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BRIEF REPORT

Neoadjuvant chemotherapy modifies serum pyrrolidone carboxypeptidase specific activity in women with breast cancer and influences circulating levels of GnRH and gonadotropins

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Abstract

Purpose Functional studies have demonstrated that gonadotropin-releasing hormone (GnRH) regulates cell proliferation, apoptosis, and tissue remodeling. GnRH is metabolized by the proteolytic regulatory enzyme pyrrolidone carboxypeptidase (Pcp) (E.C. 3.4.19.3), which is an omega peptidase widely distributed in fluids and tissues. We previously reported a decrease in both rat and human Pcp activity in breast cancer, suggesting that GnRH may be an important local hormonal factor in the pathogenesis of breast cancer. Recently, we have described that postmenopausal women with breast cancer show lower levels of serum Pcp activity than control postmenopausal women. To determine the effect of neoadjuvant chemotherapy (NACT) on serum Pcp specific activity and circulating levels of GnRH, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and steroid hormones 17-ß-estradiol and progesterone in pre- and postmenopausal women diagnosed with infiltrating ductal carcinoma.

Methods Serum Pcp activity was measured fluorometrically using pyroglutamyl-ß-naphthylamide. Circulating GnRH levels were dosed using a commercial RIA kit. Circulating LH and FSH levels were measured by enzyme immunoassays. Levels of steroid hormones were measured in serum samples by dissociation-enhanced lanthanide fluorescence immunoassay.

Results and conclusion Our results show the effect of NACT on the hypothalamic-pituitary axis, with the consequent alteration of circulating gonadotropins in premenopausal women with breast cancer. However, the results obtained in postmenopausal women with breast cancer treated with NACT, that is, the significant decrease in the concentration of GnRH and FSH compared to control postmenopausal women, differ from those obtained for premenopausal women. The only difference between pre- and postmenopausal women is their hormonal profile at the beginning of the study, that is, the presence of menopause and the consequent alteration of the hypothalamic-pituitary–gonadal axis.

Keywords Breast cancer · Neoadjuvant · Menopause · Gonadotropins · Pyrrolidone carboxypeptidase

Abbreviations

ATC	Anthracycline
AUC	Area under the curve
BMI	Body mass index
BSA	Bovine serum albumin
DELFIA	Dissociation enhanced lanthanide fluorescence immunoassay
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
LH	Luteinizing hormone
NACT	Neoadjuvant chemotherapy
PCL	Paclitaxel
PGluNNap	Pyroglutamyl-ß-naphthylamide
Pcp	Pyrrolidone carboxypeptidase
TNBC	Triple-negative breast cancers

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Introduction

Breast cancer is the most common cancer among women, being also one of the leading causes of cancer-related mortality [1, 2]. Approximately 25% of breast cancer cases occur in premenopausal women, including 12% in women between the ages of 20 and 44 years [3]. Neoadjuvant chemotherapy (NACT) is increasingly used for all breast cancer subtypes, increasing the number of conservative breast surgeries [4, 5] and it allows monitoring the treatment response. However, in young breast cancer patients, loss of ovarian function and fertility are a major limitation of NACT [6, 7]. The mechanism by which NACT, particularly alkylating agents, contribute to ovarian damage is unclear, but it could be linked to apoptotic oocyte death in primordial follicles entering the differentiation stage, which is especially susceptible to chemotherapy effects [8, 9].

The expression of the GnRH receptor has been identified in breast cancers and non-reproductive cancers. In this sense, between 50 and 64% of human breast cancers have high-affinity for GnRH receptors, as indicated by different studies [10–12]. In some studies such as Grundker et al. [13], it has been reported that GnRH receptor expression reaches 100% cases of carcinoma in situ, and in 71% cases of malignant breast cancers. In addition, approximately 10–15% of breast cancers are triple-negative breast cancers (TNBC) [14–16], with bone being the most frequent site of metastasis [17, 18]. These bone metastases were significantly inhibited in the MDA-MB-435 and MDA-MB-231 TNBC cell lines by treatment with GnRH analogs [19]. GnRH analogs could interfere with the biology of circulating breast cancer cells and they could also influence the primary steps of breast cancer metastasis, including epithelial–mesenchymal transition (EMT), migration and invasion, as already known from in vitro data [18]. Furthermore, functional studies have demonstrated that the GnRH regulates cell proliferation, apoptosis and tissue remodeling [20]. Therefore, the extra-pituitary roles of GnRH have attracted interest in the fields of tumor biology [21].

