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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Prostate cancer is the most common cancer among men in the United States (IARC, 1995) and the second most common in the European Community (IARC, 1995). The causes of prostate cancer, however, remain largely unknown, with age, race, and family history being the only established risk factors (Nomura et al., 1997). The prostate gland has historically been considered the prototype of an androgen-dependent organ. However, there is evidence that estrogens may induce mitosis of prostatic epithelial cells in many species, including humans (Leave et al., 1978; Schulze et al., 1987). This report analyzes the association between prostate cancer and estrogen metabolism investigated in a case-control study. In particular, we tested the hypothesis that the pathway favoring 2-hydroxylation over 16 $\alpha$ -hydroxylation may be associated with a decrease in prostate cancer risk. This is the final report for the study. During the final year of activity, we completed determinations of the estrogen metabolites using gas-chromatography and carried out statistical analysis. We have also prepared an article for publication of the results (see attached article).					
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## INTRODUCTION

Prostate cancer is the most common cancer among men in the United States (IARC, 1995) and the second most common in the European Community (IARC, 1995). The causes of prostate cancer, however, remain largely unknown, with age, race, and family history being the only established risk factors (Nomura et al., 1997). The prostate gland has historically been considered the prototype of an androgen-dependent organ. However, there is evidence that estrogens may induce mitosis of prostatic epithelial cells in many species, including humans (Leav et al., 1978; Schulze et al., 1987).

This report analyzes the association between prostate cancer and estrogen metabolism investigated in a case-control study. In particular, we tested the hypothesis that the pathway favoring 2-hydroxylation over 16 $\alpha$ -hydroxylation may be associated with a decrease in prostate cancer risk.

This is the annual report for the third year of the study. During the third year of activity, we completed the definition of the large dataset of the study, conducted quality control procedures on the collected data and develop new laboratory procedures for the determination of the estrogen metabolites using gas-chromatography. The determinations are in now in advanced phase of progress.

## BODY OF REPORT

We are at the final stages of conducting hormone determinations by gas-chromatography. We will then analyze the data in the next few months.

### Background

**Follow-up of the cohort:** We completed the re-call and follow-up of the Western-New York cohort (WNYHC) for the identification of the incident prostate cancer cases and their related control subjects. The follow-up was conducted in collaboration with another NIH-funded study, the “Epidemiology of Type-2 Diabetes” study, which is a prospective cohort study based on the same WNYHC cohort (RO1 DK 60587, Dr. R. Donahue, PI, Dr. P. Muti, Co-PI).

The re-call of the cohort started in January 2003. The re-call included participants without history of cancer, cardiovascular diseases, and clinically defined type-2 diabetes at baseline interviewed between 1996-2001. The re-call was also limited to those cohort participants with stored biological samples. Thus, we started the re-call and the follow-up process with a sample of **1,150 cohort participants**.

Of the 1,150 cases :-

- **52** were not eligible for medical reasons (too ill with diseases other than those mentioned before),
- **46** had died (for causes other than prostate cancer),
- **22** had moved out of the Erie and Niagara Counties,
- **117** were not contactable by mail or phone.

Overall, we had a sample of **913** re-called participants. Among the **913** participants, we identified :-

- **41** incident prostate cancer cases,
- **232** cases refused to participate in the study (they refer, in the short telephone interview, to having not been diagnosed with prostate cancer),
- **40** were scheduled but then cancelled the appointment,
- **8** were still in-process at the end of the follow-up period (September 30, 2004).

Thus, there were **592** participants available as control subjects.

During the study period, we monitored the occurrence of prostate cancer among the successfully contacted participants. All procedures to re-call, interview and collect biological specimens from the WNYHC Study were similar to the procedures used for baseline recruitment. All eligible participants were initially re-contacted by letter and then by phone (up to twelve callbacks). Participants were invited to attend our recruitment center at the Department of Social and Preventive Medicine, Buffalo, New

York for the clinical examination and to answer questions related to the occurrence of prostate cancer diagnosis between the baseline examination and the re-call time.

### **Definition of the matched case-control pairs.**

Case Identification: Incident Prostate Cancer Cases: Prostate cancer cases recruited in the study were men who have been diagnosed with incident cytologically and/or histologically confirmed prostate cancer after their recruitment (date at first interview) in the WNYHC Study and before the end of the cohort follow-up period (September, 30, 2004). Prostate cancer cases were identified by their own report at the re-call of the cohort. 32 cases have been validated by their clinical records, while the remaining 9 cases are in the process to be validated. At recruitment, each cohort member signed a consent form giving us permission to request copies of their clinical charts in cases of pathological events related to the WNYHC Study investigations. Through these clinical charts, we are validating the information collected from participants.

Control Identification: Eligible controls were all male members of the WNYHC Study who, based on their report, were not diagnosed with prostate cancer at the diagnosis of the related case. For each prostate cancer case, four controls will be randomly chosen after matching for:

- a) age (within 3 years);
- b) race;
- c) recruitment date

to control for the effect of long-term preservation of stored urine.

