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Insulin-like growth factor-1, growth hormone and daily cycling estrogen are associated with mammographic density in premenopausal women.

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Abstract

Background: Mammographic density represents epithelial and stromal proliferation, while Insulin-like growth factor (IGF)-1, IGF-binding protein (IGFBP)-3, growth hormone (GH) and estrogen, may influence cellular proliferation. However, whether these growth factors independently, or in combination with estrogen, influence mammographic density in premenopausal women remains unclear.

Material and methods: Growth factors were assessed in 202 ovulating premenopausal women participating in the Energy Balance and Breast cancer Aspects (EBBA)-I study. Estrogen was assessed in serum, and daily in saliva, throughout a menstrual cycle. Computer-assisted mammographic density (Madena) was obtained from digitized mammograms (days 7-12 of the menstrual cycle). Associations between growth factors, estrogen and percent mammographic density, were studied in regression models.

Results: Women with a mean age of 30.7 years had a mean percent mammographic density of 29.8%. Among women in the strata (above median split) of IGF-1 (>25 nmol/l) or GH (>0.80 mlU/l), we observed that an increase in salivary 17β–estradiol, was associated with a higher odds for having higher percent mammographic density (>28.5 %). The odds ratios (ORs) per standard deviation increase of 17β-estradiol, were 1.81 (95% confidence interval [CI] 1.08-3.03) in the high IGF-1 stratum, and 2.08 (95% CI 1.10-3.94) in the high GH stratum. Furthermore, women in this strata of growth factors (above median) who had an overall average 17β–estradiol above median (>16.8 pmol/l), had higher ORs for having higher percent mammographic density (>28.5%): IGF-1 4.13 (95% CI 1.33-12.83), and GH 4.17 (95% CI 1.41-12.28).

Conclusion: Growth factors, in combination with cycling estrogen, were associated with percent mammographic density, of potential clinical relevance.

Introduction

Mammographic density represents epithelial and stromal proliferation, and is a strong biomarker for breast cancer development [1]. Ovarian steroid hormones and growth factors, on the other hand, increase cellular proliferation in the breast, which may be reflected through mammographic density. Furthermore, the growth hormone (GH)–insulin-like growth factor-1 (IGF-1) signaling pathway, has been associated with breast cancer [2,3], and IGF-1 is suggested to influence cellular proliferation and inhibit apoptosis through the activation of PI3/Akt pathway, in both normal breast cells and breast cancer cell lines [4,5]. The level of IGF-1 is regulated by GH, and modulated by insulin-like growth factor-binding protein-3 (IGFBP-3). However, whether IGF-1, IGFBP-3 and GH, independently or in combination with ovarian steroid hormones, influence mammographic density among premenopausal women, remains unclear [6,7].

Mammographic density may be described by percent and absolute density. While percent mammographic density represents the fibroglandular tissue and fat tissue, absolute mammographic density represents the dense area. Mammographic density has been reported to vary during the menstrual cycle [8,9], and breast cancer risk factors, including the use of exogenous hormones, have been studied mostly in relation to, and associated with, percent mammographic density [10,11] and breast cancer development [12,13]. Recently, endogenous sex hormone levels and percent mammographic density were associated with breast cancer risk, both independently and in combination [14]. Interestingly, it has been hypothesized that a crosstalk operates between estrogen and the GH-IGF-1 signalling pathways in cells [15-18]. Thus, it is interesting to examine whether the variation in the GH-IGF axis in combination with circulating concentrations of estrogen is associated with mammographic density, described by both percent and absolute density in premenopausal women.

Previously, in the Norwegian Energy Balance and Breast cancer Aspects (EBBA)-I study, we have observed a positive association between daily circulating ovarian sex hormones and mammographic density, using a modified Wolfe classification [19]. In addition, 17β-estradiol profiles were associated with traditional breast cancer risk factors, such as age at menarche [19,20], insulin, adult height and metabolic profile in adulthood [21,19,22]. These associations also point to the need for further studies of estrogen in combination with the GH-IGF signalling pathway and mammographic density.

Thus, the purpose of this study is to examine whether IGF-1, IGFBP-3 and GH, in combination with circulating concentrations of daily 17β-estradiol, are associated with mammographic density in premenopausal women. A unique aspect of the current study is the measurement of estrogen in both serum and daily in saliva. The daily salivary 17β-estradiol

measured throughout an entire menstrual cycle represents biologically free active estrogen [23]. To facilitate comparisons with other studies and factors affecting various types of breast density, both percent and absolute density have been included.