GnRH, a hypothalamic neuronal secretory decapeptide, is crucial for human reproduction, stimulating the biosynthesis and secretion of LH and FSH from pituitary gonadotropes after binding to its related receptor (GnRH receptor). This hypothalamic release factor is metabolized by a proteolytic regulatory enzyme, the pyrrolidone carboxypeptidase (Pcp) (E.C. 3.4.19.3). This omega peptidase is widely distributed in fluids and tissues and hydrolyses N-terminal pyroglutamic residues from biologically active peptides, such as GnRH [22, 23]. We previously reported a decrease in both rat [24] and human [25] Pcp activity in breast cancer, suggesting that GnRH could also be a crucial local intracrine, autocrine and/or paracrine hormonal factor involved in the pathogenesis of breast cancer. Specifically, we have recently described that postmenopausal women with breast cancer show lower levels of serum Pcp activity than postmenopausal control women [26]. This decrease occurred concomitantly with a decrease in circulating levels of GnRH and FSH, whereas no differences in circulating levels of LH were found between groups [26]. In the present study, we also show the data of steroid hormones in these patients.

Experimental, epidemiologic, and clinical research studies have convincingly shown that endogenous estrogens are involved in the etiology of breast cancer. It is widely recognized that endogenous estrogens are related to an increased risk of postmenopausal breast cancer [27]. In this context, excess adipose tissue would be involved in the development of breast cancer, since it is believed to be the main site of estrogen production in obese postmenopausal women [28–32].

In this context, we analyzed fluorometrically serum Pcp activity in pre- and postmenopausal women diagnosed with infiltrating ductal carcinoma. The analysis was performed in women treated with NACT and women untreated with this therapy, and in pre- and postmenopausal women without breast cancer as control groups. We also determined circulating levels of GnRH, FSH and LH and the steroid hormones 17-ß-estradiol and progesterone.

Materials and methods

Subjects and study design

A total of 198 women were recruited at the Unit of Breast Pathology at the University Hospital of Jaén, and 78 volunteers women without breast cancer were also included as control groups. This study was approved by the Ethical Committee of the University Hospital of Jaén and all subjects signed a term of free, informed consent.

Patient characterization included age at diagnosis, tumor size, tumor histology, pathologic T classification, Scarff–Bloom–Richardson grade, hormonal and HER-2/neu status, molecular subtype and circulating GnRH, FSH, LH, estradiol and progesterone hormone levels. All women with breast cancer were diagnosed with ductal infiltrating carcinoma. A total of 83 of these women (39 premenopausal and 44 postmenopausal) did not receive NACT, whereas 115 of them (63 premenopausal and 52 postmenopausal) received NACT before surgery. The clinicopathological characteristics of studied patients [33, 34] are shown in Table 1. Patients treated with NACT received an anthracycline/taxane-based regimen including 4 courses of EC