To increase the power of the study (and to reduce the effects of non-diagnosed prostate cancer cases among controls), we used a 1:4 ratio for cases and controls. Therefore, the study hormone determinations will be conducted on 41 prostate cancer cases and 164 control subjects.

We are at the final stages of conducting hormone determinations by gas-chromatography. We will then analyze the data in the next few months.

### **KEY RESEARCH ACCOMPLISHMENTS**

- Completed the follow-up of the cohort
- Completed quality control procedures to ensure the completeness (reliability?) of the follow-up
- Completed quality control setting for hormone bio-assays
- Completed new analysis of secondary sexual characteristics and prostate cancer risk, based on a previously DOD-funded prostate cancer study
- Validation of prostate cancer diagnosis
- Initiated and almost completed bio-assay determinations.

## REPORTABLE OUTCOMES

### Publications and Presentations

At this time, there are no results or publications coming directly from this grant because we still completing the study. However, Dr. Muti has published, or has in press, research on hormone-related cancer using a previously collected data set on hormone and prostate cancer (the dataset was originated from a previously DOD-funded study). She has submitted a paper for publication on the relationship between Indicators of Sexual and Somatic Development and Adolescent Body Size in Relation to Prostate Cancer Risk: Results from a case-control study.

In 2006-2007, Dr. Muti has published other papers on hormones and cancer, listed below:

- 1) \*Barba M, McCann S, Nie J, Vito D, Stranges S, Fuhrmann B, Trevisan M, **Muti P**, Freudenheim JL. Perinatal Exposures and Breast Cancer Risk in the Western New York Exposures and Breast cancer (WEB) Study. *Cancer Causes Control* 17(4): 395-401, 2006
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- 4) Zeleniuch-Jacquotte A, Ludin E, Micheli A, Koenig KL, Lenner P, **Muti P**, Shore RE, Johansson I, Krogh V, Lukanova AK, Stattin P, Afanasyeva Y, Rinaldi S, Arslan A, Kaaks R, Berrino F, Hallmans G, Toniolo P, Adlercreutz H. Circulating Enterolactone and risk of endometrial cancer. *The International Journal of Cancer* 119(10):2376-81, 2006
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- 11) [Sant M](#), [Allemani C](#), [Sieri S](#), [Krogh V](#), [Menard S](#), [Tagliabue E](#), [Nardini E](#), [Micheli A](#), [Crosignani P](#), **Muti P**, [Berrino F](#). Salad vegetables dietary pattern protects against HER-2-positive breast cancer: A prospective Italian study. [Int J Cancer](#). 121(4):911-914, 2007
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- 15) Capurso G, Schünemann HJ, Terrenato I, Morretti A, Koch M, **Muti P**, Capurso L, Delle Fave G. Use of non-steroidal anti-inflammatory drugs and pancreatic cancer risk: A systematic review & meta-analysis for different exposure categories. (in press, *Aliment Pharm & Therapy*)
- 16) Barba M, Terrenato I, Schünemann H, Fuhrman B, Sperati F, Teter B, Gallucci M, D'Amato A, **Muti P**. Indicators of sexual and somatic development and adolescent body size in relation to prostate cancer risk: results from a case control study. (in press, *Urology*)



She has also presented new study results from other studies conducted, at the Meetings of the American Association for Cancer Research:

- 1) Barba M, Terrenato I, Fuhrman B, Teter B, Schunemann H, **Muti P**. *Secondary sexual characteristics and body size at different ages in relation to risk of prostate cancer: results from a case-control study* Annual Meeting American Association for Cancer Research, Washington, April 2006
- 2) McCann SE, **Muti P**, Vito D, Edge SB, Trevisan M, Freudenheim JL. *Dietary lignan intakes and risk of breast cancer by tumor estrogen receptor status.* Annual Meeting American Association for Cancer Research, Washington, April 2006
- 3) Fuhrman BJ, Teter BE, Barba M, Byrne C, Cavalleri A, Grant BJ, Horvath P, **Muti P**. *Soy intake and mammographic density in postmenopausal women: Modification by equol status.* 5<sup>th</sup> Annual Meeting American Association for Cancer Research, Boston, November, 2006
- 4) Teter BE, Fuhrman BJ, Barba M, **Muti P**. *Aspirin Use and Mammographic Breast Density as a Marker of Breast Cancer Risk in Postmenopausal Women.* 5<sup>th</sup> Annual Meeting American Association for Cancer Research , Boston, November, 2006
- 5) Teter BE, Fuhrman BJ, Barba M, **Muti P**. *Nocturnal 6-Sulfatoxymelatonin and Mammographic Breast Density as a Marker of Breast Cancer Risk in Postmenopausal Women.* 5<sup>th</sup> Annual Meeting American Association for Cancer Research, Boston, November 15, 2006

