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## Early breast development in overweight girls: does estrogen made by adipose tissue play a role?

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### Abstract

**Background**—Girls who are overweight/obese (OB) develop breast tissue but do not undergo menarche (the first menstrual period) significantly earlier than girls of normal weight (NW). It has been proposed that estrogen synthesized by adipose tissue may be contributory, yet OB do not have higher serum estrogen levels than NW matched on breast stage. We hypothesized that estrogen synthesized locally, in mammary fat, may contribute to breast development. This hypothesis would predict that breast development would be more advanced than other estrogen-sensitive tissues as a function of obesity and body fat.

**Methods**—80 pre-menarchal girls (26 OB, 54 NW), aged 8.2–14.7 yrs, underwent dual-energy x-ray absorptiometry to calculate percent body fat (%BF), Tanner staging of the breast, breast ultrasound for morphological staging, trans-abdominal pelvic ultrasound, hand x-ray (bone age), a blood test for reproductive hormones, and urine collection to determine the vaginal maturation index (VMI), an index of estrogen exposure in urogenital epithelial cells.

**Results**—When controlling for breast morphological stage determined by ultrasound, %BF was not associated with serum estrogen or gonadotropin (LH and FSH) levels or on indices of systemic estrogen action (uterine volume, endometrial thickness, bone age advancement, and VMI). Tanner breast stage did not correlate with breast morphological stage and led to misclassification of chest fatty tissue as breast tissue in some OB.

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**Conclusions**—These studies do not support the hypothesis that estrogen derived from total body fat or local (mammary) fat contributes to breast development in overweight/obese girls.

## INTRODUCTION

The past two centuries have witnessed a dramatic change in the timing of puberty in girls. The average age at menarche fell by nearly 5 years from the early 19<sup>th</sup> century to the mid-20<sup>th</sup> century, most likely reflecting improvements in socioeconomic and health conditions, but by the 1960's, the average age at menarche had stabilized at 12 to 12.5 years (1, 2). More recent studies, however, point to an emerging secular trend of earlier breast development (thelarche) in girls. The rapid pace of this change precludes a genetic explanation; instead, the contemporaneous obesity epidemic suggests that obesity may be the primary driver of earlier thelarche (3). Indeed, a number of longitudinal studies (4–7) have now demonstrated earlier thelarche in girls who are overweight/obese (OB) compared with girls of normal weight (NW).

It has long been proposed that in peri-pubertal girls with obesity, estrogen may be derived not from the ovary, but from peripheral adipose tissue which may convert adrenal androgens to estrone (E1) and, to a lesser extent, estradiol (E2), via the aromatase enzyme. The asynchrony of pubertal milestones observed in contemporary girls (earlier thelarche, relatively stable age at menarche) would be consistent with a peripheral trigger. Studies in pubertal pre-menarchal girls, however, have not identified a correlation between body fat mass, quantified by skinfold measurements (8) or dual-energy x-ray absorptiometry (DXA) (9), and serum E1 or E2 nor a decrease in E2 after significant weight loss (10).

We hypothesized that in OB, adipose tissue overlying the breast bud may be a ready source of estrogen that is sufficient to initiate thelarche. The primitive breast bud is intimately associated with the mammary fat pad during development (11, 12). Estrogen derived from mammary fat could act locally to induce thelarche but would not be detected in the circulation and therefore would not act systemically to induce uterine maturation and menses. To address this hypothesis, we measured reproductive hormones and imaged the breast, uterus, and ovaries in 80 pubertal, pre-menarchal girls. We used total body fat as determined by DXA as a proxy for mammary fat, taking advantage of the positive correlation between these two measures in studies in women and girls (13–15). We predicted that if estrogen derived from mammary fat contributes to breast maturation, then girls with more body fat (and thus more mammary fat) would demonstrate immaturity of other estrogen-sensitive tissues (e.g. uterus and endometrial lining, vaginal epithelium) compared with girls of the same breast morphological stage with less body fat.

## MATERIALS AND METHODS

### Subjects

Subjects (n=80) were 8.2–14.7-year-old healthy pre-menarchal girls with some breast development (per parental report) recruited from the Triangle region of North Carolina. Subjects had no chronic medical conditions, were not taking any medications or supplements known to affect puberty and did not have a history of precocious puberty or

premature pubarche (defined as breast development or pubic and/or axillary hair before age 8 years, respectively). The study was approved by the National Institute of Environmental Health Sciences (NIEHS) Institutional Review Board. Signed informed assent and consent was obtained from each subject and her parent, respectively.

## Protocol

All study procedures were conducted at the Clinical Research Unit (CRU) of the National Institute of Environmental Health Sciences (NIEHS) or off-site at a private medical imaging facility.

