

Hormone Therapy, Estrogen Metabolism, and Risk of Breast Cancer in the Women's Health Initiative Hormone Therapy Trial

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Abstract

Background: In the Women's Health Initiative Hormone Trials (WHI-HT), breast cancer risk was increased with estrogen plus progestin (E+P) but not with unopposed estrogen (E-alone). We hypothesized that E+P would preferentially metabolize to 16 α -hydroxyestrone (16 α -OHE1) rather than 2-hydroxyestrone (2-OHE1), and that breast cancer risk would be associated with baseline and 1 year changes in estrogen metabolites: positively for 16 α -OHE1 levels and negatively for levels of 2-OHE1 and the 2:16 ratio.

Methods: In a prospective case-control study nested in the WHI-HT, 845 confirmed breast cancer cases were matched to 1,690 controls by age and ethnicity. Using stored serum, 2-OHE1 and 16 α -OHE1 levels were measured by enzyme immunoassay at baseline, and for those randomized to active treatment ($n = 1,259$), at 1 year.

Results: The 1-year increase in 16 α -OHE1 was greater with E+P than E-alone (median 55.5 pg/mL vs. 43.5 pg/mL, $P < 0.001$), but both increased 2-OHE1 by ~ 300 pg/mL. Breast cancer risk was modestly associated with higher baseline levels of 2-OHE1 and the 2:16 ratio, and for estrogen receptor+/progesterone+ cases only, higher baseline 16 α -OHE1 levels. For those randomized to active treatment, breast cancer risk was associated with greater increase in 2-OHE1 and the 2:16 ratio, but associations were not significant.

Conclusions: Although E+P modestly increased 16 α -OHE1 more than E-alone, increase in 16 α -OHE1 was not associated with breast cancer.

Impact: Study results do not explain differences between the WHI E+P and WHI E-alone breast cancer results but metabolism of oral HT, which may explain smaller than expected increase in breast cancer compared with endogenous estrogens. *Cancer Epidemiol Biomarkers Prev*; 21(11); 2022–32. ©2012 AACR.

Introduction

Endogenous estrogen levels are positively associated with postmenopausal breast cancer for nonhormone users

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(1, 2). In the Women's Health Initiative (WHI) randomized placebo-controlled clinical trials, combined estrogen plus progestin (E+P) was associated with increased breast cancer risk [relative risk (RR) of 1.26 (1.00–1.59); 3, 4], but unopposed estrogen (E-alone) was not [RR = 0.77 (0.59–1.01); 5]. Breast cancer risk increases less with E+P postmenopausal hormone therapy (HT) than expected based on the increase in circulating estrogens (6). Furthermore, a recent case-cohort study in the WHI Hormone Trials (WHI-HT) trial showed that 1-year increase in estrogens and sex hormone binding globulin (SHBG) were similar for E+P compared with E-alone, and that progesterone decreased slightly with E+P (6). However, with both E+P and E-alone, bioavailable and free estradiol increased less than total estradiol, related to the 2.5-fold increase in SHBG with either HT regimen (6). A case-cohort study of nonhormone users in the WHI Observational Study (WHI-OS) recently reported that median levels of bioavailable estradiol were significantly higher in invasive breast cancer cases than cohort controls, especially for estrogen receptor (ER+) breast cancer, [adjusted HR 1.86 (1.00,3.45), quartiles 4 vs. 1] (7). Therefore, the difference in the risk of breast cancer between E-alone

(decreased risk) and in E+P (increased risk) in the WHI-HT do not appear to be explained by similar changes in hormone levels for E+P compared with E-alone (6).

Therefore, we hypothesized that differences in estrogen metabolism might explain differences in breast cancer risk for E+P and E-alone. Estrogens (both estradiol and estrone) metabolize along several competing pathways including of 2-hydroxyestrone (2-OHE1), 16 α -hydroxyestrone (16 α -OHE1), and 4-hydroxyestrone (4-OHE1) that may vary in estrogenic and genotoxic potential (8–10). Most studies of estrogen metabolites and breast cancer have focused on 2-OHE1, 16 α -OHE1, and their ratio (2:16). 16 α -OHE1 has strong estrogenic effects related to cell proliferation, and has a higher binding affinity for the ER than 2-OHE1, which has limited proliferation effects and possibly even antiestrogenic effects (8, 9, 11). Several studies have reported that breast cancer risk was associated with higher levels of 16 α -OHE1 (12–16), and lower levels of the 2:16 ratio (16–21). Other studies have found no associations, or a positive association of 2-OHE1 or the 2:16 ratio with breast cancer risk (22–25). Most studies have excluded hormone users. In a previous case-cohort study nested in the WHI-OS, HT users had substantially higher levels of 2-OHE1 and 16 α -OHE1 than nonusers, and combined E+P users had slightly higher 16 α -OHE1 levels than E-alone users (26). However, no prospective studies have assessed the effect of HT regimen on changes in estrogen metabolism and subsequent breast cancer risk within a randomized clinical trial of HT.

Therefore, we conducted a prospective case-control study of 2,535 postmenopausal women nested within the randomized WHI-HTs to test 3 hypotheses: (i) incident breast cancer is associated with higher baseline levels of 16 α -OHE1 and lower levels of the 2:16 ratio, (ii) compared with E-alone, E+P is associated with a greater increase in 16 α -OHE1 levels, and a smaller increase in levels of 2-OHE1 and the 2:16 ratio 1 year after randomization, and (iii) for women randomized to active HT, incident breast cancer is associated with a larger 1 year increase in 16 α -OHE1 levels, and a smaller 1 year increase in the 2:16 ratio. Finally, we hypothesized that associations are stronger for cases that are positive for ER+ and progesterone receptor (PR+).