### **Chapters in Books**

- 1) Barba M, Fuhrman B, **Muti P**. *Cancer Epidemiology. Fundamentals of Medical Oncology.* Edited by Cavalli F, Cognetti F, Costa A, Orecchia R. Milan: Elsevier; 3-13, 2006
- 2) Strano S, Barba M, **Muti P**. *Il Manifesto della Lunga Vita. L'allungamento della vita attraverso i secoli: ruolo svolto dai determinanti ambientali sul patrimonio genico e nell'insorgenza di una malattia moderna.*

### **Letters (peer-reviewed)**

- 1) Micheli A, Secreto G, Meneghini E, Krogh V, **Muti P**, Venturelli E, Berrino F. *Endogenous Steroid Hormone Concentrations and Risk of Breast Cancer Among*

**Presentations at Meetings (Peer reviewed, selected) 2006/2007**

**(Presenter in bold. Some of these are published as abstracts, citation follows presentation)**

- 6) Barba M, Terrenato I, Fuhrman B, Teter B, Schunemann H, **Muti P**. *Secondary sexual characteristics and body size at different ages in relation to risk of prostate cancer: results from a case-control study* Annual Meeting American Association for Cancer Research, Washington, April 2006
- 7) McCann SE, **Muti P**, Vito D, Edge SB, Trevisan M, Freudenheim JL. *Dietary lignan intakes and risk of breast cancer by tumor estrogen receptor status.* Annual Meeting American Association for Cancer Research, Washington, April 2006
- 8) Fuhrman BJ, Teter BE, Barba M, Byrne C, Cavalleri A, Grant BJ, Horvath P, **Muti P**. *Soy intake and mammographic density in postmenopausal women: Modification by equol status.* 5<sup>th</sup> Annual Meeting American Association for Cancer Research, Boston, November, 2006
- 9) Teter BE, Fuhrman BJ, Barba M, **Muti P**. *Aspirin Use and Mammographic Breast Density as a Marker of Breast Cancer Risk in Postmenopausal Women.* 5<sup>th</sup> Annual Meeting American Association for Cancer Research , Boston, November, 2006
- 10) Teter BE, Fuhrman BJ, Barba M, **Muti P**. *Nocturnal 6-Sulfatoxymelatonin and Mammographic Breast Density as a Marker of Breast Cancer Risk in Postmenopausal Women.* 5<sup>th</sup> Annual Meeting American Association for Cancer Research, Boston, November 15, 2006

**CONCLUSIONS**

We are about to start data analysis. Therefore, there are no conclusions to report at this time.

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- 2) Colombo C, **Muti P**, Pala V, Cavalleri A, Venturelli E, Locardi M, Berrino F, Secreto G. *Plant-based diet, serum fatty acid profile, and free radicals in postmenopausal women: the diet and androgens (DIANA) randomized trial*. Int J Biol Markers. 2005 Jul-Sep;20(3):169-76
- 3) McCann se, Kulkarni S, Trevisan M, Vito D, Nie J, Edge S, **Muti P**, Freudenheim JL *Dietary lignan intakes and risk of breast cancer by tumor estrogen receptor status (in press, Breast Cancer Research and Treatment)*

## APPENDIX

### ***Changes in 2-Hydroxyestrone and 16-Hydroxyestrone Metabolism with Flaxseed Consumption: Modification by COMT and CYP1B1 Genotype***

**Susan E. McCann<sup>1</sup>, Jean Wactawski-Wende<sup>2</sup>, Kari Kufel<sup>3</sup>, James Olson<sup>4</sup>, Bladimir Ovando<sup>4</sup>, Susan Nowell Kadlubar<sup>5</sup>, Warren Davis<sup>1</sup>, Lisa Carter<sup>1</sup>, Paola Muti<sup>6</sup>, Peter G. Shields<sup>7</sup> and Jo L. Freudenheim<sup>2</sup>**

