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# Effect of Flaxseed Consumption on Urinary Levels of Estrogen Metabolites in Postmenopausal Women

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**Flaxseed is a rich source of dietary lignans. It has been hypothesized that lignans may decrease breast cancer risk through modulation of endogenous hormone levels. The aim of this study was to determine the effect of flaxseed supplementation on urinary levels of estrogen metabolites that may be involved in the development of breast cancer. Forty-three postmenopausal women participated in this 12-wk preintervention–postintervention study. Participants consumed 7.5 g/day of ground flaxseed for 6 wk, followed by 15 g/day for an additional 6 wk. The mean urinary level of 16α–hydroxyestrone (16α-OHE1) was higher at the end of 12 wk compared to baseline (change of 1.32 ug/day, $P = 0.02$). There was no significant change in 2-OHE1 excretion. The mean urinary level of the 2-OHE1/16α-OHE1 ratio was lower at the end of 12 wk compared to baseline (change of $-1.1$, $P = 0.02$). Mean urinary excretion of 2-methoxyestradiol was also lower at 12 wk than at baseline ($P = 0.03$). Based on the current paradigm of the effects of estrogen metabolism on breast cancer risk, the regimen of dietary flaxseed intake used in this study did not appear to favorably alter breast cancer risk through shifts in estrogen metabolism pathways in postmenopausal women.**

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## INTRODUCTION

Lignans, naturally occurring compounds, are found in low levels in a wide variety of grains, fruits, and vegetables (1). In humans, plant lignans are converted by colonic microflora to the mammalian lignans, enterolactone and enterodiol (2). Because breast cancer incidence rates are lower in Asian countries where women typically consume diets rich in fruits and vegetables, it has been hypothesized that lignans may have cancer preventative properties. In particular, it has been proposed that dietary lignans may modulate breast cancer risk through their effects on estrogen metabolism.

The metabolism of estrone (E1) and estradiol (E2) occurs through three major competitive pathways: one involves 16α-hydroxylation to 16α-hyroxyestrone (16α-OHE1) and 16α-hydroxyestradiol (16α-OHE2); the second leads to 2-OHE1 and 2-OHE2; and a third leads to 4-OHE1 and 4-OHE2 (3). In laboratory studies, individual estrogen metabolites have been shown to exhibit important differences in biological activity that may differentially impact breast cancer risk. For example, the 4-hydroxy and 16α-hydroxy metabolites are relatively more estrogenic than the 2-hydroxy metabolites (4,5). In addition, the 4-hydroxy metabolites may form DNA adducts that can lead to mutations that initiate breast tumors (6). Furthermore, 2-methoxyestradiol (2-MeOHE2), formed by the conjugation of 2-OHE2 by the catechol-O-methyltransferase (COMT) enzyme, has been shown to inhibit cell proliferation (7). In epidemiologic

studies, the main estrogen metabolite exposure that has been studied is the ratio of 2-OHE1 to 16$\alpha$-OHE1 (2/16$\alpha$-OHE1). Several (8–11) but not all (12) retrospective case-control studies have reported a lower 2/16$\alpha$-OHE1 ratio in postmenopausal breast cancer cases than control study subjects. Results from prospective studies have been inconclusive (13–18). Eliassen and colleagues (18) recently suggested that future studies should include a broader panel of estrogen metabolites to fully evaluate the role of the estrogen metabolism pathway in breast cancer risk.

A recent well-designed prospective cohort study observed an inverse association between diets rich in lignans and breast cancer risk (19), although not all researchers have reported this, as reviewed by Ziegler (20). Flaxseed is a particularly rich source of lignans (21) and is thus often used in human dietary intervention trials. To date, two small randomized intervention studies (including 16 and 28 flaxseed-supplemented postmenopausal women, respectively) with moderate-length intervention periods (lasting 7–16 wk) have evaluated the effect of dietary flaxseed intake on urinary levels of estrogen metabolites (22,23). Both studies had observed a significant increase in urinary concentrations of 2-OHE1 but not of 16$\alpha$-OHE1 (22,23). Similar findings were also observed in a larger preintervention–postintervention study with an abbreviated 7-day flaxseed feeding period (24).

The purpose of this study was to examine the effects of dietary flaxseed on urinary estrogen metabolites in a study that employed a laboratory approach to reliably measure a wide range of estrogen metabolites.