**Anthropometrics and Tanner staging**—Height and weight were measured using a stadiometer (SECA AccuHite 216) and medical balance beam scale (Healthometer model 402KL), respectively. Waist-to-hip ratio was measured according to the NHANES Anthropometry Procedures Manual (16). Breast Tanner stage was assigned by a pediatric endocrinologist (NDS) based on palpation and inspection of the breast (17). To avoid misclassification of overlying adipose tissue as fibroglandular tissue when assigning a Tanner breast stage, the physical examination also included contrasting chest and abdominal fatty tissues by palpation and determining whether there was a palpable depression beneath the nipple surrounded by firm tissue (the “doughnut sign”, an indicator of fatty tissue) (18). Areolar maturity, including pigmentation and stippling (19), were recorded.

**Blood and urine sampling**—A non-fasting blood sample was collected between 8 am and 4 pm to measure reproductive hormones. Blood samples were available in all but seven participants (3 NW, 4 OB). Gonadotropins were measured by direct immunoassay using the automated Abbott ARCHITECT system (Abbott Diagnostics, USA) which has a limit of quantification of 0.06 and 0.05 IU/L for luteinizing hormone (LH) and follicle-stimulating hormone (FSH), respectively (20). The coefficients of variation for LH and FSH are <7% for the range of values under study. Estradiol (E2), estrone (E1), total testosterone (TT), and androstenedione (AD) were measured by liquid chromatography–mass spectrometry (Triple Quad™ 6500 LC/MS/MS System, AB SCIEX, Framingham, MA) at the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention (Atlanta, GA). The assay limits of detection (LOD) are: E2 1.72 pg/mL, E1 0.13 ng/dL, TT 0.57 ng/dL, and AD 0.82 ng/dL. All hormone measurements were above the LOD except for E2 in 12 NW and 10 OB girls.

Urine was collected from 43 subjects (25 NW, 18 OB) to obtain vaginal cells for calculation of a modified vaginal maturation index (mVMI). Urine (10–50 cc) was spun at 2000 rpm for 10 minutes, and the cell pellet was transferred to a 20-ml vial of ThinPrep PreservCyt preservative (Hologic, Marlborough, MA). Vials were stored at room temperature for no more than two weeks before analysis. Samples were processed according to standard clinical procedures at the National Cancer Institute (NCI) Cytopathology Section, Laboratory of Pathology (Bethesda, MD). Cells were processed into a monolayer preparation, stained with Papanicolaou stain, and at least 100 cells were counted by a cytotechnologist and reviewed by a pathologist. In contrast to a sample obtained by swabbing the vaginal mucosa, when analyzing a urine sample, desquamated parabasal and intermediate cells of vaginal origin

cannot be reliably distinguished from transitional cells of bladder origin. Cells were therefore classified as either superficial (mature, indicative of estrogen exposure) or non-superficial (other) and the percent of superficial squamous cells was calculated as a mVMI, as previously described (21). Repeat urine samples were obtained whenever inflammatory cells were present.

**Breast Ultrasound**—Breast morphological stage was determined by ultrasound using a linear transducer (Samsung H60, 6–4 Hz or Toshiba Nemio 30, 12 Hz) and classified, as stage A (no breast bud), stage B (bud < 1 cm and circular), stage C (bud > 1 cm and circular), stage D (bud with several branches extending into surrounding stromal matrix), or stage E (extensive branching) [Figure 1], as previously described (22). Breast ultrasounds were performed by a single investigator (NDS) and images were reviewed by a sonographer (JMA) blinded to the subject’s age and Tanner stage. Note that breast fat content cannot be reliably determined by breast ultrasound.

**Pelvic Ultrasound**—A trans-abdominal pelvic ultrasound was performed using the full-bladder technique and a curved, 6–4 Hz transducer (Siemens S-2000, Mountain View, CA). Maximum follicle diameter, ovarian and uterine dimensions, and endometrial thickness were recorded. Uterine and ovarian volumes were calculated using the formula for an ellipsoid ( $L \times H \times W \times 0.523$ ). Both ovaries were visualized in 67 subjects, one ovary was visualized in 11 subjects, and neither ovary was visualized in 2 subjects. All images were independently reviewed by trained research staff and a random sample of 20 scans was also reviewed by a blinded investigator.

**Dual-Energy X-ray Absorptiometry (DXA)**—Subjects underwent a DXA (GE Medical Systems Lunar, Madison, WI, USA; software version 13.60.033) to determine total percent body fat, percent gynoid fat (i.e., percent of hip and upper thigh tissue made up of fat), and percent android fat (i.e., percent of tissue between the ribs and pelvis made up of fat). A negative pregnancy test was documented before the exam.

**Bone Age (BA)**—An x-ray of the left hand was taken and skeletal age was determined by a single investigator (NDS) blinded to the subject’s age using the Greulich and Pyle atlas (23).

**Data Analysis**—Sample size calculations determined that 30 subjects per weight group were required to demonstrate a 30–40% difference in the misclassification rate of breast tissue by Tanner staging (vs ultrasound) between groups with 80% power. For this calculation, breast development was assumed to be overestimated in NW at a rate of 10–15%, as shown previously (22).