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#### **Abstract**

Consumption of the phytoestrogen lignans, structurally similar to estrogen, has been associated with alterations in gene expression and estrogen metabolism. Furthermore, lignan consumption, subsequent changes in metabolizing enzyme expression, and genetic variability in these enzymes may alter estrogen metabolism and modify disease risk. Therefore, we investigated the effect of flaxseed on hydroxyestrone metabolite excretion by catechol-O-methyltransferase (*COMT*) and cytochrome *P*450 1B1 (*CYP1B1*) genotype. We conducted an intervention among 132 healthy, postmenopausal women, ages 46 to 75 years. Participants consumed 10 g ground flaxseed daily for 7 consecutive days. Blood and urine samples were collected at baseline and after the 7-day intervention. *COMT Val*<sup>158</sup>*Met* and *CYP1B1 Leu*<sup>432</sup>*Val* genotypes were determined using PCR-RFLP methods. Urinary 2-hydroxyestrone (2OHE1) and 16-hydroxyestrone (16OHE1) were quantified by ELISA assay. The effect of genotype on intervention-related changes in estrogen metabolites was assessed with the Kruskal-Wallis test. Compared with baseline levels, postintervention levels of urinary 2OHE1 (ng/mg creatinine; mean  $\pm$  SD, 16.1  $\pm$  10.6 versus 9.3  $\pm$  6.9, postintervention and baseline, respectively;  $P < 0.01$ ) and 2OHE1/16OHE1 ratios (mean  $\pm$  SD, 2.73  $\pm$  1.47 versus 1.54  $\pm$  0.75, postintervention and baseline, respectively;  $P < 0.01$ ) were significantly higher. The change in 2OHE1/16OHE1 increased with increasing numbers of variant alleles for *COMT* (mean change: *Val/Val*, 0.90; *Val/Met*, 1.15; and *Met/Met*, 1.50;  $P = 0.17$ , Kruskal-Wallis) and especially *CYP1B1* (mean change: *Leu/Leu*, 0.89; *Leu/Val*, 1.32; and *Val/Val*, 1.51;  $P = 0.04$ , Kruskal-Wallis). Our findings suggest that

variation in hormone-related genes may modify the effect of dietary lignan exposures on estrogen metabolism. (Cancer Epidemiol Biomarkers Prev 2007;16(2):256–62.

Higher lifetime exposure to estrogen has been associated with cancers in several hormone-sensitive organs, including the breast, ovaries, and endometrium (1-3). Estrone (E1), the predominant circulating estrogen in postmenopausal women, can be metabolized and cleared from the body by the hydroxylation of estrone to hydroxyestrone with subsequent further conversion to methoxyestrone, which is excreted in the urine (4). The hydroxyestrones include several metabolites, which are differentiated by the ring position of the hydroxyl group, and possess different estrogenic activity. The 2-hydroxyestrone (2OHE1) metabolite has fairly weak estrogenic activity, whereas the 4-hydroxyestrone and 16-hydroxyestrone (16OHE1) metabolites have relatively strong estrogenic activity. Breast cancer risks are lower among women with higher urinary 2OHE1 excretion and higher ratios of 2OHE1/16OHE1 (5).

Several genes are involved in the metabolism and clearance of estrone (4). Cytochrome P450 1B1 (CYP1B1) is primarily involved in the 4-hydroxylation of E1; however, conversion to 2OHE1 has also been reported (6). CYP1B1 has several polymorphisms, at least four of which result in amino acid substitutions (7). One polymorphism, *Leu<sup>432</sup> Val*, is functional with higher activities, and subsequently higher 2OHE1 levels, reported for the variant alleles (6). The hydroxyestrones can be further metabolized and inactivated through catechol-*O*-methyltransferase (COMT)-catalyzed methylation. COMT is also polymorphic; functional changes, resulting in a 4-fold reduction in activity, have been reported for the *Val/Met* substitution at position 158/108 (8). An examination of COMT in conjunction with CYP1B1 is warranted as these two genes participate in substantial feedback inhibition (9), and both CYP1B1 and COMT polymorphisms have been associated with breast cancer in some, but not all, studies (4).

Lignans are a class of phytoestrogens that have been shown to possess anticarcinogenic, antioxidant, and hormonal properties and to affect gene expression (10). Plant lignans are ubiquitous in whole grains, seeds, vegetables, and fruits; however, flaxseed is one of the richest dietary sources. Plant lignans are metabolized in the mammalian gut to form the physiologically active forms. Enterolactone is the primary circulating mammalian lignan and is measurable in serum, plasma, urine, breast milk, and semen. Previous epidemiologic studies have suggested lower risks of hormone-related cancers, including breast cancer, associated with higher lignan exposure, although not all studies agree (10).

Although no studies, to date, have investigated the long-term effect of flaxseed consumption on cancer risks in humans, several short-term intervention studies have shown modification of 2OHE1 metabolism after flaxseed supplementation, which could have implications for future cancer development (11-13). Furthermore, genetic susceptibility to environmental exposures is increasingly recognized as an important contributor to disease risk. Dietary lignans have the potential to affect hormone metabolism, and subsequent cancer risk, through several mechanisms, including interactions with individual gene variations. Conversely, lignans may also be metabolized via steroid hormone-metabolizing enzymes, thus modifying their

bioavailability. Therefore, we designed and conducted a study to investigate the effect of variation in *CYP1B1* and *COMT* on changes in estrogen metabolism resulting from high dietary lignan intake in a short-term dietary intervention.

