Association of serum sex steroid receptor bioactivity and sex steroid hormones with breast cancer risk in postmenopausal women

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Abstract

Postmenopausal women with elevated serum sex steroids have an increased risk of breast cancer. Most of this risk is believed to be exerted through binding of the sex steroids to their receptors. For the first time, we investigate the association of estrogen receptor (ER) and androgen receptor (AR) serum bioactivity (SB) in addition to hormone levels in samples from women with breast cancer collected before diagnosis. Two hundred postmenopausal women participating in the UK Collaborative Trial of Ovarian Cancer Screening who developed ER-positive breast cancer 0.6–5 years after sample donation were identified and matched to 400 controls. ER and AR bioassays were used to measure ERα, ERβ, and AR SB. Androgen and estrogen levels were measured with immunoassays. Subjects were classified according to quintiles of the respective marker among controls and the associations between SB and hormones with breast cancer risk were determined by logistic regression analysis. ERα and ERβ SB were significantly higher before diagnosis compared with controls, while estrogens showed no difference. Women had a twofold increased breast cancer risk if ERα SB (odds ratio (OR), 2.114; 95% confidence interval (CI), 1.050–4.425; $P=0.040$) was in the top quintile >2 years before diagnosis or estrone (OR, 2.205; 95% CI, 1.104–4.586; $P=0.029$) was in the top quintile <2 years before diagnosis. AR showed no significant association with breast cancer while androstenedione (OR, 3.187; 95% CI, 1.738–6.044; $P=0.0003$) and testosterone (OR, 2.145; 95% CI, 1.256–3.712; $P=0.006$) were significantly higher compared with controls and showed a strong association with an almost threefold increased breast cancer risk independent of time to diagnosis. This study provides further evidence on the association of androgens and estrogens with breast cancer. In addition, it reports that high ER but not AR SB is associated with increased breast risk >2 years before diagnosis.

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Introduction

Breast cancer remains one of the leading causes of cancer death among women despite the huge progress that has been made in treatment (Santen *et al*. 2007, Weigel & Dowsett 2010). Many risk factors for postmenopausal breast cancer are suggested to mediate their effect through a hormonal mechanism (Henderson & Feigelson 2000). The largest meta-analysis combining nine prospective studies demonstrated that postmenopausal women with serum estrogen and

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androgen levels in the highest quintiles have a twofold increased risk of breast cancer (Key *et al*. 2002). Since then, a number of studies have reported conflicting results on the association of serum sex steroid hormones and breast cancer risk (Lamar *et al*. 2003, Manjer *et al*. 2003, Onland-Moret *et al*. 2003, Missmer *et al*. 2004, Zeleniuch-Jacquotte *et al*. 2004, 2005, Kaaks *et al*. 2005, Tworoger *et al*. 2005, Adly *et al*. 2006, Beattie *et al*. 2006, Eliassen *et al*. 2006, Sieri *et al*. 2009, Baglietto *et al*. 2010). All these reports have used conventional immunoassays to measure hormone levels. In the past few years, bioactivity assays for steroid hormone receptors have been described, enabling quantification of total hormone action (Paris *et al*. 2002, Sievernich *et al*. 2004, Roy *et al*. 2006). As estrogen and androgen hormones exert their effects through binding to sex steroid hormone receptors, we previously hypothesized that bioactivity assays might be an attractive alternative for breast cancer risk assessment. We found that estrogen receptor α (ERα) and ERβ serum bioactivity (SB) are independently associated with breast cancer using samples collected at diagnosis (Widschwendter *et al*. 2009).

To better understand the long-term effect of sex steroids and bioactivity of their receptors on breast cancer risk, it is crucial to examine levels many years before diagnosis. We were able to explore this issue using the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) biobank. Women recruited to the trial between 2001 and 2005 provided blood samples for secondary studies and continue to be followed up by cancer registration and self-reporting (Menon *et al*. 2008, 2009). We report on a nested case–control study using serum samples donated between 6 months and 5 years before diagnosis by women who developed breast cancer after joining the trial and healthy women who had not developed the disease. SB of ERα and ERβ and androgen receptor (AR) were measured using a yeast-based assay along with five sex steroid hormones (estradiol (E_2), estrone, androstenedione, testosterone, and dehydroepiandrosterone sulfate (DHEAS)), free E_2 (fE_2) and free testosterone (fT; calculated by the mass action law), and sex hormone-binding globulin (SHBG) using conventional immunoassays to examine their association with breast cancer risk.

Materials and methods

Cohort

The subjects were participants in the UKCTOCS, a multicenter randomized controlled trial of ovarian cancer screening in England, Wales, and Northern Ireland, coordinated by the Gynecological Cancer Research Centre at University College London (UCL). Women aged 50–74 were recruited through random invitation from age/sex registers of 27 participating Primary Care Trusts. At recruitment, each woman donated a blood sample, filled in a baseline questionnaire, and provided written consent giving permission to access their medical records and use their data/samples in future studies. The questionnaire included questions on demographics, height, weight, parity, hysterectomy, tubal ligation, treatment for infertility, contraceptive pill, hormone replacement treatment (HRT), and previous history of any cancer and family history of ovarian/breast cancer (Menon *et al*. 2008).

