

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

3 **Authors:** Sofia Laforest^{1,2}, Mélissa Pelletier^{1,2}, Nina Denver^{3,4}, Brigitte Poirier^{5,6},
4 Sébastien Nguyen⁵, Brian R Walker^{7,8}, Francine Durocher⁹, Natalie ZM Homer^{3,7}, Caroline
5 Diorio^{6,10}, Ruth Andrew^{3,7} and André Tchernof^{1,2}

6 **Author affiliations:** ¹CHU de Québec-Université Laval Research Center (Endocrinology
7 and Nephrology division), School of Nutrition, Faculty of Agriculture and Food Sciences,
8 Université Laval, Québec, Canada; ²Quebec Heart and Lung Institute, Québec, Canada;
9 ³Mass Spectrometry Core, Edinburgh Clinical Research Facility, Queen's Medical
10 Research Institute, Edinburgh, United Kingdom; ⁴Institute of Cardiovascular and Medical
11 Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow,
12 University Avenue, Glasgow, United Kingdom; ⁵CHU de Québec-Université Laval
13 Research Center (Oncology division), Université Laval Cancer Research Center and
14 Department of Surgery, Faculty of Medicine, Université Laval, Québec, Canada; ⁶Centre
15 des maladies du sein Deschênes-Fabia, Hôpital Saint-Sacrement, Québec, Canada;
16 ⁷University/BHF Centre for Cardiovascular Science, Queen's Medical Research Institute,
17 University of Edinburgh, 47, Little France Crescent, Edinburgh, EH16 4TJ, UK; ⁸Institute
18 of Genetic Medicine, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK; ⁹CHU
19 de Québec-Université Laval Research Center (Oncology division), Université Laval
20 Cancer Research Center and Department of Molecular Medicine, Faculty of Medicine,
21 Université Laval, Québec, Canada; ¹⁰CHU de Québec-Université Laval Research Center
22 (Oncology division), Université Laval Cancer Research Center and Department of Social
23 and Preventive Medicine, Faculty of Medicine, Université Laval, Québec, Canada.

24 **Short Title:** Steroid hormones in breast adipose tissue

25 **Keywords:** estradiol, estrone, cortisol, cortisone, breast cancer, adiposity

26 **Word count:** 5275

27 **Address all correspondence and requests for reprints to:** André Tchernof, PhD, Quebec
28 Heart and Lung Institute, 2725 Chemin Ste-Foy, Y4212, Québec, QC, Canada G1V 4G5,
29 Tel: 1-418-656-8711, E-mail: andre.tchernof@criucpq.ulaval.ca.

30 **Disclosure summary:** AT is the recipient of research grant support from Johnson &
31 Johnson Medical Companies, Medtronic and Pzifer for studies unrelated to this publication.
32 The authors have nothing to disclose.

33 This work was supported by an inter-center grant from the CMDO to AT, CD and FD. SL
34 is the recipient of PhD scholarships from *Fonds de recherche du Québec-santé* (FRQS)
35 and the Canadian Institutes of Health Research (CIHR). SL's internship in RA's lab was
36 made possible thanks to the support of the Institute of Nutrition and Functional Foods and
37 the scholarship from the International internship program of the *Fonds de recherche Nature*
38 *et Technologies du Québec* (FRQNT) as well as a Michael Smith Foreign Study
39 Supplement travel scholarship. CD holds an Investigator Awards (Senior) from the FRQS.
40 RA is funded by the Wellcome Trust. BRW is a Wellcome Trust Senior Investigator.

41

42 **ABSTRACT**

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

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

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

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

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

55 **Results:** The targeted steroids were reliably detected and quantified in mammary adipose
56 tissues. Women with ER+/PR+ tumor had higher estradiol levels than women with ER-
57 /PR- tumor ($P < 0.05$). Ratio of estradiol-to-estrone was higher in lean women compared
58 to women with a BMI ≥ 25 kg/m² ($P < 0.05$). Mixed-model analyses showed that estradiol,
59 cortisone and cortisol were negatively associated with tumor size ($P < 0.05$). Relationships
60 between glucocorticoids and tumor size remained significant after adjustment for BMI.
61 The cortisol-to-cortisone ratio was negatively associated with tumor stage ($P < 0.05$)
62 independently of BMI.

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

67 **PRÉCIS**

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

71

72 **INTRODUCTION**

73 Being overweight or obese is a well-known risk factor for postmenopausal breast cancer
74 ^{1,2}. Obesity is also linked to a poorer prognosis in women with breast cancer regardless of
75 their menopausal status ^{1,3,4}. Women with obesity have more aggressive tumors, higher
76 mortality rates, incidence of metastases and increased risk of recurrence ^{1,5-8}. Central
77 obesity, as measured by waist circumference (WC), is an emerging risk factor for both pre-
78 and postmenopausal breast cancer ⁹. Furthermore, the efficacy of chemotherapy,
79 radiotherapy, surgery and endocrine therapy is reduced in women with obesity and possibly
80 more so with increased visceral fat accumulation ¹⁰⁻¹⁴. The mechanisms underlying higher
81 risk and reduced treatment efficacy are not fully understood. Altered secretion of
82 adipokines, growth factors and steroids by dysfunctional mammary adipose tissue may
83 contribute to a pro-inflammatory, growth-promoting microenvironment for cancer cells ¹⁵.
84 Recent evidence from human studies has shown that local breast adipose tissue does
85 present an altered biological profile, as described above, concomitant with body mass index
86 (BMI) increases ¹⁶⁻²¹. Reports from Iyengar and collaborators have showed that mean
87 adipocyte cell size from breast adipose tissue was positively associated with BMI,
88 increased aromatase expression and inflammatory markers such as crown-like structures
89 and was also related to menopausal status ^{19,20}.

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

95 estradiol (E2) is five times higher in differentiated adipocytes than in preadipocytes ²³.
96 Increased mean adipocyte size is associated with higher expression level and activity of
97 11 β -HSD type 1 which locally converts cortisone to active cortisol through oxoreductase
98 activity ^{24,25}.

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

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

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

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

132

133 **METHODS**

134 *Study sample and data collection*

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

143 *Laboratory methods*

144 *Cell sizing*

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

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

149 *Standards and solvents*

150 E1 and E2 were obtained from Steraloids, Inc (Newport, USA). Cortisone, cortisol, formic
151 acid (FA; ≥ 98%), iodomethane (≥ 99%), were from Sigma-Aldrich, Inc. (Dorset, UK). 1-
152 (2,4-dinitro-5-fluorophenyl)-4-methylpiperazine (PPZ) was obtained from TCI chemicals
153 (Chuo-ku, Tokyo, Japan). HPLC grade glass distilled solvents (acetone; ethyl acetate,
154 EtOAc; water) were from Fisher Scientific UK Limited (Leicestershire, UK). Analytical

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

158 ***Instrumentation***

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

164 ***Sample preparation***

165 Following enrichment of frozen adipose tissue samples (~200 mg) with three internal
166 standards (IS), 9,11,12,12-[²H₄]-cortisol (D₄-F 5 ng; Cambridge Isotopes Laboratory,
167 England, UK), 2,3,4-[¹³C₃]-17β-estradiol and 2,3,4-[¹³C₃]-estrone (¹³C₃E₂, ¹³C₃E₁
168 respectively; 5 ng; Sigma-Aldrich, Inc (St. Louis, USA)), analytes were extracted as
169 described below.

