

Flaxseed Influences Urinary Lignan Excretion in a Dose-dependent Manner in Postmenopausal Women¹

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Abstract

Dietary estrogens, such as lignans, are similar in structure to endogenous sex steroid hormones and may act *in vivo* to alter hormone metabolism and subsequent cancer risk. The objective of this study was to examine the effect of dietary intake of a lignan-rich plant food (flaxseed) on urinary lignan excretion in postmenopausal women. This randomized, cross-over trial consisted of three 7-week feeding periods during which 31 healthy postmenopausal women, ages 52–82 years, consumed their habitual diets plus 0, 5, or 10 grams of ground flaxseed per day. Urine samples collected for 2 consecutive days during the last week of each feeding period were analyzed for lignan content (enterodiol, enterolactone, and matairesinol) by isotope dilution gas chromatography/mass spectrometry. Compared with the 0-gram flaxseed diet, consumption of 5 or 10 grams of flaxseed significantly increased excretion of enterodiol by 1,009 and 2,867 nmol/day, respectively; significantly increased excretion of enterolactone by 21,242 and 52,826 nmol/day, respectively; and significantly increased excretion of total lignans (enterodiol + enterolactone + matairesinol) by 24,333 and 60,640 nmol/day, respectively. Excretion of matairesinol was not significantly altered by flaxseed consumption. Consumption of flax, a significant source of dietary estrogens, in addition to their habitual diets increased excretion of enterodiol and enterolactone, but not matairesinol, in a dose-dependent manner in this group of postmenopausal women. Urinary excretion of lignan metabolites is a dose-dependent biomarker of flaxseed intake within the context of a habitual diet.

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Introduction

Dietary estrogens, or phytoestrogens, are compounds found in plants and plant products that possess some estrogenic or antiestrogenic activity (1–3), and growing evidence suggests that these phytoestrogens may play a role in cancer prevention (2, 4–7). Lignans, one type of phytoestrogen, are diphenolic compounds similar in structure to endogenous sex steroid hormones (Fig. 1) and are hypothesized to act *in vivo* to alter hormone metabolism and subsequent cancer risk (1, 4–8). Flaxseed is the most concentrated food source of the plant lignan secoisolariciresinol, which is converted by the colonic microflora to the mammalian lignan enterodiol (9–12). Enterodiol can then be oxidized by the colonic microflora to form the mammalian lignan enterolactone (11, 13, 14). Flaxseed also contains small quantities of the plant lignan matairesinol, which is also converted by the colonic microflora to enterolactone (11, 13, 14).

Flaxseed has been shown to reduce the early risk markers for and incidence of mammary and colonic carcinogenesis in animal models (15–18) and to affect menstrual cycle length in premenopausal women (8). Enterolactone and enterodiol, the mammalian lignans excreted in response to flaxseed consumption (19–22), have also been shown to reduce early markers for and incidence of mammary carcinogenesis in animal models (23, 24), decrease cell proliferation (25–27), increase concentrations of sex hormone-binding globulin (1, 28–30) and inhibit the activity of three enzymes [aromatase (31, 32), 5 α -reductase (33), and 17 β -hydroxysteroid dehydrogenase (33)] that play key roles in sex hormone metabolism.

Although there have been numerous studies examining the effects of various diets or dietary components on lignan excretion in a variety of populations (1, 34–40) and the effects of flaxseed consumption on lignan excretion in men and premenopausal women (19–22), to our knowledge, there have been no studies examining the effects of flaxseed consumption on urinary lignan excretion in postmenopausal women. The objective of this study was to examine the effect of dietary intake of a lignan-rich plant food (flaxseed) on urinary lignan excretion in postmenopausal women consuming a semicon-trolled diet.

