Combined effects of endogenous sex hormone levels and mammographic density on postmenopausal breast cancer risk: results from the Breakthrough Generations Study

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Background: Mammographic density and sex hormone levels are strong risk factors for breast cancer, but it is unclear whether they represent the same aetiological entity or are independent risk factors.

Methods: Within the Breakthrough Generations Study cohort, we conducted a case–control study of 265 postmenopausal breast cancer cases and 343 controls with prediagnostic mammograms and blood samples. Plasma was assayed for oestradiol, testosterone and sex hormone-binding globulin (SHBG) concentrations and mammographic density assessed by Cumulus.

Results: Oestradiol and testosterone were negatively and SHBG positively associated with percentage density and absolute dense area, but after adjusting for body mass index the associations remained significant only for SHBG. Breast cancer risk was independently and significantly positively associated with percentage density ($P = 0.002$), oestradiol ($P = 0.002$) and testosterone ($P = 0.007$) levels. Women in the highest tertile of both density and sex hormone level were at greatest risk, with an odds ratio of 7.81 (95% confidence interval (CI): 2.89–21.1) for oestradiol and 4.57 (95% CI: 1.75–11.9) for testosterone and high density compared with those who were in the lowest tertiles. The cumulative risk of breast cancer in the highest oestradiol and density tertiles, representing 8% of controls, was estimated as 12.8% at ages 50–69 years and 19.4% at ages 20–79 years, and in the lowest tertiles was 1.7% and 4.3%, respectively. Associations of breast cancer risk with tertiles of mammographic dense area were less strong than for percentage density.

Conclusions: Endogenous sex hormone levels and mammographic density are independent risk factors for postmenopausal breast cancer, which in combination can identify women who might benefit from increased frequency of screening and chemoprophylaxis.

Mammographic density is one of the strongest risk factors for breast cancer, with a four- to fivefold increased risk in women whose breasts have at least 75% radiodense tissue compared with those with no or very little radiodense tissue (Boyd *et al*, 2011). Radiodense areas of the breast constitute epithelial and connective tissues, with non-dense radiolucent areas representing fatty tissue. Percentage density is known to decline with increasing age and body mass index (BMI), and is also associated with other established breast cancer risk factors, such as parity and menopause (Boyd *et al*, 2011). Density is known to be increased in women using combined hormone therapy (HT) and decreased in tamoxifen users (Martin *et al*, 2009). There is therefore potential for stratifying risk and acting as a biomarker of effectiveness of preventive interventions (Li *et al*, 2013).

Endogenous oestradiol and testosterone levels are positively associated with postmenopausal breast cancer risk (Key *et al*, 2002), and it is unclear to what extent their effects on breast cancer risk are independent of the effect of mammographic density or to what extent density is a reflection of underlying hormone levels.

The association between sex hormone levels and mammographic density is strongly influenced by BMI because obese women have higher oestradiol levels through aromatase activity in adipose tissue, lower sex hormone-binding globulin (SHBG) levels and, on average, lower percentage mammographic density due to the presence of adipose tissue than leaner women (Folkerd *et al*, 2006; Boyd *et al*, 2011). Analyses of the relation between endogenous hormone levels and mammographic density after taking account of BMI have shown inconsistent results (Boyd *et al*, 2002b; Aiello *et al*, 2005; Greendale *et al*, 2005; Tamimi *et al*, 2005; Warren *et al*, 2006; Bremnes *et al*, 2007; Verheus *et al*, 2007; Johansson *et al*, 2008; McCormack *et al*, 2009; Sprague *et al*, 2011).

Investigation of the combined effects of prior sex hormone levels and mammographic density on breast cancer risk is important for understanding of breast cancer aetiology and identification of women at raised risk of breast cancer. Only one study has examined this question previously, a case–control study of 252 cases (Tamimi *et al*, 2007), which reported that postmenopausal endogenous sex hormone levels and mammographic density were independently related to breast cancer risk, with risks being six-fold increased in women who were in the highest tertiles for both density and testosterone and four-fold increased for density and oestradiol compared with those in the lowest tertiles for both. Thus, it highlighted a potentially high-risk group of women and therefore needs re-examination in an independent study and a different population. We conducted analyses of the combined effects of these factors based on breast cancer cases that occurred in the Breakthrough Generations Study (BGS) cohort.

MATERIALS AND METHODS

Participants. Study subjects were identified from the BGS, a United Kingdom-based cohort study with over 113 000 participants designed to investigate breast cancer aetiology (Swerdlow *et al*, 2011). Participants were initially recruited from mailing lists of supporters of the charity Breakthrough Breast Cancer and volunteers from the general population who then nominated friends and family as potential participants. They completed a postal questionnaire and, if willing (91.7%), donated a blood sample, which were predominantly taken by general practitioner practices and posted to the study's laboratory on the same day. The baseline questionnaire consisted of 44 pages including comprehensive information on many breast cancer risk factors. Participants are contacted approximately every 3 years to collect follow-up information on breast cancer diagnoses and updated risk factor information. The study was approved by the South Thames

Multicentre Research Ethics Committee and participants provided informed consent.

