

Postmenopausal circulating levels of 2- and 16 α -hydroxyestrone and risk of endometrial cancer

A Zeleniuch-Jacquotte^{*,1,2}, RE Shore^{1,3}, Y Afanasyeva¹, A Lukanova^{4,5}, S Sieri⁶, KL Koenig^{1,2}, A Idahl⁷, V Krogh⁶, M Liu^{1,2}, N Ohlson⁸, P Muti⁹, AA Arslan^{1,2,5}, P Lenner¹⁰, F Berrino¹¹, G Hallmans¹², P Toniolo^{1,2,5,13} and E Lundin⁸

¹Department of Environmental Medicine, New York University School of Medicine, 650 First Avenue, New York, NY 10016, USA; ²New York University Cancer Institute, New York University School of Medicine, 530 First Avenue, New York, NY 10016, USA; ³Radiation Effects Research Foundation, 5-2 Hijiyama Park, Minami-ku, Hiroshima, 732-0815, Japan; ⁴Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, D-69120, Heidelberg, Germany; ⁵Department of Obstetrics and Gynecology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA; ⁶Department of Preventive and Predictive Medicine, Nutritional Epidemiology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian, 1, 20133, Milan, Italy; ⁷Department of Clinical Science, Obstetrics and Gynecology, Umeå University Hospital, S-901 85, Umeå, Sweden; ⁸Department of Medical Biosciences/Pathology, Umeå University, SE-901 87, Umeå, Sweden; ⁹Istituto Nazionale Tumori Regina Elena, Via E. Chianesi 53, 00144, Rome, Italy; ¹⁰Department of Radiation Sciences, Oncology, Umeå University, S-90185, Umeå, Sweden; ¹¹Department of Preventive and Predictive Medicine, Epidemiology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian, 1, 20133, Milan, Italy; ¹²Department of Public Health and Clinical Medicine/Nutritional Research, Umeå University, SE-901 87, Umeå, Sweden; ¹³Unit of Cancer Epidemiology, Institute of Social and Preventive Medicine, Centre Hospitalier Universitaire Vaudois, Biopôle 1, 2 Route de la Corniche, CH-1066 Epalinges, Switzerland

BACKGROUND: It has been suggested that the relative importance of oestrogen-metabolising pathways may affect the risk of oestrogen-dependent tumours including endometrial cancer. One hypothesis is that the 2-hydroxy pathway is protective, whereas the 16 α -hydroxy pathway is harmful.

METHODS: We conducted a case–control study nested within three prospective cohorts to assess whether the circulating 2-hydroxyestrone:16 α -hydroxyestrone (2-OHE1:16 α -OHE1) ratio is inversely associated with endometrial cancer risk in postmenopausal women. A total of 179 cases and 336 controls, matching cases on cohort, age and date of blood donation, were included. Levels of 2-OHE1 and 16 α -OHE1 were measured using a monoclonal antibody-based enzyme assay.

RESULTS: Endometrial cancer risk increased with increasing levels of both metabolites, with odds ratios in the top tertiles of 2.4 (95% CI = 1.3, 4.6; $P_{\text{trend}} = 0.007$) for 2-OHE1 and 1.9 (95% CI = 1.1, 3.5; $P_{\text{trend}} = 0.03$) for 16 α -OHE1 in analyses adjusting for endometrial cancer risk factors. These associations were attenuated and no longer statistically significant after further adjustment for oestrone or oestradiol levels. No significant association was observed for the 2-OHE1:16 α -OHE1 ratio.

CONCLUSION: Our results do not support the hypothesis that greater metabolism of oestrogen via the 2-OH pathway, relative to the 16 α -OH pathway, protects against endometrial cancer.

British Journal of Cancer (2011) **105**, 1458–1464. doi:10.1038/bjc.2011.381 www.bjcancer.com

Published online 27 September 2011

© 2011 Cancer Research UK

Keywords: endometrial cancer; nested case–control study; oestrogen metabolites; 2-hydroxyestrone; 16 α -hydroxyestrone, post-menopause

The role of oestrogens in the development of endometrial cancer is well documented by both experimental and epidemiologic studies (Key and Pike, 1988; Akhmedkhanov *et al*, 2001). High oestrogen, relative to progesterone/progestin, exposure leads to endometrial hyperplasia, followed by atypical hyperplasia, the precursor of most endometrial cancers (Sherman, 2000). In addition to exogenous oestrogens (Grady *et al*, 1995), circulating levels of endogenous oestrogens in postmenopausal women have been

shown to be positively associated with risk of endometrial cancer in prospective studies (Zeleniuch-Jacquotte *et al*, 2001; Lukanova *et al*, 2004; Allen *et al*, 2008). Oestrogens are metabolised through various pathways, and it has been suggested that the relative importance of these pathways within an individual may affect the risk of oestrogen-dependent tumours, such as breast and endometrial cancers (Fishman *et al*, 1984; Zhu and Conney, 1998). One specific hypothesis is that the 2-hydroxyestrogen metabolite pathway is protective, whereas the 16 α -hydroxyestrogen pathway is harmful (Schneider *et al*, 1982; Fishman *et al*, 1984; Sepkovic and Bradlow, 2009). The basis for this hypothesis is that 2-hydroxyestrogens bind to oestrogen receptors but do not activate transcription, resulting in no or limited proliferative effects, whereas 16 α -hydroxyestrogens have prolonged oestrogenic

*Correspondence: Dr A Zeleniuch-Jacquotte;

E-mail: anne.jacquotte@nyumc.org

Received 27 April 2011; revised 25 August 2011; accepted 29 August 2011; published online 27 September 2011

effects including stimulation of cell proliferation (Sepkovic and Bradlow, 2009).

