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SHORT COMMUNICATION

Flaxseed-Derived Enterolactone Is Inversely Associated with Tumor Cell Proliferation in Men with Localized Prostate Cancer

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ABSTRACT Enterolactone and enterodiol, mammalian lignans derived from dietary sources such as flaxseed, sesame seeds, kale, broccoli, and apricots, may impede tumor proliferation by inhibiting activation of nuclear factor kappa B (NFκB) and vascular endothelial growth factor (VEGF). We examined the associations between urinary enterolactone and enterodiol with prostatic tumor expression of NFκB, VEGF, and Ki67 among 147 patients with prostate cancer who participated in a presurgical trial of flaxseed supplementation (30 g/day) for $\sim$30 days. Urinary enterolignans and tissue biomarkers were determined by high-performance liquid chromatography and immunohistochemistry, respectively. After supplementation, we observed significant correlations between intakes of plant lignan and urinary concentrations of total enterolignans ($\rho = 0.677$, $P < .0001$), enterolactone ($\rho = 0.676$, $P < .0001$), and enterodiol ($\rho = 0.628$, $P < .0001$). Importantly, we observed that total urinary enterolignans and enterolactone were significantly and inversely correlated with Ki67 in the tumor tissue ($\rho = -0.217$, $P = .011$, and $\rho = -0.230$, $P = .007$, respectively), and a near-significant inverse association was observed for enterodiol ($\rho = -0.159$, $P = .064$). An inverse association was observed between enterolactone and VEGF ($\rho = -0.143$, $P = .141$), although this did not reach statistical significance. We did not observe an association between enterolignans and NFκB. In conclusion, flaxseed-derived enterolignans may hinder cancer cell proliferation via VEGF-associated pathways.

KEY WORDS: • *diet* • *flaxseed* • *lignans* • *phytoestrogens* • *prostatic neoplasia*

The plant lignans secoisolariciresinol and matairesinol are present in legumes, cereals, fruits, and vegetables; however, flaxseed is the richest source of these phytoestrogens.[1] After ingestion, secoisolariciresinol and matairesinol are converted to the enterolignans, enterolactone and enterodiol, via aerobic intestinal microflora.[2] There is growing evidence suggesting that enterolactone and enterodiol may inhibit carcinogenesis. *In vitro* studies have shown that enterolignans reduce cancer cell proliferation, and animal studies have observed that animals fed with diets containing lignan have fewer tumors and reduced tumor burden.[3] Although fewer in number, several studies in human populations have supported the anticarcinogenic properties of enterolignans.[4–7]

Preclinical studies have shown that enterolignans target several molecular pathways involved in carcinogenesis. This includes nuclear factor kappa B (NFκB), a key transcription factor that controls cell growth, angiogenesis, and inflammatory response, and is frequently activated in cancer cells. Previous research suggests that enterolignans inhibit the activation of NFκB.[8] A recent investigation reported reductions in NFκB activation by enterolactone and enterodiol with decreased activation of downstream intracellular signaling targets involved in inflammation.[9] Further, it has been suggested that the transition from indolent tumor growth to more-aggressive tumor proliferation is accompanied by the onset of angiogenesis in a process coined, the angiogenic switch.[10] Thus, there is a rationale to hypothesize that therapies that inhibit angiogenesis would also

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exhibit anticarcinogenic properties. Studies have reported that enterolignans are antiangiogenic. In a xenograft breast cancer mouse model, mice fed a diet supplemented with flaxseed had reduced tumor production of the proangiogenic protein, vascular endothelial growth factor (VEGF), compared to mice fed a control diet.[11,12] In contrast, there was no effect of 25 g/day flaxseed supplementation on VEGF secretion in breast tissue from healthy women.[13]

Another key feature of tumorigenesis is uncontrolled cell growth.[14] A study on patients with postmenopausal breast cancer consuming 25 g/day flaxseed for 32 days before surgery found decreased cell proliferation in breast tumors.[15] Expanding on these findings, a previous study found that a 1-year regimen of 50 mg/day of secoisolariciresinol diglucoside significantly reduced breast cell proliferation in premenopausal women at risk for developing breast cancer.[16] Taken together, these data suggest that flaxseed-derived enterolignans have anti-carcinogenic potential, because they can inhibit inflammation, angiogenesis, and reduce tumor cell proliferation.

Similar to breast cancer, prostate cancer is a hormone-related cancer that appears to be responsive to the anticarcinogenic effects of lignan. We previously reported that flaxseed supplementation for $\sim$30 days before surgery significantly reduced the tumor proliferation rates among men who elected prostatectomy for localized prostate cancer.[17] While these findings were promising, we did not investigate the molecular targets in the tumor tissue that were associated with inflammation and angiogenesis. Therefore, the purpose of this study was to explore the correlations between dietary intake of lignan, physiologic enterolignan concentrations, and prostatic tumor tissue expression of NFκB, VEGF, and Ki67, a marker of cell proliferation, in this patient population.