## **Subjects**

Data were collected between October 2002 and March 2004 at the Center for Preventive Medicine, University at Buffalo (Buffalo, NY). The study protocol was approved by the University at Buffalo Institutional Review Board and all participants provided a signed informed consent and Health Insurance Portability and Accountability Act authorization. Participants were healthy volunteers recruited through advertisements in the western New York region (Erie and Niagara counties). Eligible women were 45 to 75 years of age, without a menstrual cycle in the past 12 months. Women with a hysterectomy but with intact ovaries were included if ages 55 years. Women were not eligible if they used antibiotics, hormone therapy, nonprescription hormones (e.g., melatonin and dehydroepiandrosterone), black cohosh, tamoxifen, raloxifene, diabetes medication, cimetidine, soy products, or flaxseed supplements within 2 months of participation. Of 422 women inquiring about participation, 122 (29%) declined, 142 (34%) were not eligible, and 158 (37%) were eligible and willing to participate. Of those women eligible and willing to participate, 134 (85%) were consented and completed the baseline visit, and 132 (84%) women completed the intervention. The characteristics of the study participants are shown in [Table 1](#).

Data were collected at several points in the study: recruitment, presupplementation (baseline) clinical visit, and postsupplementation clinical visit. The self-administered questionnaires included basic demographic information, medical and reproductive histories, prescription and over-the-counter medication use (including dietary supplements), family history of chronic disease, current physical activity, cigarette smoking, and other epidemiologic data relevant to diet and cancer. Dietary data were collected via an extensive food frequency questionnaire and two 3-day food records (week before and during the intervention). Height, weight, and waist and hip circumferences were measured at the preintervention clinical visit by trained study staff using a standardized protocol. Height and weight for all women were obtained using the same scale and stadiometer, which was periodically maintained for accuracy. Waist two inches above the umbilicus and hip at the widest point measurements were obtained using a nonflexible measuring tape.

## **Flaxseed Supplementation Trial**

We used a pre-post intervention study design, in which each woman served as her own control. Each participant's usual diet was supplemented daily for 7 days with a measured amount of commercially available ground flaxseed (10 g/d). The flaxseed was purchased in bulk from the same lot to standardize lignan content (Lexington Foods, Buffalo, NY). All participants were instructed to maintain their usual diet and activities during the 7-day intervention period. Each 10 g flaxseed supplement provided 209 kJ, 2.4 g protein, 3.6 g total fat, 2.8 g carbohydrate, 2.2 g dietary fiber, and 37 mg secoisolariciresinol (primary lignan in flaxseed). The flaxseed was kept frozen at  $-20^{\circ}\text{C}$  until consumption, and participants were given suggestions as to how to incorporate it

into their usual diets (e.g., in yogurt, stirred into beverages, etc.). For ease of consumption, we encouraged women to add the flaxseed to already prepared foods. We analyzed postintervention changes in enterolactone production by food group used to consume the flax and observed no differences. To assess compliance, the participants were asked to record daily the time of consumption and how the flaxseed was consumed, as well as any symptoms experienced that day. Reported symptoms were mild and limited primarily, as expected, to gastrointestinal complaints, such as bloating and flatulence. Although we screened potential participants for seed and nut allergies, two women reported diffuse pruritus, which resolved before the end of the intervention period, and may or may not have been related to the flaxseed. Compliance with the intervention was excellent. According to self-report, of the 132 women who completed the intervention, 127 consumed all seven doses. The remaining five women reported each missing one dose between days 3 and 5 of the intervention period.

### **Biological Samples**

All appointments were conducted between 7 and 11 a.m. after an overnight fast. Morning spot urines were collected into urine specimen cups at each clinical visit. Whole blood was collected by a standardized phlebotomy protocol by a trained phlebotomist. Biological specimens were immediately processed, and blood and urine were frozen within 60 min of collection. Serum, clots, buffy coat, plasma, and urine samples were aliquoted into cryovials and stored at  $-80^{\circ}\text{C}$ .

All assays were run in duplicate and with baseline and postintervention samples in parallel for each participant. Assays used standard quality control procedures used to describe the measurement of each biomarker for this study (i.e., standards of known concentrations and repeated samples within runs and across runs).