A number of prospective studies have examined the effects of 2-hydroxyestrone (2-OHE1), 16 α -hydroxyestrone (16 α -OHE1) and their ratio in relation to risk of breast cancer with inconsistent results (Meilahn *et al*, 1998; Muti *et al*, 2000; Cauley *et al*, 2003; Wellejus *et al*, 2005; Modugno *et al*, 2006; Eliassen *et al*, 2008; Arslan *et al*, 2009). No study, though, has been conducted in relation to endometrial cancer risk. We conducted a case-control study nested within three prospective cohorts to test the hypothesis that the circulating 2-OHE1 : 16 α -OHE1 ratio, an index of the relative importance of the 2-OH and 16 α -OH oestrogen pathways, is inversely associated with risk of endometrial cancer in postmenopausal women. We previously reported positive associations between postmenopausal circulating oestrogens and androgens and risk of endometrial cancer in a subset of the subjects included in this study (Lukanova *et al*, 2004).

MATERIALS AND METHODS

Study population and biological sample collection

The case-control study was nested within three cohorts: the Northern Sweden Disease and Health Study (NSHDS) in Umeå, Sweden (Hulten *et al*, 2001), the New York University Women's Health Study (NYUWHS) in New York City, NY, USA (Toniolo *et al*, 1995; Zeleniuch-Jacquotte *et al*, 2001) and the ORDET cohort in Milan, Italy (Muti *et al*, 2000; Sieri *et al*, 2009). The recruitment setting, eligibility criteria and case ascertainment methods for each cohort are described in Table 1. As described in Lukanova *et al* (2004), data on known risk factors for endometrial cancer were obtained at enrolment through interviews by nurses for ORDET and by a combination of self-administered baseline and follow-up questionnaires for the NSHDS and the NYUWHS. Height and weight were self-reported for the NSHDS and the NYUWHS and measured by nurses for ORDET at enrolment. Venous blood was collected at enrolment (plasma for the NSHDS, serum for the NYUWHS and both plasma and serum for ORDET). All specimens were stored at -80°C by the respective cohorts. The Institutional Review Boards of New York University School of Medicine and of the Istituto Nazionale dei Tumori in Milan and the Regional Ethical Committee of the University of Umeå, Sweden, reviewed and approved this study. Informed consent was obtained from all participants at enrolment.

Menopausal status was determined at enrolment for the NYUWHS and ORDET participants. Women were classified as postmenopausal if they had not had menstrual periods for at least 6 months (NYUWHS) or 12 months (ORDET). In the NYUWHS, women who had had a total oophorectomy and women who had had a hysterectomy without total oophorectomy and were 52 years old or older were also classified as postmenopausal. In ORDET, women who had had a bilateral oophorectomy were not eligible to enter the cohort. For NSHDS cases and controls, menopausal status at enrolment was determined retrospectively based on reported age at menopause on baseline or case-control questionnaires. In addition, FSH measurements were used for all women between the ages of 46 and 57 years. Women were classified as postmenopausal if they had FSH levels $>20\text{IU l}^{-1}$ and their questionnaire data were consistent with postmenopausal status. Finally, women taking hormone replacement therapy (HRT) in the three (ORDET), or six (NSHDS, NYUWHS), months before enrolment were not included.

Case ascertainment and control selection

Case subjects were women who were postmenopausal at enrolment and diagnosed with invasive endometrial cancer (ICD-O codes 8010, 8140, 8210, 8260, 8310, 8323, 8380, 8382, 8441, 8460, 8461, 8480, 8481, 8560 and 8570) after enrolment and before 1 January 2007 for the NSHDS, 1 July 2003 for the NYUWHS and 1 January 2004 for ORDET. Cases who had been diagnosed with another cancer (except non-melanoma skin cancer) before endometrial cancer were not eligible.

For each case, two controls were selected at random from the appropriate risk set, which consisted of all women with an intact uterus and free of cancer at the date of diagnosis of the case and matching the case on cohort, menopausal status at enrolment, age (± 6 months) at, and date (± 3 months) of, blood donation.

Laboratory analyses

All oestrogen metabolite assays were conducted in the same laboratory, Immuna Care Corporation, (Tampa, FL, USA), using monoclonal antibody-based enzyme assays (ESTRAMET 2/16, Immuna Care Corporation). The enzyme immunoassays (EIAs) were developed for urinary samples (Klug *et al*, 1994; Falk *et al*, 2000) and validated against gas chromatography-mass spectrometry (GC-MS) (Falk *et al*, 2000). They were validated for serum by conducting the assays on pooled human serum samples to

Table 1 Description of the cohorts

Cohort	Cohort size (age at enrolment, years)	Recruitment (enrolment period)	Data collection	Case ascertainment methods	Number of cases/controls
NSHDS Umeå, Sweden	> 85 000 men and women (30–70)	General population, Västerbotten County (1985–present)	Questionnaire at enrolment; data on reproductive life and hormone use updated by case-control questionnaire	Linkage to the Swedish Cancer Registry	89/164
NYUWHS New York City, NY, USA	14 274 women, no current use of OC or HRT (35–65)	Breast cancer screening centre (1985–1991)	Questionnaire at enrolment; prospective follow-up questionnaires; and case-control interview for reproductive life and hormone use	Follow-up questionnaires and linkages to state tumour registries (NY, NJ, FL) and the NDI	75/143
ORDET Milan, Italy	10 788 women, no current use of OC or HRT and no total oophorectomy (35–69)	General population, Varese Province (1987–1992)	Interview at enrolment	Linkage to the Lombardy Cancer Registry	15/29