This study utilized data from our previous multisite phase II randomized controlled trial (NCT00049309) in men with prostate cancer awaiting prostatectomy.[17] The trial randomized 161 men by race (Black vs. non-Black) and biopsy Gleason sum ($\geq$7 vs. $\leq$6) to control (n=41), flaxseed (FS; n=40), low-fat diet (LF; n=40), or FS+LF (n=40) for $\sim$30 days before surgery. Men assigned to the FS groups consumed 30 g/day of whole-ground flaxseed; men in the LF groups consumed $<$20% of calories from fat; and the control group was instructed to maintain their usual diet.[17] Pre- and posturinary enterolignan concentrations and tumor biomarkers were available from 147 men from the parent study.

All procedures were approved by the Duke University Health System, Durham Veteran's Administration Hospital, and the University of Michigan Community Clinical Oncology Program Institutional Review Boards, and written informed consent was obtained before collection of all samples and data.

The NCI Diet History Questionnaire (DHQ)[18] was administered at baseline and follow-up to determine the background dietary intake of lignans, and we used a published database developed by Thompson *et al.* to estimate the amount of plant lignan consumed from individual food items.[19] We calculated the total intake of plant lignan provided by flaxseed supplementation by multiplying the average grams per day of flaxseed consumed by the amount of plant lignan in the flaxseed (1 g provided 333 μg of plant lignan). Flaxseed was provided by ENRECO, Inc. (Manitowoc, WI, USA) in one lot (150 kg), and held in a whole-seed form in cold storage before use. It was analyzed for lignan content (Laboratory of Alister Muir, Saskatoon Research Center, Saskatoon, Saskatchewan, Canada) at the beginning and end of the study period, and the values were averaged.[17] The amount of plant lignan from flaxseed was added to the values of plant lignan that were present in the diet.

To determine the physiological levels of enterolignans, participants collected urine over a 24-h period and returned the refrigerated sample the following day, whereupon it was aliquotted and stored at -80°C. Urinary enterolignans were hydrolyzed and quantified via high-performance liquid chromatography.[20] The concentrations of urinary lignans were corrected for mg/mL of creatinine to adjust for variations in urinary volume.

As previously described elsewhere,[17,21] immunohistochemistry was used to determine the proliferation index (Ki67; Biocare, Walnut Creek, CA, USA), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and activation of NFκB (Abcam, Cambridge, MA, USA) from tissue sections cut from formalin-fixed paraffin-embedded prostatic tumor blocks. Slides were reviewed and scored by two independent pathologists who were blinded to randomization status.[17,21]

To compare the effects of a diet high in lignans against a diet not supplemented with a source of dietary lignan on urinary levels of enterolignans, we combined both flaxseed groups, that is, flaxseed alone and flaxseed/low-fat diet, into one group (Flaxseed) and combined the two groups that did not receive flaxseed, that is, control and low-fat diet (No Flaxseed). Differences in the baseline characteristics between the two study groups (Flaxseed vs. No Flaxseed) were determined using t-tests. Dietary lignan, urinary enterolignan, and tissue biomarker data were non-normally distributed, and attempts at transforming the data did not improve the distribution; therefore, the median and range

Table 1. Baseline Characteristics of Study Subjects

	No FS (n=*74*)	*FS* (n=*73*)
Age, years	58.5±7.43	59.8±7.25
Race, % (*n*)		
Caucasian	69% (51)	69% (51)
African-American	22% (16)	25% (18)
Other	9% (7)	6% (4)
BMI, kg/m^2	29.1±4.2	28.5±4.2
Serum PSA, ng/mL	5.8±3.54	6.7±3.60
Biopsy Gleason score, % (*n*)		
$\leq$6	91% (67)	89% (65)
$\geq$7	9% (7)	11% (8)

Values are given as mean±standard deviation or as percent (number).
BMI, body–mass index; PSA, prostate-specific antigen; FS, flaxseed.

Table 2. Dietary Intake and Urinary Concentrations of Lignan

	No FS (n = 74)		*FS (n = 73)*		
	Baseline	*Follow-up*	*Baseline*	*Follow-up*	P[a]
Dietary lignan, μg/day					
Lignan	262 (12–1263)	254 (1–777)	282 (59–862)	299,930 (299,720–300,448)	< .0001
Urinary enterolignan,[b] μg/day					
Enterolactone	200.7 (1.92–2731.3)	300 (2.52–3892.9)	193.2 (1.94–2787.6)	4731.9 (6.53–233,163.0)	< .0001
Enterodiol	34.5 (1.92–2040)	31.7 (2.23–2943)	45.6 (1.57–1046.9)	2724.4 (29.1–36,805.8)	< .0001
Total lignan	263.4 (3.85–3046.2)	339.11 (5.30–5079.8)	274.3 (4.35–2810.3)	10,565.3 (150–256,807)	< .0001

Values are given as median (range).