The BGS contains a nested case–control study for analyses, where data collection for the full cohort is not feasible. Cases are subjects newly diagnosed with a primary invasive or *in situ* breast cancer since study entry and ascertained by the time of the analysis. Diagnoses were generally initially self-reported on follow-up questionnaires and were then confirmed by cancer registry, general practitioner or pathology records. One control per case is randomly selected from subjects who have been breast cancer free for at least as long as the matched case, within strata of categories of year and age at study entry, ethnicity and the number of days (0–1, 2, ⩾3) between blood draw and receipt of the blood sample in the laboratory, the latter to account for the effect of processing delays on measured plasma sex hormone concentrations (Jones *et al*, 2007). The current analysis is restricted to Caucasian women who were postmenopausal at blood draw, not on postmenopausal HT or other hormonal treatments at the time of blood draw or in the period 3 months before, and for whom we were able to retrieve non-symptomatic mammograms before diagnosis for cases and before the date a control reached a matched case's diagnosis age. Being postmenopausal was defined as having had a natural menopause at least 2 years before blood draw at cohort entry or having had a bilateral oophorectomy, or, for those whose menopausal status was unknown, having reached age 55 (current smokers) or 56 (never/former smokers) years at cohort entry. The inclusion criteria for this analysis led to incomplete matched case–control pairs, as a consequence of which we analysed the data incorporating the matching based on strata of selection to maintain statistical power.

Mammograms were retrieved from breast cancer screening centres, which under the National Breast Cancer Screening Programme invite women for routine 3-yearly screening from ages 50 to 70 years. Prediagnostic mammograms were digitised with a VIDAR Diagnostic Pro Plus scanner (VIDAR Systems Corporation, Herndon, VA, USA), which covers an optical density range of 0–3.85. Cumulus software (Sunnybrook Health Sciences Centre, Toronto, ON, Canada) was used for determination of percentage mammographic density and absolute dense and non-dense area (Byng *et al*, 1994). All images were assessed by one observer, blinded to case–control status, who was trained by an experienced breast radiologist (S Allen). Two images per subject were selected, the left and right mediolateral oblique (MLO) views, from the most recent prediagnostic screening visit or most recent equivalent date for controls. The images were individually randomly allocated to batches of 100. The batches included repeats, based on which the intraclass correlation coefficient was 0.97 for percentage density. Analyses were based on density readings of the views averaged between the left and right breast. Where MLO views could not be read, we used the craniocaudal views (4.3%) or images from an earlier visit (2.1%).

Sex hormone assays. Blood samples were centrifuged, separated into plasma and buffy coat, and stored in $-80\,^\circ$C freezers on the day of receipt before transfer to liquid nitrogen tanks for long-term storage. Plasma oestradiol was measured by radioimmunoassay after organic extraction using a minor modification to a technique previously described and evaluated (Lee *et al*, 2006). Testosterone was measured using a solid-phase radioimmunoassay (Coat-a-Count SMN 10381047; Siemens Healthcare Diagnostics, Camberley, UK). Sex hormone binding globulin was measured using the Spectria SHBG IRMA kit (No. 68562; Orion Diagnostica, Espoo, Finland). The detection limits were $3\,\text{pmol}\,l^{-1}$ for total oestradiol, $0.14\,\text{nmol}\,l^{-1}$ for total testosterone and $1.3\,\text{nmol}\,l^{-1}$ for SHBG; 18 of the samples were below the limit for testosterone; for these we set the values to half the detectable limit. The intra- and interassay coefficient of variation (CV) for oestradiol at a mean of

13 pmol l^{-1} was 5.7% and 16%, respectively, for testosterone at a mean of 2.3 nmol l^{-1} the CV was 2.9% and 5.9%, respectively, and for SHBG at a mean of 72 nmol l^{-1} the CV was 3.8% and 8.3%, respectively. The technician was blinded to case–control status. Batches of samples for assay contained 29 samples on average and included cases with their matched control as far as possible. All measurements were conducted in duplicate in the same batch except for 14 assays where only a single measurement was available owing to insufficient material. Free oestradiol and testosterone were estimated using previously reported formulae (Rinaldi *et al*, 2002).

Statistical analysis. We first assessed associations of mammographic density with sex hormone and SHBG levels among controls using linear regression modelling. We then assessed the odds ratio (OR) of breast cancer in relation to tertiles or quartiles of density and sex hormone levels, determined from the distribution among controls, using an (unconditional) logistic regression model, adjusted for the matching variables, age at first birth and parity, family history of breast cancer, age at menarche, age at menopause, duration of past HT use, alcohol consumption at cohort entry and BMI. In addition, all analyses of sex hormone and SHBG levels were adjusted for laboratory batch. Body mass index was derived from self-reported current height and weight. Tests for trend were based on log base 2 transformation for sex hormone and SHBG levels and square-root transformation for mammographic density, which was needed for the residuals to be normally distributed. We tested for multiplicative interaction between tertiles of sex hormone levels and density with a log-likelihood test comparing a model including the main effects and the cross-product of the factors with a model including only the main effects. All *P*-values are based on two-sided tests.

We estimated the cumulative risk of breast cancer between ages 50–69 and 20–79 years ('lifetime') for subjects according to tertiles of both sex hormone levels and density. Cumulative risks were based on the distribution of controls among tertiles, the respective ORs of breast cancer and the population cumulative risks between ages 50–69 and 20–79 years accounting for competing risks of mortality (Gail *et al*, 1989). Background rates were derived from breast cancer incidence rates and overall mortality rates for England and Wales averaged over 2006–2010, applying a correction for the study participants being never or former HT users with exposure prevalence and ORs from published literature (Reeves *et al*, 2006; Parkin, 2009). Approximate 95% confidence intervals (CIs) were obtained by simulation.

In secondary analyses, we repeated the primary analyses restricted to never HT users. Analyses of breast cancer risk in relation to sex hormone levels were also conducted without adjustment for BMI. Analyses in relation to density were additionally adjusted for mammographic view and furthermore calculated using categories similar to those used in previous studies (Boyd *et al*, 2011). As a background check, we repeated analyses using complete matched case–control sets only. We also conducted additional analyses of combined effects of sex hormone levels and density using dichotomised categories to maximise statistical power and tested for multiplicative as well as additive interaction in this analysis (Rothman, 1986; VanderWeele and Knol, 2013).