Materials and Methods

Subjects. Healthy postmenopausal women were recruited from the Monastery of the Sisters of the Order of St. Benedict in central Minnesota. Potential subjects were screened with a detailed dietary and medical questionnaire designed to exclude those who had gastrointestinal disorders, food allergies, or alcohol intake of >2 drinks/day (equivalent to 720 ml of beer, 240 ml of wine, or 90 ml of hard liquor); smoked; had taken antibiotics within the last 6 months; had used hormone replacement therapy; or had dietary habits that were not representative of the general population (*e.g.*, exclusion of an entire food group from their diet, <20% or >45% of energy from fat, or consumption of more than three servings of flax or products

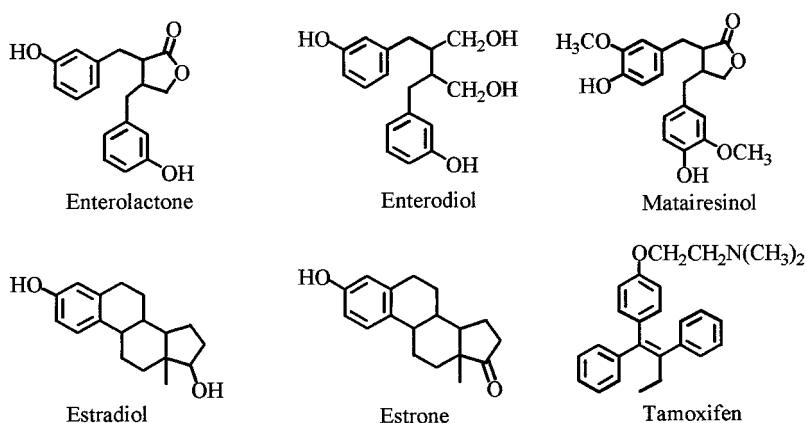


Fig. 1. Molecular structures of lignans, estrogens, and tamoxifen.

containing flaxseed per week). After screening, 34 subjects were contacted and agreed to participate by providing informed written consent. The Institutional Review Board Human Subjects Committee at the University of Minnesota approved the study. All subjects were healthy nonsmokers and were at least 1 year postmenopausal as determined by subjects' report of the date of their last menses occurring at least 1 year previous to the start of the study. Seven subjects were not taking any prescription medications; however, due to the age group of the subjects, it was not possible to exclude for all medications. Of the remaining 27 subjects, 10 were taking thyroid replacement medications, 9 were taking antihypertensive medications, 7 were taking antihyperlipidemic medications, 6 were taking diuretics, 3 were taking antidepressants, and 1 was taking a corticosteroid. In all cases, medication use was consistent throughout the duration of the study. All of the subjects in this study were nulliparous.

Of the 34 subjects who began the study, 32 completed all feeding periods. One subject withdrew from the study when she moved to another state, and another withdrew for medical reasons unrelated to study participation. During the study, one subject underwent antibiotic therapy, and her results are not included in the statistical analyses reported here. Only the results from the 31 subjects who completed the entire study were included in statistical analyses. The mean values (\pm SD) for age, height, weight, and BMI⁴ of the subjects were 66.9 ± 8.2 years, 162.2 ± 5.2 cm, 64.6 ± 12.2 kg, and 24.7 ± 4.1 kg/m², respectively.

Experimental Design. This study was designed as a randomized, cross-over trial consisting of three 7-week feeding periods with a 7-week washout period between the first and second feeding periods and a 14-week washout between the second and third feeding period. One of the three feeding periods was used as a control period during which the subjects consumed only their usual diets. During the remaining two feeding periods, subjects consumed their usual diets plus either 5 or 10 grams of ground flaxseed each day. The 5-gram flaxseed supplement provided approximately 25 kcal, 1.2 grams of protein, 1.8 grams of fat (50–60% α -linolenic acid), 1.4 grams of carbohydrate, and 1.1 grams of dietary fiber, including 0.6 gram of soluble fiber. The 10-gram flaxseed supplement provided double these amounts. During the flaxseed-supplemented feeding periods, the subjects were supplied daily with one tube con-

taining 5 or 10 grams of ground flaxseed. The flaxseed was kept frozen at -20°C until consumption, and subjects were instructed to consume the contents of one tube daily in raw form. The subjects typically consumed the flaxseed in one serving at breakfast. Used tubes were collected, and any uneaten flaxseed was measured to monitor subject compliance. Subjects were asked to maintain their usual body weight and diet and exercise habits throughout the study.