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## METHODS

The study methods have been previously published (25). Briefly, 48 study participants were recruited from the Amherst, Massachusetts, area. Eligible participants were defined as women who had undergone natural menopause (defined as the cessation of menses for at least 1 yr); were English speaking; did not have any bowel disease, cancer, or diabetes; had not taken antibiotics in the past 6 mo; had not taken certain medications within the past 6 mo (i.e., hormone replacement therapy, oral corticosteroids, anticoagulants); and were nonsmokers.

The study was explained at an in-person introductory visit, and written informed consent was obtained. For at least 3 wk prior to a baseline visit and throughout the follow-up period, participants were requested to avoid flaxseed and flaxseed-containing foods, all soy and soy products, dietary supplements, herbs, or teas known to have phytoestrogen activity; and cruciferous vegetables (specifically broccoli, broccoli sprouts, brocoflower, brocolini, brussel sprouts, cauliflower, cabbage, bok choy, Chinese cabbage, kale, Swiss chard, kohlrabi, rutabaga, turnips, collard greens, mustard greens, turnip greens, watercress) because of their potential effects on hormone levels. Initially, participants were asked to refrain from alcohol consumption because it is known to alter endogenous hormone levels. However, this requirement made subject recruitment difficult. The alcohol eligibility requirements were therefore relaxed, requiring subjects to maintain their normal alcohol consumption but to consume no more than 2 alcoholic beverages per day and no more than 7 alcoholic beverages per week. Participants were asked to maintain their usual diet and exercise patterns throughout the study.

At the baseline visit, study participants provided a 24-h urine specimen that had been collected over the previous day. In addition, study participants completed a Seven Day Dietary Recall (7DDR; University of Massachusetts Medical School, Worcester, MA) that queried dietary intake of 118 food categories or individual foods and 13 beverage items consumed over the previous week (26). Height and body weight were also measured, and a self-administered questionnaire was used to elicit information on sociodemographic factors and standard breast cancer risk factors.

Participants were instructed to consume 1 tablespoon of ground flaxseed (7.5 g) per day until the scheduled 6-wk visit. At the 6-wk visit, participants were instructed to consume 2 tablespoons of ground flaxseed per day (15 g) until the 12-wk visit. As with the baseline visit, at each of the follow-up visits, a 20 ml blood specimen was obtained, and participants provided a 24-h urine specimen that had been collected over the previous day. In addition, participants completed a 7DDR and had their height and weight measured.

Stabilized flaxseed without any additives was obtained from the Essential Nutrient Research Corporation (ENRECO, Newton, WI). All of the flaxseed used in this study originated from a single production lot and was shipped to the University of Massachusetts at Amherst in two separate shipments. Whole flaxseed was stored in cold storage and was ground by ENRECO immediately prior to shipping. A 1-lb bag of preground flaxseed and a tablespoon measure with instructions for standard measurement procedures were provided for each subject at the baseline visit, and any unused flaxseed was returned at the 6-wk visit. Two 1-lb bags of ground flaxseed were provided for each subject at the 6-wk visit, and any unused flaxseed was returned at the 12-wk visit. Participants were instructed not to cook or bake with the flaxseed but rather to consume it alone or sprinkled on foods, such as yogurt or cereal. Participants were provided with ground flaxseed to maximize adherence and were instructed to store the flaxseed in the refrigerator to prevent spoilage. The flaxseed that was returned at each follow-up visit was weighed to determine subject adherence.

Urine was collected in bottles containing 1 g ascorbic acid/l; and after recording the weight, 0.1% sodium azide was added as a preservative at a concentration of 0.1%. Urine specimens were immediately processed, frozen at $-20^{\circ}$C, and shipped on dry ice to the Department of Food Science and Nutrition, University of Minnesota, St Paul.

Frozen urine samples were thawed at room temperature, thoroughly mixed by vortex to ensure homogeneity, and centrifuged at 5$^{\circ}$C for 5 min. Duplicate 10 ml aliquots of urine were added to clean, silanized 30 ml screw-top test tubes. Deuterated standards

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(C/D/N Isotopes, Pointe-Claire, Quebec, Canada) of all estrogen metabolites assayed and enterodiol were added to the urine, and an equal volume (10 ml) of ethoximation solution was added to the test tubes, thoroughly mixed by vortex and inversion, and incubated overnight at room temperature (ca. 20°C–25°C).