Selection of the study sample

All participants are being followed up through a 'flagging study' with the NHS Information Centre for Health and Social Care. Up-to-date cancer registration data were obtained from the agencies on 2nd February 2009 (median follow-up 5.681 years and interquartile range (IQR), 1.284 years). For confirmation of diagnosis, their treating physician was sent a questionnaire requesting information regarding their diagnosis (histology) and treatment. Two hundred women who developed ER-positive invasive breast cancer after joining the UKCTOCS and were not on HRT treatment at recruitment and had donated a serum sample between 6 months and 5 years before diagnosis were chosen as 'cases' for this study. Each breast cancer case was age matched with two women who had no history of breast cancer (controls) at last follow-up and had donated serum samples on the same day and in the same clinic. The UKCTOCS was approved by the UK North West Multicentre Research Ethics Committees (North West MREC 00/8/34). Ethical approval for this nested case–control study was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research (22nd February 2007, 06/Q0505/102).

Serum sample processing

The blood samples were collected into Griener Bio one gel tubes (Cat no: 455071) at the centers, shipped overnight to the central laboratory, and centrifuged at 2000 ***g*** for 10 min. The serum was removed from the cells within 56 h of sample collection and was frozen using a two-stage freezing process: 12 h at −80 °C and then placed in liquid nitrogen (vapor phase) at −180 °C. A novel semi-automated system aliquoted serum in 500 μl straws was then heat sealed, bar coded,

data based, and stored in liquid nitrogen tanks. Two straws were retrieved, one for the measurement of hormone levels and one for the bioactivity assays. The samples were only thawed once, at the time of the assay.

Sex steroid hormone receptor bioactivity using bioassay systems

Sex steroid hormone receptor bioactivity was measured using a yeast-based reporter gene assay that not only determines whether a chemical binds to the receptor, but also whether estrogen- or androgen-dependent gene expression is stimulated. The assay has been described previously (Widschwendter *et al*. 2009). Briefly, the genetically modified yeast cells were incubated in a defined test medium with the reference substance E_2 for ERα and ERβ and dihydrotestosterone for AR test samples and negative controls. At the end of the incubation period the developed green fluorescence was determined and corrected for cell density, optical density (OD) of the cell suspension and blanks. The cell growth was determined by measuring the light absorption at 600 nm and GFP-fluorescence by measuring GFP at 535 nm, specific OD and fluorescence at $t=0$ and $t=16.5$ h for ERα and ERβ and $t=24$ h for AR in each of the 96 wells. Tests were considered as valid if the turbidity of the negative control culture increased five times during the incubation period. The control culture showed no fluorescence. The bioactivity was determined by comparison of the fluorescence development in test cultures vs the calibration curve. The dose–response curves of the reference values were fitted using the Hill equation fit and the R-function. The analysis was performed blind and cases and controls were randomly mixed. Tests were carried out with two replicates at a time on two different days (four readings in total). The lower detection limit for the ER SB is 5 pg/ml and for AR SB is 0.2 ng/ml. The inter-assay coefficients of variation were lower than 20%.

Hormone levels using immunoassay systems

For E_2, testosterone, DHEAS, and SHBG kits were obtained from Roche and the samples were run on an Elecsys 2010 analyzer (Roche Diagnostics GmbH). Androstenedione levels were measured using an ELISA kit on DPC IMMULITE 2500 analyzer (Siemens Medical Solutions Diagnostics, Munich, Germany). For estrone ELISA kit was obtained from DRG (DRG Instruments GmbH, Marburg, Germany). The samples were analyzed blind and cases and controls were randomly mixed in batches using a single lot number of reagent and calibrator. One scientist did all the measurements. Two levels of quality control (QC) material were analyzed with each run on the analyzer and standard Westgard rules applied. Two levels of QC material were included on each plate for the manual ELISA assays. FE_2 and fT were calculated using the equation based on the law of mass action (Vermeulen *et al*. 1999).

Statistical analysis

Mean and median levels of sex steroid hormones, ERα and ERβ and AR SB were calculated for all breast cancer samples and controls. Differences in the medians between the groups were tested for statistical significance using the Kruskal–Wallis test. Correlations between sex steroid hormones, and ERα and ERβ and AR SB among cases and controls were assessed by the Spearman's rank correlation coefficient. Subjects were classified according to quintiles of the respective marker among controls. The associations between ERα, ERβ, AR SB, hormone levels and the risk of breast cancer were determined by logistic regression analysis controlling for age. Finally, SB levels of each receptor were controlled for all hormones and SB in regression models to estimate their independent associations with breast cancer risk.