Subject characteristics are presented according to age-adjusted BMI (body mass index) categories of normal weight [NW] (5<sup>th</sup> to < 85<sup>th</sup> percentile) or overweight/obese (> 85<sup>th</sup>) (24) to best illustrate the composition of the cohort in a clinical context. Age-adjusted percent body fat percentiles and Z-scores were assigned based on smoothed reference curves for US children from the CDC (25). Characteristics of NW and OB were compared by t-test or Fisher’s exact test. The false positive rate for Tanner staging (i.e., girls with breast

morphological stage A on ultrasound misclassified as Tanner stage II or greater on physical exam) was reported with Wilson score confidence intervals (26).

Multiple linear regression was used to determine the relationship between percent total body fat or percent gynoid fat and: 1) measures of systemic estrogen action (uterine volume, endometrial thickness, difference between BA and chronologic age, mVMI); 2) measures of FSH action (maximum ovarian volume and follicle size) (27, 28); 3) gonadotropin and sex steroid levels (LH, FSH, AD, TT, E1, E2); and 4) indices of aromatase action (E2/TT and E1/AD). All regression models were adjusted for breast morphological stage. Homoscedasticity of residuals across breast morphological stages was confirmed using Levene's test. Uterine volume, endometrial thickness, and hormone measures were natural log-transformed before analysis due to non-Gaussian distributions. Regression models were also performed after adjusting uterine volume for age (29) and height (30); ovarian volume for age (31); and gonadotropins for the time of blood draw with no effect on the results. Tobit regression was used for analyses that included E2 because of left-censoring (i.e., E2 values below the LOD) (32). Analyses were repeated using percent gynoid fat because aromatase expression is highest in the buttocks and thighs (33). Tests for a linear trend in the outcome variable in association with increasing breast morphologic stage were conducted for each regression model. Pearson (or Spearman, where appropriate) correlation coefficients were determined between BA or BA advancement (defined as BA minus chronological age) and clinical and biochemical parameters. All analyses were performed using Stata 15.1 (College Station, TX).

## RESULTS

### Subject Characteristics: Anthropometrics and Body Composition

Eighty subjects (68% NW, 32% OB) were studied (Table 1). OB were younger than NW and were more likely to be non-Hispanic Black or Hispanic ( $p=0.03$ ). Percent body fat, determined by total body DXA, ranged from 13.1–53.5% which corresponds to the 3<sup>rd</sup>–97<sup>th</sup> percentiles (or Z-scores of  $-2.1$  to  $2.5$ ) on age-adjusted, smoothed reference curves for US girls (25). OB had a greater waist circumference, waist-to-hip ratio, total percent body fat ( $41.10 \pm 0.94$  vs.  $26.80 \pm 0.88\%$ ,  $p<0.0001$ ), percent gynoid fat ( $45.20 \pm 1.12$  vs.  $35.00 \pm 0.84\%$ ,  $p<0.0001$ ), and percent android fat ( $44.60 \pm 1.30$  vs.  $22.80 \pm 1.20\%$ ,  $p<0.001$ ) than NW. Bone age was advanced by one year, on average, in OB. Bone age was positively correlated with age, Tanner breast and pubic hair stages, breast morphological stage, maximum ovarian volume, maximum follicle size, uterine volume, endometrial thickness, mVMI, as well as AD, TT, and E2 levels (Supplementary Table 1), whereas BA advancement correlated with waist-hip ratio ( $r=0.27$ ,  $p=0.01$ ) and BMI Z-score ( $r=0.36$ ,  $p=0.001$ ).

The correlation between BMI and adiposity varies by age, weight, and race/ethnicity (34–36), and indeed, we found that only 77.0% of girls classified as overweight/obese according to BMI had high adiposity (defined as a body fat percentile  $\geq 80^{\text{th}}$  based on a higher risk of metabolic syndrome; modified from Weber et al. who used the fat mass index 80<sup>th</sup> percentile as a cut point (37)), whereas 3.8% of girls classified as normal weight had high adiposity.

Thus, total percent body fat was utilized in all analyses to best address the specific effect of body fat on pubertal development.

### Pubertal Development

The distribution of Tanner breast and pubic hair stages was similar in NW and OB (Table 1). Although parents reported that their daughter had breast development at the time of enrollment, a small number of subjects (3 NW and 4 OB) were found to have Tanner I breasts on physical exam.

### Relationship of Breast Morphology, Other Indices of Estrogen Activity, and Percent Body Fat

Breast morphological stage was assigned according to an ultrasound-based grading system established by Bruni et al. (22) where stage A represents the pre-pubertal breast and stage E a fully mature breast. The full spectrum of breast development (A-E) was observed among subjects with stages D and E being the most common stages (59%) overall (Figure 1, Table 1). Consistent with previous studies (22), there was not a 1:1 relationship between Tanner breast stage and breast morphological stage: Tanner III breasts corresponded to breast ultrasound stages B-E, Tanner IV to stages D-E, and Tanner V to stages C-E, indicating the relative imprecision of physical examination in the determination of breast maturity. Ultrasound confirmed the pre-pubertal status of six of seven girls classified as Tanner I; the remaining girl, who was NW, was found to have a small (< 1 cm) breast bud on ultrasound (stage B). In four OB girls, adipose tissue in the chest area was mistaken for true breast tissue by inspection and palpation (Tanner II/III on exam, breast stage A on ultrasound), representing a false positive rate of 40% (95% CI 16–69%). Characteristics of these four OB are presented in Table 2. NW tended to have more advanced breast morphology than OB, but this difference was attenuated after accounting for the older age in NW ( $p=0.1$ ) (Table 1).

