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Research Article

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Endogenous Sex Hormones and Breast Density in Young Women

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Abstract

Background: Breast density is a strong risk factor for breast cancer and reflects epithelial and stromal content. Breast tissue is particularly sensitive to hormonal stimuli before it fully differentiates following the first full-term pregnancy. Few studies have examined associations between sex hormones and breast density among young women.

Methods: We conducted a cross-sectional study among 180 women ages 25 to 29 years old who participated in the Dietary Intervention Study in Children 2006 Follow-up Study. Eighty-five percent of participants attended a clinic visit during their luteal phase of menstrual cycle. Magnetic resonance imaging measured the percentage of dense breast volume (%DBV), absolute dense breast volume (ADBV), and absolute nondense breast volume (ANDBV). Multiple-linear mixed-effect regression models were used to evaluate the association of sex hormones and sex hormone–binding globulin (SHBG) with %DBV, ADBV, and ANDBV.

Results: Testosterone was significantly positively associated with %DBV and ADBV. The multivariable geometric mean of %DBV and ADBV across testosterone quartiles increased from 16.5% to 20.3% and from 68.6 to 82.3 cm^3, respectively ($P_{trend} \leq 0.03$). There was no association of %DBV or ADBV with estrogens, progesterone, non–SHBG-bound testosterone, or SHBG ($P_{trend} \geq 0.27$). Neither sex hormones nor SHBG was associated with ANDBV except progesterone; however, the progesterone result was nonsignificant in analysis restricted to women in the luteal phase.

Conclusions: These findings suggest a modest positive association between testosterone and breast density in young women.

Impact: Hormonal influences at critical periods may contribute to morphologic differences in the breast associated with breast cancer risk later in life. *Cancer Epidemiol Biomarkers Prev; 24(2); 369–78.* ©2014 AACR.

Introduction

Breast density is a measure of the relative proportion of glandular and stromal tissue to fatty tissue in the breasts and is a strong risk factor for breast cancer (1, 2). In a meta-analysis, women with dense breasts were at a 4-fold excess risk of developing breast cancer (2). Reproductive and menstrual factors (e.g., nulliparity, late menopause, and late first pregnancy; ref. 3) and exogenous hormone use (4, 5) have been associated with greater breast density. These findings are consistent with an estrogenic hormonal proliferative effect on breast tissue (6–10) that is reflected in the amount of dense tissue and breast density (11). However, endogenous serum sex hormones have not been consistently associated with breast density in premenopausal (12–18) or postmenopausal women (18–28).

Breast tissue differs in its response to stimuli over the life course (29, 30). Most research on breast density in healthy premenopausal women has used screening mammograms and consequently includes women 40 years and older. Few studies have examined endogenous sex hormones in relation to breast density among women younger than 40 years old. In 280 women with a mean age of 18 years old, Boyd and colleagues observed positive associations of serum estradiol, testosterone, progesterone, and sex hormone–binding globulin (SHBG) concentrations with percentage of breast water, a measure of the proportion of the fibroglandular breast tissue to fatty tissue, but only SHBG results were statistically significant (31). Chen and colleagues reported a significant positive correlation of estradiol and progesterone with breast density at the third week of the menstrual cycle in a small study ($N = 24$) of Asian women with a mean age of 29.4 years old. When saliva was alternatively used to measure sex hormones, Frydenberg and colleagues found that high mammographic density was significantly associated with high progesterone levels among women ages 25 to 35 years old (32). We also reported positive associations between breast density and nulliparity and the duration of hormonal contraceptive use, proxies of higher cumulative estrogen exposure to breast tissue (6, 7, 9), among women ages 25 to 29 years (33). In sum, despite some suggestive positive associations of sex hormones with breast density among young women, evidence is sparse necessitating further study.

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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We hypothesized that higher endogenous sex hormone levels are associated with higher breast density among young women in their twenties. To test this hypothesis, we conducted a cross-sectional analysis of the Dietary Intervention Study in Children (DISC) 2006 Follow-Up Study (33–35) to evaluate associations of serum estrogens, androgens, progesterone, and SHBG concentrations with breast density among women ages 25 to 29 years.

Materials and Methods

Study design

The DISC was a two-armed, multicenter, randomized controlled clinical trial sponsored by the National Heart, Lung, and Blood Institute (NHLBI, Bethesda, MD) to assess the safety and efficacy of a lipid-lowering diet in children with elevated low-density lipoprotein cholesterol (LDL-C). Details of the DISC study design have been previously described (36, 37). Between 1988 and 1990, DISC enrolled 663 children (301 girls and 362 boys) at six clinical centers: Children's Hospital (New Orleans, LA), Johns Hopkins University Hospital (Baltimore, MD), Kaiser Permanente Center for Health Research (Portland, OR), University of Medicine and Dentistry of New Jersey (Newark, NJ), Northwestern University Medical School (Chicago, IL), and University of Iowa Hospital and Clinics (Iowa City, IA). Children in the DISC met the following inclusion criteria: (i) age 7–10 years, ii) in the 80th to 90th percentiles for serum LDL-C level, (iii) in the $\geq$5th percentile for height and 5th to 90th percentiles for weight for height, (iv) Tanner Stage 1 of sexual maturation, and (v) no major illnesses and not taking any medication influencing blood lipids or growth. Children were randomized to either a behavioral diet intervention to promote a diet lower in saturated fat or a usual care control group. The intervention continued until 1997, when the mean age of the participants was 16.7 years old (38). From 2006 to 2008, the DISC06 Follow-Up Study evaluated the effect of diet intervention during puberty on biomarkers associated with breast cancer risk among a subgroup of 260 DISC women ages 25–29 years (34, 35). Assents from study participants and informed consents from their parents or guardians were obtained before the DISC trial and informed consents from participants were obtained before the DISC06 Follow-Up Study. The institutional review boards at all participating centers approved the protocols of both original and follow-up DISC studies. An NHLBI-appointed independent data and safety monitoring committee oversaw the original DISC trial.

