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Estrogen Biosynthesis in Breast Adipose Tissue during Menstrual Cycle in Women with and without Breast Cancer

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Abbreviations: BMI, body mass index; 17β-HSD, 17β-hydroxysteroid dehydrogenase; E_1, estrone; E_2, estradiol; E_2-FAE, E_2 fatty acyl ester; FEI, free estradiol index; IQR, interquartile range; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; STS, steroid sulfatase; WHR, waist to hip ratio.

ABSTRACT

Circulating estrogens fluctuate during the menstrual cycle but it is not known whether this fluctuation is related to local hormone levels in adipose tissue. We analyzed estrogen concentrations and gene expression of estrogen-regulating enzymes in breast subcutaneous adipose tissue in premenopausal women with (n=11) and without (n=17) estrogen receptor-positive breast cancer. Estrone (E_1) was the predominant estrogen in premenopausal breast adipose tissue, and E_1 and mRNA expression of *CYP19A1* in adipose tissue correlated positively with BMI. Adipose tissue estradiol (E_2) concentrations fluctuated during the menstrual cycle, similarly to the serum concentrations. In women with breast cancer median adipose tissue E_1 (1519 vs. 3244, $P<0.05$) and E_2 (404 vs. 889 pmol/kg, $P<0.05$) levels were lower in the follicular than in the luteal phase whereas in control women no significant differences were observed. In the follicular phase, mRNA expressions of *HSD17B1* (median 0.06; interquartile range 0.05-0.07 vs. 0.17; 0.03-0.2, $P=0.010$) and *CYP19A1* (0.08; 0.07-0.14 vs. 0.22; 0.09-0.54, $P=0.025$) were lower in women with breast cancer than in controls. In conclusion, the changes in adipose tissue E_1 and E_2 concentrations and the estrogen-regulating *CYP19A1* and *HSD17B1* during the menstrual cycle may be related to dysfunctional local estrogen metabolism in women with breast cancer.

Words: 200

Key words: adipose tissue, aromatase, breast cancer, estrogen, menstrual cycle

Main text: 1964

Introduction

In premenopausal women, estrogens are produced both in the ovaries and through peripheral conversion of adrenal precursor androgens [1, 2]. Increased circulating estrogen and androgen concentrations have been linked to premenopausal breast cancer [3], but the breast cancer risk, unlike in postmenopausal women, may be inversely associated with adiposity [4, 5].

In the breast, the mammary gland and the ductal system are abundantly surrounded by adipose tissue (AT). Therefore, AT might provide a local source of estrogen. In AT, aromatase converts androstenedione and testosterone to estrone (E_1) and estradiol (E_2), respectively [6, 7]. Estrone may also be formed from E_1 sulfate by steroid sulfatase (STS) and be further converted to E_2 by 17β-hydroxysteroid dehydrogenases (17β-HSDs) [8, 9]. Estradiol fatty acyl esters (E_2-FAE) may be released by hormone-sensitive lipase (LIPE) [10]. Although estrogen metabolism has been studied to some extent in postmenopausal AT [11-13], little is known about the menstrual cycle -related estrogen concentrations in the breast AT of premenopausal women [14]. Yet, there is evidence that the circulating estrogen concentrations during the follicular phase of the menstrual cycle may be associated with an increased breast cancer risk [15, 16]. On the other hand, breast tumor proliferation is increased in the luteal phase [17]. Thus, exploring the regulation of estrogen synthesis in AT during the menstrual cycle becomes of interest.

We assessed E_1, E_2, and E_2-FAE concentrations in the breast subcutaneous AT according to the phase of the menstrual cycle. We also studied adipose tissue mRNA expressions of aromatase (*CYP19A1*), *STS*, 17β-HSDs *(HSD17B1*, *HSD17B7* and *HSD17B12)*, and *LIPE*, which all drive toward the formation of biologically active E_2. Since the fluctuation of estrogens during the menstrual cycle may play a role in premenopausal breast cancer, we compared AT estrogen levels and the estrogen-metabolizing gene expressions in women with

and without breast cancer.

Methods

Subjects and study design

We collected AT samples from premenopausal women operated for estrogen receptor-positive breast cancer (mastectomy; n=11) and women undergoing reduction mammoplasty (control; n=17). Detailed clinical information (Table 1) was gathered at the preoperative visit and from medical records. Women using oral contraceptives were excluded.