Table 1 Clinicopathological description of the patients involved in this study

Characteristics	Without neoadjuvant treatment		With neoadjuvant treatment	
	Premenopausal	*Postmenopausal*	*Premenopausal*	*Postmenopausal*
	n (%)	*n* (%)	*n* (%)	*n* (%)
Age (years)				
Mean	45.2 ± 1.2	65.3 ± 0.9	45.1 ± 0.8	65.3 ± 0.90
Median	48	64	46	63
Range	27–54	57–78	29–53	56–78
Tumor histology				
Ductal	39 (100%)	44 (100%)	63 (100%)	52 (100%)
Lobular	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Molecular subtypes				
Luminal A	23 (59.0%)	27 (61.4%)	34 (54.0%)	27 (51.9%)
Luminal B	10 (25.6%)	6 (13.6%)	7 (11.1%)	12 (23.1%)
Her-2	2 (5.1%)	4 (9.1%)	18 (28.6%)	0 (0%)
Triple-negative	4 (10.3%)	7 (15.9%)	4 (6.3%)	13 (25.0%)
Pathologic tumor size (cm)				
Mean ± SEM	1.31 ± 0.09	1.52 ± 0.14	3.02 ± 0.17	3.36 ± 0.15
Median	1.20	1.30	3.00	3.00
Range	0.5–3.1	0.8–5.0	0.8–5.6	1.4–5.0
Classification				
0	0 (0%)	0 (0%)	0 (0%)	0 (0%)
1	35 (89.7%)	40 (90.9%)	18 (28.6%)	6 (11.5%)
2	4 (10.3%)	4 (9.1%)	40 (63.5%)	43 (82.7%)
3	0 (0%)	0 (0%)	5 (7.9%)	3 (5.8%)
Scarff–Bloom–Richardson grade				
I	19 (48.7%)	10 (22.7%)	8 (12.7%)	13 (25%)
II	20 (51.3%)	34 (77.3%)	55 (87.3%)	39 (75%)
II	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Hormonal status				
ER+	33 (84.6%)	33 (75.0%)	41 (65.1%)	36 (69.2%)
ER−	6 (15.4%)	11 (25.0%)	22 (34.9%)	16 (30.8%)
PgR+	25 (64.1%)	27 (61.4%)	41 (65.1%)	33 (63.5%)
PgR−	14 (35.9%)	17 (38.6%)	22 (34.9%)	19 (36.5%)
HER-2/neu status				
Negative	29 (74.4%)	34 (77.3%)	38 (60.3%)	49 (94.2%)
Positive	10 (25.6%)	10 (22.7%)	25 (39.7%)	3 (5.8%)

(epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days), followed by 8 courses of 100 mg/m^2 paclitaxel once a week or 4 courses of 75 mg/m^2 docetaxel every 21 days. Patients with a HER-2/neu-overexpressing tumor also received trastuzumab (14 courses at 6 mg/kg every 21 days). Women with triple-negative breast cancer received 6 cycles of 75 mg/m^2 docetaxel plus carboplatin (AUC 6). No significant differences were found between treatments in the several parameters assayed (data not shown).

Control groups consisted of 78 women, aged 28 to 69 years old (premenopausal women with regular menstrual periods $n = 38$; postmenopausal women with spontaneous menopause for at least one year, $n = 40$), with no previous history of any type of cancer, chemotherapy, hormonal or antioxidant therapy, or chronic diseases.

Women were excluded if they were current smokers, regular alcohol consumers, antioxidant supplement users, pregnant or lactating, presented hepatic, cardiac or renal dysfunction, hormonal therapy, use of drugs, hypertension, diabetes, and other eventual chronic conditions.

Sample acquisition

Samples from patients treated with NACT were obtained after completion of chemotherapy treatment and in parallel to samples from patients not treated with NACT and control volunteers in order to be processed under the same conditions.

Blood samples were obtained after an overnight fast by venous arm puncture in tubes without anticoagulants. Blood specimens were allowed to cloth and centrifuged at 3000×*g*, for 10 min, at 4 °C to obtain the serum. Serum samples were collected, rapidly frozen in liquid nitrogen and kept on − 80 °C until usage for assays.

Pyrrolidone carboxypeptidase assay

Serum Pcp activity was measured fluorometrically using pyroglutamyl-ß-naphthylamide (pGluNNap) as the substrate, according to the previously described by us [35]. Briefly, 10 µL of each sample were incubated in triplicate for 30 min at 37 °C with 100 µL of the substrate solution containing 100 µM of pGLUNNap, 0.65 mM dithiothreitol (DTT) and 1.3 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM of phosphate buffer at pH 7.4. All the reactions were stopped by adding 100 µL of 0.1 M acetate buffer at pH 4.2. The amount of ß-naphthylamine released as the result of the enzymatic activity was measured fluorometrically at 412 nm emission wavelength with an excitation wavelength of 345 nm. Proteins were quantified also in triplicate using bovine serum albumin (BSA) as standard.