Materials and methods:

Participants and study design

The Norwegian EBBA–I study was conducted in 2000–2002 in Tromsø. It included 204 healthy women aged 25-35 years, with regular menstrual cycles (length 22-38 days) [19,22]. The women did not use any daily medication, or steroid contraceptives, in the 6 months prior to recruitment, they were not pregnant or lactating, and had no gynaecological or chronic disorders (e.g. diabetes, hypothyroidism) [19]. Participants' characteristics, including reproductive and lifestyle factors, were collected by a trained nurse using questionnaires and interviews at the time of recruitment. Recall and memory-probing aids, including a lifetime calendar, were used to date specific life events. Two women were excluded due to missing mammographic data, resulting in 202 participants in the present study.

Clinical examination

All participants underwent clinical examinations at the Clinical Research Centre, University Hospital of North Norway, Tromsø. They attended three scheduled visits during their menstrual cycle, after onset of the menstrual bleeding (first visit days 1-5, second visit days 7-12 and third visit days 21-25). Anthropometric measurements were conducted with participants wearing light clothing and no footwear. Height was measured to the nearest 0.5 cm, and weight to the nearest 0.1 kg on a regularly calibrated electronic scale. Body mass index (BMI) was calculated as weight in kilograms per height in square meter (kg/m^2). Waist circumference (WC) was measured to the nearest 0.5 cm, 2.5 cm above the umbilicus. A whole body scan was obtained at the second visit for the estimation of the total percentage of fat tissue, using dual-energy X-ray absorptiometry (DEXA; DPLX-L 2288, Lunar Radiation Corporation, Madison, WI, USA). The percentage of fat tissue was estimated using Lunar software.

Collection and analysis of fasting serum samples

Fasting serum samples were drawn in the morning from the antecubital vein at the three scheduled visits. Serum glucose was measured enzymatically using the hexokinase method at the Department of Clinical Chemistry, University Hospital of North Norway, Tromsø.

Collection of hormones – IGF-1, IGFBP-3, GH and estrogen

Fasting morning blood samples of insulin, IGF-1 and IGFBP-3, were obtained at the first scheduled visit, and fasting serum concentrations of GH was obtained at the second scheduled visit. Insulin, IGF-1 and IGFBP-3, were measured in serum stored at –70^{o}C for up to 3 years until analysis took place at the Hormone Laboratory, University Hospital of Oslo, Aker. Serum insulin was measured by radioimmunoassay (RIA) using kits from Linco Research Inc (St.Charles, MO, USA). IGF-1 and IGFBP-3, measured as glycosylated, were determined by ILMA, Immulite 2000 (Diagnostic Products Co, Los Angeles, CA, USA). GH was measured in serum, stored at –70^{o}C for up to 10 years until analysis, which took place at the Hormone Laboratory, University Hospital of Oslo, Aker. DELFIA kits from PerkinElmer Life Sciences (Wallac Oy, Turku, Finland) were used for the GH analysis. The coefficients of variation (CVs) derived from the laboratories were as follows: 7-10% for IGF-1 and 6% for IGFBP-3. For GH the average intra-assay variability was 1.9%, and the inter-assay variability ranged from 5.5% for low pools (0.29 mlU/l) to 2.5% for high pools (38.7 mlU/l).

Fasting serum 17β-estradiol concentrations were measured consecutively, by direct immunometric assay (Immuno-1, Bayer Diagnostics, Norway), at the three scheduled visits during the menstrual cycle.

The participants self-collected daily morning saliva samples, into plastic tubes pretreated with sodium azide, starting on the first day of bleeding, for one menstrual cycle, according to collection protocols previously established at the Reproductive Ecology Laboratory, Harvard University, USA [19,24,25]. Levels of salivary 17β-estradiol concentrations, were measured in daily saliva samples from 20 days (reverse cycle days −5 to −24; with the last day of the menstrual cycle designated -1) using ^{125}I-labelled RIA kits (#39100, Diagnostic Systems Laboratories, Webster, TX, USA), along with published modifications of the manufacturer's protocols [19]. Overall average 17β-estradiol concentrations, was calculated using daily levels of 17β salivary- estradiol. All samples were run in duplicate, and from a single participant, all samples were run together in the same assay with women randomly assigned to assay batches. The CVs were calculated based on the high and low value pools included in each assay.

Salivary assays have higher variability than serum assays, because their measuring levels are one to two orders of magnitude lower in concentration. In the present study measurements of 17β-estradiol at the start and end of the menstrual cycles had higher CV's. The sensitivity of the 17β-estradiol assay (the lowest 17β-estradiol concentration distinguishable from 0 at a 95% level) was 4 pmol/l. Average intra-assay variability was 9%, and inter-assay variability ranged from 23% for low pools, to 13% for high pools. Therefore, we included 17β-estradiol measurements from aligned cycle days -7 to +6 in the linear models. All cycles of the participants were aligned based on the identification of the mid-cycle drop in salivary 17β-estradiol concentration (aligned cycle day 0). The drop provides an estimate of the day of ovulation [26]. A drop in 17β-estradiol could not be identified for 14 women, hence their cycles could not be aligned and they were not included in the statistical analysis. Overall salivary 17β-estradiol concentrations were calculated for all 202 women, whereas hormonal indices (e.g. follicular, mid-menstrual and luteal phases) were calculated only for the women with aligned cycles (n=188).

