Estrogens and glucocorticoids in mammary adipose tissue: Relationships with body mass index and breast cancer features

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ABSTRACT

Context: Adipose tissue is an important site for extragonadal steroid hormone biosynthesis through the expression and activity of P450 aromatase, 11β-HSD1 and 17β-HSDs. The contribution of steroid hormones produced by adjacent adipose tissue for the progression and survival of breast tumors is unknown.

Objective: To quantify estrogens (estradiol, estrone) and glucocorticoids (cortisol, cortisone) in breast adipose tissue from both healthy and diseased women and their relationships with adiposity indices and breast cancer prognostic markers.

Design and setting: Breast adipose tissue was collected at time of surgery.

Patients: Pre- and post-menopausal women undergoing partial mastectomy for treatment of breast cancer (n=17) or reduction mammoplasty (n=6) were studied.

Interventions: Estrogen and glucocorticoid relative amounts were determined by liquid chromatography-tandem mass spectrometry.

Results: The targeted steroids were reliably detected and quantified in mammary adipose tissues. Women with ER+/PR+ tumor had higher estradiol levels than women with ER-/PR- tumor ($P < 0.05$). Ratio of estradiol-to-estrone was higher in lean women compared to women with a BMI ≥ 25 kg/m^2 ($P < 0.05$). Mixed-model analyses showed that estradiol, cortisone and cortisol were negatively associated with tumor size ($P < 0.05$). Relationships between glucocorticoids and tumor size remained significant after adjustment for BMI. The cortisol-to-cortisone ratio was negatively associated with tumor stage ($P < 0.05$) independently of BMI.

Conclusions: We reliably quantified estrogens and glucocorticoids in breast adipose tissue from healthy women and women suffering from breast cancer. Our findings suggest that smaller breast tumors are associated with higher levels of estradiol and cortisol in adipose tissue.

PRÉCIS

Estrogens and glucocorticoids were measured by LC-MS/MS in breast fat tissue from women with or without breast cancer. Tumor size was negatively associated with estradiol and glucocorticoid levels.

INTRODUCTION

Being overweight or obese is a well-known risk factor for postmenopausal breast cancer [1,2]. Obesity is also linked to a poorer prognosis in women with breast cancer regardless of their menopausal status [1,3,4]. Women with obesity have more aggressive tumors, higher mortality rates, incidence of metastases and increased risk of recurrence [1,5-8]. Central obesity, as measured by waist circumference (WC), is an emerging risk factor for both pre- and postmenopausal breast cancer [9]. Furthermore, the efficacy of chemotherapy, radiotherapy, surgery and endocrine therapy is reduced in women with obesity and possibly more so with increased visceral fat accumulation [10-14]. The mechanisms underlying higher risk and reduced treatment efficacy are not fully understood. Altered secretion of adipokines, growth factors and steroids by dysfunctional mammary adipose tissue may contribute to a pro-inflammatory, growth-promoting microenvironment for cancer cells [15]. Recent evidence from human studies has shown that local breast adipose tissue does present an altered biological profile, as described above, concomitant with body mass index (BMI) increases [16-21]. Reports from Iyengar and collaborators have showed that mean adipocyte cell size from breast adipose tissue was positively associated with BMI, increased aromatase expression and inflammatory markers such as crown-like structures and was also related to menopausal status [19,20].

Expression and activity of several steroidogenic enzymes present in adipose tissue have been linked to increased adiposity [22]. For example, higher rates of androgen-to-estrogen conversion through aromatization in adipose tissue [22] have been proposed as a mechanism for the obesity-related increase in breast cancer risk. Findings from our group suggest that known estrogenic 17β-HSD (type 1, 7 and 12)-mediated conversion of estrone (E1) to

estradiol (E2) is five times higher in differentiated adipocytes than in preadipocytes [23]. Increased mean adipocyte size is associated with higher expression level and activity of 11β-HSD type 1 which locally converts cortisone to active cortisol through oxoreductase activity [24,25].

Despite their well-known anti-inflammatory effects, glucocorticoids (GC) could contribute to breast cancer initiation, progression and survival via the activation of the glucocorticoid receptor (GR) or by increasing aromatase expression via the GC response element (GRE) on exon I.4 of the CYP19A1 gene [26]. Moreover, 11β-HSD1 expression increases with estrogen receptor β (ERβ) activation [27]. In a rodent model of breast cancer, increased GC levels nurtured the transition from DCIS (ductal carcinoma *in situ*) to IDC (invasive ductal carcinoma) while administration of RU-486 was able to partially block this effect, i.e. prevent breast cancer progression to IDC [28]. Considering the slow turnover of GC in adipose tissue [29], there is biological plausibility for autocrine and paracrine effects of active GC, such as cortisol, in the tumoral microenvironment.

Hence, steroid dynamics in breast adipose tissue and cancer appear to be involve more than overexpression and increased activity of aromatase. We currently have very little information about the relative importance of adipose tissue dysfunction markers such as altered steroid conversion to tumor progression and aggressiveness or patient prognosis. Moreover, considering the complexity of potential enzymatic hormone conversion in adipose tissues, direct measurement of active hormone levels and their precursors has become highly relevant.

Liquid-chromatography followed by tandem mass spectrometry (LC-MS/MS) is recognized as the gold standard to quantify endogenous steroids in plasma, however, tissue

steroids are harder to measure than those in plasma due to the complexity of the matrix and the need to homogenize uniformly. Measurements are confounded by the low abundance and the poor ionisation profile of steroids as well as the higher concentrations of lipids in adipose tissue than in plasma, which result in higher susceptibility of so-called matrix effects. We aimed to characterize the relationship of locally-produced cortisol and E2 as well as inactive steroid cortisone and main E2 precursor E1, with adiposity and prognostic markers in a sample of women with or without breast cancer. We hypothesize that independently of menopausal status: i) E2 and cortisol breast adipose tissue levels as well as the ratio E2/E1 and cortisol on cortisone are increased with adiposity; ii) worst clinical breast cancer features, such as tumor stage, size and grade are associated with lower adipose tissue steroid levels; and iii) estrogen relative amounts are increased in women with ER+/PR+ tumors. We also investigated if these relationships were independent of total adiposity i.e. reflecting the micro-environment or a characteristic of the adiposity state.

METHODS

Study sample and data collection

The study protocol was approved by the Research Ethics Committees of Laval University Medical Center (DR-002-136). All patients signed a written, informed consent prior to surgery. Breast adipose tissue was obtained from women undergoing partial or total mastectomy for treatment of breast cancer (n = 17) or reduction mammoplasty (n = 6). Fresh tissue specimens were acquired from residual resected breast tissues that were not required for clinical diagnosis at least 1 cm away from the tumor margins. Information on clinicopathologic and anthropometric factors was collected from in-person interviews, phone-call interviews and medical records.

Laboratory methods

Cell sizing

Breast adipocyte size was measured as previously described [30,31] in formalin-fixed adipose tissue. Briefly, 250 breast adipocytes from 10 randomly chosen areas at × 40 magnification using Calopix software (Tribvn) were measured for each subject, in a blinded fashion.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Standards and solvents

E1 and E2 were obtained from Steraloids, Inc (Newport, USA). Cortisone, cortisol, formic acid (FA; ≥ 98%), iodomethane (≥ 99%), were from Sigma-Aldrich, Inc. (Dorset, UK). 1-(2,4-dinitro-5-fluorophenyl)-4-methylpiperazine (PPZ) was obtained from TCI chemicals (Chuo-ku, Tokyo, Japan). HPLC grade glass distilled solvents (acetone; ethyl acetate, EtOAc; water) were from Fisher Scientific UK Limited (Leicestershire, UK). Analytical

reagent (AR) grade ethanol (EtOH) and HPLC grade glass distilled solvents (acetonitrile; methanol, MeOH) and LCMS grade (acetonitrile; formic acid, FA; water) solvents were from VWR (England, UK).