Results

The median age of the 200 women with breast cancer (cases) was 61.33 (IQR, 11.32) and 62.33 (IQR, 9.57), in the 400 healthy women (matched controls). Breast tumor characteristics of the cases were similar to a typical breast cancer cohort (Table 1). None of the traditional risk factors (family history, age at menarche, menopause, number of pregnancies, contraceptive pill use, hysterectomy, infertility, body mass index, and height) were significantly different between cases and controls except for fallopian tube ligation (odds ratio (OR) for breast cancer, 0.57; 95% confidence interval (CI), 0.35–0.94; $P=0.029$).

Using all samples, correlations of sex steroid hormones and SHBG with sex steroid receptor SB were investigated. FE_2 and fT showed a statistical significant positive correlation and SHBG a negative correlation with ERα, ERβ, and AR SB. All three sex steroid hormone receptor SB correlated with each other (Table 2).

For the purposes of the analysis, women were stratified into groups based on whether their sample was obtained 6 months to ≤ 2 or >2–5 years before breast cancer diagnosis. We decided to use the same cut off as that used in the largest reanalysis by

Table 1 Characteristics of the breast cancer cases

	No.
Histology	
Ductal	156
Ductal and lobular	6
Lobular	25
Mucinous	1
NST	3
Tubular	1
Other	8
Stage	
1	96
2	39
3	10
Unknown	55
Grading	
1	32
2	111
3	53
Unknown	4
Estrogen receptor (ER)	
ER-positive	200
Progesterone receptor (PR)	
PR-negative	32
PR-positive	100
Unknown	68
HER2	
HER2-negative	79
HER2-positive	16
Unknown	105

NST, no specified type; HER2, human epidermal growth factor receptor 2.

Key *et al*. (2002) that included nine prospective studies. For those women who had given a sample >2 years before diagnosis, the serum androgens: androstenedione, testosterone, and fT, and both ERα and ERβ SB showed significant differences between cases and controls (Table 3). We further analyzed the data based on quintiles with subjects being classified according to quintiles of the respective marker among controls. Women with serum ERα bioactivity in the top quintile had a 2.15 (95% CI, 1.05–4.43; $P<0.05$)-fold breast cancer risk (Table 4). No association was shown between breast cancer risk and ERβ and AR SB (Table 4). Women with serum levels in the top quintile of androstenedione, testosterone, and fT were significantly associated with 4.36 (95% CI, 1.87–11.55)-, 2.53 (95% CI, 1.24–5.41)-, and 2.84 (95% CI, 1.30–6.64)-fold risk for breast cancer respectively (Table 4). Other hormones tested did not show any significant association with breast cancer risk (Table 4). To test whether serum sex steroid receptor bioactivity is independently associated with breast cancer logistic regression analysis was performed adjusting for all hormones and SB. ERα bioactivity was independently associated with breast cancer after adjustment for all hormones and AR and borderline significant after adjustment for ERβ for those women who had given a sample >2 years before diagnosis. Furthermore, after adjustment for all hormones and SB both androstenedione and testosterone were independently associated with breast cancer risk (data not shown).

For those women who had given samples ≤ 2 years before diagnosis, ERα, ERβ, and AR SB did not show any significant association with breast cancer and did not predict risk (Tables 3 and 4). This observation did not change after adjusting for all hormones and SB. SHBG and serum fT showed significant differences between cases and controls (Table 3). Serum levels in the top quintile of androstenedione, testosterone, fT, and estrone were significantly associated with 2.49 (95% CI, 1.20–5.46)-, 1.870 (95% CI, 0.97–3.70)-, 2.02 (95% CI, 0.09–4.24)-, and 2.21 (95% CI, 1.10–4.59)-fold risk for breast cancer respectively (Table 4). The association of androstenedione, testosterone, and estrone with breast cancer risk remained statistically significant after adjustment for all hormones and SB (data not shown). In addition, women who had serum levels in the top quintile of SHBG had a reduced risk of breast cancer (0.32; 95% CI, 0.13–0.73; $P=0.001$; Table 4). Other hormones tested did not show any significant association with breast cancer risk (Table 4).

Analysis was also undertaken combining both groups. For the 11 hormones and sex steroid receptor SB, differences between cases and controls were observed for serum androstenedione, testosterone, and fT levels (Table 3). ERα, ERβ, and AR SB did not show any significant association with breast cancer and did not predict risk (Table 4). This observation did not change after adjusting for all hormones and SB. Women who had serum levels in the top quintile of androstenedione, testosterone, and fT had 3.187 (95% CI, 1.74–6.04)-, 2.15 (95% CI, 1.26–3.71)-, and 2.35 (95% CI, 1.33–4.26)-fold breast cancer risk respectively (Table 4). The association of androstenedione and testosterone with breast cancer risk remained statistically significant after adjustment for all hormones and SB (data not shown). Other hormones examined did not show any significant association with breast cancer risk (Table 4).