RESULTS

We successfully retrieved prediagnostic mammograms for 75% of cases and 78% of controls who reported attendance at mammographic screening. Women for whom we retrieved mammograms were older and therefore more likely to be postmenopausal than women for whom we could not retrieve a mammogram, reflecting a higher retrieval rate in women with multiple historical triennial

screening visits. The age difference was greater for cases (mean 58.2 *vs* 53.5 years) than for controls (mean 58.4 *vs* 56.0 years) (Supplementary Table S1). Among subjects with retrieved mammograms, 265 cases (52.8%) and 343 controls (61.7%) met the eligibility criteria to be assayed for sex hormone levels and were therefore included in the analysis. Among included subjects, the interval from the prediagnostic screening mammogram to the matching index date was greater for cases than for controls, and cases were on average younger at screening than controls owing to the most recent mammogram for cases frequently being diagnostic and therefore an earlier mammogram having to be considered (Table 1). The cases included 227 invasive and 38 *in situ* breast cancers. Cases and controls were similar with regard to the matching factors (Table 1). For three cases and nine controls images could not be read owing to poor resolution or implants.

Among controls, women with higher percentage mammographic density were younger and leaner (Table 2). Total oestradiol levels decreased by 10.0% (*P*<0.001) and free oestradiol by 12.4% (*P*<0.001) per quartile increase in density when adjusted for laboratory batch and age at mammogram, which reduced to 3.2% (*P*=0.261) and 4.6% (*P*=0.114), respectively, after further adjustment for BMI. Decreases in density with testosterone levels were more modest: 4.3% (*P*=0.205) and 9.3% (*P*=0.009) for total and free concentrations, respectively, reduced to nonsignificant decreases of 1.7% and 4.6%, respectively, after additional adjustment for BMI. Sex hormone-binding globulin levels were positively associated with density (9.9%, *P*<0.001), with the association remaining significant after adjustment for BMI (5.9%, *P*=0.006), or BMI plus total (5.9%, *P*=0.007) or free (5.1%, *P*=0.015) oestradiol, or BMI plus total (6.0%, *P*=0.005) or free (5.2, *P*=0.013) testosterone (not in table). Absolute non-dense area was most strongly positively associated with sex hormone levels, in particular free-circulating oestradiol (25.3%, *P*<0.001) and free-circulating testosterone (14.7%, *P*<0.001) and was negatively associated with SHBG (10.7%, *P*<0.001) (Supplementary Table S2b). Associations of absolute dense area in relation with the analytes were weaker than those of percentage density, but statistically significant for most (Supplementary Table S2a).

Breast cancer risk was positively associated with mammographic percentage density (Table 3), with the OR in the highest quartile (median density 40.2%) compared with the lowest quartile (median density 3.2%) being 2.75 (95% CI: 1.57–4.79). These associations became somewhat stronger after adjustment for sex hormones or SHBG. Odds ratios in relation to frequently reported categories of density were 1.81 (95% CI: 1.13–2.90) for 10–24%, 2.32 (95% CI: 1.40–3.86) for 25–49% and 2.37 (95% CI: 1.04–5.41) for ⩾50% compared with <10% density. Associations with absolute dense area were somewhat weaker than for percentage dense area (Supplementary Table S3).

Total and free oestradiol and testosterone levels were positively associated with breast cancer risk (Table 4). Odds ratios in the highest quartile compared with the lowest were greatest for free oestradiol (OR=2.42; 95% CI: 1.27–4.61) and of similar magnitude for total oestradiol (OR=2.07; 95% CI: 1.11–3.84), total testosterone (OR=2.11; 95% CI: 1.20–3.70) and free testosterone (OR=2.07; 95% CI: 1.15–3.74). Additional adjustment for percentage density (Table 4) or absolute dense area (not in table) did not materially affect these associations. Breast cancer risk was borderline significantly inversely associated with SHBG and significantly so after adjustment for percentage density (OR=0.58; 95% CI: 0.31–1.10 for the highest quartile). Odds ratios of breast cancer in relation to sex hormone levels without adjustment for BMI were somewhat lower for oestradiol and stronger for testosterone than those reported after adjustment (not in table).

In analyses of the combined effects of percentage density and total sex hormone levels, the OR in women in the top tertile for both was 7.81 (95% CI: 2.89–21.1) for oestradiol and 4.57

Table 1. Characteristics at recruitment of cases and controls, Breakthrough Generations Study, United Kingdom

Characteristic	Cases No.[a]	Cases %	Controls No.[a]	Controls %
Age at entry (years)				
<60	121	45.7	152	44.3
60–69	130	49.1	165	48.1
⩾70	14	5.3	26	7.6
Year of entry to the study				
<2006	132	49.8	155	45.2
2006	71	26.8	103	30.0
2007	40	15.1	55	16.0
⩾2008	22	8.3	30	8.8
Days blood sample in post				
0–1	216	82.1	278	81.3
2	23	8.8	28	8.2
⩾3	24	9.1	36	10.5
Age at mammogram (years)				
<60	123	46.4	146	42.7
60–69	131	49.4	175	51.2
⩾70	11	4.2	21	6.1
Time of mammogram compared with diagnosis or index date				
<1.0 year earlier	16	6.0	76	22.2
1–1.9 years earlier	39	14.7	101	29.5
2–2.9 years earlier	96	36.2	103	30.1
⩾3.0 years earlier	114	43.0	62	18.1
Timing of mammogram compared with blood draw				
2–3 years earlier	61	23.0	46	13.5
1–1.9 years earlier	57	21.5	60	17.5
Within 12 months	99	37.4	149	43.6
1–1.9 years later	22	8.3	32	9.4
⩾2.0 years later	26	9.8	55	16.1
Age at menarche (years)				
<13	94	39.2	143	46.4
13	67	27.9	74	24.0
⩾14	79	32.9	91	29.6
Age at first birth and parity				
Nulliparous	27	10.2	36	10.5
<25 years/1–2 children	55	20.8	85	24.8
<25 years/⩾3 children	30	11.3	69	20.1
25–29 years/1–2 children	67	25.3	84	24.5
25–29 years/⩾3 children	39	14.7	29	8.5
⩾30 years/1–4 children	47	17.7	40	11.7
Age at menopause (years)				
<50	51	28.7	81	35.7
50–54	99	55.6	123	54.2
⩾55	28	15.7	23	10.1
Former use of postmenopausal hormone therapy (years)				
Never	144	56.7	160	50.2
<5	32	12.6	51	16.0
5–9	45	17.7	56	17.6
⩾10	33	13.0	52	16.3