Blood samples were obtained preoperatively and processed as described [13]. Two subcutaneous AT samples (1 g each) from the breast specimens were immediately snap frozen in liquid nitrogen [13]. The phase of the menstrual cycle was based on serum progesterone and E_2 (luteal phase concentrations >7 nmol/L and 0.37-0.77 nmol/L, respectively).

Women with and without breast cancer were comparable in the primary clinical characteristics only waist to hip ratio (WHR) was slightly higher in control women (Table 1). Five women with cancer and six controls (45% vs. 35%, *P*=0.34) had a levonorgestrel-releasing intrauterine device (Mirena®). Due to the very low systemic absorption of levonorgestrel into systemic circulation, these women were included in the study. Serum follicle stimulating hormone (FSH) and sex-hormone binding globulin (SHBG) concentrations were similar in women with or without Mirena (data not shown). Women in the follicular (n=12) or the luteal phase (n=11) of the menstrual cycle did not differ in their primary clinical characteristics.

Ethical approval

The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of Helsinki University Hospital. Informed consent was obtained from all subjects.

Quantification of hormones and SHBG

For determination of E_1, 30 µL of $^{13}C_3$-E_1 (2.5 nM; IsoSciences) was added as an internal standard (IS) to a 250 mL aliquot of serum or 200 mg of AT homogenized in 1 mL of distilled water. The AT samples were then processed as described in [18]. E_1 in serum and purified tissue samples was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described [18]. The limit of quantification (LOQ) of E_1 was 10 pmol/L (signal to noise-ratio S/N=10). For tissue E_1, interassay variation of the control samples [18] was 1.8% and 4.9% in three consequent assays.

For E_2 and E_2-FAE, serum and AT samples were processed as described [19]. Pooled subcutaneous breast AT [18], and serum with added E_2-17-stearate [20] were used as control. E_2 and hydrolyzed E_2-FAE were analyzed by E_2 LC-MS/MS as described [2] with a LOQ of 15 pmol/L (S/N=10). Interassay imprecision of endogenous E_2-FAE in pooled AT was 15% in nine consequent assays. Interassay variation of the serum control samples was 11% and 7.1% for E_2, and 6.2% and 18% for E_2-FAE, respectively, in nine or ten consequent assays.

For serum progesterone, $^{13}C_3$-progesterone was added as an IS (IsoSciences) and the samples were extracted with diethylether. Extracts and calibrators (Riedel-deHaën, Buchs, Switzerland) were analyzed on a LC-MS/MS system equipped with an API 3000 triple quadrupole mass spectrometer (PE Sciex, Foster City, CA), an Agilent series 1200 HPLC system with a binary pump (Waldbronn, Germany) and a SunFire C18 column (2.1 x 50 mm; Waters, Milford, MA). The mobile phase was a linear gradient consisting of methanol and 50 mM ammonium acetate in water, at a flow rate of 250 uL/min and the gradient was: 0 min, 50% methanol; 1.5 min 95% methanol; 5 min 95% methanol; and 5.5-10 min 50% methanol. Progesterone was detected as protonated ion in the positive mode with the following transitions: m/z 315 to m/z 109 and IS, m/z 318 to m/z 112. The data was processed with the

Analyst Software (Ver. 1.6, Sciex).

Serum SHBG was measured by immunoassay [13].

Preparation and quantification of mRNA

Total RNA was isolated and purified [19], and 1.0 μg of RNA was reverse transcribed into cDNA. Real-time PCR was performed as described [19]. Data were normalized to the geometric mean of two reference genes, importin 8 *(IPO8)* and lysine-specific demethylase 2B *(KDM2B)*.

Statistical analysis

Data are expressed as median (range) or median (IQR) unless otherwise stated. The statistical tests were done with SPSS Statistics software, version 22.0. Normality was assessed with the Shapiro-Wilk test. Between-group differences were evaluated with the Student's t test, or for nonparametric variables, with Mann-Whitney U test. For multiple comparisons, analysis of variance with the LSD post hoc test (parametric variables) or the Kruskal-Wallis test (non-parametric variables) were used. For comparisons between the cancer and control groups we used analysis of with adjustment for WHR. For pairwise comparisons, paired samples t test was used (parametric) and Wilcoxon signed ranks test (nonparametric). Correlation was assessed using Spearman's correlation. The level of significance was $P < 0.05$.