Overall, the cohort demonstrated the expected estrogen-induced changes in the uterus and endometrium with increasing maturity of breast morphological stage, with a linear increase in uterine length, uterine volume, and endometrial thickness ( $p<0.001$  for all) (Figure 2). Uterine length and volume were also positively correlated with height ( $\beta=0.05$ ,  $p=0.001$  and  $\beta=0.03$ ,  $p=0.004$ , respectively) but were not related to subject age ( $p=0.3$  and  $p=0.8$ , respectively). Maximum ovarian volume ( $p=0.02$ ) and maximum follicle diameter ( $p<0.001$ ) increased with advancing breast maturity, and ovarian volume also appeared related to age ( $\beta=0.45$ ,  $p=0.06$ ). The mVMI tended to increase with advancing breast maturity ( $p=0.08$ ), and bone age advancement did not correlate with breast maturity ( $p=0.8$ ). Neither percent body fat nor percent gynoid fat influenced uterine dimensions or endometrial thickness [Table 3] when adjusted for breast morphological stage. Body fat also had no effect on BA advancement in girls matched on breast morphological stage.

### Relationship of Breast Morphology, Reproductive Hormones, and Percent Body Fat

Sex steroids (E1, E2, AD, TT) and gonadotropins (LH, FSH) increased linearly with advancing breast morphological stage (Table 4) and were not affected by total or gynoid body fat (Table 3). Maximum ovarian volume and follicle size, which reflect FSH action, were also independent of total and gynoid fat (Table 3). Total body fat and gynoid fat

similarly had no effect on indices of aromatase activity (E1/AD and E2/TT) in analyses controlled for breast morphological stage (Table 3).

## DISCUSSION

We performed detailed reproductive phenotyping in pubertal girls with either overweight/obesity or normal weight to understand how excess body fat may contribute to earlier breast development in girls (Fig 3). While thelarche is typically the result of activation of the neuroendocrine components of the reproductive axis (LH and FSH) and estrogen production by the ovary, it has been proposed that adipose tissue, which expresses aromatase, may also be a relevant source of estrogen in OB. In the current studies, however, we found no differences in serum E1 and E2 levels or in indices of aromatase action (AD/E1, TT/E2) as a function of body fat in girls of the same pubertal stage. These findings are consistent with previous studies (8–10) in suggesting that peripheral adipose tissue may not explain earlier thelarche in OB. By using a novel, non-invasive approach, we showed further that local production of estrogen by breast adipose tissue is also unlikely to account for earlier breast development in OB.

Indeed, the breast is unique among estrogen-sensitive organs (e.g. uterus, bone) in that it has an adipose depot all its own - the mammary fat pad. The potential role of this particular fat pad in inducing breast development in OB has not been previously investigated. Studies in the rodent have shown that early in development, the mammary bud elongates and invades the mammary fat pad in response to a signaling cascade between these two structures (12, 38). Studies of human infant breast tissue have also identified developing ducts in close proximity to or encased by fat (11). This early rudimentary ductal structure remains in place until puberty, when ovarian steroids drive ductal elongation, branching, and the formation of lobuloalveolar units. As mammary fat expresses aromatase and synthesizes estrogen *de novo* (39), we hypothesized that in OB girls, increased mammary fat could provide a source of local estrogen and induce isolated breast growth independent from the hypothalamic-pituitary-ovarian (HPO) axis.

Mammary fat estrogen content cannot be measured in healthy pediatric research subjects. We therefore examined the relationship between breast morphological stage and other indices of estrogen action (uterine dimensions, endometrial thickness, BA, and mVMI) in girls over a range of body fat. We assumed that a higher total percent body fat, as determined by DXA, was associated with a higher breast fat content in girls based on previous studies demonstrating a positive correlation between BMI and breast fat in women and girls undergoing breast reduction mammoplasty (40–42). Studies in healthy women and girls have reported a similar relationship using non-invasive imaging techniques. Novotny et al., for example, used whole-body DXA and breast-specific DXA and a 2-compartment model of the breast (a fat compartment and fibroglandular compartment) to demonstrate that percent body fat was inversely correlated with breast fibroglandular density in girls (and therefore, positively correlated with breast fat) (13). MRI studies of the breast in girls and young women (43) and in adults (14, 15) have shown a positive association between body weight and total breast fat. In further support of a relationship between total body fat and breast fat, percent truncal fat (as determined by DXA) is correlated with breast adipocyte

size (44) and there is a proportionate decrease in breast, visceral, and subcutaneous fat mass after diet-induced weight loss (14). If estrogen derived from mammary fat influences breast development in girls, we expected to find that, in girls with the same breast morphological stage, those with more body fat would demonstrate immaturity of other estrogen-sensitive tissues compared with girls with less body fat. Instead, we observed that girls who differed in total body fat had comparable end-organ markers of systemic estrogen exposure. Thus, these findings do not support the hypothesis that excessive mammary fat in OB girls contributes to breast development.