Discussion

The study adds to the ongoing effort to better understand the association of sex steroid hormones with breast cancer. This report is the first we are aware of that examines the role of sex steroid hormone receptor

Table 2 Spearman's correlation coefficients among estrogens, androgens, SHBG, and serum bioactivity of estrogen and androgen receptors for cases and controls combined

	Correlation coefficients				
	ERα	**Erβ**	**AR**	**Body mass index**	***n***
Estradiol (E_2)	0.059	0.062	0.055	0.313	573
	$P=0.181$	$P=0.16$	$P=0.214$	***P*=0.000**	
Free E_2	0.124	0.148	0.109	0.444	555
	***P*=0.005**	***P*=0.001**	***P*=0.013**	***P*=0.000**	
Estrone	0.025	0.066	0.080	0.098	582
	$P=0.565$	$P=0.132$	$P=0.067$	***P*=0.021**	
Androstenedione	0.058	0.081	0.002	0.097	581
	$P=0.186$	$P=0.064$	$P=0.963$	***P*=0.022**	
Testosterone	0.024	0.051	0.034	0.132	575
	$P=0.592$	$P=0.244$	$P=0.443$	***P*=0.001**	
Free testosterone	0.102	0.139	0.090	0.545	558
	***P*=0.021**	***P*=0.002**	***P*=0.041**	***P*=0.000**	
DHEAS	0.020	0.010	0.012	0.010	580
	$P=0.647$	$P=0.814$	$P=0.785$	$P=0.803$	
SHBG	−0.220	−0.242	−0.128	−0.423	580
	***P*=0.005**	***P*=0.000**	***P*=0.004**	***P*=0.000**	
ERα		0.507	0.307	0.074	588
		***P*=0.000**	***P*=0.000**	$P=0.073$	
ERβ	0.507		0.330	0.126	589
	***P*=0.000**		***P*=0.000**	***P*=0.002**	
AR	0.307	0.330		0.045	588
	***P*=0.000**	***P*=0.000**		$P=0.279$	

AR, androgen receptor; DHEAS, dehydroepiandrosterone sulfate; ER, estrogen receptor; SHBG, sex hormone-binding globulin.

bioactivity using a yeast-based bioassay and sex steroid hormones using conventional immunoassays before breast cancer diagnosis within a well-defined cohort of women diagnosed with estrogen-sensitive breast cancer and healthy controls. Serum ERα and ERβ were significantly higher in postmenopausal women before diagnosis, with women having a twofold increased breast cancer risk if ERα SB was in the top quintile more than 2 years before diagnosis. Estrogens were not found to be significantly different between cases and controls but women with estrone levels in the top quintile <2 years before diagnosis had a twofold increased breast cancer risk. Testosterone and androstenedione were significantly higher among cases compared with controls and showed a strong association with an almost threefold increased breast cancer risk independent of time to diagnosis. However, this was not reflected in serum AR bioactivity that was not associated with breast cancer.

The strengths of this study are 1) the nested case–control design within a well-defined cohort with prospective identification of breast cancer cases, 2) use of standardized protocol for serum sample collection and storage with protocol adherence confirmed by the lack of any difference in mean hormone or steroid receptor SB levels between the different trial centers (data not shown), 3) confirmation of breast cancer diagnosis and receptor status from the treating physicians that eliminated possible misidentification of cases from use of cancer registry data or self-reporting alone, 4) well-defined homogenous cases through use of strict eligibility criteria (women not on HRT with ER-positive invasive breast cancer), and 5) selection of controls from the same population as those with breast cancer.

Our observations that ERα and ERβ SB were significantly higher in postmenopausal women before diagnosis of invasive ER-positive breast cancer extend our previous findings of elevated bioactivity in women with breast cancer at the time of clinical diagnosis (Widschwendter *et al*. 2009). The receptor SB showed statistically significant correlation with fE_2 that has the highest known affinity for ERα (Lippman *et al*. 1977). This is in keeping with the meta-analysis results that women with high E_2 levels more than 2 years before diagnosis had a higher breast cancer risk compared with those who had high E_2 levels closer to diagnosis (Key *et al*. 2002). Serum receptor activation is probably modulated by other surrogates as well. In our previous study, receptor SB was two- to threefold higher than the actual E_2 concentration (Widschwendter *et al*. 2009). This may explain the increased breast cancer risk in

Table 3 Comparison of sex steroid hormones and sex steroid receptor serum bioactivity levels 6 months to 5 years before breast cancer diagnosis between cases and controls

