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Title

Circulating growth and sex hormone levels and breast-tissue composition in young nulliparous women

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Sex hormones, growth factors and breast-tissue composition

Running title

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Abbreviations

ALSPAC: Avon Longitudinal Study of Parents and Children (ALSPAC)

BMI: body mass index

CI: confidence interval

DHEA: dehydroepiandrosterone

GH: growth hormone

HT: hormone therapy

IGF-I: insulin-like growth factor-I

IGF-II: insulin-like growth factor-II

IGFBP-3: insulin-like growth factor-binding protein 3

IQR: inter-quartile range

MRI: magnetic resonance imaging

RD: relative difference in MRI breast measure associated one standard deviation increase in the exposure of interest (e.g. hormone levels)

SD: standard deviation

SHBG: sex-hormone binding globulin

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Abstract (250 words)

Background

Endogenous hormones are associated with breast cancer risk, but little is known about their role on breast-tissue composition, a strong risk predictor. This study aims to investigate the relationship between growth and sex hormone levels and breast-tissue composition in young nulliparous women.

Methods

A cross-sectional study of 415 young (aged ~21.5 years) nulliparous women from an English pre-birth cohort underwent a magnetic resonance imaging examination of their breasts to estimate percent-water (a proxy for mammographic percent-density) and provided a blood sample to measure plasma levels of growth factors (insulin-like growth-factor-I, insulin-like growth-factor-II, insulin-growth-factor-binding-protein-3, growth-hormone) and, if not on hormonal contraception (n=117) sex-hormones (dehydroepiandrosterone, androstenedione, testosterone, estrone, estradiol, sex-hormone-binding-globulin, prolactin). Testosterone (n=330) and sex-hormone-binding-globulin (n=318) were also measured at age 15.5 years. Regression models were used to estimate the relative difference (RD) in percent-water associated with one standard deviation increment in hormone levels.

Results

Estradiol at age 21.5 and sex-hormone-binding-globulin at ages 21.5 were positively associated with body mass index (BMI)-adjusted percent-water (RD (95% CI): 3% (0%, 7%) and 3% (1%, 5%), respectively). There was a positive non-linear association between androstenedione at age 21.5 and percent-water. Insulin-like-growth-factor-I and growth-hormone at age 21.5 were also positively associated with BMI-adjusted percent-water (RD (95% CI): 2% (0%, 4%) and 4% (1%, 7%), respectively).

Conclusion

The findings suggest that endogenous hormones affect breast-tissue composition in young nulliparous women.

Sex hormones, growth factors and breast-tissue composition

Impact

The well-established associations of childhood growth and development with breast cancer risk may be partly mediated by the role of endogenous hormones on breast-tissue composition.

Introduction

The risk of developing breast cancer has been associated with several hormone-related exposures. Early age of menarche, late age of menopause and use of hormonal contraceptives and menopausal hormone therapy (HT), factors that act as proxy measures for an increased lifetime exposure to endogenous and exogenous sex hormone levels, are well-established risk factors for breast cancer (1-3). Such associations are in line with findings from prospective studies which have consistently shown that higher circulating levels of estrogens and/or androgens in both pre- (4, 5) and post-menopausal women (6) are associated with increased breast cancer risk. Associations of risk with other hormones have been less consistent. Previous studies have reported positive associations between prolactin levels and breast cancer risk, particularly amongst postmenopausal women (7) and post-menopausal HT users (8). Circulating levels of growth factors, such as insulin-like growth factor-1 (IGF-I), have been associated with increased breast cancer risk in both pre- and post-menopausal women (9, 10).

Mammographic density, a measure of the relative amounts of radio-dense fibro-glandular tissue to fat tissue in the breast as seen on a mammogram (i.e. the relative amounts of white radio-dense areas to black non-radio-dense areas), for a woman's age and body mass index (BMI) is one of the strongest predictors of breast cancer risk (11). Mammographic percent-density increases with combined estrogen-progesterone HT use (12, 13) and decreases with tamoxifen use (14) and the menopause (15). However, current evidence on the relationship between breast-tissue composition and circulating levels of endogenous sex hormones and growth factors is mixed. BMI strongly influences the relationship between endogenous sex hormones and mammographic density as obese women have, on average, higher estradiol levels and lower sex hormone-binding globulin (SHBG) (6) as well as lower percent mammographic density (15). Studies that have accounted for BMI have found sex hormone levels to be positively associated with mammographic density in premenopausal women (16-18). Higher circulating levels of IGF-I, insulin growth factor binding protein-3 (IGFBP-3), IGF-I/IGFBP-3 molar ratio and growth hormone (GH) have been associated with increased mammographic density in pre-menopausal women in some (19), but not all (20), studies.

To our knowledge no study has yet investigated the role of circulating sex hormones and growth factors on breast-tissue composition in women whose breast-tissue has not been influenced

by reproductive-related events, partly because mammography involves exposure to ionising radiation and hence cannot be performed in healthy young women. Herein, we investigate the relationship between sex and growth hormone measures collected in adolescence and early adulthood and breast-tissue composition measured by radiation-free magnetic resonance imaging (MRI)(16, 21, 22), in young nulliparous women within a British pre-birth cohort.