Results

Estrone was the predominant estrogen in premenopausal AT, the concentration being approximately three times higher than that of E_2 (Figure 1A, Table 2). Adipose tissue concentrations of E_1 ($P<0.0001$), E_2 ($P<0.0001$), and E_2-FAE ($P<0.0001$) were higher than the corresponding serum levels (Figure 1), and AT E_1 and E_2 correlated positively with their

corresponding serum concentrations (r=0.77, *P*=0.001 and r=0.73, *P*<0.0001, respectively).

Adipose tissue E_1, but not E_2 or E_2-FAE, correlated positively with mRNA expressions of *HSD17B12* (r=0.42, *P*<0.05*), CYP19A1 (*r=0.57, *P*<0.01*)*, *STS (*r=0.47*, P*<0.05*)* and *LIPE* (r=0.44, *P*<0.05). Furthermore, AT E_1 (r= 0.52, P=0.008) and expression of *CYP19A1* (r=0.60, *P*<0.001) correlated positively with BMI.

During the menstrual cycle, the E_2 levels in AT followed the changes detected in serum, while E_1 and E_2-FAE levels did not change significantly (Table 2). Serum progesterone did not correlate with AT hormone levels (data not shown). Stratification of the AT hormone concentrations according to the cancer status demonstrated that E_1 and E_2 concentrations were lower in the follicular than the luteal phase of the menstrual cycle in women with breast cancer but not in the control women (Figure 1B-C). In the luteal phase, the E_2 level tended to be higher in women with cancer than in control women, but the difference did not reach statistical significance (*P*=0.06 when adjusted for WHR).

In the follicular phase, the expression of *HSD17B1* (median 0.06; interquartile range 0.05-0.07 vs. 0.17; 0.03-0.2, cancer vs. control, *P*=0.010) and *CYP19A1* mRNA (0.08; 0.07-0.14 vs. 0.22; 0.09-0.54, cancer vs. control, *P*=0.025) were lower in women with breast cancer than in control women. There were no statistically significant differences in the expressions of *STS, LIPE, HSD17B7* or *HSD17B12*. Serum FSH correlated negatively with the expression of *CYP19A1* (r= -0.65, *P*=0.03), and with AT E_1 (r= -0.80, *P*=0.01), and E_2 (r= -0.81, *P*=0.005) in women with cancer.

Discussion

Although ovarian follicles are the principal site of estrogen biosynthesis in premenopausal women, our data indicate that AT synthesizes a considerable amount of estrogens, and AT

158 estrogen levels change significantly during the menstrual cycle. We found that E_1 levels in
159 premenopausal breast subcutaneous AT were about eight times higher than in serum. Adipose
160 tissue E_1 concentration correlated positively with BMI and mRNA expressions of aromatase
161 (*CYP19A1), HSD17B12* and *STS* genes, all related to E_1 bioavailability. This supports active
162 local synthesis of E_1, an important precursor for E_2, in premenopausal AT.

163 In theory, there are two possible explanations for the novel finding of fluctuating E_2
164 concentrations in AT reflecting the menstrual cycle serum E_2 levels. First, we cannot rule out
165 the possibility that circulating estrogens would directly influence their AT concentrations.
166 However, no active transport mechanism transferring estrogen from serum to AT against a
167 concentration gradient, has been reported so far. Second, it is theoretically possible that AT
168 estrogen is regulated by mechanisms resembling those in the ovaries. The synthesis of E_2 in
169 the ovaries is controlled by pituitary gonadotropins, as well as cytokines and growth factors
170 [21]. FSH induces estrogen biosynthesis by regulating the transcription of *CYP19A1* and
171 *HSD17B1* in ovarian granulosa cells [22, 23], and the mRNA expression of these genes is
172 decreased during the luteinization process [24, 25]. Furthermore, FSH receptors have been
173 found in the human AT [26]. Data on the regulation of estrogen concentrations in AT during
174 menstrual cycle, however, is scarce: in one previous study, AT E_2 level as determined with
175 immunological methods correlated with the time since last menses [14]. While our current
176 findings indicate that estrogen levels may be regulated in AT during the menstrual cycle, our
177 data do not give direct evidence that these fluctuations could be gonadotropin-controlled.

