0.2	0.2	–	0.4	0.1	0.2	*54.4 ± 1.6*	*1.0*

a) SEM values are only reported in cases where they differ from 0.0.
b) I, isoflavones.
c) L, lignans.

Table 3. Concentration (nM) of lignans and isoflavones in serum ($n = 6$) (median (range))

	GEN	DAID	GLY	EQUOL	*O*-DMA	SECO	END	ENL
AIN	nd	nd	nd	nd	nd	nd (nd-28.7)	22.5 (3.5 – 63.9)	4.0 (nd-34.1)
Wheat	nm	nm	nm	nm	nm	nd	6.5 (nd-77.3)	11.0 (4.4 – 37.9)
Rye	nm	nm	nm	nm	nm	nd	7.6 (4.0 – 14.0)	13.3 (3.5 – 31.1)
Flax	nm	nm	nm	nm	nm	221.2 (35.3 – 1431)	4720** (3048 – 9034)	335.8** (222.8 – 554.4)
Soy	1295 (832.7 – 1750)	35.8 (7.7 – 117.1)	26.9 (8.2 – 76.0)	205.5 (85.5 – 487.1)	97.2 (nd-300.3)	nm	nm	nm

nd, not detected; nm, not measured.
Asterisks indicate significant difference to AIN at $p < 0.01$ (**).

culated (Table 2), and exposure confirmed by serum analysis after acute and sub-chronic exposure (Table 3). As the concentrations in serum did not significantly differ between the two-time point the data were pooled.

Only the soy diet contained isoflavones (Table 1). The total isoflavone content of the soy diet was 20.4 mg/100 g (Table 1), which is comparable to reported values for soy beans [2], considering that the diet contained 22% soy. The intake of isoflavones in the soy group was 54 – 55 mg/kg/40 h, consisting mainly of GEN (Table 2), which is approximately 20-fold higher than intake values reported for Asian populations (up to 50 mg/day/person) [33]. However, the serum concentration of GEN in serum of the soy mice (1 – 2 μM) (Table 3) is attainable in humans as well [34]. In accordance with earlier reports, all mice were equol producers [35].