Hormones and serum bioactivity	Controls				More than 2 years before breast cancer diagnosis				<2 years before breast cancer diagnosis				All samples			
	No.[a]	Mean	Median	STD	No.[a]	Mean	Median	STD	*P* value[b]	No.[a]	Mean	Median	STD	*P* value[b]	No.[a]	Mean	Median	STD	*P* value[b]
SHBG (μg/ml)	385	596.00	560.53	274.63	100	555.7	502.21	224.8	0.50	95	510.95	489.95	215.68	**0.02**	195	533.36	500.53	220.84	0.12
Testosterone (ng/ml)	382	0.28	0.25	0.16	99	0.38	0.29	0.34	**0.01**	94	0.31	0.27	0.18	0.08	193	0.35	0.28	0.28	**0.04**
Free testosterone (ng/dl)	365	0.12	0.09	0.20	100	0.16	0.14	0.16	**0.03**	93	0.13	0.11	0.10	**0.03**	193	0.15	0.11	0.13	**0.00**
Androstenedione (ng/dl)	386	96.85	89.68	50.43	100	120.1	106.30	66.76	**0.00**	95	113.18	96.56	65.04	0.19	195	116.62	102.87	65.90	**0.01**
DHEAS (μg/ml)	385	111.83	100.6	61.15	100	116.5	93.90	75.31	1.00	95	121.3	103	70.78	0.25	195	118.87	97.95	72.92	0.58
AR (ng/ml)	391	2.33	2.32	1.01	103	2.44	2.38	0.88	0.19	94	2.28	2.26	0.85	0.20	197	2.36	2.29	0.86	0.97
Estradiol (E_2; pg/ml)	379	18.44	16.03	13.81	100	19.2	16.87	9.96	0.20	93	17.93	16.24	11.19	0.93	194	18.57	16.51	10.59	0.47
Free E_2 (pg/ml)	362	0.91	0.79	0.62	100	1.00	0.84	0.57	0.17	93	0.93	0.84	0.44	0.17	193	0.98	0.84	0.51	0.07
Estrone (pg/ml)	384	99.74	80.93	80.63	103	108.7	81.16	118.2	0.46	95	116.56	83.14	132.79	0.09	198	112.42	81.79	125.44	0.11
ERα (pg/ml)	390	70.74	62.09	60.45	103	85.60	74.85	67.19	**0.05**	95	74.86	57.60	69.81	0.78	198	80.24	64.17	68.54	0.30
ERβ (pg/ml)	391	59.95	43.87	67.63	103	82.26	59.64	80.79	**0.01**	95	61.10	37.56	85.81	0.26	198	71.69	48.22	83.79	0.41

AR, androgen receptor; DHEAS, dehydroepiandrosterone sulfate; ER, estrogen receptor; SHBG, sex hormone-binding globulin; STD, standard deviation.
[a]Numbers do not always add up to 200 cases and 400 controls due to some missing values.
[b]Kruskal–Wallis for difference in median value between cases and controls.

women with ERα SB in the highest quintile more than 2 years before diagnosis in the absence of a correlation with individual estrogens. The potential advantage of using SB assays for steroid receptors is that their levels reflect the sum of all the factors in the serum that transactivate the two different ERs. Furthermore, previous data based on cell-based assays have shown ERβ to be less active on gene transcription than ERα (Fox *et al*. 2008). This could explain our findings that while ERβ SB is different among cases and controls, levels in the top quintile are not associated with an increased breast cancer risk.

Lack of association between E_2 and breast cancer risk may also be attributed to the assay performance. E_2 levels in postmenopausal women are very low and over the last few years there have been concerns about the sensitivity of direct immunoassays to measure such hormones (Santen *et al*. 2007). Estrone (the main circulating estrogen in postmenopausal women) in the top quintile was associated with increased risk 2 years before breast cancer diagnosis. This observation of estrone rather than E_2 having a stronger association with increased breast cancer risk has been reported by other authors (Zeleniuch-Jacquotte *et al*. 2004). After adjustment for all the other hormones and SB, estrone remained associated with breast cancer risk indicating an independent role. It has weak and low affinity to ERα (Bonofiglio *et al*. 1999) and may exert its effect on breast carcinogenesis by inducing ERK phosphorylation via binding to the estrogen G protein-coupled receptor 30 (GPR30; Maggiolini *et al*. 1999*b*, Yager 2000). If a significant ER-independent pathway is confirmed, it could have implications for hormone therapy in prevention and treatment of breast cancer in postmenopausal women.