[a]*P* for change score.

[b]Urinary enterolignan concentrations adjusted for creatinine.

are presented for these variables. Wilcoxon signed-rank tests were used to test for differences in change scores for dietary and urinary lignans. Spearman correlation coefficients were generated to determine the associations between dietary lignan, urinary lignan, and tissue biomarkers. JMP 8.0 software (Cary, NC, USA) was used in the analyses; all *P* values were 2-sided, and an alpha of < 0.05 was considered statistically significant.

At baseline, there were no differences in characteristics between the Flaxseed ($n = 73$) and No Flaxseed ($n = 74$) groups (Table 1). Dietary intake of plant lignan also was similar across the study groups at baseline, but as expected, the intake of plant lignan markedly increased in the flaxseed-supplemented groups over the study period. Similarly, there were no between-group differences in the concentrations of urinary lignan metabolites at baseline; however, after flaxseed supplementation, marked increases in urinary enterolignans were observed in the Flaxseed group (Table 2). In addition, there was a highly significant correlation overall between the dietary intake of plant lignan and urinary excretion of enterolactone ($\rho = 0.676$, $P < .0001$), enterodiol ($\rho = 0.628$, $P < .0001$), and total enterolignans ($\rho = 0.677$, $P < .0001$) in the follow-up time period.

Correlations between urinary enterolignans and prostate tumor expression of Ki67, VEGF, and NFκB are shown in Table 3. Urinary concentrations of enterolactone and total enterolignan were significantly and inversely associated with Ki67. However, associations with enterodiol were weaker. Prostatic tissue expression of VEGF was lower in patients with higher enterolactone, although this did not reach statistical significance. In contrast, there was no apparent correlation between the urinary enterolignans and NFκB.

Table 3. Spearman Correlations Between Urinary Enterolignans and Prostatic Tumor Biomarkers

	NFκB	*Ki67*	*VEGF*
Enterolactone	−0.109	−0.230*	−0.143
Enterodiol	−0.117	−0.159	−0.07
Total lignan	−0.132	−0.217*	−0.132

*$P < .05$.

NFκB, nuclear factor kappa B; VEGF, vascular endothelial growth factor.

In this study, we observed that the total enterolignans and enterolactone were associated with the antiproliferative effects in the target tissue, as indicated by a significant inverse correlation between these plant lignan metabolites and Ki67 staining in prostate tumor tissue. The magnitude of these associations ranged from modest to moderate in significance. There also was a suggestion that higher enterolactone was associated with hindered tumor angiogenesis by lower tumor expression of VEGF. Conversely, we observed no association between enterolignans and reduced activation of inflammatory pathways in the prostate as manifested by NFκB activity, a finding consistent with a study by Heymach *et al.*[21] Therefore, these study findings support that enterolignans, and specifically enterolactone, are likely to exert chemoprotective effects by hindering cell proliferation and possibly reduced tumor angiogenesis.

As previously observed in a recent meta-analysis, higher dietary intake of foods high in plant lignan may reduce the risk for cancer.[4] Similarly, some observational studies,[6,7,22] but not all,[23] have reported that higher physiological levels of enterolignans are correlated with a reduced risk for cancer, improved cancer prognosis, and reduced risk for cancer-related mortality, suggesting that enterolignans are chemoprotective. In contrast, earlier studies in preclinical models suggested that enterolactone may stimulate cell proliferation in some hormone-sensitive cell lines, including MCF-7 breast cancer cells.[24] In the present study, we found no evidence that enterolignans stimulated tumor growth in men with localized prostate cancer. Indeed, our findings support the hypothesis that enterolignans are antiproliferative *in vivo*. These findings expand on the knowledge regarding the molecular targets through which these dietary constituents may exert their antineoplastic potential. Based on our findings, increased dietary intake of lignans via flaxseed supplementation significantly increased physiological enterolignan concentrations and hindered tumor cell growth.

Prostate cancer is unique in that most cases will remain indolent, while only some will become virulent. Therefore, some men will choose active surveillance rather than more aggressive treatment. Flaxseed supplementation has yet to be tested in men who choose active surveillance, but based

on our findings that it is safe, inexpensive, easy to obtain, and its metabolic products reduce cell proliferation, flaxseed supplementation may present an opportunity for future chemopreventive research. Additional studies using flaxseed supplementation in this unique patient population are therefore warranted.

In conclusion, this study provides direct evidence that dietary intake of plant lignans via flaxseed supplementation inhibits cancer cell growth and possibly reduces tumor angiogenesis in patients with prostate cancer. The consumption of food items, such as flaxseed, which are potential sources of enterolignans, should be explored further, given their chemoprotective properties.

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AUTHOR DISCLOSURE STATEMENT

The authors of this article have no conflicts of interest to declare.

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