Table 1. (Continued)

Characteristic	Cases No.[a]	Cases %	Controls No.[a]	Controls %
Body mass index (kg m⁻²)				
<25.0	120	45.8	162	47.8
25.0–29.9	97	37.0	128	37.8
⩾30.0	45	17.2	49	14.5
Alcohol consumption (g per day)				
None	52	20.0	68	20.1
<5	36	13.9	48	14.2
5–14	88	33.9	91	26.9
⩾15	84	32.3	131	38.8
Family history of breast cancer (yes)	66	24.9	57	16.6
History of benign breast disease (yes)	93	35.4	86	25.2
Total	265	100.0	343	100.0

[a]Numbers do not always add up to the total owing to missing values.

(95% CI: 1.75–11.9) for testosterone compared with those who were in the lowest tertile for both (Table 5). For SHBG, the OR was greatest in the lowest tertile of SHBG and the highest tertile of density (OR = 2.23; 95% CI: 0.92–5.42). There was no statistical evidence for interaction between these factors on breast cancer risk. Analyses of absolute dense area instead of percentage dense area showed weaker associations (Supplementary Table S4).

Estimates of cumulative risk of breast cancer based on the above ORs for combinations of tertiles and background population rates for England and Wales were 12.8% (95% CI: 4.9–30.7%) from ages 50 to 69 years and 19.4% (95% CI: 8.8–42.0%) between ages 20 and 79 years for women in the top tertiles of both oestradiol and density, and 1.7% (95% CI: 0.61%–4.9%) and 4.3% (95% CI: 2.7%–8.6%) for those in the bottom tertiles of both, whereas those in the general population were 6.0% and 10.2%, respectively (Table 6).

In analyses restricted to never HT users, ORs for breast cancer were somewhat lower in relation to percentage density and to total oestradiol than for all study subjects combined, were similar for free oestradiol, free testosterone and SHBG, but stronger for total testosterone. Analyses of combined effects in this group were not conducted owing to small numbers. Analyses of density on breast cancer risk adjusted for mammographic view did not affect the results. Restricting the analysis to matched sets only did not materially affect the conclusions but affected the precision of the estimates. Analyses repeated with dichotomised categories of density and sex hormone levels did not reveal statistically significant interactions (Supplementary Table S5).

DISCUSSION

In our study, circulating oestradiol and testosterone levels and mammographic density were each independent risk factors for breast cancer. Women in the highest tertile of both mammographic percentage density and total oestradiol or testosterone were at over seven- and fourfold increased risk, respectively, compared with those in the lowest for both. On the basis of United Kingdom national cancer incidence rates, we estimate that women in the top tertile for both oestradiol and percentage density might be at cumulative risk of 12.8% between ages 50 and 69 years and 19.4% between 20 and 79 years, although approximate CIs were wide reflecting limited number of subjects in the tertile groups. Such risk

Table 2. Association between plasma oestradiol, testosterone and SHBG levels and percentage mammographic density among control subjects: Breakthrough Generations Study, United Kingdom

Characteristic	Percentage mammographic density (%), quartiles[a]				% Increase per quartile[b]	P-value trend[b]
	1st	2nd	3rd	4th		
No. of controls	84	83	84	83		
Median percentage density (%)	3.2	11.8	22.2	40.2		
Mean age at mammogram (years)	62.0	60.8	60.7	60.6		
Mean BMI at cohort entry (kg m^{-2})	28.0	25.4	26.5	24.1		
Geometric mean, adjusted for laboratory batch and age at mammogram						
Oestradiol (pmol l^{-1})	22.6	19.9	20.8	15.6	− 10.0	<0.001
Free oestradiol (pmol l^{-1})	0.55	0.47	0.49	0.35	− 12.4	<0.001
Testosterone (nmol l^{-1})	0.64	0.56	0.62	0.53	− 4.3	0.205
Free testosterone (nmol l^{-1})	0.010	0.008	0.009	0.007	− 9.3	0.009
SHBG (nmol l^{-1})	37.3	42.3	42.5	51.0	9.9	<0.001
Geometric mean, additionally adjusted for BMI						
Oestradiol (pmol l^{-1})	19.7	20.8	20.2	17.9	− 3.2	0.261
Free oestradiol (pmol l^{-1})	0.47	0.49	0.48	0.41	− 4.6	0.114
Testosterone (nmol l^{-1})	0.62	0.57	0.62	0.56	− 1.7	0.638
Free testosterone (nmol l^{-1})	0.009	0.008	0.009	0.007	− 4.6	0.202
SHBG (nmol l^{-1})	39.9	41.5	43.1	47.8	5.9	0.006

Abbreviations: BMI = body mass index; SHBG = sex hormone-binding globulin.
[a]Q1: <6.58; Q2: 6.58–16.75; Q3: 16.76–29.60; Q4: >29.60 percentage density.
[b]Percentage change in sex hormone or SHBG level per quartile increase in percentage mammographic density, P-value based on Wald test.