Methods

Overview of the WHI-HT

Between 1993 and 1998, the WHI recruited 27,347 postmenopausal women aged 50 to 79 years at 40 centers in the United States into 2 randomized clinical trials: E+P and E-alone. Details of the methods and results of these trials have been published (27, 28). Briefly, the E+P trial randomized 16,608 postmenopausal women to conjugated equine estrogen (CEE; 0.625 mg/day) plus medroxyprogesterone acetate (2.5 mg/day) or placebo. The E-alone trial randomized 10,738 women with a prior hysterectomy to CEE (0.625 mg/day) or placebo. Women using hormones at the screening visit underwent a 3-month wash-

out period before study randomization. The protocol and consent forms were approved by the institutional review boards of the participating institutions, and all women provided written informed consent. Follow-up contacts by telephone or clinic visit occurred every 6 months with clinic visits required annually. Covariates were collected via questionnaires or examinations and included age, ethnicity, body mass index (BMI), waist circumference, reproductive history, alcohol intake, smoking status, physical activity, prior HT use, family history of breast cancer, and oophorectomy status. BMI and waist circumference were measured at clinic visits.

Outcomes

Baseline and annual mammograms were required for women in the WHI-HT to identify potential breast cancer cases. Details of breast cancer endpoints were abstracted and confirmed by review of medical records and pathology reports (available on 98.2% of participants) by physician adjudicators at local clinics masked to treatment assignments. ER/PR tumor status was obtained by pathology reports. Only ER+, PR+ and ER-, PR- status was specifically evaluated because of limited sample size in other groups.

Study design and participants

The current study selected breast cancer cases and controls from both the E+P and E-alone WHI-HT, to compare changes in metabolites and their associations with subsequent breast cancer risk according to HT regimen (E+P trial vs. E-alone trial). Estrogen metabolites were measured on breast cancer cases and controls at baseline, and for participants randomized to active treatment, metabolites were also measured at 1-year postrandomization. Cases ($N = 845$) include all women diagnosed with breast cancer after study enrollment through August 2006 who met the following study eligibility criteria: diagnosis of breast cancer during WHI follow-up, breast cancer diagnosis that occurred at least 5 years after menopause, no diagnosis of other cancers during follow-up, no use of medications that could affect estrogen levels (i.e., antimycobacterial, imidazole-related antifungals, androgen-anabolic steroids, adrenal steroid inhibitors, hormone receptor modulators, histamine H₂ receptor antagonists, cyclosporine analogs, and herbal estrogens), self-reported race or ethnicity other than "unknown," and adequate serum available at baseline and year 1. To preserve the temporal relationship between exposure and incident breast cancer, the study design called for the exclusion of breast cancer cases with diagnosis ≤ 1 year postrandomization $n = 48$ at baseline, reducing the total number of cases to $n = 797$ with baseline estrogen metabolites ($n = 627$ invasive).

Potential controls were selected from participants who were free of breast cancer diagnosis through August 2006 and met the following criteria: postmenopausal for at least 5 years, no use of medications that could affect estrogen levels, self-reported race or ethnicity other than

"unknown," and with adequate serum available. For each case, 2 controls ($N = 1,690$) were selected, frequency-matched for age and ethnicity. Approximately half of the controls were selected from existing WHI case-control studies of breast cancer and hip fracture that include measurements of both baseline and 1-year follow-up sex steroid hormone levels, with the remainder ($n = 827$) selected from the remaining risk set as of August 2006. Among women randomized to active treatment ($n = 1,259$), 21 breast cancer cases were excluded for occurring ≤ 1 year follow-up, and 24 participants did not have 1 year samples available. Therefore, 1 year change in estrogen metabolites were available on $n = 1,214$, including $n = 401$ breast cancer cases.

Laboratory analyses

At baseline and 1 year postrandomization blood was drawn after a minimum 10 hours of fasting. Serum and plasma samples were shipped to a central repository and stored at -70°C and not previously thawed (29). For the current study, stored serum from baseline (all participants) and 1 year (participants randomized to active HT) was shipped on dry ice to Immuna Care, Inc., for measurement of estrogen metabolites under the direction of Dr. Thomas Klug. Using serum (150 μL per assay) $16\alpha\text{-OHE1}$ and 2-OHE1 were measured by immunoassay (ESTRAMET 2/16 kits, Immuna Care). The limits of detection are ~ 10 pg/mL for the $16\alpha\text{-OHE1}$ assay and ~ 20 pg/mL for the 2-OHE1 assay. Postmenopausal women screened for WHI provided 210 additional samples that were split into duplicates for quality control (QC) testing. Vials were coded to provide no participant information to study investigators or laboratory staff. Cases, controls, intervention groups, and QC samples were randomly distributed throughout each batch of specimens, and to minimize potential differences between cases and controls that might arise from batching on a plate, each plate batch contained a ratio of 1 breast cancer case to 2 controls (masked). Coefficients of variation (CV) and intraclass correlation coefficients (ICC) were calculated using the 210 pairs of split QC samples.