The following day, the ethoximated samples were applied to Bond Elute LRC C-18 columns (Varian, Inc., Lake Forest, CA; 500 mg/column). The C-18 columns had been preconditioned with 1) 5 ml methanol and 2) 10 ml of deionized-distilled (DD) water immediately prior to sample introduction. Columns were then washed with 5 ml of 0.15 M acetate buffer, pH 3.0. Samples were eluted into a clean, silanized test tube with 3.0 ml of methanol and evaporated to dryness under nitrogen. The dry samples were hydrolyzed by dissolving in 5 ml of a solution containing 25 mg ascorbic acid, 50 ul $\beta$-glucuronidase (Sigma #G-7770, crude extract from *Helix pomatia)* (St. Louis, MO) in 0.15 M acetate buffer, pH 4.1, and incubated overnight at 37°C.

The following day, the hydrolyzed samples were applied to conditioned (as previously) C-18 columns, washed with 5 ml of DD water, and eluted into clean, silanized test tubes with 4.0 ml of MeOH. Samples were evaporated to dryness under nitrogen and derivatized to their trimethyl silyl components with 200 ul of a 15% MSTFA+ TMCS solution in acetonitrile (MSTFA+ 1% TMCS; Pierce Biotechnology Inc., Rockford, IL; product #48915). Chromatographic analysis was performed on an HP 5890 Series II gas chromatograph equipped with an HP-1MS 15 m column (0.25 mm inside diameter, 0.25 um film thickness) interfaced to an HP 5970 mass selective detector. Instrumental programmed control and quantitative analysis was performed using HP Chemstation software (27–29). The intraassay and interassay coefficients of variations (CVs) were as follows: estradiol (3.7%, 14.8%), estrone (4.7%, 7.8%), estriol (4.9%, 5.5%), 2-OHE1 (5.9%, 9.8%), 16$\alpha$-OHE1 (5.4%, 6.7%), 2-OHE2 (5.1%, 9.1%), 2-MeOHE1 (4.4%, 8.8%), 2-MeOHE2 (4.3%, 7.1%), and 4-OHE2 (5.2%, 10.1%). This analysis is based on 43 women with three complete urine collections, and all samples from the same individual were analyzed within the same batch. Values below the level of detection were assigned a value of 0.5 ng/ml.

### Statistical Analyses

Repeated measures analysis of variance was performed to assess the significance of changes over time in levels of estrogen metabolites. Results are presented in ng/day; findings were similar when analyzed as ng/mg creatinine. Repeated measures analysis of covariance (ANCOVA) was used to determine changes in the estrogen metabolite levels after adjustment for selected dietary factors and weight. Dietary variables were created from the 7DDR administered at each of the three visits. Macronutrient and micronutrient intake in grams per day were adjusted for total energy intake using the residual method (30). Dietary factors evaluated included total energy intake and energy adjusted macronutrients and micronutrients (total fat, total saturated fat, total protein, vegetable protein, animal protein, total carbohydrates, alcohol, total fiber, insoluble fiber, linoleic acid, and linolenic acid). The decision to retain potential confounders in the repeated measures ANCOVA was based on initial assessments of changes over time in each of the preceding covariates and utilized the $F$ test ($P \leq 0.05$) in a repeated measures analysis.

## RESULTS

As shown in Table 1, the study population was mainly non-Hispanic White (98%) and college educated (70%), with a mean age of 57.3 $\pm$ 4.8 yr. Based on an analysis of the weights of distributed and returned flaxseed, mean daily flaxseed intake ($\pm$ SD) was 8.35 (3.22) g/day and 15.0 (2.65) g/day during the first and second follow-up periods, respectively. Mean ($\pm$ SD) urinary enterodiol levels at baseline, 6 wk, and 12 wk were 631 (2,670) ug/day, 2,427 (2,915) ug/day, and 5,327 (5,324) ug/day, showing a rise as expected when higher doses of flaxseed were ingested. As shown in Table 2, there were no significant changes in study subject weight, physical activity, or total energy intake over the intervention. There was a suggestion of a decline in total energy intake between the first and second follow-up visits, consistent with small statistically significant declines in several macronutrient and micronutrients (total fat, total protein, vegetable protein, carbohydrates, total fiber, insoluable fiber, linoleic acid, and linolenic acid).