Mammograms and mammographic density

Bilateral two-view mammograms were obtained from all women, during the second scheduled visit (between cycle days 7 and 12 after onset of bleeding), at the Centre of Breast Imaging, University Hospital of North Norway, Tromsø, using a standard protocol [19,27].

The left craniocaudal mammograms were digitized and imported into a computerized mammographic density assessment program (Madena), developed at the University of Southern California School of Medicine (Los Angeles, CA, USA) [28,29]. The density measurements were conducted by a trained reader (G. Ursin). These were done as follows: First a region of interest (ROI) [13] that included the entire breast, but excluded light artifacts such as the pectoralis muscle, prominent veins and fibrous strands was outlined. The mammogram reader then used a tinting tool to tint pixels considered to represent areas of mammographic density. The Madena software estimated the number of tinted pixels within the ROI. Absolute mammographic density represents the tinted pixels within the ROI, and percent mammographic density is the ratio of absolute mammographic density to the total breast area multiplied by 100. The mammograms were read in four batches, with an equal number of mammograms in each batch. A duplicate reading of 26 randomly selected mammograms from 2 of the batches showed a Pearson's correlation coefficient of 0.97. The reader was blinded to any characteristics of the study population.

Statistical analysis

To study the associations between characteristics of the women and percent and absolute mammographic density, we used Students t-test. Linear mixed models for repeated measures were used to investigate the association between fasting high or low serum concentrations of IGF-1, IGFBP-3 and GH, in combination with salivary 17β-estradiol concentrations throughout an entire menstrual cycle, and the study outcomes; percent and absolute mammographic density. This was done to take into account a potential combined effect of growth hormones and cycling estrogen throughout the menstrual cycle among premenopausal women in relation to mammographic density.

Based on plausible biological mechanisms, and previous findings suggesting a threshold effect between growth factors and breast cancer development [2], we stratified the regression analysis by median split of IGF-1 (25 nmol/l) and GH (0.80 mlU/l). Multivariable logistic and linear regression models were used to assess the associations between GH, IGF-1, estrogens and mammographic density. In the logistic regression models median split of percent and absolute mammographic density were used as dependent variables, >28.5% [yes/no], > 32.4 cm^2 [yes/no] and the GH-IGF-1 axis, and measures of 17β-estradiol (serum, salivary; overall, mid-menstrual, follicular, luteal and area under curve) throughout a menstrual cycle as independent variables. The 17β-estradiol levels were included as categorical variables (median split) and as continuous variables with one standard deviation (SD) increase. In the linear regression models, both percent and absolute mammographic density were used as dependent variables, and the GH-IGF-1 axis, and measures of 17β-estradiol throughout a menstrual cycle as independent variables. The 17β-estradiol levels were included as continuous variables with one SD increase.

The variables, including the breast density measures, were approximately normally distributed, thus no transformations were needed. Moreover, there were no observations of any outliers that could be driving the associations. Based on previously established observations, including results from the same study population [19] and suggested biological mechanisms, which may influence breast density, growth factors or levels of estradiol, several models were tested, including a variety of potentially confounding variables. We tested whether adjustments for potentially confounding factors such as age (continuous in years), BMI (continuous in kg/m^2), age at menarche (continuous in years), number of children (continuous in numbers), previous oral contraceptive use (categorical, yes/no), alcohol intake (continuous in units/week), smoking habits (categorical, yes/no), energy intake (continuous in kJ/day) and leisure time activity (continuous in metabolic equivalents [METs] hours/week)

influenced our estimates. Previous oral contraceptive use, age at menarche, alcohol intake, smoking habits, energy intake and leisure time activity were all tested as potential confounders, but as these factors did not influence our results they were not included in the final model. The adjustment factors in the final model were age, BMI and number of children. IGF-1 and IGFBP-3 were adjusted for each other when appropriate. The area under the curve (AUC) for estradiol was calculated for each participant with an aligned cycle (days -10 to +9) using the trapezium rule [30]. The results were considered statistically significant when two-sided $p < 0.05$. The analyses were conducted with SPSS version 21.0.

Ethical considerations

All the participating women signed an informed consent form. The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate approved the study.

Results

Selected general characteristics of the study participants are provided in Table 1. Among women with a mean age of 30.7 years, a mean salivary 17β-estradiol concentration of 17.9 pmol/l, a mean percent mammographic density of 29.8% (median 28.5%), and a mean absolute mammographic density of 34.7 cm^2 (median 32.4 cm^2) was observed (Results not presented in table). Age, parity and body composition (BMI, waist circumference and total tissue fat) were inversely associated with both percent mammographic density (>28.5 %) and absolute mammographic density (>32.4 cm^2). We observed IGF-1, IGFBP-3 and growth hormone independently, and in association with percent and absolute mammographic density, and no trends were observed (Table 1).