The ground flaxseed was prepared weekly from commercially available whole flaxseed (Frontier Whole Flax Seed, Norway, IA). The whole flaxseed was ground to a coarse texture in a household blender for 1 min. The ground flaxseed was then aliquoted immediately into 5- or 10-gram doses and frozen at -20°C . To determine the approximate plant lignan content of the ground flaxseed, the secoisolariciresinol-diglycoside content was measured by high-performance liquid chromatography (41) by Kenneth D. R. Setchell (Children's Hospital Medical Center, Cincinnati, OH). According to the analysis, the 5- and 10-gram doses of ground flaxseed provided 10 and 20 mg of secoisolariciresinol (2 mg or $5.5 \mu\text{M}$ secoisolariciresinol/gram ground flaxseed), respectively. The other primary plant lignan, matairesinol, was not measured because it comprises only about 0.3% of the total lignans in flaxseed (42).

Sample Collection and Analysis. During the last week of each feeding period, subjects completed self-reported 3-day diet records to monitor food intake, body weights were measured, and 24 h urine samples were collected on 2 consecutive days of the 3-day diet record period. Each urine sample was collected separately in individual plastic tubs containing 200–350 mg of ascorbic acid and stored at 4°C until processed. The amount of ascorbic acid per tub was estimated to provide a final concentration of approximately 1 gram/liter when all urine collections from a 24-h period were combined. However, due to variability in urine volume and frequency, actual ascorbic acid concentrations averaged 1.3 grams/liter. The final volume of each 24 h urine collection was recorded, and an aliquot from each 24 h urine sample was frozen for subsequent creatinine analysis to monitor the completeness of the collection. Pooled aliquots of the two 24 h urine collections were then stored at -20°C after the addition of 10% sodium azide (final w/v, 0.1% sodium azide).

The urine samples were analyzed for lignan (enterodiol, enterolactone, and matairesinol) content by isotope dilution gas chromatography-mass spectrometry according to the method developed by Adlercreutz *et al.* (43). The urine was first extracted using Bond Elute LRC C-18 columns (Chrom Tech,

⁴ The abbreviations used are: BMI, body mass index; CV, coefficient of variation.

Table 1 Subject weight and BMI during each feeding period

	Values are means \pm SD. There were no significant differences.		
	Control	Flaxseed	
		5 grams	10 grams
Weight (kg)	65.0 \pm 12.1	64.6 \pm 12.5	64.2 \pm 12.1
BMI (kg/m ²)	25.0 \pm 4.1	24.7 \pm 4.3	24.6 \pm 4.1

Apple Valley, MN) and further purified on a DEAE-Sephadex A-25 (Sigma Chemical Co., St. Louis, MO) anion-exchange column in the acetate form. Deuterated internal standards of the compounds [provided by Dr. T. Hase, Dr. K. Wähälä, and T. Mäkelä (Department of Chemistry, University of Helsinki, Helsinki, Finland) in collaboration with Dr. H. Adlercreutz (Department of Clinical Chemistry, University of Helsinki, Helsinki, Finland) and Dr. J. Lampe, Cancer Prevention Research Program, Fred Hutchinson Cancer Research Center, Seattle, WA)] were added. The conjugates were hydrolyzed, isolated using Bond Elute C-18 columns, and applied to a QAE-Sephadex A-25 (Sigma Chemical Co.) anion-exchange column in the acetate form. Two fractions were collected; the first fraction contained the lignans (enterodiol, enterolactone, and matairesinol) that were purified further on a QAE-Sephadex A-25 anion-exchange column in the carbonate form. Trimethyl-silyl derivatives of the samples were analyzed by gas chromatography-mass spectrometry in the selective ion-monitoring mode. The second fraction contained isoflavones, and those results are not reported in this study.