Study population

The current study included women with data on both endogenous sex hormones and breast tissue composition measured by magnetic resonance imaging (MRI) from the DISC06 Follow-Up Study. Of the 260 DISC06 Follow-Up Study participants, we excluded women who were pregnant or breastfeeding at or within 12 weeks before the visit ($n = 30$), had breast implant or breast reduction surgery ($n = 16$), had a technically unacceptable MRI ($n = 21$) or missing MRI images ($n = 11$), or had no information on any of the sex hormones studied ($n = 2$). After all exclusions, 180 women remained in the analysis; these women were similar to the entire DISC06 population, regarding lifestyle characteristics (33, 39).

Data collection

Each participant attended a single clinic visit between 2006 and 2008; 85% of the visits occurred during the luteal phase of the menstrual cycle, within 14 days of onset of the next menses. Participants completed questionnaires regarding demographics and lifestyle characteristics, such as age, race, education, physical activities, smoking, alcohol use, medical histories, reproductive factors, menstrual histories, and medication use. At the visit, participants were given postcards to record date of start of next menses and return by mail to the clinic. Participants who did not return postcards were called weekly until start of next menses. Weight and height were measured using a standard protocol (39), and body mass index (BMI) was calculated as weight(kg)/height (m^2). Diet was assessed via three nonconsecutive 24-hour dietary recalls over a 2-week period. All data were collected on the same day except the 24-hour dietary recall and date of start of next menses.

A centralized data collection training session was held before initiation of data collection to train and certify individuals responsible for the different types of data collection except breast density (described below). Each clinical center had at least one person centrally trained and certified to collect each type of data who trained and certified others at their center locally. In addition, clinical centers had access, via the study website, to the study manual of operations that included details about data and sample collection procedures.

Blood sampling

Hormones were measured in serum that was collected at the clinic visit in the morning after an overnight fast by venipuncture using standard procedures. Blood was allowed to stand at room temperature for 45 minutes to allow complete clotting. Blood was then centrifuged and serum was separated and pipetted in 0.5 mL aliquots into cryovials. Cryovials were labeled and stored at −80°C.

Testosterone and SHBG were measured in serum from all blood samples collected, while estradiol and progesterone were measured among 76 women not currently using hormonal contraceptives because hormonal contraceptives contains synthetic forms of estrogen and/or progestin (40), which may influence endogenous levels of estrogens and progesterone.

Laboratory assays

All analyses were conducted following standard procedures at the Reproductive Endocrine Research Laboratory, University of Southern California Keck School of Medicine (Los Angeles, CA; ref. 34). Unconjugated estradiol (41), progesterone (42), and testosterone (43) were quantified by specific radioimmunoassays (RIA) following extraction and Celite column partition chromatography. SHBG was measured by a chemiluminescent immunoassay on the Immulite analyzer (Siemens Healthcare Corporation). The concentrations of non–SHBG-bound estradiol and non–SHBG-bound testosterone were calculated using laws of mass action (44, 45). The overall coefficients of variation estimated from the masked quality control samples were 14.7% for estradiol, 7.8% for progesterone, 7.8% for testosterone, and 6.7% for SHBG. These coefficients of variation are comparable with those from a recent large pooled analysis of premenopausal women from seven prospective cohort studies (46).

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Measurement of breast density

Breast density was measured at the clinic visit using noncontrast MRI (39). Equipment standards were consistent with American College of Radiology guidelines for breast MRI (47) and required that imaging be performed using a whole-body MRI scanner of 1.5 Tesla or higher field strength and a dedicated breast imaging radiofrequency coil. A standard image acquisition protocol was followed at all clinics as described previously (39). To insure accuracy and uniformity of data acquisition at the different clinical centers, MRI technologists at the sites were individually trained (by C. Klifa) to recognize and correct failures due to incomplete fat suppression, motion artifacts, and inadequate breast coverage. In addition, acceptable image quality on three volunteers was required for site certification. Participant scans that were inaccurate due to artifacts, motion, or technique were excluded ($n = 21$).

All MRI image data were processed at University of California San Francisco (San Francisco, CA) by the same investigator (C. Klifa) using customized software to identify the chest wall–breast tissue boundary and skin surface, and to separate breast fibroglandular and fatty tissue using a segmentation method based on fuzzy C-means clustering (48). In problematic cases that could not be segmented with automated fuzzy C-means methods, manual delineation was used.

Fibroglandular tissue content was estimated as the absolute dense breast volume (ADBV) and fatty tissue content was estimated as the absolute nondense breast volume (ANDBV) from the MRI image. The percentage of dense breast volume (%DBV) was calculated as ADBV divided by total breast volume. ADBV, ANDBV, and %DBV were assessed separately for each breast and averaged for analysis; correlations of density parameters of both breasts ranged from 0.94 for ADBV to 0.98 for %DBV.