178 In women with breast cancer, follicular phase AT E_1 and E_2 levels were lower than luteal
179 phase levels, whereas there was no significant difference in control women. Also, in cancer
180 patients, the follicular phase gene expressions of the two estrogen regulating enzymes,
181 *CYP19A1* and *HSD17B1* were lower than in controls. These findings may imply that estrogen
182 synthesis in the tumor-bearing breast AT is dysregulated. There is evidence that adipocytes

near the tumor go through a phenotypic change into cancer-associated adipocytes [27], which could possibly affect local estrogen production, at least in part explaining our findings. Contrary to our data on postmenopausal women [13] the overall E_2 or E_2-FAE levels in women with and without breast cancer did not differ. This emphasizes the differences between premenopausal and postmenopausal breast cancer, and the need to take into account hormonal fluctuations when studying premenopausal women.

Our study has limitations. We acknowledge the relatively small number of women in our study, which may affect the power in the comparisons between the subgroups. Also, the limited amount of samples did not allow us to analyze protein levels or activities of the steroidogenic enzymes, or the concentrations of other possible regulators of hormone synthesis, such as luteinizing hormone or AT progesterone levels. Although our findings are preliminary in nature, to our knowledge this is the first study to use LC-MS/MS to show menstrual cycle-dependent fluctuation in AT estrogen levels, and furthermore, comparisons between premenopausal women with and without breast cancer.

In conclusion, E_1, E_2, and the E_2 fatty acyl ester concentrations were higher in the breast subcutaneous AT than in serum. E_2 concentrations fluctuated during the menstrual cycle, not only in serum, but also in AT. The changes in E_1 and E_2 concentrations and the relative mRNA expression levels of estrogen-regulating *CYP19A1* and *HSD17B1* in AT during the menstrual cycle may be related to dysfunctional local estrogen metabolism in women with breast cancer.

Disclosure of interest: H.S-P. has been a speaker for Mylan and received funding for congress trips from Mylan and MSD. T.M. has been a speaker and/or received consulting fees from Mylan and Novo Nordisk. The remaining authors report no conflicts of interest in this work.

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Figure legends

Fig. 1. A. Serum and adipose tissue estrone (E_1), estradiol (E_2), and E_2-fatty acyl ester (FAE) concentrations (pmol/l in serum and pmol/kg in adipose tissue) in all premenopausal women (n=28). The hormone concentrations in women with (n=11) and without (n=17) breast cancer were comparable, thus, thus data from all women were combined. The data are expressed as median and interquartile range. **P* <0.0001, adipose tissue compared with the respective serum concentrations (paired samples t test and the Wilcoxon signed ranks test). **B.** Adipose tissue estrone (E_1) and **C.** estradiol (E_2), concentrations (pmol/kg) in premenopausal women with and without breast cancer stratified according to the phase of menstrual cycle. Six women in both groups were in the follicular phase of the cycle and six control women and five women with cancer in the luteal phase of the cycle. The data are expressed as median and interquartile range. **P* <0.05, student's t test for parametric and Mann-Whitney U test for non-parametric variables

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307 Table 1. Clinical characteristics.

	Breast cancer N=11	**Control N=17**	***P*-value**
Age[1], years	45 (27-49)	40 (21-50)	0.18
Body mass index[1], kg/m^2	24 (22-34)	28 (19-33)	0.15
Waist to hip ratio[1]	0.82 (0.69-0.91)	0.88 (0.76-1.00)	0.006
Serum follicle stimulating hormone[1], IU/L	3.8 (0.9-12.7)	4.3 (1.7-10.5)	0.69
Sex hormone binding globulin[1], nmol/L	60.0 (14.8-92)	59.9 (18.9-122)	0.92
Age at menarche[1], years	13 (11-15)	13 (11-16)	0.23
Age at first labour[1], years	29 (20-34)	29 (17-36)	0.75
Number of labours[1]	2 (0-2)	2 (0-3)	0.41
History of hormonal contraception[1], years	0 (0-14)	0 (0-15)	0.17
Ductal breast cancer histology[2]	10 (91)		
Estrogen receptor positive breast cancer[2]	11 (100)		
Progesterone receptor positive breast cancer[2]	10 (91)		

