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## Adipose tissue estrogen production and metabolism in premenopausal women

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## ABSTRACT

**Objective:** Although the ovaries produce the majority of estrogens in women before menopause, estrogen is also synthesized in peripheral tissues such as adipose tissue (AT). The typical female AT distribution, concentrated in subcutaneous and femoro-gluteal regions, is estrogen-mediated, but the significance of estrogen synthesis in AT of premenopausal women is poorly understood.**Design and Methods:** Serum and subcutaneous and visceral AT homogenates from 28 premenopausal women undergoing non-malignant surgery were analyzed for estrone, estradiol, and serum estrone sulfate (E<sub>1</sub>S) concentrations with liquid chromatography-tandem mass spectrometry. Isotopic precursors were used to measure enzyme activities of estrone-producing steroid sulfatase and estradiol-producing 17 $\beta$ -hydroxysteroid dehydrogenases (17 $\beta$ -HSD). Messenger RNA (mRNA) expression levels of genes for estrogen-metabolizing enzymes were analyzed using real-time reverse transcription quantitative polymerase chain reaction.**Results:** While estradiol was the predominant circulating active estrogen, estrone dominated in AT, with a higher concentration in visceral than subcutaneous AT (median, 2657 vs 1459 pmol/kg;  $P = 0.002$ ). Both AT depots converted circulating E<sub>1</sub>S to estrone, and estrone to estradiol. Median levels of estrone were five to ten times higher in subcutaneous and visceral AT than in serum ( $P < 0.001$ ) and the estradiol level in visceral AT was 1.3 times higher than in serum ( $P < 0.005$ ). The local estrone concentration in visceral AT correlated positively with mRNA expression of estrone-producing enzyme aromatase ( $r = 0.65$ ,  $P = 0.003$ ). Waist circumference correlated positively with increased estradiol production in subcutaneous AT ( $r = 0.60$ ,  $P = 0.039$ ).**Conclusions:** Premenopausal AT demonstrated high estrogenic enzyme activity and considerable local estrogen concentrations. This may be a factor promoting female-typical AT distribution in premenopausal women.

## 1. Introduction

Adipose tissue (AT) is a key site for the peripheral production and metabolism of estrogens in women [1–4]. While the ovaries are the major source of estrogens in premenopausal women, it is known that

extragonadal sites can also take up and convert circulating precursor molecules, such as dehydroepiandrosterone sulfate (DHEAS) and estrone sulfate (E<sub>1</sub>S) to dehydroepiandrosterone (DHEA) and estrone (E<sub>1</sub>), respectively [5–8]. The significance of the contribution of estrogens produced and metabolized in AT in premenopausal women is,

**Abbreviations:** 17 $\beta$ -HSD, 17 $\beta$ -hydroxysteroid dehydrogenase; AT, adipose tissue; BMI, body mass index; cDNA, complementary DNA; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; E<sub>1</sub>, estrone; E<sub>1</sub>S, estrone sulfate; E<sub>2</sub>, 17 $\beta$ -estradiol; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOQ, limit of quantification; mRNA, messenger RNA; PCR, polymerase chain reaction; SHBG, sex hormone-binding globulin; STS, steroid sulfatase.

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however, poorly understood.

The development and regulation of the different fat depots in the female body are controlled by complex interactions between AT genes and ovarian hormones [9]. Prior to the menopause, AT is preferentially localized in the subcutaneous and gluteo-femoral regions, whereas in the transition to the postmenopausal phase AT distribution shifts increasingly to the visceral compartment. Visceral AT has been strongly associated with metabolic dysregulation, arising from adipocyte hypertrophy, infiltrating macrophages, and a proinflammatory cytokine and adipokine profile, and therefore poses the greatest cardiometabolic risk [10,11].

We showed in postmenopausal women that abdominal subcutaneous and visceral AT converted  $E_1S$  to  $E_1$  and further on to estradiol ( $E_2$ ) through the action of the steroid sulfatase (STS) and reductive  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD) enzymes, respectively [12]. Visceral AT had a higher concentration of  $E_1$  compared to subcutaneous AT, and  $E_2$  production increased with increasing visceral adiposity. Moreover, STS in AT also catalyzed the conversion of DHEAS to active DHEA, with postmenopausal women exhibiting higher activity compared to premenopausal women in this reaction [5]. Supposing that the active estrogen metabolism and production in postmenopausal AT could be compensatory for the relative lack of circulating ovarian estrogens, it would be of interest to study AT estrogen metabolism in premenopausal women with functioning menstrual cycles. Accordingly, we set out to study whether AT estrogen production might differ from that in postmenopausal women, hypothesizing that it might be suppressed in the presence of active ovarian steroid hormone production.