Determination of circulating levels of GnRH, FSH and LH

Hormone concentrations in all serum samples were assayed using a pool of several kits. Circulating LH and FSH were measured by enzyme immunoassays (Bioserv Diagnostics, Rostock, Germany). Circulating GnRH was dosed using a commercial RIA kit (Phoenix Pharmaceuticals, Inc., USA) according to the manufacturer's instructions. The lowest detection limit was 25.4 pg/mL. The detection range was 10–1280 pg/mL. The sensitivities of the enzyme immunoassays were 0.3 IU/L for LH and FSH. The values of 0.2 IU/L for LH and FSH were assigned to samples below the detection limit.

Assessment of sex hormones

Serum samples were measured by dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) for 17-ß-estradiol and progesterone (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland), according to manufacturer's instructions. For E2, the lowest limit of the assay detection is 0.05 nmol/L (13.6 pg/mL); inter-assay coefficients of variation are between 1.8 and 2.1%. For P, the lowest limit of the assay detection is 0.8 nmol/L (0.25 ng/mL); inter-assay coefficients of variation are between 1.9 and 4.5%.

Statistical analysis

To analyze the differences between groups, we have used multiple analysis of variance in addition to the Newman–Keuls post hoc test. Eta-squared (η^2) has been used to measure the effect size. Statistics were performed using IBM SPSS V.24 software. All comparisons with p-values below 0.05 were considered significant.

Results

Figure 1 shows serum Pcp specific activity levels found in pre- and postmenopausal control women and in women diagnosed with infiltrating ductal carcinoma treated or untreated with NACT. Circulating levels of GnRH and gonadotropins (FSH and LH) found in the several groups of women are also shown in Fig. 1.

Regarding Pcp specific activity, no significant changes are found in premenopausal women with breast cancer when compared with premenopausal control women. However, we observe a significant increase ($p < 0.001$) in serum Pcp specific activity in premenopausal women with breast cancer treated with NACT. On the contrary, no changes are observed in postmenopausal women with breast cancer treated with NACT, although untreated women show a slightly significant decrease ($p < 0.05$) in Pcp specific activity when compared with postmenopausal control women (Fig. 1a). An effect size between groups for Pcp specific activity of $\eta^2 = 0.075$ was found.

Figure 1b shows a significant ($p < 0.001$) lower concentration of circulating GnRH in both premenopausal and postmenopausal women with breast cancer treated with NACT when compared to their respective controls. Similar results were previously described in untreated pre- and postmenopausal women with breast cancer when compared to their corresponding controls [26]. An effect size between groups for GnRH of $\eta^2 = 0.697$ was found.

Regarding FSH, no significant changes are found in untreated premenopausal women with breast cancer when compared with premenopausal control women. However, we observe a significant increase ($p < 0.001$) in circulating FSH in premenopausal women with breast cancer treated with NACT. On the contrary, we observe a significant decrease in FSH levels ($p < 0.001$) in both postmenopausal women with breast cancer treated or untreated with NACT (Fig. 1c). An effect size between groups for FSH of $\eta^2 = 0.239$ was found.