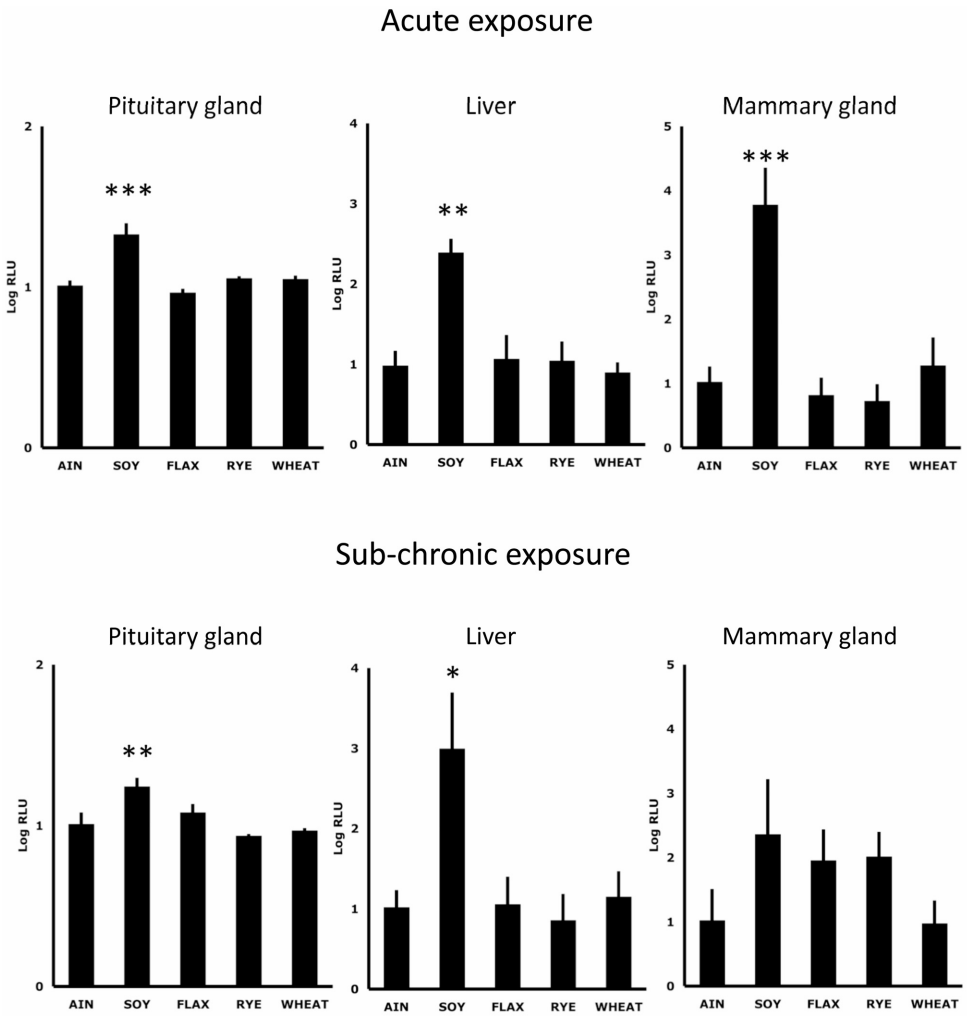


Figure 2. Luc response in the pituitary gland, liver, and mammary gland after acute and sub-chronic maintenance on the experimental diets. Asterisks indicate statistically significant difference to AIN evaluated with one-way ANOVA and Tukey's *post hoc* test using log-transformed data. Statistical differences are marked as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). RLU.

All diets contained lignans (Table 1). As expected, the concentration of plant lignans in the flax diet, 52.6 mg/100 g, was substantially higher than the concentrations in wheat (0.2 mg/100 g) and rye (0.3 mg/100 g) diets (Table 1). All the values are in agreement with published data [2, 3, 29], considering that the diets contained 14% flax, 10% wheat, and 11% rye. Also the soy diet contained lignans (Table 1). Although soy beans contain lignans, the concentration in our soy diet, containing soy granules, was higher than anticipated based on the literature concerning whole soy beans [2, 36]. In accordance with the earlier reports, SYR was the major lignan in cereal bran, SECO in flaxseed, and LAR and SYR in soy bean (Table 1) [2, 3, 36, 37]. Calculated intake of plant lignans varied from 0.5 – 1.0 mg/kg/40 h in the wheat, rye, and soy groups to 125 – 155 mg/kg/40 h in the flax group (Table 2). These values are higher than reported for the general Western population (≈1 mg/day/person), although certain individuals may consume as much as 600 mg of plant lignans *per* day [5, 38].

Rye and wheat diets did not significantly affect serum enterolignan concentration, while the mice on the flax diet had markedly higher concentrations of END and ENL in their serum (Table 3). It was somewhat surprising that rye and wheat bran supplementation did not elevate serum ENL concentration. However, similar observations were recently reported for rye bran in rats and in an *in vitro* fermentation model [39]. These results may suggest low bioavailability of cereal lignans. The concentration of ENL in the circulation of the flax-fed mice, 200 – 600 nM, is higher than in general human population, but can be achieved after flaxseed supplementation [40, 41]. The ratio END/ENL was high (≈15:1) in our study (Table 3). In humans, the metabolism of END to ENL is a feature of subdominant microbial species in the gut while the conversion of plant lignans to

END occurs more readily [42]. Gut microbe composition could perhaps explain the high END/ENL ratio in the present study. SECO was detected in all mice consuming flax diet (median 221 nM, range 35 nM – 1.4 μM) while other plant lignans were present only in some individual mice at concentrations <50 nM (Table 3 and data not shown).

3.3 Acute and sub-chronic effects of the diets

Flax, rye, and wheat diets did not induce luc expression at acute or sub-chronic exposure (Fig. 2 and data not shown). The lack of reporter gene activity in the flax-fed mice, having high circulating level of ENL, was surprising, since ENL given as a pure compound (10 mg/kg) induced luc expression in the uterus and vagina of the same mouse model in our previous study [10]. Hence, it seems that the activity of ENL is different when derived through intake of lignan-rich diets compared to the activity of pure ENL injected as such. When ENL is derived through ingestion of plant lignans, the activity could be reduced by phase II metabolism. For instance in humans, the majority of ENL circulates as glucuronic acid conjugates [34], and the conjugation is suggested to occur already upon absorption from the gut [43]. Since we injected ENL *i.p.* in our previous study [10] it might have been more available to tissues before conjugation than in the present study where ENL was derived through dietary intake of plant lignans. The presence of high concentrations of END and SECO in the serum of the flax-fed mice may also have affected the activity of ENL, as biological activity of mixtures can differ from the activity of the single compounds alone [44]. The effects of SECO and END on ER-signaling should be addressed in future studies.