To study the concentrations of estrogens and their metabolic pathways in premenopausal women, levels of  $E_1$  and  $E_2$  in serum and AT, and serum  $E_1S$  were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Furthermore, AT homogenates were incubated with isotopic precursors to determine the activity of STS in converting  $E_1S$  to  $E_1$  and of  $17\beta$ -HSD enzymes in converting  $E_1$  to  $E_2$ . In order to verify the activity of STS in AT, we used a specific STS inhibitor. Finally, relative messenger RNA (mRNA) expression levels of genes for steroid regulating enzymes (STS; aromatase;  $17\beta$ -HSD types 2, 7, and 12) were determined with real-time reverse transcription quantitative polymerase chain reaction (PCR).

## 2. Subjects and methods

### 2.1. Subjects and study design

Samples of abdominal subcutaneous and visceral AT were collected at laparoscopic or abdominal surgery from premenopausal women ( $n = 28$ ; Table 1) at the Department of Obstetrics and Gynecology of the Helsinki University Hospital. Subcutaneous AT samples were obtained from AT adjacent to the surgical wound, and visceral AT from the omentum. The Ethics committee of the Helsinki University Hospital accepted the study protocol, and subjects gave their signed informed consent prior to surgery. The non-malignant surgery indications included: uterine fibroids ( $n = 21$ ), adenomyosis ( $n = 1$ ), heavy menstrual bleeding ( $n = 1$ ), ovarian cysts ( $n = 3$ ), vaginal prolapse ( $n = 1$ ), and ovariectomy due to family history of breast malignancies ( $n = 1$ ).

**Table 1**

Clinical characteristics. SHBG, sex hormone-binding globulin. Data are expressed as median (range).

Clinical characteristics	$n = 28$
Age, y	46 (30–52)
Body mass index, $\text{kg}/\text{m}^2$	25 (17–40)
Waist/hip ratio	0.83 (0.71–0.99)
Waist circumference, cm	88 (65–115)
Serum follicle stimulating hormone, IU/L	6.1 (1.5–21.5)
Serum progesterone, nmol/L	0.9 (0.1–54.8)
Serum SHBG, nmol/L	62 (18–183)

Women using oral contraceptives or gonadotropin-releasing hormone agonists 4 weeks or less prior to surgery, and women with endometriosis, were excluded. We included 12 women using the levonorgestrel-releasing intrauterine system (LNG-IUS; Mirena©, Bayer AG, Leverkusen, Germany), as the systemic absorption of levonorgestrel was considered low [13,14]. No differences were observed between users and non-users of the LNG-IUS in the local and circulating estrogen levels, levels of follicle stimulating hormone (FSH), sex hormone-binding globulin (SHBG), or the activities or the relative mRNA expression levels of estrogen-producing enzymes studied (data not shown).

Premenopausal status was confirmed by information on the menstrual cycle and by measuring the level of FSH ( $\leq 26.6$  IU/L for premenopausal women, HUSLAB). The stage of menstrual cycle (follicular,  $n = 14$ ; periovulatory,  $n = 5$ ; luteal,  $n = 7$ ; not determined,  $n = 2$ ) was assessed by using the date of last menses, serum progesterone (follicular phase concentration, 0.3–2.5 nmol/L; luteal phase concentration, 7–80 nmol/L, HUSLAB), and serum  $E_2$  (follicular phase concentration, 0.11–0.44 nmol/L, preovulatory concentration, 0.55–1.29 nmol/L, luteal phase concentration 0.37–0.77 nmol/L, HUSLAB). Information on the pathologic-anatomic diagnosis of the endometrium was used as supporting information where available. Body mass index (BMI), waist/hip ratio, and waist circumference were recorded as measures of adiposity.

Blood samples were drawn preoperatively, serum was separated by centrifugation within 1 h, and the samples were stored at  $-80^\circ\text{C}$ . During surgery, AT samples ranging from 200 mg to 8 g each were obtained, immediately snap-frozen in liquid nitrogen, and subsequently stored at  $-80^\circ\text{C}$  until analysis.