We examined women with high and low levels of growth factors (median split of IGF-1, IGFBP-3 and GH) in combination with mean salivary 17β-estradiol concentrations, throughout the mid-menstrual phase in relation to percent mammographic density. When we compared women with high IGF-1 (>25 nmol/l) and lower percent mammographic density (≤28.5%), with women with high IGF-1 (>25 nmol/l) and higher percent mammographic density (>28.5%); we observed a 38.3% difference in overall average 17β-estradiol ($p = 0.023$). Similarly, women with either high IGFBP-3 (>100 nmol/l) or high GH (>0.80 mlU/l) and lower percent mammographic density (≤28.5%), compared with women with higher percent mammographic density (>28.5%), we observed a difference in overall average 17β-estradiol of 34.1% ($p = 0.024$) and 34.2% ($p = 0.013$), respectively. Among women with low IGF-1 (≤25 nmol/l), low IGFBP-3 (≤100 nmol/l) or low GH (≤0.80 mlU/l), we observed no

difference in mean salivary 17β-estradiol concentrations for those with higher or lower percent mammographic density (Figure 1). The IGF-1:IGFBP-3 ratio showed the same pattern as described for IGF-1 and IGFBP-3 alone (data not shown).

When we stratified by median split of IGF-1, IGFBP-3 and GH, and examined the mean salivary 17β-estradiol concentrations throughout the mid-menstrual phase in relation to absolute mammographic density, (≤ 32.4 cm^2, > 32.4 cm^2) no differences were observed (results not shown in table).

In stratified analysis of growth factors (median split), the association between 17β-estradiol (both as a continuous and binary [median split] variable) and percent mammographic density was further evaluated in multivariable analysis (adjusted by age, BMI and number of children, and IGFBP-3 or IGF-1 when appropriate). In women with high IGF-1 (>25 nmol/l) or high GH (>0.80 mlU/l), a one SD increase in overall average salivary 17β–estradiol was associated with a 1.81 (95% confidence interval [CI] 1.08-3.03) and 2.08 (95% CI 1.10-3.94) times higher odds for having higher percent mammographic density (>28.5%), respectively. Similarly, in women with high levels of IGF-1 or GH, overall average 17β–estradiol above versus below median (16.8 pmol/l), was associated with a 4.13 (95% CI 1.33-12.83) and 4.17 (95% CI 1.41-12.28) times higher odds for having higher percent mammographic density (>28.5%), respectively. In women with IGFBP-3 (>100 nmol/l), the corresponding odds was 3.62 (95% CI 1.15-11.38) (results not shown in table). When we subdivided the menstrual cycle into mid-menstrual, follicular and luteal phases, we observed adjusted ORs comparable to the results listed above for overall salivary 17β-estradiol. In contrast, serum estrogen was not associated with percent mammographic density (Tables 2 and 3). The current study did not reveal any association between IGF-1, IGFBP-3 and GH in combination with 17β-estradiol and absolute mammographic density (Results not shown in table).

Linear regression was also performed with percent and absolute mammographic density as continuous variables. There were no associations between high IGF-1, estrogens and mammographic density in the linear regression models. However, among women with high GH (>0.80 mlU/l), a one SD increase in overall average and follicular phase salivary 17β–estradiol was positively associated with percent mammographic density. Thus, no associations were found when we used absolute mammographic density as the dependent variable (Supplementary table 1 and 2). The association between percent mammographic density and different categories of 17β-estradiol and IGF-1 (low 17β-estradiol –low IGF-1, low 17β-estradiol –high IGF-1, high 17β-estradiol –low IGF-1, and high 17β-estradiol –high IGF-1) were also tested. The category with both high 17β-estradiol and high IGF-1 showed an

increased OR for having high percent mammographic density; however the other categories were not associated with having high percent mammographic density (results not shown in table).

To test for interaction between the 17β-estradiol variables, and IGF-1, IGFBP-3 or GH, all variables were first dichotomized and then entered into logistic regression models with interaction terms. In addition, we tested for interaction between 17β-estradiol as a continuous variable, and growth factors as dichotomized variables. We observed statistically significant interactions between the IGF-1 and AUC of salivary 17β-estradiol, and mid-menstrual salivary 17β-estradiol, and between GH and overall salivary 17β-estradiol (Tables 2 and 3).