Abbreviations: OC = oral contraceptives; HRT = hormone replacement therapy; NDI = national death index; NSHDS = Northern Sweden Disease and Health Study; NYUWHS = New York University Women's Health Study.

which known amounts of 2-OHE1 and 16 α -OHE1 had been added (Klug, personal communication). Serum samples were used for the NYUWHS and ORDET, and EDTA-plasma samples for the NSHDS. Assay variability was assessed by including 10% blinded quality control samples (samples from a pool for the NSHDS and duplicates for the NYUWHS). For serum, the within- and between-batch coefficients of variation were 4% and 6% for 2-OHE1 and 1% and 2% for 16 α -OHE1, respectively. For EDTA-plasma, the within- and between-batch coefficients of variation were 2% and 2% for 2-OHE1 and 1% and 4% for 16 α -OHE1, respectively.

As described previously (Lukanova *et al*, 2004), oestrone and oestradiol were measured by a radioimmunoassay following organic extraction and celite chromatography for 129 (76%) of the NYUWHS samples. For all other samples, oestrone was measured by double antibody RIA and oestradiol by ultrasensitive double antibody RIA with reagents from Diagnostic Systems Laboratories (Webster, TX, USA).

Statistical methods

The intra-class correlation coefficient (Donner, 1986) was used to assess the temporal reliability of the oestrogen metabolites and their ratio in a pilot study where oestrogen metabolites were measured in three serum samples collected at approximately yearly intervals in 30 NYUWHS participants postmenopausal at blood donation and free of cancer at latest follow-up.

Because serum was used for the NYUWHS and the ORDET cohorts and EDTA plasma for the NSHDS, a substudy was conducted to compare levels of oestrogen metabolites in serum with levels in EDTA-plasma samples collected at the same visit from 17 NSHDS participants. We found that plasma samples led to much higher values than serum samples: for 2-OHE1 the median was 526 pg ml⁻¹ using plasma and 183 pg ml⁻¹ using serum, and for 16 α -OHE1 the median was 618 pg ml⁻¹ using plasma and 518 pg ml⁻¹ using serum. The median 2-OHE1:16 α -OHE1 ratio was 1.06 using plasma samples and 0.43 using serum samples. Although the absolute values were quite different, the Pearson correlation coefficients were high between plasma and serum levels (0.96 for 2-OHE1, 0.99 for 16 α -OHE1 and 0.81 for the 2-OHE1:16 α -OHE1 ratio). Further analyses showed that serum levels (on the natural logarithmic scale) of metabolites could be accurately predicted from plasma levels (also on the logarithmic scale) using a linear regression model ($R^2 = 0.85$ for 2-OHE1 and 0.99 for 16 α -OHE1). The observed serum 2-OHE1:16 α -OHE1 ratio was slightly more strongly correlated ($r = 0.86$) with the predicted serum ratio (calculated as the ratio of the predicted serum 2-OHE1 and 16 α -OHE1 levels) than with the plasma ratio ($r = 0.81$). Therefore, and in order to facilitate the combination of the data from the three cohorts as well as the interpretation of the results, we used predicted serum values for the NSHDS participants in all analyses. To predict the serum levels of 2-OHE1 and 16 α -OHE1 from the plasma levels for the NSHDS samples, we created 10 imputed data sets using a multiple imputation procedure with the linear regression models fitted in the substudy. We then calculated the serum 2-OHE1:16 α -OHE1 ratio using the imputed serum levels of 2-OHE1 and 16 α -OHE1. The SAS PROC MI and MIANALYZE in SAS 9.2 were used (SAS Institute, Inc., Cary, NC, USA).

The conditional logistic regression model was used to take into account the matched design. The analysis was conducted after classifying participants according to cohort-specific metabolite tertiles using the distribution of cases and controls combined. The lowest tertile was used as reference. Because analyses were conducted using 10 imputed data sets, the means of the odds ratio estimates are reported. The within-imputation variance and between-imputation variances were calculated and combined to yield the total variance using Rubin's method (Rubin, 1987). The 95% confidence intervals and *P*-values were then calculated using

the *t*-distribution (SAS PROC MI and MIANALYZE). In addition to analyses by tertiles, analyses were conducted with the oestrogen metabolites and their ratio on the continuous (log transformed) scale. The log₂ transformation, which leads to an odds ratio for a doubling in level, was used.

The following known endometrial cancer risk factors were considered as potential confounders: age at menarche, parity, OC use, HRT use, body mass index (BMI) and smoking. Analyses adjusting simultaneously for all these variables are presented in addition to unadjusted analyses. Analyses stratified by age at sampling (<60 and ≥ 60 years), age at diagnosis (<65 and ≥ 65 years), lag time between blood donation and diagnosis (<7 and ≥ 7 years) and BMI (<25 and ≥ 25 kg m⁻²) were also conducted. Interaction tests were conducted to assess whether the associations of the oestrogen metabolites or their ratio with endometrial cancer risk varied according to strata of these factors. We also assessed whether the oestrogen metabolite-endometrial cancer risk associations differed by cohort.