Table 3. Odds ratios for incident breast cancer in relation to percentage mammographic density, Breakthrough Generations Study, United Kingdom

	Percentage mammographic density (%), quartiles[a]				P-value trend[b]
	1st	2nd	3rd	4th	
Number (%)					
Cases	40 (15.3)	69 (26.3)	56 (21.4)	97 (37.0)	
Controls	84 (25.2)	83 (24.9)	84 (25.2)	83 (24.9)	
Odds ratio (95% CI)					
Multivariate[c]	1.00	2.09 (1.20–3.64)	1.43 (0.82–2.50)	2.75 (1.57–4.79)	0.002
Additional adjustment for					
Oestradiol[d]	1.00	2.15 (1.18–3.93)	1.52 (0.82–2.82)	2.72 (1.49–4.96)	0.006
Free oestradiol[d]	1.00	2.17 (1.19–3.97)	1.50 (0.81–2.79)	2.79 (1.52–5.10)	0.005
Testosterone[d]	1.00	2.26 (1.23–4.15)	1.61 (0.87–2.98)	2.70 (1.48–4.94)	0.008
Free testosterone[d]	1.00	2.31 (1.27–4.21)	1.63 (0.88–3.02)	2.83 (1.55–5.18)	0.006
SHBG[d]	1.00	2.32 (1.27–4.22)	1.61 (0.87–3.00)	3.03 (1.64–5.61)	0.004

Abbreviations: CI = confidence interval; SHBG = sex hormone-binding globulin.
[a]Q1: <6.58; Q2: 6.58–16.75; Q3: 16.76–29.60; Q4: >29.60 percentage density.
[b]On the basis of Wald test on square-root-transformed percentage density, continuous.
[c]Covariates: year of entry to the study (⩽2005, 2006, 2007, ⩾2008); age at entry (5-year age groups); blood in post (0–1, 2, ⩾3 days, not known); years breast cancer free in study (0–6); body mass index (kg m^{-2}) (continuous); parity (nulliparous; age at first birth <25 years/1–2 children; age at first birth <25 years/⩾3 children; age at first birth 25–29 years/1–2 children; age at first birth 25–29 years/⩾3 children; age at first birth ⩾30 years/1–4 children); family history of breast cancer (yes, no); age at menarche (⩽12, 13,14,15–20, not known); age at menopause (<45, 45–49, 50–54, ⩾55, not known); years of postmenopausal hormone therapy use (never, <2, 2–5, 6–9, ⩾10, not known); alcohol units (0, <5, 5–14, ⩾15 g per day, not known).
[d]Adjustment for covariates and quartile of respective sex hormone or SHBG level and laboratory batch.

estimates in the top tertiles, representing 8.2% of controls in our study, are within the range of risk levels for which the National Institute for Health and Care Excellence recommends increased frequency of breast cancer screening and chemoprophylaxis (National Institute for Health and Care Excellence, 2013). On the other hand, women in the lowest tertile for both have estimated cumulative risks of 1.7% at ages 50–69 years and 4.3% lifetime, considerably lower than that in the general population.

Table 4. Odds ratios for incident breast cancer in relation to quartiles of plasma oestradiol, testosterone and SHBG, Breakthrough Generations Study, United Kingdom

Sex hormone or SHBG level/adjustment factors	Plasma hormone level, quartiles[a]				
	1st	2nd	3rd	4th	
		OR[c] (95% CI)	OR[c] (95% CI)	OR[c] (95% CI)	P-value trend[b]
Oestradiol					
Multivariate[c]	1.00	1.18 (0.67–2.09)	1.51 (0.83–2.75)	2.07 (1.11–3.84)	0.002
+ Percentage density[d]	1.00	1.21 (0.68–2.16)	1.49 (0.81–2.75)	2.03 (1.08–3.81)	0.004
Free oestradiol					
Multivariate[c]	1.00	1.32 (0.74–2.36)	1.72 (0.95–3.13)	2.42 (1.27–4.61)	0.001
+ Percentage density[d]	1.00	1.46 (0.81–2.63)	1.78 (0.97–3.27)	2.48 (1.29–4.78)	0.001
Testosterone					
Multivariate[c]	1.00	1.34 (0.75–2.37)	1.42 (0.79–2.52)	2.11 (1.20–3.70)	0.007
+ Percentage density[d]	1.00	1.21 (0.67–2.18)	1.36 (0.76–2.44)	2.01 (1.14–3.54)	0.009
Free testosterone					
Multivariate[c]	1.00	1.47 (0.82–2.64)	1.82 (1.01–3.28)	2.07 (1.15–3.74)	0.001
+ Percentage density[d]	1.00	1.53 (0.85–2.76)	1.87 (1.03–3.40)	2.15 (1.18–3.91)	0.001
SHBG					
Multivariate[c]	1.00	1.10 (0.63–1.90)	0.86 (0.48–1.56)	0.64 (0.35–1.20)	0.055
+ Percentage density[d]	1.00	1.11 (0.63–1.95)	0.74 (0.40–1.37)	0.58 (0.31–1.10)	0.024