Statistical analysis

Summary statistics were used to describe the study population with means (SDs) or percentages. Hormones and breast density measures were reported as medians with their interquartile ranges. Spearman correlation coefficients were calculated to examine the relationships of all analytes.

%DBV, ADBV, and ANDBV were log-transformed to improve normality. Associations for %DBV, ADBV, and ANDBV were quantified separately for each hormone; geometric means and 95% confidence intervals (CI) of %DBV, ADBV, and ANDBV were calculated for each quartile category of the hormones. Because of the established association between percent breast density and breast cancer risk (2), %DBV was the primary outcome of interest; ADBV (49) and ANDBV (49, 50) were also analyzed to investigate whether a specific breast tissue compartment (dense vs. nondense) was responsible for observed associations with %DBV.

To evaluate associations of sex hormones with %DBV, ADBV, and ANDBV, we used multiple linear mixed-effects models (51) with robust SEs; the clinic was included as a random intercept to account for the potential correlation among participants from the same clinic. Other variables that were potential confounders, selected *a priori* based on previous DISC06 analyses (33, 39, 52) and on knowledge about breast cancer risk factors (53), were modeled as fixed effects and included race, education, BMI, duration of hormonal contraceptive use, and parity. The number of days from blood draw to next menses was included as a cubic spline in the analyses of estrogens and progesterone, which are known to vary over the menstrual cycle (54). Adding other factors, such as assay batch, family history of breast cancer, age at menarche, breastfeeding, physical activity, childhood BMI, smoking, drinking status, and intake of fat and fiber into the multivariable model did not change the result appreciably, but reduced the precision. The age distribution was narrow (<5 years) and also did not affect the results. Thus, those variables were not included in our final model. No highly influential observations were identified (55). Tests for trend were conducted on the continuous measure of each sex hormone using the Wald statistic (56).

In sensitivity analyses, we restricted women to those who were nulliparous or whose blood was collected during the luteal phase of menstrual cycle (57, 58). Furthermore, stratified analyses were conducted by hormonal contraceptive use (5, 59, 60) and treatment assignment. Differences in these subgroups' associations were tested using the Wald statistic of the cross-product term between continuous sex hormones and stratification variables in the fully adjusted linear mixed-effects model.

Analyses were conducted with SAS version 9.3 and STATA version 13.0. All P values were two sided and considered significant if <0.05.

Results

This study included 180 young women with a mean age of 27 years (range 25–29 years; Table 1). Their mean BMI was 25.5 kg/m^2. The majority of women were white (90%), had a college degree (66%), and had no family history of breast cancer (96%). Seventy-one percent of women were nulliparous. Few had never used hormonal contraceptives, 37% were past users, and 57% were current users. The median and interquartile range (IQR) of breast density measures were 24.3% (9.6%–40.6%) for %DBV, 92.0 cm^3 (48.7–141.0 cm^3) for ADBV, and 301.9 cm^3 (162.0–486.7 cm^3) for ANDBV.

Endogenous sex hormones and SHBG were correlated (Table 2). Estrogens (estradiol and non–SHBG-bound estradiol) were positively correlated with progesterone and androgens (testosterone and non–SHBG-bound testosterone; $r \geq 0.25$; $P \leq 0.03$). Progesterone was positively correlated with SHBG ($r = 0.38$; $P < 0.001$) and inversely correlated with non–SHBG-bound testosterone ($r = -0.33$; $P = 0.004$).

Associations of endogenous sex hormone levels and SHBG with %DBV are shown in Table 3. In the unadjusted model, %DBV was significantly positively associated with estradiol and SHBG, whereas non–SHBG-bound testosterone was inversely associated with %DBV. However, in multivariable models, particularly after adjustment for BMI, these associations were substantially attenuated and no longer significant. Only testosterone was significantly positively associated with %DBV in multivariable adjusted models; geometric mean (95% CI) for %DBV increased from 16.5% (12.9%–21.1%) in the lowest testosterone quartile to 20.3% (14.9%–27.6%) in the highest quartile ($P_{trend} = 0.03$). Restricting analyses to women in the luteal phase of their menstrual cycle did not alter results materially.

Similar to the results from %DBV, ADBV was significantly positively associated with testosterone (Table 4). The multivariable geometric mean of ADBV across increasing quartiles of testosterone increased from 68.6 to 82.3 cm^3 ($P_{trend} = 0.001$). None of the other sex hormones or SHBG was associated

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Table 1. Study population

Characteristics	N	Mean (SD)
Age, y	180	27.2 (1.0)
BMI (kg/m^2)	180	25.5 (5.3)
Duration of hormonal contraceptive use, y	180	5.2 (3.6)
Age started using hormonal contraceptives, y	169	19.3 (3.1)
Age at menarche, y	180	12.9 (1.3)
Days from blood draw to start of next menses, days	168	9.1 (10.6)

	N	Percentage
Race		
White	162	90
Non-white	18	10
Education		
Bachelor's degree	95	53
Graduate degree	23	13
Other	62	34
Number of pregnancies		
0	127	71
1	30	17
2–4	23	12
Family history of breast cancer		
No	169	96
Yes	7	4
Hormonal contraceptive use		
Never	11	6
Former	66	37
Current	103	57