Statistical analysis

The characteristics of study participants according to HT arm and case/control status were compared using t tests and Wilcoxon tests for normally and nonnormally distributed continuous variables, respectively, and χ^2 and Fisher exact tests for categorical variables (Table 1). Associations between the estrogen metabolites and breast cancer risk were assessed using conditional logistic regression models to account for the primary matching variables (age, ethnicity). Quartiles were defined based on the controls' estrogen metabolite levels. For continuous models, metabolite values were log-transformed, and 1-year changes in metabolites were increased by a constant to create all positive values and then log transformed. Differences in by trial and active versus placebo assignment were tested by including an interaction term in the

logistic regression models. Sensitivity analyses included adjusting for BMI and treatment assignment, and for baseline levels in models assessing change in metabolites, and also stratifying by ER+/PR+ status, and restricting to invasive cancer only ($n = 697$). All P values are 2-sided. SAS 9.2 (SAS Institute) was used for analyses.

Results

The overall CVs for 2-OHE1 , $16\alpha\text{-OHE1}$, and the 2:16 ratio were 26.4%, 15.2%, and 28.0%, respectively, with ICCs of 0.94, 0.86, and 0.90, respectively.

Spearman correlations between 2-OHE1 and $16\alpha\text{-OHE1}$ were $\rho = 0.18$, $P < 0.0001$ at baseline, not on HT, and $\rho = 0.24$, $P < 0.0001$ at 1 year for women randomized to HT.

Table 1 presents baseline characteristics of the study population, stratified by E+P versus E-alone trials and breast cancer case-control status. Study participants from the E-alone arm differed from those from the E+P arm, (e.g., $\sim 60\%$ of study participants had partial or total oophorectomy in addition to their eligibility requirement of hysterectomy, compared with $\sim 4\%$ in the E+P arm). Women in the E-alone arm were also more likely to be non-White. These characteristics are similar to the overall sample of women in the HT clinical trials. For both trials, breast cancer cases were heavier, had larger waist circumference, and were more likely to have a family history of breast cancer than controls ($P < 0.05$). Mean (SD) time to breast cancer was 4.6 (2.4) years, with similar overall follow-up time for cases [9.1 (1.6)] and controls [9.0 (1.5)] years, respectively.

Relationship of baseline estrogen metabolite levels to incident breast cancer

The median levels of serum $16\alpha\text{-OHE1}$, 2-OHE1 , and the 2:16 ratio at baseline were not significantly different between women who developed breast cancer during follow-up compared with controls in both E-alone and E+P arms (Table 2). In logistic models that accounted for age and ethnicity matching, breast cancer risk was significantly higher for quartile 4 versus 1 of baseline levels of both 2-OHE1 and the 2:16 ratio, but not associated with baseline levels of $16\alpha\text{-OHE1}$ (Table 3). Higher baseline levels of 2-OHE1 and the 2:16 ratio (but not $16\alpha\text{-OHE1}$) were associated with breast cancer risk in models with the estrogen metabolites as continuous variables. These associations persisted after further adjustment for BMI, and for treatment assignment. The association of higher baseline $16\alpha\text{-OHE1}$ with increased odds of breast cancer was slightly stronger (i) if restricted to invasive cancer only, or (ii) for women randomized to active treatment in the E-alone trial, but associations and interactions by treatment assignment, were not statistically significant (data not shown).

Baseline estrogen metabolite-breast cancer risk stratified by ER+/PR+ versus ER-/PR- status

Associations between baseline estrogen metabolites and breast cancer risk were further evaluated after

Table 1. Baseline demographics of study participants by WHI-HT

Variable	E-alone trial			E+P trial		
	Breast cancer n = 269	Control n = 639	P	Breast cancer n = 528	Control n = 1051	P
Oophorectomy status—a part, one, or both ovaries removed, N, %	145, 54.31	375, 58.6	0.06 ^c	21, 4.0	41, 3.6	0.73 ^d
Age, years	63.9 (0.43)	65.3 (0.29)	0.01 ^a	64.1 (0.29)	64.6 (0.22)	0.16 ^a
BMI, kg/m ²	31.3 (0.37)	29.2 (0.22)	<0.0001 ^a	29.3 (0.25)	28.4 (0.18)	0.001 ^a
Waist circumference, cm	95.1 (0.93)	90.2 (0.51)	<0.0001 ^a	90.3 (0.57)	87.8 (0.41)	0.0005 ^a
Family history breast cancer, N, %	64, 46.7	101, 37.8	0.09 ^c	110, 45.3	167, 36.0	0.02 ^c
Active treatment assignment, N, %	115, 42.8	323, 50.6	0.03 ^c	310, 58.7	490, 46.6	<0.0001 ^c
Age at first birth, N, %						
No births	8, 3.7	11, 2.0		14, 3.3	33, 3.8	
<20 years old	59, 27.4	128, 23.8		66, 15.8	120, 13.7	
20–<30 years old	139, 64.7	361, 67.1		286, 68.3	640, 72.9	
30 or more years old	9, 4.2	38, 7.1	0.19 ^c	53, 12.7	85, 9.7	0.24 ^c
Ethnicity, N, %						
American Indian/Alaskan Native	2, 0.7	3, 0.5		1, 0.2	2, 0.2	
Asian/Pacific Islander	5, 1.9	10, 1.6		17, 3.2	23, 2.2	
Black/African American	39, 14.5	77, 12.1		38, 7.2	60, 5.7	
Hispanic/Latino	10, 3.7	23, 3.6		15, 2.8	26, 2.5	
White	213, 79.2	525, 82.2		457, 86.6	940, 89.4	
Other	0, 0	1, 0.2	0.85 ^d	0, 0	0, 0	0.45 ^d
Alcohol intake, N, %						
Nondrinker	46, 17.4	81, 12.8		56, 10.8	112, 10.8	
Past drinker	63, 23.9	132, 20.8		91, 17.5	168, 16.2	
<1 drink/month	33, 12.5	98, 15.5		72, 13.9	164, 15.8	
<1 drink/week	47, 17.8	151, 23.8		103, 19.9	192, 18.5	
1–6 drinks/week	46, 17.4	122, 19.2		128, 24.7	297, 28.6	
7 or more drinks/week	29, 11.0	50, 7.9	0.07 ^c	69, 13.3	107, 10.3	0.28 ^c
Current smoker, N, %	20, 7.5	55, 8.7	0.79 ^c	44, 8.5	93, 8.9	0.04 ^c
Parity, children, N, %						
Never pregnant	21, 7.8	52, 8.2		44, 8.4	75, 7.2	
Never had term	8, 3.0	11, 1.7		14, 2.7	33, 3.2	
Pregnancy						
1	16, 6.0	39, 6.2		48, 9.2	68, 6.5	
2	51, 19.0	114, 18.0		115, 22.0	210, 20.1	
3	62, 23.1	143, 22.6		131, 25.1	252, 24.1	
4	59, 22.0	123, 19.4		95, 18.2	203, 19.4	
5 or more	51, 19.0	152, 24.0	0.64 ^c	76, 14.5	206, 19.7	0.10 ^c
Recreational physical activity, minutes/week (SEM)	132.9 (9.6)	160.3 (7.30)	0.07 ^b	154.6 (7.56)	171.6 (5.56)	0.08 ^b
Prior E+P use, years (SEM)	2.7 (0.69)	5.2 (1.06)	0.57 ^b	4.3 (0.55)	3.9 (0.35)	0.70 ^b
Previous hormone use at baseline, N, %						
Never used	144, 53.5	331, 51.9		401, 76.0	782, 74.4	
<5 years ago	24, 8.9	73, 11.4		38, 7.2	72, 6.9	
5–<10 years ago	17, 6.3	29, 4.6		24, 4.6	41, 3.9	
10 or more years ago	49, 18.2	125, 19.6		41, 7.8	94, 8.9	
Current user	35, 13.0	80, 12.5	0.62 ^c	24, 4.6	62, 5.9	0.68 ^c