170 Briefly, frozen adipose tissue samples were homogenized (Model Pro 200, ProScientific,
171 Inc, Monroe, CT, USA) in EtOH:EtOAc (1 mL; 1:1) and immediately frozen on dry ice
172 and stored at -80°C overnight. The following morning, samples were thawed on wet ice
173 and sonicated (8 x 15 second bursts with 1-minute gaps; Ultrasonic cleaner, Branson
174 Ultrasonic Inc, Danbury, CT, USA). Samples were subjected to centrifugation (3200 g,
175 45 minutes, 4°C; Heraeus Megafuge 16R, ThermoFisher Scientific, Germany). The
176 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 H₂O (2 x 10 mL)). The adipose
180 extract was loaded, and the column was washed with H₂O (10 mL) followed by aqueous
181 MeOH (5%, 10 mL). Steroids were eluted with MeOH:CH₃CN (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 described³²⁻³⁴. Derivatization was carried out by incubating (1h, 60°C) with
185 (CH₃)₂CO (70 µL), NaHCO₃ (10 µL, 1M, Sigma-Aldrich, Inc (St-Louis, USA)) and PPZ
186 (10 µL, 1 mg/mL, dissolved in (CH₃)₂CO) followed by addition of CH₃I; (100 µL) and
187 further incubation (2h, 40°C) as previously described^{33,34}. Samples were dissolved in
188 H₂O:CH₃CN (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 H₂O with 0.1% FA (solution A) and
193 CH₃CN 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

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

209 ***Calculated ratios***

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

214 ***Gene expression***

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

223 by the manufacturer. The conditions for PCR reactions were: 45 cycles, denaturation at
224 95°C for 10 sec, annealing at 58-60°C for 10 sec, elongation at 72°C for 14 sec and then
225 72°C for 5 sec (reading). Oligoprimer pairs were designed by GeneTool 2.0 software
226 (Biotools Inc, Edmonton, AB, CA) and their specificity was verified by blast in the
227 GenBank database. The synthesis was performed by IDT (Integrated DNA Technology,
228 Coralville, IA, USA) (**Table 1**). Normalization was performed using the following
229 reference genes ATP synthase O subunit (ATP5O), hypoxanthine guanine phosphoribosyl
230 transferase 1 (HPRT1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
231 Quantitative Real-Time PCR measurements were performed by the CHU de Québec
232 Research Center (CHU) Gene Expression Platform, Quebec, Canada and were compliant
233 with MIQE guidelines.

234 *Statistical analyses*

235 Differences in breast adipocyte diameter between women with breast cancer (cases) and
236 women without breast cancer (controls) or according to menopausal status were assessed
237 by Student's t-test. Cell size frequency distribution differences between case and control
238 subjects or according to menopausal status were assessed by the Kolmogorov–Smirnov
239 (KS) test. Women were subdivided in categories of BMI [lean (< 25 kg/m²) or overweight
240 and obese (≥ 25 kg/m²)] or according to their estrogen and progesterone receptor (ER/PR)
241 status. Hormone levels or ratios between those subgroups were assessed by Student's t-
242 test. Satterthwaite approximation was used when variances were deemed unequal
243 according to a conservative Folded F statistic (P < 0.10). Exact p-values computed using
244 non-parametric Wilcoxon tests showed similar results. Women were subdivided according
245 to their respective tumor size, according to tertiles of the distribution. A mixed-model was

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

259

260 **RESULTS**

261 **Table 2** shows the characteristics of the study sample. Women were overweight with a
262 median BMI of 25.6 kg/m² and a median age of 55 years. Postmenopausal status was
263 equally balanced across case and control women. **Table 3** presents the clinicopathological
264 features of pre-menopausal and post-menopausal breast cancer patients. Most women
265 presented with a unilateral breast lesion. Breast tumors were mainly of ductal histology
266 (82%). Only one woman presented a HER2+ tumor and 13 women had an ER+/PR+ tumor.
267 Half of the women had stage 2 breast cancer as classified by TNM score ³⁵.

268 Women with or without breast cancer were comparable for primary clinical characteristics,
269 except for mean fat cell size which was 13.3 μm higher in the control women (*t-test*,
270 $P = 0.0072$). This difference was also shown by the adipocyte size distribution curve
271 comparison (*KS*, $P = 0.0041$) (**Figure 1A**). Postmenopausal women had a 9.7 μm higher
272 mean adipocyte size compared to premenopausal women as well as a right-shifted
273 adipocyte size distribution, however this difference was not statistically significant (**Figure**
274 **1B**). The distribution remained similar when considering only cancer cases (**Figure 1C**).

275 ***Quantification of steroids***

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

280 11β-HSD1 mRNA abundance was positively correlated with the cortisol:cortisone ratio
281 ($r = 0.4929$; $P = 0.0198$) and negatively associated with the cortisone relative amount

282 ($r = -0.4850$; $P = 0.0221$). In women with ER+/PR+ tumor, 17 β -HSD12 transcript amount
283 ($r = 0.5804$; $P = 0.0479$) was correlated with E1 relative amount in breast adipose tissue.
284 17 β -HSD12, 17 β -HSD7 or CYP19A1 were not correlated with E2 relative amount.
285 Women with cancer had higher expression level of 17 β -HSD12 mRNA compared to
286 controls ($P = 0.0231$). E2 relative amount was positively associated with ER β mRNA
287 expression ($r = 0.6083$, $P = 0.0358$), but not ER α mRNA expression in women with
288 ER+/PR+ tumor. In women with breast cancer, abundance of CYP19A1 transcript was
289 positively correlated with 11 β -HSD1 mRNA expression ($r = 0.5353$, $P = 0.0326$).

290 ***Adiposity***

291 The ratio of E2 to E1 was higher in lean women compared to women with a BMI ≥ 25
292 kg/m² ($P = 0.0335$) (**Figure 2A**) (postmenopausal, $n=15$, $P=0.0072$; premenopausal, $n=8$,
293 $P=NS$) in the entire cohort, even if CYP19A1 expression was higher in women with a
294 BMI ≥ 25 kg/m² ($p<0.05$). This difference remained significant when considering only
295 cancer cases ($P = 0.0393$, $n = 17$), or only ER+/PR+ patients ($P = 0.0436$, $n = 13$). The
296 difference, although the same magnitude as above, was no longer statistically significant
297 when considering ER+/PR+ patients with invasive carcinoma (stage ≥ 1) ($P = 0.0900$,
298 $n = 11$). E2 levels were higher in lean women than in overweight and obese women in
299 cancer cases ($P = 0.0494$) (**Figure 2B**) (postmenopausal, $n=11$, $P=0.0325$; premenopausal,
300 $n=6$, $P=NS$). cancer cases ($P = 0.0494$) (**Figure 2B**). There was no difference in levels of
301 cortisol and cortisone between lean and overweight/obese women (data not shown).