Sex hormones and SHBG	N	Median (IQR)
Estradiol (pg/mL)[a]	76	137.9 (86.8–205.7)
Non–SHBG-bound estradiol (pg/mL)[a]	76	85.1 (52.3–130.9)
Progesterone (ng/mL)[a]	76	6.4 (0.2–12.8)
Testosterone (ng/dL)	180	40.6 (32.5–51.5)
Non–SHBG-bound testosterone (ng/dL)	180	12.3 (6.8–18.4)
SHBG (nmol/L)	180	94.2 (46.6–204.0)

Breast density measures	N	Median (IQR)
Percentage of dense breast volume	180	24.3 (9.6–40.6)
Absolute dense breast volume (cm^3)	180	92.0 (48.7–141.0)
Absolute nondense breast volume (cm^3)	180	301.9 (162.0–486.7)

[a]Estradiol, non–SHBG-bound estradiol, and progesterone were measured among women who were not using hormonal contraceptives.

with ADBV in multivariable adjusted models. Results from women in the luteal phase were similar to those observed for all women.

Associations of ANDBV with hormones were null with the exception of progesterone, which was significantly positively associated. Multivariable adjusted geometric means of ANDBV across increasing quartiles of progesterone were from 292.2 to 358.4 cm^3 ($P_{trend} = 0.01$; Table 5). However, as expected, progesterone levels differed markedly by menstrual cycle phase at blood collection, the median value of progesterone was 7.5 ng/mL in the luteal phase and 0.15 ng/mL in the follicular phase of the menstrual cycle. Among women in the luteal phase, the association between progesterone and ANDBV fluctuated and was no longer significant ($P_{trend} = 0.11$).

Repeating all analyses in nulliparous women only did not change the results substantially (data not shown). No significant effect modification by hormonal contraceptive use ($P_{interaction} \geq 0.33$) or intervention status ($P_{interaction} \geq 0.37$) was observed for the associations of sex hormones or SHBG with %DBV, ADBV, and ANDBV. However, associations between testosterone and breast density measures were apparent among women who were current hormonal contraceptive users but not among noncurrent users (Supplementary Table S1). Among current users, %DBV and ADBV increased across increasing quartiles of testosterone from 14.4% to 18.9% and from 58.2 to 82.0 cm^3, respectively ($P_{trend} \leq 0.04$). Among noncurrent users, the associations were flat and nonsignificant ($P_{trend} \geq 0.55$).

Discussion

In this cross-sectional study, a higher level of testosterone was significantly associated with higher %DBV. This finding resulted from the positive association of testosterone with ADBV and the absence of association with ANDBV. Progesterone was significantly positively associated with ANDBV among all women; however, the result was attenuated and became nonsignificant in analysis restricted to women in the luteal phase of their menstrual cycles. Estrogens (total and non–SHBG-bound estradiol) and SHBG were not significantly associated with %DBV, ADBV, or ANDBV.

We observed a significant positive association between testosterone and breast density, particularly because of a positive association with the absolute amount of dense breast tissue. This finding is consistent with animal and experimental studies that have demonstrated that testosterone stimulates mammary epithelial proliferation by serving as a precursor of estrogen (61, 62) and as a ligand to androgen receptors (63–65). However, in previous population-based studies, testosterone was not significantly associated with breast density (14–16, 20,

Table 2. Spearman correlations[a] of sex hormones and SHBG

	Estradiol	Non–SHBG-bound estradiol	Progesterone	Testosterone	Non–SHBG-bound testosterone	SHBG
Estradiol	1.00	0.94 ($P < 0.001$)	0.50 ($P < 0.001$)	0.32 ($P = 0.005$)	0.25 ($P = 0.03$)	0.08 ($P = 0.52$)
Non–SHBG-bound estradiol	—	1.00	0.36 ($P = 0.002$)	0.26 ($P = 0.02$)	0.41 ($P < 0.001$)	−0.19 ($P = 0.10$)
Progesterone	—	—	1.00	−0.08 ($P = 0.51$)	−0.33 ($P = 0.004$)	0.38 ($P < 0.001$)
Testosterone	—	—	—	1.00	0.49 ($P < 0.001$)	0.04 ($P = 0.63$)
Non–SHBG-bound testosterone	—	—	—	—	1.00	−0.83 ($P < 0.001$)
SHBG	—	—	—	—	—	1.00

[a]Spearman correlations of estradiol, non–SHBG-bound estradiol, and progesterone with other hormones and SHBG were calculated from 76 women who were not current users of hormonal contraceptives, whereas Spearman correlations among testosterone, non–SHBG-bound testosterone, and SHBG were calculated from 180 women.