### **Genotyping for *CYP1B1* and *COMT***

Genotyping for *COMT* was conducted using a method similar to that of Kocabas et al. (14). Total genomic DNA was isolated from the buffy coat layer of blood according to the manufacturer's instructions for the QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA). DNA was quantified by spectrophotometry at 260 nm using a Beckman Coulter (Fullerton, CA) DU-530 spectrophotometer and stored at  $-80^{\circ}\text{C}$ . Genomic DNA was subjected to PCR and genotyping for *COMT* was done by RFLP. PCR amplification of genomic DNA resulted in the formation of a 115-bp amplicon. The PCR mixture (total volume 25  $\mu\text{L}$ ) consisted of the following: 5  $\mu\text{L}$  DNA (0.1-0.4  $\mu\text{g}/\mu\text{L}$ ), 1.25  $\mu\text{L}$  of 50 mmol/L  $\text{MgCl}_2$ , 0.5  $\mu\text{L}$  of 10 mmol/L deoxynucleotide triphosphate, 0.5  $\mu\text{L}$  of 100  $\mu\text{mol}/\text{L}$  *COMT* sense primer (5'-GGCGAGGCTCATCACCATCG-3'), 0.5  $\mu\text{L}$  of 100  $\mu\text{mol}/\text{L}$  *COMT* antisense primer (5'-CAGGTCTGACAACGGGTCAG-3'), 2.5  $\mu\text{L}$  of 10x PCR buffer, 0.5  $\mu\text{L}$  Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 14.25  $\mu\text{L}$  molecular grade  $\text{H}_2\text{O}$ . PCR amplification was as follows: initial denaturation at  $95^{\circ}\text{C}$  for 4 min followed by 30 cycles of  $95^{\circ}\text{C}$  for 1 min,  $58^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min. This was followed by a 10-min extension step at  $72^{\circ}\text{C}$ . A total of 10  $\mu\text{L}$  PCR product was separated by acrylamide gel (20%) electrophoresis to identify samples that had amplified correctly. Restriction enzyme genotyping was carried out in 10- $\mu\text{L}$  reaction containing 5  $\mu\text{L}$  PCR product, 1  $\mu\text{L}$  bovine serum albumin, 1  $\mu\text{L}$  NE buffer 4, 0.5  $\mu\text{L}$  *Nla*III (New England Biolabs, Beverly MA), and 2.5  $\mu\text{L}$  molecular grade  $\text{H}_2\text{O}$ . The

enzymatic mixture was incubated for 2 h at 37°C. Products of the restriction digest were viewed on a 20% acrylamide gel stained with ethidium bromide. High-activity *COMT* was identified by the presence of a 94-bp fragment and low-activity *COMT* was identified by a 74-bp fragment.

The *CYP1B1* *Leu/Val* variant was determined by PCR-RFLP. Briefly, a fragment of the *CYP1B1* containing the polymorphic base was amplified by PCR using gene-specific primers (forward primer 5'-CTGCCAACACCTCTGTCTTG-3' and reverse primer 5'-CTGAAATCGCACTGGTGAGC-3'). The resulting PCR product (271 bp) was digested by exposure to the restriction endonuclease *Eco57I* and the subsequent fragments were resolved by agarose gel electrophoresis and visualized by transillumination after ethidium bromide staining. Homozygous *Leu* alleles generate fragments of 105 and 166 bp, whereas homozygous *Val* is unaffected by exposure to the enzyme. DNA from heterozygous individuals will display all three bands 9271, 166, and 105 bp.

### **Enterolactone Quantification**

Enterolactone was determined from preintervention and postintervention serum samples using the Labmaster Enterolactone Time-Resolved FluoroImmunoAssay (Labmaster, Turku, Finland). Briefly, the assay is conducted as follows: goat anti-rabbit IgG immobilized to the walls of low fluorescence microtiter plate will bind the anti-enterolactone antibody. Europium-labeled enterolactone and sample enterolactone compete for the antibody. Enhancement solution is added, which dissociates the europium ions from the labeled enterolactone into solution to form highly fluorescent chelates with components in the enhancement solution. The fluorescence from the sample is inversely proportional to the concentration of enterolactone in the sample. For the present study, we observed intra-assay coefficients of variation (CV) ranging from 2% to 18% (mean CV, 11.6%) and interassay CVs ranging from 32% to 56% (mean CV, 40%).

### **Estrogen Metabolite Analysis**

The majority of the literature addressing the relationship between estrogen metabolism and either breast cancer or flaxseed interventions has focused on 2OHE1, 16OHE1, and their ratio. Therefore, we limited our analyses to those two metabolites. Urinary 2OHE1 and 16OHE1 excretion was quantified using a competitive inhibition ELISA (Immuna Care Corp., Bethlehem, PA). This ELISA kit was developed to measure metabolite levels as low as 0.625 ng/mL. The assay is reproducible, with CV ranging from 10% to 20% and intraclass correlation coefficients ranging from 80% to 95% in both premenopausal and postmenopausal urines. In the present study, the intra-assay CVs ranged from 1.1% to 12.9% (mean CV, 4.9%) for 2OHE1 and 1.4% to 9.2% (mean CV, 4.6%) for 16OHE1. Interassay CVs ranged from 5.3% to 14.4% (mean CV, 9.5%) for 2OHE1 and 3.2% to 6.7% (mean CV, 4.7%).

### **Dietary Nutrient Intake**

For an assessment of diet, each participant recorded all foods and beverages consumed for three randomly selected days (2 weekdays and 1 weekend day) during the week before the intervention and again during the intervention. Assignment of recording days was made on a purely random assignment basis, although the majority (>75%) of selected days were nonconsecutive. Subjects were instructed on completion



of the food records, and records were reviewed for completeness by study personnel. Mean daily nutrient intakes were calculated using Nutritionist Pro (15) nutrient analysis software, which uses standard nutrient calculation algorithms (grams of food x nutrient content / 100 g, summed across all reported foods) and United States Department of Agriculture food composition data (16).

