



Sex hormone measurements using mass spectrometry and sensitive extraction radioimmunoassay and risk of estrogen receptor negative and positive breast cancer: Case control study in UK Collaborative Cancer Trial of Ovarian Cancer Screening (UKCTOCS)



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ABSTRACT

Introduction: Associations of endogenous sex hormone levels and all as well as estrogen-receptor (ER)-positive breast cancers are well described. However, studies investigating their association with ER-negative tumours are limited and none use accurate assays such as mass spectrometry.

Methods: Within the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), a nested case-control study was undertaken of postmenopausal-women who developed ER-negative (n = 92) or ER-positive (n = 205) breast cancer after sample donation and 297 (1:1) age-matched controls. Androgens (testosterone and androstenedione) were measured using mass spectrometry and estradiol by extraction radioimmunoassay (RIA). Bioavailable estradiol and testosterone were calculated using the total hormone level and the sex hormone-binding globulin concentration. Subjects were classified according to the quartile range among controls. Logistic regression was used to estimate odds-ratio (OR) and 95% confidence-intervals (CI) of the associations between two factors and breast cancer risk. A separate analysis was done by stratifying the women based on whether they provided their samples less than or more than 2 years before diagnosis.

Results: Estradiol and free estradiol were significantly higher prior to diagnosis of ER-negative breast cancer compared with controls while androgens and SHBG did not show any difference. Estradiol, free estradiol, free testosterone and SHBG were significantly higher before ER-positive breast cancer diagnosis compared with controls. Women had a twofold increased ER-negative breast cancer risk if estradiol and free estradiol were in the top quartile but not androgens (testosterone and androstenedione) or SHBG. These associations remained significant only when samples closer (median 1.1 y before) to diagnosis were analyzed rather than farther from diagnosis (median 2.9 y before). Women had a 2.34 (95% CI: 1.21–4.61, p = 0.001), 2.21 (95% CI: 1.14–4.38, p = 0.001), 2 (95% CI: 1.05–3.89, p = 0.005) fold increased ER-positive breast cancer risk if estradiol, free estradiol and free testosterone respectively were in the top quartile. These associations remained significant regardless of whether the samples were collected less than or more than 2 years prior to diagnosis.

Conclusion: In postmenopausal women increased estrogens but not androgens are associated with ER-negative breast cancer. Previously reported associations of estradiol and free testosterone with ER-positive breast cancer are confirmed. The use of mass spectrometry and sensitive RIA add validity to these findings.

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1. Introduction

Breast cancer remains one of the leading causes of cancer death among women despite the huge progress that has been made in treatment [1,2]. It is a complex and heterogeneous disease with multiple histopathological and molecular subtypes which have varying clinical outcome [3]. Currently, estrogen (ER), progesterone (PR) and herceptin-2 (HER2) receptor [4] status in the tumour is used to guide breast cancer treatment and provide prognostic information.

A key aspect of trying to improve disease outcome has been identification of risk factors [5]. Many of these, such as early menarche and late menopause [6], mediate their effect through a hormonal mechanism [7]. This has led to the investigation of associations of endogenous sex hormone levels and breast cancer risk [8–12]. The majority of such studies have explored overall breast cancer [8,10,11] or ER-positive breast cancer risk [9,12]. There are only five studies that have reported the association by hormone receptor status [13–17]. They are characterized by relatively small numbers and conflicting results.

Additional issues are the assays used to measure sex steroid hormones. The most commonly used methods are direct immunoassays, which tend to overestimate concentrations and suffer from cross-reactivity with other steroids [18]. In the last few years, assays with an organic extraction step and mass spectrometry are emerging as the 'gold standard' [19]. Mass spectrometry has improved specificity and has a wide analytical range compared to the immunoassays and allows the measurement of multiple hormones during a single run [20]. There are two published studies that we are aware of which have used mass spectrometry to examine the association of sex steroid hormones and breast cancer risk and this was limited to estrogens [21,22]. There is growing consensus of the need for more studies evaluating breast cancer risk by hormone receptor status [4] using more accurate hormone assays.

We have previously shown the relationship between postmenopausal sex steroid hormone levels with hormone receptor positive breast cancer risk using immunoassays in the UK Collaborative Trial of Ovarian Cancer Risk (UKCTOCS) [9]. The current analysis expands this nested case-control study to include ER-negative cases with androgens measured using mass spectrometry and the estrogens using sensitive and specific extraction radioimmunoassay.