Discussion

In the present study, we observed that premenopausal women with higher levels of growth factors, in combination with daily salivary 17β-estradiol, have higher odds for having higher percent mammographic density, independent of age, BMI and number of children. In women with high IGF-1 (>25 nmol/l), or high GH (>0.80 mlU/l), there was a positive relationship between 17β-estradiol and percent mammographic density, with up to a doubling in odds by one SD increase of salivary 17β-estradiol. These findings were further strengthened in women with high IGF-1 or high GH, combined with levels above median of daily cycling 17β – estradiol (>16.8 pmol/l), as these women had a three- to four times higher odds for having higher percent mammographic density (>28.5%).

The present results extend our own [19], and previous reports [31-33], as we observed a positive association between growth factors in combination with estrogen and mammographic density, not only an association between estrogen alone and mammographic density. Interestingly, a crosstalk between IGF-1 and estrogen in breast cancer development has been hypothesized [15-18]. Recently, GH action was studied in a panel of estrogen receptor-positive breast cancer cell lines, and GH significantly enhanced 17β-estradiol-stimulated proliferation in these cells. Interestingly, the combination of GH and 17β-estradiol overcame inhibition of IGF-I receptor activity to restore proliferation [18]. These observations support a potential joint effect of growth factors and estrogen on breast cancer development. Our observations are also partly supported by others [33]. Among premenopausal women, positive associations were observed both between IGF-1 and between estrogen and percent mammographic density. However, estrogen was assessed on six consecutive days in relation

to ovulation and one day in the luteal phase in urine, but interestingly when they adjusted for BMI, the association was attenuated [33].

Interestingly, we did not observe any clear association between any of the growth factors alone and percent mammographic density, which is consistent with other studies. Recently, no association between GH, IGF-1 and percent and absolute mammographic density was observed for either premenopausal or postmenopausal women [6], which correspond to the results ofothers [34-36]. Furthermore, our observation of an association between IGF-1 and percent mammographic density, mainly seen among women with higher levels of IGF-1 suggest a threshold effect. This association is indirectly supported by Hankinson and co-authors as they observed an increased breast cancer risk among premenopausal women in the upper tertile of IGF-1 only [2]. Whether a linear association or a threshold effect exists between growth factors and percent mammographic density may be questioned, but our findings of no linear association with an interaction between growth factors, in combinations with continuous estrogen and percent mammographic density lends support to a threshold effect between growth factors and mammographic density. Other findings may also indirectly support a threshold effect only among women with high percent mammographic density. In a cohort study from the Netherlands, women aged 35 and older with percent mammographic density above 25%, had a 2 times increased odds ratio for breast cancer development [37]. Moreover, recently percent mammographic density above 25% was associated with later increased postmenopausal breast cancer risk [1]. These results partly support a threshold effect, and support our cut off of 28.5% for percent mammographic density as an appropriate cut off level.

To note, our observed association between growth factors in combination with estrogen and percent mammographic density, were not observed with absolute mammographic density. These findings can partly be explained by the fact that absolute mammographic density reflect the dense breast tissue, while percent mammographic density reflects fibroglandular and fat tissue [38]. Moreover, such an association between growth factors and percent mammographic density has been suggested to reflect cumulative exposure of hormones and growth factors in breast stroma and epithelium to stimulate cell division [39,38]. Thus, estrogen and growth factors may influence not only the dense area, but the stroma and the surrounding adipose tissue. Importantly, others have observed that fibroglandular and fat tissue may have independent effects on breast cancer development [40].

Altogether, even if plausible mechanisms have been suggested, and has been supported by experimental studies [41], less is known about the association of the GH-IGF

signaling pathway, estrogen and mammographic density. Recently, estrogen and IGF-1 have been observed to have synergistic effects on the growth of breast cancer cells [41]. Moreover, GH regulates the production of IGF-1, and around 99% of circulating IGF-1 is bound to IGFBP-3. IGF binds to the tyrosine kinase receptor, which induces an intracellular signalling cascade. IGF-1 receptor activation primarily leads to proliferation and differentiation [42]. The GH, IGF-1 and IGFBP-3 levels are age dependent and decrease after puberty. Some studies have examined the GH-IGF signaling pathway in association with mammographic density, for both premenopausal and postmenopausal women, but the results are indifferent [43,44,34]. For postmenopausal women, however, the growth factor levels, estrogen levels and mammographic densities are lower than that for premenopausal women. This may explain in part, why no associations between these growth factors and mammographic density have been observed for postmenopausal women [34,35,43]. Thus, the higher levels of cyclic estrogen and serum level of growth factors may be more likely to capture the aetiologically relevant exposure period, and may explain in part why the associations are more pronounced among premenopausal women [45]. Furthermore, estrogen and mammographic density varies throughout the menstrual cycle and this could partly explain why some studies that measure estrogen in serum did not show associations with mammographic density [31,46]. Variations in the populations may also be explained by the fact that variations in IGF-1 levels have been observed in relation to single-nucleotide polymorphisms as well as lifestyle factors (such as age, nutrition, hepatic function) [47].