Instrumentation

Cortisone, cortisol, E1 and E2 were measured by LC-MS/MS, using a UHPLC Shimadzu Nexera X2 system (UK) coupled to a Sciex QTRAP® 6500+ (SCIEX, Warrington, UK) equipped with an electrospray ionization interface (ESI). Mass spectrometry conditions were previously described in conjunction with ion spray voltage (5500 V) and source temperature (500°C) [32].

Sample preparation

Following enrichment of frozen adipose tissue samples (~200 mg) with three internal standards (IS), 9,11,12,12-[$^{2}H_4$]-cortisol (D_4-F 5 ng; Cambridge Isotopes Laboratory, England, UK), 2,3,4-[$^{13}C_3$]-17β-estradiol and 2,3,4-[$^{13}C_3$]-estrone ($^{13}C_3$E2, $^{13}C_3$E1 respectively; 5 ng; Sigma-Aldrich, Inc (St. Louis, USA)), analytes were extracted as described below.

Briefly, frozen adipose tissue samples were homogenized (Model Pro 200, ProScientific, Inc, Monroe, CT, USA) in EtOH:EtOAc (1 mL; 1:1) and immediately frozen on dry ice and stored at -80°C overnight. The following morning, samples were thawed on wet ice and sonicated (8 x 15 second bursts with 1-minute gaps; Ultrasonic cleaner, Branson Ultrasonic Inc, Danbury, CT, USA). Samples were subjected to centrifugation (3200 *g*, 45 minutes, 4°C; Heraeus Megafuge 16R, ThermoFisher Scientific, Germany). The supernatant was transferred into a new glass tube and dried down under oxygen-free

177 nitrogen (OFN; 60°C). Samples were resuspended in aqueous MeOH (30% v/v, 5 mL).
178 Solid-phase extraction was carried out after conditioning C18 Sep-Pak columns (12cc, 2g;
179 Waters, Wilmslow, UK; MeOH (2 x 10 mL), followed by H_2O (2 x 10 mL)). The adipose
180 extract was loaded, and the column was washed with H_2O (10 mL) followed by aqueous
181 MeOH (5%, 10 mL). Steroids were eluted with MeOH:CH_3CN (1:1, 10 mL). The eluent
182 was dried down under OFN at 60°C prior to derivatization of estrogens. Generation of 1-
183 (2,4-dinitrophenyl)-4,4-dimethylpiperazinium (MPPZ) derivatives of E1 and E2 has
184 already been described [32-34]. Derivatization was carried out by incubating (1h, 60°C) with
185 $(CH_3)_2CO$ (70 µL), $NaHCO_3$ (10 µL, 1M, Sigma-Aldrich, Inc (St-Louis, USA)) and PPZ
186 (10 µL, 1 mg/mL, dissolved in $(CH_3)_2CO$) followed by addition of CH_3I; (100 µL) and
187 further incubation (2h, 40°C) as previously described [33,34]. Samples were dissolved in
188 H_2O:CH_3CN (70 µL; 70:30) and transferred to LC vials.

189 ***Liquid chromatography parameters***

190 Following injection (30 µL), analytes were separated on an ACE 2 Excel C18-PFP
191 (150 × 2.1 mm, 2 µm; HiChrom, Reading, England, 40°C) column. The elution process
192 started with mobile phase compositions of 90:10 H_2O with 0.1% FA (solution A) and
193 CH_3CN with 0.1% FA (solution B) which was maintained for 1 minute. This was followed
194 by an 11-minute linear gradient to 50% solution B, which was maintained for 2 minutes,
195 before returning to 10% solution B at 15 minutes and maintained for 3 minutes, all at a
196 constant flow rate of 0.5 mL/min.

197 ***Linearity and lower limit of quantitation (LLOQ)***

198 Blank samples and aliquots containing estrogens (5, 7.5, 10, 15, 25, 50, 100, 200, 500,
199 1000 pg/sample), GC (50, 75, 100, 150, 250, 500, 1000, 2000, 5000, 10000 pg/sample) and

IS (500 pg) were analysed by LC–MS/MS. Calibration curves were plotted as the peak area ratio (standard/IS) versus amount of analytes (GC or estrogens). Calibration lines of best fit were acceptable if the regression coefficient, r, was > 0.98. Weightings of 1/x were used for all four steroids. LLOQs were 50 pg, 15 pg, 100 pg and 75 pg for E2, E1, cortisol and cortisone, respectively. Values below the confirmed LLOQ were calculated as LLOQ divided by 3 i.e. the lowest acceptable signal-to-noise ratio. The values were then converted to pmol/kg according to the weight of the corresponding adipose tissue sample. This transformation was performed to avoid null values to calculate steroid ratios as described below.

Calculated ratios

All steroid amounts were converted into pmol/kg. Those values were then used to calculate product and substrate ratios, as described below. The ratio of cortisol:cortisone was used as a marker of 11β-HSD1 enzyme activity. The ratio of E2:E1 was used as a marker of estrogenic 17β-HSD enzyme activity.

Gene expression

Tissues were homogenized in Qiazol buffer (Qiagen, Germantown, MD, USA) and total RNA was extracted using the RNeasy mini kit on-column DNase (Qiagen, Hilden, DE) treatment following the manufacturer's instructions. First-strand cDNA synthesis was accomplished using 1 ug of RNA in a reaction containing 200 U of Superscript IV Rnase H-RT (Invitrogen Life Technologies, Burlington, ON, CA). cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based Realtime PCR quantification using the LightCycler 480 (Roche Diagnostics, Mannheim, DE). Reagent LightCycler 480 SYBRGreen I Master (Roche Diagnostics, Indianapolis, IN, USA) was used as described

by the manufacturer. The conditions for PCR reactions were: 45 cycles, denaturation at 95°C for 10 sec, annealing at 58-60°C for 10 sec, elongation at 72°C for 14 sec and then 72°C for 5 sec (reading). Oligoprimer pairs were designed by GeneTool 2.0 software (Biotools Inc, Edmonton, AB, CA) and their specificity was verified by blast in the GenBank database. The synthesis was performed by IDT (Integrated DNA Technology, Coralville, IA, USA) (**Table 1**). Normalization was performed using the following reference genes ATP synthase O subunit (ATP5O), hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative Real-Time PCR measurements were performed by the CHU de Québec Research *Center* (CHU) Gene Expression Platform, Quebec, Canada and were compliant with MIQE guidelines.

Statistical analyses

Differences in breast adipocyte diameter between women with breast cancer (cases) and women without breast cancer (controls) or according to menopausal status were assessed by Student's t-test. Cell size frequency distribution differences between case and control subjects or according to menopausal status were assessed by the Kolmogorov–Smirnov (KS) test. Women were subdivided in categories of BMI [lean (< 25 kg/m^2) or overweight and obese (≥ 25 kg/m^2)] or according to their estrogen and progesterone receptor (ER/PR) status. Hormone levels or ratios between those subgroups were assessed by Student's t-test. Satterthwaite approximation was used when variances were deemed unequal according to a conservative Folded F statistic ($P < 0.10$). Exact p-values computed using non-parametric Wilcoxon tests showed similar results. Women were subdivided according to their respective tumor size, according to tertiles of the distribution. A mixed-model was

performed to evaluate the relationship between hormone levels and tumor size (in tertiles or continuous; as determined by the best AIC fit for the model), grade (categorical) and stage (categorical). A repeated statement was incorporated into the model to account for the non-constant variance among the residuals i.e. specifying a variance component covariance structure in the model. Non-normally distributed variables were log-transformed to achieve normality and linearity. Models were further adjusted for BMI (as a continuous variable) to account for total adiposity. Adjustments for menopausal status and current use of hormonal derivatives (as combined indicator variables) were also performed as they are identified as confounders in the breast cancer literature. Spearman correlation coefficients were computed to assess the relationship between steroid relative amount, prognostic factors and relative expression of genes. P-values < 0.05 were considered significant. All statistical analyses were performed with SAS software (SAS Institute, Cary, NC, USA).

RESULTS

Table 2 shows the characteristics of the study sample. Women were overweight with a median BMI of 25.6 kg/m^2 and a median age of 55 years. Postmenopausal status was equally balanced across case and control women. **Table 3** presents the clinicopathological features of pre-menopausal and post-menopausal breast cancer patients. Most women presented with a unilateral breast lesion. Breast tumors were mainly of ductal histology (82%). Only one woman presented a HER2+ tumor and 13 women had an ER+/PR+ tumor. Half of the women had stage 2 breast cancer as classified by TNM score [35].