302 ***Breast cancer clinical features***

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

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

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

324 No difference was detected between control women and cancer-positive women with
325 respect to cortisol (data not shown). Both log-transformed cortisol and cortisone levels
326 were inversely associated with tumor size [$(\beta = -0.01873$, $P = 0.0007)$ and $(\beta = -0.05048$,
327 $P < 0.0001)$] independent of BMI, menopausal status and current use of hormonal

328 derivatives [$\beta = - 0.02135$, $P = 0.0027$] and [$\beta = - 0.03636$, $P < 0.0001$] (**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: ($\beta = - 0.02376$, $P = 0.0078$); ($\beta_{adj} = - 0.02977$, $P = 0.0079$) and
334 Cortisone ($\beta = - 0.03328$, $P < 0.0001$); ($\beta_{adj} = - 0.03650$, $P < 0.0001$)]. 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

348 **DISCUSSION**

349 To our knowledge, this is the first study to report a LC-MS/MS quantification method
350 combining analysis of cortisone, cortisol, E1 and E2 extracted from breast adipose tissue
351 from both healthy women and women with breast cancer. Several lines of evidence showed
352 that adipose tissue might play an active role in tumor initiation and progression^{18,36,37}. As
353 such, the notion of an active cross-talk between adipose and tumor cells has been put
354 forward in the literature^{15,38,39}. Yet, the contribution of adipose tissue to the steroid
355 hormone relative amounts and their possible actions remain to be fully elucidated. Most of
356 the steroidogenic pathways have been studied directly in breast tumors or *in vitro*. Reports
357 of endogenous steroid hormones in breast adipose tissue are mostly comprised of analyses
358 of estrogens (E1 and E2) and their corresponding fatty acyl-esters as well as androgen
359 precursors, namely androstenedione and testosterone⁴⁰⁻⁴³. Of note, a recent study
360 characterised more than 20 steroids, including androgens, progestogens and estrogens in
361 breast adipose tissue by gas chromatography-tandem mass spectrometry (GC-MS/MS)⁴⁴.
362 A limitation of that study is the lack of data regarding normal breast adipose tissue from
363 healthy controls. *In vitro* uptake of E1, E2 and cortisol from culture media by female
364 abdominal adipose tissue explants was reported as more than two-fold lower compared to
365 progesterone and testosterone, highlighting the possible contribution of steroid conversion
366 in adipose tissue as a source of estrogens and GC for the tumor⁴⁵.

367 ***Quantification of steroids***

368 We have been able to quantify these four steroids in most of our breast adipose samples.
369 Interestingly, we found similar ranges in relative amounts for estrogens as those reported
370 by Honma and collaborators in breast cancer tissue, using LC-MS/MS⁴⁶. These findings

371 suggest that breast adipose tissue is a potent source of sex hormones for the tumor. One of
372 the strengths of our study is the use of stable isotope dilution LC-MS/MS instead of the
373 historically used ELISAs (enzyme-linked immunosorbent assay). ELISAs for steroid
374 measurements have several drawbacks such as nonspecific antibody interactions,
375 inconsistent reproducibility and inadequate sensitivity⁴⁷. Moreover, they usually require
376 separate assays for each compound of interest, demanding a large quantity of tissue. Using
377 three stable isotope labelled standards in our protocol allowed us to normalize for loss of
378 analytes during the extraction process.

379 Contrary to our hypothesis, our data, reported as pmol/kg of whole adipose tissue, showed
380 a decrease in the ratio of E2/E1 and a decrease of E2 with increasing adiposity, as assessed
381 with the BMI, suggesting little impact of aromatase conversion per mass unit in adipose
382 tissue. This can also be explained by the higher affinity of androstenedione as a substrate
383 for aromatase compared to testosterone, as previously reported⁴⁸. Marchand et al. reported
384 that higher circulating E2 level is directly associated with increased fat mass⁴⁹. Simpson
385 et al. reported that increased aromatization in obesity was due to a higher number of cells
386 and not to higher conversion activity per adipose tissue mass unit⁵⁰. Our results are also
387 consistent with another study where the authors found a positive correlation between E1/E2
388 ratio from visceral fat and BMI in postmenopausal women⁵¹. Contrary to Savolainen-
389 Peltonen, we found that 17 β -HSD type 12 mRNA expression is higher in adipose tissue
390 from cancer patients compared to controls⁴³. We cannot exclude that the lower levels of
391 E2 observed in adipose tissue of overweight and obese women are due to increased uptake
392 by the tumor cells as previously proposed by Savolainen-Peltonen⁴³. However, we
393 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 collaborators¹⁹. 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 11 β -HSD1, because our gene expression
406 results suggest that 11 β -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 11 β -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 groups⁵². No difference in tissue relative amount
414 of cortisol could be explained by concomitant higher clearance by 5 α -reductase, also
415 increased in obesity⁵³. However, our data showed no decrease of cortisone with higher

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

418 Some steroids were previously proposed to be increased in adipose tissue during obesity
419 such as cortisol, which could also act as an immune suppressor in breast tissue. As reported
420 by Cirillo et al., many different tumor types produce active cortisol which inhibits tumor-
421 specific CD8+ T proliferation *in vitro*⁵⁴. Infiltration of CD8+ cells was linked to improved
422 cancer-specific survival by Mohammed and collaborators⁵⁵. However, breast cancer was
423 one of the types of cancer not showing any difference between 11 β -HSD1/2 expression
424 between cancer and matched normal epithelial tissues, which suggest a paracrine, possibly
425 by adjacent adipose tissue instead of an autocrine effect by cortisol⁵⁴. In the same order of
426 ideas, immunohistochemistry of 11 β -HSD1 showed a presence of the enzyme in 64% of
427 breast tumors and 97% of matched adjacent tissue⁵⁶ and GR protein level was higher in
428 breast tumor vs normal epithelial tissue⁵⁷. Increases in GC, particularly cortisol, can induce
429 aromatase expression via the GRE on exon I.4²⁶. On the other hand, cortisol via binding
430 to GR is an activator of the estrogen sulfotransferase, which inactivates estrogens by adding
431 a sulfate group and limiting its binding to ER⁵⁸.