Recently, both an independent and a combined effect of endogenous sex hormone and percent mammographic density were observed on breast cancer risk [14]. Postmenopausal women in the highest tertile of estradiol and with the highest percent mammographic density (>24.0%) had an increased breast cancer risk [14]. These observations support that, including circulating endogenous estrogen, may add additional information about the complexity using mammographic density as a biomarker for breast cancer development.

Thus, our findings of an association between growth factors, estrogen and percent mammographic density with a threshold effect are supported by plausible biological mechanisms, and suggested as a crosstalk in cells between the signalling pathways for estrogens and IGF-1[15,16]. Importantly, GH and IGF-1 signalling, together with estrogens, is essential for the development of the mammary gland, particularly the terminal end-buds. It has also been found that during lactation, IGF-1 plays an important role in the maintenance of the adult mammary gland [48-50]. Furthermore, percent mammographic density refers to the structure of the adipose, epithelial and connective tissue in the breast [51]. Thus, the growth

factors and estrogens in combination, are key factors in proliferation of breast cells and are potential drivers for breast cancer development [41].

In the present study, we observed a positive association between IGFBP-3 and percent mammographic density, which is in contrast to others [3]. However, the association between IGFBP-3 and breast cancer risk may differ according to whether IGFBP-3 is measured as intact, fragmented or total [52]. In our study, serum IGF-1 and IGFBP-3 are measured once in serum, but have long half-lives and little daily variations. This seems to be adequate for measuring the long-term levels of these peptides [53]. However, GH is normally secreted episodically with 7-10 peaks per day, but importantly with a more constant fasting morning level, as is the case for many other cycling biological markers and the half-life in serum is shorter than that for IGF-1. To minimize these variations, GH was assessed in fasting morning samples, as is the case for all other growth factors. However, caution should be exerted when interpreting the results.

The current study benefited from several unique features, such as salivary measurements of concentrations of unbound 17β-estradiol collected daily across an entire menstrual cycle [23,54], following strict procedures [19] and validated methods [26]**.** Among ovulating premenopausal women, the estrogen levels vary considerably throughout the menstrual cycle, and by using daily salivary samples, we were able to measure the free biologically active form of estrogen, which is considered to be the ideal measure among ovulating premenopausal women [23,26]. Thus, we were able to capture the continuous estrogen exposure of the women. Moreover, standardized repeated hormone levels in serum were also included.

Furthermore, mammograms were taken during a narrow time frame in the late follicular phase (days 7-12)**,** thereby avoiding the bias of variation in mammographic density during the menstrual cycle [55]. The validated computer-assisted method has been observed to quantify mammographic density, and shown to give a superior prediction of breast cancer risk compared with qualitative methods [28]. All mammograms were read by one experienced blinded reader, and the assessed mammographic density was negatively associated with age, BMI and number of children [56,57]. Thus, we have adjusted for these confounders in the multiple analyses. The study population was homogenous with healthy women aged 25-35 years from the same cultural background. However, the small sample size of the current study and small number of earlier reports underlines the need for further studies.

In conclusion, based on the biological mechanisms suggested and previous reports, the present findings are unique in character, supporting that IGF-1, IGFBP-3 and GH, in

combination with cycling bioactive estrogen, may be associated with percent mammographic density in premenopausal women. This combination of biomarkers may also be important in clinical settings. However, our results are based on a relatively small sample size and should be interpreted with caution. Hence, there is a need for replication in larger studies.

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Table 1 Characteristics of the study population by median split of percent and absolute mammographic density: The Norwegian Energy Balance and Breast cancer Aspects (EBBA)-I study (n=202) [a]