Women with or without breast cancer were comparable for primary clinical characteristics, except for mean fat cell size which was 13.3 µm higher in the control women (*t-test*, P = 0.0072). This difference was also shown by the adipocyte size distribution curve comparison (*KS*, P = 0.0041) (**Figure 1A**). Postmenopausal women had a 9.7 µm higher mean adipocyte size compared to premenopausal women as well as a right-shifted adipocyte size distribution, however this difference was not statistically significant (**Figure 1B**). The distribution remained similar when considering only cancer cases (**Figure 1C**).

Quantification of steroids

Table 4 shows adipose tissue steroid relative amounts and calculated ratios. E1 and cortisol relative amounts were quantified in each of the 23 samples of mammary adipose tissue. E2 level was below the limit of quantification for 2 samples. Cortisone level was below the limit of quantification for 5 samples, 4 of which were obtained from control women.

11β-HSD1 mRNA abundance was positively correlated with the cortisol:cortisone ratio (r = 0.4929; P = 0.0198) and negatively associated with the cortisone relative amount

(*r* = - 0.4850; P = 0.0221). In women with ER+/PR+ tumor, 17β-HSD12 transcript amount (*r* = 0.5804; P = 0.0479) was correlated with E1 relative amount in breast adipose tissue. 17β-HSD12, 17β-HSD7 or CYP19A1 were not correlated with E2 relative amount. Women with cancer had higher expression level of 17β-HSD12 mRNA compared to controls (P = 0.0231). E2 relative amount was positively associated with ERβ mRNA expression (*r* = 0.6083, P = 0.0358), but not ERα mRNA expression in women with ER+/PR+ tumor. In women with breast cancer, abundance of CYP19A1 transcript was positively correlated with 11β-HSD1 mRNA expression (*r* = 0.5353, P = 0.0326).

Adiposity

The ratio of E2 to E1 was higher in lean women compared to women with a BMI $\geq$ 25 kg/m^2 (P = 0.0335) (**Figure 2A**) (postmenopausal, n=15, P=0.0072; premenopausal, n=8, P=NS) in the entire cohort, even if CYP19A1 expression was higher in women with a BMI $\geq$ 25 kg/m^2 ($p<0.05$). This difference remained significant when considering only cancer cases (P = 0.0393, n = 17), or only ER+/PR+ patients (P = 0.0436, n = 13). The difference, although the same magnitude as above, was no longer statistically significant when considering ER+/PR+ patients with invasive carcinoma (stage $\geq$ 1) (P = 0.0900, n = 11). E2 levels were higher in lean women than in overweight and obese women in cancer cases (P = 0.0494) (**Figure 2B)** (postmenopausal, n=11, P=0.0325; premenopausal, n=6, P=NS). cancer cases (P = 0.0494) (**Figure 2B)**. There was no difference in levels of cortisol and cortisone between lean and overweight/obese women (data not shown).

Breast cancer clinical features

Adipose tissue E2 level (log-transformed) was inversely associated with tumor size (categorical variable) (**Figure 3A**) (P = 0.0281, n = 17) (postmenopausal, n=11, P=0.0283;

premenopausal, no convergence), but this relationship did not reach statistical significance when tumor size was treated as a continuous variable (β = - 0.0199, P = 0.1089, n = 17). Further adjustment for menopausal status and use of hormonal derivatives did not alter the results (P = 0.0488, n = 17) whereas the relationship was no longer significant after adjusting for BMI (P = 0.1569, n = 17) or when considering only patients with invasive carcinoma (stage ≥ 1) (P = 0.1157, n = 14). Including only women with ductal carcinoma generated similar effect size. Although the adjusted models were no longer significant, the non-adjusted model remained significant (data not shown). CYP19A1 mRNA expression level was positively correlated with tumor size (r = 0.4875, P < 0.05), whereas expression of ERα mRNA was negatively associated with this variable (r = - 0.5435, P = 0.0296). There was no relationship between adipose tissue estrogen levels and tumor stage or tumor grade in our sample.

The ratio of cortisol to cortisone was lower in women with cancer compared to control women (P < 0.0001). This difference was driven by higher cortisone levels and not lower cortisol levels. In fact, cortisone levels were higher in cancer patients than in control patients (P = 0.0055). These differences remained when combining controls with women with ER-/PR- breast cancer, who shared similar characteristics (**Table 5**), and comparing them to women with ER+/PR+ tumor (P = 0.0097 and P = 0.0171, respectively) (data not shown).

No difference was detected between control women and cancer-positive women with respect to cortisol (data not shown). Both log-transformed cortisol and cortisone levels were inversely associated with tumor size [(β = - 0.01873, P = 0.0007) and (β = - 0.05048, P < 0.0001)] independent of BMI, menopausal status and current use of hormonal

328 derivatives [(β = - 0.02135, P = 0.0027) and (β = - 0.03636, P < 0.0001)] (**Figure 3BC**)
329 when tumor size was treated as a continuous variable. When we stratified according to
330 menopausal status, the mixed-models remained significant for postmenopausal women
331 (n=11) (**Figure 3BC**; P=0.0055, P<0.0001) but did not converge for premenopausal
332 women (n=6). Those relationships were still significant when including only patients with
333 invasive tumors [Cortisol: (β = - 0.02376, P = 0.0078); (β_{adj} = - 0.02977, P = 0.0079) and
334 Cortisone (β = - 0.03328, P < 0.0001); (β_{adj} = - 0.03650, P < 0.0001)]. Contrary to
335 estradiol, cortisone and cortisol were not associated with tumor size (all TNM stages
336 included or with only invasive tumors) (P = 0.0904 and P = 0.1894). Tumor stage was
337 negatively associated with cortisol/cortisone ratio independent of BMI ($\beta 2$ = - 1.8720,
338 $\beta 3$ = - 1.8316; P = 0.0410) (**Figure 3D**) (postmenopausal, n=11, P=0.0660;
339 premenopausal, n=6, P=0.3096). In sensitivity analyses, including only women presenting
340 a ductal carcinoma histology phenotype, the models generate similar results (data not
341 shown). There was no relationship between adipose tissue glucocorticoid levels and tumor
342 grade in our sample.

343 Higher levels of E2 and E1 were detected in the adipose tissue of women with ER+/PR+
344 tumor compared to women with ER-/PR- tumor (P = 0.0163 and P = 0.0134, respectively)
345 (**Figure 3E**) (postmenopausal, n=11, P=0.1077 and P=0.4324, respectively;
346 premenopausal, no convergence).

347

DISCUSSION

To our knowledge, this is the first study to report a LC-MS/MS quantification method combining analysis of cortisone, cortisol, E1 and E2 extracted from breast adipose tissue from both healthy women and women with breast cancer. Several lines of evidence showed that adipose tissue might play an active role in tumor initiation and progression [18,36,37]. As such, the notion of an active cross-talk between adipose and tumor cells has been put forward in the literature [15,38,39]. Yet, the contribution of adipose tissue to the steroid hormone relative amounts and their possible actions remain to be fully elucidated. Most of the steroidogenic pathways have been studied directly in breast tumors or *in vitro*. Reports of endogenous steroid hormones in breast adipose tissue are mostly comprised of analyses of estrogens (E1 and E2) and their corresponding fatty acyl-esters as well as androgen precursors, namely androstenedione and testosterone [40-43]. Of note, a recent study characterised more than 20 steroids, including androgens, progestogens and estrogens in breast adipose tissue by gas chromatography-tandem mass spectrometry (GC-MS/MS) [44]. A limitation of that study is the lack of data regarding normal breast adipose tissue from healthy controls. *In vitro* uptake of E1, E2 and cortisol from culture media by female abdominal adipose tissue explants was reported as more than two-fold lower compared to progesterone and testosterone, highlighting the possible contribution of steroid conversion in adipose tissue as a source of estrogens and GC for the tumor [45].