Method

Study population

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a prospective pre-birth cohort of 14,775 children born in Avon, England, between April 1st 1991 and December 31st 1992 (23, 24). The cohort represents 72% of the eligible population (23). For the present primarily cross-sectional study, nulliparous women born from singleton pregnancies who participated in at least one follow-up survey were invited to attend an MRI examination of their breasts at the University of Bristol Clinical Research and Imaging Centre between June 2011 and November 2014. Women who had MRI contra-indications (e.g. pregnancy, metal implants), or who had been diagnosed with cancer or a hormone-related disease, were excluded. In all, 500 of 2,530 (20%) eligible women invited to participate attended an MRI examination (Figure 1). The highly demanding nature of the study (e.g. time and travel to the MRI examination centre) and relocation away from the study area (i.e. to attend university) contributed to the low response rate. Nevertheless, participants and non-participants were similar in relation to body size and available hormone measurements. For example, mean BMI and median testosterone blood levels at ~15.5 years were 21.3kg/m² (standard deviation (SD)=3.1kg/m²) and 0.82nmol/L (inter-quartile range (IQR)=0.42nmol/L), respectively, amongst women who underwent the MRI examination, and 21.8kg/m² (SD=3.8kg/m²) and 0.80nmol/L (IQR=0.45nmol/L), respectively, amongst those who did not.

The study received approval from the South West Frenchay NRES Committee (11/SW/0051), the ALSPAC Law and Ethics Committee, and the London School of Hygiene and Tropical Medicine ethics committee. Participants provided written informed consent.

Blood sample collection at age 21.5 years

A blood sample was taken from the participants on the same day they had their MRI breast examination, aged, on average, 21.5 (mean=21.5 (SD=0.92); Table 1) years. The samples were processed within 24 hrs, and plasma aliquots stored at -80°C. The blood sample for 2% of participants was collected after the MRI examination at their general practitioner clinics and sent by post to the ALSPAC laboratory. Figure 1 provides details on the number of samples that contributed to each hormone measurement, and reasons for exclusion.

Hormone measurements at age 15.5 years

Previously-conducted serum measurements of total testosterone and SHBG levels at age 15.5 (mean=15.4 (SD=0.25)) years were available for a subset of participants (Figure 1). Total testosterone was measured using Agilent triple quadrupole 6410 liquid chromatography/mass spectrometry equipment with an electrospray ionization source operating in positive ion mode (Agilent Technologies, Wilmington, DE, USA). SHBG was measured using a Cobas Auto Analyzer (Roche Diagnostic, West Sussex, UK), and SHBG reagent using the manufacturer's calibrators and quality control material.

Hormone measurements at age 21.5 years

Measurements of plasma sex steroid hormones and SHBG at age 21.5 were restricted to women who had not used hormone contraception during the 3 months prior to blood sample collection (n=117, Figure 1), and were performed at the Royal Marsden Hospital (laboratory of Prof. M Dowsett). Radioimmunoassay was used to determine plasma concentrations of testosterone (Indirect RIA, IM1087, Beckman Coulter), SHBG (IRMA-RIA-4184, DRG International, Inc), dehydroepiandrosterone (DHEA, RIA DSL 8900, Beckman Coulter), androstenedione (DSL 3800, Beckman Coulter) and prolactin (MG12161, IBL International). Assay sensitivities were 0.10nmol/L, 0.41nmol/L, 0.21nmol/L, 0.31nmol/L and 10mIU/L, respectively. Between and within assay variation were, respectively, 5.1% and 13% for testosterone at 1.1nmol/l, 3.6% and 1.0% for SHBG at 75nmol/l, 2.4% and 0.9% for DHEA at 21nmol/l, 8.3% and 4.9% for androstenedione at 3.1nmol/l, and 5.7% and 2.5% for prolactin at 503mIU/L. Estrone and estradiol were measured by indirect radioimmunoassay methods (25, 26) and the sensitivity of the assays was 15pmol/L and 3.0pmol/L,

respectively; their between and within assay variation were 16.0% and 6.6% at 118pmol/l estrone, and 12.0% and 5.9% at 266pmol/l estradiol.

Plasma levels of IGF-I, insulin-like growth-factor-II (IGF-II), IGFBP-3 and GH were measured in samples from 406 participants (Figure 1) at the University of Bristol (laboratory of Prof. J Holly) by in-house radioimmunoassay method (IGF-I, IGF-II, IGFBP-3), and an ELISA (GH, Quantikine kit DGH00, R&D Systems), respectively. Measurement errors for IGFs are known to be low/moderate, with within and between coefficients of variation of 3.4% and 13.7%, 2.8% and 7.4%, 3.9% and 11.7%, and 3.1% and 7.8% for IGF-I, IGF-II, IGFBP-3 and GH, respectively (27). Molar ratios of IGF-I and IGF-II to IGFBP-3 were calculated as $(0.13 \times (\text{IGF-I or IGF-II}) / (0.025 \times \text{IGFBP-3}))$.

If the sample volume was too small for all laboratory measurements priority was given to the IGFs, GH and prolactin assays. Laboratory staff were blind to the characteristics of the participants.

Participants in the MRI study for whom adolescent sex hormone measurements were, and were not available, were similar with respect to anthropometric and breast-measures. For example, mean age at menarche, BMI and percent-water at age 21.5 years were 12.7 (SD=1.00) years, 24.1 (SD=4.3) kg/m² and 42.0% (SD=10.6%), respectively amongst those with testosterone measurement at age 15.5 (n=330; Table 1), compared to 12.8 (SD=1.1) years, 23.6 (SD=4.4) kg/m², and 41.4% (SD=9.8%) amongst those without such measurements. Similarly, among women who contributed to the hormone analyses, those with any sex hormone measurements (n=117; Table 1) were comparable to those for whom these were not possible (mean BMI=23.3 (SD=3.8) kg/m² and percent-water=42.8% (SD=10.4%) at age 21.5 compared to BMI=23.8 (SD=4.1) kg/m² and percent-water of 42.2% (SD=9.8%), respectively).