Aberrant central regulation of the HPO axis may also be responsible for the observation of earlier thelarche without significantly earlier menarche in OB. That is, the reproductive axis is activated earlier but takes longer to reach mature function (45). In support of this hypothesis, secondary analysis of LH levels in a small group of pre-pubertal girls (46) demonstrated that the OB were more likely to demonstrate increased LH secretion during sleep than their NW peers (45), suggesting earlier central activation in OB. A study using NHANES III data also found that pre-adolescent weight gain predicts earlier central activation, but only in OB born small for gestational age (47). In contrast, studies in early/mid-pubertal girls have consistently demonstrated a blunted nocturnal rise in LH in OB compared with NW of the same Tanner breast stage (46, 48), suggesting an attenuation of neuroendocrine activity in OB by mid-puberty. An alternative explanation for lower nocturnal LH levels in OB, however, is that pre-pubertal OB may have been misclassified as early pubertal using Tanner staging, as discussed below. We found no association between percent body fat and daytime LH or FSH levels or two indices of FSH action (ovarian volume and follicle size) in girls in whom pubertal stage was precisely determined by ultrasound-based breast morphological staging. With data from a single point in time, however, we cannot comment on the potential influence of body fat on the pace of puberty as girls with more body fat may have spent a longer time at a given breast morphological stage than those with less body fat.

The current studies suggest that misclassification of adipose tissue as breast tissue may in part explain reports of earlier breast development among OB. The difficulty in distinguishing glandular from adipose chest tissue on physical exam was recognized well before the current obesity epidemic and led to a recommendation to use additional physical exam findings such as the palpable depression beneath the nipple that is present in chest adipose but not glandular tissue (18) and the Garn and Falkner areolar staging system (19, 49), which takes into account areolar diameter, pigmentation, and shape. In the current studies, none of the OB girls who were misjudged to be pubertal (categorized as Tanner breast stage II/III but had breast morphological stage A) had areolar stippling, which is typically associated with Tanner III breasts (50). However, as noted by others, areolar diameter shows too much inter-individual variability to be a useful criterion for thelarche in cross-sectional studies (51, 52) and is related not only to estrogen action but also to truncal subcutaneous fat thickness (53). The absence of areolar pigmentation scales appropriate for minorities also limits the utility of this measure. While a recent study comparing breast MRI (gold standard) and Tanner staging found that Tanner staging could reliably detect glandular breast tissue in girls, this study was limited by the inclusion of only nine OB out of 100 participants studied (54). Consistent with our findings, another study focused on obese,



Tanner II/III girls found that Tanner staging overestimated breast maturation as determined by ultrasound in 35% of cases (55). Thus, misclassification bias in pubertal staging of OB is a potential confounder in studies of pubertal timing.

Obesity affects not only pubertal development, but also linear growth and growth plate maturation. Consistent with previous studies, we found that BA advancement was positively correlated with BMI Z-score (9, 56–59) and not with E2 (57, 59). We also found no relationship to E1, TT, and AD levels, but did not measure other proposed mediators of BA advancement such as dehydroepiandrosterone sulfate (DHEAS), insulin-like growth factor 1 (IGF-1), insulin, leptin, or the rate of weight gain in participants (57, 59).

This study had several limitations. We could not measure breast adipose tissue or breast estrogen content directly in these healthy pediatric subjects. As such, we cannot completely rule-out the possibility of paracrine estrogen action in the adolescent breast. While breast ultrasound affords a more quantitative and objective assessment of breast maturation than Tanner staging, there have been no studies to specifically investigate the reproducibility of this technique, and in the current studies, both Tanner staging and breast ultrasounds were performed by a single, unblinded investigator.