### 2.2. Quantitation of $E_1$ , $E_1S$ , $E_2$ and progesterone in serum

Serum  $E_1$  and  $E_2$  were quantified in one run by LC-MS/MS. Assay calibrators of 10–1000 pmol/L  $E_1$  (Vetrinal, Sigma-Aldrich) and 13–1275 pmol/L of  $E_2$  (Sigma-Aldrich) were prepared in 50 v-% aqueous methanol. To 250  $\mu\text{L}$  of calibrators, 30  $\mu\text{L}$  of internal standard (IS) containing 3 nmol/L  $^{13}\text{C}_3$ - $E_1$  (IsoSciences) and 3 nmol/L  $^{13}\text{C}_3$ - $E_2$  (IsoSciences) in 5 v-% methanol was added prior to LC-MS/MS-analysis. To 250  $\mu\text{L}$  of serum, 30  $\mu\text{L}$  of IS and 4 mL of diethyl ether were added, followed by 15  $\mu\text{L}$  of 12.5 % aqueous ammonia. Samples were mixed for 3 min. The upper phase was transferred into another borosilicate glass tube, followed by 200  $\mu\text{L}$  of water. After mixing, the upper phase was transferred into another tube and evaporated under nitrogen. The residue was dissolved in 100  $\mu\text{L}$  of 50 v-% methanol. All steps were performed using certified borosilicate glassware to minimize the risk of contamination from plastics or surroundings.

Aliquots of 50  $\mu\text{L}$  of calibrators and sample extracts were analyzed by an LC-MS/MS equipped with an Agilent 1200 HPLC and an AB Sciex Triple Quad 5500 mass spectrometer. Separation was performed on a tandem column where a Discovery HS F5–3 column ( $2.1 \times 100$  mm, 3  $\mu\text{m}$ ; Supelco, Bellefonte, PA, U.S.A) was coupled with a SunFire C18 column ( $2.1 \times 50$  mm, 3.5  $\mu\text{m}$ ; Waters, Milford, MA, U.S.A). The mobile phase was a linear gradient of 200  $\mu\text{mol}/\text{L}$  ammonium fluoride (A) and methanol (B), at a flow rate of 300  $\mu\text{L}/\text{min}$ . The gradient was: 0 min 50 % B, 4.5 min 100 % B, 10 min 100 % B and 10.5–19 min 50 % B. Ions were detected by multiple reaction monitoring in the negative ion mode with the following transitions:  $E_1$   $m/z$  269.1 to  $m/z$  269.1 and  $m/z$  145.0;  $E_2$   $m/z$  271.2 to  $m/z$  271.2 and  $m/z$  183.1;  $^{13}\text{C}_3$ - $E_1$   $m/z$  272.1 to  $m/z$  272.1 and  $m/z$  148.0; and with  $^{13}\text{C}_3$ - $E_2$   $m/z$  274.2 to  $m/z$  274.2 and  $m/z$  186.1. In addition to one product ion, the precursor ion was monitored for quantification. Calibration curves of  $E_1$  and  $E_2$  showed linear extrapolation up to 5 nmol/L. The between-run imprecision (coefficient of variation, CV) for human serum based samples (Bio-Rad, Lyphochek Immunoassay Plus Controls) was for  $E_1$  9.1 % at 99 pmol/L and 12.5 % at 112 pmol/L, and for  $E_2$  4.5 % at 317 pmol/L, 5.7 % at 898 pmol/L, and 4.9 % at 1947 pmol/L in 14 runs. The limit of quantification (LOQ) for  $E_1$  and  $E_2$  was 10 pmol/L.

Serum E<sub>1</sub>S was quantified using LC–MS/MS as described in [12]. The LOQ was 0.1 nmol/L.

Serum progesterone concentration was determined by LC–MS/MS as described [15]. The LOQ was 0.1 nmol/L.

### 2.3. Quantitation of E<sub>1</sub> and E<sub>2</sub> in AT

Paired samples of AT from individual patients were analyzed within the same assay. E<sub>1</sub> extracted from AT was determined by LC–MS/MS as described [16], and inter-assay CV was 4% and 6% for low and high control samples in five consecutive assays. The LOQ for determination of E<sub>1</sub> in AT was 15 pmol/L.

E<sub>2</sub> concentration in AT extracts was determined by a LC–MS/MS quantification method as described [12]. Inter-assay CV was 11 % for control serum in 10 consecutive assays, and 24 % and 5% for two pools of female AT in seven and three consecutive assays, respectively. The LOQ for determination of E<sub>2</sub> in AT was 20 pmol/L.