Characteristics	Percent mammographic density[e] (%)			Absolute mammographic density[e] (cm^2)		
	≤28.5 (n=101)[a]	*>28.5 (n=101)*[a]		*≤32.4 (n=101)*[a]	*>32.4 (n=101)*[a]	
	Mean (SD)	**Mean (SD)**	***p*-value**	**Mean (SD)**	**Mean (SD)**	***p*-value**
Age (years)	31.54 (2.72)	29.88 (3.18)	<0.001	31.48 (2.80)	29.94 (3.14)	<0.001
Education (total years)	16.14 (3.20)	16.01 (2.88)	0.771	15.92 (2.99)	16.23 (3.09)	0.467
Body composition[b]						
BMI (kg/m^2)	26.15 (3.90)	22.66 (2.73)	<0.001	25.42 (4.07)	23.39 (3.19)	<0.001
Waist circumference (cm)	84.43 (9.92)	74.83 (6.93)	<0.001	82.45 (10.35)	76.81 (8.35)	<0.001
Tissue fat, DEXA scan (%)[e]	37.95 (6.72)	30.54 (6.55)	<0.001	36.39 (7.50)	32.10 (7.10)	<0.001
Reproductive factors						
Parity (no children)	1.35 (1.21)	0.49 (0.84)	<0.001	1.25 (1.14)	0.58 (1.02)	<0.001
Age at menarche (years)	12.87 (1.31)	13.35 (1.40)	0.011	12.91 (1.34)	13.31 (1.39)	0.038
Cycle length (days)	27.87 (3.00)	28.67 (3.22)	0.069	27.78 (3.10)	28.76 (3.10)	0.026
Serum concentrations[c]						
Glucose (mmol/l)	5.15 (0.59)	4.90 (0.51)	0.002	5.10 (0.62)	4.95 (0.49)	0.049
Insulin (pmol/l)	90.42 (61.26)	80.96 (57.60)	0.260	91.06 (68.26)	80.32 (48.97)	0.200
IGF-1 (nmol/l)	24.66 (6.27)	25.36 (6.41)	0.439	24.23 (6.00)	25.79 (6.59)	0.079
IGFBP-3 (nmol/l)	100.51 (15.93)	100.38 (14.17)	0.951	98.63 (15.17)	102.27 (14.75)	0.085
Growth hormone (mlU/l)	3.03 (4.90)	4.21 (7.02)	0.175	3.13 (5.19)	4.11 (6.85)	0.260
Serum hormones[c]						
Estradiol (pmol/l)	149.60 (72.54)	144.60 (48.67)	0.567	153.07 (73.06)	141.10 (47.24)	0.170
Salivary hormones[d]						
Overall average 17β-estradiol (pmol/l)	17.55 (8.78)	18.50 (8.73)	0.441	17.63 (8.42)	18.43 (9.09)	0.515
Lifestyle factors						
Energy intake (kJ/day)	7.94 (1.94)	8.27 (1.85)	0.218	7.89 (1.93)	8.32 (1.85)	0.106
Previous use of OC (%)	1.16 (0.37)	1.18 (0.39)	0.708	1.18 (0.39)	1.16 (0.37)	0.708
Leisure time (MET hours/week)	62.49 (121.61)	53.03 (37.64)	0.456	59.99 (121.14)	55.50 (39.55)	0.724
Alcohol (units/week)	2.56 (3.10)	3.24 (3.61)	0.154	2.50 (3.13)	3.30 (3.57)	0.091
Current smokers (%)	1.76 (0.43)	1.78 (0.42)	0.710	1.77 (0.42)	1.77 (0.42)	0.970

NOTE: All analyses have used Students t-test
BMI, body mass index; DEXA, dual-energy X-ray; IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein-3; OC, oral contraceptives; MET, metabolic equivalents; SD, standard deviations.

[a]Numbers may vary due to missing information.
[b]Measurements at days 1-5 after onset of menstrual cycle.
[c]Serum samples in early follicular phase: days 1-5 after onset of menstrual cycle.
[d]Daily saliva samples throughout an entire menstrual cycle.
[e]Mammograms and total tissue fat (DEXA) were taken days 7-12 (mid-cycle phase).

Table 2 Odds ratios (ORs) with 95 % Confidence interval (CI) for higher percent mammographic density (>28.5%) by estrogen among premenopausal women, stratified by median split of insulin-like growth factor (IGF)-1.

	IGF-1				
	≤25 nmol/l n=105[a]		>25 nmol/l n=99[a]		Interaction
Hormones	**OR**	**95% CI**	**OR**	**95% CI**	***p*-value**
Serum 17β-estradiol (pmol/l)					
Early follicular[b]					
≤130.0	1.00	(Referent)	1.00	(Referent)	
>130.0	1.15	(0.43-3.12)	1.57	(0.56-4.43)	0.776
Estradiol per 1 SD (61.59 pmol/l)	0.84	(0.47-1.52)	1.22	(0.77-1.95)	0.461
Salivary 17β -estradiol (pmol/l)					
Overall[c]					
≤16.8	1.00	(Referent)	1.00	(Referent)	
>16.8	1.21	(0.42-3.46)	4.13	(1.33-12.83)	0.056
Estradiol per 1 SD (8.79 pmol/l)	1.52	(0.81-2.82)	1.81	(1.08-3.03)	0.356
Mid-menstrual[c]					
≤16.9	1.00	(Referent)	1.00	(Referent)	
>16.9	1.10	(0.35-3.44)	4.35	(1.42-13.35)	0.028
Estradiol per 1 SD (8.98 pmol/l)	1.38	(0.67-2.86)	1.80	(1.09-2.97)	0.183
Follicular[e]					
≤17.5	1.00	(Referent)	1.00	(Referent)	
>17.5	1.35	(0.44-4.16)	3.84	(1.20-12.26)	0.057
Estradiol per 1 SD (9.58 pmol/l)	1.39	(0.69-2.80)	1.99	(1.14-3.48)	0.101
Luteal[f]					
≤15.5	1.00	(Referent)	1.00	(Referent)	
>15.5	1.91	(0.61-6.00)	5.26	(1.67-16.56)	0.071
Estradiol per 1 SD (9.22 pmol/l)	1.28	(0.64-2.57)	1.62	(1.02-2.58)	0.282
AUC[g] (pmol/l x cycle)					
≤251.25	1.00	(Referent)	1.00	(Referent)	
>251.25	1.25	(0.40-3.90)	4.35	(1.42-13.35)	0.038
AUC per 1 SD (133.01 pmol/l x cycle)	1.38	(0.67-2.86)	1.81	(1.10-3.01)	0.176