2. Methods

UKCTOCS is a multicentre randomized controlled trial of ovarian cancer screening. The trial design has been previously described [23]. In brief, women aged 50–74 were recruited from England, Northern Ireland and Wales through random invitation from age/sex registers of 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 of their data/samples in future studies [9,23]. All participants continue to be followed up through a 'flagging study' with the National Health System Information Centre for Health and Social Care who provide data on cancer registrations and deaths.

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).

3. Subjects

The treating physician of women diagnosed with breast cancer during the trial were sent a questionnaire requesting information

regarding their diagnosis (histology), hormone receptor status and treatment [24]. From those in whom complete data were available, women who developed ER-positive or ER-negative invasive breast cancer after joining UKCTOCS, were not on hormone replacement therapy treatment at recruitment and had donated a serum sample between 6 months and 5 years before diagnosis were chosen as 'cases'. Controls were women who were not on HRT at recruitment and had no history of cancer at last follow-up. Each breast cancer case was age matched (± 5 years) to one control woman who had donated serum samples on the same day and in the same clinic.

4. Serum processing

All blood samples were collected into Greiner Bio one gel tubes (Cat no: 455071) at the participating centers, shipped overnight to the central laboratory and centrifuged at 2000g 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 that were heat sealed, bar coded, data based, and stored in liquid nitrogen tanks. The samples were only thawed once, at the time of the assay.

5. Measurement of hormone levels

5.1. Estradiol by extraction radioimmunoassay

Unconjugated estradiol was measured in 100 μL of sample in duplicate using an in-house radioimmunoassay [25]. Estradiol was extracted into 1 mL diethyl ether (Vickers) by vortex mixing for 3 min (after addition of 10 μL 2 M sodium hydroxide). After freezing the aqueous layer in a dry-ice methanol bath, the organic layer was decanted into a glass tube. Solvent was removed by vacuum oven. Competitive radioimmunoassay was performed on the dried extract using an antiserum raised in a goat against estradiol-6-BSA conjugate and iodinated estradiol label (125I, Siemens). Unbound estradiol was adsorbed onto dextran-coated charcoal, leaving the bound fraction in solution. The bound fraction was decanted and counted in the gamma counter (Wallac Wizard). The estradiol values for quality control material and study samples were obtained by interpolation of the counts bound using a standard curve of estradiol concentrations from 5 to 1000 pmol/L by Multicalc software. Significant cross-reactions are seen with Estrone (10%) and Estriol (1.3%). Inter-assay imprecision of estradiol in quality control material is shown in [Supplementary Table 1](#).

5.2. Testosterone and androstenedione by tandem mass spectrometry

Testosterone and androstenedione were measured in 100 μL sample using tandem mass spectrometry (LC-MS/MS). Tri-deuterated (d_3) testosterone and hepta-deuterated (d_7) androstenedione were used as the internal standards. The steroids were extracted into 1 mL of *tert*-butyl methyl ether (MTBE, Fisher) by vortex mixing for 5 min. After freezing the aqueous layer in a dry-ice methanol bath, the organic layer was decanted into a glass tube (10 \times 75 mm). Solvent was removed using a vacuum oven at 40°C . The dried residue was reconstituted in 200 μL of aqueous methanol (1:1 solution by volume, Fisher) and mixed thoroughly. The extracts were transferred to a deep well micro-titre plate (Fisher), which was then sealed with film. Extract (30 μL) was injected onto the LC column (Sunfire 2.5 μM C18 50 \times 4.6 mm, Waters). The LC-MS/MS system consisted of a Shimadzu Prominence LC (LC-20AD XR) with auto-sampler and column oven connected to an AB Sciex API 5000 triple quadrupole mass

spectrometer. The mobile phase conditions were as described previously [25]. Ions were generated by positive electrospray ionisation. The ion transitions used for testosterone and internal standard were 289 > 97 and 292 > 109 respectively; and for androstenedione and internal standard were 287 > 97 and 294 > 100 respectively. Androstenedione and internal standard eluted at 4.9 min. Testosterone and internal standard eluted at 5.2 min. The peak area for androstenedione or testosterone relative to the peak area for the internal standard was plotted against the concentration of the calibrators by the Analyst software. Calibrator ranges were 0.2–39.1 nmol/L for testosterone and 0.2–50 nmol/L for androstenedione. Results for quality control material and study samples were interpolated from the calibration lines. Inter-assay imprecision of androstenedione and testosterone in quality control material was shown in [Supplementary Table 2](#).