### Statistical Analyses

All analyses were conducted using SPSS for Windows, version 11.0. All statistical tests were two sided and considered statistically significant at  $P < 0.05$ . Descriptive characteristics of the study participants are presented as means and SDs for continuous variables and number and percentage for categorical variables.

Baseline and postintervention measures of urinary 2OHE1, 16OHE1, 2OHE1/16OHE1, and serum enterolactone were compared with the Wilcoxon signed-rank test. The distributions of each biomarker across genotype were compared with the Kruskal-Wallis test. To assess the combined effect of *CYP1B1* and *COMT*, we computed a new variable that expressed the cumulative number of variant alleles an individual possessed. For example, a participant with the *Val/Val* *COMT* genotype and *Leu/Leu* *CYP1B1* genotype (homozygous common for both genes) would have a value of 0, whereas the *Met/Met* *COMT* genotype and *Val/Val* *CYP1B1* genotype (homozygous variant for both genes) would have a value of 4. The relationship between combined *COMT* and *CYP1B1* alleles on the estrogen metabolites was assessed as described for the individual genes. Finally, to quantify the intervention related changes in metabolites by genotype, we conducted separate linear regressions with each metabolite as the dependent variable and each gene as the independent variable. Regression models were adjusted for age and mutually adjusted for genotype.

### Statistical Power

The current study was designed to have at least 80% power at  $P = 0.05$  to detect a paired *t* test difference in 2OHE1 from preintervention to postintervention of 0.5 ng/mg creatinine. We estimated slightly reduced power (between 60-80%) to observe differences of this magnitude across category of *COMT* and *CYP1B1* genotypes, assuming 25 women per category.

The effect of the flaxseed intervention on urinary estrogen metabolite excretion and serum enterolactone levels is shown in [Fig. 1](#). Participants were compliant with the intervention, as is evidenced by the large increase in serum enterolactone from preintervention to postintervention. Similarly, we observed a statistically significant increase in 2OHE1 (mean change, 6.8 ng/mg creatinine) and consequently the 2OHE1/16OHE1 ratio, related to the intervention. There was a slight increase in 16OHE1, but the difference was not statistically significant.

Baseline and postintervention urinary 2OHE1, 16OHE1, and 2OHE1/16OHE1 by *COMT* and *CYP1B1* genotype are shown in [Fig. 2](#). There was little association between either the preintervention levels or the intervention related changes in the 2OHE1 or 16OHE1 by *COMT* genotype. Although not statistically significant, we observed a slight increase

in 2OHE1/16OHE1 by *COMT* genotype. Urinary excretion of 2OHE1 for women with at least one variant *CYP1B1* allele was borderline significantly higher than that observed for women with the common *CYP1B1* genotype ( $P = 0.06$ , Kruskal-Wallis). This increase in 2OHE1 resulted in significantly higher 2OHE1/16OHE1 levels for women with the variant *CYP1B1* genotypes compared with those with the common genotypes ( $P = 0.04$ , Kruskal-Wallis).

We also examined the joint effect of the two genes on hydroxyestrone excretion ([Fig. 3](#)). In general, urinary 2OHE1 increased with increasing numbers of variant alleles ( $P = 0.03$ ). This trend was not maintained for women homozygous for both genes, but our ability to detect differences in this group of women was limited because of sample size ( $n = 4$ ). However, we observed a statistically significant intervention related increase in the 2OHE1/16OHE1 ratio related to increasing numbers of variant alleles ( $P = 0.009$ , Kruskal-Wallis), although the trend across categories was not linear.

Finally, we conducted separate linear regressions to estimate the quantitative change in hydroxyestrone excretion by *COMT* and *CYP1B1* genotype ([Table 2](#)). We also assessed the potential for confounding by several personal and dietary characteristics, including age, previous hormone use, body mass index, total energy intake, and dietary fiber intake. Only age and genotype were significant in the regression models. After adjustment for age and *CYP1B1* genotype, the associations between *COMT* genotype and 2OHE1 or 16OHE1 were attenuated, although there was a borderline significant slight increase in 2OHE1 with each additional variant *COMT* allele ( $P = 0.06$ ). On the other hand, each additional *CYP1B1* variant was associated with an increase of 2.94 ng/mg creatinine in postintervention 2OHE1 ( $P = 0.03$ ) and a borderline significant increase of 0.34 in 2OHE1/16OHE1 ( $P = 0.05$ ).