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Table 3. Unadjusted and adjusted geometric mean and 95% CI of %DBV according to quartiles of sex hormones and SHBG at the DISC06 follow-up visit

	All women				Luteal phase[a]	
	Unadjusted model		MV-adjusted model[b]		MV-adjusted model[b]	
Quartiles (cutpoints)	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]
A. Estradiol, non-SHBG estradiol, and progesterone[d]						
Estradiol (n = 76, 70, 58)						
Q1 (4–86.2)	12.1 (7.3–20.2)	0.02	18 (14.3–22.7)	0.78	22.6 (9.2–55.3)	0.82
Q2 (87.4–137.9)	16.2 (10.1–25.9)		16.3 (13.1–20.3)		18.8 (10.6–33.4)	
Q3 (138.0–204.1)	16.2 (9.6–27.2)		17.3 (13.5–22.3)		20.8 (7.4–58.5)	
Q4 (207.4–547.4)	26.3 (18.5–37.6)		19.3 (12.8–29.0)		22.2 (8.1–61.4)	
Non–SHBG-bound estradiol (n = 76, 70, 58)						
Q1 (0.8–52.0)	12.1 (7.3–20.2)	0.32	17.1 (11.9–24.6)	0.82	18.9 (6.0–59.8)	0.46
Q2 (52.7–84.7)	22.2 (14.4–34.4)		18.7 (12.7–27.4)		19.6 (10.8–35.6)	
Q3 (85.5–129.9)	16.6 (9.7–28.1)		18.7 (13.1–26.8)		20.2 (6.4–63.4)	
Q4 (131.8–337.0)	18.7 (12.4–28.3)		16.3 (10.0–26.4)		16.6 (5.8–47.7)	
Progesterone (n = 76, 70, 58)						
Q1 (0.2–0.2)	12.3 (7.2–21.1)	0.10	20.3 (14.0–29.6)	0.48	22.8 (10.5–49.6)	0.08
Q2 (0.6–6.2)	13.4 (8.0–22.5)		16 (10.6–24.1)		19.2 (10.1–36.3)	
Q3 (6.6–12.5)	21.8 (14.3–33.4)		17.5 (12.7–24.3)		19.2 (9.0–41.3)	
Q4 (13.2–37.4)	23.2 (16.1–33.6)		17.1 (13.8–21.0)		18.7 (8.8–39.8)	

	All women				Luteal phase	
	Unadjusted model		MV-adjusted model[e]		MV-adjusted model[e]	
Quartiles (cutpoints)	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]
B. Testosterone, non–SHBG-bound testosterone, and SHBG						
Testosterone (n = 180, 180, 143)						
Q1 (10.7–32.5)	15 (11.1–20.4)	0.19	16.5 (12.9–21.1)	0.03	18.1 (13.2–24.8)	0.06
Q2 (32.6–40.6)	16.4 (11.8–22.6)		16.6 (11.6–23.7)		15.9 (11.2–22.7)	
Q3 (40.6–51.3)	24.6 (18.8–32.1)		21.1 (19.1–23.4)		22.8 (19.7–26.4)	
Q4 (51.7–97.8)	19.4 (14.3–26.3)		20.3 (14.9–27.6)		21.9 (16.7–28.7)	
Non–SHBG-bound testosterone (n = 180, 180, 143)						
Q1 (2.2–6.7)	20.9 (16.0–27.3)	0.01	18.9 (16.7–21.3)	0.86	18.8 (16.2–21.8)	0.60
Q2 (6.8–12.2)	22.4 (17.7–28.3)		19.1 (16.5–22.1)		18.8 (14.8–24.0)	
Q3 (12.0–18.4)	19 (13.8–26.1)		17.6 (13.3–23.4)		20.0 (16.4–24.3)	
Q4 (18.4–51.4)	13.2 (9.2–19.0)		18.4 (12.6–27.0)		20.5 (14.7–28.6)	
SHBG (n = 180, 180, 143)						
Q1 (12–46.4)	9.1 (6.5–12.8)	<0.001	14.7 (9.6–22.3)	0.43	16.4 (11.0–24.4)	0.74
Q2 (46.7–94)	26 (20.1–33.6)		20.4 (17.2–24.2)		21.4 (18.6–24.7)	
Q3 (94.4–204)	20 (15.2–26.1)		20.2 (16.2–25.2)		20.9 (16.1–27.1)	
Q4 (208–473)	24.9 (19.6–31.7)		19.3 (16.9–22.1)		19.2 (16.3–22.7)	

[a]Blood samples collected 1 through 14 days before the start of next menses were classified as luteal samples.

[b]Geometric means and 95% CI were estimated from linear mixed effects models including clinic as a random effect and adjusted for BMI (kg/m^2, continuous), parity (0 and >0), duration of hormone use (years, continuous), race (white and nonwhite), education (bachelor's degree, graduate school, and other), and days from blood draw to next menses (days, cubic splines) as fixed effects.

[c]P_{trend} was calculated from the Wald test of continuous term for sex hormones and SHBG in the linear mixed effects models.

[d]Analyses for estradiol, non–SHBG-bound estradiol, and progesterone included women who were not using hormonal contraceptives.

[e]Geometric means and 95% CI were estimated from linear mixed effects models including clinic as a random effect and adjusted for BMI (kg/m^2, continuous), parity (0 and >0), duration of hormone use (years, continuous), race (white and nonwhite), and education (bachelor's degree, graduate school, and other) as fixed effects.

22–24, 26, 28, 31, 66) or absolute amount of dense breast tissue (15, 16, 19, 22, 24, 31, 66) in either premenopausal (14–16, 31) or postmenopausal women (20, 22–24, 26, 28, 66).