NOTE: P values reflect comparisons between cases and controls within each HT trial arm.

SEM, standard error of the mean.

^at test.^bWilcoxon test.^c χ^2 test.^dFisher exact test.

Table 2. Median baseline levels of 16 α -OHE1, 2-OHE1, and the 2:16 ratio by treatment assignment and breast cancer case/control status among study participants from the WHI-HT

	E-alone		E-alone Placebo		E+P		E+P Placebo	
N cases/controls	115/323		154/316		310/490		218/561	
16α-OHE1 (pg/mL)	Median	P	Median	P	Median	P	Median	P
Cases	349.8	0.71	345.3	0.62	338.5	0.96	345.5	0.38
Controls	353.5		351.4		335.8		339.8	
2-OHE1 (pg/mL)								
Cases	99.0	0.22	104.3	0.52	102.9	0.48	106.0	0.26
Controls	93.5		102.5		99.8		100.8	
2:16 ratio								
Cases	0.30	0.38	0.32	0.28	0.31	0.51	0.31	0.77
Controls	0.29		0.30		0.31		0.30	

NOTE: P value from Wilcoxon test.

Table 3. Association of baseline estrogen metabolite levels with breast cancer risk among study participants in the WHI-HT

	N cases/controls	Median (IQR)	OR (95% CI)	P
16 α -OHE1 (pg/mL)				
Quartile 1 ^a	177/423	239.0 (212.1, 257.3)	1.00 (reference)	0.21 ^d
Quartile 2 ^a	219/420	309.5 (291.5, 327)	1.19 (0.94–1.52)	
Quartile 3 ^a	217/425	377.5 (359, 397.5)	1.19 (0.93–1.51)	
Quartile 4 ^a	184/422	489.5 (450, 564.5)	0.98 (0.76–1.26)	
Continuous ^a			0.96 (0.73–1.25)	0.76
Continuous ^b			0.89 (0.68–1.17)	0.40
Continuous ^c			0.91 (0.70–1.20)	0.52
2-OHE1 (pg/mL)				
Quartile 1 ^a	179/428	60 (49.5, 69)	1.00 (reference)	0.11 ^d
Quartile 2 ^a	194/420	88 (81.7, 93.5)	1.15 (0.90–1.47)	
Quartile 3 ^a	195/419	116 (107.5, 124.5)	1.15 (0.90–1.47)	
Quartile 4 ^a	229/423	173.3 (151.6, 227.8)	1.35 (1.06–1.71)	
Continuous ^a			1.23 (1.04–1.45)	0.01
Continuous ^b			1.20 (1.02–1.42)	0.03
Continuous ^c			1.18 (0.99–1.40)	0.05
2:16 ratio				
Quartile 1 ^a	184/422	0.17 (0.13, 0.19)	1.00 (reference)	0.20 ^d
Quartile 2 ^a	186/421	0.26 (0.24, 0.28)	1.04 (0.81–1.33)	
Quartile 3 ^a	203/422	0.35 (0.32, 0.38)	1.13 (0.89–1.44)	
Quartile 4 ^a	220/420	0.53 (0.47, 0.68)	1.28 (1.00–1.63)	
Continuous ^a			1.21 (1.04–1.41)	0.02
Continuous ^b			1.21 (1.04–1.42)	0.02
Continuous ^c			1.19 (1.01–1.39)	0.03

NOTE: All models include matching factors: age ethnicity. Estrogen metabolite levels are log-transformed for continuous models.