Breast-tissue composition assessment

Women underwent an examination of both breasts using a 3T Siemens Skyra MRI system. For each participant, a set of T1-weighted VIBE 3-D images (≈ 176 images/woman), with a voxel size of 0.76x0.76x0.90mm³, and T2-weighted trans-axial images (≈ 40 images/woman), with in-plane resolution 0.85x0.85mm² and slice thickness of 4mm were obtained. Fully-automated algorithms were developed to estimate breast volume using T1-weighted and T2-weighted images, whilst fat/water segmentations were completed on T2-weighted images. Left-right average estimates of volumes (cm³) of breast, water and fat (the latter two correspond to mammographic dense and non-dense

tissues, respectively), as well as percent-water, were generated. In the same women, percent-water and mammographic percent-density are highly correlated (range $r=0.76$ to 0.85) (16, 21, 22). Of the 500 participants who had an MRI examination, valid breast parameters were produced for 491. A full description of the methodology, and of its validity, is given in Doran et al. (28).

Covariates

Information on anthropometric measures were collected from annual clinical assessments participants attended between ages 7 to 13 years and at ages 15 and 17 years. Annual questionnaires sent to participants between the ages 8 to 17 years collected pubertal and menstruation data. At the time of the MRI examination and blood sample collection for this study, participants completed a short questionnaire on menstrual-related variables, and anthropometric measurements were taken using a standard protocol. The study website contains details of all the data that are available through a fully searchable data dictionary (29).

Statistical analysis

MRI percent-water was the primary outcome of interest in the analysis given its well-established associations with breast cancer risk but, whenever appropriate, hormone associations with water and fat volumes were also investigated. Continuous hormone measurements were standardised (z-scores), and MRI breast-tissue measures were log-transformed to achieve near-normal distributions. The relationship between participants' hormone measurements and breast-tissue measures were examined using linear regression models. Exponentiated regression coefficients are shown; these coefficients represent the relative difference (RD) in breast-tissue measures associated with a unit increase in the exposure of interest (i.e. one SD for hormone levels). Non-linear relationships between hormone levels and breast-tissue measures were investigated and, if appropriate, are reported. Two types of models were fitted to the data. The first adjusted for assay batch number and storage time (number of months between blood sample being taken and analysed), and age, phase of menstrual cycle and hormone contraceptive-use at MRI examination and blood sample collection (Table 1). Phase of menstrual cycle (follicular, luteal, irregular) was estimated for women not using hormone contraception by calculating the number of days since last menstrual period (date of MRI – start of last menstrual period), and luteal (day 14-17 to 28-31) and follicular (day 0 to 14-17) phase, or an 'irregular period' (32+ days) was defined using average length of menstrual cycle. BMI at age 15.5

years, when the adolescent blood sample was taken, age of menarche, and history of contact with a doctor regarding their periods, were also investigated as possible confounding factors but inclusion of these additional variables in the models had little effect on the magnitude of the estimates – hence, these models are not presented. The second type of models were further adjusted for BMI at age 21.5 years as it is unclear whether this variable should be treated as a confounder or a mediator for the hormone – breast-tissue associations. We also examined whether BMI at 21.5 years modified the hormone – breast-tissue associations.

Data analyses were conducted in STATA, version 14 (StataCorp LP, College Station, TX). All tests of significance are two-sided.

Results

Study subjects

Table 1 presents the characteristics of the participants and their distributions of hormone measurements and MRI breast-tissue composition estimates.

Age of menarche was weakly correlated with circulating estradiol ($r=0.19$, $p=0.044$), whilst BMI at age 21.5 years was inversely correlated with SHBG at ages 15.5 ($r=-0.16$, $p=0.005$) and 21.5 years ($r=-0.29$, $p=0.001$), and with IGF-II at 21.5 years ($r=-0.10$, $p=0.040$) (Table S1). BMI at age 21.5 years was associated with MRI breast-measures, with a unit increase in BMI being associated with a 4% (RD=-0.04; 95% CI: -0.05, -0.04) lower percent-water but a 14% (0.14; 0.13, 0.15) and a 7% (0.07; 0.06, 0.08) higher fat and water volumes, respectively (unadjusted estimates).

Plasma sex hormones and MRI breast-measures

Estradiol levels at age 21.5 were positively associated with percent-water, reflecting a more marked inverse association of this hormone with fat than water volume (Figure 1-model 1). These associations strengthened upon further adjustment for BMI at 21.5 years (Figure 1-model 2), such that a one SD (=283.6pmol/L) increase in circulating estradiol was associated with a 3% (RD=1.03; 95% CI 1.00, 1.07) higher percent-water reflecting a 12% (0.88; 0.81, 0.98) lower fat volume and a 7% (0.93; 0.86, 1.01) lower water volume. Overall, there was no association between estrone levels and MRI breast-measures; however, analyses stratified according to BMI at MRI showed evidence that this variable modified the estrone – percent-water association, with estrone being positively

associated with percent-water (1.11; 1.03, 1.20) in overweight/obese (BMI \geq 25 kg/m²) participants but not in underweight/normal weight women (p for interaction=0.018; Table S2).