The urine samples were analyzed singly, in batches, with all of the samples from one subject run in the same batch. Two quality control urine samples were included with each batch. The mean values and mean intra-assay imprecision for the quality control urine samples were as follows: (a) enterodiol, 3,108 nmol/day (CV, 2.7%); (b) enterolactone, 43,904 nmol/day (CV, 10.4%); and (c) matairesinol, 48 nmol/day (CV, 12.6%).

Creatinine excretion was measured by a vitrof analyzer (Johnson and Johnson Clinical Diagnostics, Inc.).

Diet analyses were performed using the Minnesota Nutrition Data System software (Food Database version 6A, Nutrient Database 21, 1992) developed by the Nutrition Coordinating Center, University of Minnesota (Minneapolis, MN; Ref. 44).

Statistical Analysis. Statistical analyses were performed using the Statistical Analysis System (SAS Proprietary Software Release 6.11; SAS Institute, Cary, NC). Results were analyzed using a repeated-measure ANOVA within subject. To correct for data that were not normally distributed, urinary lignan excretion analyses and *P* value computations were performed on a logarithmic scale. The linear regression analyses of enterodiol, enterolactone, and total lignans were performed on the logarithmic scale, adjusted for subject, and regressed on the amount of flaxseed in the diet. For reporting purposes, data summaries were transformed back to the original scale. For all measurements, results were considered statistically significant at *P* < 0.05.

Results

Body weight measurements and BMI at the end of each feeding period are presented in Table 1. Because the subjects maintained their body weights throughout the duration of the study, there were no significant differences in these measurements between any of the feeding periods.

Table 2 Nutrient intakes during diet treatments (n = 31)

	Values, which include nutrient contribution of flaxseed, are means \pm SD.		
	Control	Flaxseed	
		5 grams	10 grams
Energy (kcal)	1957 \pm 386	1908 \pm 441	1815 \pm 369
CHO (grams) ^a	280 \pm 63	274 \pm 62	260 \pm 61
CHO (% energy)	57 \pm 6	58 \pm 6	57 \pm 5
Protein (grams)	77 \pm 17	73 \pm 16	75 \pm 13
Protein (% energy)	16 \pm 3	16 \pm 3	17 \pm 3
Fat (grams)	63 \pm 19	63 \pm 22	58 \pm 18
Fat (% energy)	29 \pm 5	29 \pm 6	28 \pm 5
Total dietary fiber (grams)	23.0 \pm 5.5	24.2 \pm 6.2	24.8 \pm 7.3
Soluble fiber (grams)	8.3 \pm 2.0	9.0 \pm 2.2 ^b	9.3 \pm 2.3 ^c
Insoluble fiber (grams)	14.5 \pm 3.9	14.9 \pm 4.3	15.2 \pm 5.6

^a CHO, carbohydrate.

^b Significantly different from control, *P* = 0.0358.

^c Significantly different from control, *P* = 0.0028.

Intakes of total energy, carbohydrate, protein, fat, and dietary fiber during each feeding period are presented in Table 2. There were no significant differences in total energy, carbohydrate, protein, fat, or total fiber intake between any of the feeding periods. For soluble fiber, intakes for the 5-gram flaxseed feeding period (9.0 \pm 2.2 grams) and the 10-gram flaxseed feeding period (9.3 \pm 2.3 grams) were significantly higher than that for control (8.3 \pm 2.0 grams; *P* = 0.0358 and *P* = 0.0028, respectively).

Compared to the 0-gram flaxseed diet, consumption of 5 or 10 grams of flaxseed significantly increased excretion of enterodiol by 1,009 and 2,867 nmol/day, respectively; significantly increased excretion of enterolactone by 21,242 and 52,826 nmol/day, respectively; and significantly increased excretion of total lignans (enterodiol + enterolactone + matairesinol) by 24,333 and 60,640 nmol/day, respectively. Additionally, the enterodiol, enterolactone, and total lignan excretion showed a linear, dose-response increase between the control, 5-gram flaxseed, and 10-gram flaxseed feeding periods (*P* = 0.0001 and *r*² = 0.5285; *P* = 0.0001 and *r*² = 0.6378; *P* = 0.0001 and *r*² = 0.6975, respectively; Fig. 2). Excretion of matairesinol was not significantly altered with flaxseed consumption. Complete urinary lignan excretion data are presented in Table 3.