432 We did observe higher cortisone levels in adipose tissue from women with cancer with no
433 change in cortisol. As per our initial hypothesis, we did observe a decrease of estradiol,
434 cortisol and cortisone adipose tissue levels according to tumor size and a decrease of
435 cortisol/cortisone ratio with increasing tumor stage. As stated previously, the decrease of
436 estradiol, but not estrone with tumor size could represent an increased uptake by the tumor
437 cells⁴². The higher cortisol/cortisone ratio between cancer patients in comparison to control
438 women and the further decrease of this ratio with tumor stage point to a dual effect of

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

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

461 Limitations of our study comprised the relatively low number of participants which is
462 counterbalanced by the wide range of BMI and prognostic marker values of our cohort. Of
463 note, our total sample number is in the same order of magnitude compared to previous
464 literature on breast adipose tissue steroid measurements⁴⁰⁻⁴³, representing the difficulty in
465 obtaining those samples for research purposes. Moreover, we have included in our analysis,
466 control samples i.e. samples from healthy women.

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

484 The use of a cross-sectional design does not allow for causal inferences and we
485 acknowledge that there might be reverse causality as higher tumor stage and size may cause
486 changes in steroid metabolism rather than the opposite. Another constraint is that we cannot
487 quantify the distance from the tumor at which the adipose tissue samples were taken.
488 However, as the defined margins were all included in paraffin blocks for
489 clinicopathological assessment directly at the hospital, we can attest that our adipose
490 samples were taken at least 1 cm from the tumor extremities. Hennig et al reported no
491 difference between steroid levels between two sample locations (less than 0.5 cm and more
492 than 5 cm), except for E2⁴⁴. Due to standard of care and acceptable limit margins in
493 Quebec for clinicopathological assessment, it was not possible to include adipose tissue
494 less than 0.5 cm away from the tumor in a research project. Therefore, the variation of E2
495 due to the relative distance from the tumor was likely limited.

496

497 **CONCLUSION**

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

507

508 **ACKNOWLEDGMENTS**

509 We would like to acknowledge the collaboration of the study participants. The authors
510 thank Kaoutar Ennour-Idrissi and Lucie Tellier for data collection from the medical
511 records. We are also grateful to Scott Denham from the Mass Spectrometry Core,
512 Edinburgh Clinical Research Facility and to Serge Simard, MSc for technical and scientific
513 expertise.

514

515 **REFERENCES**

- 516 1. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and
517 incidence of cancer: a systematic review and meta-analysis of prospective
518 observational studies. *Lancet*. 2008;371(9612):569-578.
- 519 2. Neuhouser ML, Aragaki AK, Prentice RL, Manson JE, Chlebowski R, Carty C L,
520 Ochs-Balcom HM, Thomson CA, Caan BJ, Tinker LF, Urrutia RP, Knudtson J,
521 Anderson GL. Overweight, Obesity, and Postmenopausal Invasive Breast Cancer
522 Risk: A Secondary Analysis of the Women's Health Initiative Randomized
523 Clinical Trials. *JAMA Oncol*. 2015;1(5):611-621.
- 524 3. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and
525 mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J*
526 *Med*. 2003;348(17):1625-1638.
- 527 4. Chan DS, Vieira AR, Aune D, Bandera EV, Greenwood DC, McTiernan A,
528 Navarro Rosenblatt D, Thune I, Vieira R, Norat T. Body mass index and survival
529 in women with breast cancer-systematic literature review and meta-analysis of 82
530 follow-up studies. *Ann Oncol*. 2014;25(10):1901-1914.
- 531 5. Ewertz M, Jensen MB, Gunnarsdottir KA, Hojris I, Jakobsen EH, Nielsen D,
532 Stenbygaard LE, Tange UB, Cold S. Effect of obesity on prognosis after early-
533 stage breast cancer. *J Clin Oncol*. 2011;29(1):25-31.
- 534 6. Jiralerspong S, Kim ES, Dong W, Feng L, Hortobagyi GN, Giordano SH.
535 Obesity, diabetes, and survival outcomes in a large cohort of early-stage breast
536 cancer patients. *Ann Oncol*. 2013;24(10):2506-2514.
- 537 7. Biganzoli E, Desmedt C, Fornili M, de Azambuja E, Cornez N, Ries F, Closon-
538 Dejardin MT, Kerger J, Focan C, Di Leo A, Nogaret JM, Sotiriou C, Piccart M,
539 Demicheli R. Recurrence dynamics of breast cancer according to baseline body
540 mass index. *Eur J Cancer*. 2017;87:10-20.
- 541 8. Protani M, Coory M, Martin JH. Effect of obesity on survival of women with
542 breast cancer: systematic review and meta-analysis. *Breast Cancer Res Treat*.
543 2010;123(3):627-635.
- 544 9. Chen GC, Chen SJ, Zhang R, Hidayat K, Qin JB, Zhang YS, Qin LQ. Central
545 obesity and risks of pre- and postmenopausal breast cancer: a dose-response meta-
546 analysis of prospective studies. *Obes Rev*. 2016;17(11):1167-1177.
- 547 10. Sestak I, Distler W, Forbes JF, Dowsett M, Howell A, Cuzick J. Effect of body
548 mass index on recurrences in tamoxifen and anastrozole treated women: an
549 exploratory analysis from the ATAC trial. *J Clin Oncol*. 2010;28(21):3411-3415.
- 550 11. Pfeiler G, Konigsberg R, Fesl C, Mlineritsch B, Stoeger H, Singer CF, Postlberger
551 S, Steger GG, Seifert M, Dubsky P, Taucher S, Samonigg H, Bjelic-Radisic V,
552 Greil R, Marth C, Gnant M. Impact of body mass index on the efficacy of
553 endocrine therapy in premenopausal patients with breast cancer: an analysis of the
554 prospective ABCSG-12 trial. *J Clin Oncol*. 2011;29(19):2653-2659.

