Serum estrogen receptor bioactivity and breast cancer risk among postmenopausal women

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Abstract

The estrogen levels of Asian women are different from those of Western women, and this could affect estrogen receptor (ER) bioactivity and breast cancer risk. We conducted a case-control study in 169 postmenopausal breast cancer cases and 426 matched controls nested within a population-based prospective cohort study, the Singapore Chinese Health Study, to evaluate the serum levels of estrogens and their receptor (ER α and ER β)-mediated estrogenic activities in relation to breast cancer risk. Breast cancer cases had higher levels of estrogens and ER-mediated bioactivities in baseline serum than the controls. Compared with those in the lowest quartile, women in the highest quartile for estrone (E₁) or ER α -mediated bioactivity had increased breast cancer risk. After additional adjustment for ER β bioactivity, free estradiol, and E₁ levels, serum ER α -mediated bioactivity remained associated with increased breast cancer risk. Compared with those in the lowest quartile, women in the highest quartile for ER α -mediated bioactivity had an odds ratio of 2.39 (95% CI = 1.17–4.88; *P* for trend = 0.016). Conversely, the positive association between E₁ and cancer risk became null after adjustment for ER α -mediated bioactivity, suggesting that the effect of E₁ could be mediated through ER α . Factor(s) contributing to increased ER α -mediated estrogenic bioactivity in serum and its role as a predictor for breast cancer risk need to be validated in future studies.

Key Words

- ▶ breast cancer
- ▶ estrogen
- ▶ estrogen receptor
- ▶ estrogen receptor bioassay

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Introduction

There is ample experimental, epidemiological, and clinical evidence linking estrogens and breast cancer risk (Henderson & Feigelson 2000, Russo & Russo 2006). Several established risk factors for breast cancer are

strongly associated with sex hormone levels, suggesting that these factors affect estrogen signaling pathways to impact breast cancer risk (Key *et al.* 2011). Endogenous hormone levels have been used to estimate breast cancer

risk in nine previous prospective studies carried out in postmenopausal women (Key *et al.* 2002). An analysis of pooled data from these studies indicated that postmenopausal women with relatively high serum levels of sex hormones such as estradiol (E_2) and testosterone had a roughly twofold higher risk of breast cancer compared with those with lower levels. Of these studies, all, but one in Japan (Kabuto *et al.* 2000), were conducted in North America or Europe where women are known to have a higher BMI compared with their leaner Asian counterparts and also more likely to use hormone replacement therapy. Obesity could contribute to higher estrogen levels, as adipose tissues are a source of estrogens in postmenopausal women (Siiteri 1987). Furthermore, marked differences in circulating estrogen levels have been reported between Asian and Caucasian women (Bernstein *et al.* 1990, Shimizu *et al.* 1990, Wu & Pike 1995).

Estrogen receptor α (ER α) and ER β bioassays are classically used to identify estrogenic compounds present in the environment or to detect persistent organic pollutants in the serum of humans (Hjelmborg *et al.* 2006, Kanno *et al.* 2007, Kruger *et al.* 2008, Djiogue *et al.* 2010, Du *et al.* 2010). Recently, using the ER α assay, it has been demonstrated that ER α -mediated bioactivity in serum can predict hip fracture risk in menopausal women (Lim *et al.* 2012). The strength of such ER bioassays is that the overall effects of all factors (known and unknown) on ER activation can be quantified. Therefore, these ER bioassays measure the summated activity of all agonists and antagonists that either activate or inhibit the ERs.

In terms of breast cancer, the bioactivities of both ER α and ER β have been shown to be independently associated with increased risk in European studies (Widschwendter *et al.* 2009, Fourkala *et al.* 2012). Specifically, in a case-control study conducted in Germany, women in the highest quintile of both serum ER α and ER β bioactivities had approximately seven times higher breast cancer risk compared with those in the lower quintile (Widschwendter *et al.* 2009). More recently, in a case-control study nested in a cohort study of women in the UK, ER α bioactivity has been shown to be independently associated with breast cancer risk using serum collected more than 2 years before diagnosis for breast cancer cases (Fourkala *et al.* 2012). These studies suggest that assays for serum ER α and ER β bioactivities may be useful for the prediction and management of breast cancer at a population level as well as in a clinical setting.

Singapore Chinese women are currently experiencing one of the highest rates of increase in breast cancer

incidence globally. Over the past 35 years from 1970 to 2005, the incidence of breast cancer in Singapore has tripled from 20.0 to 60.0/100 000 (National Registry of Diseases Office 2008). Factors causing the rapid increase in this historically low-risk population are largely unclear. Utilizing prospectively collected questionnaire data and serum collected from women of a population-based cohort study in Singapore, in the present study, we investigated the associations between estrogen and ER-mediated bioactivity levels and breast cancer risk among postmenopausal women in an Asian country. We provide evidence to suggest that factors other than estrone (E_1) and E_2 may activate ER α -mediated signaling pathways to increase breast cancer risk.

Subjects and methods

Study population

Both cases and controls were participants of the Singapore Chinese Health Study, a population-based prospective cohort study carried out in 63 257 Chinese subjects (including 35 298 women) who were aged 45–74 years during recruitment between April 1993 and December 1998 (Hankin *et al.* 2001). All the participants of this cohort study were residents of government-subsidized housing schemes, in which the majority (86%) of Singaporeans resided at the time of recruitment. Study subjects were restricted to the two major Chinese dialect groups: Hokkien and Cantonese, who originated from two contiguous prefectures in southern China. The Institutional Review Board of the National University of Singapore approved this study.

During recruitment, the subjects were interviewed in-person using a structured questionnaire to collect information on tobacco use and medical history as well as a dietary component to assess current intake patterns. In addition, information on menstrual (including menopausal status) and reproductive (including menopausal hormone therapy use) histories was collected from the women. Between April 1994 and December 1999, blood and single-void urine specimens were collected from a random 3% sample of study enrollees. Details of biospecimen collection, processing, and storage procedures have been described previously (Koh *et al.* 2003). Between January 2000 and April 2005, we extended our biospecimen collection to all surviving cohort members and collected biospecimens (blood/buccal cells and urine) from 32 575 participants, representing a consent rate of about 60% of surviving cohort participants at that time.

Among the 35 298 women in this cohort, 15 415 (44%) donated blood for research. Only postmenopausal women without a history of breast cancer during recruitment were included in the present study.