NOTE: All analyses have used multivariable logistic regression models, and are adjusted for age (continuous), body mass index (continuous), number of children (continuous), and IGFBP-3 (continuous).
AUC, area under curve; CI, confidence interval; IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein-3; OR, odds ratio; SD, standard deviation.

[a]Numbers may vary due to missing information.
[b]Serum samples in early follicular phase: days 1-5 after onset of menstrual cycle.
[c]Daily saliva samples throughout an entire menstrual cycle.
[d]Daily saliva samples in mid-menstrual phase: aligned cycle days -7,+6
[e]Daily saliva samples in follicular phase: aligned cycle days -7,-1.
[f]Daily saliva samples in luteal phase: aligned cycle days 0,+6
[g]AUC estimated from aligned cycle days -10,+9

Table 3 Odds ratios (ORs) with 95 % Confidence interval (CI) for higher percent mammographic density (>28.5 %) by estrogen among premenopausal women, stratified by median split of growth hormone (GH).

	GH				
	≤0.80 mIU/l n=102[a]		>0.80 mIU/l n=93[a]		Interaction
Hormones	**OR**	**95% CI**	**OR**	**95% CI**	***p*-value**
Serum 17β -estradiol (pmol/l)					
Early follicular[b]					
≤130.0	1.00	(Referent)	1.00	(Referent)	
>130.0	1.00	(0.35-2.78)	2.27	(0.76-6.73)	0.321
Estradiol per 1 SD (61.59 pmol/l)	1.08	(0.59-1.98)	1.02	(0.63-1.63)	0.743
Salivary 17β -estradiol (pmol/l)					
Overall[c]					
≤16.8	1.00	(Referent)	1.00	(Referent)	
>16.8	0.88	(0.30-2.61)	4.17	(1.41-12.28)	0.024
Estradiol per 1 SD (8.79 pmol/l)	1.57	(0.91-2.69)	2.08	(1.10-3.94)	0.256
Mid-menstrual[d]					
≤16.9	1.00	(Referent)	1.00	(Referent)	
>16.9	1.16	(0.37-3.59)	3.05	(1.02-9.12)	0.170
Estradiol per 1 SD (8.98 pmol/l)	1.56	(0.89-2.75)	2.06	(1.06-4.00)	0.328
Follicular[e]					
≤17.5	1.00	(Referent)	1.00	(Referent)	
>17.5	1.19	(0.38-3.73)	4.09	(1.27-13.12)	0.083
Estradiol per 1 SD (9.58 pmol/l)	1.40	(0.83-2.37)	2.78	(1.29-6.03)	0.063
Luteal[f]					
≤15.5	1.00	(Referent)	1.00	(Referent)	
>15.5	2.34	(0.71-7.77)	3.70	(1.25-10.96)	0.361
Estradiol per 1 SD (9.22 pmol/l)	1.69	(0.94-3.03)	1.58	(0.88-2.84)	0.949
AUC[g] (pmol/l x cycle)					
≤251.25	1.00	(Referent)	1.00	(Referent)	
>251.25	1.33	(0.43-4.12)	3.05	(1.02-9.12)	0.586
AUC per 1 SD (133.01 pmol/l x cycle)	1.60	(0.91-2.83)	2.06	(1.05-4.04)	0.348

NOTE: All analyses have used multivariable logistic regression models, and are adjusted for age (continuous), body mass index (continuous), and number of children (continuous).
AUC, area under curve; CI, confidence interval; GH, growth hormone; OR, odds ratio; SD, standard deviation.

[a]Numbers may vary due to missing information.
[b]Serum samples in early follicular phase: days 1-5 after onset of menstrual cycle.
[c]Daily saliva samples throughout an entire menstrual cycle.
[d]Daily saliva samples in mid-menstrual phase: aligned cycle days -7,+6
[e]Daily saliva samples in follicular phase: aligned cycle days -7,-1.
[f]Daily saliva samples in luteal phase: aligned cycle days 0,+6
[g]AUC estimated from aligned cycle days -10,+9