- 555 12. Iwase T, Sangai T, Nagashima T, Sakakibara M, Sakakibara J, Hayama S,
556 Ishigami E, Masuda T, Miyazaki M. Impact of body fat distribution on
557 neoadjuvant chemotherapy outcomes in advanced breast cancer patients. *Cancer*
558 *Med.* 2016;5(1):41-48.
- 559 13. Karatas F, Erdem GU, Sahin S, Aytakin A, Yuce D, Sever AR, Babacan T, Ates
560 O, Ozisik Y, Altundag K. Obesity is an independent prognostic factor of
561 decreased pathological complete response to neoadjuvant chemotherapy in breast
562 cancer patients. *Breast.* 2017;32:237-244.
- 563 14. Carmichael AR. Obesity as a risk factor for development and poor prognosis of
564 breast cancer. *Bjog.* 2006;113(10):1160-1166.
- 565 15. Nieman KM, Romero IL, Van Houten B, Lengyel E. Adipose tissue and
566 adipocytes support tumorigenesis and metastasis. *Biochim Biophys Acta.*
567 2013;1831(10):1533-1541.
- 568 16. Balaban S, Shearer RF, Lee LS, van Geldermalsen M, Schreuder M, Shtein HC,
569 Cairns R, Thomas KC, Fazakerley DJ, Grewal T, Holst J, Saunders DN, Hoy AJ.
570 Adipocyte lipolysis links obesity to breast cancer growth: adipocyte-derived fatty
571 acids drive breast cancer cell proliferation and migration. *Cancer Metab.*
572 2017;5:1.
- 573 17. Berstein LM, Kovalevskij AY, Poroshina TE, Kotov AV, Kovalenko IG, Tsyrlina
574 EV, Leenman EE, Revskoy SY, Semiglazov VF, Pozhariski KM. Signs of
575 proinflammatory/genotoxic switch (adipogenotoxicosis) in mammary fat of breast
576 cancer patients: role of menopausal status, estrogens and hyperglycemia. *Int J*
577 *Cancer.* 2007;121(3):514-519.
- 578 18. Fletcher SJ, Sacca PA, Pistone-Creydt M, Colo FA, Serra MF, Santino FE, Sasso
579 CV, Lopez-Fontana CM, Caron RW, Calvo JC, Pistone-Creydt V. Human breast
580 adipose tissue: characterization of factors that change during tumor progression in
581 human breast cancer. *J Exp Clin Cancer Res.* 2017;36(1):26.
- 582 19. Iyengar NM, Morris PG, Zhou XK, Gucalp A, Giri D, Harbus MD, Falcone DJ,
583 Krasne MD, Vahdat LT, Subbaramaiah K, Morrow M, Hudis CA, Dannenberg
584 AJ. Menopause is a determinant of breast adipose inflammation. *Cancer Prev Res*
585 *(Phila).* 2015;8(5):349-358.
- 586 20. Morris PG, Hudis CA, Giri D, Morrow M, Falcone DJ, Zhou XK, Du B, Brogi E,
587 Crawford CB, Kopelovich L, Subbaramaiah K, Dannenberg AJ. Inflammation
588 and increased aromatase expression occur in the breast tissue of obese women
589 with breast cancer. *Cancer Prev Res.* 2011;4(7):1021-1029.
- 590 21. Wang F, Gao S, Chen F, Fu Z, Yin H, Lu X, Yu J, Lu C. Mammary fat of breast
591 cancer: gene expression profiling and functional characterization. *PLoS One.*
592 2014;9(10):e109742.
- 593 22. Tchernof A, Mansour MF, Pelletier M, Boulet MM, Nadeau M, Luu-The V.
594 Updated survey of the steroid-converting enzymes in human adipose tissues. *J*
595 *Steroid Biochem Mol Biol.* 2015;147:56-69.

- 596 23. Bellemare V, Laberge P, Noel S, Tchernof A, Luu-The V. Differential estrogenic
597 17beta-hydroxysteroid dehydrogenase activity and type 12 17beta-hydroxysteroid
598 dehydrogenase expression levels in preadipocytes and differentiated adipocytes. *J*
599 *Steroid Biochem Mol Biol.* 2009;114(3-5):129-134.
- 600 24. Veilleux A, Rheaume C, Daris M, Luu-The V, Tchernof A. Omental adipose
601 tissue type 1 11 beta-hydroxysteroid dehydrogenase oxoreductase activity, body
602 fat distribution, and metabolic alterations in women. *J Clin Endocrinol Metab.*
603 2009;94(9):3550-3557.
- 604 25. Veilleux A, Laberge PY, Morency J, Noel S, Luu-The V, Tchernof A. Expression
605 of genes related to glucocorticoid action in human subcutaneous and omental
606 adipose tissue. *J Steroid Biochem Mol Biol.* 2010;122(1-3):28-34.
- 607 26. Zhao Y, Nichols JE, Bulun SE, Mendelson CR, Simpson ER. Aromatase P450
608 gene expression in human adipose tissue. Role of a Jak/STAT pathway in
609 regulation of the adipose-specific promoter. *J Biol Chem.* 1995;270(27):16449-
610 16457.
- 611 27. McInnes KJ, Andersson TC, Simonyte K, Soderstrom I, Mattsson C, Seckl JR,
612 Olsson T. Association of 11beta-hydroxysteroid dehydrogenase type I expression
613 and activity with estrogen receptor beta in adipose tissue from postmenopausal
614 women. *Menopause.* 2012;19(12):1347-1352.
- 615 28. Zubeldia-Plazaola A, Recalde-Percas L, Moragas N, Alcaraz M, Chen X,
616 Mancino M, Fernandez-Nogueira P, Prats de Puig M, Guzman F, Noguera-
617 Castells A, Lopez-Plana A, Enreig E, Carbo N, Almendro V, Gascon P, Bragado
618 P, Fuster G. Glucocorticoids promote transition of ductal carcinoma in situ to
619 invasive ductal carcinoma by inducing myoepithelial cell apoptosis. *Breast*
620 *Cancer Res.* 2018;20(1):65.
- 621 29. Hughes KA, Reynolds RM, Andrew R, Critchley HO, Walker BR.
622 Glucocorticoids turn over slowly in human adipose tissue in vivo. *J Clin*
623 *Endocrinol Metab.* 2010;95(10):4696-4702.
- 624 30. Laforest S, Michaud A, Paris G, Pelletier M, Vidal H, Geloën A, Tchernof A.
625 Comparative analysis of three human adipocyte size measurement methods and
626 their relevance for cardiometabolic risk. *Obesity.* 2017;25(1):122-135.
- 627 31. Michaud A, Tordjman J, Pelletier M, Liu Y, Laforest S, Noel S, Le Naour G,
628 Bouchard C, Clement K, Tchernof A. Relevance of omental pericellular adipose
629 tissue collagen in the pathophysiology of human abdominal obesity and related
630 cardiometabolic risk. *Int J Obes.* 2016;40(12):1823-1831.
- 631 32. Laforest S, Pelletier M, Denver N, Poirier B, Nguyen S, Walker BR, Durocher F,
632 Homer NZM, Diorio C, Tchernof A, Andrew R. Simultaneous quantification of
633 estrogens and glucocorticoids in human adipose tissue by liquid-chromatography-
634 tandem mass spectrometry. *J Steroid Biochem Mol Biol.* 2019;195:105476.
- 635 33. Nishio T, Higashi T, Funaishi A, Tanaka J, Shimada K. Development and
636 application of electrospray-active derivatization reagents for hydroxysteroids. *J*
637 *Pharm Biomed Anal.* 2007;44(3):786-795.