Abbreviations: CI = confidence interval; OR = odds ratio; SHBG = sex hormone-binding globulin.
[a]1st, 2nd, 3rd, 4th quartile, respectively, oestradiol: < 13.18, 13.18–18.86, 18.87–29.38, > 29.38 pmol l^{-1}; free oestradiol: < 0.29, 0.29–0.44, 0.45–0.69, > 0.69 pmol l^{-1}; testosterone: < 0.40, 0.40–0.63, 0.64–0.91, > 0.91 nmol l^{-1}; free testosterone: < 0.0055, 0.0055–0.0087, 0.0088–0.0138, > 0.0138 nmol l^{-1}; SHBG: < 31.30, 31.30–43.45, 43.46–59.10, > 59.10 nmol l^{-1}.
[b]On the basis of Wald test on log base 2 transformation of respective sex hormone or SHBG value.
[c]Covariates: year of entry to the study (⩽ 2005, 2006, 2007, ⩾ 2008); age at entry (5-year age groups); blood in post (0–1, 2, ⩾ 3 days, not known); years breast cancer free in study (0–6); body mass index (kg m^{-2}) (continuous); parity (nulliparous; age at first birth < 25 years/1–2 children; age at first birth < 25 years/⩾ 3 children; age at first birth 25–29 years/1–2 children; age at first birth 25–29 years/⩾ 3 children; age at first birth ⩾ 30 years/1–4 children); family history of breast cancer (yes, no); age at menarche (⩽ 12, 13, 14, 15–20, not known); age at menopause (< 45, 45–49, 50–54, ⩾ 55, not known); years of postmenopausal hormone therapy use (never, < 2, 2–5, 6–9, ⩾ 10, not known); alcohol units (0, < 5, 5–14, ⩾ 15 g per day, not known); laboratory batch.
[d]Further adjustment for mammographic percentage density quartiles.

Among the three mammographic density parameters we analysed, we observed the strongest associations between sex hormone levels and SHBG on the absolute area of non-dense mammographic tissue. Non-dense tissue on the mammogram consists of adipose tissue and is highly correlated with BMI (Haars et al, 2005; Boyd et al, 2006). In postmenopausal women, oestrogen production mostly arises through aromatase activity converting androgens in adipose tissue after cessation of ovarian function (Folkerd et al, 2006). Women with high BMI have therefore higher aromatase levels and lower levels of SHBG and as a consequence higher total and free oestradiol levels than women with lower BMI. They also have, for unclear reasons, increased testosterone and androstenedione levels (Folkerd et al, 2006; Key et al, 2011). For percentage density, we observed significant inverse associations with sex hormone levels but not significant associations after adjustment for BMI. The influence of BMI on relative density is thought to be entirely due to an effect of non-dense tissue (Haars et al, 2005) and it is conventional to adjust percentage density for BMI (and age) because a particular level of percentage density in a woman with low BMI does not represent the same amount of dense tissue as in a woman with high BMI, owing to high BMI being associated with more non-dense area and larger total breast size. The use of an absolute rather than relative measure of dense area can reduce or eliminate confounding by BMI (Assi et al, 2012). In our study, absolute dense area was less strongly negatively associated with oestradiol and similarly associated with

testosterone levels as percentage dense area, suggesting an influence of sex hormone levels on dense area. Additional adjustment for BMI to explore residual confounding showed weaker nonsignificant associations, but it is questionable whether inclusion of BMI represents overadjustment because BMI and sex hormone levels are on the same pathway. Previous studies of the association between sex hormone levels and density have shown inconsistent results, with some reporting an inverse (Boyd et al, 2002b; Aiello et al, 2005), positive (Greendale et al, 2005; Bremnes et al, 2007; Johansson et al, 2008) or no association (Tamimi et al, 2005; Warren et al, 2006; Verheus et al, 2007; McCormack et al, 2009; Sprague et al, 2011) with circulating sex hormone levels after adjusting for adiposity.

The positive association of SHBG levels with percentage and absolute dense area in our study, which remained after adjusting for BMI, is supported by some (Boyd et al, 2002b; Greendale et al, 2005; Tamimi et al, 2005; Warren et al, 2006; Bremnes et al, 2007; Johansson et al, 2008; Sprague et al, 2011; Woolcott et al, 2013), but not all (Aiello et al, 2005; Verheus et al, 2007; McCormack et al, 2009) past studies. Our findings suggest that SHBG independently affects density because the association remained significant after adjustment for oestradiol or testosterone. Other hormones could potentially also affect density, such as progesterone (Sprague et al, 2011; Lee et al, 2012), because associations with density have been stronger for combined HT than solely for oestrogen HT (Martin et al, 2009), or prolactin, which has been

Table 5. Odds ratios for incident breast cancer in relation to tertiles of plasma sex hormone and SHBG level and percentage mammographic density, Breakthrough Generations Study, United Kingdom

Sex hormone or SHBG level (tertiles)	Percentage mammographic density (%), tertiles[a]					
	1st		2nd		3rd	
	Cases/controls	OR[b] (95% CI)	Cases/controls	OR[b] (95% CI)	Cases/controls	OR[b] (95% CI)
Oestradiol[c]						
1st	11/31	1.00	25/34	2.60 (0.96–7.05)	38/45	2.97 (1.16–7.60)
2nd	17/38	1.53 (0.54–4.37)	28/33	4.18 (1.55–11.3)	33/37	2.58 (0.98–6.80)
3rd	28/41	2.88 (1.04–8.02)	35/44	3.68 (1.39–9.76)	47/27	7.81 (2.89–21.1)
						P interaction[d] = 0.276
Free oestradiol[c]						
1st	12/34	1.00	25/30	3.15 (1.18–8.36)	44/46	3.34 (1.37–8.15)
2nd	15/38	1.21 (0.43–3.42)	32/37	4.02 (1.57–10.3)	28/34	2.53 (0.97–6.57)
3rd	29/38	3.17 (1.17–8.63)	31/44	2.88 (1.11–7.74)	46/29	6.23 (2.40–16.2)
						P interaction[d] = 0.116
Testosterone[c]						
1st	10/36	1.00	31/35	3.91 (1.46–10.5)	33/39	2.96 (1.10–7.95)
2nd	23/35	2.18 (0.80–5.95)	26/44	2.61 (0.95–7.11)	40/33	4.60 (1.75–12.1)
3rd	23/40	2.18 (0.79–5.99)	31/32	4.23 (1.59–11.3)	45/39	4.57 (1.75–11.9)
						P interaction[d] = 0.434
Free testosterone[c]						
1st	12/36	1.00	28/35	3.33 (1.28–8.67)	34/39	2.85 (1.11–7.31)
2nd	15/34	1.65 (0.59–4.62)	22/39	2.74 (1.02–7.35)	37/39	3.44 (1.38–8.57)
3rd	29/41	2.35 (0.91–6.07)	38/37	3.60 (1.45–8.93)	47/33	5.00 (2.00–12.55)
						P interaction[d] = 0.768
SHBG[c]						
1st	31/46	1.00	31/42	1.20 (0.55–2.63)	30/21	2.23 (0.92–5.42)
2nd	16/33	0.62 (0.25–1.57)	36/36	1.68 (0.75–3.76)	45/41	1.52 (0.68–3.38)
3rd	9/32	0.35 (0.13–0.97)	21/33	1.08 (0.43–2.71)	43/49	1.20 (0.53–2.73)
						P interaction[d] = 0.517