^aUnadjusted.^bAdjusted for BMI.^cAdjusted for BMI and treatment arm.^dP value for Wald χ^2 test for differences among quartiles.

Table 4. Association of baseline estrogen metabolite levels with breast cancer risk by ER/PR status among women in the WHI-HT

	ER/PR status								
	ER+/PR+				ER-/PR-				
	N case/ control	Median (IQR)	OR (95% CI)	P	N case/ control	Median (IQR)	OR (95% CI)	P	
16α-OHE1 (pg/mL)					16α-OHE1 (pg/mL)				
Quartile 1 ^a	78/423	239.5 (212.3–257.5)	1.00 (reference)	0.09 ^d	Quartile 1 ^a	18/423	238.8 (211.0, 257.0)	1.00 (reference)	0.86 ^d
Quartile 2 ^a	109/420	309.5 (291.0–327.0)	1.41 (1.02–1.95)		Quartile 2 ^a	23/420	309.5 (291.0, 326.5)	1.23 (0.65–2.36)	
Quartile 3 ^a	115/425	378.0 (358.0–398.0)	1.48 (1.07–2.04)		Quartile 3 ^a	20/425	377.5 (359.0, 398.0)	1.09 (0.56–2.12)	
Quartile 4 ^a	97/422	492.0 (450.5–570.0)	1.23 (0.88–1.72)		Quartile 4 ^a	17/422	491.5 (450.5, 569.0)	0.93 (0.46–1.88)	
Continuous ^a			1.23 (0.87–1.74)	0.24	Continuous ^a		0.75 (0.36–1.59)	0.46	
Continuous ^b			1.12 (0.79–1.59)	0.53	Continuous ^b		0.75 (0.35–1.58)	0.44	
Continuous ^c			1.17 (0.82–1.67)	0.40	Continuous ^c		0.77 (0.36–1.64)	0.50	
2-OHE1 (pg/mL)					2-OHE1 (pg/mL)				
Quartile 1 ^a	86/428	60.0 (49.0, 69.0)	1.00 (reference)	0.12 ^d	Quartile 1 ^a	17/428	59.5 (48.5, 69.0)	1.00 (reference)	0.35 ^d
Quartile 2 ^a	94/420	88.5 (82.0, 93.5)	1.17 (0.84–1.62)		Quartile 2 ^a	25/420	88.5 (82.0, 93.5)	1.64 (0.84–3.20)	
Quartile 3 ^a	105/419	116.0 (107.5, 125.0)	1.35 (0.97–1.86)		Quartile 3 ^a	15/419	115.5 (107.3, 125.0)	0.97 (0.47–2.01)	
Quartile 4 ^a	114/423	174.5 (151.5, 227.0)	1.44 (1.05–1.98)		Quartile 4 ^a	21/423	174.0 (153.0, 228.5)	1.35 (0.68–2.67)	
Continuous ^a			1.35 (1.09–1.68)	0.006	Continuous ^a		1.15 (0.75–1.76)	0.54	
Continuous ^b			1.34 (1.08–1.66)	0.008	Continuous ^b		1.12 (0.73–1.73)	0.60	
Continuous ^c			1.31 (1.05–1.63)	0.02	Continuous ^c		1.13 (0.72–1.75)	0.60	
2:16 ratio					2:16 ratio				
Quartile 1 ^a	87/420	0.17 (0.13–0.19)	1.00 (reference)	0.48 ^d	Quartile 1 ^a	24/420	0.17 (0.13–0.19)	1.00 (reference)	0.31 ^d
Quartile 2 ^a	99/422	0.26 (0.24–0.28)	1.13 (0.82–1.56)		Quartile 2 ^a	11/422	0.26 (0.24–0.28)	0.57 (0.27–1.20)	
Quartile 3 ^a	107/422	0.35 (0.32–0.38)	1.26 (0.92–1.74)		Quartile 3 ^a	19/422	0.35 (0.33–0.38)	0.80 (0.42–1.51)	
Quartile 4 ^a	102/421	0.53 (0.47–0.67)	1.24 (0.89–1.71)		Quartile 4 ^a	24/421	0.53 (0.47–0.67)	1.10 (0.60–2.03)	
Continuous ^a			1.21 (0.99–1.49)	0.06	Continuous ^a		1.22 (0.82–1.82)	0.33	
Continuous ^b			1.24 (1.01–1.53) ^e	0.04	Continuous ^b		1.20 (0.81–1.79)	0.37	
Continuous ^c			1.20 (0.98–1.49)	0.08	Continuous ^c		1.20 (0.79–1.81)	0.39	

NOTE: All models account for age and ethnicity matching. Estrogen metabolite levels are log-transformed for continuous models.

^aUnadjusted.

^bAdjusted for BMI.

^cAdjusted for BMI and treatment arm.

^dP value for Wald χ^2 test for differences among quartiles.

stratifying cases by ER and PR tumor status (ER+/PR+ vs. ER-/PR-; Table 4). Associations were somewhat stronger for (ER+/PR+) cases and weaker for ER-/PR- cases. For ER+/PR+ cases, 16 α OHE-1 was positively associated with breast cancer risk. Stratified by trial and treatment group, positive associations of 16 α OHE-1 with breast cancer were stronger for women randomized to placebo in both trials, although interactions with treatment assignment were not statistically significant (data not shown). Among ER+/PR+ cases, higher baseline 2-OHE1 was also associated with increased breast cancer risk ($P < 0.05$) for the highest versus lowest quartile, and also in continuous models adjusted for BMI and treatment assignment. Compared with the overall results, associations with the 2:16 ratio were slightly attenuated for ER+/PR+ cases and seemed absent for ER-/PR- cases. Overall results were similar when restricted to invasive cancer only, or when cases were stratified by median follow-up time (data not shown).