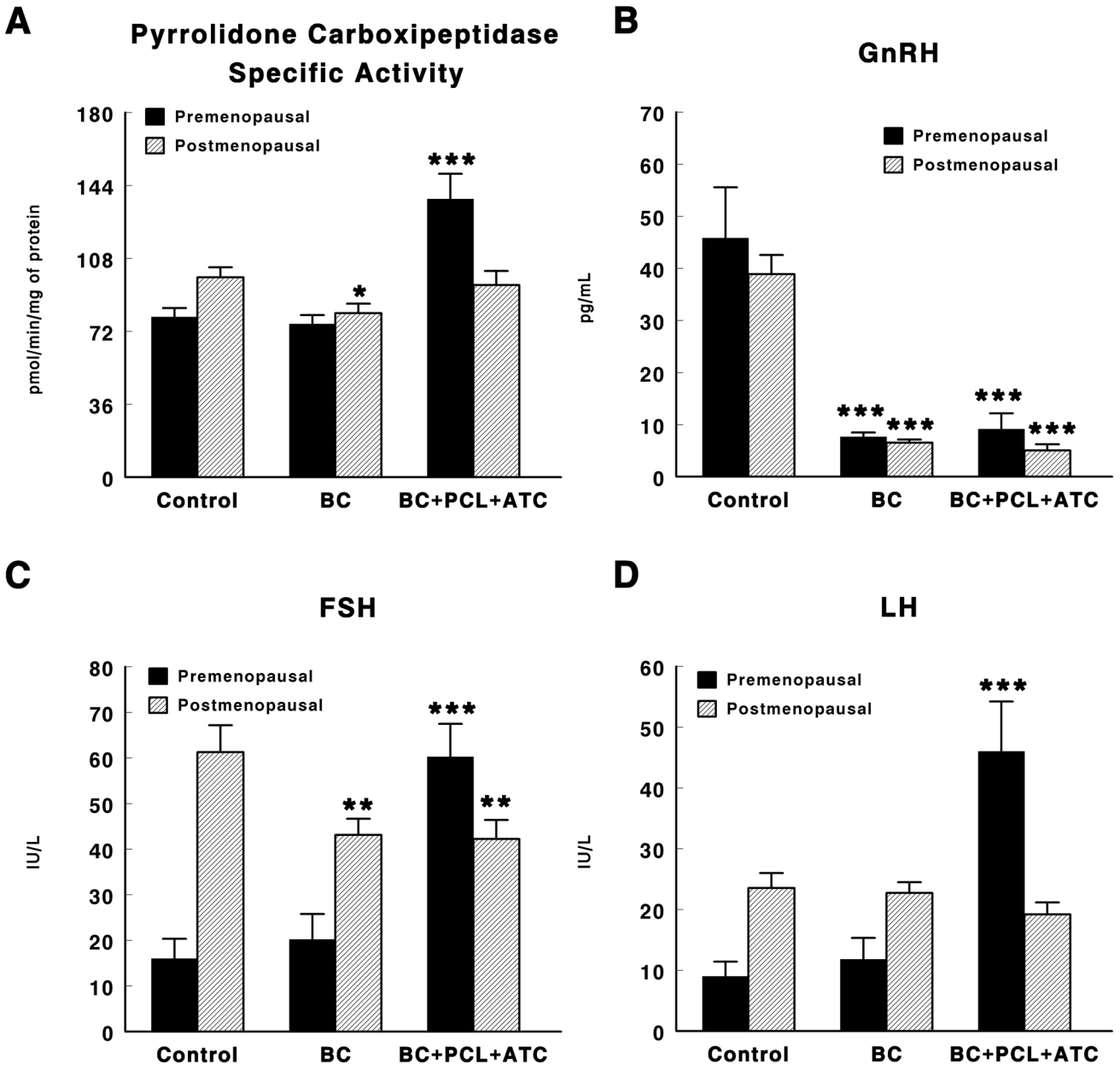

Fig. 1 Pyrrolidone carboxypeptidase specific activity and circulating levels of GnRH, FSH and LH measured in serum of control premenopausal and postmenopausal control women, premenopausal and postmenopausal women with breast cancer and premenopausal and postmenopausal women with breast cancer treated with neoadjuvant chemotherapy (NACT) (Mean ± SEM; $*p<0.05$; $**p<0.01$; $***p<0.001$.)

Regarding LH, no significant changes are found either in untreated premenopausal women with breast cancer when compared with premenopausal control women. However, we newly observe a significant increase ($p<0.001$) in circulating LH in premenopausal women with breast cancer treated with NACT. On the contrary, no significant differences in LH levels are observed in both treated or untreated postmenopausal women with breast cancer (Fig. 1d). An effect size between groups for LH of $\eta^2=0.153$ was found.