5.3. SHBG measurement by immunoassay

SHBG kits were obtained from Roche and the samples were run on an Elecsys 2010 analyzer (Roche Diagnostics GmbH). Two levels of quality control (QC) material were analyzed with each run on the analyzer and standard Westgard rules applied. 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.

5.4. Calculation of free estradiol and testosterone

For the calculation of free estradiol (fE2) and free testosterone (fT) the equation based on the law of mass action by Vermeulen was used [26]. The equation relies on the assumption that the concentration of fE2 and fT in blood is determined mainly by interaction with SHBG and albumin, and that other hormones present in the blood exert little influence on this equilibrium.

6. Statistical analysis

Mean and median levels of sex steroid hormones and SHBG were calculated for ER-positive and -negative breast cancer cases and controls. Women were stratified into two groups based on whether their sample was obtained between 6 months and 2 years or between 2 and 5 years before breast cancer diagnosis, similar to Key et al. [8]. Differences in the medians between the groups were tested for statistical significance using the Mann-Whitney test. Subjects were classified according to quartiles of the respective marker among controls. The associations between hormone levels and the risk of breast cancer were determined by logistic regression analysis controlling for age.

7. Results

Cases included 202 women who developed ER-positive invasive breast cancer and 92 who developed ER-negative invasive breast cancer after joining UKCTOCS and 292 healthy controls. All reported no HRT use at recruitment. 6 were excluded from analysis as the assays failed in 3 women with ER-positive breast cancer and 5 controls. The median age of the 202 women with ER-positive breast cancer (cases) was 62 (IQR 9) and 62 (IQR 9) in the 200 healthy women (matched controls). The median age of the 92 women with ER-negative breast cancer (cases) was 61 (IQR 9.25) and 60.5 (IQR 9) in the 92 healthy women (matched controls). Breast tumour characteristics of the cases were similar to a typical breast cancer cohort – the majority of the ER-positive group were Stage 1 (39.5%), ductal (82.9%) and Grade 2 (59.5%) and of the ER-negative group were Stage 1 (40.2%), ductal (98.9%) and Grade 3

(71.7%). Different lifestyle and anthropometric characteristics are presented in [Table 1](#) to provide the baseline information between cases and controls. Of the traditional risk factors investigated family history and body mass index were significantly different between cases and controls ([Table 1](#)). Mean values were chosen as cut-points for continuous covariates in [Table 1](#).

Using all samples, correlations of sex steroid hormones and SHBG were investigated between ER-positive and ER-negative groups. Among the ER-positive group, estradiol showed a statistically significant positive correlation with free estradiol, androstenedione, testosterone and free testosterone; testosterone showed a statistically significant positive correlation with free testosterone and androstenedione; and free testosterone showed a statistically significant positive correlation with androstenedione and a negative correlation with SHBG. Among the ER-negative group, estradiol showed a statistically significant positive correlation with free estradiol and androstenedione; androstenedione showed a statistically significant positive correlation with free estradiol, testosterone, free testosterone and a negative correlation with SHBG; and free testosterone showed a statistically significant positive correlation with testosterone and a negative correlation with SHBG ([Supplementary Table 3](#)).

For the ER-negative group, estradiol and free estradiol showed significant differences between cases and controls ([Table 2](#)). Women who provided their samples more than 2 years before cancer diagnosis had significantly higher levels of free estradiol compared to controls but women who provided their samples less than 2 years before cancer diagnosis did not show any significant differences ([Table 2](#)). For the ER-negative group as a whole, those with serum free estradiol level in the top quartile had 2.52 (95% CI: 1.04–6.3, $p = 0.03$) fold increased breast cancer risk. The association remained significant after adjustment for body mass index and family history (data not shown). However on subgroup analysis, the increased risk remained significant only in women who provided samples less than 2 years before diagnosis ([Table 3a](#)).