Quantification of steroids

We have been able to quantify these four steroids in most of our breast adipose samples. Interestingly, we found similar ranges in relative amounts for estrogens as those reported by Honma and collaborators in breast cancer tissue, using LC-MS/MS [46]. These findings

suggest that breast adipose tissue is a potent source of sex hormones for the tumor. One of the strengths of our study is the use of stable isotope dilution LC-MS/MS instead of the historically used ELISAs (enzyme-linked immunosorbent assay). ELISAs for steroid measurements have several drawbacks such as nonspecific antibody interactions, inconsistent reproducibility and inadequate sensitivity [47]. Moreover, they usually require separate assays for each compound of interest, demanding a large quantity of tissue. Using three stable isotope labelled standards in our protocol allowed us to normalize for loss of analytes during the extraction process.

Contrary to our hypothesis, our data, reported as pmol/kg of whole adipose tissue, showed a decrease in the ratio of E2/E1 and a decrease of E2 with increasing adiposity, as assessed with the BMI, suggesting little impact of aromatase conversion per mass unit in adipose tissue. This can also be explained by the higher affinity of androstenedione as a substrate for aromatase compared to testosterone, as previously reported [48]. Marchand et al. reported that higher circulating E2 level is directly associated with increased fat mass [49]. Simpson et al. reported that increased aromatization in obesity was due to a higher number of cells and not to higher conversion activity per adipose tissue mass unit [50]. Our results are also consistent with another study where the authors found a positive correlation between E1/E2 ratio from visceral fat and BMI in postmenopausal women [51]. Contrary to Savolainen-Peltonen, we found that 17β-HSD type 12 mRNA expression is higher in adipose tissue from cancer patients compared to controls [43]. We cannot exclude that the lower levels of E2 observed in adipose tissue of overweight and obese women are due to increased uptake by the tumor cells as previously proposed by Savolainen-Peltonen [43]. However, we observed this difference in our entire sample and with all women with cancer, including

394 those with ER- status. Effect size were not modified when stratification by ER+/PR+ was
395 performed.

396 We were unable to find a significant association between BMI and cortisol levels in breast
397 adipose tissue. We acknowledged that this may be due to our limited sample size. However,
398 we found lower and non-quantifiable cortisone levels in breast adipose tissue of our control
399 women (4 out of six). Our group of control women had higher mean adipocyte size than
400 our women with cancer. This is not surprising, as our control women tended to have higher
401 BMI than the women with cancer, although not statistically significant probably because
402 of our small sample. The adipocyte size difference reported between pre- and post-
403 menopausal women is similar to the findings of Iyengar and collaborators[19]. The lower
404 levels of cortisone could be partially explained by a higher activation rate of cortisol or a
405 lower inactivation of cortisol to cortisone by 11β-HSD1, because our gene expression
406 results suggest that 11β-HSD1 expression is positively associated with the
407 cortisol:cortisone ratio and negatively associated with the cortisone relative amount These
408 results are consistent with previous findings from our team which showed that 11β-HSD1
409 activity and expression is positively associated with adipocyte size, at least in the
410 abdominal subcutaneous and omental depots [24,25]. A previous study with obese subjects
411 undergoing bariatric surgery has indeed found lower levels of cortisone in adipose tissue
412 of obese subjects before weight loss and when compared to control with no difference in
413 adipose tissue cortisol levels among those groups [52]. No difference in tissue relative amount
414 of cortisol could be explained by concomitant higher clearance by 5α-reductase, also
415 increased in obesity [53]. However, our data showed no decrease of cortisone with higher

BMI which suggests a different catabolism of cortisol and cortisone in women with breast cancer.

Some steroids were previously proposed to be increased in adipose tissue during obesity such as cortisol, which could also act as an immune suppressor in breast tissue. As reported by Cirillo et al., many different tumor types produce active cortisol which inhibits tumor-specific CD8+ T proliferation *in vitro* [54]. Infiltration of CD8+ cells was linked to improved cancer-specific survival by Mohammed and collaborators [55]. However, breast cancer was one of the types of cancer not showing any difference between 11β-HSD1/2 expression between cancer and matched normal epithelial tissues, which suggest a paracrine, possibly by adjacent adipose tissue instead of an autocrine effect by cortisol [54]. In the same order of ideas, immunohistochemistry of 11β-HSD1 showed a presence of the enzyme in 64% of breast tumors and 97% of matched adjacent tissue [56] and GR protein level was higher in breast tumor vs normal epithelial tissue [57]. Increases in GC, particularly cortisol, can induce aromatase expression via the GRE on exon I.4 [26]. On the other hand, cortisol via binding to GR is an activator of the estrogen sulfotransferase, which inactivates estrogens by adding a sulfate group and limiting its binding to ER [58].

We did observe higher cortisone levels in adipose tissue from women with cancer with no change in cortisol. As per our initial hypothesis, we did observe a decrease of estradiol, cortisol and cortisone adipose tissue levels according to tumor size and a decrease of cortisol/cortisone ratio with increasing tumor stage. As stated previously, the decrease of estradiol, but not estrone with tumor size could represent an increased uptake by the tumor cells [42]. The higher cortisol/cortisone ratio between cancer patients in comparison to control women and the further decrease of this ratio with tumor stage point to a dual effect of

glucocorticoids in breast cancer related to stage of the disease. Increases in cortisol/cortisone ratio at the lower stages could increase estrogen production via activation of aromatase, but at a later stage, decreases in cortisol/cortisone could be explained by a negative feedback loop through increased estrogen production by the tumor and a concomitant lower expression of 11β-HSD1. However, we were unable to demonstrate a relationship between cortisol/cortisone ratio with E2/E1 ratio or E2 and E1 in our sample, contrary to previous results in visceral adipose tissue, but similar to results in subcutaneous tissue [51].

Despite our relatively low number of patients in both groups, we found that median E2 adipose tissue level was higher in women ER+/PR+ than ER-/PR-. Hennig et al. reported higher adipose tissue E2, androstenedione and androsterone levels in women with ER+ breast cancer compared to ER-, but no difference in E1 or any of the other androgens and progestagens [44]. Falk et al. measured sex steroids by radioimmunoassay and found a significant difference only for testosterone and a trend for higher E2, E1 and androstenedione adipose tissue levels in ER+/PR+ compared to ER-/PR- samples [40]. We found that E2 relative amount was associated with ERβ mRNA, but not that of ERα in women with ER+/PR+ tumor. ERβ expression is known to be increased with E2 production, not ERα, either suggesting a negative feedback or no effect depending on depot origin [59]. ERβ is only expressed in mature adipocytes in adipose tissue whereas ERα is present in both the stroma-vascular fraction and in mature adipocytes [60]. Lower ERα expression has been linked with adipose tissue dysfunction [61]. However, a relationship with adipocyte cell size and ERα expression level was not observed in our study.

Limitations of our study comprised the relatively low number of participants which is counterbalanced by the wide range of BMI and prognostic marker values of our cohort. Of note, our total sample number is in the same order of magnitude compared to previous literature on breast adipose tissue steroid measurements [40-43], representing the difficulty in obtaining those samples for research purposes. Moreover, we have included in our analysis, control samples i.e. samples from healthy women.

Data on steroid concentration in breast adipose tissue from healthy control and women with cancer is scarce in the literature. Most publications investigated this relationship in postmenopausal women [40,41,43] with the exception of Hennig et al. [44], who did include premenopausal women (n=6 out of 51) in their analyses. They did not, however, separate according to menopausal status due their low number of premenopausal women. We acknowledge the potential difference in hormone metabolism between pre- and postmenopausal women. Because the main source of estrogens shifts from gonads to peripheral tissues in menopause, we adjusted for menopausal status and age in our analysis. We also performed stratification to further alleviate concerns about the influence of menopausal status, but these analyses should be interpreted with caution. It should be noted that inference in our study is limited to postmenopausal women, as our number of premenopausal women did not allow us to investigate fully their steroid concentration as a separate group. Most of our findings were still significant when including only postmenopausal women. Of note, tissue steroid concentration in our study did not vary as a function of menopause. Our cohort represents normal demographics in breast cancer, i.e. increased prevalence in older, postmenopausal women. Hence, we suggest that these findings are relevant in this context.