Figure 2 shows circulating levels of steroid hormones in pre- and postmenopausal control women and in women diagnosed with infiltrating ductal carcinoma treated or untreated with NACT.

A significant ($p<0.001$) lower level of 17-ß-estradiol was found in both treated or untreated premenopausal women when compared to their control group, being these levels in treated premenopausal women even significantly lower ($p<0.01$) than in untreated. No changes in 17-ß-estradiol

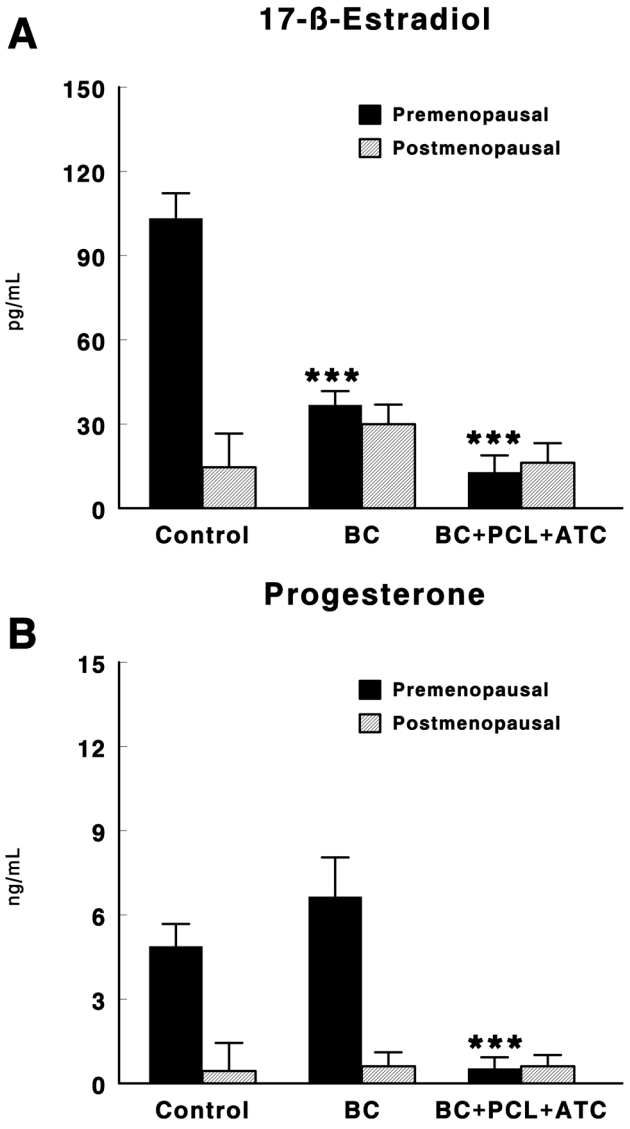

Fig. 2 Circulating levels of 17-ß estradiol and progesterone measured in serum of control premenopausal and postmenopausal control women, premenopausal and postmenopausal women with breast cancer and premenopausal and postmenopausal women with breast cancer treated with neoadjuvant chemotherapy (NACT) (Mean ± SEM; ***$p < 0.001$.)

levels are found in treated or untreated postmenopausal women with breast cancer (Fig. 2a). An effect size between groups for 17-ß-estradiol of $\eta^2 = 0.336$ was found.

Similar results are observed in postmenopausal women regarding circulating levels of progesterone, without differences between groups (Fig. 2b). However, in premenopausal women, only patients treated with NACT show a significant decrease ($p < 0.001$) in the circulating levels of progesterone. Untreated premenopausal women with breast cancer show no significant differences with premenopausal control women. Finally, an effect size between groups for progesterone of $\eta^2 = 0.106$ was found.

Discussion

In the present study, we find that serum Pcp activity is modified by NACT, affecting the hormonal profile of premenopausal women with breast cancer, with a significant increase in circulating gonadotropins and a decrease in circulating estradiol and progesterone. These changes in the hormonal profile of premenopausal women with breast cancer treated with NACT may be due to an alteration of the hypothalamic-pituitary–gonadal axis and/or ovarian dysfunction produced by the chemotherapy treatment. Either way, this diminution of circulating steroid hormones would have a protective effect on the development and/or proliferation of the disease.