For the ER-positive group, the serum estradiol, free estradiol, free testosterone and SHBG showed significant differences between cases and controls ([Table 2](#)). Women who provided their samples less than 2 years before cancer diagnosis had significant higher levels of estradiol and free estradiol and lower of SHBG compared to controls. Women who provided their samples more than 2 years had significantly higher levels of estradiol, free estradiol and free testosterone and lower levels of SHBG compared to controls. For the ER-positive group, when all women were investigated, those with serum estradiol levels, free estradiol, free testosterone and SHBG in the top quartile had a 2.26 (95% CI: 1.28–4.05, $p = 0.001$), 2.77 (1.5–5.21, $p \leq 0.001$); 2.23 (95% CI: 1.23–4.1, $p = 0.004$), 0.35 (95% CI: 0.19–0.64, $p = 0.001$) breast cancer risk, respectively ([Table 3b](#)). Similar associations were observed on subgroup analysis based on whether their sample was obtained less than or more than 2 years before breast cancer diagnosis ([Table 3b](#)). All logistic regression analysis provided in [Tables 3a and 3b](#) were adjusted for age. No association was shown between breast cancer risk and androstenedione ([Table 3b](#)). Additionally, the association of the hormones with breast cancer risk remained significant after adjustment for body mass index and family history (data not shown).

8. Discussion

The current study adds to the ongoing effort to better understand the association of sex steroid hormones and breast cancer risk. This report is the first we are aware of that examines the role of sex steroid hormones measured using mass spectrometry for the androgens and an extraction RIA for estrogens in ER-positive and

Table 1
Risk factors for cases and controls in the ER POSITIVE and ER NEGATIVE groups.

	Category	ER POSITIVE									ER NEGATIVE								
		Case, N	%	Control, N	%	Total, N	OR	L95% CI	U95% CI	p-Value	Case, N	%	Control, N	%	Total, N	OR	L95% CI	U95% CI	p-Value
Ethnicity	Non-white	2	0.01%	7	0.04%	9	1				1	0.01%	3	0.03%	4	1			
	White	200	0.99%	193	0.97%	393	3.63	0.86	24.5	0.11	91	0.99%	89	0.97%	180	3.07	0.39	62.7	0.34
Height	<1.6	61	0.30%	65	0.33%	126	1				35	0.38%	29	0.32%	64	1			
	1.6+	140	0.70%	134	0.67%	274	1.11	0.73	1.7	0.62	57	0.62%	63	0.69%	120	0.75	0.41	1.38	0.35
BMI	<24	52	0.26%	73	0.37%	125	1				23	0.25%	45	0.49%	68	1			
	24+	149	0.74%	126	0.63%	275	1.66	1.09	2.55	0.02	69	0.75%	47	0.51%	116	2.87	1.55	5.43	0.001
Age last period	<50	85	0.42%	94	0.47%	179	1				47	0.51%	47	0.51%	94	1			
	50+	117	0.58%	106	0.53%	223	1.22	0.82	1.81	0.32	45	0.49%	45	0.49%	90	1	0.56	1.78	1
Age first period	<12	34	0.17%	38	0.19%	72	1				25	0.27%	15	0.16%	40	1			
	12+	166	0.83%	161	0.81%	327	1.15	0.69	1.93	0.59	67	0.73%	77	0.84%	144	0.52	0.25	1.06	0.08
Pill use	No	81	0.40%	85	0.43%	166	1				40	0.44%	37	0.40%	77	1			
	Yes	121	0.60%	115	0.58%	236	1.1	0.74	1.64	0.63	52	0.57%	55	0.60%	107	0.88	0.49	1.57	0.65
Pregnancies <6Mth	0	139	0.70%	134	0.68%	273	1				64	0.70%	64	0.70%	128	1			
	1+	61	0.31%	62	0.32%	123	0.95	0.62	1.45	0.81	28	0.30%	27	0.30%	55	1.04	0.55	1.96	0.91
Pregnancies >6Mth	0	24	0.12%	13	0.07%	37	1				10	0.11%	11	0.12%	21	1			
	1+	178	0.88%	186	0.94%	364	0.52	0.25	1.03	0.07	81	0.89%	81	0.88%	162	1.1	0.44	2.78	0.84
Sterilization	No	163	0.81%	147	0.74%	310	1				75	0.82%	75	0.82%	150	1			
	Yes	39	0.19%	53	0.27%	92	0.66	0.41	1.06	0.09	17	0.19%	17	0.19%	34	1	0.47	2.12	1
Number of relatives breast	0	139	0.69%	155	0.78%	294	1				63	0.69%	80	0.87%	143	1			
	1+	63	0.31%	45	0.23%	108	1.56	1	2.45	0.05	29	0.32%	12	0.13%	41	3.07	1.48	6.7	0.003
Alcohol	0	41	0.20%	28	0.21%	69	1				19	0.21%	16	0.18%	35	1			
	1+	160	0.80%	107	0.79%	267	1.02	0.59	1.75	0.94	73	0.79%	75	0.82%	148	0.82	0.39	1.72	0.6
Smoker	0	115	0.57%	152	0.76%	267	1				49	0.53%	59	0.64%	108	1			
	1	87	0.43%	48	0.24%	135	2.4	1.57	3.69	<0.001	43	0.47%	33	0.36%	76	1.57	0.87	2.85	0.14
Hysterectomy	0	170	0.84%	178	0.89%	348	1				72	0.78%	80	0.87%	152	1			
	1	32	0.16%	22	0.11%	54	1.52	0.86	2.76	0.16	20	0.22%	12	0.13%	32	1.85	0.86	4.15	0.12