RESULTS

The ICC measuring the temporal reliability over a 2-year period was 0.62 (95% CI = 0.42, 0.78) for 2-OHE1, 0.95 (95% CI = 0.91, 0.97) for 16 α -OHE1 and 0.69 (95% CI = 0.52, 0.82) for their ratio. These results were quite similar to those observed by Eliassen *et al* who reported intra-class correlation coefficients of 0.63 for 2-OHE1, 0.80 for 16 α -OHE1 and 0.73 for their ratio, indicating that a single measurement can be used as a marker of average exposure to these metabolites in epidemiologic studies (Eliassen *et al*, 2008).

A total of 179 cases and 336 controls were included (89 cases and 164 controls from the NSHDS, 75 cases and 143 controls from the NYUWHS and 15 cases and 29 controls from ORDET). Table 2 reports on the case and control subject characteristics. The median age at blood donation was 59.9 years and at diagnosis 66.5 years. The vast majority of participants (94% of cases and 97% of controls) were Caucasians. Associations with known risk factors for endometrial cancer were in the expected directions. Compared with controls, cases had younger age at menarche and older age at menopause, and were heavier. Cases were more likely than controls to have used HRT but less likely to have used oral contraceptives and to have ever smoked. Levels of both oestrogen metabolites were significantly higher in cases than in controls, whereas the 2-OHE1:16 α -OHE1 ratio was not statistically different in cases and controls.

The 2-OHE1 and 16 α -OHE1 were moderately correlated with each other (cohort- and age-adjusted Spearman correlation $r = 0.20$ in controls and 0.19 in cases). The 2-OHE1:16 α -OHE1 ratio was strongly correlated with levels of 2-OHE1 ($r = 0.85$ in controls and 0.87 in cases) and negatively, but less strongly, correlated with 16 α -OHE1 ($r = -0.30$ in controls and -0.26 in cases). Correlations were low between oestrone and 2-OHE1 ($r = 0.13$ in controls and 0.19 in cases), 16 α -OHE1 ($r = 0.15$ in controls and 0.21 in cases) and their ratio ($r = 0.03$ in controls and 0.10 in cases). Correlations of the oestrogen metabolites and their ratio with oestradiol were in the same direction as with oestrone but weaker (data not shown).

Table 3 reports odds ratios for the associations of the two oestrogen metabolites and their ratio, with risk of endometrial cancer. In unadjusted analyses, the risk of endometrial cancer increased significantly with increasing tertiles of 2-OHE1 and 16 α -OHE1. The associations remained significant after adjusting for known endometrial cancer risk factors, with odds ratios in the top tertile of 2.4 (95% CI = 1.3–4.6; $P_{\text{trend}} = 0.007$) for 2-OHE1 and 1.9 (95% CI = 1.1–3.5; $P_{\text{trend}} = 0.03$) for 16 α -OHE1. Although the odds ratios in the top two tertiles were somewhat elevated, no significant association was observed with the 2-OHE1:16 α -OHE1 ratio in unadjusted, as well as in adjusted, analyses.

Table 2 Description of case and control subjects

Characteristic	Cases (n = 179)			Controls (n = 336)			P-value
	N	%	Median (10th, 90th)	N	%	Median (10th, 90th)	
Age at blood donation, years			59.9 (51.6, 65.4)			59.9 (51.8, 65.4)	Matched
Age at diagnosis, years			66.5 (58.9, 73.8)				
Lag time between blood donation and diagnosis			7.3 (1.9–13.0)				
Race							0.41
Caucasian	163	94		312	97		
Other	11	6		13	3		
Missing	5			11			
Height, cm			162.6 (154.0, 170.2)			161.5 (154.0, 169.0)	0.49
Weight, kg			71.3 (55.0, 94.0)			65.8 (54.4, 84.4)	<0.0001
Body mass index, kg m ⁻²							0.001
<25	53	34		154	50		
25–<30	57	37		101	33		
≥30	46	29		54	17		
Missing, n	23			27			
Age at menarche, years							0.01
<12	33	19		33	10		
12–13	82	46		161	49		
>13	61	35		132	41		
Missing, n	3			10			
Age at menopause, years			51.5 (46.0, 56.0)			50.0 (45.0, 55.0)	0.004
Missing, n			23			43	
Nulliparous	29	16		58	18		0.70
Missing, n	0			5			
Ever used OC	33	19		75	23		0.05
Missing, n	7			13			
Ever used HRT	59	37		80	26		0.01
Missing, n	23			27			
Ever smoked	57	34		134	42		0.09
Missing, n	12			17			
History of diabetes	13	8		17	5		0.54
Missing, n	10			11			
2-OHE1 (pg ml ⁻¹)			130.2 (67.0–221.4)			113.5 (61.3, 208.3)	0.03
16 α -OHE1 (pg ml ⁻¹)			335.3 (255.0, 460.2)			314.0 (225.6, 436.9)	0.02
2-OHE1 : 16 α -OHE1 ratio			0.40 (0.19, 0.69)			0.37 (0.19, 0.70)	0.58

Abbreviations: OC = oral contraceptives; HRT = hormone replacement therapy; 16 α -OHE1 = 16 α -hydroxyestrone; 2-OHE1 = 2-hydroxyestrone.