In accordance with the luc data, flax and rye diets did not affect the uterine weight (data not shown) or uterine morphology at acute or sub-chronic exposure (Fig. S1 of Supporting Information). The wheat diet significantly reduced relative uterine weight at acute exposure (0.0007 g/g *vs.* 0.0006 g/g, $p < 0.01$). However, wheat did not affect the absolute weights (0.016 g *vs.* 0.015 g, $p > 0.05$) nor did it regulate uterine weight in sub-chronic exposure, or uterine morphology at either time point (Fig. S1 of Supporting Information, and data not shown). No changes were observed in the gross morphology of the mammary glands (glandular structures assessed in whole mount preparations) in any of the test groups (Fig. S2 of Supporting Information).

Soy diet induced luc expression in the mammary gland, liver, and pituitary gland (Fig. 2). The activity in the mammary gland is in accordance with observations in humans and rodents. In humans, soy supplementation induces estrogenic effects in breast epithelium [45] and in rodents, GEN and soy stimulate mammary gland and promote carcinoma growth [17, 22, 23]. However, the roles of soy and GEN in breast cancer are still a matter of controversy [46]. Our study indicates that dietary soy can activate ER signaling in the mammary gland. Interestingly, the effect on the reporter gene was only transient and the observed ER activation did not lead to stimulation of glandular structures in sub-chronic exposure (Fig. 2 and Fig. S2 of Supporting Information), implying to lack of sustained estrogenicity of soy in the mammary gland. In contrast, the soy-induced luc expression was significant at both acute and sub-chronic exposure in the liver and pituitary gland (Fig. 2). GEN-induced estrogen reporter gene activity in liver has been documented before [18], but estrogenicity of GEN in the pituitary gland *in vivo* has not been described.

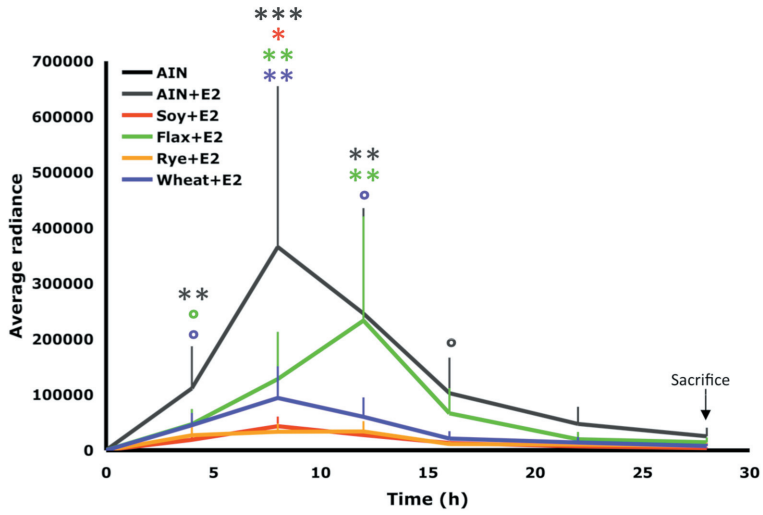
As soy has been suggested to exert estrogenic effects on bone [20, 21], we measured luc expression in femur and lumbar vertebra in addition to tibia. No luc induction was observed in these bone types either (data not shown), suggesting that the effects of soy in bone are not mediated through direct modulation of ER-activity on ERE-driven genes.

In accordance with the lack of luc induction in the uterus, soy did not affect uterine weight or morphology at acute or sub-chronic exposure (Fig. S1 of Supporting Information, and data not shown).