Table 2
Characteristics of the hormones' values in different time groups.

ER POSITIVE																			
	Controls				Cases – All samples					Cases – Less than 2 years before diagnosis					Cases – More than 2 years before diagnosis				
	No	Mean	Median	STD	No	Mean	Median	STD	p-Value	No	Mean	Median	STD	p-Value	No	Mean	Median	STD	p-Value
Estradiol (pmol/l)	182	39.2	28	56.3	189	39.1	32	33.4	<0.01	93	40.1	32	42.8	0.02	96	38.1	32.5	20.8	0.01
Free estradiol (pmol/l)	181	1.07	0.71	1.83	189	1.16	0.86	2.02	<0.01	93	1.27	0.86	2.8	0.01	96	1.05	0.85	0.65	<0.01
Testosterone (nmol/l)	190	0.71	0.61	0.39	196	0.72	0.66	0.37	0.38	98	0.74	0.67	0.42	0.4	98	0.7	0.63	0.32	0.56
Free testosterone (nmol/l)	190	0.008	0.007	0.006	196	0.009	0.008	0.006	0.01	98	0.009	0.008	0.005	0.04	98	0.01	0.008	0.007	0.06
Androstenedione (nmol/L)	192	1.49	1.36	0.63	200	1.61	1.48	0.75	0.13	99	1.6	1.48	0.71	0.23	101	1.61	1.47	0.79	0.2
SHBG (nmol/l)	200	68.9	64.4	29.6	202	59.9	54.5	26	<0.01	100	61.1	55.1	26.4	0.03	102	58.7	54.3	25.7	<0.01
ER NEGATIVE																			
	Controls				Cases – All samples					Cases – Less than 2 years before diagnosis					Cases – More than 2 years before diagnosis				
	No	Mean	Median	STD	No	Mean	Median	STD	p-Value	No	Mean	Median	STD	p-Value	No	Mean	Median	STD	p-Value
Estradiol (pmol/l)	88	31.3	27	21.5	82	36.8	31	32.7	0.04	41	40.3	31	44.8	0.14	41	33.2	31	11.2	0.06
Free estradiol (pmol/l)	88	0.84	0.72	0.77	82	1.09	0.82	1.78	0.03	41	1.29	0.82	2.49	0.13	41	0.88	0.82	0.33	0.04
Testosterone (nmol/l)	85	0.52	0.49	0.33	90	0.56	0.46	0.41	0.89	47	0.55	0.45	0.49	0.72	43	0.56	0.53	0.32	0.54
Free testosterone (nmol/l)	85	0.006	0.005	0.004	90	0.007	0.006	0.005	0.34	47	0.007	0.006	0.005	0.11	43	0.006	0.006	0.004	0.98
Androstenedione (nmol/L)	90	1.21	1.15	0.54	92	1.22	1.16	0.58	0.89	49	1.2	1.15	0.62	0.72	43	1.23	1.22	0.53	0.87
SHBG (nmol/l)	92	73.2	68.9	31.5	92	67.3	59.3	33.1	0.13	49	70	60.9	37.3	0.28	43	64.4	57.9	27.8	0.16

Table 3a

Quartile analysis in the ER NEGATIVE subgroup as a whole, and stratified by time to diagnosis.