### 2.4. Steroid sulfatase activity assay

Whole AT samples of ~200 mg were homogenized in 1 mL of 0.1 mol/L Tris–HCl (pH, 7.5), labelled with  $5.3 \cdot 10^5$  dpm (or 6.0 pmol per 200 mg AT; weighted mean) of purified tritium-labeled E<sub>1</sub>S ((6,7-<sup>3</sup>H (N)), specific activity 1.48 T Bq/mmol, Perkin Elmer), incubated for 3 h at +37 °C, and tritium-labeled E<sub>1</sub> formed was determined by liquid-scintillation counting (Rack-beta, Wallac, Turku, Finland) as described [12]. Incubation of tritium-labeled E<sub>1</sub>S in 0.1 mol/L Tris–HCl was used as a blank control. The activity of STS was expressed as tritium-labeled E<sub>1</sub> (nmol) formed from tritium-labeled E<sub>1</sub>S per mass of AT (kg) per time of incubation (h).

Inhibition of STS was studied in a similar incubation using pooled subcutaneous and visceral AT samples from eight patients. 1 μmol/L STX64 (Irosustat 667COUMATE; kindly supplied by Professor Barry Potter, University of Oxford, United Kingdom) in dimethyl sulfoxide (DMSO; final concentration 1%) was added to AT homogenates. Incubation of tritium-labeled E<sub>1</sub>S in 0.1 mol/L Tris–HCl (with and without STX64 in DMSO) were used as blank controls.

### 2.5. Activity of reductive 17β-HSD enzymes in converting E<sub>1</sub> to E<sub>2</sub>

Whole AT samples of ~200 mg were homogenized and incubated with 75 nmol/L <sup>13</sup>C<sub>3</sub>-E<sub>1</sub> (IsoSciences LLC), 150 nmol/L E<sub>1</sub> (1,3,5(10)-estratrien-3-OL-17-one, Steraloids, Newport, RI) and 3.7 mmol/L nicotinamide adenine dinucleotide phosphate, reduced form (Roche Diagnostics, Indianapolis, IN) for 3 h at +37 °C, after which the samples were extracted, purified, and processed for determination of <sup>13</sup>C<sub>3</sub>-E<sub>2</sub> using LC–MS/MS as described [12]. The LOQ was 10 pmol/L. The reductive 17β-HSD enzyme activity was expressed as <sup>13</sup>C<sub>3</sub>-E<sub>2</sub> (nmol) formed from <sup>13</sup>C<sub>3</sub>-E<sub>1</sub> per mass of AT (kg) per time of incubation (h).

### 2.6. Preparation of RNA and cDNA, and quantitation of mRNA

Real-time reverse transcription quantitative PCR was used to measure the relative mRNA expression levels of *STS*, *CYP19A1*, *HSD17B2*, *HSD17B7*, and *HSD17B12*. We isolated, purified, and reverse-transcribed total mRNA into complementary DNA (cDNA) as described [12]. Data were normalized to the geometric mean of three reference genes, *importin 8 (IPO8)*, *lysine-specific demethylase 2B (KDM2B)*, and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* using the Qbase + qPCR data analysis software (Biogazelle NV, Zwijnaarde, Belgium).

### 2.7. Other analyses

Serum FSH and serum SHBG were measured as described [5,17].

### 2.8. Statistical analysis

Statistical analysis was carried out using the IBM SPSS Statistics 22.0 software. The Shapiro-Wilk test was used to assess the normality of variables. For nonparametric variables, pairwise comparisons were performed with the Wilcoxon signed rank test, and between-group differences were assessed with the independent-samples Mann-Whitney *U* test and the independent-samples Kruskal-Wallis test. Unless otherwise stated, data are expressed as median (interquartile range) or median (range). Correlation analyses were evaluated using Spearman's nonparametric correlation. The level of significance was  $P < 0.05$ .

## 3. Results

### 3.1. Serum and AT estrogen concentrations

The median level of E<sub>1</sub> was higher in visceral than in subcutaneous AT (2657 vs 1459 pmol/kg;  $P = 0.002$ ), while E<sub>2</sub> concentrations did not differ (558 vs 651 pmol/kg;  $P = 0.55$ ) (Fig. 1). The median levels of E<sub>1</sub> in AT were five to ten times higher ( $P < 0.001$  for both subcutaneous and visceral AT, respectively) than in the circulation (serum E<sub>1</sub>, 279 pmol/L). For E<sub>2</sub>, the median levels in visceral AT were 1.3-fold higher than in serum (serum E<sub>2</sub>, 432 pmol/L;  $P = 0.005$ ), but the E<sub>2</sub> concentrations in subcutaneous AT did not differ from those in serum ( $P = 0.14$ ) (Fig. 1). E<sub>1</sub>S was the most abundant circulating estrogen (4360 pmol/L), the median level being 16 times higher than that of serum E<sub>1</sub>.