Case ascertainment

Incident breast cancer cases were identified through the population-based cancer registry in Singapore (National Registry of Diseases Office 2008). All cases were further verified by manual checking of pathological and medical records. As of 28 June 2010, among 25 584 women who were postmenopausal during recruitment, 573 had developed breast cancer. Among them, 169 women with breast cancer had donated blood before cancer diagnosis and were included as cases in this study. Compared with the other breast cancer patients who did not donate a blood sample, cases in this study were younger at diagnosis (66.7 vs 64.9 years). Patients who did not donate blood samples were less educated (45.3% had no formal education) than those who did (20.7% had no formal education). Those who did not donate biospecimens were also more likely to have advanced stage of breast cancer, but less likely to use hormone replacement therapy compared with those who donated. Otherwise, there was no significant difference in BMI or prevalence of positive family history for breast cancer between the two groups of breast cancer patients.

Control selection

For each of the 169 cases, up to three control subjects were randomly selected among all the female cohort participants who had donated blood samples and who were alive and free of breast cancer at the time of cancer diagnosis of their index case. The chosen controls were matched to the index case on age at study enrollment (± 3 years), dialect group (Hokkien and Cantonese), and dates of study enrollment (± 2 years) and of blood collection (± 6 months). Among the 169 cases, there were 19 cases having only one eligible control each and 43 cases having only two controls each. The other 107 cases had three controls each.

Blood analysis

Serum samples of a given matched set (containing the samples from the case and one to three matched controls) were arranged in a random order, identified only by unique codes, and tested in the same laboratory batch for all measurements. Laboratory personnel were blinded to case

or control status of the samples. Serum samples were thawed at 4 °C and individually filtered via 0.22 μ m sterile cartridges. Filtered sera were collected in aliquots for measurements of E₁, E₂, sex hormone-binding globulin (SHBG), and ER α - and ER β -mediated bioactivity levels.

Serum E₁ and E₂ levels were measured with liquid chromatography–tandem mass spectrometry (LC–MS/MS) using d₄-E₁ and d₅-E₂ as internal standards, as reported previously (Nelson *et al.* 2004). The respective intra-assay and inter-assay variability (coefficient of variation (%)) ranges for E₂ were 3.9–14.5% (mean=8.7%) and 1.2–13.3% (mean=6.3%) under the condition of 10–1000 pM E₂ respectively. The corresponding values for E₁ were 1.3–15.7% (mean=8.2%) and 1.7–11.5% (mean=5.4%) under the condition of 25–1000 pM E₁.

Serum SHBG levels were quantified with a solid-phase, two-site chemiluminescent immunoassay using the Immulite Analyzer (Siemens Medical Solutions USA Inc., Malvern, PA, USA). The solid phase was a polystyrene bead with a MAB specific for SHBG. The intra-assay and inter-assay relative standard deviation (RSD) values for SHBG in the range of 2–18 nM were 6.7 and 2.0% respectively. The percent of free E₂ was calculated from serum SHBG levels based on the following regression model: percent of free E₂ = $-0.01533 \times \text{serum SHBG} + 2.921$ (Langley *et al.* 1985). Free E₂ level for each subject was then computed as the product of percent of free E₂ and total E₂ levels.

The biological activity of estrogens in serum was assessed using a validated estrogen-driven recombinant cell bioassay (Wong *et al.* 2007, Li *et al.* 2009). Human uterine cervical HeLa cells (ATCC, Manassas, VA, USA) were transformed to stably express either ER α (ESR1) or ER β (ESR2). When exposed to test sera, luciferase activity expressed from these transformed HeLa cells reflects estrogenic bioactivity through four tandem copies of a consensus estrogen response element coupled to a luciferase gene stably incorporated into the genome. Such luciferase activity reflects the summated bioactivity of estrogenic ligands in serum. The expression levels of ER α or ER β in these recombinant cell lines were confirmed with immunoblotting. Strong expressions of ER α or ER β protein were detected in the ER α or ER β cell lines respectively (Fig. 1A). HeLa cells do not express ER α , and the ER β -stable cell line does not contain any detectable ER α protein. HeLa cells do express low levels of ER β , and the ER α -stable cell line reflects this, although the predominant receptor is ER α . In the presence of the natural ligands E₁ and E₂, the ER α and ER β cell lines exhibit dose-dependent bioactivity (Fig. 1B).

Figure 1

Estrogen receptor (ER) bioassay. (A) Immunoblot of ERα and ERβ proteins in stably transfected HeLa cells. Total cellular proteins were extracted from ERα or ERβ stably transfected HeLa cells, and the presence of ERα and ERβ proteins was detected with specific MABs. MCF-7 and MDA-MB-231 cell lysates were used as positive and negative controls for ERα respectively. HeLa cells are original cells without any transfection of ERα or ERβ.

(B) Dose-dependent activation of luciferase reporter gene in ERα and ERβ cells by estradiol (E₂) and estrone (E₁). Medium with 10% CT FBS was spiked with E₂ and E₁. Readings are expressed as the percent of highest activation of E₂ at 10 nM. (C) Representative calibration curves of ER-driven reporter gene bioassays for the quantification of ER-mediated bioactivity of serum samples.

Recombinant cells were passaged, plated in a 96-well plate in 10% charcoal-treated (CT) fetal bovine serum (FBS), and allowed to adhere overnight. Culture medium was then removed and replaced with incremental amounts of E₂ in 10% male human serum (Sigma) stripped of steroids using charcoal treatment for calibration standards. Stock solutions of E₂ were prepared in serum and diluted to 10% using culture medium (Eagle's Minimum Essential Medium), and duplicates of each concentration were included in the plate. Similarly, test serum was also diluted to 10% using culture medium and added to the cells in duplicates. Baseline level of E₂ in CT human serum is about 20 pM tested using LC-MS/MS. After an incubation period of 24 h, the medium was decanted, and the cells were washed with PBS and lysed with the Mammalian Protein Extraction Reagent (MPER) obtained from Thermo Scientific (Rockford, IL, USA). Luciferase activity was measured using the Luciferase Assay System (Promega) on the Glomax 96-well Microplate Luminometer (Promega).

All test sera were tested on the same day using the same batch of cells. Calibration standards and quality control (QC) samples were included in every 96-well plate. Readings from the plate were used if QC samples in that plate had relative errors <30%. If the relative error was >30%, the assay was repeated. The mean (s.d.) of relative errors for ERα bioassay was 5.85% (5.16) and for ERβ bioassay was 11.51% (7.87).