The use of a cross-sectional design does not allow for causal inferences and we acknowledge that there might be reverse causality as higher tumor stage and size may cause changes in steroid metabolism rather than the opposite. Another constraint is that we cannot quantify the distance from the tumor at which the adipose tissue samples were taken. However, as the defined margins were all included in paraffin blocks for clinicopathological assessment directly at the hospital, we can attest that our adipose samples were taken at least 1 cm from the tumor extremities. Hennig et al reported no difference between steroid levels between two sample locations (less than 0.5 cm and more than 5 cm), except for E2 [44]. Due to standard of care and acceptable limit margins in Quebec for clinicopathological assessment, it was not possible to include adipose tissue less than 0.5 cm away from the tumor in a research project. Therefore, the variation of E2 due to the relative distance from the tumor was likely limited.

CONCLUSION

We were able to quantify estrogens and glucocorticoids in breast adipose tissue from both healthy women and women suffering from breast cancer. There is clear indication that steroid hormone metabolism is different among those two subgroups. Moreover, relative amounts of sex steroids in adipose tissue appear to be related to BMI, especially for E2, whereas differences in glucocorticoids levels appear to be more closely related to cancer progression. As such, estradiol levels were lower in women with larger tumors independently of age and menopausal status and glucocorticoid breast adipose tissue levels were negatively associated with tumor size, independently of age, menopausal status and BMI.

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FIGURE HEADINGS

Figure 1: Size distribution of breast adipocytes. (A) Comparison between control and case women (*t-test*, P=0.0072; KS, P=0.0041; n=22). (B) Comparison between premenopausal and postmenopausal women (*t-test*, P=0.0510; *KS*, P=0.0864; n=22). (C) Comparison between premenopausal and postmenopausal women with cancer (*t-test*, P=0.0552; *KS*, P=0.0480; n=16).

Figure 2: Adiposity and estrogens in breast adipose tissue. (A) Difference of the estradiol/estrone ratio according to BMI status using 25 kg/m^2 as a cut-off (P=0.0335, n=23). (B) Difference of estradiol levels in women with cancer according to BMI status using 25 kg/m^2 as a cut-off (P=0.0494, n=17). Data on graphs are mean. Open circles represent premenopausal women data point.

Figure 3: Breast cancer clinical features and breast adipose tissue steroid levels. (A) Mixed-model regression between estradiol relative amount (log-transformed) and tertiles of tumor size adjusted for menopausal status and current intake of hormonal derivatives (β_2 = - 0.9785, β_3 = - 1.1197*;* P=0.0488). (B) Mixed-model regression between cortisol relative amount (log-transformed) and tumor size (continuous) adjusted for BMI, menopausal status and current intake of hormonal derivatives (β = - 0.02135, P=0.0027). (C) Mixed-model regression between cortisone relative amount (log-transformed) and tumor size (continuous) adjusted for BMI, menopausal status and current intake of hormonal derivatives (β = - 0.03636, P < 0.0001). (D) Mixed-model between cortisol/cortisone ratio (log-transformed) and tumor stage adjusted for BMI (β_2 = - 1.8720, β_3 = - 1.8316; P=0.0410). (E) Difference in estradiol and estrone levels in ER-/PR- vs ER+/PR+ women (P=0.0134 and P=0.0454). Data on graphs are mean. n=17. Open circles represent premenopausal women data point.

LIST OF ABBREVIATIONS

11β-HSD1/2: 11β-hydroxysteroid dehydrogenase type 1 and 2

$^{13}C_3$E1: 2,3,4-[$^{13}C_3$]-estrone

$^{13}C_3$E2: 2,3,4-[$^{13}C_3$]-17β-estradiol

17β-HSD: 17β-hydroxysteroid dehydrogenase

AR: Analytical reagent

BMI: Body mass index

CD8+ T: Cytotoxic T cell

CI: Confidence interval

D_4F: 9,11,12,12 [$^2H_4$]-cortisol

DCIS: Ductal carcinoma *in situ*

E1: Estrone

E2: Estradiol

ELISA: Enzyme-linked immunosorbent assay

ER: Estrogen receptor

ERβ: Estrogen receptor beta

ESI: Electrospray ionization

EtOAc: Ethyl acetate

EtOH: Ethanol

FA: Formic acid

GC: Glucocorticoids

GC-MS/MS: Gas chromatography-tandem mass spectrometry

GR: Glucocorticoid receptor

GRE: Glucocorticoid response element

HER2: Human epidermal growth factor receptor 2

HPLC: High-performance liquid chromatography

HRT: Hormonal replacement therapy

IDC: Invasive ductal carcinoma

IQR: Interquartile range

IS: Internal standards

KS: Kolmogorov-Smirnov

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LLOQ: Lower limit of quantitation

MeOH: Methanol

MPPZ: 1-(2,4-dinitrophenyl)-4,4-dimethylpiperazinium

MRM: Multiple reaction monitoring

OFN: Oxygen-free nitrogen

PPZ: 1-(2,4-dinitro-5-fluorophenyl)-4-methylpiperazine

PR: Progesterone receptor

UHPLC: Ultra-high-performance liquid chromatography

WC: Waist circumference

TABLES

Table 1: Sequence primers and gene description

Gene Symbol	Description	GenBank	size (pb)	Primer sequence 5'→3' S/AS
HSD11B1	Homo sapiens hydroxysteroid 11-beta dehydrogenase 1 (HSD11B1), 3 transcripts	NM_005525	85	TGTGCCCTGGAGATCATCAAA/TGATCAGAAGAGTGGTCCAGAGTG
CYP19A1	Homo sapiens cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), 11 transcripts	NM_000103	123	AAGAGGCAATAATAAAGGAAATCCAGAC/CGACAGGCTGGTACCGCATGCTC
HSD17B12	Homo sapien hydroxysteroid (17-beta) dehydrogenase 12 (HSD17B12)	NM_016142	145	CCCACTCTTGACCATCTATTCTG/CTTCCGGATTTTAGCCAGTTTTGTA
HSD17B7	Homo sapiens hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7)	NM_016371	293	TCCACCAAAAGCCTGAATCTCTC/GGGCTCACTATGTTTCTCAGGC
Er alpha	Homo sapiens estrogen receptor 1 (ESR1), 6 transcripts	NM_000125	293	TGCAAAATCTAACCCCTAAGGAAGTG/CTCCCAGTACCCACAGTCCATCTC
Er beta	Homo sapiens estrogen receptor 2 (ESR2), 5 transcripts	NM_001437	114	ACGCCGTGACCGATGCTTTGG/TCGCATGCCTGACGTGGGACA
Atp5o	Homo sapiens ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (ATP5O)	NM_001697	267	ATTGAAGGTCGCTATGCCACAG/AACGACTCCTTGGGTATTGCTTAA
Hprt1	Homo sapiens hypoxanthine phosphoribosyltransferase 1 (HPRT1)	NM_000194	157	AGTTCTGTGGCCATCTGCTTAGTAG/AAACAACAATCCGCCCAAAGG
GAPDH	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046	194	GGCTCTCCAGAACATCATCCCT/ACGCCTGCTTCACCACCTTCTT

Table 2: Clinical characteristics of the women with breast cancer and control women

	Women		
Variables Median (Q1-Q3)	**All (n=23)**	**Controls (n=6)**	**Cases (n=17)**
Age (years)	55.0 (50.1-62.9)	53.7 (44.9-57.5)	55.9 (53.2-63.2)
Menopausal status			
Premenopausal n (%)	8 (35)	2 (33)	6 (35)
Postmenopausal n (%)	15 (65)	4 (67)	11 (65)
Anthropometrics			
BMI (kg/m^2)	25.6 (24.3-28.2)	27.1 (24.3-29.4)	25.4 (24.5-26.8)
WC (cm)	–	–	94 (86-99)
Breast adipocyte mean diameter (µm)	75.5 (67.3-87.5)[a]	87.9 (86.2-89.5)	74.1 (66.1-80.3)[b]
Hormonal derivatives			
Current oral contraceptive use (yes) n (% of premenopausal)	6 (75)	1 (50)	5 (83)
Current HRT use (yes) n (% of postmenopausal)	5 (33)	2 (50)	3 (27)

[a]n=22, [b]n=16. **Key:** BMI, body mass index; HRT, hormone replacement therapy; WC, waist circumference.