The limited number of subcutaneous AT samples obtained at different menstrual cycle stages did not allow for reliable statistical analysis. However, in visceral AT periovulatory E<sub>1</sub> levels were higher than those in the follicular [5667 (5419–14550),  $n = 5$  vs 1867 (1192–3011),  $n = 13$  pmol/kg; median (interquartile range);  $P < 0.001$ ] or luteal stage [5667 (5419–14550),  $n = 5$  vs 2603 (1958–4498),  $n = 6$  pmol/kg;  $P = 0.03$ ]. The periovulatory E<sub>2</sub> level in visceral AT was also higher than that determined at the follicular stage [1569 (1465–9428),  $n = 5$  vs 475 (361–526),  $n = 10$  pmol/kg;  $P = 0.001$ ]. AT or circulating estrogen levels were not related to BMI or waist circumference (data not shown), but serum SHBG correlated negatively with waist circumference ( $r = -0.51$ ,  $P = 0.005$ ,  $n = 28$ ).

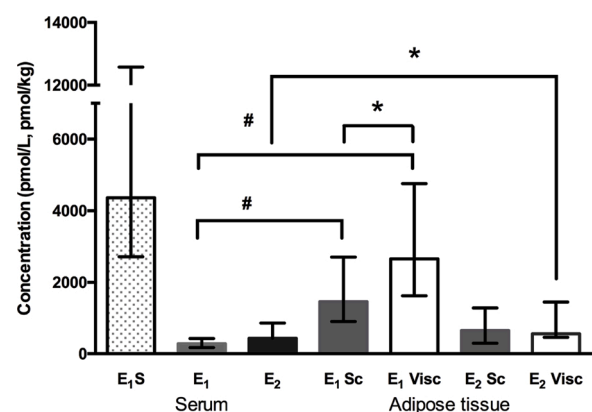


Fig. 1. Concentrations of estrogens in serum and subcutaneous (Sc) and visceral (Visc) AT.

\*,  $P < 0.01$ , visceral AT E<sub>1</sub> vs subcutaneous AT E<sub>1</sub> ( $n = 17$ ), and serum E<sub>2</sub> vs visceral AT E<sub>2</sub> ( $n = 22$ ), Wilcoxon signed rank test; #,  $P < 0.001$ , subcutaneous and visceral AT E<sub>1</sub> vs serum E<sub>1</sub> ( $n = 17–25$ ), Wilcoxon signed rank test. E<sub>1</sub>S, estrone sulfate; serum E<sub>1</sub>, serum estrone; serum E<sub>2</sub>, serum estradiol. Data are expressed as median (interquartile range).

### 3.2. Steroid sulfatase and reductive 17 $\beta$ -hydroxysteroid dehydrogenase activities in AT

STS activity in converting E<sub>1</sub>S to E<sub>1</sub> was similar in subcutaneous and visceral AT [5.2 (4.8–5.4) vs 5.0 (4.8–5.4) nmol/kg AT/hour; median (interquartile range);  $P = 0.47$ ;  $n=15$ ]. STS activity was reduced by 90 % in subcutaneous and by 92 % in visceral AT, when using the specific STS inhibitor STX64 (Fig. 2).

The production of <sup>13</sup>C<sub>3</sub>-E<sub>2</sub> from <sup>13</sup>C<sub>3</sub>-E<sub>1</sub> showed no difference between subcutaneous and visceral AT [0.56 (0.45–0.87) vs 0.60 (0.28–0.82) nmol/kg AT/hour; median (interquartile range);  $P = 0.66$ ;  $n=12$ ]. In subcutaneous, but not in visceral AT, E<sub>2</sub> production increased with waist circumference ( $r = 0.60$ ;  $P = 0.039$ ;  $n=12$ ).