Table 3 ORs and 95% CIs for endometrial cancer according to circulating levels of 2-OHE1, 16 α -OHE1 and the 2-OHE1 : 16 α -OHE1 ratio

Oestrogen metabolites	Tertiles			P _{trend}
	1	2	3	
2-OHE1				
Cases/controls	45/125	61/112	73/99	
Crude OR (95% CI)	1.00 (reference)	1.55 (0.89, 2.70)	2.24 (1.28, 3.91)	0.005
Multivariate OR (95% CI) ^a	1.00 (reference)	1.87 (0.98, 3.55)	2.41 (1.27, 4.58)	0.007
16 α -OHE1				
Cases/controls	44/127	66/107	69/102	
Crude OR (95% CI)	1.00 (reference)	1.71 (1.01, 2.91)	2.01 (1.21, 3.35)	0.007
Multivariate OR (95% CI) ^a	1.00 (reference)	2.06 (1.14, 3.70)	1.89 (1.05, 3.41)	0.03
2-OHE1 : 16 α -OHE1 ratio				
Cases/controls	49/121	69/104	61/111	
Crude OR (95% CI)	1.00 (reference)	1.56 (0.92, 2.66)	1.34 (0.78, 2.31)	0.28
Multivariate OR (95% CI) ^a	1.00 (reference)	1.74 (0.95, 3.20)	1.33 (0.73, 2.44)	0.35

Abbreviations: BMI = body mass index; CI = confidence interval; OR = odds ratio; 16 α -OHE1 = 16 α -hydroxyestrone; 2-OHE1 = 2-hydroxyestrone. ^aAdjusted for age at menarche (<12, 12, 13, >13 years and missing), age at menopause (<50, 50–52, >52 years and missing), parity (ever, never and missing), oral contraceptive use (ever, never and missing), hormone replacement therapy use (ever, never and missing), smoking (ever, never and missing) and BMI (<25, 25–30, ≥30 kg m⁻² and missing).

Table 4 Odds ratios associated with a doubling in levels of circulating oestrogens, oestrogen metabolites and 2-OHE1 : 16 α -OHE1 ratio

Model	Oestrone	Oestradiol	2-OHE1	16 α -OHE1	2-OHE1 : 16 α -OHE1 ratio
All subjects					
Adjusted for known risk factors ^a					
OR (95% CI)			1.39 (1.01, 1.90)	1.73 (1.05, 2.85)	1.05 (0.50, 2.20)
P-value			0.04	0.03	0.90
Subjects with oestrogen measurements					
Adjusted for known risk factors ^a					
OR (95% CI)	2.25 (1.36, 3.71)	2.51 (1.57, 4.02)	1.76 (1.07, 2.92)	1.92 (0.93, 3.96)	1.07 (0.41, 2.81)
P-value	0.002	0.0001	0.03	0.08	0.89
Adjusted for known risk factors, 2-OHE1 and 16 α -OHE1 ^a					
OR (95% CI)	1.98 (1.18, 3.32)	2.26 (1.40, 3.66)			
P-value	0.009	0.001			
Adjusted for known risk factors and oestrone ^a					
OR (95% CI)			1.51 (0.89, 2.55)	1.58 (0.80, 3.15)	1.00 (0.37, 2.73)
P-value			0.13	0.19	0.99
Adjusted for known risk factors and oestradiol ^a					
OR (95% CI)			1.51 (0.89, 2.57)	1.59 (0.79, 3.22)	1.04 (0.37, 2.91)
P-value			0.12	0.19	0.94

Abbreviations: BMI = body mass index; CI = confidence interval; OR = odds ratio; 16 α -OHE1 = 16 α -hydroxyestrone; 2-OHE1 = 2-hydroxyestrone. ^aAge at menarche (< 12, 12, 13, > 13 years and missing), age at menopause (< 50, 50–52, > 52 years and missing), parity (ever, never and missing), oral contraceptive use (ever, never and missing), hormone replacement therapy use (ever, never and missing), smoking (ever, never and missing), and BMI (< 25, 25–30, \geq 30 kg m⁻² and missing).

Table 4 shows odds ratios for endometrial cancer risk associated with a doubling in levels of each of the two oestrogen metabolites and of their ratio, for all subjects included in the study as well as for the subset of 101 cases and 183 controls for whom oestrone and oestradiol measurements were available. Overall results were similar in the two groups, that is significant trends of increasing risk with increasing levels of 2-OHE1 and 16 α -OHE1 and no association with the ratio, although odds ratios for 2-OHE1 and 16 α -OHE1 were slightly higher in the subgroup than in the whole-group analyses. The table also shows that adjusting for either oestrone or oestradiol, in addition to adjusting for known risk factors, resulted in an attenuation of the odds ratios associated with 2-OHE1 and 16 α -OHE1, which were no longer statistically significant. On the other hand, the strong associations of oestrone and oestradiol with endometrial cancer risk remained highly significant after adjusting for the two oestrogen metabolites.

Results in analyses limited to the 142 sets whose case subject had an endometrioid-type tumour were similar to the overall results (data not shown). There was no evidence of heterogeneity by cohort in any of the analyses. There was also no evidence of effect modification by age at blood donation, age at diagnosis, lag time between blood donation and diagnosis or BMI.

DISCUSSION

Contrary to our hypothesis, women with a higher 2-OHE1 : 16 α -OHE1 ratio did not have a decreased risk of endometrial cancer as compared with women with a lower ratio. Regarding absolute levels of 2-OHE1 and 16 α -OHE1, we found that both were directly associated with risk in unadjusted analyses as well as in analyses adjusting for known risk factors of endometrial cancer. These associations, though, were no longer significant after adjusting for oestrone, which is positively associated with both risk of endometrial cancer (Lukanova *et al*, 2004; Allen *et al*, 2008) and levels of metabolites, and therefore a positive confounder. Similar results were observed when adjusting for oestradiol, a more potent oestrogen than oestrone. On the other hand, the odds ratios associated with the 2-OHE1 : 16 α -OHE1 ratio were not materially

affected by adjustment for oestrone. This was not surprising, as the ratio was not correlated with the level of oestrone, which confirmed that the ratio is an index of the relative importance of these two metabolic pathways independent of the levels of oestrone.