Change in estrogen metabolites following 1-year active HT treatment

Among those randomized to active hormone treatment ($n = 1,259$), estrogen metabolites were also measured in serum from the 1 year follow-up after starting HT (Table 5). As hypothesized, the increase in 16 α -OHE1 was greater with E+P versus E-alone (median increase 58 pg/mL vs. 44 pg/mL, $P = 0.0002$). However, the increase in 16 α -OHE1 was dwarfed by the approximately 4-fold increase in 2-OHE1 (median increase = ~300 pg/mL) that occurred with both E-alone and E+P. This is consistent with previous studies. Following 1 year of HT, levels of 2-OHE1 were similar to levels of 16 α -OHE1, resulting in a 2:16 ratio = 1.0 for both treatment arms.

Associations of 1 year change in estrogen metabolites to incident breast cancer

Overall, none of the 1-year changes in estrogen metabolites showed a clear pattern of association with breast cancer risk, evaluated in quartiles or per (log-

Table 5. Median (IQR) baseline, 1-year, and 1-year change in 16 α -OHE1, 2-OHE1, and the 2:16 ratio among study participants randomized to active treatment in the E-alone and E+P WHI-HT

N	E-alone 431	E+P 783	P^a
16α-OHE1 (pg/mL)			
Baseline	352.0 (277.5, 434.5)	337.5 (272.0, 412.0)	0.045
1-year	393.5 (322.5, 489.5)	398.0 (327.5, 495.5)	0.49
Change	43.8 (-4.5, 93.5)	58.0 (12.0, 122.5)	0.0002
2-OHE1 (pg/mL)			
Baseline	96.0 (74.5, 134.7)	101.0 (77.0, 137.5)	0.17
1-year	390.5 (245.0, 537.5)	410.8 (247.3, 573.0)	0.14
Change	273.3 (142.0, 423.0)	305.0 (151.5, 457.5)	0.17
2:16 Ratio			
Baseline	0.30 (0.21, 0.40)	0.31 (0.23, 0.43)	0.02
1-year	1.00 (0.60, 1.40)	1.00 (0.57, 1.42)	0.53
Change	0.66 (0.28, 1.06)	0.66 (0.27, 1.08)	0.94

^aWilcoxon test.

transformed) unit change (Table 6). For E-alone, breast cancer risk was associated with smaller increase in 16 α -OHE-1, 2-OHE-1, and the 2:16 ratio, although ORs were generally not significant. ORs for a 1 ln-unit increase in estrogen metabolites generally showed a similar pattern that was relatively unaffected by adjusting for BMI. However, greater 1 year increase in both metabolites and the 2:16 ratio was associated with lower odds of breast cancer in models that adjusted for baseline levels, particularly for the E-alone arm. However, confidence intervals were wide and results were not significant. Results were similar if restricted to ER+/PR+ total or ER+/PR+ invasive cancer only (not shown). However, when stratified by median follow-up time (Supplementary Table S1), unadjusted ORs for quartiles of 1 year change in both 2-OHE-1 and the 2:16 ratio showed a stronger protective association with breast cancer that occurred before median follow-up time compared with cases that occurred postmedian follow-up time, or overall.

Discussion

This is the first study to evaluate estrogen metabolites in relation to incident breast cancer within the context of a randomized clinical trial of HT. Incident breast cancer risk was modestly associated with higher baseline levels of 16 α -OHE-1 among ER+/PR+ cases, but was also associated with higher baseline levels of 2-OHE-1 and the 2:16 ratio. For those randomized to active treatment, the 1-year increase in 16 α -OHE1 was greater for E+P than for E-alone, but incident breast cancer was not associated with greater 1-year increases in 16 α -OHE1 for either the E+P or the E-alone trial. Finally, greater 1-year increase in 2-OHE-1 or the 2:16 ratio were associated with lower odds of breast cancer but were generally not significant, although associations were stronger in the E-alone trial or for cases occurring before median follow-up time.

Although E+P treatment increased serum levels of 16 α -OHE1 more than E-alone, this difference does not appear to explain the increased breast cancer risk with active treatment in the WHI E+P (E+P) trial versus the decreased breast cancer risk in the WHI E-alone (E-alone) clinical trial. A noteworthy finding is that randomization to active treatment in either trial (E+P or E-alone) similarly increased 2-OHE1 ~4-fold, raising 2-OHE-1 to levels similar to 16 α -OHE1, and changing the 2:16 ratio from ~0.35 at baseline to 1.0 at 1 year. The substantial increase in 2-OHE-1 compared with 16-OHE1 is consistent with our previous reports from nonrandomized HT (26, 30) and the Woman On the Move through Activity and Nutrition (WOMAN) study, a randomized trial of weight loss among women on HT (31). At baseline, compared with women not on HT, women on HT had 2-OHE1 levels 5 times higher but similar 16 α -OHE1 levels. The WHI-HT trial results were published shortly after randomization in the WOMAN study, and most of the participants stopped their HT, mostly E+P. There was a very substantial decline in levels of 2-OHE1 between baseline and the 18-month follow-up visit for women who had stopped their HT. In contrast, for women not using HT at either visit, correlations between 2-OHE1 and 16 α -OHE1 levels measured ~18 months apart were 0.54 and 0.85, respectively.