Calibration curves were fitted with a regression model that visually best fits the points within the range of the test samples and maximizes the correlation coefficient (R^2) value. The R^2 values for ERα and ERβ ranged from 0.96 to 0.99. Total estrogen-mediated activity was calculated based on the average luminescence readings for each test serum, and it is expressed as pM E₂ equivalent obtained by interpolation from calibration curves in each plate. Microsoft Excel Software was used for curve fitting and interpolation. For the ERα bioassay, the calibration curve ranged from 5 to 70 pM, with intra-assay and inter-assay RSD values of 6 and 14% respectively (Fig. 1C, upper panel). For the ERβ bioassay, the calibration curve ranged from 5 to 250 pM, and the intra-assay and inter-assay RSD values were 8.1 and 16% respectively (Fig. 1C, lower panel). Five serum samples had ERβ values below the detection limit and were thus omitted from the analysis.

Statistical analysis

The distributions of all biomarkers measured were markedly skewed with a long tail toward high values, which were corrected, to a large extent, by transforming the original values to logarithmic values. Therefore, a formal statistical test was carried out on logarithmically transformed values and geometric (as opposed to arithmetic) means were obtained. The analysis of covariance (ANCOVA) method was used to examine the differences in

Table 1 Baseline characteristics of breast cancer cases and controls (mean (s.d.) or number (%)), the Singapore Chinese Health Study

	Cases (n=169)	Controls (n=426)
Mean age at blood draw (years)	60.9 (7.2)	60.0 (6.5)
BMI (kg/m ²)		
<20	16 (9.5)	45 (10.6)
20–24	86 (50.9)	246 (57.8)
24–28	50 (29.6)	109 (25.6)
28+	17 (10.1)	26 (6.1)
Dialect (%)		
Cantonese	96 (56.8)	249 (58.5)
Hokkien	73 (43.2)	177 (41.5)
Level of education (%)		
No formal education	35 (20.7)	109 (25.6)
Primary school	81 (47.9)	195 (45.8)
Secondary and above	53 (31.4)	122 (28.6)
Age at menarche (years)		
<13	27 (16.0)	82 (19.3)
13–14	77 (45.6)	164 (38.5)
15–16	52 (30.8)	139 (32.6)
17+	13 (7.6)	41 (9.6)
Number of live births		
None	20 (11.8)	32 (7.5)
1–2	61 (36.1)	134 (31.5)
3–4	54 (31.9)	184 (43.2)
5+	34 (20.1)	76 (17.8)
Use of menopausal hormone therapy (%)	18 (10.7)	26 (6.1)
Family history of breast cancer	4 (2.4)	5 (1.2)

the levels of serum biomarkers between breast cancer cases and control subjects with adjustment for other covariates, namely BMI (<20, 20–<24, 24–<28, and 28+ kg/m²), number of live births (none, one to two, three to four, and five or more), age at menarche (<13, 13–14, 15–16, and 17+ years), use of hormone replacement (yes, no) and family history of breast cancer (yes, no). We also included set number as a covariate to account for the matched case–control design in this study.

We used the conditional logistic regression method to examine the associations between serum biomarkers

measured and breast cancer risk in our main analysis. For subanalysis involving stratification by receptor positivity of breast cancer cases and time between blood draw and diagnosis, unconditional logistic regression models that included all the controls in this study were used to examine the association between serum biomarkers and breast cancer risk. In this study, our aim was to determine how estrogen and bioactivity levels in cases compared with those in the controls might affect breast cancer risk. Hence, study subjects were grouped into quartiles of individual serum parameters based on their distributions among control subjects because they formed the baseline or comparison group. Furthermore, in the general population, as the number of women without breast cancer far exceeds that of those with breast cancer, the levels in a population are essentially defined by the levels in the controls. The magnitude of the association was assessed by odds ratio (OR) and its corresponding 95% CI and *P* value. An additional analysis also included quartile levels of E₁, free E₂, SHBG, and ERα- and ERβ-mediated bioactivities in the same model. For the unconditional logistic regression analyses, age at blood draw was included as a covariate. All the analyses were carried out using the SAS, version 9.1 (SAS Institute, Inc., Cary, NC, USA). All *P* values reported are two sided. The statistical significance level was set at a two-sided *P* value of 0.05.

Results

For the 169 breast cancer cases, the mean time interval from blood draw to breast cancer diagnosis was 4.0 years (s.d. 2.5 years), and only 43 cases (25%) had blood drawn within 2 years of cancer diagnosis. The mean age at cancer diagnosis was 64.9 (s.d. 7.5; range 48.3–82.0) years. Compared with the controls, a higher proportion of women with breast cancer had BMI ≥24 kg/m², had secondary school education or higher, were nulliparous or had fewer live births, and were older at the first live

Table 2 Geometric means (95% CI) of serum biomarkers in postmenopausal breast cancer cases and controls, the Singapore Chinese Health Study

	Cases (n=169)	Controls (n=426)	Two-sided <i>P</i> ^a
Estrone (pM)	404.70 (355–461.36)	335.96 (308.28–366.12)	0.02
Estradiol (E ₂ ; pM)	66.26 (57.26–76.66)	58.82 (53.46–64.74)	0.19
SHBG (nM)	44.60 (41.32–48.16)	47.62 (45.3–50.08)	0.16
Free E ₂ (pM)	1.38 (1.2–1.6)	1.16 (1.06–1.28)	0.05
ERα activity (pM E ₂ equivalent)	25.54 (24.66–26.44)	24.40 (23.84–24.96)	0.03
ERβ activity (pM E ₂ equivalent)	25.22 (24.24–26.24)	24.22 (23.6–24.86)	0.10

^aAdjusted for BMI, number of live births, age at menarche, use of hormone replacement therapy, and family history of breast cancer.

birth, which were observations similar to previous results published for this cohort of women (Koh *et al.* 2003). Only 6.1% among the controls and 10.7% among the cases used hormone replacement therapy (Table 1).