3.4 Effects of the diets on E_2-induced events

When measured with *in vivo* imaging, the luc expression in the positive Ctrl group (AIN + E_2) was significantly elevated compared to the negative Ctrl (AIN + vehicle) 4 – 12 h post E_2 injection, and the activity peaked at 8 h (Fig. 3). Interestingly, all supplemented diets modulated the E_2-induced luc response. In the mice consuming flax diet, luc activity was significantly elevated 8 – 12 h post E_2 injection, and the time of peak activity was delayed by 2 h (Fig. 3). Rye, wheat, and soy diets did not alter the time of the peak activity, but attenuated E_2-induced luc expression (Fig. 3). Luc activity was significantly elevated only at 8 h post E_2 injection in mice consuming wheat and soy diets, and surprisingly, no significant luc induction was observed at any time point in mice consuming the rye diet (Fig. 3). The total luc response (area under the curve) was reduced by all diets (Fig. 3). However, due to the high individual variation these differences did not reach statistical significance between the groups. Interestingly, the individual variation of the luc response was significantly reduced in the mice consuming the soy diet (Fig. 3). It is unclear how dietary priming with soy (*i.e.*, continuous presence of weak ER modulators) can lead to more homogenous response to E_2 stimulation. However, one might speculate that attenuation of the E_2 response in the most sensitive individuals could lead to a smaller risk of estrogen-dependent disease in populations consuming soy.

The possible role of soy consumption in the low risk of breast cancer in Asian populations has been under debate



	AIN+E_2	Soy+E_2	Flax+E_2	Rye+E_2	Wheat+E_2
Time of peak activity, h (SEM)	8.0 (0.9)	9.1 (0.7)	10.3 (0.8)*	9.1 (0.7)	8.6 (0.6)
Area under the curve, RLU*h (SEM)	4,313,541 (2,682,526)	552,915 (154,992)	2,438,859 (1,517,878)	595,642 (276,867)	1,157,152 (579,582)
Coefficient of variation, % (SEM)	59.9 (2.8)	30.1 (2.9)***	57.7 (2.9)	48.7 (4.0)	52.5 (2.7)

Figure 3. Luc response after a single injection of E_2 (50 μg/kg *i.p.*) as a function of time, quantified with *in vivo* imaging, and time of maximal induction (*h*), area under the curve (RLU**h*), and CV (%) of the luc response. All data, except for the time of maximal induction, were log-transformed and analyzed with one- or two-way ANOVA followed by Tukey's *post hoc* test. Time point of maximal luc induction was analyzed with Chi Square test. Statistical differences to AIN (imaging curves) or AIN + E_2 (data in the table) are marked as $p < 0.1$ (°), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

for at least two decades. In our study, dietary soy was antiestrogenic in the presence of E_2 and estrogenic in the absence of E_2. This is in agreement with the suggested mixed estrogen agonist/antagonist activity of soy: for instance in immature mice dietary soy promotes uterine growth in the absence of estrogens, but reduces the growth in the presence of diethylstilbestrol [47]. The estrogenic effect of soy is not surprising, considering the presence of isoflavones, known ER agonists. E_2 antagonism, on the other hand, could be attributed to not only isoflavones but also to glyceollins, previously shown to act as antiestrogens [48]. Taken together, these results suggest that consumption of soy in premenopause *versus* postmenopause could differ in terms of breast cancer risk modulation.

At the end of the imaging, tissues were collected for *ex vivo* luc analysis. The response in liver homogenates was significantly correlated to the *in vivo* imaging data ($y = 0.9x + 1.1$, $R^2 = 0.8$). The modulation of E_2-induced luc activity by diets was not observed in any of the analyzed tissues (data not shown), which is not surprising, considering the time response curves obtained by *in vivo* imaging, demonstrating a clear decline in the response at this time point (28 h). It should be pointed out that this study was specifically designed for the *in vivo* assessment and based on our results, future studies addressing the modulation of E_2-induced ER transactivation on tissue/organ level should focus on earlier time points (6–8 h) showing the peak E_2 activity [25, 49, 50].

Although modulation of E_2-induced luc expression by the diets was not detected on tissue level at 28 h post E_2 injection, the attenuation of the E_2-signaling was observed in uterine weights. The average weight increase was smaller in soy, flax, wheat, and rye groups compared to the positive Ctrl, and the difference gained statistical significance in the case of the rye diet (Fig. 4), confirming a role for rye consumption in regulation of endogenous estrogen responses. The diets did not affect the morphology of the uterus (Fig. 4 and Fig. S1 of Supporting Information).