Table 3: Characteristics of the tumor

	Premenopausal (n=6)	Postmenopausal (n=11)	All (n=17)
Characteristics n (%)			
Lesion			
unilateral	5 (83.33)	10 (90.91)	15 (88.24)
bilateral	1 (16.67)	1 (9.09)	2 (11.76)
Histology			
Ductal	6 (100.00)	8 (72.73)	14 (82.35)
Others[a]	0 (0.00)	3 (27.27)	3 (17.65)
Receptor status			
ER+/PR+	5 (83.33)	8 (72.73)	13 (76.47)
TNM status			
0	0 (0.00)	3 (27.27)	3 (17.65)
1	2 (33.33)	4 (36.36)	6 (35.29)
2	4 (66.67)	4 (36.36)	8 (47.06)

[a]includes lobular, mucinous and metaplastic carcinomas. **Key:** ER, estrogen receptor; PR, progesterone receptor.

819 **Table 4: Relative amounts of steroid and associated ratios in adipose**
820 **tissue**

	Women			
Steroids **Median (Q1-Q3)**	**All (n=23)**	**Controls (n=6)**	**Cases (n=17)**	***p-value**
Cortisone (pmol/kg)	5501 (1829-16748)	326 (298-1829)	7817 (2811-16748)	0.0055
Cortisol (pmol/kg)	22671 (16040-33719)	22142 (16040-46171)	22671 (18473-32688)	0.5697[#]
Estrone (pmol/kg)	3744 (2847-9899)	7855 (3062-18998)	3744 (2514-7415)	0.1611
Estradiol (pmol/kg)	2320 (1537-4160)	2747 (1796-2937)	2306 (1537-4160)	0.4211
Ratio Cortisol/Cortisone	4.13 (2.47-15.09)	42.28 (15.09-56.09)	3.35 (2.26-5.54)	<0.0001
Ratio Estradiol/Estrone	0.46 (0.32-0.66)	0.54 (0.15-0.65)	0.44 (0.33-0.63)	0.6059

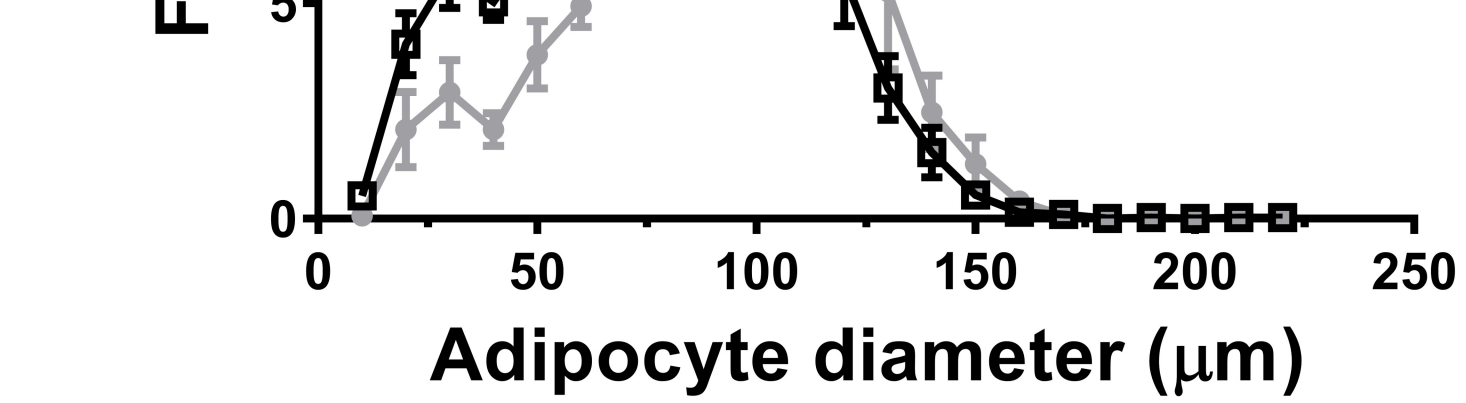
821 *Student t-test p-values calculated with log-transformed variables. [#]Satterthwaite adjusted p-value

822

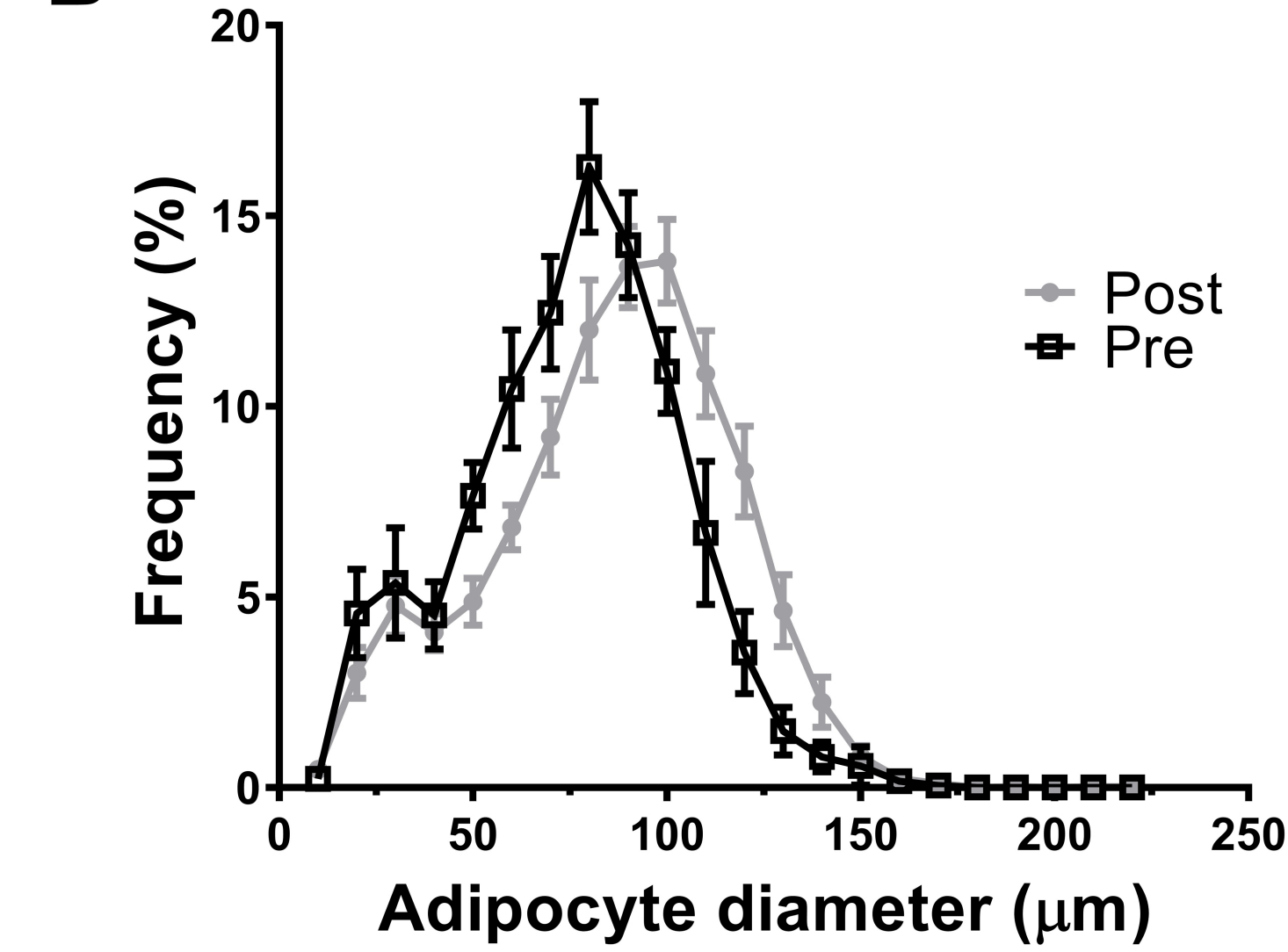
Table 5: Clinical characteristics of the women with ER-PR- breast cancer and control women