The ratio of enterodiol:enterolactone excretion did not change with flaxseed consumption. The mean enterodiol:enterolactone excretion ratio was 0.16 \pm 0.15 (mean \pm SD) on the subjects' habitual diet, 0.19 \pm 0.41 on the 5-gram flaxseed diet, and 0.17 \pm 0.31 on the 10-gram flaxseed diet. Of the 31 women, 19 showed a decrease and 6 showed an increase in the ratio of enterodiol:enterolactone excretion with flaxseed feeding. Of the remaining six women, two had an increase in the enterodiol:enterolactone excretion ratio on the 5-gram flaxseed diet but a decrease on the 10-gram flaxseed diet, three had a decrease in the ratio on the 5-gram flaxseed diet and an increase on the 10-gram flaxseed diet, and the remaining subject had no change in the ratio on the 5-gram flaxseed diet and an increase on the 10-gram flaxseed diet.

Discussion

Consumption of flaxseed, a significant source of plant lignans, in addition to their habitual diets significantly increased the excretion of enterodiol, enterolactone, and total lignans, but not matairesinol, in a linear, dose-response manner in this group of

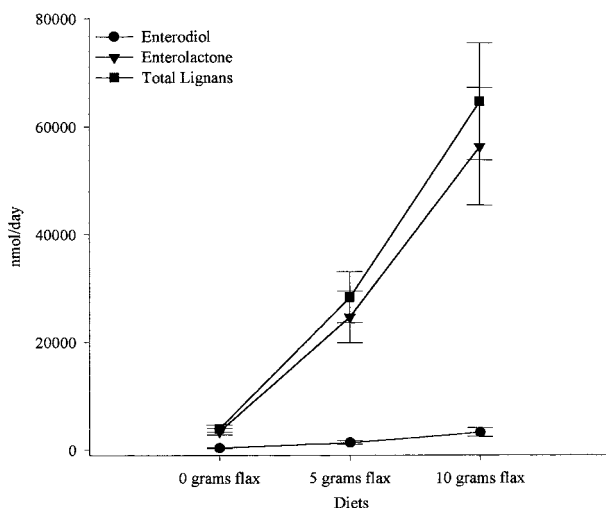


Fig. 2. Dose-response relationship between flaxseed intake and lignan excretion. Data presented as geometric mean (95% confidence interval).

postmenopausal women. The results of this study suggest that urinary excretion of lignan metabolites can be used as a dose-dependent biomarker of flaxseed intake, and potentially other plant food intake, within the context of a semicontrolled diet.

Mean urinary enterolactone excretion by subjects in this study while consuming their habitual diets was comparable to that reported for postmenopausal omnivorous women (3284 nmol/day; Ref. 34) and postmenopausal vegetarian women (3180 nmol/day) living in the Boston area (45), premenopausal omnivores (3160 nmol/day) in Minnesota (20), and premenopausal lacto-vegetarians living in Helsinki (3650 nmol/day; Ref. 35). Mean urinary enterodiol excretion by subjects in this study was also similar to that of the postmenopausal vegetarian women (400 nmol/day) in Boston and premenopausal lacto-vegetarians (368 nmol/day) in Helsinki but was lower than that of the premenopausal omnivores (1090 nmol/day) in Minnesota. Excretion of enterolactone and enterodiol in this group of postmenopausal omnivores may be comparable to that of the postmenopausal vegetarians and premenopausal lacto-vegetarians due to the similarities in fiber intake. The plant lignan precursors of enterolactone and enterodiol are believed to be constituents of the fiber component of plants typically found in the roots and rhizomes and in the woody parts, stems, leaves, seeds, and fruits (40). In grains, they are more likely localized in the bran layer and in the aleuronic layer of the grain right below the bran (4, 46). In several studies, Adlercreutz *et al.* (1, 4, 29, 34) have reported positive correlations between total fiber intake and enterolactone, enterodiol, and total mammalian lignan excretion in a variety of populations. The subjects in this study consumed an average of 23 grams of dietary fiber each day, similar to the consumption reported for the postmenopausal vegetarians (24 grams/day) and higher than that of the postmenopausal omnivores (15 grams/day) in the Boston area. However, despite similar enterolactone excretion between the postmenopausal omnivores in this study and the premenopausal omnivores in Minnesota, the premenopausal omnivores had one-third of the dietary fiber intake (8 grams/day) of the subjects in this study. Therefore, factors other than dietary fiber intake may be related to the formation and excretion of enterolactone and enterodiol in humans.