Several potential explanations can account for the inconsistency between our findings and those of other studies. First, previous studies were mostly conducted among middle aged to older women, whereas our study was conducted in younger women. With aging, women's breasts undergo lobular involution, which replaces epithelium initially with stroma and subsequently with fat (67, 68) resulting in decreased breast density (69, 70). The tempo and degree of lobular involution varies considerably among women, but the frequency and extent of involution increases with aging (68). Consequently, the contribution of age-related lobular involution to breast density is considerably greater in previous studies of older women than in our study of younger women and could, in part, contribute to different hormonal associations (71). Second, the majority of previous studies measured testosterone using direct RIAs (14, 16, 19, 20, 22, 23, 28, 31), electrochemiluminescence immunoassays (18) or enzyme immunoassays (15), whereas we measured testosterone by RIA following solvent extraction and Celite column partition chromatography, which improves assay accuracy and precision (72). Third, previous studies excluded current hormonal contraceptive users, whereas in our study slightly over half of

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Table 4. Unadjusted and adjusted geometric mean and 95% CI of absolute DBV according to quartiles of sex hormones and SHBG at the DISC06 follow-up visit

	All women				Luteal phase[a]	
	Unadjusted model		MV-adjusted model[b]		MV-adjusted model[b]	
Quartiles (cutpoints)	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]
A. Estradiol, non-SHBG estradiol, and progesterone[d]						
Estradiol (n = 76, 70, 58)						
Q1 (4–86.2)	68.9 (46.4–102.4)	0.17	87.5 (59.2–120.2)	0.91	112.9 (48.9–260.3)	0.46
Q2 (87.4–137.9)	71.9 (50.4–102.4)		76.2 (65.2–89.0)		85.0 (51.2–141.2)	
Q3 (138.0–204.1)	72.0 (51.0–101.7)		81.2 (65.0–101.5)		89.7 (34.8–231.5)	
Q4 (207.4–547.4)	100.2 (76.8–130.8)		82.4 (59.5–114.1)		79.3 (29.4–213.9)	
Non–SHBG-bound estradiol (n = 76, 70, 58)						
Q1 (0.8–52.0)	66 (43.7–99.6)	0.30	81.2 (49.8–132.5)	0.93	95.0 (30.7–293.7)	0.30
Q2 (52.7–84.7)	79.1 (58.3–107.4)		80.6 (54.3–119.6)		82.5 (48.1–141.7)	
Q3 (85.5–129.9)	78.1 (55.7–109.5)		90.2 (65.6–124.1)		94.8 (34.2–252.6)	
Q4 (131.8–337.0)	87.7 (63.3–121.4)		74.6 (48.1–115.8)		69.6 (23.6–205.9)	
Progesterone (n = 76, 70, 58)						
Q1 (0.2–0.2)	73.3 (51.0–105.3)	0.60	92.8 (75.6–113.9)	0.92	98.0 (44.7–214.6)	0.31
Q2 (0.6–6.2)	68.3 (45.7–102.0)		69.5 (44.3–108.9)		80.5 (44.7–130.1)	
Q3 (6.6–12.5)	81.6 (61.3–108.5)		80.1 (59.5–107.8)		75.7 (44.1–130.1)	
Q4 (13.2–37.4)	87.2 (62.3–122.1)		84.8 (70.1–102.7)		80.1 (40.2–159.7)	

	All women				Luteal phase	
	Unadjusted model		MV-adjusted model[e]		MV-adjusted model[e]	
Quartiles (cutpoints)	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]
B. Testosterone, non–SHBG-bound testosterone, and SHBG						
Testosterone (n = 180, 180, 143)						
Q1 (10.7–32.5)	65.6 (49.9–86.2)	0.12	68.6 (53.7–87.7)	0.001	68.7 (50.5–93.4)	0.03
Q2 (32.6–40.6)	75.2 (56.3–100.4)		75.6 (47.8–119.5)		71.8 (44.2–116.6)	
Q3 (40.6–51.3)	91.6 (75.0–111.8)		89.4 (75.8–105.4)		98.8 (80.2–121.8)	
Q4 (51.7–97.8)	84.4 (66.9–106.5)		82.3 (66.0–102.5)		94.1 (76.4–115.9)	
Non–SHBG-bound testosterone (n = 180, 180, 143)						
Q1 (2.2–6.7)	89.8 (72.6–111.1)	0.35	85.8 (75.5–97.5)	0.84	82.3 (67.8–99.8)	0.19
Q2 (6.8–12.2)	82.1 (65.9–102.4)		80.1 (72.4–88.7)		73.4 (60.1–89.5)	
Q3 (12.0–18.4)	68 (50.3–91.9)		67.6 (48.9–93.6)		80.9 (67.6–97.0)	
Q4 (18.4–51.4)	76.1 (58.5–98.9)		82.0 (57.4–117.2)		95.2 (68.2–133.0)	
SHBG (n = 180, 180, 143)						
Q1 (12–46.4)	55.8 (41.3–75.4)	0.009	62.1 (42.0–91.9)	0.27	67.0 (45.6–98.6)	0.63
Q2 (46.7–94)	92.9 (75.0–115.1)		88.5 (71.2–109.8)		94.8 (75.2–119.5)	
Q3 (94.4–204)	79.3 (62.0–101.3)		80.9 (67.6–96.8)		85.1 (68.9–105.2)	
Q4 (208–473)	93.1 (75.3–115.1)		85.8 (70.5–104.5)		83.2 (67.5–102.5)	

[a]Blood samples collected 1 through 14 days before the start of next menses were classified as luteal samples.