Abbreviations: CI = confidence interval; OR = odds ratio; SHBG = sex hormone-binding globulin.
[a]Tertiles: <9.99, 9.99–24.09, >24.09%.
[b]Covariates: year of entry to the study (⩽2005, 2006, 2007, ⩾2008); age at entry (5-year age groups); blood in post (0–1, 2, ⩾3 days, not known); years breast cancer free in study (0–6); body mass index, kg m^{-2} (continuous); parity (nulliparous; age at first birth <25 years/1–2 children; age at first birth <25 years/⩾3 children; age at first birth 25–29 years/1–2 children; age at first birth 25–29 years/⩾3 children; age at first birth ⩾30 years/1–4 children); family history of breast cancer (yes, no); age at menarche (⩽12, 13, 14, 15–20, not known); age at menopause (<45, 45–49, 50–54, ⩾55, not known); years of postmenopausal hormone therapy use (never, <2, 2–5, 6–9, ⩾10, not known); alcohol units (0, <5, 5–14, ⩾15 g per day, not known); laboratory batch.
[c]1st, 2nd, 3rd tertile, respectively, oestradiol: <14.77, 14.77–24.45, >24.45 pmol l^{-1}; free oestradiol: <0.35, 0.35–0.61, >0.61 pmol l^{-1}; testosterone: <0.49, 0.49–0.84, >0.84 nmol l^{-1}; free testosterone: <0.0064, 0.0064–0.0115, >0.0115 nmol l^{-1}; SHBG: <35.2, 35.2–52.7, >52.7 nmol l^{-1}.
[d]On the basis of log-likelihood ratio test.

positively associated with density in some, but not all, studies (Boyd *et al*, 2002b; Tamimi *et al*, 2005; Bremnes *et al*, 2007; Greendale *et al*, 2007). Recent reports also suggest that the extent and pathway of oestrogen metabolism is associated with density (Fuhrman *et al*, 2012).

The mechanisms through which sex hormones, BMI and density contribute to breast cancer risk are complex and incompletely understood. It is thought that oestrogens promote breast cancer progression by increasing proliferation of breast epithelial tissue, but it has also been suggested that oestrogens and their hydroxylated metabolites may act as carcinogens (Folkerd *et al*, 2006). The association of BMI with breast cancer risk is probably due to increased sex hormone levels rather than an effect of BMI *per se* (Key *et al*, 2003). Mammographic density has been proposed to represent the cumulative exposure of tissue to hormones and growth factors that stimulate cell division and it has been hypothesised that

such exposures at young ages, during the greatest susceptibility of the breast according to the Pike Model (Pike *et al*, 1983), is related to tissue composition (Boyd *et al*, 2010). The well-established associations of density with parity, combined HT use and tamoxifen (Martin *et al*, 2009; Boyd *et al*, 2011) provide evidence for both endogenous and exogenous sex hormone levels as important determinants of density. The extent of dense tissue might also simply raise cancer risk because such tissue has more epithelial cells and thus a higher risk of transformation, and because there is more communication of epithelial cells with connective tissue, which produces hormones and growth factors (Polyak and Kalluri, 2010). An independent protective role of non-dense tissue on breast carcinogenesis has recently been reported (Pettersson *et al*, 2011), despite adipose tissue being an important source of oestrogens.

The independence in effects of postmenopausal sex hormone levels and density on breast cancer risk in our study suggests that

Table 6. Cumulative risks (%) of incident breast cancer between various ages by tertile of percentage mammographic density and plasma sex hormone levels		
Percentage mammographic density (%), tertiles[a]		
1st	**2nd**	**3rd**
Cumulative risk of breast cancer between ages		
Oestradiol, tertiles[b]		
Age: 50–69 years		
1st 1.7 (0.61–4.9)	4.5 (1.7–11.7)	5.1 (2.0–12.4)
2nd 2.7 (0.95–7.4)	7.1 (2.7–17.9)	4.4 (1.7–11.3)
3rd 4.9 (1.8–13.2)	6.3 (2.4–15.7)	12.8 (4.9–30.7)
Age: 20–79 years		
1st 4.3 (2.7–8.6)	8.1 (4.2–17.8)	9.0 (4.7–18.9)
2nd 5.6 (3.2–11.9)	11.7 (5.6–25.9)	8.0 (4.2–17.2)
3rd 8.8 (4.4–19.9)	10.6 (5.2–23.2)	19.4 (8.8–42.0)

[a]Tertiles for mammographic density: <9.99, 9.99–24.09, >24.09%.
[b]Tertiles for oestradiol: <14.77, 14.77–24.45, >24.45 pmol l^{-1}.

current sex hormone levels are a poor proxy for cumulative hormonal exposures, or that other forms of oestrogens not measured in this study have a role or that there are other important pathways involved. Positive associations of density with IGF I and IGFBP3 levels have been reported in some studies, although these associations appear to be confined to premenopausal women (dos Santos Silva et al, 2006; Bremnes et al, 2007; Becker and Kaaks, 2009; McCormack et al, 2009; Rice et al, 2012). The heritability of density is estimated as 63% from twin studies (Boyd et al, 2002a) and breast cancer susceptibility variants in genes LSP1, RAD51L1 (Vachon et al, 2012) and ZNF365 (Lindstrom et al, 2011), and at 12q24 (Stevens et al, 2012) have also been associated with density; therefore, density and breast cancer might have a shared genetic basis.