More than 75% of breast tumors express the estrogen receptor (ER), suggesting that the huge majority of breast cancers are hormone-dependent and grow in response to estrogens [36], playing 17-ß-estradiol and its receptor an essential role in the etiology and development of breast cancer [37, 38]. Several epidemiological and clinical studies have confirmed this correlation between hormones and breast malignancy by reporting higher serum levels of estrogens in women with breast cancer compared to control cases [39, 40]. However, no increase in circulating estrogen levels was found in our study in any of the patient groups, although differences in steroid hormone levels were found between the control group of premenopausal and postmenopausal women. The major site of estrogen biosynthesis differs between premenopausal and postmenopausal women. In premenopausal women, estrogens are abundantly produced in the granulosa cells of the ovary with every menstrual cycle, while lower levels of estrogens are also produced in other organs including bone, adipose tissue, vascular endothelium, aortic smooth muscle or brain [41–43]. Extragonadal sites become the main source of estrogen during menopause, due to the definitive cessation of ovarian function during this stage [29]. Therefore, the main site of estrogen production in postmenopausal obese women could be the excess of adipose tissue, thus contributing to the increase in circulating estrogen concentrations [31]. In our study, premenopausal women with breast cancer non-treated and treated with NACT and postmenopausal women without breast cancer presented a BMI ≈ 25–29. 9 kg/m^2, whereas in postmenopausal women with breast cancer treated or untreated was BMI ≥ 30 kg/m^2 or more [44]. However, a modification in serum estradiol and progesterone levels in postmenopausal women with breast cancer treated and untreated with NACT respect to postmenopausal women without breast cancer (control group) was not found.

In general, node-positive breast cancer is treated systemically with chemotherapy, endocrine therapy (for