Breast cancer cases tended to have increased circulating serum E₁, E₂, free E₂, and ER α - and ER β -mediated bioactivity levels, but lower serum SHBG levels than the controls, although only the differences for E₁, free E₂, and ER α -mediated bioactivity levels reached statistical significance (Table 2). After adjusting for BMI, number of live births, age at menarche, use of hormone replacement, and family history of breast cancer, borderline dose-dependent activity was observed for E₁, which exhibited an association with a 60% increase in risk for women

in the highest quartile relative to those in quartile 1 (OR, 1.60; 95% CI, 0.94–2.72; *P* for trend=0.05; Table 3). There were no clear dose-dependent associations between serum total E₂, free E₂, SHBG, and ER β -mediated activity levels and breast cancer risk. Strikingly, compared with that in women in the lowest quartile, ER α -mediated bioactivity was associated with a dose-dependent increase in breast cancer risk, ranging from a 45% higher risk in women in quartile 2 to a 159% higher risk in those in the highest quartile (OR, 2.59; 95% CI, 1.44–4.65; *P* for trend=0.001; Table 3). Reanalysis done with a minimal value (20 pM) assigned to five values of ER β below the limit of detection yielded essentially the same results; there was no significant association between serum ER β

Table 3 Association between serum estrogenic parameters and postmenopausal breast cancer risk, the Singapore Chinese Health Study

Biomarker	Range	Cases	Controls	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)
Estrone						
Q1	23.65–205.5	37	106	1.00	1.00	1.00
Q2	207.5–284.0	33	106	0.86 (0.49–1.51)	0.87 (0.49–1.54)	0.83 (0.45–1.51)
Q3	285.5–455.5	39	107	1.02 (0.59–1.75)	1.10 (0.63–1.92)	0.90 (0.49–1.66)
Q4	≥457.5	60	105	1.63 (0.97–2.73)	1.60 (0.94–2.72)	1.32 (0.63–2.75)
<i>P</i> for trend				0.04	0.05	0.54
Estradiol						
Q1	4.365–34.3	33	106	1.00	1.00	1.00
Q2	34.4–50.25	42	107	1.21 (0.69–2.14)	1.15 (0.64–2.06)	1.01 (0.54–1.89)
Q3	50.35–76.6	47	105	1.42 (0.80–2.50)	1.43 (0.80–2.54)	1.17 (0.61–2.26)
Q4	≥76.95	47	106	1.40 (0.80–2.46)	1.33 (0.75–2.36)	0.78 (0.37–1.66)
<i>P</i> for trend				0.21	0.26	0.60
SHBG						
Q1	8.39–33.5	55	106	1.00	1.00	1.00
Q2	33.7–47.4	34	106	0.62 (0.37–1.05)	0.65 (0.38–1.11)	0.72 (0.41–1.24)
Q3	47.5–67.53	46	106	0.81 (0.49–1.33)	0.80 (0.47–1.34)	0.94 (0.55–1.61)
Q4	≥68.1	34	106	0.61 (0.36–1.04)	0.65 (0.37–1.12)	0.74 (0.41–1.34)
<i>P</i> for trend				0.16	0.20	0.52
Free estradiol						
Q1	0.065–0.668	30	106	1.00	1.00	1.00
Q2	0.673–1.04	42	106	1.33 (0.75–2.37)	1.29 (0.73–2.30)	1.07 (0.58–1.96)
Q3	1.045–1.661	51	106	1.71 (0.97–3.00)	1.72 (0.97–3.03)	1.37 (0.74–2.54)
Q4	≥1.666	46	106	1.56 (0.88–2.76)	1.47 (0.83–2.61)	0.78 (0.37–1.63)
<i>P</i> for trend				0.09	0.13	0.77
ER α activity						
Q1	12.39–21.01	26	107	1.00	1.00	1.00
Q2	21.03–23.63	38	106	1.47 (0.81–2.66)	1.45 (0.79–2.64)	1.30 (0.69–2.45)
Q3	23.66–26.64	41	106	1.64 (0.91–2.96)	1.60 (0.88–2.92)	1.44 (0.74–2.81)
Q4	≥26.65	64	106	2.80 (1.58–4.97)	2.59 (1.44–4.65)	2.39 (1.17–4.88)
<i>P</i> for trend				0.0004	0.001	0.016
ER β activity						
Q1	11.03–20.16	37	104	1.00	1.00	1.00
Q2	20.22–24.46	40	104	1.07 (0.59–1.91)	1.01 (0.56–1.84)	0.99 (0.54–1.82)
Q3	24.47–27.48	33	104	0.96 (0.48–1.94)	0.96 (0.47–1.95)	0.94 (0.46–1.94)
Q4	≥27.51	54	104	1.74 (0.89–3.41)	1.53 (0.77–3.04)	1.41 (0.69–2.88)
<i>P</i> for trend				0.08	0.19	0.31

Model 1, unadjusted model; Model 2, adjusted for BMI (<20, 20–<24, 24–<28, and 28+ kg/m²), number of live births (none, one to two, three to four, and five or more), age at menarche (<13, 13–14, 15–16, and 17+ years), use of hormone replacement therapy (yes, no), and family history of breast cancer (yes, no); Model 3, adjusted for covariates in Model 1 and also quartile values of serum estrone, free estradiol or total estradiol (for SHBG), SHBG, ER α activity, and ER β activity; OR, odds ratio.

Table 4 Association between serum ER α -mediated bioactivity and postmenopausal breast cancer risk according to ER status of cancer cases, the Singapore Chinese Health Study

Biomarker	Cases	Controls	OR (95% CI)*
ER-positive cancer			
ER α activity			
Q1	11	107	1.00
Q2	17	106	1.46 (0.63–3.35)
Q3	13	106	0.95 (0.39–2.34)
Q4	28	106	2.43 (1.00–5.94)
P for trend			0.10
ER-negative cancer			
ER α activity			
Q1	5	107	1.00
Q2	7	106	2.28 (0.66–7.88)
Q3	9	106	3.26 (0.97–10.95)
Q4	9	106	3.63 (1.01–13.08)
P for trend			0.04
ER unknown cancer			
ER α activity			
Q1	10	107	1.00
Q2	14	106	1.27 (0.53–3.07)
Q3	19	106	1.56 (0.66–3.70)
Q4	27	106	1.71 (0.70–4.20)
P for trend			0.21

*Adjusted for BMI (<20, 20–<24, 24–<28, and 28+ kg/m²), number of live births (none, one to two, three to four, and five or more), age at menarche (<13, 13–14, 15–16, and 17+ years), use of hormone replacement therapy (yes, no), family history of breast cancer (yes, no), and quartile value of estrone and free estradiol levels. OR, odds ratio.

levels and breast cancer risk. To examine the independent effect of individual estrogenic factors measured on breast cancer risk, further adjustment for E₁, free E₂, SHBG, and ER α - and ER β -mediated bioactivity levels was done (Table 3, last column). After adjusting for ER α -mediated activity, the dose-dependent relationship between E₁ and breast cancer risk was no longer evident; the ORs (95% CI) for those in quartiles 2, 3, and 4 were 0.80 (0.45–1.42), 0.88 (0.49–1.57), and 1.08 (0.58–1.99) respectively (*P* for trend=0.74), suggesting that ER α -mediated bioactivity was primarily responsible for the effect of E₁ on breast cancer risk. Additional adjustment for free E₂, SHBG, and ER β bioactivity levels did not materially change the risk estimates (Table 3). By contrast, even after adjusting for E₁, free E₂, SHBG, and ER β bioactivity levels, the strong association with ER α -mediated activity was essentially unaltered with a 2.4-fold increase (OR, 2.39; 95% CI, 1.17–4.88; *P*=0.016) in breast cancer risk (Table 3). To further delineate the role of serum ER α bioactivity, we also carried out an analysis to compare risk in ER α -positive or ER α -negative tumor cases. Data on ER status were available for

58.6% of the breast cancer cases. There were 69 ER α -positive and 30 ER α -negative breast cancer diagnoses. The results suggested that the positive association between ER α -mediated activity and breast cancer risk was present in both subtypes of breast cancer. Compared with those of women in the lowest quartile, the ORs (95% CI) of women in the highest quartile were statistically significant at 2.43 (1.00–5.94) for ER-positive breast cancer and 3.63 (1.01–13.08) for ER-negative breast cancer (Table 4).