	Quartile	Range	All cases		Cases – Less than 2 years		Cases – More than 2 years				
			N Controls	N Cases	OR	N Controls	N Cases	OR	N Controls	N Cases	OR
Estradiol (pmol/l)	1st	12–21.8	22	13	1	22	5	1	22	8	1
	2nd	21.8–27	23	16	1.18 (0.46–3.03)	23	9	1.72 (0.51–6.35)	23	7	0.84 (0.25–2.71)
	3rd	27–37	22	26	1.97 (0.82–4.91)	22	13	2.59 (0.81–9.31)	22	13	1.63 (0.57–4.86)
	4th	37–206	21	26	2.2 (0.9–5.57)	21	14	3.04 (0.96–11)	21	12	1.65 (0.56–5.07)
											P-value for trend = 0.06
											P-value for trend = 0.05
											P-value for trend = 0.25
Free estradiol (pmol/l)	1st	0.29–0.54	22	12	1	22	4	1	22	8	1
	2nd	0.54–0.72	22	17	1.42 (0.55–3.71)	22	10	2.51 (0.72–10.3)	22	7	0.87 (0.26–2.85)
	3rd	0.72–0.94	22	22	1.86 (0.75–4.76)	22	12	3.07 (0.9–12.4)	22	10	1.29 (0.43–4.01)
	4th	0.94–7.34	22	30	2.52 (1.04–6.3)	22	15	3.76 (1.15–14.9)	22	15	1.86 (0.66–5.47)
											P-value for trend = 0.03
											P-value for trend = 0.04
											P-value for trend = 0.17
Androstenedione (nmol/l)	1st	0.21–0.84	23	27	1	23	12	1	23	15	1
	2nd	0.84–1.15	22	19	0.74 (0.32–1.69)	22	7	0.61 (0.19–1.82)	22	12	0.85 (0.32–2.21)
	3rd	1.15–1.57	22	21	0.83 (0.36–1.88)	22	12	1.17 (0.42–3.3)	22	9	0.59 (0.2–1.62)
	4th	1.57–2.64	23	24	0.865 (0.385–1.934)	23	12	1 (0.37–2.72)	23	12	0.76 (0.28–2.01)
											P-value for trend = 0.82
											P-value for trend = 0.79
											P-value for trend = 0.54
Testosterone (nmol/l)	1st	0.03–0.34	22	25	1	22	9	1	22	16	1
	2nd	0.34–0.49	21	22	0.93 (0.4–2.13)	21	12	1.43 (0.5–4.22)	21	10	0.65 (0.24–1.74)
	3rd	0.49–0.67	21	19	0.8 (0.34–1.86)	21	11	1.29 (0.44–3.86)	21	8	0.52 (0.18–1.44)
	4th	0.67–2.62	21	23	0.96 (0.42–2.2)	21	11	1.28 (0.44–3.78)	21	12	0.79 (0.3–2.05)
											P-value for trend = 0.85
											P-value for trend = 0.72
											P-value for trend = 0.53
Free testosterone (nmol/l)	1st	0–0.004	22	24	1	22	8	1	22	16	1
	2nd	0.004–0.005	21	15	0.66 (0.27–1.59)	21	7	0.95 (0.28–3.156)	21	8	0.5 (0.17–1.43)
	3rd	0.005–0.008	21	19	0.83 (0.35–1.94)	21	11	1.42 (0.48–4.36)	21	8	0.53 (0.18–1.46)
	4th	0.008–0.023	21	32	1.39 (0.63–3.12)	21	17	2.23 (0.81–6.54)	21	15	0.92 (0.36–2.35)
											P-value for trend = 0.33
											P-value for trend = 0.08
											P-value for trend = 0.95
SHBG (nmol/l)	1st	26–48.8	23	28	1	23	11	1	23	17	1
	2nd	48.8–68.9	23	28	0.99 (0.45–2.17)	23	16	1.55 (0.59–4.2)	23	12	0.71 (0.27–1.82)
	3rd	68.9–90.9	23	14	0.54 (0.22–1.3)	23	8	1.05 (0.32–3.48)	23	6	0.35 (0.11–1.01)
	4th	90.9–167	23	19	0.68 (0.29–1.54)	23	7	0.65 (0.2–1.94)	23	12	0.74 (0.28–1.91)
											P-value for trend = 0.17
											P-value for trend = 0.25
											P-value for trend = 0.28