In conclusion, comprehensive reproductive phenotyping of pubertal girls across a range of body weight and fat indicates a positive relationship between breast morphological stage and other markers of peripheral estrogen action but does not support the hypothesis that estrogen derived from mammary fat contributes to breast development in OB. We also find no evidence that estrogen derived from total body fat influences breast development as serum estrogen levels and indices of aromatase action were independent of percent body fat. It remains possible that obese girls may be more vulnerable to an environmental agent that preferentially activates estrogen receptors in breast tissue. Longitudinal follow-up of this cohort is now underway to investigate the potential effect of body fat on the maturational trajectory of the neuroendocrine components of the female reproductive axis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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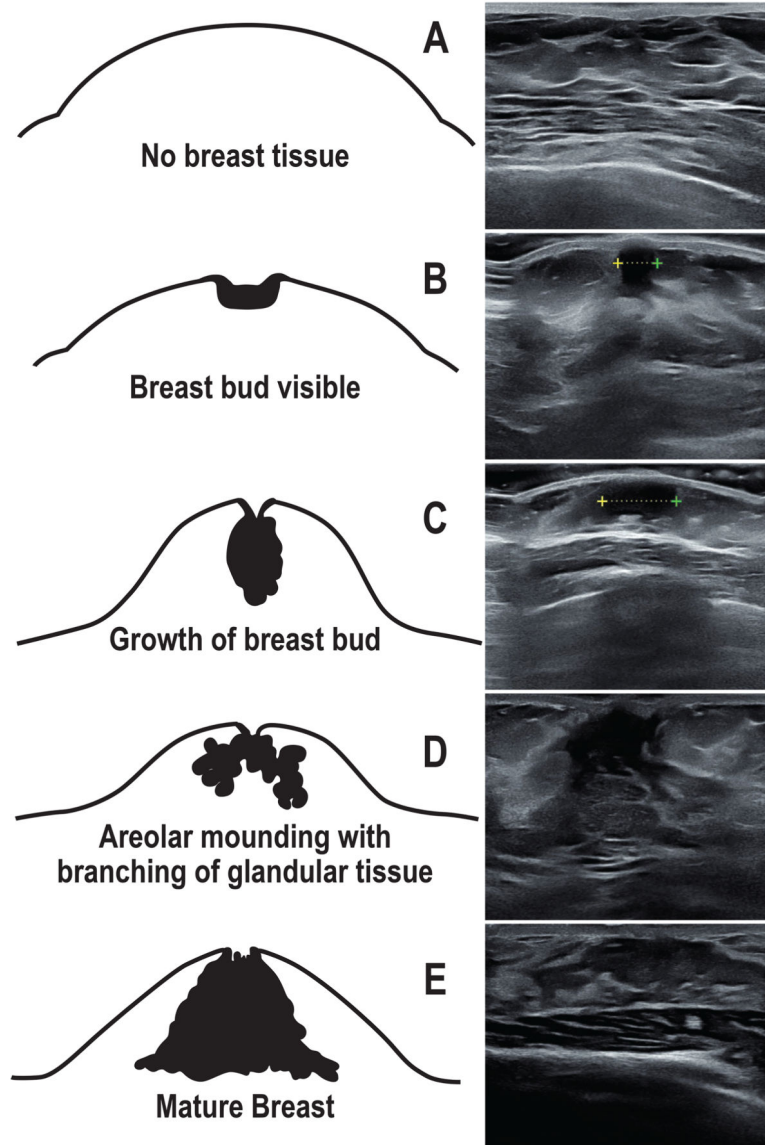
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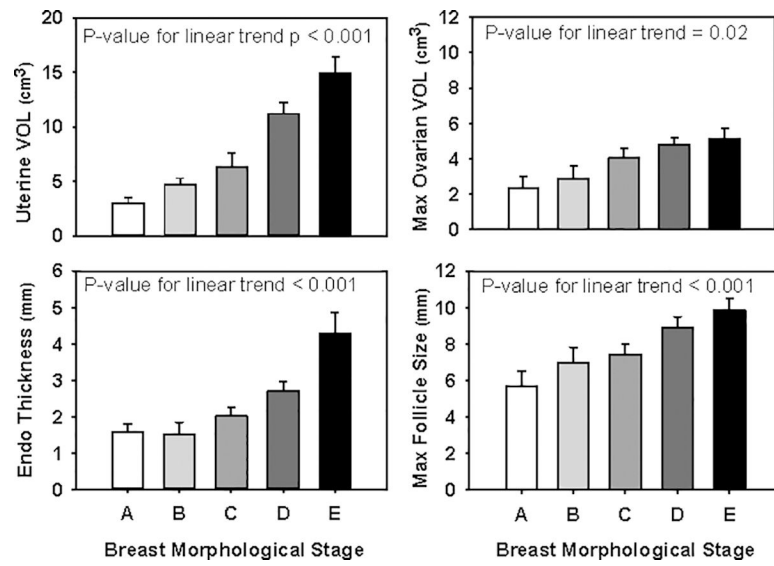
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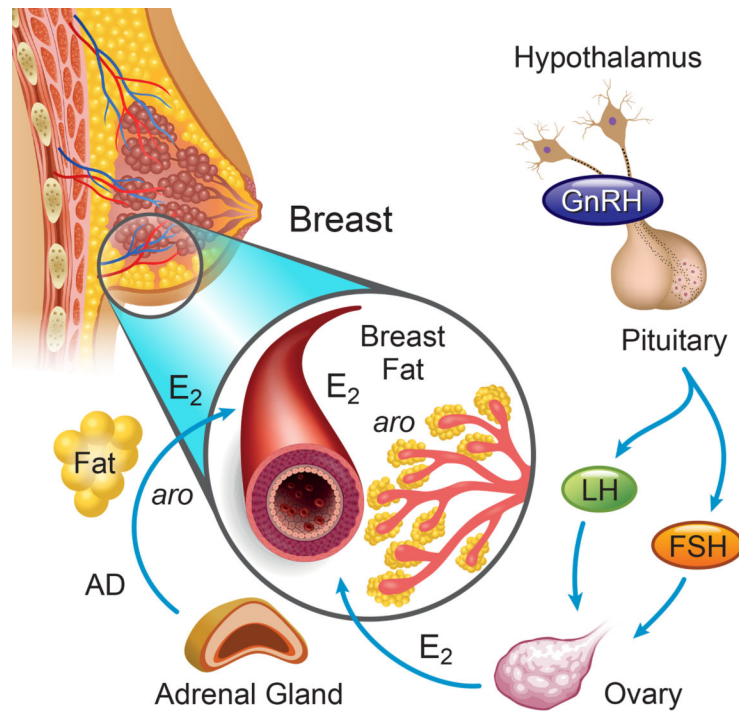
## Breast Morphological Staging by Ultrasound