Finally, we examined the association between ER α -mediated activity and breast cancer risk by time between blood draw to cancer diagnosis. Risk estimates obtained from the analysis of cases with blood drawn within 2 years of cancer diagnosis were essentially similar to those obtained from the analysis of cases with blood drawn more than 2 years before cancer diagnosis. Although the *P* for trend was of borderline statistical significance (*P*=0.08) for the analysis limited to cases with blood drawn more than 2 years before cancer diagnosis, relative to those in the lowest quartile, women in the highest quartile in this group still had significantly increased breast cancer risk (OR, 2.15; 95% CI, 1.07–4.33; Table 5).

Discussion

This is the first study to investigate the effect of estrogens and ER α - and ER β -mediated bioactivities on the risk of breast cancer in Chinese postmenopausal women.

Table 5 Association between serum ER α -mediated bioactivity and postmenopausal breast cancer risk according to time between blood draw and cancer diagnosis for cases, the Singapore Chinese Health Study

Biomarker	Cases	Controls	OR (95% CI)*
Within 2 years of blood draw and cancer diagnosis			
ER α activity			
Q1	8	107	1.00
Q2	4	106	0.57 (0.16–2.02)
Q3	11	106	1.46 (0.53–4.03)
Q4	20	106	2.67 (0.94–7.54)
P for trend			0.02
More than 2 years between blood draw and cancer diagnosis			
ER α activity			
Q1	18	107	1.00
Q2	34	106	1.83 (0.95–3.50)
Q3	30	106	1.48 (0.75–2.91)
Q4	44	106	2.15 (1.07–4.33)
P for trend			0.08

*Adjusted for BMI (<20, 20–<24, 24–<28, and 28+ kg/m²), number of live births (none, one to two, three to four, and five or more), age at menarche (<13, 13–14, 15–16, and 17+ years), use of hormone replacement therapy (yes, no), family history of breast cancer (yes, no), and quartile value of estrone and free estradiol levels. OR, odds ratio.

Our data demonstrated that higher levels of ER α -mediated bioactivity in sera were associated with an increased risk of cancer and that estrogens, especially E₁, influenced cancer risk via interaction with ER α in the pathogenesis of postmenopausal breast cancer.

The geometric means of total free E₂, E₁, and ER α bioactivity levels were significantly higher in pre-disease serum collected from breast cancer cases than in that from controls. However, after dividing the biomarker levels into quartiles and assessing their association with breast cancer risk, a borderline dose-dependent relationship was also observed with the highest quartile of E₁ exhibiting a 60% higher risk of breast cancer. Although high E₂ levels are known to be associated with a higher risk of breast cancer in postmenopausal women (Kabuto *et al.* 2000, Key *et al.* 2003, Manjer *et al.* 2003, Missmer *et al.* 2004, Zeleniuch-Jacquotte *et al.* 2004, Kaaks *et al.* 2005, Eliassen *et al.* 2006, Baglietto *et al.* 2010, Farhat *et al.* 2011), the role of E₁ in breast cancer carcinogenesis is less well appreciated. There is evidence that E₁ levels, but not E₂, can be higher depending on lifestyle factors. Japanese women born in the USA have mean E₁ levels higher than those in Caucasian counterparts, contrasting with the largely similar mean E₂ levels between these two populations (Probst-Hensch *et al.* 2000). In that study, differences in E₁ levels were still evident after adjustment for age, weight, and androstenedione levels. On the other hand, Japanese women living in rural areas were found to have 43% lower E₁ levels compared with weight- and age-matched Caucasian women living in California (Wu & Pike 1995), suggesting that the transition from a rural to an urban lifestyle may have a contributory effect on high E₁ levels. One lifestyle factor may be shift work, as women working graveyard shifts have been reported to have significantly higher E₁ levels (20 vs 11.5 pg/ml) compared with those who never worked night shifts (Nagata *et al.* 2008). Although E₁ has 20–80% of the bioactivity of E₂ depending on the assay used (Fang *et al.* 2000), its higher levels indicate that its contribution to overall estrogenicity and breast cancer risk is significant in the postmenopausal condition in our cohort. The challenge is to define the role of lifestyle modifications that may lower E₁ levels and breast cancer risk. The loss of statistically significant association with E₁ levels in this study was primarily due to adjustment for ER α activity, and we deduced that the effect of E₁ in the pathogenesis of postmenopausal breast cancer could be mediated via its binding to ER α .

Conversely, ER α -mediated bioactivity appeared to be an independent risk factor for breast cancer risk. Logistic regression analyses indicated that even after adjustment

for the known factors of estrogenic action such as E₂, E₁, and BMI, women whose ER α -mediated bioactivity was in the highest quartile still had significantly higher breast cancer risk compared with those in the lowest quartile, suggesting that other factor(s), besides E₁ and E₂, was acting via the ER α -mediated genomic signaling systems to increase breast cancer risk. This dose-dependent increase in risk with high ER α -mediated bioactivity was evident in periods before 4 years and within 2 years of blood draw, indicating the robustness of the association. To our knowledge, this is the first study to use mammalian cell-based bioassays to prospectively examine the relationship between ER α -mediated bioactivity and breast cancer risk. One recent UK study, utilizing a yeast-based reporter gene assay system, did not observe any overall relationship between cancer risk and receptor bioactivity. In this study (Fourkala *et al.* 2012), an association between ER α -mediated bioactivity and breast cancer risk was only present in the subset of cases whose blood was collected more than 2 years before cancer diagnosis (Fourkala *et al.* 2012). This contradicted the findings of a case-control study that the same group of investigators had conducted in Germany using blood collected from cases after clinical diagnosis and showing a strong association between ER α bioactivity and cancer risk (Widschwendter *et al.* 2009). Differences between our study and the study carried out by the UK group could be due to the use of mammalian cells by us, which are more physiologically relevant to differentiate between agonists and antagonists compared with yeast cell-based bioassays used by other investigators (Widschwendter *et al.* 2009, Fourkala *et al.* 2012). In addition, the co-regulators involved in the transactivation of estrogen-sensitive reporter genes has been reported to differ between yeast and mammalian systems (Kohnno *et al.* 1994).