ER-negative breast cancers. Women had a fourfold increased ER-negative breast cancer risk if free estradiol was in the top quartile in the two years prior to diagnosis but not androgens or SHBG. Estradiol, free estradiol and free testosterone were significantly higher and SHBG significantly lower before ER-positive breast cancer diagnosis compared with controls. Women had a two fold increased ER-positive breast cancer risk if estradiol, free estradiol and free testosterone were in the top quartile and SHBG in the bottom quartile, with the association remaining significant regardless of whether the samples were collected less or over 2 years prior to diagnosis.

The strengths of this study are the nested case control design within a large and well-characterized cohort of postmenopausal women with donation of serum samples prior to diagnosis. In keeping with previous reports, BMI [8] and family history were associated with ER-positive and negative breast cancer risk. Data on hormone receptor status was mainly from a breast cancer questionnaire sent to the treating physician. Endogenous estrogen and androgen were measured by the most accurate tests currently

available to our laboratory – extraction RIA and mass spectrometry, respectively. Limitations include inability to stratify using tumour PR status because of small numbers and restricted generalizability of results to postmenopausal women using hormone therapy, non-white racial groups and premenopausal women.

In ER-negative breast cancer, we found an association with serum estradiol and free estradiol but not with testosterone, free testosterone and androstenedione with women with higher levels of these hormones having increased risk. Currently there are 7 studies that have reported on the association of sex steroid hormone levels with breast cancer risk by receptor status [12–15,17,25,26]. Initial studies that investigated ER-negative breast cancers included only 20–45 women with little power to show any significant association [12,15,17,27,28]. More recently there have been two larger studies by James et al. [13], who investigated 172 ER-negative breast cancers and by Fharat et al. [14], who investigated 111 ER-negatives breast cancers, which have shown conflicting results. The first study reported serum androgens (testosterone and free testosterone) and estrogens (estradiol and

Table 3b
Quartile analysis in the ER POSITIVE subgroup as a whole, and stratified by time to diagnosis.