Wide ranges in the excretion of total mammalian lignans

have been observed on various habitual diets in both animals and humans (1, 34–40) and in relation to a flaxseed challenge (19–22). We observed the same variations in excretion in this group of postmenopausal women (Table 3) who were consuming similar but not identical diets. Several researchers have proposed that differences in the concentrations of lignans excreted may be due to the composition of the colonic microflora (11, 40, 47–49), differences in intestinal transit time (11, 47), or the redox level of the large intestine (11), all factors related to the habitual diets of the subjects (1, 36, 40, 49). Factors such as these may account for the variations in excretion observed in our study, despite the fact that these subjects have stable, long-standing habitual dietary patterns. The subjects in this study consume foods prepared in a central kitchen and served in a central dining room and do not frequently leave the monastery to eat elsewhere, and food items are offered based on a 7-week cycle menu that has been used for many years. Although the specific foods chosen at each meal vary among the subjects, the variation is much less than that encountered in the general population. Therefore, we hypothesized that the variation in mammalian lignan excretion would be less in this group of subjects than that which has been reported for other populations (1, 34–40). However, mammalian lignan excretion did vary greatly between the subjects, even on the subjects' habitual diets, and the variation increased with the addition of flaxseed (Table 3).

Researchers have also proposed that the variability observed in lignan excretion in humans may be due to the existence of several alternate pathways for lignan metabolism (40, 49–51) or the existence of mammalian lignan metabolites that are not routinely measured (52). In a recent report, Jacobs *et al.* (52) identified six enterolactone metabolites and three enterodiol metabolites in the urine of two male and two female subjects. Although they did not quantify the excretion of these metabolites, the existence of these metabolites supports the hypothesis that additional lignan metabolites that are not routinely measured do exist. Additional studies that address the effects of these metabolites *in vivo* and the effects of gastrointestinal function and colonic microfloral differences on lignan metabolism are needed.

We observed a significant dose-response increase in enterolactone, enterodiol, and total lignan excretion in this group of postmenopausal women (Table 3). Nesbitt *et al.* (21) also reported a dose-dependent increase in urinary lignan excretion ($P \leq 0.001$, $r^2 = 0.5184$) in response to the consumption of 5, 15, or 25 grams of raw flaxseed in a group of premenopausal women. However, Nesbitt *et al.* (21) reported that enterodiol was the mammalian lignan produced in the highest concentration after flaxseed consumption. The results reported by Nesbitt *et al.* (21) are in agreement with a study by Cunnane *et al.* (19) that reported that enterodiol was excreted in greater concentrations than enterolactone with the consumption of 50 grams of flaxseed/day. In contrast, enterolactone was excreted in much higher concentrations than enterodiol in our study and in flaxseed feeding studies by Shultz *et al.* (22) and Lampe *et al.* (20), although the primary plant lignan in flaxseed is secoisolariciresinol, the direct precursor of enterodiol. Research studies have suggested that enterodiol is synthesized from secoisolariciresinol (9–12) by facultative anaerobes in the colon and can be further oxidized by the colonic microflora to enterolactone (11, 13, 14). The conflicting results reported by these studies may be due to differences in the metabolism of plant lignans by individuals, a hypothesis supported by the results of Kirkman *et al.* (37), who reported gender differences in the excretion of enterolactone and enterodiol on controlled diets supplemented

Table 3 Urinary lignan excretion during each feeding period (nmol/day)

Data presented as geometric mean (95% confidence interval) [range].