A recent study indicates that intake of fiber, cereal fiber in particular, is inversely associated with the risk of premenopausal breast cancer (high circulating estrogen levels), but not with postmenopausal breast cancer (low circulating estrogens levels) [51]. In accordance with this, the cereal fiber diets lowered the effects of E_2 in our study. The mechanisms of fiber-mediated protection against breast cancer are not well understood. According to one theory, fiber interferes with the enterohepatic circulation of endogenous estrogens by binding them in the gut and thereby lowers the level of estrogens in the circulation [52, 53]. This is supported by the finding that wheat, rye, flaxseed, and soy can bind E_2 in vitro [54]. However, binding of E_2 in the gut by plant fiber in our study is not a likely mechanism of action as a single dose of E_2 was administered *i.p.* According to another theory, bioactive compounds bound to fiber, such as lignans, protect against breast cancer by modulating the estrogen signaling pathway [7]. In our study, however, the

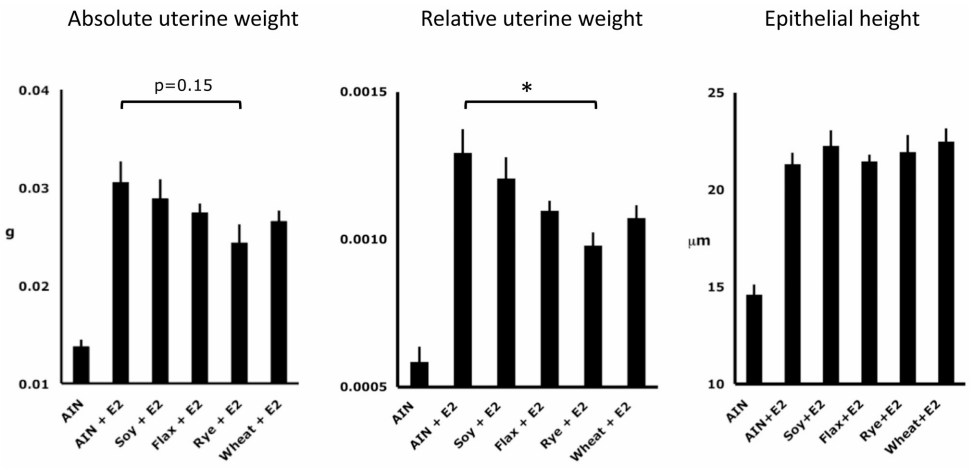


Figure 4. Absolute and relative uterine weights and the height of the luminal epithelium 28 h post E_2 exposure. All E_2-treated groups differed significantly from the untreated AIN group ($p < 0.01$ for all). Statistically significant differences between the E_2-treated dietary groups and AIN + E_2 are indicated in the figure. The data were evaluated with one-way ANOVA and Tukey's *post hoc* test. Statistical differences are marked as $p < 0.05$ (*).

only diet giving rise to considerable concentration of lignans in serum, flax diet, showed more modest effects on estrogen signaling than soy, wheat, and rye (Fig. 3). Therefore, lignans are perhaps not in a central role in the effects reported here, and the presence of other possibly bioactive compounds in rye and wheat should be considered. For example, rye and wheat bran are good sources of alkylresorcinols; phenolic lipids that possess a phenolic hydroxyl group considered as an important structural determinant for ER binding [55, 56]. Modulation of ER-signaling by alkylresorcinols has not been studied so far.

Of the tested diets, the rye bran diet was the most efficient down-regulator of E_2-induced estrogenicity. An interesting feature of rye bran is the production of SCFAs, especially butyrate, in the gut [39, 57]. Butyrate is a histone deacetylase inhibitor that inhibits E_2-induced expression of estrogen target genes and proliferation of MCF-7 cells [58, 59]. Perhaps butyrate production explains some of the estrogen antagonism in the rye-fed mice in our study. The role of rye-derived butyrate in the modulation of estrogen signaling should be addressed in future studies.

4 Concluding remarks

In this study we show for the first time that diet can modulate E_2-induced ER-mediated responses *in vivo*. The food items tested here, flaxseed, rye bran, wheat bran, and soy granules, are often classified as "health foods" and contain many non-nutrient compounds with suggested bioactivity, but little is known about the net effects of these food items on intracellular signaling pathways critical for disease development. Here we show that consumption of crushed flaxseed, rye bran, wheat bran, and soy granules modulate E_2-induced ER activity in reporter mice. Attenuation of estrogen responses by diet is clearly of interest when trying to identify factors reducing breast cancer risk.

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