Although the two most abundant estrogens present in the serum (E₂ and E₁) can bind to ER α and activate it, many other compounds in the serum are also known to be capable of activating the receptor. These include estrogen metabolites from birth control pills and endocrine-disrupting compounds (EDCs) that have estrogenic activity. A wide range of synthetic endocrine-disrupting chemicals such as dioxins and polychlorinated biphenyls, bisphenol A, and pesticides (endosulfan, toxaphene, and dieldrin) are estrogenic compounds that can exert biological effects at trace concentrations and have the potential to provoke additive estrogenic mixture effects at low doses, even at no observed effect levels (Diamanti-Kandarakis *et al.* 2009, Kandaraki *et al.* 2011). Thus, ER α -mediated bioactivity could be a more comprehensive

measurement of the combined effects of all known and unknown estrogenic compounds present in the sera of women, all of which could affect breast cancer risk. Our data indicating that the risk due to increased ER α -mediated bioactivity was independent of endogenous estrogens support the hypothesis that environmental factors may have a contributory role in breast cancer risk in our cohort. More research is warranted to identify these EDCs. This is consistent with the rapid increase in breast cancer rates in rapidly modernizing Singapore.

While the UK study included only ER-positive cases (Fourkala *et al.* 2012), our study included both ER-positive and ER-negative breast cancer cases and showed that ER α -mediated bioactivity was associated with increased risk for both subtypes of cancers, suggesting that ER-negative cancer also depends on estrogenic activity for growth. Intriguingly, risk was highest in patients with ER α -negative tumors, increasing 15-fold within the highest quartile of ER α bioactivity, suggesting that compounds that were activating the ER α genomic signaling cascade were also capable of activating other estrogenic signaling pathways in breast cancer tissues with absent or significantly lower levels of ER α . Reports from earlier studies showed that ovariectomy prevented the formation of both ER α -positive and ER α -negative breast cancers, thus suggesting that the estrogen-driven pathway could also play a role in the development of ER-negative breast cancer (Early Breast Cancer Trialists' Collaborative Group 1992). Hence, we hypothesize that our mammalian reporter gene assay has reflected the presence of estrogenic compounds present in the serum of ER α -negative breast cancer patients that may activate estrogen signaling in the absence of intact ER α . One example is membrane-bound ER α 36, a truncated estrogen-sensitive receptor present in ER-negative breast cancer, which can mediate nongenomic estrogen signaling in the carcinogenesis of ER-negative tumors (Rao *et al.* 2011).

Although our data were suggestive of a 1.5-fold increase in the risk of breast cancer for women in the highest quartile, the association of breast cancer risk with ER β bioactivity did not reach statistical significance and was weaker than that with ER α bioactivity. The role of ER β in breast cancer has been controversial. Breast cancer patients who are treated with tamoxifen and have high expression levels of ER β were found to have better response and longer survival time (Esslimani-Sahla *et al.* 2004, Hopp *et al.* 2004). On the other hand, in ER α -negative breast cancer, high ER β expression is positively correlated with poor prognostic phenotypes (Skiris *et al.* 2006). Some of these ligands with preferential binding to

ER β have been shown to be associated with decreased breast cancer risk. For example, genistein is a soy phytoestrogen possessing high affinity for ER β (Lee *et al.* 2004), and higher soy intake has been shown to be associated with a reduced risk of breast cancer (Iwasaki *et al.* 2008, Wu *et al.* 2008).

The strength of this study is the nesting of the study within a population-based prospective cohort study that allows the use of questionnaire data and blood specimens collected before the occurrence of breast cancer to reduce recall and reverse causality bias. Cancer cases were identified using a comprehensive nationwide cancer registry. The limitations of this study are that the results are based on blood samples collected at a single time point, as the natural fluctuation of biomarkers that most probably occurred equally in both cases and controls could lead to the underestimation of the true associations with breast cancer risk. In this cohort, among postmenopausal women who gave blood for research, we limited the selection of cases to women who donated blood before the occurrence of breast cancer. Differences in factors such as age and hormone replacement therapy use between breast cancer cases included and those excluded in this study were accounted for either by matching or by statistical adjustment in our analyses. Furthermore, willingness to donate blood for research did not influence the biomarker–breast cancer association in this study. In addition, we acknowledge that the presence of existing occult tumors could play a role in the association between ER α -mediated bioactivity and breast cancer risk in this group of patients with an average follow-up period of 4 years. Finally, the relatively small sample size, especially in the stratification by ER positivity, does not give us sufficient power to detect significant differences in the levels of most of the blood parameters between cases and controls.

In conclusion, ER α -mediated bioactivity in sera was independently associated with a significantly increased risk of postmenopausal breast cancer. The measurement of this serum biomarker in postmenopausal women may have the potential to be developed into a clinical index for the prediction of breast cancer risk or the monitoring of patients on chemopreventive therapy for breast cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

W-P Koh and E L Yong conceived the study. V W Lim, J Li, Y Gong, and E L Yong carried out the biomarker assays. A Jin, J-M Yuan, and W-P Koh carried out the statistical analysis. V W Lim, J-M Yuan, W-P Koh, and E L Yong drafted the manuscript. All authors edited and approved the final manuscript.