### 3.3. mRNA expression levels of estrogen-forming enzymes

The relative mRNA expression levels of genes for enzymes involved in the synthesis of estrogens in AT are shown in Fig. 3. STS gene mRNA expression in visceral AT increased with BMI ( $r = 0.50$ ,  $P = 0.017$ ,  $n=22$ ) and waist circumference ( $r = 0.56$ ,  $P = 0.007$ ,  $n=22$ ), but did not correlate with STS activity ( $r=0.19$ ,  $P = 0.46$ ,  $n=17$ ). High BMI and waist circumference correlated positively with subcutaneous AT mRNA expression of *HSD17B2* ( $r = 0.61$ ,  $P = 0.016$ ,  $n=15$ , and  $r=0.59$ ,  $P = 0.02$ ,  $n=15$ , respectively) and *CYP19A1* ( $r = 0.81$ ,  $P < 0.001$ ,  $n=14$ , for both BMI and waist circumference). BMI correlated negatively with mRNA expression of *HSD17B12* in subcutaneous AT ( $r = -0.55$ ,

$P = 0.03$ ,  $n=15$ ). In visceral AT, the local E<sub>1</sub> level correlated positively with mRNA expression of *CYP19A1* ( $r = 0.65$ ,  $P = 0.003$ ,  $n=19$ ).

## 4. Discussion

This study shows that both subcutaneous and visceral AT produced active estrogens in premenopausal women, and that these were stored within the tissue at considerable concentrations. While E<sub>2</sub> was the predominant active circulating estrogen in premenopausal women, E<sub>1</sub> was the dominant AT estrogen in both fat depots, with a higher concentration in visceral than subcutaneous AT, and a remarkably higher concentration in AT compared to serum. Both AT depots could use circulating E<sub>1</sub>S to produce E<sub>1</sub> through the action of STS, and E<sub>1</sub> was converted to E<sub>2</sub> by reductive 17 $\beta$ -HSD enzymes at similar rates in both

subcutaneous and visceral AT. Waist circumference was associated with increased E<sub>2</sub> production in subcutaneous AT.

The body AT deposition pattern changes during a woman's lifespan. The typical female AT distribution, with an emphasis in the subcutaneous and femoro-gluteal regions, is estrogen-mediated [9]. After menopause, decreased systemic estrogen levels are associated with preferential fat deposition in the abdominal and visceral compartments, and at the same time carry an increase in risk for metabolic disorders such as cardiovascular disease and type 2 diabetes [18]. Estrogen receptor-positive breast cancer risk is also elevated in postmenopausal women with central obesity, while the link between obesity and premenopausal breast cancer is less clear and may be inverse [19]. In postmenopausal obesity, the visceral AT depot and its pathophysiology are thus highlighted. We have reported that in postmenopausal women, the E<sub>1</sub> concentration was higher in visceral compared to subcutaneous AT, and that increasing BMI was related to a higher E<sub>1</sub> concentration in visceral AT [12]. Data on local E<sub>1</sub> and E<sub>2</sub> concentrations, their interconversions, and mRNA expression levels of estrogen-forming enzymes in pre- and postmenopausal women provide an opportunity to get an insight into changes occurring in AT estrogen metabolism during the menopausal transition.

Serum levels of E<sub>1</sub>S and both serum and AT levels of E<sub>1</sub> and E<sub>2</sub> were high in premenopausal women, when compared to the values reported previously in postmenopausal women [12,17,20,21]. The pattern was similar, however, with E<sub>1</sub>S being the dominant circulating estrogen, and E<sub>1</sub> concentration being higher in visceral than in subcutaneous AT. The E<sub>1</sub> concentrations in AT of premenopausal women were also five to ten times higher than circulating E<sub>1</sub> levels. In the present study, E<sub>2</sub> concentrations in premenopausal visceral and subcutaneous AT were higher than those in serum, but the difference reached statistical significance for visceral AT only. The tissue to serum ratios of E<sub>1</sub> and E<sub>2</sub> were in line with previously reported values [20,22]. In postmenopausal women, tissue to serum ratios of E<sub>1</sub> and especially E<sub>2</sub> tend to be higher when compared to premenopausal women [12,20,21].

Local estrogen biosynthesis in AT takes place through a complex network of steroidogenic enzymes. We have previously demonstrated the activity of STS in converting DHEAS to DHEA in subcutaneous and visceral AT [5], and in this reaction, STS activity was higher in postmenopausal compared to premenopausal women in both AT depots. This study shows that STS also converts E<sub>1</sub>S to E<sub>1</sub> in premenopausal AT. We also studied the conversion of E<sub>1</sub> to E<sub>2</sub> by reductive 17 $\beta$ -HSD enzymes, showing that both subcutaneous and visceral AT produced E<sub>2</sub> at similar rates in premenopausal women. In postmenopausal women, the reductive 17 $\beta$ -HSD activity was higher in subcutaneous compared to visceral AT [12]. Waist circumference was associated with increased E<sub>2</sub> production in subcutaneous AT of premenopausal women whereas in postmenopausal women it was associated with increased E<sub>2</sub> production in visceral AT [12]. This may reflect the distinct AT distribution and the relative importance of the different AT depots before and after menopause.