	Control	Flaxseed	
		5 grams	10 grams
Enterodiol	365 (291–458) ^a [171–1,031]	1,374 (1,095–1,723) ^a [326–8,211]	3,232 (2,577–4,054) ^{a,b} [505–19,439]
Enterolactone	3,421 (2,866–4,083) ^a [358–16,934]	24,663 (20,662–29,439) ^a [2,465–148,736]	56,247 (47,122–67,140) ^{a,c} [6,416–309,773]
Matairesinol	45 (36–55) [0–213]	52 (42–65) [0–498]	60 (47–75) [0–2,961]
Total lignans	3,983 (3,412–4,649) ^a [647–17,447]	28,316 (24,258–33,053) ^a [7,660–151,288]	64,623 (55,361–75,433) ^{a,d} [17,691–310,965]

^a Significantly different from other diets, $P = 0.0001$.^b Linear trend between control, 5 grams of flaxseed, and 10 grams of flaxseed ($P = 0.0001$; $r^2 = 0.5285$).^c Linear trend between control, 5 grams of flaxseed, and 10 grams of flaxseed ($P = 0.0001$; $r^2 = 0.6378$).^d Linear trend between control, 5 grams of flaxseed, and 10 grams of flaxseed ($P = 0.0001$; $r^2 = 0.6975$).

with vegetables or soy. However, the studies by Cunnane *et al.* (19) and Lampe *et al.* (53) reported that lignan excretion did not differ significantly between men and women.

Physiological implications of the wide variation in enterodiol and enterolactone excretion and the differences in the enterodiol:enterolactone ratio are not currently known. However, researchers have found differences in the biological activity of these mammalian lignans. Enterolactone has approximately 10 times the estrogenic activity of enterodiol (2), was more effective than enterodiol at inhibiting the growth of MCF-7 breast cancer cells (54) and LS174T, Caco-2, HCT-15, and T-84 human colon tumor cell lines (27), and more effectively displaced both estradiol and testosterone binding by sex hormone-binding globulin (30). Enterolactone was also more effective than enterodiol at inhibiting human placental aromatase (31, 32), 5 α -reductase (33), and 17 β -hydroxysteroid dehydrogenase activity (33). These results suggest that enterolactone may be more effective against the prevention of some types of cancer, in particular, colon cancer and hormone-dependent breast cancer, than enterodiol. Therefore, subjects who excrete higher concentrations of enterolactone and presumably have higher plasma enterolactone concentrations may have more protection against hormone-dependent cancers than subjects who excrete higher concentrations of enterodiol.

In conclusion, consumption of 5 or 10 grams of flaxseed by postmenopausal women on a semicontrolled diet increased the excretion of enterolactone, enterodiol, and total lignans in a dose-dependent manner without changing the ratio of enterodiol:enterolactone excretion, suggesting that urinary excretion of lignan metabolites can be used as a dose-dependent biomarker of flaxseed consumption. Because flaxseed and its mammalian lignan products enterolactone and, to a lesser extent, enterodiol have been shown to influence the early risk markers for and incidence of mammary and colonic carcinogenesis in animal models, decrease cell proliferation *in vitro*, and influence factors that effect the hormone concentrations in humans, increases in the metabolism and excretion of these compounds may offer increased protection against hormone-dependent cancers. Wide variations in the excretion of enterodiol, enterolactone, and total lignans and the enterodiol:enterolactone ratio suggest that the factors that may influence individual variations in mammalian lignan formation, absorption, and excretion need to be examined so that we may have a better understanding of the metabolism of these compounds.

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