Variables Median (Q1-Q3)	**Control (n=6)**	**ER-/PR- (n=4)**
Age (years)	53.7 (44.9-57.5)	54.9 (53.6-60.29)
BMI (kg/m^2)	27.1 (24.3-29.4)	26.0 (25.5-26.6)
Premenopausal n (%)	2 (33)	1 (25)
Postmenopausal n (%)	4 (67)	3 (75)

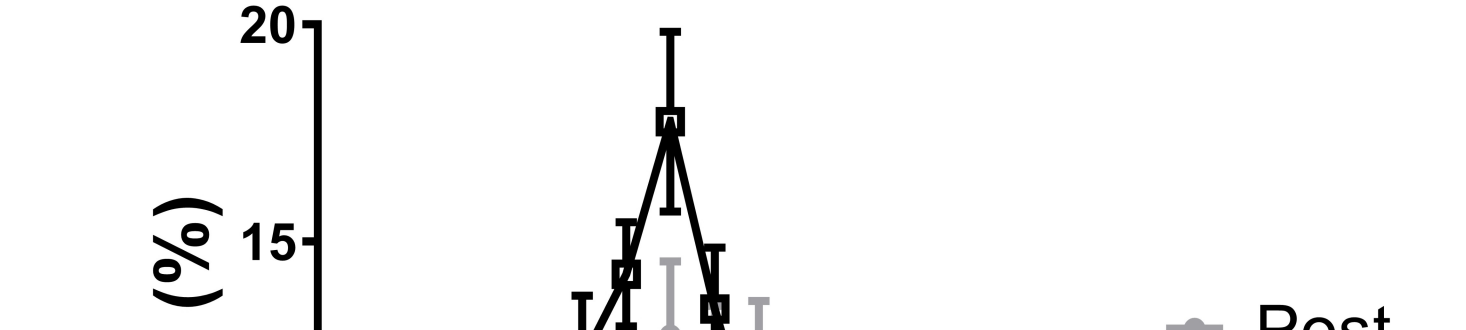
Key: BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor.

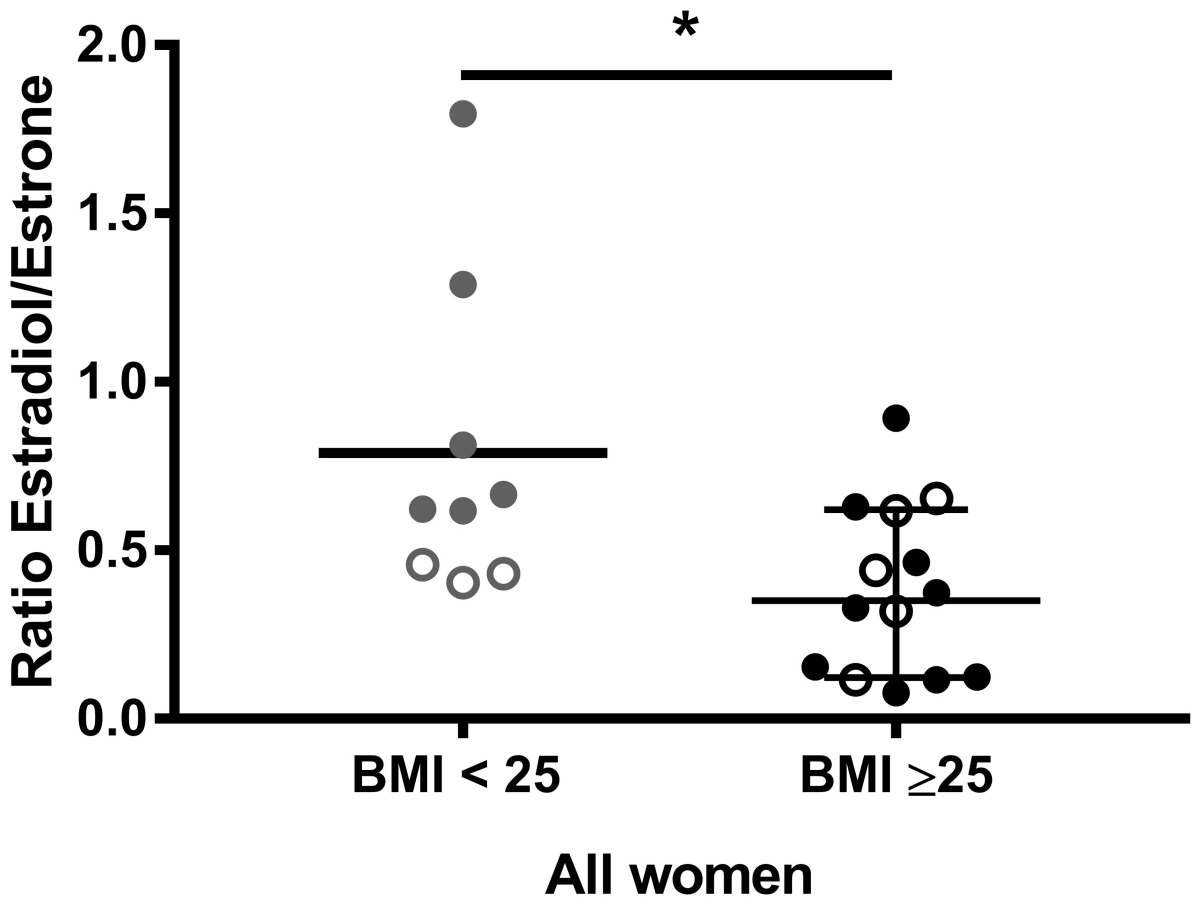

0
50
100
150
200
250
Adipocyte diameter (μm)