It is perhaps counterintuitive that AT should exhibit such high estrogenic activity in premenopausal women, as the vast majority of estrogens are produced by the ovaries [23]. One might also consider premenopausal AT as a storage site for steroid hormones secreted from the ovaries and the adrenal glands. Contrary to this, the current results exhibiting much higher E<sub>1</sub> and E<sub>2</sub> levels compared to postmenopausal AT, support an active role of intracrine and paracrine estrogen-producing enzymatic pathways in AT, such as those regulated by STS and the 17 $\beta$ -HSD enzymes, already during the premenopause. We have previously reported that breast subcutaneous AT in premenopausal women with and without estrogen receptor-positive breast cancer also produces a considerable amount of estrogens, with breast adipose tissue E<sub>1</sub> levels exceeding those in the serum by eight times [15]. The importance of local estrogen production by nonmalignant premenopausal breast AT in the development of breast cancer warrants further research.

In premenopausal women, the mRNA expression patterns of

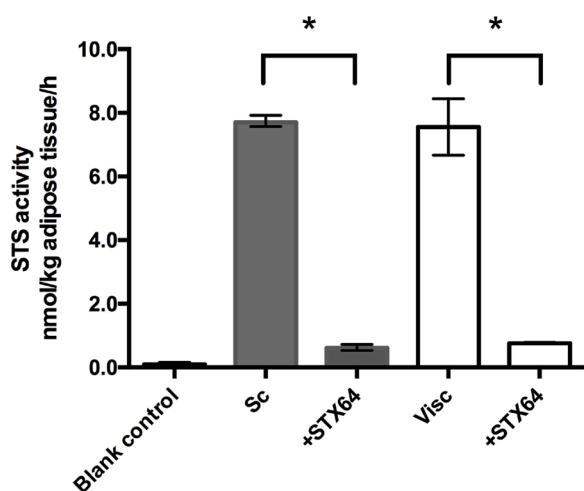
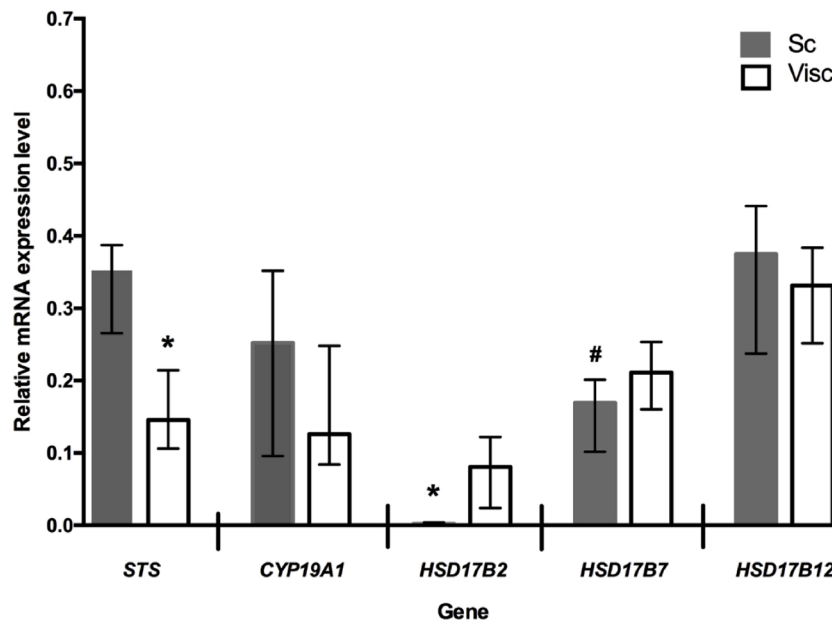


Fig. 2. Formation of [<sup>3</sup>H]-E<sub>1</sub> from [<sup>3</sup>H]-E<sub>1</sub>S in abdominal subcutaneous (Sc) and visceral (Visc) AT with and without the presence of STS inhibitor STX64. Activity is expressed as the amount of [<sup>3</sup>H]-E<sub>1</sub> formed from [<sup>3</sup>H]-E<sub>1</sub>S per mass of AT (kg) per time of incubation (h).