- 638 34. Denver N, Khan S, Stasinopoulos I, Church C, Homer ZM N, MacLean RM,
639 Andrew R. Derivatization enhances analysis of estrogens and their bioactive
640 metabolites in human plasma by liquid chromatography tandem mass
641 spectrometry. *Analytica Chimica Acta*. 2019;1054:84-94.
- 642 35. *AJCC cancer staging manual*. 7th edition ed. New York, NY: Springer; 2010.
- 643 36. Bougaret L, Delort L, Billard H, Lequeux C, Goncalves-Mendes N, Mojallal A,
644 Damour O, Vasson MP, Caldefie-Chezet F. Supernatants of Adipocytes From
645 Obese Versus Normal Weight Women and Breast Cancer Cells: In Vitro Impact
646 on Angiogenesis. *J Cell Physiol*. 2017;232(7):1808-1816
- 647 37. D'Esposito V, Passaretti F, Hammarstedt A, Liguoro D, Terracciano D, Molea G,
648 Canta L, Miele C, Smith U, Beguinot F, Formisano P. Adipocyte-released insulin-
649 like growth factor-1 is regulated by glucose and fatty acids and controls breast
650 cancer cell growth in vitro. *Diabetologia*. 2012;55:2811-2822.
- 651 38. Tan J, Buache E, Chenard MP, Dali-Youcef N, Rio MC. Adipocyte is a non-
652 trivial, dynamic partner of breast cancer cells. *Int J Dev Biol*. 2011;55(7-9):851-
653 859.
- 654 39. Matafome P, Santos-Silva D, Sena CM, Seica R. Common mechanisms of
655 dysfunctional adipose tissue and obesity-related cancers. *Diabetes Metab Res Rev*.
656 2013;29(4):285-295.
- 657 40. Falk RT, Gentschein E, Stanczyk FZ, Garcia-Closas M, Figueroa JD, Ioffe OB,
658 Lissowska J, Brinton LA, Sherman ME. Sex steroid hormone levels in breast
659 adipose tissue and serum in postmenopausal women. *Breast Cancer Res Treat*.
660 2012;131(1):287-294.
- 661 41. Vihma V, Wang F, Savolainen-Peltonen H, Turpeinen U, Hamalainen E,
662 Leidenius M, Mikkola TS, Tikkanen MJ. Quantitative determination of estrone by
663 liquid chromatography-tandem mass spectrometry in subcutaneous adipose tissue
664 from the breast in postmenopausal women. *J Steroid Biochem Mol Biol*.
665 2016;155(Pt A):120-125.
- 666 42. Wang F, Vihma V, Badeau M, Savolainen-Peltonen H, Leidenius M, Mikkola T,
667 Turpeinen U, Hamalainen E, Ikonen E, Wahala K, Fledelius C, Jauhiainen M,
668 Tikkanen, MJ. Fatty acyl esterification and deesterification of 17beta-estradiol in
669 human breast subcutaneous adipose tissue. *J Clin Endocrinol Metab*.
670 2012;97(9):3349-3356.
- 671 43. Savolainen-Peltonen H, Vihma V, Leidenius M, Wang F, Turpeinen U,
672 Hamalainen E, Tikkanen MJ, Mikkola TS. Breast adipose tissue estrogen
673 metabolism in postmenopausal women with or without breast cancer. *J Clin*
674 *Endocrinol Metab*. 2014;99(12):E2661-2667.
- 675 44. Hennig K, Antignac JP, Bichon E, Morvan ML, Miran I, Delalogue S, Feunteun J,
676 Le Bizec B. Steroid hormone profiling in human breast adipose tissue using semi-
677 automated purification and highly sensitive determination of estrogens by GC-
678 APCI-MS/MS. *Anal Bioanal Chem*. 2018;410(1):259-275.

- 679 45. Bleau G, Roberts KD, Chapdelaine A. The in vitro and in vivo uptake and
680 metabolism of steroids in human adipose tissue. *J Clin Endocrinol Metab.*
681 1974;39(2):236-246.
- 682 46. Honma N, Saji S, Hirose M, Horiguchi S, Kuroi K, Hayashi S, Utsumi T, Harada,
683 N. Sex steroid hormones in pairs of tumor and serum from breast cancer patients
684 and pathobiological role of androstene-3beta, 17beta-diol. *Cancer Sci.*
685 2011;102(10):1848-1854.
- 686 47. Hoofnagle AN, Wener MH. The fundamental flaws of immunoassays and
687 potential solutions using tandem mass spectrometry. *J Immunol Methods.*
688 2009;347(1-2):3-11.
- 689 48. Forney JP, Milewich L, Chen GT, Garlock JL, Schwarz BE, Edman CD,
690 MacDonald PC. Aromatization of androstenedione to estrone by human adipose
691 tissue in vitro. Correlation with adipose tissue mass, age, and endometrial
692 neoplasia. *J Clin Endocrinol Metab.* 1981;53(1):192-199.
- 693 49. Marchand GB, Carreau AM, Weisnagel SJ, Bergeron J, Labrie F, Lemieux S,
694 Tchernof A. Increased body fat mass explains the positive association between
695 circulating estradiol and insulin resistance in postmenopausal women. *Am J*
696 *Physiol Endocrinol Metab.* 2018;314(5):E448-e456.
- 697 50. Cleland WH, Mendelson CR, Simpson ER. Effects of aging and obesity on
698 aromatase activity of human adipose cells. *J Clin Endocrinol Metab.*
699 1985;60(1):174-177.
- 700 51. Yamatani H, Takahashi K, Yoshida T, Takata K, Kurachi H. Association of
701 estrogen with glucocorticoid levels in visceral fat in postmenopausal women.
702 *Menopause.* 2013;20(4):437-442.
- 703 52. Methlie P, Dankel S, Myhra T, Christensen B, Gjerde J, Fadnes D, Vage V, Lovas
704 K, Mellgren G. Changes in adipose glucocorticoid metabolism before and after
705 bariatric surgery assessed by direct hormone measurements. *Obesity (Silver*
706 *Spring).* 2013;21(12):2495-2503.
- 707 53. Tsilchorozidou T, Honour JW, Conway GS. Altered cortisol metabolism in
708 polycystic ovary syndrome: insulin enhances 5alpha-reduction but not the
709 elevated adrenal steroid production rates. *J Clin Endocrinol Metab.*
710 2003;88(12):5907-5913.
- 711 54. Cirillo N, Morgan DJ, Pedicillo MC, Celentano, A, Lo Muzio L, McCullough MJ,
712 Prime SS. Characterisation of the cancer-associated glucocorticoid system: key
713 role of 11beta-hydroxysteroid dehydrogenase type 2. *Br J Cancer.*
714 2017;117(7):984-993.
- 715 55. Mohammed ZM, Going JJ, Edwards J, Elsberger B, McMillan DC. The
716 relationship between lymphocyte subsets and clinico-pathological determinants of
717 survival in patients with primary operable invasive ductal breast cancer. *Br J*
718 *Cancer.* 2013;109(6):1676-1684.
- 719 56. Lu L, Zhao G, Luu-The V, Ouellet J, Fan Z, Labrie F, Pelletier G. Expression of
720 11beta-hydroxysteroid dehydrogenase type 1 in breast cancer and adjacent non-