B

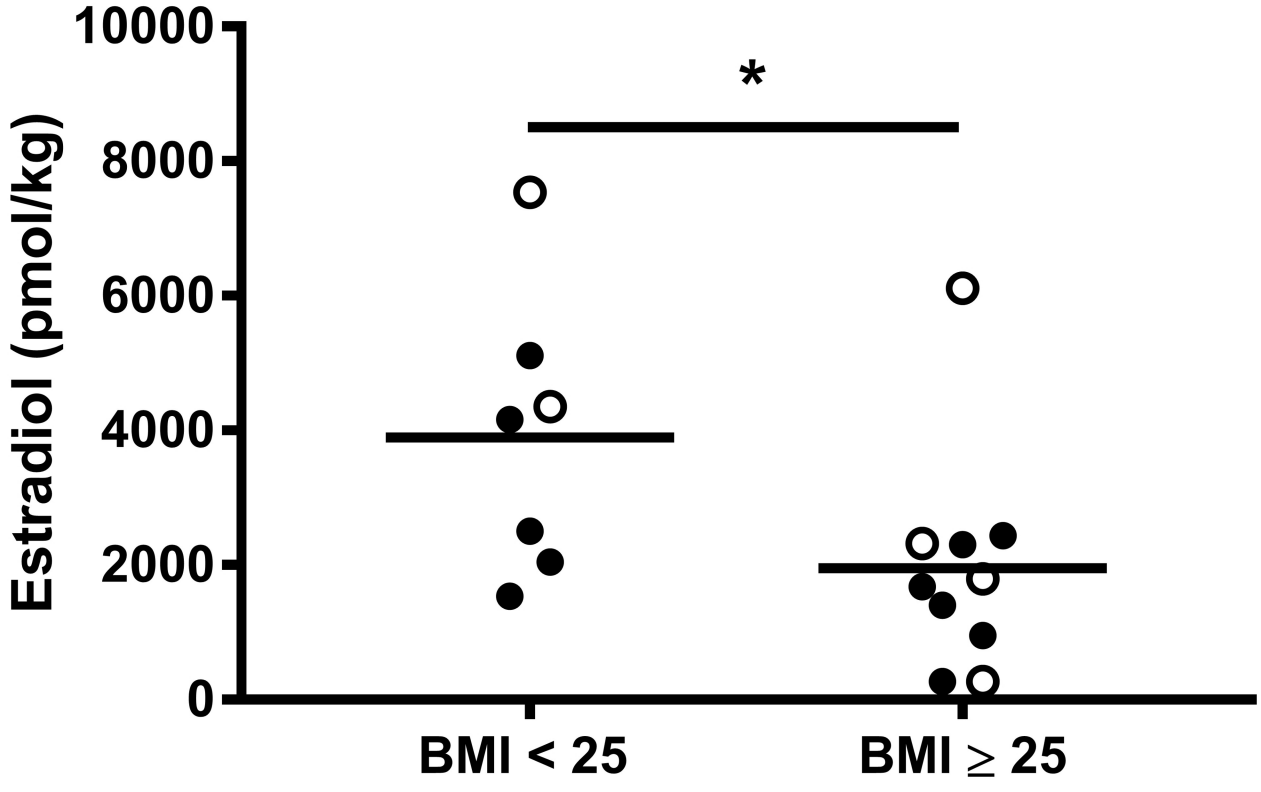

20
15
10
5
0
Frequency (%)
Post
Pre
0
50
100
150
200
250
Adipocyte diameter (μm)

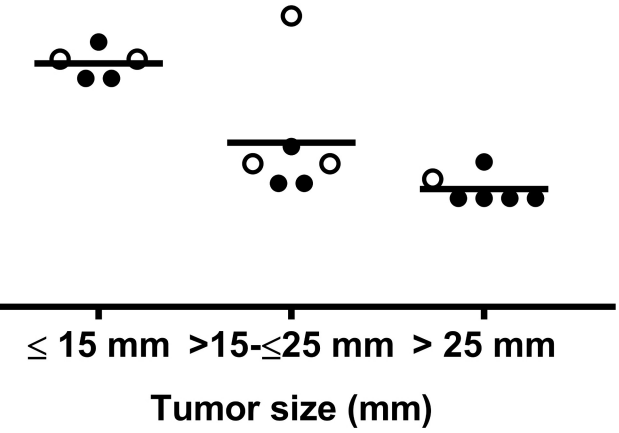
C

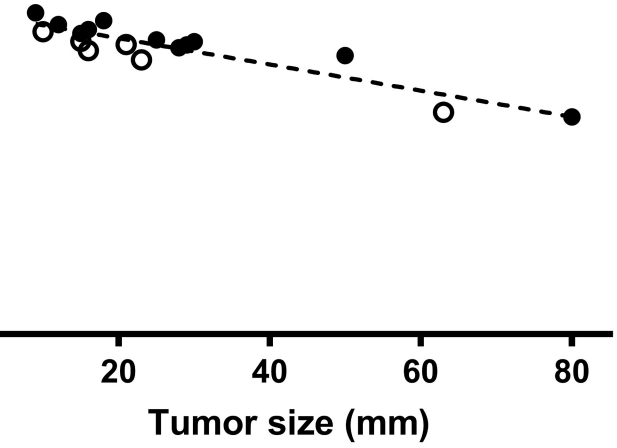

20
15
(%)
Post

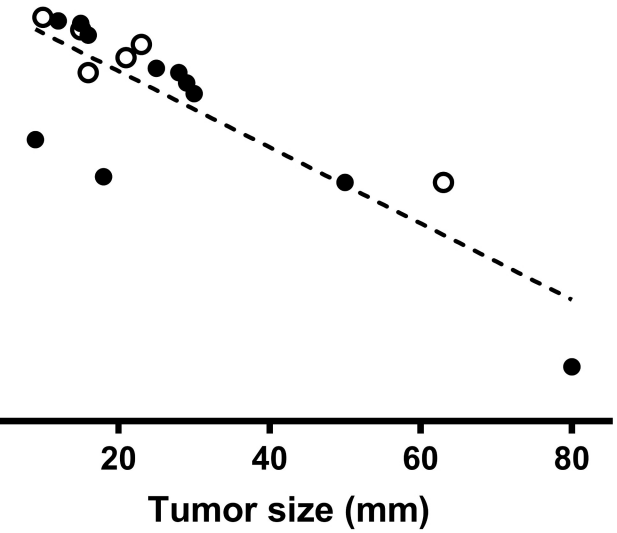

*
2.0
1.5
1.0
0.5
0.0
Ratio Estradiol/Estrone
BMI < 25
BMI ≥25
All women

B

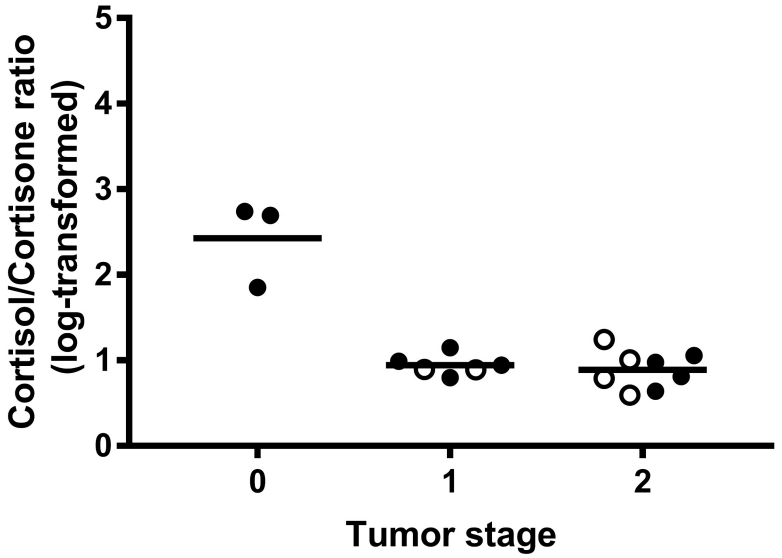

*
10000
8000
6000
4000
2000
0
Estradiol (pmol/kg)
BMI < 25
BMI ≥ 25


≤ 15 mm
>15-≤25 mm
> 25 mm
Tumor size (mm)

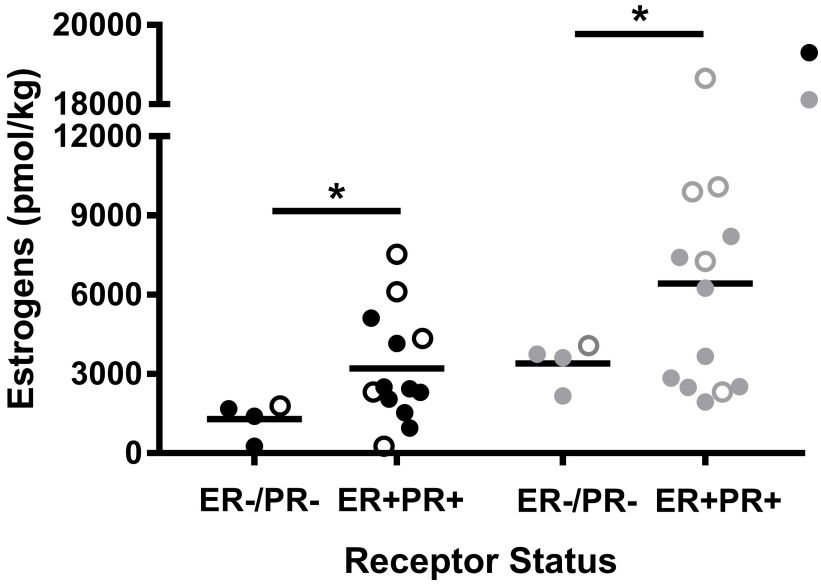

20
40
60
80
Tumor size (mm)


20
40
60
80
Tumor size (mm)

D


Cortisol/Cortisone ratio
(log-transformed)
0
1
2
3
4
5
0
1
2
Tumor stage

E


Estrogens (pmol/kg)
0
3000
6000
9000
12000
18000
20000
*
*
ER-/PR-
ER+PR+
ER-/PR-
ER+PR+
Receptor Status