SHBG levels at ages 15.5 and 21.5 were only weakly correlated (r=0.36, p<0.001). Nevertheless, SHBG levels at both these ages were positively associated with percent-water, driven mainly by inverse associations with fat volume (Figure 2-model 1). A one SD increase in SHBG levels at ages 15.5 (SD=32.0nmol/L) and at 21.5 (SD=32.9nmol/L) was accompanied, respectively, by a 6% (1.06; 1.03, 1.09) and a 7% (1.07; 1.02, 1.12) higher percent-water. These SHBG – percent-water associations were, however, attenuated upon further adjustment for BMI at MRI, particularly so for adult SHBG (Figure 2-model 2).

There was no clear evidence of an association between DHEA and percent-water before (0.96; 0.92, 1.01; Figure 1-model 1) or after further adjustment for BMI at MRI. There was, however, an inverse association between DHEA and water volume which was strengthened upon further adjustment for BMI at MRI (0.91; 0.84, 0.99). There was also evidence of a non-linear association between androstenedione and percent-water; relative to women in the lowest quartile those in the second lowest had a 4% (0.96; 0.87, 1.06) lower percent-water whilst those in the second and first highest quartiles had, respectively, a 2% (1.02; 0.92, 1.13) and 1% (1.01; 0.92, 1.12) higher percent-water (Table S3). Plasma testosterone levels at age 15.5 were weakly correlated with levels at age 21.5 (r=0.45; p<0.001) despite the different measurement methodologies used (30). Neither adolescent nor young adult testosterone levels were found to be associated with MRI breast-measures.

Circulating levels of prolactin were not associated with MRI breast-measures overall, but there was evidence that BMI at MRI modified the association between prolactin and fat volume, with high levels of this hormone being associated with lower fat volume in overweight/obese participants only ((p for interaction=0.04; Table S2).

There was no evidence that phase of menstrual cycle modified the sex hormone–percent-water associations but the power of the study to detect interactions was low.

Plasma growth factors and MRI breast-measures

Plasma levels of IGF-I and IGF-II were weakly correlated with each other (r=0.37, p<0.001) and with IGFBP-3 (r=0.27, p<0.001 and 0.39, p<0.001, respectively). GH was not correlated with IGF-I (r=0.01, p=0.56), IGF-II (r=-0.01, p=0.91) or IGFBP-3(r=0.04, p=0.38). The relationship between

circulating levels of growth factors and MRI breast-measures are presented in Figure 3. IGF-I and its IGF-I/IGFBP-3 molar ratio (molar ratio I) were both positively associated with percent-water, with these associations persisting upon further adjustment for BMI at MRI (1.02; 1.00, 1.04 and 1.02; 1.00, 1.04, respectively). The positive relationship between IGF-I and percent-water was driven by a more markedly lower fat volume (0.94; 0.90, 0.99) than water volume (0.97; 0.92, 1.02). Neither IGF-II, nor its IGF-II/IGFBP-3 molar ratio (molar ratio II), were found to be associated with MRI breast-measures (Figure 3). GH levels were also associated, albeit in a non-linear fashion, with percent-water (Figure 4). In BMI-adjusted models, the top two GH quartiles were positively associated with percent-water, compared to the lowest GH quartile, and appeared to have a plateau effect (Table S3). Collapsing quartiles into a binary category, GH levels ≥ 1.65 ng/ml were associated with a 4% (1.04; 1.01, 1.08) higher percent-water relative to levels < 1.65 ng/ml.

There was weak evidence that IGFBP-3 was inversely associated with percent-water, with the magnitude of this association being little affected by further adjustment for BMI at MRI. This association was driven by a non-linear inverse association between IGFBP-3 and water volume, with young women in the second and third highest quartiles of the IGFBP-3 distribution having, respectively, a 5% (0.95; 0.90, 1.00) and a 4% (0.94; 0.90, 0.99) lower water volume compared to those in the lowest quartile in BMI-adjusted models (Table S3).

Variability in MRI percent-water

Sex hormones explained little of the variability in percent-water being lowest for testosterone at age 15.5 (r^2 adjusted for age, assay batch, storage time and the other hormone levels: 0.06%) and highest for SHBG at age 21.5 (adjusted- $r^2=7.7\%$). Growth hormone levels accounted for even less (adjusted- $r^2<1.6\%$ for all). In contrast, BMI at MRI explained 54.4% of the variability in percent-water.

Discussion

Main findings

In this unique study with endogenous sex and growth hormones measurements taken in adolescence and young adulthood, we found evidence of positive associations between estradiol at age 21.5 years, and SHBG at ages 15.5 and 21.5 years, with MRI percent-water at age 21.5 years. The magnitude of the young adult SHBG – percent-water association was slightly attenuated upon

adjustment for BMI at MRI; in contrast, the magnitude of the adolescent SHBG and young adult estradiol associations with percent-water were little affected upon such adjustment. Testosterone levels at ages 15.5 or 21.5 were not associated with any MRI breast-measures, but androstenedione level at 21.5 years was associated, albeit in a non-linear fashion, with percent-water. Young adult circulating levels of IGF-I and of GH were also associated with increases in percent-water, with these associations persisting after adjustment for concurrent BMI.