- 721 malignant tissue. An immunocytochemical study. *Pathol Oncol Res.*
722 2011;17(3):627-632.
- 723 57. Alyusuf R, Wazir JF, Brahmi UP, Fakhro AR, Bakhiet M. The Immunoexpression
724 of Glucocorticoid Receptors in Breast Carcinomas, Lactational Change, and
725 Normal Breast Epithelium and Its Possible Role in Mammary Carcinogenesis. *Int*
726 *J Breast Cancer.* 2017;2017:1403054.
- 727 58. Gong H, Jarzynka MJ, Cole TJ, Lee JH, Wada T, Zhang B, Gao J, Song WC,
728 DeFranco DB, Cheng SY, Xie W. Glucocorticoids antagonize estrogens by
729 glucocorticoid receptor-mediated activation of estrogen sulfotransferase. *Cancer*
730 *Res.* 2008;68(18):7386-7393.
- 731 59. Anwar A, McTernan PG, Anderson LA, Askaa J, Moody CG, Barnett AH, Eggo
732 MC, Kumar S. Site-specific regulation of oestrogen receptor-alpha and -beta by
733 oestradiol in human adipose tissue. *Diabetes Obes Metab.* 2001;3(5):338-349.
- 734 60. Dieudonne MN, Leneuve MC, Giudicelli Y, Pecquery R. Evidence for functional
735 estrogen receptors alpha and beta in human adipose cells: regional specificities
736 and regulation by estrogens. *Am J Physiol Cell Physiol.* 2004;286(3):C655-661.
- 737 61. Bluher M. Importance of estrogen receptors in adipose tissue function. *Mol*
738 *Metab.* 2013;2(3):130-132.
- 739
- 740

741 **FIGURE HEADINGS**

742 **Figure 1: Size distribution of breast adipocytes.** (A) Comparison between control and
 743 case women (*t-test*, $P=0.0072$; *KS*, $P=0.0041$; $n=22$). (B) Comparison between
 744 premenopausal and postmenopausal women (*t-test*, $P=0.0510$; *KS*, $P=0.0864$; $n=22$). (C)
 745 Comparison between premenopausal and postmenopausal women with cancer (*t-test*,
 746 $P=0.0552$; *KS*, $P=0.0480$; $n=16$).

747 **Figure 2: Adiposity and estrogens in breast adipose tissue.** (A) Difference of the
 748 estradiol/estrone ratio according to BMI status using 25 kg/m^2 as a cut-off ($P=0.0335$,
 749 $n=23$). (B) Difference of estradiol levels in women with cancer according to BMI status
 750 using 25 kg/m^2 as a cut-off ($P=0.0494$, $n=17$). Data on graphs are mean. Open circles
 751 represent premenopausal women data point.

752 **Figure 3: Breast cancer clinical features and breast adipose tissue steroid levels.** (A)
 753 Mixed-model regression between estradiol relative amount (log-transformed) and tertiles
 754 of tumor size adjusted for menopausal status and current intake of hormonal derivatives
 755 ($\beta_2 = -0.9785$, $\beta_3 = -1.1197$; $P=0.0488$). (B) Mixed-model regression between cortisol
 756 relative amount (log-transformed) and tumor size (continuous) adjusted for BMI,
 757 menopausal status and current intake of hormonal derivatives ($\beta = -0.02135$, $P=0.0027$).
 758 (C) Mixed-model regression between cortisone relative amount (log-transformed) and
 759 tumor size (continuous) adjusted for BMI, menopausal status and current intake of
 760 hormonal derivatives ($\beta = -0.03636$, $P < 0.0001$). (D) Mixed-model between
 761 cortisol/cortisone ratio (log-transformed) and tumor stage adjusted for BMI ($\beta_2 = -1.8720$,
 762 $\beta_3 = -1.8316$; $P=0.0410$). (E) Difference in estradiol and estrone levels in ER-/PR- vs
 763 ER+/PR+ women ($P=0.0134$ and $P=0.0454$). Data on graphs are mean. $n=17$. Open circles
 764 represent premenopausal women data point.

765

766

767 **LIST OF ABBREVIATIONS**

768 11 β -HSD1/2: 11 β -hydroxysteroid dehydrogenase type 1 and 2

769 ¹³C₃E1: 2,3,4-[¹³C₃]-estrone

770 ¹³C₃E2: 2,3,4-[¹³C₃]-17 β -estradiol

771 17 β -HSD: 17 β -hydroxysteroid dehydrogenase

772 AR: Analytical reagent

773 BMI: Body mass index

774 CD8⁺ T: Cytotoxic T cell

775 CI: Confidence interval

776 D₄F: 9,11,12,12 [²H₄]-cortisol

777 DCIS: Ductal carcinoma *in situ*

778 E1: Estrone

779 E2: Estradiol

780 ELISA: Enzyme-linked immunosorbent assay

781 ER: Estrogen receptor

782 ER β : Estrogen receptor beta

783 ESI: Electrospray ionization

784 EtOAc: Ethyl acetate

785 EtOH: Ethanol

786 FA: Formic acid

787 GC: Glucocorticoids

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

789 GR: Glucocorticoid receptor

790 GRE: Glucocorticoid response element
791 HER2: Human epidermal growth factor receptor 2
792 HPLC: High-performance liquid chromatography
793 HRT: Hormonal replacement therapy
794 IDC: Invasive ductal carcinoma
795 IQR: Interquartile range
796 IS: Internal standards
797 KS: Kolmogorov-Smirnov
798 LC-MS/MS: Liquid chromatography-tandem mass spectrometry
799 LLOQ: Lower limit of quantitation
800 MeOH: Methanol
801 MPPZ: 1-(2,4-dinitrophenyl)-4,4-dimethylpiperazinium
802 MRM: Multiple reaction monitoring
803 OFN: Oxygen-free nitrogen
804 PPZ: 1-(2,4-dinitro-5-fluorophenyl)-4-methylpiperazine
805 PR: Progesterone receptor
806 UHPLC: Ultra-high-performance liquid chromatography
807 WC: Waist circumference
808

809 **TABLES**

810 **Table 1: Sequence primers and gene description**

Gene Symbol	Description	GenBank	size (pb)	Primer sequence 5'→3' S/AS
<i>HSD11B1</i>	Homo sapiens hydroxysteroid 11-beta dehydrogenase 1 (HSD11B1), 3 transcripts	NM_005525	85	TGTGCCCTGGAGATCATCAA A/TGATCAGAAGAGTGGTCC AGAGTG
<i>CYP19A1</i>	Homo sapiens cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), 11 transcripts	NM_000103	123	AAGAGGCAATAATAAAGGAA ATCCAGAC/CGACAGGCTGG TACCGCATGCTC
<i>HSD17B12</i>	Homo sapien hydroxysteroid (17-beta) dehydrogenase 12 (HSD17B12)	NM_016142	145	CCCACTCTTGACCATCTATT CTG/CTTCCGGATTTTAGCCA GTTTTGTA
<i>HSD17B7</i>	Homo sapiens hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7)	NM_016371	293	TCCACCAAAGCCTGAATCT CTC/GGGCTCACTATGTTTCT CAGGC
<i>Er alpha</i>	Homo sapiens estrogen receptor 1 (ESR1), 6 transcripts	NM_000125	293	TGCAAAATCTAACCCTAAG GAAGTG/CTCCCAGTACCCA CAGTCCATCTC
<i>Er beta</i>	Homo sapiens estrogen receptor 2 (ESR2), 5 transcripts	NM_001437	114	ACGCCGTGACCGATGCTTTG G/TCGCATGCCTGACGTGGG ACA
<i>Atp5o</i>	Homo sapiens ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (ATP5O)	NM_001697	267	ATTGAAGGTCGCTATGCCAC AG/AACGACTCCTTGGGTATT GCTTAA
<i>Hprt1</i>	Homo sapiens hypoxanthine phosphoribosyltransferase 1 (HPRT1)	NM_000194	157	AGTTCTGTGGCCATCTGCTT AGTAG/AAACAACAATCCGC CCAAAGG
<i>GAPDH</i>	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046	194	GGCTCTCCAGAACATCATCC CT/ACGCCTGCTTCACCACC TTCTT