Our study found that the effect of exposure to dietary lignans from flaxseed is modified by polymorphisms in two genes associated with estrogen metabolism. Previous studies have clearly shown that consumption of flaxseed, which contains large amounts of the phytoestrogen lignans, can affect the metabolism of estrogen, usually resulting in higher urinary excretion of the 2OHE1 metabolite and subsequently higher 2OHE1/16OHE1 ratios ([11-13](#)). Several epidemiologic studies have associated higher urinary 2OHE1 excretion with lower breast cancer risks ([5](#)); therefore, interventions that could positively affect 2OHE1 excretion have been hypothesized to be potentially useful in reducing breast cancer development. On the other hand, although lignan exposure has been shown to increase 2OHE1, investigations of dietary lignan exposure in association with breast cancer have been inconsistent ([17-26](#)).

Genetic variation is becoming increasingly recognized as an important modifier of the effects of environmental exposures on disease risk ([27](#)). The functional changes produced by polymorphisms in genes responsible for metabolism may partially explain the inconsistencies reported in many studies of diet and disease. Several studies have reported that the effect of phytoestrogen intakes on disease-associated biomarkers may be related to polymorphisms of estrogen metabolizing genes. Using data from the

European Prospective Investigation of Cancer-Norfolk study, Low et al. (28) reported negative associations between urinary soy isoflavone levels and estradiol, primarily among women with the CC genotype for the estrogen receptor gene *ESR1 PvuII* polymorphism and, in another study, reported that the associations between serum and urinary phytoestrogens and plasma androgen concentrations in men differed by *CYP19* genotype (29). To our knowledge, before our study, only two experimental studies investigating phytoestrogen-gene interactions have been published. In the first, 117 healthy postmenopausal women participated in a randomized, double blind, placebo-controlled, crossover trial investigating the effect of isoflavone-enriched cereal bars on inflammatory biomarkers (30). Overall, there was little effect of the intervention on the inflammatory biomarkers, except that differences in vascular cell adhesion molecule-1 response differed by estrogen receptor  $\beta$  genotype. In another study by these authors, isoflavones were found to increase high-density lipoprotein only in women with a variant estrogen receptor  $\beta$  polymorphism. These studies, as well as the current study, highlight the importance of genetic variation in determining response to an exposure, such as diet.

The implications of our current findings are reflected in several recent studies investigating gene-environment interactions in breast cancer. We had reported previously a significantly decreased risk of breast cancer among premenopausal women with at least one A2 allele for *CYP17* and higher dietary lignan intakes (31). Similar interactions between lignans and *CYP17* were reported by Piller et al. (32) who reported a significant inverse relationship between plasma enterolactone levels and breast cancer risk only among women with the A2 allele. Additional interactions have been reported for fruit and vegetable intakes and both catalase and myeloperoxidase gene polymorphisms (33, 34).

The current study included a relatively large number of participants for a metabolic study. Most previous flaxseed interventions included fewer than 50 subjects. We designed our study to have at least 80% power at  $P = 0.05$  to detect a postintervention difference in 2OHE1 of at least 0.5 ng/mg creatinine. In fact, we observed a mean difference of 6.8 ng/mg creatinine, suggesting that the study had more than adequate statistical power to test the stated hypotheses. We recruited a larger sample to increase our power to investigate genetic differences. Again, we observed larger than expected differences in 2OHE1 changes across genotype. Interestingly, we also obtained a slightly higher than expected proportion of women homozygous for the *COMT* variant alleles, although the distribution of this gene remained in Hardy-Weinberg equilibrium. Because we were interested in hormone metabolism, we excluded women taking hormone therapy within 2 months of the intervention. *COMT* is responsible for the methylation and inactivation of estrone, and enzyme activity is greatly reduced for individuals with variant alleles. Decreased inactivation would result in higher levels of bioavailable estrone, which could possibly reduce the need for hormone therapy. Similar interactions have been reported for *CYP17* wherein women with A2 alleles were less likely to be hormone therapy users (35). The current findings reinforce that future studies should consider the potential effect of genetic variation on disease related lifestyle factors.

A potential limitation of this study is the lack of a control group. Although it is possible that intraindividual variability in estrone metabolism may account for our findings, this is not likely. We observed very little difference in baseline metabolite levels across *COMT* or *CYP1B1* genotype ([Fig. 2](#)). On the other hand, postintervention 2OHE1 and 2OHE1/16OHE1 levels clearly increased with increasing numbers of variant alleles for both genes. These findings support a true intervention-related effect by genotype.

Although the intervention was limited (1 week), we observed significant and substantial changes in hormone metabolism supporting an immediate effect of diet on hormone metabolism. Whether these changes persist in the long-term setting remains to be shown. Notwithstanding the short intervention period, the postintervention hydroxyestrone levels in our participants are comparable with other, longer flaxseed interventions supporting an intervention-related effect ([11](#), [12](#)).

In conclusion, our study provides further support that the effect of dietary exposures may be modified by variation in metabolism-related genes. Individual susceptibility to environmental exposures, such as diet, may partially explain the inconsistencies in epidemiologic studies and should provide guidance in developing targeted interventions for reducing chronic disease risks.

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