Androstenedione and testosterone were associated with an almost threefold increase in breast cancer risk independent of time from diagnosis. The meta-analysis of nine studies in postmenopausal women confirmed that high testosterone and androstenedione levels were associated with increased risk (Key *et al*. 2002). The more recent report from EPIC (Kaaks *et al*. 2005) also confirmed that androgens were associated with breast risk independent of time to diagnosis. After adjustment for estrogens, the association of the androgens with breast cancer risk remained, indicating that they may have an estrogen-independent effect on the breast, an observation that has been reported by other authors (Key *et al*. 2002, Missmer *et al*. 2004, Kaaks *et al*. 2005). One of the possible pathways that androgens may influence breast cancer risk is by directly binding to AR, stimulating or inhibiting breast cell growth (Maggiolini *et al*. 1999*a*, Cox *et al*. 2006) but we were

Table 4 Sex steroid receptor serum bioactivity (A) sex steroid hormones (B) and breast cancer risk

			Controls	Cases: More than 2 years			Controls	Cases: <2 years			Controls	Cases: All		
	Quintile	Range	*n*	*n*	OR (95% CI)	*P* value	*n*	*n*	OR (95% CI)	*P* value	*n*	*n*	OR (95% CI)	*P* value
(A) Serum bioactivity														
ERα (pg/ml)														
	1st	0–27.06	77	14	1.00 (ref.)		77	22	1.00 (ref.)		77	36	1.00 (ref.)	
	2nd	27.06–52.73	76	17	1.22 (0.56–2.68)	0.617	76	20	0.92 (0.46–1.83)	0.817	76	37	1.04 (0.593–1.816)	0.898
	3rd	52.73–70.01	76	15	1.08 (0.48–2.41)	0.853	76	16	0.74 (0.36–1.50)	0.405	76	31	0.87 (0.484–1.538)	0.621
	4th	70.01–104.36	76	20	1.49 (0.70–3.23)	0.306	76	21	0.97 (0.492–1.92)	0.935	76	41	1.16 (0.669–2.016)	0.598
	5th	104.36–459.22	76	29	2.11 (1.05–4.43)	**0.040**	76	21	0.91 (0.46–1.81)	0.793	76	49	1.38 (0.807–2.360)	0.243
					P for trend = 0.039				*P* for trend = 0.854				*P* for trend = 0.304	
ERβ (pg/ml)	1st	0–2.97	77	13	1.00 (ref.)		77	23	1.00 (ref.)		77	36	1.00 (ref.)	
	2nd	2.97–34.36	76	13	0.98 (0.42–2.23)	0.959	76	23	1.03 (0.53–1.99)	0.934	76	36	1.01 (0.57–1.78)	0.967
	3rd	34.36–55.80	76	18	1.41 (0.65–3.13)	0.392	76	19	0.84 (0.42–1.67)	0.622	76	37	1.05 (0.60–1.83)	0.878
	4th	55.80–98.96	76	25	1.95 (0.94–4.20)	0.079	76	20	0.87 (0.44–1.72)	0.692	76	45	1.27 (0.74–2.189)	0.387
	5th	98.96–477.56	77	26	1.99 (0.97–4.27)	0.067	77	20	0.61 (0.29–1.27)	0.191	77	40	1.11 (0.641–1.93)	0.706
					P for trend = 0.015				*P* for trend = 0.227				*P* for trend = 0.565	
AR (pg/ml)	1st	0.36–1.59	77	17	1.00 (ref.)		77	24	1.00 (ref.)		77	41	1.00 (ref.)	
	2nd	1.59–2.10	76	10	0.60 (0.25–1.37)	0.231	76	18	0.76 (0.38–1.51)	0.441	76	28	0.69 (0.39–1.23)	0.212
	3rd	2.10–2.45	76	23	1.38 (0.68–2.81)	0.374	76	25	1.06 (0.56–2.02)	0.863	76	48	1.19 (0.71–2.02)	0.509
	4th	2.45–2.87	76	21	1.26 (0.62–2.59)	0.533	76	12	0.50 (0.23–1.05)	0.074	76	33	0.81 (0.47–1.42)	0.47
	5th	2.87–7.45	77	23	1.38 (0.68–2.82)	0.375	77	12	0.84 (0.42–1.64)	0.604	77	43	1.06 (0.62–1.82)	0.824
					P for trend = 0.110				*P* for trend = 0.310				*P* for trend = 0.744	
(B) Hormone														
Estradiol (E_2; pg/ml)	1st	0–11.47	76	16	1.00 (ref.)		76	20	1.00 (ref.)		76	36	1.00 (ref.)	
	2nd	11.47–14.74	75	13	0.84 (0.37–1.88)	0.677	75	16	0.81 (0.39–1.69)	0.578	75	29	0.82 (0.46–1.48)	0.514
	3rd	14.74–17.98	75	22	1.39 (0.68–2.90	0.366	75	25	1.29 (0.66–2.57)	0.453	75	47	1.33 (0.78–2.29)	0.304
	4th	17.98–22.66	75	20	1.27 (0.61–2.67)	0.522	75	20	1.02 (0.50–2.05)	0.965	75	40	1.12 (0.65–1.95)	0.685
	5th	22.66–209.40	75	23	1.46 (0.72–3.03)	0.298	75	20	0.91 (0.44–1.86)	0.799	75	41	1.15 (0.67–2.00)	0.613
					P for trend = 0.141				*P* for trend = 0.973				*P* for trend = 0.304	
Free E_2 (pg/ml)	1st	0–0.50	76	14	1.00 (ref.)		76	13	1.00 (ref.)		76	27	1.00 (ref.)	
	2nd	0.50–0.69	75	15	1.07 (0.48–2.40)	0.861	75	17	1.20 (0.67–2.21)	0.546	75	32	1.32 (0.60–2.96)	0.546
	3rd	0.69–0.90	75	27	1.95 (0.96–4.10)	0.071	75	28	2.08 (1.19–3.68)	0.017	75	55	2.19 (1.07–4.67)	0.011
	4th	0.90–1.19	75	15	1.10 (0.49–2.46)	0.821	75	19	1.30 (0.71–2.37)	0.399	75	34	1.50 (0.70–3.33)	0.399
	5th	1.19–6.62	75	23	1.66 (0.80–3.54)	0.180	75	19	1.69 (0.96–3.03)	0.075	76	45	1.70 (0.81–3.71)	0.075
					P for trend = 0.225				*P* for trend = 0.169				*P* for trend = 0.073	
Estrone (pg/ml)	1st	0–56.28	76	14	1.00 (ref.)		76	14	1.00 (ref.)		76	28	1.00 (ref.)	
	2nd	56.28–72.63	75	23	1.65 (0.78–3.52)	0.183	75	17	1.24 (0.57–2.75)	0.583	75	40	1.44 (0.81–2.59)	0.218
	3rd	72.63–90.14	75	22	1.58 (0.76–3.39)	0.229	75	19	1.35 (0.63–2.95)	0.437	75	41	1.46 (0.82–2.62)	0.202
	4th	90.14–115.53	75	15	1.07 (0.48–2.436)	0.862	75	19	1.33 (0.62–2.89)	0.472	75	34	1.20 (0.66–2.19)	0.559
	5th	115.53–779.83	75	21	1.53 (0.73–3.31)	0.267	75	19	2.21 (1.10–4.59)	**0.029**	76	52	1.86 (1.07–3.29)	**0.030**
					P for trend = 0.624				*P* for trend = 0.062				*P* for trend = 0.122	