\*,  $P < 0.001$ , incubation of [<sup>3</sup>H]-E<sub>1</sub>S in subcutaneous and visceral AT without and with STX64 (Wilcoxon signed rank test,  $n = 8$ ). Data are expressed as mean (range).



**Fig. 3.** Relative mRNA expression levels of genes for estrogen-metabolizing enzymes in abdominal subcutaneous (Sc) and visceral (Visc) AT. \*,  $P < 0.001$  and #,  $P < 0.005$ , subcutaneous vs visceral AT (Wilcoxon signed rank test,  $n=14-16$ ). STS, steroid sulfatase; CYP19A1, aromatase; HSD17B2, HSD17B7, and HSD17B12, hydroxysteroid 17- $\beta$  hydroxysteroid 2, 7, and 12. Data are expressed as median (interquartile range) and represent relative expression levels.

estrogen-metabolizing enzymes in AT were very similar to those we have previously reported in postmenopausal women [5,12]. Thus, based on the present data, it does not seem likely that differences in local AT estrogen concentrations are due to changes in gene expression patterns associated with the menopausal transition. Posttranscriptional modification of enzymes and other factors relating to local tissue environments, such as adipokines and low-grade inflammation leading to AT dysfunction, are likely to play a role [4,24–27].

Local, intra-tissue estrogen production and resulting high AT estrogen levels might promote and help maintain the typical female AT distribution of the premenopausal age. Although the mechanisms behind the hormonal regulation of AT distribution are complex and still incompletely characterized, they may arise from effects on lipolysis and lipogenesis, adipocyte differentiation, and insulin sensitivity [9,24,28–30]. We also noted that in premenopausal women, estrogen concentrations in visceral AT showed a periovulatory peak possibly reflecting circulating levels of estrogens. This is in line with our recent report showing fluctuation of estrogen concentrations during the menstrual cycle in the AT of the female breast [15], the mechanism of which remains unclear. Whether there is interplay between circulating estrogens and estrogen production within the AT remains, nevertheless, poorly understood.

Our study has limitations. Due to the variable amount (from 200 mg to 8 g per sample) of AT material available, it did not always suffice for all of the analyses in the study. As the samples were collected during elective surgery, it was not possible to control for the stage of menstrual cycle. Furthermore, there was not sufficient material for protein level analysis, or for measurement of the activity of aromatase, another key enzyme in AT estrogen production. The sample size of 28 premenopausal women in the current study is substantial, however, when compared with those in previous studies by other groups reporting on abdominal AT analyses. We cite four previous studies, with the following numbers of female subjects included: six women [7], 12 premenopausal and four postmenopausal women [20], 15 premenopausal and 25 postmenopausal women [21], and 12 postmenopausal women [28]. Only a few studies to date have been able to address the possible differences in estrogen production according to the stage of menstrual cycle. We studied the activity of two consecutively acting key enzymes in AT estrogen production, showing that both subcutaneous and visceral

AT could convert  $E_1S$  to  $E_1$ , and  $E_1$  on to  $E_2$ . We were also able to verify STS activity in subcutaneous and visceral AT by its inhibition by the specific STS inhibitor STX64. A major strength of our study is the use of specific and sensitive mass-spectrometric methods for quantification of estrogens in both serum and AT.

In conclusion, the current results demonstrated high estrogenic enzyme activity and metabolism in premenopausal AT, with higher AT estrogen concentrations compared to the postmenopause. The high local  $E_1$  and  $E_2$  concentrations within the AT likely result from active intracrine and paracrine estrogen-producing pathways, such as those regulated by STS and 17 $\beta$ -HSD enzymes. This intra-tissue estrogen activity, not circulating estrogen levels, may be a key factor which promotes and helps maintain the typical female subcutaneous and gluteo-femoral AT distribution in premenopausal women.

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## CRedit authorship contribution statement

**Natalia Hetemäki:** Conceptualization, Investigation, Formal analysis, Writing - original draft, Visualization. **Tomi S. Mikkola:** Conceptualization, Supervision, Funding acquisition. **Matti J. Tikkanen:** Conceptualization, Supervision, Writing - review & editing. **Feng Wang:** Methodology, Investigation. **Esa Hämäläinen:** Methodology, Resources. **Ursula Turpeinen:** Methodology, Validation. **Mikko Haanpää:** Methodology, Validation. **Veera Vihma:** Methodology, Validation, Data curation, Writing - review & editing. **Hanna Savolainen-Peltonen:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors report no declarations of interest.

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