**Figure 1.** Bruni scale of breast morphological stages during female pubertal development with corresponding breast ultrasound images from representative subjects in the current study. The breast bud diameter is marked by calipers (+---+) in ultrasound images of stages B and C. Adapted and reprinted with permission from “Breast Development in Adolescent Girls” by Bruni et al., *Adolescent and Pediatric Gynecology*, 3, Fig 1, p.203, Copyright Elsevier 1990 and from “Breast Disorders” by Mann et al. (eds.), *Imaging of Gynecological Disorders in Infants and Children*, Fig 5, p.229, Copyright Springer 2012.



**Figure 2.** Uterine volume, endometrial thickness, maximum ovarian volume, and maximum follicle size increased linearly with breast morphological stage maturity. Data is presented as means with standard error bars.



**Figure 3.**

Potential sources of increased estradiol (E<sub>2</sub>) responsible for earlier breast development in obese/overweight girls compared with normal weight girls. The ovary, once activated by the hypothalamic-pituitary unit, is the primary source of E<sub>2</sub> during normal pubertal development. Adipose tissue expresses aromatase (aro) and adipocytes (yellow spheres) in peripubertal girls that reside either in the periphery or near the developing mammary epithelium may also influence systemic or local E<sub>2</sub> exposure, respectively. Evidence to date, however, does not support a contribution of estrogen derived from adipose tissue in breast development in overweight/obese and normal weight girls. AD, androstenedione.

**Table 1.**

Baseline characteristics of the cohort.

	Normal Weight (NW)	Overweight/Obese (OB)	P-value
n	54	26	
Age (yrs; mean, SD)	11.3 (1.3)	10.1 (1.1)	<0.001
Race (n, %)			0.05
White, Non-Hispanic	36 (66.7)	11 (42.3)	
Black, Non-Hispanic	12 (22.2)	11 (42.3)	
Hispanic	3 (5.6)	4 (15.4)	
Other	3 (5.6)	0 (0)	
Anthropometrics (mean, SD)			
BMI (kg/m <sup>2</sup> )	17.6 (1.7)	23.8 (3.1)	#
BMI Z-score	-0.12 (0.7)	1.70 (0.4)	#
Waist Circumference (cm)	65.3 (5.1)	81.8 (7.4)	<0.001
Waist-to-Hip Ratio	0.8 (0.05)	0.9 (0.04)	<0.001
Bone age (yrs; mean, SD)	11.7 (1.0)	11.1 (1.3)	0.02
Bone age - Chronologic age (yrs; mean, SD)	0.39 (1.0)	1.0 (1.1)	0.01
Breast Tanner Stage (n, %)			0.12
I	3 (5.6)	4 (15.4)	
II	0 (0)	2 (7.7)	
III	38 (70.4)	13 (50.0)	
IV	7 (13.0)	4 (15.4)	
V	6 (11.1)	3 (11.5)	
Pubic Hair Tanner Stage (n, %)			0.35
I	7 (13.0)	8 (30.8)	
II	8 (14.8)	2 (7.7)	
III	20 (37.0)	7 (26.9)	
IV	15 (27.8)	6 (23.1)	
V	4 (7.4)	3 (11.5)	
Breast morphological stage (n, %)			0.001
A	2 (3.7)	8 (30.8)	
B	3 (5.6)	6 (23.1)	
C	11 (20.4)	3 (11.5)	
D	21 (38.9)	4 (15.4)	
E	17 (31.5)	5 (19.2)	



**Table 2**

Characteristics of participants with Tanner 2 breasts and breast morphological stage A on ultrasound.

Subject	Age (yrs)	Race	Age-adjusted BMI Z-score	Waist-Hip Ratio	Age-adjusted Body Fat Percentile	Tanner Stage: Breast	Areolar Diameter (cm)	Areolar Stippling	Arcolar Pigmentation	Tanner Stage: Pubic Hair
1	11.449	White, non-Hispanic	1.2	0.92	75	II	2.0	No	Pale Pink	I
2	11.017	White, non-Hispanic	1.6	0.96	97	III	1.5	No	Pale Pink	I
3	8.465	Black, non-Hispanic	2.1	0.86	97	III	3.0	No	Light Brown	III
4	9.868	Black, non-Hispanic	1.9	0.92	80	III	2.0	No	Light Brown	III

**Table 3.**

Results of multiple linear regression models testing the relationship between hormones or indices of estrogen action and either total percent body fat or percent gynoid fat controlled for breast morphological stage.