Table 4 *continued*

			Controls	Cases: More than 2 years			Controls	Cases: <2 years			Controls	Cases: All		
	Quintile	Range	*n*	*n*	OR (95% CI)	*P* value	*n*	*n*	OR (95% CI)	*P* value	*n*	*n*	OR (95% CI)	*P* value
Androstenedione (ng/dl)	1st	0–52.44	76	7	1.00 (ref.)		76	12	1.00 (ref.)		76	19	1.00 (ref.)	
	2nd	52.44–77.94	76	18	3.09 (1.23–8.65)	**0.022**	76	23	2.18 (1.01–4.95)	0.054	76	41	2.52 (1.33–4.95)	**0.006**
	3rd	77.94–102.87	74	17	2.84 (1.13–7.88)	**0.033**	74	18	1.57 (0.71–3.60)	0.271	74	35	2.02 (1.06–3.94)	**0.036**
	4th	102.87–132.09	75	23	3.64 (1.51–9.84)	**0.006**	75	13	1.09 (0.46–2.61)	0.845	75	36	2.01 (1.06–3.94)	**0.037**
	5th	132.09–383.95	76	30	4.36 (1.87–11.55)	**0.001**	75	13	2.49 (1.20–5.46)	**0.018**	76	60	3.19 (1.74–6.04)	**0.0003**
					***P* for trend = 0.001**				*P* for trend = 0.310				***P* for trend = 0.007**	
Testosterone (ng/ml)	1st	0–0.16	77	13	1.00 (ref.)		77	18	1.00 (ref.)		77	31	1.00 (ref.)	
	2nd	0.16–0.22	75	15	1.22 (0.54–2.80)	0.636	75	19	1.15 (0.55–2.39)	0.714	75	34	1.16 (0.65–2.10)	0.613
	3rd	0.22–0.29	75	18	1.38 (0.63–3.10)	0.427	75	18	1.02 (0.48–2.13)	0.969	75	36	1.17 (0.65–2.10)	0.590
	4th	0.29–0.38	76	15	1.19 (0.53–2.72)	0.669	76	9	0.49 (0.20–1.13)	0.104	76	24	0.78 (0.42–1.45)	0.441
	5th	0.38–1.07	76	33	2.53 (1.24–5.41)	**0.013**	76	9	1.87 (0.97–3.68)	**0.05**	76	68	2.15 (1.26–3.71)	**0.006**
					***P* for trend = 0.011**				*P* for trend = 0.087				***P* for trend = 0.005**	
Free testosterone (ng/dl)	1st	0–0.05	79	10	1.00 (ref.)		79	14	1.00 (ref.)		79	24	1.00 (ref.)	
	2nd	0.05–0.08	76	18	1.93 (0.845–4.62)	0.126	76	24	1.81 (0.88–3.86)	0.112	76	42	1.85 (1.03–3.38)	0.044
	3rd	0.08–0.11	74	18	1.96 (0.86–4.67)	0.115	74	12	0.92 (0.39–2.12)	0.843	74	30	1.34 (0.72–2.53)	0.355
	4th	0.11–0.16	75	22	2.26 (1.02–5.29)	0.050	75	20	1.51 (0.72–3.26)	0.283	75	42	1.83 (1.02–3.35)	0.046
	5th	0.16–3.48	76	26	2.84 (1.30–6.64)	**0.011**	75	20	2.02 (0.99–4.24)	0.057	76	55	2.35 (1.33–4.26)	**0.004**
					***P* for trend = 0.013**				*P* for trend = 0.078				***P* for trend = 0.007**	
DHEAS (μg/ml)	1st	0–58.44	77	17	1.00 (ref.)		77	16	1.00 (ref.)		77	33	1.00 (ref.)	
	2nd	58.44–85.52	77	22	1.29 (0.64–2.66)	0.483	77	15	0.96 (0.44–2.09)	0.92	77	37	1.14 (0.65–2.02)	0.654
	3rd	85.52–119.16	76	24	1.44 (0.71–2.98)	0.312	76	28	1.79 (0.89–3.70)	0.106	76	52	1.62 (0.93–2.84)	0.089
	4th	119.16–162.04	77	14	1.05 (0.45–2.44)	0.908	77	20	1.58 (0.72–3.55)	0.26	77	34	1.31 (0.70–2.47)	0.396
	5th	162.04–459.60	77	18	1.40 (0.60–3.28)	0.438	77	20	1.53 (0.65–3.64)	0.325	77	39	1.49 (0.77–2.90)	0.237
					P for trend = 0.695				*P* for trend = 0.341				*P* for trend = 0.838	
SHBG (μg/ml)	1st	0–346.53	77	15	1.00 (ref.)		77	25	1.00 (ref.)		77	40	1.00 (ref.)	
	2nd	346.15–485.79	77	30	1.99 (1.00–4.09)	0.053	77	20	0.81 (0.41–1.57)	0.53	77	50	1.24 (0.734–2.10)	0.418