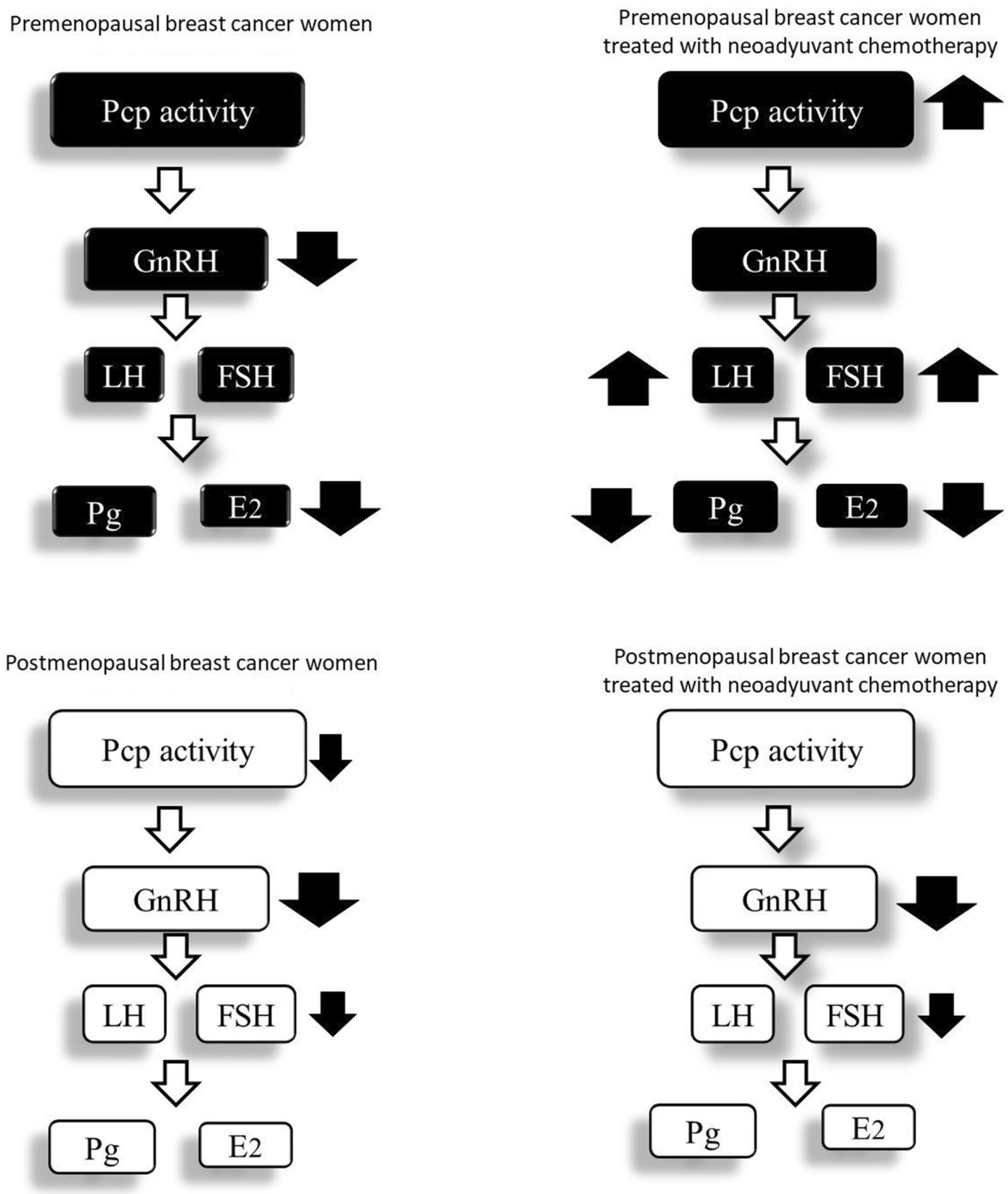

Fig. 3 Summary of the role of specific pyrrolidone carboxypeptidase (Pcp) activity and circulating levels of GnRH, FSH, LH, 17-ß estradiol and progesterone in premenopausal women (**a**) and postmenopausal women (**b**) with breast cancer, treated or not with neoadjuvant chemotherapy (NACT)

hormone receptor-positive cancer), and trastuzumab (for cancer overexpressing ERBB2) [45]. However, in TNBC, that is, in those cancers that do not exhibit the estrogen receptor α (ERα) or the progesterone receptor and do not overexpress the HER-2–neu gene, current therapeutic options are incredibly limited [46–50].

In this sense, some authors state that more than 70% of TNBCs have GnRH receptors, [51] while others confirm that 100% of them express these receptors [52]. Since breast cancers express both GnRH and its receptor, it seems plausible to consider that there may be a regulatory system locally based on GnRH in many of these tumors [53–55]. In both postmenopausal women with breast cancer treated and untreated with NACT, we found an alteration in the circulating levels of GnRH, as well as in treated and untreated premenopausal women with breast cancer.

In conclusion, our results show the effect of NACT on the hypothalamic-pituitary axis, with the consequent alteration of circulating gonadotropins in premenopausal women with breast cancer. However, the results obtained in postmenopausal women with breast cancer treated with chemotherapy, that is, the significant decrease in the concentration of GnRH and FSH compared to control postmenopausal women, differ from those obtained for premenopausal women, being the only difference the starting hormonal profile, i.e., the presence of menopause and the consequent alteration of the hypothalamic-pituitary–gonadal axis (Fig. 3).

Limitations of the study

The present study shows limitations concerning circulating GnRH levels, which could be altered by factors like stress or obesity as well as the menstrual cycle. Indeed, we have observed an increase in cortisol levels in all patients diagnosed with breast cancer respect to women without breast cancer. However, neither BMI nor cortisol levels significantly influences the parameters assayed here. Stress is a complex factor to deal with and even more so in patients who have been diagnosed with breast cancer, a situation full of uncertainty. Hence, it is a difficult field to tackle, not only determined by the hormonal profile of the patients, which undoubtedly has decisive importance, but also by other factors that could influence the development of the disease, therefore requiring a study of greater complexity.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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