Strengths and weaknesses

To our knowledge, this is the first study to examine breast-tissue composition in young women prior to it being affected by reproductive-related events (e.g. pregnancies, breastfeeding). Other strengths of the study include the use of objective, and previously-validated, methods of assessing volumetric breast-tissue composition (28). The unique pre-birth cohort design meant that prospective standardised data on a wide range of childhood and adolescence variables, including certain adolescent hormone measurements, could be used alongside hormone measurements performed when participants were aged 21.5. But the study had some weaknesses. First, the participation rate was low ($\approx 20\%$), albeit comparable to that of a similar MRI breast-tissue composition study of young women by Boyd et al. (16), reflecting the demanding nature of the study for participants (time and travel to the MRI examination centre). Second, blood samples could not be collected, or the amount collected was insufficient, for 16% of the participants. In addition, a high proportion of the participants had been on hormonal contraception in the 3 months prior to blood collection and hence were excluded from the sex hormone analyses. However, there was no evidence that participants for whom hormone measurements were available were a biased sample as they did not differ from the rest of the active ALSPAC cohort with regard to anthropometric or adolescent hormone measures, although by age 15.5 years the cohort was affected by socially-driven attrition (31). Furthermore, although response biases might have affected the sample's representativeness it is unlikely that it would have distorted the associations between hormone levels and MRI breast-measures. Third, hormone and MRI breast measurements were taken at a single point in time and may not characterise long-term average levels. Plasma sex hormone levels change throughout the menstrual cycle, some markedly and some modestly. Small variations in breast density throughout the menstrual cycle have also been reported (32). It was logistically impossible to time the blood collection and the MRI examination to the menstrual cycle, but the participants' menstrual phase was accounted for in the analysis. A previous

investigation (33) has shown that a single blood sample is sufficient to reliably characterise average levels of androgens, estrone sulfate, prolactin, and IGFs in pre-menopausal women over a 2-3 year period; however, reproducibility of a single measurement of estradiol and estrone was somewhat lower. GH secretion by the pituitary gland occurs in pulses throughout the day but again it was logistically impossible to time the blood collection to a particular time of the day (e.g. fasting morning samples). However, any errors in the measurement of plasma hormone/growth factors levels is likely to be non-differential as they were performed independently of the MRI ascertainment and hence the observed magnitude of the hormone – breast-measure associations is likely to be an under-estimation of the true magnitude of these associations. Fourth, hormone levels in young adulthood were measured at the time of the MRI breast examination making it impossible to establish temporality. The only exceptions were for testosterone and SHBG as plasma levels of these hormones at ages 15.5 years were also available, with adolescent and young adult levels yielding associations with percent-water of similar direction and magnitude. Finally, the high number of statistical tests performed may have led to spurious associations.

Interpretation of the findings

The study reveals positive associations between MRI percent-water in young women and concurrent circulating levels of IGF-I, and its molar ratio, consistent with mammography- and MRI-based findings reported by others (18, 19, 34), albeit not all (16, 20, 35), studies in pre-menopausal women. These associations are also in line with the reported positive association between IGF-I and breast cancer risk, albeit possibly restricted to oestrogen-receptor-positive tumours (9, 10), suggesting that the role of these hormones in the aetiology of this cancer may be mediated through their effect on breast-tissue composition at young ages. This study also revealed a positive non-linear association between GH and percent-water. GH is the main mediator of postnatal somatic growth, exerting its effect directly by binding to receptors located on the membrane of target cells as well as indirectly by stimulating the production of IGF factors by the liver and other target organs, including the breast. A positive GH – percent-water association was reported by another study on the determinants of MRI breast-tissue composition in young women (16) although, in contrast to our study, it found no evidence of an association with IGF-I. Our findings are also consistent with previously reported positive associations of childhood growth and adult height with breast cancer risk (36) and breast density, as ascertained by percent mammographic density (37, 38) or MRI percent-water (16),

including with our previous findings from the ALSPAC cohort showing strong associations between childhood growth and MRI percent-water (39) .

Our finding of a positive estradiol – percent-water association is consistent with results from two other studies which examined sex hormone levels throughout the menstrual cycle in relation to percent mammographic density (17, 34). However, the only other study to our knowledge to have examined the role of circulating sex hormones on MRI breast-tissue composition in young women found that percent-water was not associated with follicular levels of this hormone, but was inversely associated with luteal levels of free estradiol and free testosterone (16). Studies of the associations between sex hormone levels and mammographic density in postmenopausal women have produced mixed results (40, 41).

The present study identified positive associations between SHBG at ages 15.5 and 21.5, and MRI percent-water at age 21.5, consistent with Boyd et al. (16). The direction of the observed association is, however, the opposite of the reported SHBG – breast cancer risk association (4, 5). The biological basis for the opposing effects on breast-tissue composition and cancer risk is unclear. Plasma SHBG not only binds to circulating steroids, thus regulating their bioavailability and access to target cells, but it also mediates the uptake of steroid molecules into cells through binding to cell-membrane receptors. The latter might stimulate intracellular messengers to cell proliferation and thus contribute to mitogenesis in the breast.

Conclusion

The study findings suggest that endogenous sex hormones and growth factors affect breast-tissue composition in young women. In particular, the associations of MRI percent breast water with GH and IGF-I, which are key mediators of postnatal somatic growth, suggests a potential biological mechanism for the well-established associations of childhood growth and adult height with breast-tissue composition and, ultimately, breast cancer risk.