[b]Geometric means and 95% CI were estimated from linear mixed effects models including clinic as a random effect and adjusted for BMI (kg/m^2, continuous), parity (0 and >0), duration of hormone use (years, continuous), race (white and nonwhite), education (bachelor's degree, graduate school, and other), and days from blood draw to next menses (days, cubic splines) as fixed effects.

[c]P_{trend} was calculated from the Wald test of continuous term for sex hormones and SHBG in the linear mixed effects models.

[d]Analyses for estradiol, non–SHBG-bound estradiol, and progesterone included women who were not using hormonal contraceptives.

[e]Geometric means and 95% CI were estimated from linear mixed effects models including clinic as a random effect and adjusted for BMI (kg/m^2, continuous), parity (0 and >0), duration of hormone use (years, continuous), race (white and nonwhite), and education (bachelor's degree, graduate school, and other) as fixed effects.

participants were currently using hormonal contraceptives. In our data, a positive association of testosterone with breast density was consistently observed among current hormone users, but not among past and never users. The altered steroid environment of hormonal contraceptive users (73–75) could potentially modify responsiveness of the breast to testosterone (76, 77). Even so, test for interaction between hormonal contraceptive use and testosterone in relation to breast density was not significant, and this may be a chance finding.

Despite strong support for the proliferative and growth-promoting effects of estrogens on mammary epithelial cells from animal and experimental studies (78, 79), previous studies on the associations between estrogens and breast density showed largely null or inconsistent results. Similar to our results, six (12, 14–16, 18, 31) of seven studies (12–16, 18, 31) in premenopausal women reported no significant association between estrogens and breast density. Only one small study (n = 24) in premenopausal women (13) found a significant positive association of estradiol with breast density. The large fluctuations in estrogen concentration over the menstrual cycle challenge its characterization by a single measurement, potentially attenuating associations.

The lack of association of progesterone with %DBV or ADBV in our study is consistent with several prior studies

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Table 5. Unadjusted and adjusted geometric mean and 95% CI of absolute non-DBV according to quartiles of sex hormones and SHBG at the DISC06 follow-up visit

	All women				Luteal phase[a]	
	Unadjusted model		MV-adjusted model[b]		MV-adjusted model[b]	
Quartiles (cutpoints)	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]
A. Estradiol, non-SHBG estradiol, and progesterone[d]						
Estradiol (n = 76, 70, 58)						
Q1 (4–86.2)	448.1 (303.6–661.5)	0.11	342.9 (287.7–408.8)	0.83	330.9 (209.9–521.6)	0.71
Q2 (87.4–137.9)	317.4 (207.9–484.6)		329.5 (256.9–422.8)		312 (192.2–506.6)	
Q3 (138.0–204.1)	304.4 (191.9–483.1)		317.1 (265.9–378.2)		277.7 (180.5–427.3)	
Q4 (207.4–547.4)	246.3 (167.2–362.8)		308.6 (22.8–427.5)		251.1 (203.5–309.8)	
Non–SHBG-bound estradiol (n = 76, 70, 58)						
Q1 (0.8–52.0)	429.7 (294.0–628.1)	0.80	342.0 (317.7–368.2)	0.85	348.3 (249.4–486.4)	0.69
Q2 (52.7–84.7)	227.9 (148.6–349.6)		291.2 (211.3–401.3)		286.5 (170.7–481.1)	
Q3 (85.5–129.9)	322.4 (198.7–523.2)		319.6 (236.3–432.2)		297.1 (166.1–531.5)	
Q4 (131.8–337.0)	337.7 (236.1–483.0)		348.1 (245.8–492.9)		325.5 (266.8–397.2)	
Progesterone (n = 76, 70, 58)						
Q1 (0.2–0.2)	442.0 (277.2–704.8)	0.05	292.2 (210.0–406.6)	0.01	259.6 (138.1–488.1)	0.11
Q2 (0.6–6.2)	395.4 (262.5–595.5)		321.0 (291.3–353.9)		294.5 (208.7–415.6)	
Q3 (6.6–12.5)	250.2 (170.3–367.5)		329.3 (267.6–405.4)		271.5 (165.1–446.5)	
Q4 (13.2–37.4)	242.4 (168.8–348.3)		358.4 (307.3–417.9)		296.1 (184.6–475.1)	

	All women				Luteal phase	
	Unadjusted model		MV-adjusted model[e]		MV-adjusted model[e]	
Quartiles (cutpoints)	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]	Mean (95% CI)	P_{trend}[c]
B. Testosterone, non–SHBG-bound testosterone, and SHBG						
Testosterone (n = 180, 180, 143)						
Q1 (10.7–32.5)	327.5 (260.5—411.8)	0.44	301.2 (275.9–328.8)	0.11	263.8 (238.6–291.6)	0.73
Q2 (32.6–40.6)	334.4 (266.2—420.1)		332.9 (306.2–362.0)		331.3 (308.8–355.4)	
Q3 (40.6–51.3)	230.1 (175.3—302.0)		272.1 (228.9–323.5)		268.6 (224.5–321.4)	
Q4 (51.7–97.8)	291.3 (219.5—386.4)		269.1 (220.2–328.9)		282.0 (220.9–360.0)	
Non—SHBG-bound testosterone (n = 180, 180, 143)						
Q1 (2.2–6.7)	291.2 (225.8—375.5)	0.004	316.7 (276.1–363.3)	0.29	309.6 (276.6–346.6)	0.76
Q2 (6.8–12.2)	248.5 (196.9—313.5)		293.5 (239.4–359.8)		267.0 (231.6–307.7)	
Q3 (12.0–18.4)	242.0 (192.6—304.0)		268.1 (232.5–309.3)		267.8 (221.6–323.7)	
Q4 (18.4–51.4)	419.3 (317.0—554.7)		294.6 (253.8–342.0)		296.8 (239.5–367.8)	
SHBG (n = 180, 180, 143)						
Q1 (12–46.4)	510.4 (409.4–636.2)	0.002	307.1 (269.5–349.9)	0.92	280.3 (241.3–325.7)	0.81
Q2 (46.7–94)	220.0 (174.2–277.8)		288.0 (247.0–337.0)		288.5 (245.9–338.6)	
Q3 (94.4–204)	275.1 (213.8–354.1)		274.7 (244.7–308.3)		275.6 (238.8–318.0)	
Q4 (208–473)	236.9 (185.2–302.9)		302.9 (257.5–356.4)		297.4 (259.5–340.7)	