308 The data are expressed as median (range)[1] or n (%)[2].

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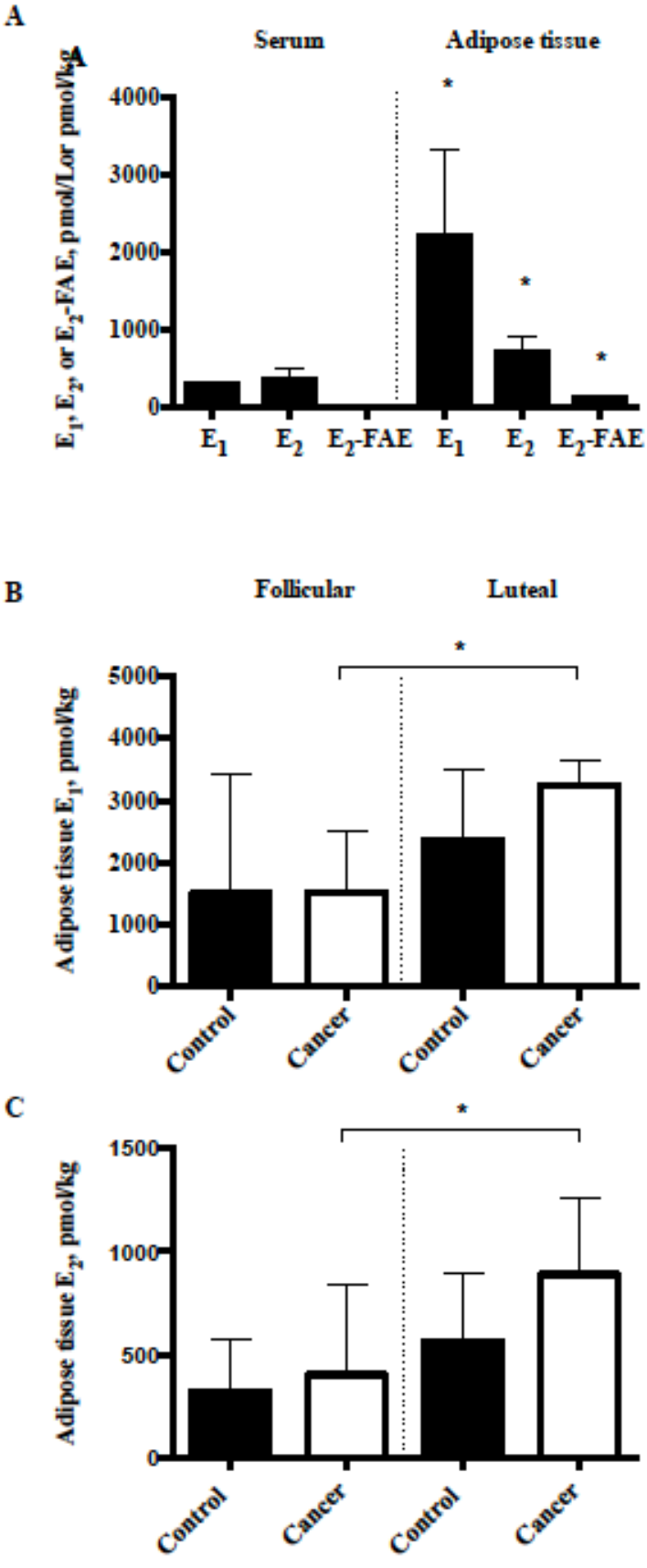
Table 2. Serum and adipose tissue E_1, E_2, and E_2-FAE levels in premenopausal women according to the phase of menstrual cycle.

	Phase of Menstrual cycle			
	Follicular	**Periovulatory**	**Luteal**	***P*-value**
	N=12	**N=5**	**N=11**	
Adipose tissue				
E_1	1519 (1119-3055)	2213 (1904-3574)	2616 (1992-3592)	0.14
E_2	326 (204-700)^{**,*1}	975 (880-1539)^{**,*2}	767 (495-916)^{*1,*2}	0.003
E_2-FAE	91 (60-128)	133 (100-250)	115 (101-179)	0.088
Serum				
E_1	200 (104-290)	315 (304-417)	271 (161-336)	0.10
E_2	182 (104-336)$^{\#,*}$	575 (506-820)$^{\#,**}$	399 (282-457)*,**	<0.0001
E_2-FAE	8 (5-11)**	23 (13-177)**,*	9 (5-17)*	0.024

The data are expressed as median (interquartile range), pmol/L in serum and pmol/kg in adipose tissue. **P*<0.05, ***P*<0.01, ****P*<0.001, #*P*<0.0001

311

312 Figure
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