The findings from this first prospective epidemiological study of oestrogen metabolites and endometrial cancer are in line with results from prospective studies on breast cancer, another oestrogen-related cancer. None of the seven studies on breast cancer reported significant associations overall (Meilahn *et al*, 1998; Muti *et al*, 2000; Cauley *et al*, 2003; Wellejus *et al*, 2005; Modugno *et al*, 2006; Eliassen *et al*, 2008; Arslan *et al*, 2009). Some studies reported significant associations in subgroups, but the subgroup results were not consistent across studies (Eliassen *et al*, 2008). On the whole, prospective epidemiological data do not support the hypothesis that the 2-hydroxyestrogen pathway is protective, and the 16 α -hydroxyestrogen pathway harmful, in hormone-dependent cancers.

Although no longer significant, odds ratios associated with 2-OHE1 remained somewhat elevated after adjusting for oestrone or oestradiol. It is possible that these elevations were due to remaining confounding by the main oestrogens affecting endometrial cancer, that is, oestrone and oestradiol. However, a significant increase in risk of breast cancer with levels of 2-OHE1 has also been reported previously, although it was limited to hormone receptor-negative tumours (Eliassen *et al*, 2008). Although there is a substantial amount of experimental data supporting the hypothesis that the 2-hydroxyestrogen pathway may be protective against oestrogen-driven cancers, not all studies agree. One study showed that uterine adenocarcinomas were induced in CD-1 mice by both 2-hydroxyestradiol and 4-hydroxyestradiol (Newbold and Liehr, 2000). Both 2- and 4-hydroxyestrogens are catecholestrogens, and it has been suggested that catecholestrogens increase risk of oestrogen-mediated cancers through direct genotoxic effects, rather than through stimulation of cell proliferation via binding to oestrogen receptors (Jefcoate *et al*, 2000; Yager, 2000; Liehr and Jones, 2001; Cavalieri *et al*, 2002). Although the evidence is stronger for 4-hydroxyestrogens than for 2-hydroxyestrogens (Gaikwad *et al*, 2008), the hypothesis that catecholestrogens,

including 2-hydroxyestrogens, increase risk of endometrial cancer deserves to be evaluated. Further, oestrogen can be metabolised through other pathways. New methods are now available to measure a broad range of oestrogen metabolites (Xu *et al*, 2005, 2007), which may help assess the role of various pathways in the development of breast and endometrial cancers.

Strengths of our study include its prospective design, with blood samples collected before diagnosis, and the results of our pilot study, which showed that oestrogen metabolites had good temporal reliability in our population, indicating that a single measurement reflects reasonably well a woman's average level over several years, relative to other women. Associations with known risk factors for endometrial cancer were observed in our study. Finally, in addition to adjusting for these known risk factors, we were able to control for circulating levels of the two main oestrogens, that is, oestrone and oestradiol.

Our study also has some limitations. First, the specificity and accuracy of the EIAs assays have been recently questioned (Faupel Badger *et al*, 2010). An early study comparing the EIAs with GC-MS for urinary samples from postmenopausal women had showed high correlations between the two methods for 2-OHE1, 16 α -OHE1 and their ratio (Spearman correlation coefficients ≥ 0.70 ; Falk *et al*, 2000). A recent, much larger study, though, found that in postmenopausal women the mean urinary concentration of 2-OHE1 was 6-fold higher, and that of 16 α -OHE1 12-fold higher when measured by the EIAs than when measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Further, the Spearman correlation coefficients for the ratio based on the two methods were only 0.17 for the 2-OHE1:16 α -OHE1 ratio and 0.25 for the 2-pathway:16 α -pathway ratio (Faupel Badger, Fuhrman *et al*, 2010). Although these correlations are weak, suggesting limited specificity of the EIA assays, it is unlikely that we would have observed an inverse association between the 2-OHE1:16 α -OHE1 ratio and endometrial cancer risk if we had used LC-MS/MS rather than EIAs because, although not significant, we observed a positive association between the EIA ratio and

risk of endometrial cancer and because the correlation of the ratio estimates obtained by the two methods is positive.

Another limitation of our study is that concentrations of oestrogen metabolites were higher when measured in EDTA plasma (for the NSHDS) than when measured in serum (for the NYUWHS and ORDET) samples. Several reasons may have contributed to the higher concentrations observed with EDTA-plasma samples, such as binding of EDTA with cations (e.g., calcium) which are cofactors in the stabilisation of the enzymes used in assay (e.g., alkaline phosphatase) and differences in protein composition. Because the validation study we conducted showed that serum concentrations could be accurately predicted from plasma concentrations in Swedish participants for whom EDTA-plasma and serum samples collected at the same time were available, we predicted serum values for the NSHDS participants and presented results combining the three cohorts. It should be noted that analyses conducted within each cohort separately showed similar results, suggesting that the type of samples did not affect the conclusions.

In conclusion, our results do not support the hypothesis that greater metabolism of oestrogen via the 2-OH pathway, relative to the 16 α -OH pathway, protects against endometrial cancer. Indeed our results are more suggestive of an increase in risk, rather than a decrease, with higher levels of 2-OHE1. Assays measuring a broad panel of oestrogen metabolites, including catecholestrogens, should be used in future studies to assess the role of various oestrogen metabolic pathways in the development of oestrogen-related cancers.