[a]Blood samples collected 1 through 14 days before the start of next menses were classified as luteal samples.
[b]Geometric means and 95% CI were estimated from linear mixed effects models including clinic as a random effect and adjusted for BMI (kg/m^2, continuous), parity (0 and >0), duration of hormone use (years, continuous), race (white and nonwhite), education (bachelor's degree, graduate school, and other), and days from blood draw to next menses (days, cubic splines) as fixed effects.
[c]P_{trend} was calculated from the Wald test of continuous term for sex hormones and SHBG in the linear mixed effects models.
[d]Analyses for estradiol, non–SHBG-bound estradiol, and progesterone included women who were not using hormonal contraceptives.
[e]Geometric means and 95% CI were estimated from linear mixed effects models including clinic as a random effect and adjusted for BMI (kg/m^2, continuous), parity (0 and >0), duration of hormone use (years, continuous), race (white and nonwhite), and education (bachelor's degree, graduate school, and other) as fixed effects.

(12, 16, 18, 31) but not with all (23, 26). The finding of a positive association of progesterone with ANDBV was unexpected, but was attenuated and no longer significant in analysis restricted to women in the luteal phase of their menstrual cycles and could have been due to uncontrolled confounding by cycle day. To date, three studies (15, 16, 31) on premenopausal women have examined the association between progesterone and nondense breast tissue, but none reported a significant association between the two.

We observed no association of SHBG with breast density. This result agrees with most studies in premenopausal (12, 16, 18) and postmenopausal women (20–25, 27), but not all (14, 15, 19, 31). SHBG sequesters bioavailable estradiol and testosterone, while exerting a cell membrane-associated agonistic effect on the steroid signaling pathway (26, 80). Our result may reflect the net effect of both the inhibiting and enhancing hormonal actions of SHBG.

A major strength of this study was the large sample of young women ages 25 to 29 years, providing insights into hormonal influences on breast morphology at young ages. Another strength was the measurement of breast density by MRI, which provides information on breast composition not impaired by high parenchymal breast density typical of young women (81). Even so, breast density measured by MRI and mammography are highly

correlated ($r > 0.75$; refs. 82, 83) and high breast density, whether assessed by a volumetric method or by mammography, is similarly positively associated with breast cancer risk (2, 84). Additional strengths included using highly sensitive and specific RIAs that included extraction and chromatographic separation to measure sex hormones (72), and collecting blood samples on the same day that the breast was imaged by MRI.

Our study also has several limitations. Blood collection was not timed to the menstrual cycle. Nonetheless, 79% of blood samples were collected during the luteal phase of the menstrual cycle and we adjusted for days until start of next menses in the multivariable model; restricting analyses to the samples collected during the luteal phase did not substantially change the results except for progesterone. Single hormone measurements may not precisely represent long-term hormone levels. However, measurements of sex hormones were reported to be reproducible from blood samples collected from premenopausal women over 2 to 3 years (85). Circulating levels of hormones do not take into account peripheral conversions of hormones in breast tissue, which could attenuate associations between hormones and breast density measures.

Another limitation is that breast density measures and hormone levels were not collected from all DISC06 participants. Nonetheless, the distribution of lifestyle characteristics among our study participants was similar to that of all DISC06 participants (33, 39). Participants had elevated LDL-C and were slightly heavier than the general population when they were randomized in the DISC as children (36, 37). However, at the time of DISC06 visit, only 8% of the study participants had elevated LDL-C, and the prevalence of overweight in our analyses (44%) was similar to the U.S. average for women in twenties (45.6%; ref. 86). Although the study participants were randomized to a diet intervention or usual care control group, intervention assignment had no long-term effect on breast density (34) and did not modify associations of sex hormones with breast density measures. Although data was obtained at different sites, all our data were collected using standardized procedures to ensure data integrity. The power to detect association might have been limited due to small sample size. We cannot rule out residual confounding.

In conclusion, we observed that testosterone was modestly positively associated with %DBV particularly among hormonal contraceptive users, and this association appeared to be driven by a positive association with ADBV. Our finding adds to the limited literature on hormonal influences on breast density in younger women. Further research is warranted to confirm and extend these findings.

Disclosure of Potential Conflicts of Interest

F.Z. Stanczyk is a consultant/advisory board member for Merck and Agile Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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