In the current study, the association of metabolites and risk of breast cancer were somewhat stronger among ER+/PR+ cases than ER-/PR- cases. For example, breast cancer risk was associated with higher levels of 16 α -OHE1 among ER+/PR+ cases (not significant). ER+/PR+ breast cancer also showed stronger associations with higher baseline 2-OHE1 levels, but there were $n = 423$ ER+/PR+ cases and only $n = 67$ ER-/PR- cases, so we had reduced power to detect a significant risk for ER-/PR- cases.

Table 6. Association between 1-year HT-induced change in estrogen metabolites and breast cancer risk among study participants randomized to active treatment, stratified by WHI-HT (E-alone vs. E+P)

		<i>N</i> cases/controls ^a	Median (IQR)	OR (95% CI)	<i>P</i>
Change in 16 α -OHE1 (pg/mL)					
E-alone	Quartile 1 ^b	32/80	-36.0 (-64.5, -14.0)	1.00 (reference)	0.77 ^e
	Quartile 2 ^b	29/82	28.0 (8.0, 36.0)	0.76 (0.39-1.48)	
	Quartile 3 ^b	22/81	66.0 (55.0, 81.0)	0.71 (0.36-1.40)	
	Quartile 4 ^b	24/80	152.8 (117.8, 204.3)	0.84 (0.43-1.65)	
	Continuous ^b			0.34 (0.008-13.8)	0.57
	Continuous ^c			0.42 (0.01-17.66)	0.65
E+P	Quartile 1 ^b	74/122	-20.0 (-45.5, -2.5)	1.00 (reference)	0.45 ^e
	Quartile 2 ^b	69/123	32.8 (23.0, 45.1)	0.91 (0.59-1.39)	
	Quartile 3 ^b	85/125	87.0 (73.0, 104.4)	1.20 (0.79-1.82)	
	Quartile 4 ^b	65/120	185.0 (149.5, 240.0)	0.88 (0.57-1.35)	
	Continuous ^b			0.39 (0.07-2.08)	0.27
	Continuous ^c			0.48 (0.09-2.40)	0.37
Change in 2-OHE1 (pg/mL)					
E-alone	Quartile 1 ^b	32/80	18.5 (-8.6, 93.8)	1.00 (reference)	0.52 ^e
	Quartile 2 ^b	29/81	220.1 (181.5, 252.0)	0.81 (0.42-1.56)	
	Quartile 3 ^b	27/81	344.8 (313.7, 389.5)	0.67 (0.34-1.35)	
	Quartile 4 ^b	20/81	606.0 (503.5, 725.5)	0.61 (0.30-1.22)	
	Continuous ^b			0.69 (0.18-2.64)	0.58
	Continuous ^c			0.70 (0.18-2.77)	0.62
E+P	Quartile 1 ^b	69/122	17.0 (-10.7, 85.5)	1.00 (reference)	0.71 ^e
	Quartile 2 ^b	85/123	228.0 (182.3, 272.0)	1.15 (0.75-1.77)	
	Quartile 3 ^b	71/121	372.0 (340.0, 415.8)	1.06 (0.68-1.64)	
	Quartile 4 ^b	68/123 ^f	582.5 (506.0, 738.7)	0.90 (0.58-1.40)	
	Continuous ^b			0.88 (0.50-1.54)	0.65
	Continuous ^c			1.02 (0.57-1.84)	0.94
Change in 2:16 ratio					
E-alone	Quartile 1 ^b	32/81	0.08 (-0.02, 0.17)	1.00 (reference)	0.07 ^e
	Quartile 2 ^b	34/80	0.49 (0.40, 0.59)	0.85 (0.44-1.64)	
	Quartile 3 ^b	18/81	0.87 (0.77, 0.96)	0.40 (0.19-0.82)	
	Quartile 4 ^b	23/81	1.38 (1.20, 1.70)	0.63 (0.32-1.26)	
	Continuous ^b			0.57 (0.08-3.90)	0.57
	Continuous ^c			0.59 (0.08-4.17)	0.59
E+P	Quartile 1 ^b	71/123	0.04 (-0.03-0.14)	1.00 (reference)	0.99 ^e
	Quartile 2 ^b	73/122	0.46 (0.37-0.54)	1.06 (0.69-1.63)	
	Quartile 3 ^b	73/122	0.83 (0.75-0.95)	1.03 (0.67-1.59)	
	Quartile 4 ^b	75/122	1.38 (1.21-1.74)	1.04 (0.67-1.61)	
	Continuous ^b			1.01 (0.36-2.86)	0.99
	Continuous ^c			1.41 (0.47-4.23)	0.54
	Continuous ^d			0.60 (0.11, 3.38)	0.56

NOTE: 1-year changes assessed only among those assigned to active treatment, not those assigned to placebo. All models account for age and ethnicity matching. For continuous models, change in estrogen metabolite levels are log-transformed (after adding a constant).

^a*N*'s differ from total because of missing values: 16-OHE-1 (1 case and 1 control), 2-OHE-1 (1 control), and 2:16 ratio (2 cases, 1 control).

^bUnadjusted.

^cAdjusted for BMI.

^dAdjusted for ln-transformed baseline levels.

^e*P* value for Wald χ^2 test for differences among quartiles.

Several studies have reported results similar to our baseline estrogen metabolite associations. A large nested case-control study of postmenopausal breast cancer ($n = 426$ cases and 426 controls) with urinary 2-OHE and 16 α -OHE1 also found a positive association of 2-OHE with ER+ breast cancer, but among HT users only, with no associations among non-HT users or ER- cases (24). Among postmenopausal non-HT users, the Nurses' Health Study plasma levels of 2-OHE1 and the 2:16 ratio were positively associated with breast cancer, but only for ER-/PR- cases (32). Studies that did not stratify by ER status have also reported a positive association of the 2:16 ratio with incident breast cancer for premenopausal women (25) and postmenopausal women (18). Among postmenopausal non-HT users, Cauley and colleagues found a nonsignificant increased risk with increasing quartile of both 2-OHE1 and 16 α -OHE1 but not with the 2:16 ratio (23). The analyses did not stratify by ER status.