ACKNOWLEDGEMENTS

This study was supported by the National Institute of Health R01 CA81212, R01 CA098661, center Grant CA16087 and National Institute of Environmental Health Sciences center Grant ES00260.

REFERENCES

- Akhmedkhanov A, Zeleniuch-Jacquotte A, Toniolo P (2001) Role of exogenous and endogenous hormones in endometrial cancer: review of the evidence and research perspectives. *Ann N Y Acad Sci* **943**: 296–315
- Allen NE, Key TJ, Dossus L, Rinaldi S, Cust A, Lukanova A, Peeters PH, Onland-Moret NC, Lahmann PH, Berrino F, Panico S, Larranaga N, Pera G, Tormo MJ, Sanchez MJ, Ramon Quiros J, Ardanaz E, Tjonneland A, Olsen A, Chang-Claude J, Linseisen J, Schulz M, Boeing H, Lundin E, Palli D, Overvad K, Clavel-Chapelon F, Boutron-Ruault MC, Bingham S, Khaw KT, Bas Bueno-de-Mesquita H, Trichopoulou A, Trichopoulos D, Naska A, Tumino R, Riboli E, Kaaks R, Allen NE, Key TJ, Dossus L, Rinaldi S, Cust A, Lukanova A, Peeters PH, Onland-Moret NC, Lahmann PH, Berrino F, Panico S, Larranaga N, Pera G, Tormo M-J, Sanchez M-J, Ardanaz E, Tjonneland A, Olsen A, Chang-Claude J, Linseisen J, Schulz M, Boeing H, Lundin E, Palli D, Overvad K, Clavel-Chapelon F, Boutron-Ruault M-C, Bingham S, Khaw K-T, Trichopoulou A, Trichopoulos D, Naska A, Tumino R, Riboli E, Kaaks R (2008) Endogenous sex hormones and endometrial cancer risk in women in the European Prospective Investigation into Cancer and Nutrition (EPIC). *Endocr Relat Cancer* **15**(2): 485–497
- Arslan AA, Shore RE, Afanasyeva Y, Koenig KL, Toniolo P, Zeleniuch-Jacquotte A, Arslan AA, Shore RE, Afanasyeva Y, Koenig KL, Toniolo P, Zeleniuch-Jacquotte A (2009) Circulating estrogen metabolites and risk for breast cancer in premenopausal women. *Cancer Epidemiol Biomarkers Prev* **18**(8): 2273–2279
- Cauley JA, Zmuda JM, Danielson ME, Ljung BM, Bauer DC, Cummings SR, Kuller LH, Cauley JA, Zmuda JM, Danielson ME, Ljung B-M, Bauer DC, Cummings SR, Kuller LH (2003) Estrogen metabolites and the risk of breast cancer in older women. *Epidemiology* **14**(6): 740–744
- Cavalieri EL, Rogan EG, Chakravarti D (2002) Initiation of cancer and other diseases by catechol ortho-quinones: a unifying mechanism. *Cell Mol Life Sci* **59**(4): 665–681
- Donner A (1986) A review of inference procedures for the intraclass correlation coefficient in the one-way random effects model. *Int Stat Rev* **54**: 67–82
- Eliassen AH, Missmer SA, Tworoger SS, Hankinson SE (2008) Circulating 2-hydroxy- and 16 α -hydroxy estrone levels and risk of breast cancer among postmenopausal women. *Cancer Epidemiol Biomarkers Prev* **17**(8): 2029–2035
- Falk RT, Rossi SC, Fears TR, Sepkovic DW, Migella A, Adlercreutz H, Donaldson J, Bradlow HL, Ziegler RG (2000) A new ELISA kit for measuring urinary 2-hydroxyestrone, 16 α -hydroxyestrone, and their ratio: reproducibility, validity, and assay performance after freeze-thaw cycling and preservation by boric acid. *Cancer Epidemiol Biomarkers Prev* **9**(1): 81–87
- Faupel Badger J, Fuhrman B, Xu X, Falk R, Keefer L, Veenstra T, Hoover R, Ziegler R (2010) Comparison of liquid chromatography-tandem mass spectrometry, RIA, and ELISA methods for measurement of urinary estrogens. *Cancer Epidemiol Biomarkers Prev* **19**(1): 292–300
- Fishman J, Schneider J, Hershcopf RJ, Bradlow HL (1984) Increased estrogen-16 α -hydroxylase activity in women with breast and endometrial cancer. *J Steroid Biochem* **20**: 1077–1081
- Gaikwad NW, Yang L, Muti P, Meza JL, Pruthi S, Ingle JN, Rogan EG, Cavalieri EL (2008) The molecular etiology of breast cancer: evidence from biomarkers of risk. *Int J Cancer* **122**(9): 1949–1957
- Grady D, Gebretsadik T, Kerlikowske K, Ernster V, Petitti D (1995) Hormone replacement therapy and endometrial cancer risk: a meta-analysis. *Obstet Gynecol* **85**(2): 304–313
- Hulten K, Van Kappel AL, Winkvist A, Kaaks R, Hallmans G, Lenner P, Riboli E (2001) Carotenoids, alpha-tocopherols, and retinol in plasma and breast cancer risk in northern Sweden. *Cancer Causes Control* **12**(6): 529–537
- Jefcoate CR, Liehr JG, Santen RJ, Sutter TR, Yager JD, Yue W, Santner SJ, Tekmal R, Demers L, Pauley R, Naftolin F, Mor G, Berstein L (2000)