	3rd	485.79–634.32	76	17	1.15 (0.54–2.49)	0.722	76	31	1.26 (0.68–2.36)	0.459	76	48	1.22 (0.72–2.07)	0.46
	4th	634.32–843.47	77	22	1.48 (0.72–3.11)	0.295	77	16	0.65 (0.3127–1.30)	0.228	77	38	0.96 (0.56–1.67)	0.891
	5th	843.47–1533.7	77	11	0.71 (0.30–1.64)	0.422	77	16	0.32 (0.13–0.73)	**0.001**	77	19	0.48 (0.25–0.89)	**0.022**
					P for trend = 0.290				***P* for trend = 0.015**				***P* for trend = 0.004**	

OR values for quintiles based on controls only being age adjusted. OR with *P* values ≤0.05 marked with bold. AR, androgen receptor; DHEAS, dehydroepiandrosterone sulfate; ER, estrogen receptor; OR, odds ratio; SHBG, sex hormone-binding globulin.

unable to demonstrate such an association. While fT is the best ligand of AR, androgens have also been shown to bind and activate ERs (Maggiolini *et al*. 1999*a*). Our data showing a statistically significant correlation between fT and both ERs favor the view for the existence of the latter pathway where androgens promote breast cell proliferation by binding directly to ER.

To summarize, our findings provide further evidence of the association between sex steroid hormones and breast cancer risk. Testosterone and estrone were shown to be associated with increased breast cancer risk. Based on that, it would be interesting to evaluate the association of key enzymes in steroidogenesis such as aromatase and 17β-hydroxysteroid dehydrogenases and breast cancer. In addition, our report provides novel insight into the role of sex steroid receptor SB in breast cancer with ER but not AR SB associated with increased risk more than 2 years before diagnosis. Further development of these assays might appear promising for giving greater insight into the role of sex hormones in relation to breast cancer risk but on the basis of the current results the assays do not appear to have a stronger association with breast cancer risk compared with this and previous studies using conventional assays. If ER SB results are validated in other studies, it may also prove beneficial in individualizing and monitoring breast cancer chemopreventive strategies using antiestrogens such as tamoxifen (Cuzick *et al*. 2003), raloxifene (Fabian & Kimler 2005), and aromatase inhibitors (Kalidas & Brown 2005).

Declaration of interest

I Jacobs has consultancy arrangements with Becton Dickinson, who have an interest in tumor markers and ovarian cancer. They have provided consulting fees, funds for research, and staff but are not directly related to this study. U Menon has a financial interest through UCL Business and Abcodia Ltd. in the third party exploitation of clinical trials biobanks, which have been developed through the research at UCL. No other financial disclosures.

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