Our results are supported by the only previous report of combined effects of density and sex hormones on breast cancer risk, a case–control analysis including 253 cases from the Nurses' Health Study, which reported that women in the top tertile of percentage density and oestradiol or testosterone were at four- and six-fold risk of breast cancer, respectively, compared with those in the lowest tertiles for both (Tamimi et al, 2007). Associations of breast cancer risk with sex hormone levels, irrespective of density, were of similar magnitude in our study to that by Tamimi et al (2007), but associations of risk with density, irrespective of sex hormone levels, were weaker, despite a very similar distribution of percentage density among controls. However, when we categorised density using cut-points frequently used by others rather than quartiles of our data, our results were comparable to those of a pooled analyses of three Canadian studies (Boyd et al, 2011).

We could not retrieve prediagnostic mammograms for all cases and controls, and women with retrieved mammograms were older and therefore more often postmenopausal than those for whom we were unsuccessful, likely to be a reflection of older women having longer asymptomatic screening histories and therefore a higher probability that a set of mammograms can be retrieved from a screening centre. For cases this age difference was more pronounced than for controls because younger cases had a shorter or no asymptomatic screening history owing to screen-detected breast cancers. There was, however, no appreciable difference in

mean age at entry or menopausal status between cases and controls with retrieved mammograms. A smaller proportion of cases than controls were eligible for hormone analyses owing to cases being more likely to be on HT at blood draw, which resulted in a lower prevalence of past HT use among included cases compared with controls.

Limitations of our study are that sex hormone assessments were based on a single plasma sample and density was evaluated at a single time point and that the sex hormone and mammographic density measures were collected at a median of 1.2 (controls) or 1.5 (cases) years apart, which is likely to have weakened associations. For sex hormone levels the observed OR of association is likely to be an underestimate of the true association because a single measurement is an imperfect estimate of the true pattern over several years that presumably matters, owing to within-person variation and assay variation. Zhang et al (2013) recently assessed the reliability of a single measurement of plasma oestradiol and testosterone by measuring these hormones in samples taking from postmenopausal women 10 years apart and observed an intraclass correlation coefficient of 0.69 for total oestradiol and 0.71 for total testosterone. They concluded that single plasma measurements of oestradiol and testosterone were predictive of breast cancer for up to 16–20 years. Mammographic density measurements have inter- and intrareader variability (Boyd et al, 2011), although readings with Cumulus have been shown to be highly reliable (McCormack et al, 2007). Associations of postmenopausal breast cancer risk with mammographic density have been reported to persist for up to 10 years after the mammogram (Yaghjyan et al, 2013).

Our study had very limited precision to estimate combined effects in subgroups of women. It is possible that ORs for combined effects are higher in women who have never used HT, as suggested by the previous study by Tamimi et al (2007) and by stronger associations with endogenous oestradiol in such women in a large pooled analysis (Key et al, 2002). Furthermore, ORs are likely to be higher for ER-positive breast cancer than for breast cancer overall, because it has been suggested that sex hormone associations are limited to ER-positive cancer (Tamimi et al, 2007; Zhang et al, 2013), but receptor status is not currently available for enough subjects in our data set to confirm or refute this. Pooling of data sets will be needed to investigate combined effects by hormone receptor status of the breast cancer, former HT use or other factors such as BMI and family history of breast cancer.

Mammographic density information is not yet routinely collected at screening in the United Kingdom and has not yet been included in published risk prediction models or been incorporated into breast cancer screening strategies. The use of Cumulus or similar programmes is labour intensive and requires thorough operator training, making it unsuitable for high volume processing. The introduction of digital mammography into the National Breast Cancer Screening Programme and recently developed high-throughput methods to assess mammographic density could help facilitate collection of such information on a large scale (Li et al, 2012; Skippage et al, 2013). Our study suggests that information on density and sex hormone levels combined might have additional predictive power, but a standardised assay for oestradiol would need to be developed for postmenopausal women which is suitable for incorporation into clinical practice (Dowsett and Folkerd, 2005; Lee et al, 2006). This could help tailor screening frequencies as well as screening modalities, and could aid clinical decision-making with regard to prophylactic measures.

We conclude that prior circulating oestradiol and testosterone levels as well as mammographic density appear to be independent risk factors for postmenopausal breast cancer, and that women with high levels of both sex hormones and density have levels of cumulative breast cancer risk that might require increased screening frequencies and chemoprophylaxis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DISCLAIMER

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation or writing of the report. The authors had full access to the data in the study and had the full responsibility for the decision to submit the manuscript for publication.

AUTHOR CONTRIBUTIONS

MD and AJS designed and obtained funding for the study, MD and EJF designed and supervised the laboratory assays and MR carried out the laboratory work. SA contributed to data collection and mammographic density readings. AA, AJS, MEJ and MJS set up and collected data in the Breakthrough Generations Study and AJS and MEJ contributed to data collection for the substudy. MJS oversaw data collection, conducted the analyses and drafted the manuscript. All authors contributed to data interpretation and writing of the manuscript.

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