- Chapter 5: tissue-specific synthesis and oxidative metabolism of estrogens. *J Natl Cancer Inst Monogr* 27: 95–112
- Key TJA, Pike MC (1988) The dose-effect relationship between unopposed estrogens and endometrial mitotic rate: its central role in explaining and predicting endometrial cancer risk. *Br J Cancer* 57: 205–212
- Klug TL, Bradlow HL, Sepkovic DW (1994) Monoclonal antibody-based enzyme immunoassay for simultaneous quantitation of 2- and 16 α -hydroxyestrone in urine. *Steroids* 59: 648–655
- Liehr JG, Jones JS (2001) Role of iron in estrogen-induced cancer. *Curr Med Chem* 8: 839–849
- Lukanova A, Lundin E, Micheli A, Arslan AA, Ferrari P, Rinaldi S, Krogh V, Lenner P, Shore RE, Biessy C, Muti P, Riboli E, Koenig KL, Levitz M, Stattin P, Berrino F, Hallmans G, Kaaks R, Toniolo P, Zeleniuch-Jacquotte A (2004) Circulating levels of sex steroid hormones and risk of endometrial cancer in postmenopausal women. *Int J Cancer* 108: 425–432
- Meilahn EN, De Stavola B, Allen DS, Fentiman IS, Bradlow HL, Sepkovic DW, Kuller LH (1998) Do urinary oestrogen metabolites predict breast cancer? Guernsey III cohort follow-up. *Br J Cancer* 78: 1250–1255
- Modugno F, Kip KE, Cochrane B, Kuller L, Klug TL, Rohan TE, Chlebowski RT, Lasser N, Stefanick ML (2006) Obesity, hormone therapy, estrogen metabolism and risk of postmenopausal breast cancer. *Int J Cancer* 118: 1292–1301
- Muti P, Bradlow HL, Micheli A, Krogh V, Freudenheim JL, Schunemann HJ, Stanulla M, Yang J, Sepkovic DW, Trevisan M, Berrino F (2000) Estrogen metabolism and risk of breast cancer: a prospective study of the 2:16 α -hydroxyestrone ratio in premenopausal and postmenopausal women. *Epidemiology* 11: 635–640
- Newbold RR, Liehr JG (2000) Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. *Cancer Res* 60: 235–237
- Rubin DB (1987) *Multiple Imputation for Nonresponse in Surveys*. J. Wiley & Sons: New York
- Schneider J, Kinne D, Fracchia A, Pierce V, Anderson KE, Bradlow HL, Fishman J (1982) Abnormal oxidative metabolism in women with breast cancer. *Proc Natl Acad Sci USA* 79: 3047–3051
- Sepkovic DW, Bradlow HL (2009) Estrogen hydroxylation – the good and the bad. *Ann N Y Acad Sci* 1155: 57–67
- Sherman ME (2000) Theories of endometrial carcinogenesis: a multi-disciplinary approach. *Mod Pathol* 13: 295–308
- Sieri S, Krogh V, Bolelli G, Abagnato CA, Grioni S, Pala V, Evangelista A, Allemani C, Micheli A, Tagliabue G, Schunemann HJ, Menard S, Berrino F, Muti P, Sieri S, Krogh V, Bolelli G, Abagnato CA, Grioni S, Pala V, Evangelista A, Allemani C, Micheli A, Tagliabue G, Schunemann HJ, Menard S, Berrino F, Muti P (2009) Sex hormone levels, breast cancer risk, and cancer receptor status in postmenopausal women: the ORDET cohort. *Cancer Epidemiol Biomarkers Prev* 18(1): 169–176
- Toniolo P, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig KL, Shore RE, Strax P, Pasternack BS (1995) A prospective study of endogenous estrogens and breast cancer in postmenopausal women. *J Natl Cancer Inst* 87: 190–197
- Wellejus A, Olsen A, Tjonneland A, Thomsen BL, Overvad K, Loft S, Wellejus A, Olsen A, Tjonneland A, Thomsen BL, Overvad K, Loft S (2005) Urinary hydroxyestrogens and breast cancer risk among postmenopausal women: a prospective study. *Cancer Epidemiol Biomarkers Prev* 14(9): 2137–2142
- Xu X, Roman JM, Issaq HJ, Keefer LK, Veenstra TD, Ziegler RG (2007) Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography-tandem mass spectrometry. *Anal Chem* 79(20): 7813–7821
- Xu X, Veenstra TD, Fox SD, Roman JM, Issaq HJ, Falk R, Saavedra JE, Keefer LK, Ziegler RG (2005) Measuring fifteen endogenous estrogens simultaneously in human urine by high-performance liquid chromatography-mass spectrometry. *Anal Chem* 77(20): 6646–6654
- Yager JD (2000) Endogenous estrogens as carcinogens through metabolic activation. *J Natl Cancer Inst Monogr* 27: 67–73
- Zeleniuch-Jacquotte A, Akhmedkhanov A, Kato I, Koenig KL, Shore RE, Kim MY, Levitz M, Mittal KR, Raju A, Banerjee S, Toniolo P (2001) Postmenopausal endogenous oestrogens and risk of endometrial cancer: results of a prospective study. *Br J Cancer* 84: 975–981
- Zhu BT, Conney AH (1998) Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* 19: 1–27

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.