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References

1. Collaborative Group on Hormonal Factors in Breast C. Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. *Lancet Oncol.* 2012;13(11):1141-51.
2. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer. *Lancet.* 1997;350(9084):1047-59.
3. Collaborative Group on Hormonal Factors in Breast C. Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. *Lancet.* 1996;347(9017):1713-27.
4. Endogenous H, Breast Cancer Collaborative G, Key TJ, Appleby PN, Reeves GK, Travis RC, et al. Sex hormones and risk of breast cancer in premenopausal women: a collaborative reanalysis of individual participant data from seven prospective studies. *Lancet Oncol.* 2013;14(10):1009-19.
5. Kaaks R, Tikk K, Sookthai D, Schock H, Johnson T, Tjonneland A, et al. Premenopausal serum sex hormone levels in relation to breast cancer risk, overall and by hormone receptor status - results from the EPIC cohort. *Int J Cancer.* 2014;134(8):1947-57.
6. Key TJ, Appleby PN, Reeves GK, Roddam A, Dorgan JF, Longcope C, et al. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *J Natl Cancer Inst.* 2003;95(16):1218-26.
7. Tworoger SS, Eliassen AH, Zhang X, Qian J, Sluss PM, Rosner BA, et al. A 20-year prospective study of plasma prolactin as a risk marker of breast cancer development. *Cancer Res.* 2013;73(15):4810-9.
8. Tikk K, Sookthai D, Johnson T, Rinaldi S, Romieu I, Tjonneland A, et al. Circulating prolactin and breast cancer risk among pre- and postmenopausal women in the EPIC cohort. *Ann Oncol.* 2014;25(7):1422-8.
9. Endogenous H, Breast Cancer Collaborative G, Key TJ, Appleby PN, Reeves GK, Roddam AW. Insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and breast cancer risk: pooled individual data analysis of 17 prospective studies. *Lancet Oncol.* 2010;11(6):530-42.
10. Kaaks R, Johnson T, Tikk K, Sookthai D, Tjonneland A, Roswall N, et al. Insulin-like growth factor I and risk of breast cancer by age and hormone receptor status-A prospective study within the EPIC cohort. *Int J Cancer.* 2014;134(11):2683-90.
11. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer Epidemiol Biomarkers Prev.* 2006;15(6):1159-69.
12. Crandall C, Palla S, Reboussin BA, Ursin G, Greendale GA. Positive association between mammographic breast density and bone mineral density in the Postmenopausal Estrogen/Progestin Interventions Study. *Breast Cancer Res.* 2005;7(6):R922-8.
13. Byrne C, Ursin G, Martin CF, Peck JD, Cole EB, Zeng D, et al. Mammographic Density Change With Estrogen and Progestin Therapy and Breast Cancer Risk. *J Natl Cancer Inst.* 2017;109(9).
14. Martin LJ, Minkin S, Boyd NF. Hormone therapy, mammographic density, and breast cancer risk. *Maturitas.* 2009;64(1):20-6.

15. Burton A, Maskarinec G, Perez-Gomez B, Vachon C, Miao H, Lajous M, et al. Mammographic density and ageing: A collaborative pooled analysis of cross-sectional data from 22 countries worldwide. *PLoS Med.* 2017;14(6):e1002335.
16. Boyd N, Martin L, Chavez S, Gunasekara A, Salleh A, Melnichouk O, et al. Breast-tissue composition and other risk factors for breast cancer in young women: a cross-sectional study. *Lancet Oncol.* 2009;10(6):569-80.
17. Iversen A, Frydenberg H, Furberg AS, Flote VG, Finstad SE, McTiernan A, et al. Cyclic endogenous estrogen and progesterone vary by mammographic density phenotypes in premenopausal women. *Eur J Cancer Prev.* 2016;25(1):9-18.
18. Boyd NF, Stone J, Martin LJ, Jong R, Fishell E, Yaffe M, et al. The association of breast mitogens with mammographic densities. *Br J Cancer.* 2002;87(8):876-82.
19. dos Santos Silva I, Johnson N, De Stavola B, Torres-Mejia G, Fletcher O, Allen DS, et al. The insulin-like growth factor system and mammographic features in premenopausal and postmenopausal women. *Cancer Epidemiol Biomarkers Prev.* 2006;15(3):449-55.
20. Rice MS, Tworoger SS, Rosner BA, Pollak MN, Hankinson SE, Tamimi RM. Insulin-like growth factor-1, insulin-like growth factor-binding protein-3, growth hormone, and mammographic density in the Nurses' Health Studies. *Breast Cancer Res Treat.* 2012;136(3):805-12.
21. Khazen M, Warren RM, Boggis CR, Bryant EC, Reed S, Warsi I, et al. A pilot study of compositional analysis of the breast and estimation of breast mammographic density using three-dimensional T1-weighted magnetic resonance imaging. *Cancer Epidemiol Biomarkers Prev.* 2008;17(9):2268-74.
22. Thompson DJ, Leach MO, Kwan-Lim G, Gayther SA, Ramus SJ, Warsi I, et al. Assessing the usefulness of a novel MRI-based breast density estimation algorithm in a cohort of women at high genetic risk of breast cancer: the UK MARIBS study. *Breast Cancer Res.* 2009;11(6):R80.
23. Boyd A, Golding J, Macleod J, Lawlor DA, Fraser A, Henderson J, et al. Cohort Profile: the 'children of the 90s'--the index offspring of the Avon Longitudinal Study of Parents and Children. *Int J Epidemiol.* 2013;42(1):111-27.
24. Fraser A, Macdonald-Wallis C, Tilling K, Boyd A, Golding J, Davey Smith G, et al. Cohort Profile: the Avon Longitudinal Study of Parents and Children: ALSPAC mothers cohort. *Int J Epidemiol.* 2013;42(1):97-110.
25. Brand JS, Chan MF, Dowsett M, Folkard E, Wareham NJ, Luben RN, et al. Cigarette smoking and endogenous sex hormones in postmenopausal women. *J Clin Endocrinol Metab.* 2011;96(10):3184-92.
26. Dowsett M, Goss PE, Powles TJ, Hutchinson G, Brodie AM, Jeffcoate SL, et al. Use of the aromatase inhibitor 4-hydroxyandrostenedione in postmenopausal breast cancer: optimization of therapeutic dose and route. *Cancer Res.* 1987;47(7):1957-61.
27. Allen NE, Roddam AW, Allen DS, Fentiman IS, Dos Santos Silva I, Peto J, et al. A prospective study of serum insulin-like growth factor-I (IGF-I), IGF-II, IGF-binding protein-3 and breast cancer risk. *Br J Cancer.* 2005;92(7):1283-7.
28. Doran SJ, Hipwell JH, Denholm R, Eiben B, Busana M, Hawkes DJ, et al. Breast MRI segmentation for density estimation: Do different methods give the same results and how much do differences matter? *Med Phys.* 2017.
29. Bristol Uo. [cited 2016 07/01/2016]. Available from: <http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/>.