TABLE 1
Baseline demographic characteristics of enrolled participants in flaxseed intervention study

| Characteristic | Mean (SD) | No.[a] | % |
|---|---|---|---|
| Age (yr) | 57.3 (4.8) | | |
| Race | | | |
| White | | 41 | 97.7 |
| Other | | 1 | 2.4 |
| Education | | | |
| High school or some college | | 13 | 30.2 |
| College graduate | | 30 | 69.8 |
| Postmenopausal hormone use | | | |
| Never | | 24 | 55.8 |
| <5 yr | | 11 | 25.6 |
| ≥5yr | | 7 | 16.3 |
| Ever smoked cigarettes | | | |
| No | | 30 | 69.8 |
| Yes | | 13 | 30.2 |
| Family history of breast cancer | | | |
| No | | 36 | 83.7 |
| Yes | | 7 | 16.3 |
| History of benign breast disease | | | |
| No | | 34 | 79.1 |
| Yes | | 9 | 20.9 |

[a]May not add up to 43 (100%) due to missing data.

TABLE 2
Body Mass Index and Dietary Intake and Change Over Time Among Postmenopausal Women (n = 43)

| All women (n = 43 ) | Baseline Level Mean | Change from Baseline to Follow-up 1 | | Change from Follow-up 1 to Follow-up 2 | |
|---|---|---|---|---|---|
| | | Mean | *p*-value | Mean | *p*-value |
| Weight (kg)[1] | 67.9 | +0.03 | .87 | −0.05 | .55 |
| Body mass index (kg/m$^2$)[1] | 25.6 | +0.03 | .70 | −0.05 | .47 |
| Physical activity (minutes/week) | 257 | −12.2 | .57 | −21.0 | .25 |
| Total energy intake (kcal/day) | 1697 | 17.1 | .84 | −96.6 | .15 |
| Energy-Adjusted Nutrients | | | | | |
| Total fat (g/day) | 75.8 | 2.2 | .24 | −7.7 | ≤.01 |
| Total saturated fat (g/day) | 24.2 | −0.2 | .71 | −0.7 | .26 |
| Total protein (g/day) | 69.9 | 1.9 | .35 | −4.7 | .03 |
| Animal protein (g/day) | 47.9 | 1.4 | .55 | −1.1 | .62 |
| Vegetable protein (g/day) | 21.3 | 0.5 | .69 | −3.5 | ≤.01 |
| Carbohydrates (g/day) | 202.7 | −11.4 | .02 | −10.7 | .02 |
| Alcohol (g/day) | 4.02 | 0.7 | .13 | −0.2 | .71 |
| Total fiber (g/day) | 13.8 | 0.2 | .71 | −1.5 | ≤.01 |
| Insoluable fiber (g/day) | 8.9 | 0.1 | .75 | −1.0 | ≤.01 |
| Linoleic acid (g/day) | 15.5 | 1.0 | .23 | −2.9 | ≤.01 |
| Linolenic acid (g/day) | 1.51 | −0.02 | .80 | −0.13 | .04 |

[1]One person with missing data on weight.

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As shown in Table 3, mean 24-h urinary excretion of estradiol tended to be higher at the 6- and 12-wk visits than at the baseline visit. There was a suggestion that mean 24-h excretion of estrone was lower at 12 wk than at baseline. Mean 24-h urinary excretion of estriol was unchanged over the three visits.

Mean 24-h urinary excretion of 2-OHE1 and 2-OHE2 did not change over the three visits. There was an increase in the mean 24-h urinary excretion of 16$\alpha$-OHE1 over the three visits, and the increase from baseline to 12 wk was statistically significant. Corresponding with the observed increase in 16$\alpha$-OHE1 excretion, the urinary ratio of 2/16$\alpha$-OHE1 declined over the three visits. The decrease from baseline to 12 wk was statistically significant. Mean 24-h urinary excretion of 2-MeOHE2 also declined over the three visits, and the change between the baseline and 12 wk was statistically significant. Mean 24-h urinary excretion of 2-MeOHE1 and 4-MeOHE2 was unchanged over the three visits. Although mean intake of certain dietary factors changed significantly during the intervention, particularly between the first and second follow-up visits (as shown in Table 2), the results in Table 3 were essentially unchanged after adjustment for these factors. Results in Table 3 were also similar when examined separately by body mass index category ($\leq$25 kg/m$^2$, $>$25 kg/m$^2$).

## DISCUSSION

This study showed a decline in the 2/16$\alpha$-OHE1 excretion ratio with a 12-wk flaxseed intervention, corresponding with an increase in the urinary excretion of 16$\alpha$-OHE1 from baseline to 12 wk. These results are not consistent with others (22–24) and suggest that flaxseed may increase levels of 16$\alpha$-OHE1, which has been proposed to be the more estrogenic metabolite. In our study, flaxseed intake was not correlated with a decline in the presumably more optimal 2-hydroxestrogen metabolites as has been observed by others in flaxseed intervention studies. We also observed a significant decline in urinary levels of 2-methyoxyestradiol, a metabolite that is considered to be a promising cancer preventative agent (7).