	Quartile	Range	All cases		OR	N	Cases – Less than 2 years		N	Cases – More than 2 years	
			Controls	Cases			N	Cases		Controls	N
Estradiol (pmol/l)	1st	11–22	53	34	1	53	17	1	53	17	1
	2nd	22–28	45	32	1.11 (0.59–2.08)	45	18	1.25 (0.58–2.72)	45	14	0.97 (0.43–2.18)
	3rd	28–38	39	57	2.28 (1.27–4.16)	39	25	2.02 (0.97–4.32)	39	32	2.54 (1.25–5.3)
	4th	38–612	45	65	2.26 (1.28–4.05)	45	36	2.49 (1.25–5.1)	45	29	2.02 (0.99–4.21)
											P-value for trend = 0.001
											P-value for trend = 0.004
											P-value for trend = 0.01
Free estradiol (pmol/l)	1st	0.26–0.54	46	24	1	46	11	1	46	13	1
	2nd	0.54–0.71	45	32	1.36 (0.7–2.67)	45	19	1.77 (0.77–4.26)	45	13	1 (0.41–2.43)
	3rd	0.71–1.02	45	66	2.81 (1.52–5.29)	45	31	2.889 (1.32–6.68)	45	35	2.75 (1.31–6.02)
	4th	1.02–215	46	66	2.77 (1.5–5.21)	46	35	3.19 (1.48–7.3)	46	31	2.38 (1.12–5.26)
											P-value for trend = <0.001
											P-value for trend = 0.002
											P-value for trend = 0.003
Androstenedione (nmol/l)	1st	0.42–1.04	49	49	1	49	22	1	49	27	1
	2nd	1.04–1.36	47	32	0.69 (0.38–1.25)	47	18	0.85 (0.4–1.78)	47	14	0.54 (0.25–1.16)
	3rd	1.36–1.77	48	44	0.94 (0.53–1.68)	48	20	0.94 (0.45–1.95)	48	24	0.95 (0.48–1.9)
	4th	1.77–3.8	48	72	1.48 (0.86–2.55)	48	38	1.79 (0.93–3.52)	48	34	1.29 (0.68–2.46)
											P-value for trend = 0.08
											P-value for trend = 0.07
											P-value for trend = 0.26
Testosterone (nmol/l)	1st	0.22–0.46	48	49	1	48	25	1	48	24	1
	2nd	0.46–0.61	48	37	0.72 (0.4–1.3)	48	21	0.79 (0.38–1.62)	48	16	0.62 (0.29–1.32)
	3rd	0.61–0.79	46	45	0.94 (0.52–1.67)	46	20	0.84 (0.4–1.73)	46	25	1.01 (0.5–2.05)
	4th	0.79–2.73	48	64	1.29 (0.74–2.25)	48	32	1.32 (0.67–2.62)	48	32	1.25 (0.63–2.48)
											P-value for trend = 0.23
											P-value for trend = 0.45
											P-value for trend = 0.22
Free testosterone (nmol/l)	1st	0.002–0.005	49	28	1	49	15	1	49	13	1
	2nd	0.005–0.007	46	43	1.66 (0.89–3.12)	46	19	1.41 (0.64–3.17)	46	24	1.95 (0.89–4.43)
	3rd	0.007–0.01	47	60	2.19 (1.2–4.03)	47	36	2.47 (1.21–5.22)	47	24	1.83 (0.84–4.14)
	4th	0.01–0.051	48	63	2.23 (1.23–4.1)	48	28	1.86 (0.89–4)	48	35	2.64 (1.25–5.79)
											P-value for trend = 0.004
											P-value for trend = 0.03
											P-value for trend = 0.01
SHBG (nmol/l)	1st	17.8–46	50	68	1	50	34	1	50	34	1
	2nd	46–64.4	50	58	0.85 (0.50–1.44)	50	33	0.97 (0.52–1.81)	50	25	0.74 (0.38–1.41)
	3rd	64.4–88.2	50	50	0.73 (0.43–1.25)	50	29	0.85 (0.45–1.6)	50	21	0.62 (0.31–1.2)
	4th	88.2–165	50	24	0.35 (0.19–0.64)	50	5	0.15 (0.05–0.38)	50	19	0.55 (0.28–1.09)
											P-value for trend = 0.001
											P-value for trend = 0.001
											P-value for trend = 0.07

free estradiol) to be associated with increased risks of both ER-positive and ER-negative breast cancer. The latter study reported a significant reduction of breast cancer with increasing levels of testosterone and no association with estradiol. The current study, although itself of limited size, includes substantially more ER-negative cases of breast cancer than most previous studies. It needs to be noted though that the confidence intervals of the ORs are wide even though there is a trend for increased risk with increasing levels of the hormones, possibly due to the number of the cases investigated.

All previous studies [12–15,17,27,28] have generally shown an association between estrogen and/or androgens with ER-positive breast cancer type in postmenopausal women. Our study confirmed the association with estradiol, free estradiol and free testosterone but not androstenedione. This is in contrast to our previous findings in a separate group of 200 ER-positive women from the same cohort where there was a significant association of serum testosterone and androstenedione with increased breast cancer risk [9]. These differences can only be explained by the fact that androgens were measured in this current study by mass

spectrometry and in the previous by a direct immunoassay. It highlights the critical importance of assays used in postmenopausal women with low levels of circulating sex steroid hormones.

Currently, the role of estrogens and androgens in tumour development is still controversial with conflicting data as to whether estrogens are responsible for the promotion of the early development of a breast tumour or the growth of an already established breast tumour at later stages [11]. Furthermore, the fact that we show a significant association between estrogens and ER-negative breast cancer suggests that the estrogens exert their effects through different pathways other than through the ER. The role of androgens has been controversial in breast cancer and the mechanism of action still remains obscure. It is unclear as to whether they directly stimulate proliferation of breast cells or simply serve as estrogen precursors [15].

In conclusion, this study provides evidence that in postmenopausal women higher levels of estradiol and free estradiol increase ER-negative and -positive breast cancer risk while higher levels of free testosterone increase ER-positive but not ER-negative. Androstenedione measured with mass spectrometry did not show

significant association with either type of breast cancer. Further research is required to better understand the effect of the estrogens and androgens in breast tumour promotion and progression.

Conflict of interest statement

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.steroids.2016.04.003>.

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