30. Tran TS, Center JR, Seibel MJ, Eisman JA, Kushnir MM, Rockwood AL, et al. Relationship between Serum Testosterone and Fracture Risk in Men: A Comparison of RIA and LC-MS/MS. *Clin Chem*. 2015;61(9):1182-90.
31. Howe LD, Tilling K, Galobardes B, Lawlor DA. Loss to follow-up in cohort studies: bias in estimates of socioeconomic inequalities. *Epidemiology*. 2013;24(1):1-9.
32. White E, Velentgas P, Mandelson MT, Lehman CD, Elmore JG, Porter P, et al. Variation in mammographic breast density by time in menstrual cycle among women aged 40-49 years. *J Natl Cancer Inst*. 1998;90(12):906-10.
33. Missmer SA, Spiegelman D, Bertone-Johnson ER, Barbieri RL, Pollak MN, Hankinson SE. Reproducibility of plasma steroid hormones, prolactin, and insulin-like growth factor levels among premenopausal women over a 2- to 3-year period. *Cancer Epidemiol Biomarkers Prev*. 2006;15(5):972-8.
34. Walker K, Fletcher O, Johnson N, Coupland B, McCormack VA, Folkard E, et al. Premenopausal mammographic density in relation to cyclic variations in endogenous sex hormone levels, prolactin, and insulin-like growth factors. *Cancer Res*. 2009;69(16):6490-9.
35. Rinaldi S, Biessy C, Hernandez M, Lesueur F, dos-Santos-Silva I, Rice MS, et al. Circulating concentrations of insulin-like growth factor-I, insulin-like growth factor-binding protein-3, genetic polymorphisms and mammographic density in premenopausal Mexican women: results from the ESMAestras cohort. *Int J Cancer*. 2014;134(6):1436-44.
36. Ahlgren M, Melbye M, Wohlfahrt J, Sorensen TI. Growth patterns and the risk of breast cancer in women. *N Engl J Med*. 2004;351(16):1619-26.
37. Lope V, Perez-Gomez B, Moreno MP, Vidal C, Salas-Trejo D, Ascunce N, et al. Childhood factors associated with mammographic density in adult women. *Breast Cancer Res Treat*. 2011;130(3):965-74.
38. Sellers TA, Vachon CM, Pankratz VS, Janney CA, Fredericksen Z, Brandt KR, et al. Association of childhood and adolescent anthropometric factors, physical activity, and diet with adult mammographic breast density. *Am J Epidemiol*. 2007;166(4):456-64.
39. Denholm R, De Stavola B, Hipwell JH, Doran SJ, Busana MC, Leach MO, et al. Growth Trajectories, Breast Size, and Breast-Tissue Composition in a British Pre-Birth Cohort of Young Women. *Am J Epidemiol*. 2017.
40. Warren R, Skinner J, Sala E, Denton E, Dowsett M, Folkard E, et al. Associations among mammographic density, circulating sex hormones, and polymorphisms in sex hormone metabolism genes in postmenopausal women. *Cancer Epidemiol Biomarkers Prev*. 2006;15(8):1502-8.
41. Tamimi RM, Byrne C, Colditz GA, Hankinson SE. Endogenous hormone levels, mammographic density, and subsequent risk of breast cancer in postmenopausal women. *J Natl Cancer Inst*. 2007;99(15):1178-87.

Table 1: Baseline characteristics of the participants, MRI breast-tissue composition measures, and plasma levels of sex hormones and growth factors

	N	Mean/ %	SD	Median	IQR
Participants characteristics at MRI examination					
Age (months) ^a	439	257.6	11.1	258.0	33.0
BMI (kg/m ²)	435	23.9	4.3	23.0	5.1
Menstrual phase ^b	Follicular	66	15		
	Luteal	44	10		
Hormone contraceptives ^c		299	69		
	Irregular period	26	6		
MRI breast-measures ^d					
Left-right average breast volume (cm ³)	438	649.8	465.3	507.8	471.8
Left-right average breast fat volume (cm ³)	438	408.9	353.8	293.9	332.3
Left-right average breast water volume (cm ³)	438	240.8	131.3	209.2	173.4
Left-right average breast percent-water (%)	439	41.8	10.4	41.7	16.3
Sex hormones					
At age 15.5 years					
Testosterone (nmol/L)	330	0.91	0.53	0.82	0.42
SHBG (nmol/L)	318	64.1	32.0	58.1	37.5
At age 21.5 years ^e					
DHEA (nmol/L)	115	28.6	12.8	26.0	16.0
Androstenedione (nmol/L)	116	7.1	3.1	6.7	2.9
Testosterone (nmol/L)	117	1.6	0.7	1.5	0.9
Estrone (pmol/L)	109	260.7	159.9	223.0	150.0
Estradiol (pmol/L)	115	318.6	283.6	196.0	255.0
SHBG (nmol/L)	117	67.5	32.9	62.0	38.0
Prolactin (mIU/L) ^f	399	264.1	135.4	234.0	135.0
Growth factors at age 21.5 years					
IGF-I (ng/ml)	400	244.8	84.2	234.9	121.4
IGF-II (ng/ml)	400	668.6	184.9	650.0	255.0
IGFBP-3(ng/ml)	400	5509.9	1442.3	5607.1	1934.4
IGF-I/IGFBP-3molar ratio ^g	400	0.25	0.13	0.23	0.12
IGF-II/IGFBP-3molar ratio ^h	400	0.67	0.28	0.62	0.26
GH (ng/ml)	400	3.8	4.8	1.7	5.7