Our results are also in agreement with those of the Prostate, Lung, Colon and Ovary (PLCO) screening trial, which recently used new technologies to evaluate multiple estrogen metabolites in blood and urine (10). The PLCO longitudinal study included 277 postmenopausal incident breast cancers and 433 controls, none of whom were using HT, and also found that incident breast cancer was associated with higher levels of serum 2-OHE1 and 16 α -OHE1 (not statistically significant) as well as higher levels of total estrogens and unconjugated estradiol (33). However, in the PLCO study, the ratio of the 2-OHE1 pathway to total estrogens was inversely related to breast cancer risk, as was the ratio of the entire 2-hydroxylation pathway to the 16-hydroxylation pathway, consistent with the current study's original hypothesis that preferential metabolism to the 2-hydroxylation pathway is associated with lower breast cancer risk. In addition, both 4-OHE-1 and the ratio of 4-hydroxy pathway catechols to methylated catechols were positively related to breast cancer risk (33). These results from the PLCO study suggest that the evaluation of estrogen metabolites may need to be done in the context of total estrogen or unconjugated estrogen levels, and may explain, in part, the negative results in this study and previous studies.

Our results showing that in among women randomized to HT, 2-OHE1 increases much more than 16 α -OHE1 suggests that differences in metabolism of exogenous estrogens may be a partial explanation for the apparent smaller increased risk of breast cancer with oral HT, compared with the more substantial increase in breast cancer associated with higher levels of endogenous estrone and estradiol (1). It is possible that other estrogen metabolites, i.e., 4-OH pathway, not measured in this study could contribute to an increased or decreased risk of breast cancer. Some of the metabolites resulting from HT could be acting as a selective estrogen receptor modulator, decreasing both breast cancer risk and osteoporotic fracture with small effect on coronary heart disease. Another possible contributor to smaller than expected HT-related increased breast cancer risk is that although

total estradiol increases substantially with both E-alone and E+P, free or bioavailable estradiol increases only slightly because of the concomitant increase in SHBG. Oral estrogen therapy clearly has estrogenic effects on bone, uterus, and other tissues. It is very possible that the balance of the effects of the specific metabolites of oral E-alone and E+P, both estrogenic and antiestrogenic effects, the effects of oral estrogens by their first pass from gut to liver, i.e., increased SHBG and other proteins, and host susceptibility (genetics), play a key role in the specific health effects of oral E-alone or E+P therapy. The WHI is currently measuring unconjugated estradiol, estrone, progesterone, testosterone, and SHBG in the HT arm at baseline and 1 year. Further evaluation of estrogen metabolites, especially in relation to tumor characteristics, may provide a better rationale for effects of oral E-alone or E+P on breast cancer risks. Most important, it may be possible to individualize therapy based on both risk and metabolism of hormones.

A possible limitation of the study is the measurement of the metabolites in serum, whereas many previous studies have used plasma or urine specimens. The correlation between plasma and urine estrogen metabolites has been studied and found to be only fair (0.52 for the 2:16 ratio, 0.60 for 2-OHE1, and only 0.22 for 16 α -OHE1) and to differ according to baseline urinary 2:16 ratio and other covariates (34). The relationship of serum, plasma, or urinary metabolites to metabolite levels in tissue is relatively unknown, although a recent study showed a fair correlation between urinary 2:16 ratio and breast tissue ratio levels (35). Reproducibility of the estrogen metabolites was very good, judging by high ICCs (86%–94%), which were higher than in a previous study (32), but %CVs (15%–28%), although in the acceptable range, were lower than previous reports. A possible explanation for both is probable HT use by some of the women providing QC samples, particularly for the 2:16 ratio, for which paired samples would cluster around 1.0 for HT users and around 0.3 for nonusers. Another reason for higher ICCs in our study is that our ICCs include only interassay variation of a single sample, whereas the previous study evaluated paired samples collected up to 3 years apart (32).

In summary, to our knowledge, this study of more than 2,500 women is the largest of its kind exploring breast cancer and its relationship to serum estrogen metabolites in relation to HT. This sample from the WHI study population represents a very large, geographically diverse cohort of U.S. women, with valuable incident and prevalent epidemiologic and clinical information. Furthermore, this is the only study, which evaluates the change in estrogen metabolites with randomization to E+P versus E-alone. These results show that although 16 α -OHE1 was increased more by E+P than by E-alone, the increase in the 2:16 ratio was similar for both HT regimens, because of ~4-fold greater increase in 2OHE-1 than 16 α -OHE1 for both HT regimens. Second, baseline and 1 year change in 16 α -OHE1 showed little relationship to incident breast cancer. In contrast, higher baseline 2-

OHE1 and the 2:16 ratio were modestly associated with higher odds of incident breast cancer, but larger 1 year increase in 2-OHE-1 and the 2:16 ratio were also weakly associated with lower odds of breast cancer, even if adjusted for baseline metabolite levels. It is possible that differences in metabolism of oral estrogens, (i.e., as shown in this study, the increase primarily in the 2-OHE1, a less potent estrogen with regard to breast cancer, and also by the previously reported increase in SHBG), may be important determinants of risk of breast cancer with oral E-alone or E+P.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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