811

812

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

Variables Median (Q1-Q3)	Women		
	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)

815 ^an=22, ^bn=16. **Key:** BMI, body mass index; HRT, hormone replacement therapy; WC, waist circumference.

816 **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)

817 ^aincludes lobular, mucinous and metaplastic carcinomas. **Key:** ER, estrogen receptor; PR, progesterone
818 receptor.

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

Women				
Steroids	All (n=23)	Controls (n=6)	Cases (n=17)	*p-value
Median (Q1-Q3)				
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

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

Variables	Control (n=6)	ER-/PR- (n=4)
Median (Q1-Q3)		
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)

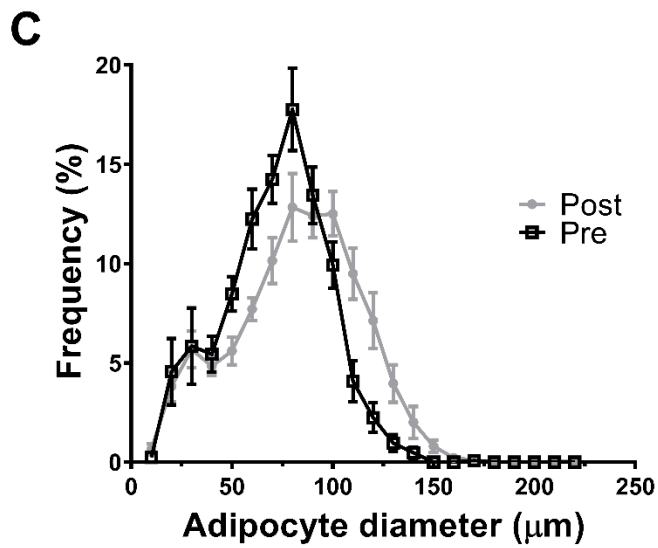
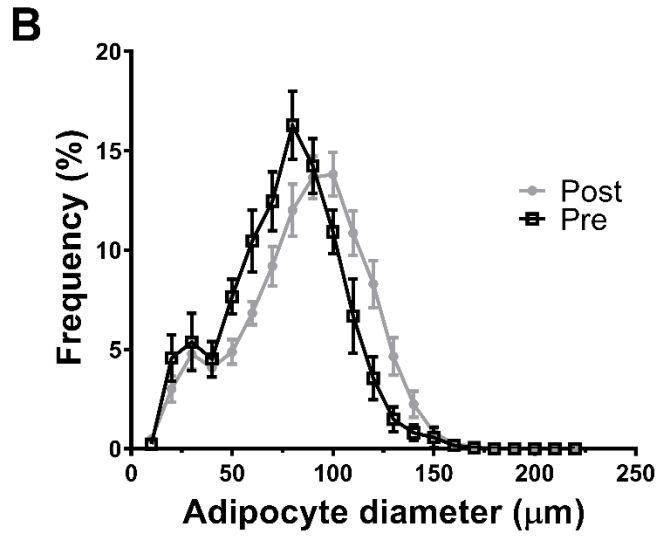
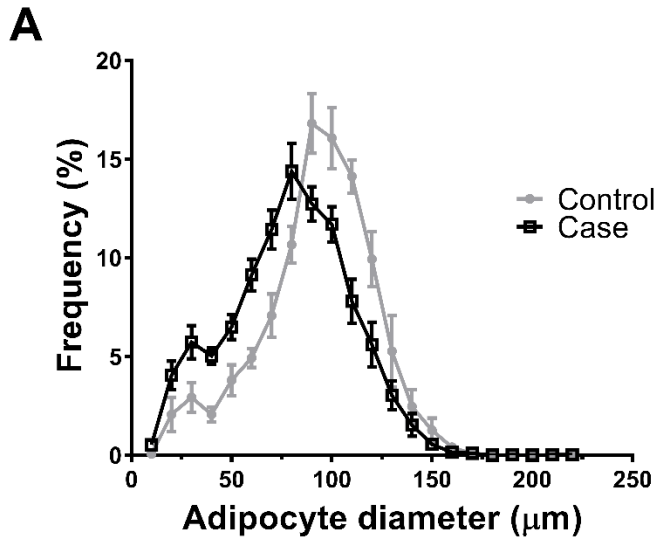
825 **Key:** BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor.

826

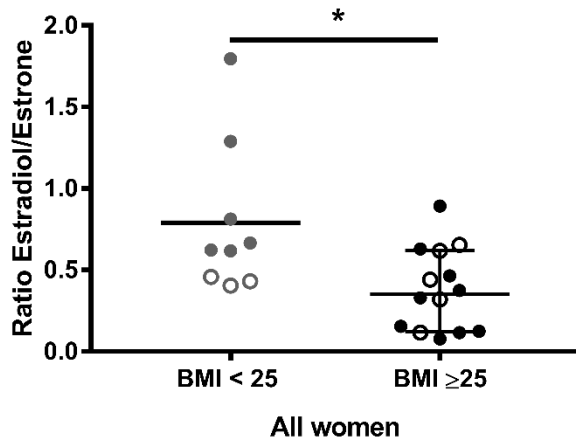
827

828

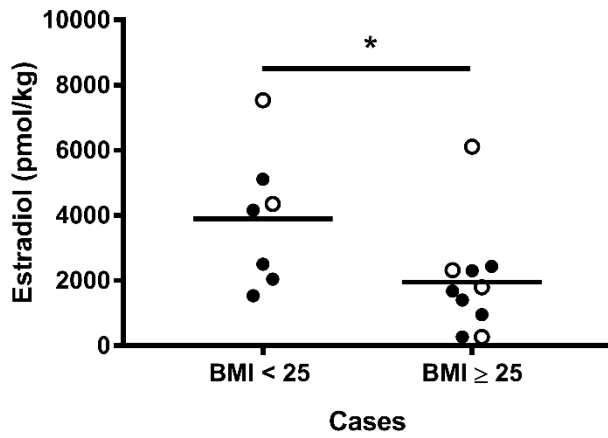
829



A



B



831

832