IQR: inter-quartile range; SD: standard deviation; BMI: body mass index; MRI: magnetic resonance imaging; SHBG: sex-hormone binding globulin; DHEA: dehydroepiandrosterone; IGF: insulin-like growth factor; IGFBP-3: insulin-like growth factor-binding protein 3; GH: growth hormone

^a After exclusion of 6 women with invalid MRI breast-measures, 439 participants had at least one hormone measurement in adolescence and/or at age 21 years

^b Estimated for women not using hormone contraception by calculating the number of days since last menstrual period (date of MRI – start of last menstrual period). Luteal (day 14-17 to 28-31) and follicular (day 0 to 14-17) phase, and an 'irregular period' (32+ days) was defined using average length of menstrual cycle.

^c Women who used hormone contraception at any time during the three months prior to blood collection.

^d Sections of the breast missing in the MRI images for one participant, thus volumetric measures cannot be ascertained and percent-water only used.

^e Sex hormone analysis, excluding prolactin, were carried out in women who had not used hormone contraception at any time during the 3 months prior to blood collection.

^f An outlier of 1,212mIU/L removed

^g Molar ratio I calculated as $(0.13 \cdot \text{IGF-I}) / (0.025 \cdot \text{IGFBP-3})$. An outlier of 1.79 removed

^h Molar ratio II calculated as $(0.13 \cdot \text{IGF-II}) / (0.025 \cdot \text{IGFBP-3})$. An outlier of 3.81 removed

Figure Legends

Figure 1. Flowchart detailing patient selection, blood sample collection and hormone measurements.

Figure 1 contains a flowchart detailing the inclusion of ALSPAC participants and collection of data for the study.

Figure 2: Associations between plasma levels of sex hormones at ages 15.5 and 21.5 years (z-scores) and MRI breast-tissue composition measures at age 21.5 years

CI: confidence intervals; DHEA: dehydroepiandrosterone; MRI: magnetic resonance imaging; RD: relative difference per one standard deviation (SD) increase in plasma hormone levels; SHBG: sex-hormone binding globulin.

* Non-linear relationship results presented in Table 2.

Plasma levels of sex hormones were standardised and MRI breast-measures were log transformed. Exponentiated estimated regression parameters are presented, with 95% CIs calculated by exponentiating the original 95% CIs. Model 1 adjusted for assay batch number, storage time, and age and menstrual phase at MRI examination; Model 2 further adjusted for BMI at MRI examination. Numbers contributing to these analyses are reported in Table 2: DHEA n=114; Androstenedione n=115; Testosterone 15.5 years n=321; Testosterone 21.5 years n=116; Estrone n=108; Estradiol n=114; SHBG 15.5 years n=311; SHBG 21.5 years n=116; Prolactin n=389.

Figure 3: Associations between plasma levels of growth factors (z-scores) at age 21.5 years and concurrent MRI breast-tissue composition measures

CI: confidence intervals; IGF-I: insulin-like growth factor-I; IGF-II: insulin-like growth factor-II; IGFBP-3: insulin-like growth factor-binding protein 3; GH; growth hormone; MRI: magnetic resonance imaging; RD: relative difference per one standard deviation (SD) increase in plasma growth factor levels

* Non-linear relationship – results presented in Figure 3 and Table S3.

Plasma levels of growth factors were standardised and MRI breast-tissue composition measures were log transformed. Exponentiated estimated regression parameters are presented, with 95% CI calculated by exponentiating the original 95% CIs. Model 1 adjusted for assay batch number, storage time, and age and menstrual phase at MRI examination, and IGFBP-3 for IGF-I and IGF-II models, IGF-I for IGFBP-3 model, and IGF-II and IGFBP-3 for molar ratio I and IGF-I and IGFBP-3 for molar ratio II models. Model 2 further adjusted for BMI at MRI examination.

^a Molar ratio calculated as $(0.13 \cdot \text{IGF-I}) / (0.025 \cdot \text{IGFBP-3})$. An outlier of 1.79 removed.

^b Molar ratio II calculated as $(0.13 \cdot \text{IGF-II}) / (0.025 \cdot \text{IGFBP-3})$. An outlier of 3.81 removed

Numbers contributing to these analyses are reported in Table 2: IGF-I n=396; IGF-II n=396; IGFBP-3 n=396; Molar Ratio I n=395; Molar Ratio II n=395; GH n=396.

Figure 4: Non-linear associations between plasma levels of growth hormone (GH) at age 21.5 years (z-scores) and concurrent MRI percent-water

MRI: magnetic resonance imaging

Plasma levels of growth hormone (GH) were standardised and MRI breast-tissue composition measures were log transformed. The fitted model shows the predicted estimates taking into account

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the log transformation of MRI percent-water, and adjusting for assay batch number, storage time, and age, menstrual phase and BMI at MRI examination.