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References

- Baglietto L, Severi G, English DR, Krishnan K, Hopper JL, McLean C, Morris HA, Tilley WD & Giles GG 2010 Circulating steroid hormone levels and risk of breast cancer for postmenopausal women. *Cancer Epidemiology, Biomarkers & Prevention* **19** 492–502. (doi:10.1158/1055-9965.EPI-09-0532)
- Bernstein L, Yuan JM, Ross RK, Pike MC, Hanisch R, Lobo R, Stanczyk F, Gao YT & Henderson BE 1990 Serum hormone levels in premenopausal Chinese women in Shanghai and white women in Los Angeles: results from two breast cancer case-control studies. *Cancer Causes & Control* **1** 51–58. (doi:10.1007/BF00053183)
- Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT & Gore AC 2009 Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine Reviews* **30** 293–342. (doi:10.1210/er.2009-0002)
- Djogue S, Njamen D, Halabalaki M, Kretzschmar G, Beyer A, Mbanya JC, Skaltsounis AL & Vollmer G 2010 Estrogenic properties of naturally occurring prenylated isoflavones in U2OS human osteosarcoma cells: structure-activity relationships. *Journal of Steroid Biochemistry and Molecular Biology* **120** 184–191. (doi:10.1016/j.jsbmb.2010.04.014)
- Du G, Shen O, Sun H, Fei J, Lu C, Song L, Xia Y, Wang S & Wang X 2010 Assessing hormone receptor activities of pyrethroid insecticides and their metabolites in reporter gene assays. *Toxicological Sciences* **116** 58–66. (doi:10.1093/toxsci/kfq120)
- Early Breast Cancer Trialists' Collaborative Group 1992 Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet* **339** 71–85.
- Eliassen AH, Missmer SA, Tworoger SS & Hankinson SE 2006 Endogenous steroid hormone concentrations and risk of breast cancer: does the association vary by a woman's predicted breast cancer risk? *Journal of Clinical Oncology* **24** 1823–1830. (doi:10.1200/JCO.2005.03.7432)
- Esslimani-Sahla M, Simony-Lafontaine J, Kramar A, Lavaill R, Mollevi C, Warner M, Gustafsson J-A & Rochefort H 2004 Estrogen receptor β (ER β) level but not its ER β cx variant helps to predict tamoxifen resistance in breast cancer. *Clinical Cancer Research* **10** 5769–5776. (doi:10.1158/1078-0432.CCR-04-0389)
- Fang H, Tong W, Perkins R, Soto AM, Prechtel NV & Sheehan DM 2000 Quantitative comparisons of *in vitro* assays for estrogenic activities. *Environmental Health Perspectives* **108** 723–729. (doi:10.1289/ehp.00108723)
- Farhat GN, Cummings SR, Chlebowski RT, Parimi N, Cauley JA, Rohan TE, Huang AJ, Vitolins M, Hubbell FA, Manson JE et al. 2011 Sex hormone levels and risks of estrogen receptor-negative and estrogen receptor-positive breast cancers. *Journal of the National Cancer Institute* **103** 562–570. (doi:10.1093/jnci/djr031)
- Fourkala EO, Zaikin A, Burnell M, Gentry-Maharaj A, Ford J, Gunu R, Soromani C, Hasenbrink G, Jacobs I, Dawnay A et al. 2012 Association of serum sex steroid receptor bioactivity and sex steroid hormones with breast cancer risk in postmenopausal women. *Endocrine-Related Cancer* **19** 137–147. (doi:10.1530/ERC-11-0310)
- Hankin JH, Stram DO, Arakawa K, Park S, Low SH, Lee HP & Yu MC 2001 Singapore Chinese Health Study: development, validation, and calibration of the quantitative food frequency questionnaire. *Nutrition and Cancer* **39** 187–195. (doi:10.1207/S15327914nc392_5)
- Henderson BE & Feigelson HS 2000 Hormonal carcinogenesis. *Carcinogenesis* **21** 427–433. (doi:10.1093/carcin/21.3.427)
- Hjelmborg PS, Ghisari M & Bonefeld-Jorgensen EC 2006 SPE-HPLC purification of endocrine-disrupting compounds from human serum for assessment of xenoestrogenic activity. *Analytical and Bioanalytical Chemistry* **385** 875–887. (doi:10.1007/s00216-006-0463-9)
- Hopp TA, Weiss HL, Parra IS, Cui Y, Osborne CK & Fuqua SA 2004 Low levels of estrogen receptor β protein predict resistance to tamoxifen therapy in breast cancer. *Clinical Cancer Research* **10** 7490–7499. (doi:10.1158/1078-0432.CCR-04-1114)
- Iwasaki M, Inoue M, Otani T, Sasazuki S, Kurahashi N, Miura T, Yamamoto S, Tsugane S & Japan Public Health Center-based prospective study group 2008 Plasma isoflavone level and subsequent risk of breast cancer among Japanese women: a nested case-control study from the Japan Public Health Center-based prospective study group. *Journal of Clinical Oncology* **26** 1677–1683. (doi:10.1200/JCO.2007.13.9964)
- Kaaks R, Rinaldi S, Key TJ, Berrino F, Peeters PH, Biessy C, Dossus L, Lukanova A, Bingham S, Khaw KT et al. 2005 Postmenopausal serum androgens, oestrogens and breast cancer risk: the European prospective investigation into cancer and nutrition. *Endocrine-Related Cancer* **12** 1071–1082. (doi:10.1677/erc.1.01038)
- Kabuto M, Akiba S, Stevens RG, Neriishi K & Land CE 2000 A prospective study of estradiol and breast cancer in Japanese women. *Cancer Epidemiology, Biomarkers & Prevention* **9** 575–579.
- Kandaraki E, Chatzigeorgiou A, Livadas S, Palioura E, Economou F, Koutsilieris M, Palimeri S, Panidis D & Diamanti-Kandarakis E 2011 Endocrine disruptors and polycystic ovary syndrome (PCOS): elevated serum levels of bisphenol A in women with PCOS. *Journal of Clinical Endocrinology and Metabolism* **96** E480–E484. (doi:10.1210/jc.2010-1658)
- Kanno Y, Okada H, Kobayashi T, Takenaka T & Suzuki H 2007 Effects of endocrine disrupting substance on estrogen receptor gene transcription in dialysis patients. *Therapeutic Apheresis and Dialysis* **11** 262–265. (doi:10.1111/j.1744-9987.2007.00472.x)
- Key T, Appleby P, Barnes I, Reeves G & Endogenous Hormones and Breast Cancer Collaborative Group 2002 Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *Journal of the National Cancer Institute* **94** 606–616. (doi:10.1093/jnci/94.8.606)
- Key TJ, Appleby PN, Reeves GK, Roddam A, Dorgan JF, Longcope C, Stanczyk FZ, Stephenson HE, Falk RT, Miller R et al. 2003 Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *Journal of the National Cancer Institute* **95** 1218–1226. (doi:10.1093/jnci/djg022)
- Key TJ, Appleby PN, Reeves GK, Roddam AW, Helzlsouer KJ, Alberg AJ, Rollison DE, Dorgan JF, Brinton LA, Overvad K et al. 2011 Circulating sex hormones and breast cancer risk factors in postmenopausal women:

- reanalysis of 13 studies. *British Journal of Cancer* **105** 709–722. (doi:10.1038/bjc.2011.254)
- Koh W-P, Yuan J-M, Sun C-L, van den Berg D, Seow A, Lee H-P & Yu MC 2003 Angiotensin I-converting enzyme (ACE) gene polymorphism and breast cancer risk among Chinese women in Singapore. *Cancer Research* **63** 573–578.
- Kohn H, Gandini O, Curtis SW & Korach KS 1994 Anti-estrogen activity in the yeast transcription system: estrogen receptor mediated agonist response. *Steroids* **59** 572–578. (doi:10.1016/0039-128X(94)90050-7)
- Kruger T, Spano M, Long M, Eleuteri P, Rescia M, Hjelmberg PS, Manicardi GC, Bizzaro D, Giwerzman A, Toft G *et al.* 2008 Xenobiotic activity in serum and sperm chromatin integrity in European and Inuit populations. *Molecular Reproduction and Development* **75** 669–680. (doi:10.1002/mrd.20747)
- Langley MS, Hammond GL, Bardsley A, Sellwood RA & Anderson DC 1985 Serum steroid binding proteins and the bioavailability of estradiol in relation to breast diseases. *Journal of the National Cancer Institute* **75** 823–829.
- Lee GS, Choi KC, Kim HJ & Jeung EB 2004 Effect of genistein as a selective estrogen receptor β agonist on the expression of Calbindin-D9k in the uterus of immature rats. *Toxicological Sciences* **82** 451–457. (doi:10.1093/toxsci/kfh296)
- Li J, Lee L, Gong Y, Shen P, Wong SP, Wise SD & Yong EL 2009 Bioassays for estrogenic activity: development and validation of estrogen receptor (ER α /ER β) and breast cancer proliferation bioassays to measure serum estrogenic activity in clinical studies. *Assay and Drug Development Technologies* **7** 80–89. (doi:10.1089/adt.2008.154)
- Lim VW, Li J, Gong Y, Yuan JM, Wu TS, Hammond GL, Jin A, Koh WP & Yong EL 2012 Serum free estradiol and estrogen receptor- α mediated activity are related to decreased incident hip fractures in older women. *Bone* **50** 1311–1316. (doi:10.1016/j.bone.2012.03.006)
- Manjer J, Johansson R, Berglund G, Janzon L, Kaaks R, Agren A & Lenner P 2003 Postmenopausal breast cancer risk in relation to sex steroid hormones, prolactin and SHBG (Sweden). *Cancer Causes & Control* **14** 599–607. (doi:10.1023/A:1025671317220)
- Missmer SA, Eliassen AH, Barbieri RL & Hankinson SE 2004 Endogenous estrogen, androgen, and progesterone concentrations and breast cancer risk among postmenopausal women. *Journal of the National Cancer Institute* **96** 1856–1865. (doi:10.1093/jnci/djh336)
- Nagata C, Nagao Y, Yamamoto S, Shibuya C, Kashiki Y & Shimizu H 2008 Light exposure at night, urinary 6-sulfatoxymelatonin, and serum estrogens and androgens in postmenopausal Japanese women. *Cancer Epidemiology, Biomarkers & Prevention* **17** 1418–1423. (doi:10.1158/1055-9965.EPI-07-0656)
- National Registry of Diseases Office 2008 Singapore Cancer Registry Report No 7: Trends in Cancer Incidence in Singapore 1968–2007. Singapore: Health Promotion Board.
- Nelson RE, Grebe SK, OKane DJ & Singh RJ 2004 Liquid chromatography–tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. *Clinical Chemistry* **50** 373–384. (doi:10.1373/clinchem.2003.025478)
- Probst-Hensch NM, Pike MC, McKean-Cowdin R, Stanczyk FZ, Kolonel LN & Henderson BE 2000 Ethnic differences in post-menopausal plasma oestrogen levels: high oestrone levels in Japanese-American women despite low weight. *British Journal of Cancer* **82** 1867–1870. (doi:10.1054/bjoc.1999.1082)
- Rao J, Jiang X, Wang Y & Chen B 2011 Advances in the understanding of the structure and function of ER- α 36, a novel variant of human estrogen receptor- α . *Journal of Steroid Biochemistry and Molecular Biology* **127** 231–237. (doi:10.1016/j.jsmb.2011.08.004)
- Russo J & Russo IH 2006 The role of estrogen in the initiation of breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **102** 89–96. (doi:10.1016/j.jsmb.2006.09.004)
- Shimizu H, Ross RK, Bernstein L, Pike MC & Henderson BE 1990 Serum oestrogen levels in postmenopausal women: comparison of American whites and Japanese in Japan. *British Journal of Cancer* **62** 451–453. (doi:10.1038/bjc.1990.316)
- Siiteri PK 1987 Adipose tissue as a source of hormones. *American Journal of Clinical Nutrition* **45** 277–282.
- Skliris GP, Leygue E, Curtis-Snell L, Watson PH & Murphy LC 2006 Expression of oestrogen receptor- β in oestrogen receptor- α negative human breast tumours. *British Journal of Cancer* **95** 616–626. (doi:10.1038/sj.bjc.6603295)
- Widschwendter M, Lichtenberg-Frate H, Hasenbrink G, Schwarzer S, Dawnay A, Lam A, Menon U, Apostolidou S, Raum E, Stegmaier C *et al.* 2009 Serum oestrogen receptor α and β bioactivity are independently associated with breast cancer: a proof of principle study. *British Journal of Cancer* **101** 160–165. (doi:10.1038/sj.bjc.6605106)
- Wong SP, Li J, Shen P, Gong Y, Yap SP & Yong EL 2007 Ultrasensitive cell-based bioassay for the measurement of global estrogenic activity of flavonoid mixtures revealing additive, restrictive, and enhanced actions in binary and higher order combinations. *Assay and Drug Development Technologies* **5** 355–362. (doi:10.1089/adt.2007.056)
- Wu AH & Pike MC 1995 Dietary soy protein and hormonal status in females. *American Journal of Clinical Nutrition* **62** 151–153.
- Wu AH, Koh WP, Wang R, Lee HP & Yu MC 2008 Soy intake and breast cancer risk in Singapore Chinese Health Study. *British Journal of Cancer* **99** 196–200. (doi:10.1038/sj.bjc.6604448)
- Zeleniuch-Jacquotte A, Shore RE, Koenig KL, Akhmedkhanov A, Afanasyeva Y, Kato I, Kim MY, Rinaldi S, Kaaks R & Toniolo P 2004 Postmenopausal levels of oestrogen, androgen, and SHBG and breast cancer: long-term results of a prospective study. *British Journal of Cancer* **90** 153–159. (doi:10.1038/sj.bjc.6601517)

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