Prior studies in postmenopausal women of the effects of flaxseed supplementation on estrogen metabolism have varied in prescribed dose and length of the intervention (22–24). Haggans and colleagues (23) reported that 10 g flaxseed for 7 wk increased urinary excretion of 2-OHEI by 34%. With an abbreviated week-long flaxseed supplementation, McCann and colleagues (24) observed that a similar prescribed dose (10 g) increased 2-OHE1 excretion levels by 73%. Brooks and colleagues (22) observed a 103% increase in 2-OHE1 with supplementation with a muffin (25 g ground flaxseed) for 16 wk. In sum, the prescribed dose and length of our intervention was relatively comparable to other studies, and thus facets of the intervention do not appear to explain the discrepant findings between our study and others. As in our study, there were also no reported differences in body weight measurements or energy intake over the intervention in the three prior studies. In general, statistically significant differences in dietary factors were also not observed over the intervention periods in

TABLE 3
Urinary Sex Hormone Levels at Baseline, and Change Over Time Among Postmenopausal Women (n = 43)

| All women (n = 43 ) | Baseline Level Mean | Change from Baseline to Follow-up 1 Mean (95% CI) $p$-value | Change from Baseline to Follow-up 2 Mean (95% CI) $p$-value |
|---|---|---|---|
| Estradiol (ug/day) | 10.41 | 2.38 (−0.11,4.86) p = .06 | 2.30 (−0.33,6.32) p = .07 |
| Estrone (ug/day) | 4.50 | 0.52 (−0.44,1.48) p = .28 | −0.82 (−1.74, 0.11) p = .08 |
| Estriol (ug/day) | 8.01 | 0.06 (−1.95,2.07) p = .95 | 2.76 (−2.95, 3.50) p = .86 |
| 2-hydroxyestrone (ug/day) | 4.18 | −0.62 (−2.30, 1.07) p = .46 | −1.04 (−2.72,0.64) p = .22 |
| 2-hydroxyestradiol(ug/day) | 1.65 | −0.21 (−0.77, 0.35) p = .46 | −0.38 (−1.03, 0.25) p = .23 |
| 16$\alpha$-hydroxyestrone (ug/day) | 2.60 | 0.69 (−0.42, 1.80) p = .22 | 1.32 (0.26, 2.40) p = .02 |
| 2/16$\alpha$-hydroxyestrone | 2.85 | −.75 (−1.79, 0.28) p = .15 | −1.10 (−2.00, −0.21) p = .02 |
| 2-methoxyestradiol (ug/day) | 11.49 | −0.99 (−5.83, 3.84) p = .68 | −4.02 (−7.67, −0.36) p = .03 |
| 2-methoxyestrone (ug/day) | 2.45 | −0.45 (−2.39, 1.50) p = .65 | −0.34 (−2.40, 1.73) p = .75 |
| 4-hydroxyestradiol (ug/day ) | 0.96 | −0.56 (−0.38, 0.26) p = .63 | −0.87 (−0.45, 0.28) p = .85 |

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these studies but this may, at least in part, reflect small sample sizes.

Our study has several strengths. With the exception of the one study with an abbreviated intervention period, it was relatively larger than other studies. We used gas chromatography mass spectrometry to measure broad range of estrogen metabolites in 24-h urine. The emphasis of this study was on changes over time measured in the same individual, and thus the high assay reproducibility is reassuring.

Our study does lack a control group, and so it is not known if there were temporal changes in metabolite levels that may explain our findings. However, adjustment for dietary-related factors did not alter our findings.

Different estrogen metabolites have different biological activities, but it is still unclear what the optimal estrogen metabolism pattern is with respect to breast cancer. For example, a large prospective study reported that higher urinary levels of 2-OHE1 were associated with a significant increase in breast cancer risk in women using hormone replacement, with no association in nonusers (15). In addition, recent findings from the Nurses' Health Study reported no association between circulating levels of 2-OHE1 and breast cancer overall and an increase in risk for estrogen- and progesterone-negative tumors among women with higher 2-OHE1 levels (18). In summary, the findings of the present study do not suggest that dietary flaxseed intake has a favorable effect on the urinary estrogen metabolite profile based on the current paradigm of the effects of estrogen metabolism on breast cancer risk.

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