Indices of Estrogen Action	Total Percent Body Fat		Percent Gynoid Fat	
	$\beta$ coefficient (95% CI) <sup>b</sup>	P-Value	$\beta$ coefficient (95% CI) <sup>b</sup>	P-Value
Uterine volume (cm <sup>3</sup> )	0.005 (-0.01, 0.02)	0.60	0.005 (-0.016, 0.025)	0.65
Endometrial thickness (mm)	-0.0005 (-0.18, 0.02)	0.95	0.0009 (-0.02, 0.021)	0.93
Bone age - Chronological age (yrs)	0.02 (-0.01, 0.05)	0.20	0.024 (-0.015, 0.06)	0.22
Maximum ovarian volume (cm <sup>3</sup> )	0.03 (-0.04, 0.10)	0.34	0.037 (-0.04, 0.12)	0.37
Maximum follicle size (mm)	0.002 (-0.08, 0.08)	0.97	-0.006 (-0.11, 0.092)	0.90
Modified VMI (% superficial cells)	-0.13 (-0.69, 0.43)	0.64	-0.36 (-0.95, 0.23)	0.22
<b>Hormones<sup>a</sup></b>				
E1	0.008 (-0.013, 0.03)	0.44	0.007 (-0.018, 0.032)	0.57
E2	0.01 (-0.03, 0.05)	0.52	0.017 (-0.031, 0.065)	0.48
AD	0.003 (-0.01, 0.02)	0.72	0.008 (-0.009, 0.024)	0.37
TT	0.01 (-0.005, 0.02)	0.21	0.02 (-0.002, 0.03)	0.07
LH	0.01 (-0.03, 0.05)	0.59	0.019 (-0.03, 0.06)	0.39
FSH	-0.002 (-0.02, 0.01)	0.82	-0.004 (-0.02, 0.01)	0.63
E1/AD	0.004 (-0.007, 0.02)	0.46	0.003 (-0.01, 0.02)	0.70
E2/TT	0.004 (-0.02, 0.03)	0.76	0.004 (-0.03, 0.04)	0.80

Percent gynoid fat (i.e., percent of hip and upper thigh tissue made up of fat) was tested separately because aromatase expression is highest in the buttocks and thighs.

<sup>a</sup>All hormones were natural log(ln)-transformed before analysis.

<sup>b</sup>Partial correlation coefficients ( $\beta$ ) indicate the unit change in the dependent variable for every 1 percentage point increase in the independent variable (percent body fat or gynoid fat). For natural log(ln)-transformed variables,  $\beta \times 100$  represents the percent change in the independent variable for every 1 percentage point increase in percent body fat. VMI, vaginal maturation index; E1, estrone; E2, estradiol; AD, androstenedione; TT, total testosterone. To convert E2 to SI units (pmol/L), multiply by 3.67; for E1 (pmol/L), multiply by 3.69; for AD (nmol/L), multiply by 0.0349; for TT (nmol/L), multiply by 0.0347.

**Table 4.**

Gonadotropin and sex steroid levels according to breast morphological stage as determined by ultrasound.

Breast Morphological Stage	LH (IU/L)	FSH (IU/L)	E2 (pg/mL)	E1 (ng/dL)	TT (ng/dL)	AD (ng/dL)
A	0.05 (0.04–0.07)	1.14 (0.98–1.77)	1.22 (1.22–3.08)	0.46 (0.39–0.59)	6.59 (3.09–8.53)	22.39 (13.47–30.31)
B	0.10 (0.06–1.42)	1.64 (0.94–2.77)	2.21 (1.22–5.01)	0.78 (0.52–3.01)	8.76 (5.53–11.21)	23.67 (18.12–34.04)
C	0.27 (0.08–1.52)	2.54 (1.87–4.03)	5.96 (1.22–11.38)	1.04 (0.63–1.72)	9.19 (5.96–11.58)	35.72 (26.73–50.44)
D	1.81 (1.22–3.37)	4.31 (3.48–5.36)	15.85 (8.78–33.65)	2.23 (1.70–2.86)	16.60 (13.75–22.99)	68.93 (51.56–89.53)
E	1.95 (0.77–3.04)	3.96 (2.86–5.31)	20.46 (6.28–29.58)	2.12 (1.54–2.95)	11.79 (9.27–20.07)	61.58 (46.84–81.37)

Data is presented as median (IQR). All hormones increased linearly with breast maturation,  $p < 0.001$ . To convert E2 to SI units (pmol/L), multiply by 3.67; for E1 (pmol/L), multiply by 3.69; for AD (nmol/L), multiply by 0.0349; for TT (nmol/L), multiply by 0.0347.