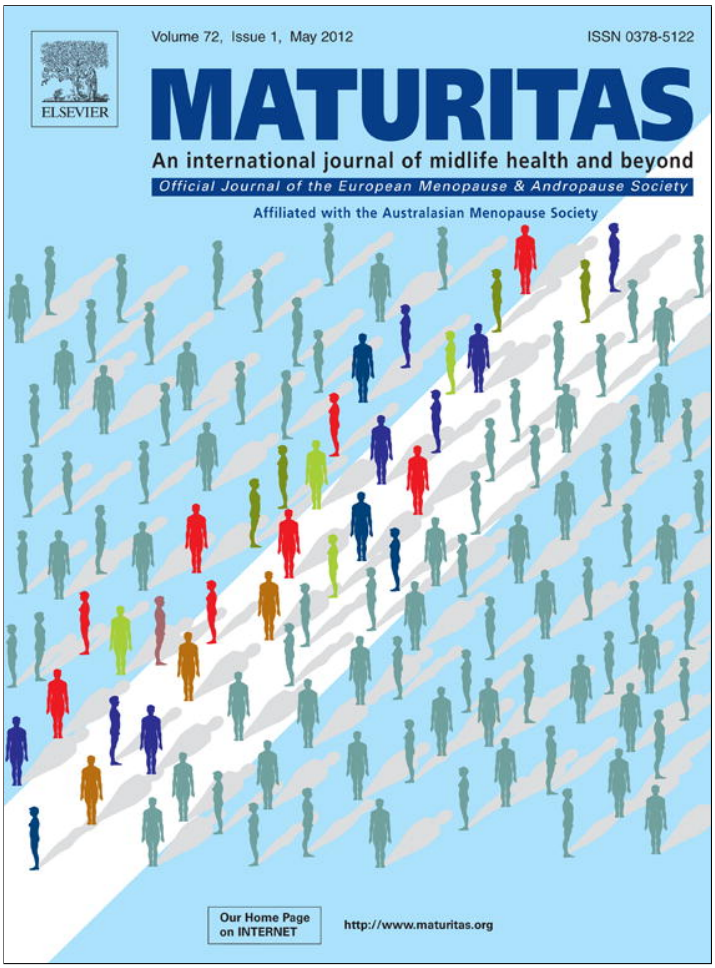

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Neoadjuvant chemotherapy modifies serum angiotensinase activities in women with breast cancer

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ABSTRACT

Purpose: The aim of this study was to investigate the putative changes in serum angiotensinase activities (aminopeptidase N, APN; aminopeptidase B, APB; aminopeptidase A, APA; aspartyl aminopeptidase, ASAP) involved in the renin–angiotensin system (RAS) in women with breast cancer treated or not with a neoadjuvant therapy of paclitaxel and anthracycline and in healthy women volunteers.
Methods: We fluorometrically analysed serum APN, APB, APA and ASAP activities using their corresponding aminoacyl-β-naphthylamides as substrates in women with breast cancer treated with a neoadjuvant therapy of paclitaxel and anthracycline.
Results: When compared with healthy controls, women with breast cancer not treated with neoadjuvant chemotherapy, showed a decrease in angiotensinase activity, which support the putative increase of angiotensin II (Ang II) levels, indicating that the tumour process would favour the development of the disease. Also, an increase in APN and APB activities was observed, which support a role for angiotensin IV (Ang IV). In women treated with a neoadjuvant therapy, we described an increase in ASAP and APA activities, supporting the idea that this treatment increases Ang II catabolism. The resulting decrease in Ang II level could lead to an inhibition of the tumour growth.
Conclusion: Present results show changes in serum angiotensinase activities in women with breast cancer and in women with breast cancer treated with a neoadjuvant therapy of paclitaxel and anthracycline. Therefore, considerable attention should be focused on the development of RAS blockade therapy as a new strategy for breast cancer treatment.

1. Introduction

The renin–angiotensin system (RAS) has classically been identified in reno-cardiovascular organs including the kidney and the heart and vessel walls, where its enzymatic actions and released peptides lead to blood pressure regulation and electrolyte/fluid homeostasis. In addition, it has been demonstrated that the RAS participates in the regulation of angiogenesis in breast cancer cells via the up-regulation of various angiogenesis-associated genes.

In the RAS, angiotensin II degradation begins with the action of aspartyl aminopeptidase (ASAP) and aminopeptidase A (APA), which remove the N-terminal Asp to produce angiotensin III (Ang III), a less potent vasoconstrictor peptide than Ang II [1]. Ang III is also produced from angiotensin I (Ang I) through the production of des-Asp1-Ang I, which is further converted to Ang III by the action of angiotensin-converting enzyme (ACE). Ang III is further converted to angiotensin IV (Ang IV) by aminopeptidase B or aminopeptidase N [2]. Ang II mediates its effects by binding to two different receptors, Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R) [3]. The inhibition of AT1R suppresses the Ang II-dependent gene regulation.

On the other hand, aminopeptidase A and N (APA and APN) are considered cell-surface peptidases in the form of integral membrane proteins, which are involved in the control of cell proliferation and differentiation by modulating the access of peptides to their membrane receptors [4–6]. Besides this angiotensinase function, APN and APA are directly involved in the tumoural process. In fact, APN has previously been considered to be a proteolytic enzyme with ability to facilitate the tumour cell invasion through the extracellular matrix [7,8]. Moreover, a recent report indicated that APA was up-regulated and enzymatically active in the blood vessels of

Abbreviations: APN, aminopeptidase N; APB, aminopeptidase B; ASAP, aspartil aminopeptidase; APA, aminopeptidase A; RAS, renin–angiotensin system; Ang II, angiotensin II; Ang IV, angiotensin IV.

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Table 1
Clinicopathological characterisation of patients.

	Patients without neoadjuvant chemotherapy ($n = 66$)	Patients after neoadjuvant chemotherapy ($n = 46$)
Age at diagnosis (years ± SEM)	56.42 ± 1.36	60.36 ± 1.64
Median	54	59.5
Range	39–84	39–78
Body mass index	30.49 ± 0.50	30.38 ± 0.68
Median	31.1	30.4
Range	23.7–37.4	23–38.6
Histological type		
Ductal	66 (100%)	46 (100%)
Lobular	None	None
Mixed	None	None
Molecular receptor status		
ER+	83.33%	48.57%
PR+	68.18%	62.85%
HER-2/neu+	63.63%	82.85%
Scarf–Bloom–Richardson grade		
I	22.0%	13.63%
II	60.0%	36.36%
III	18.0%	50.00%
TNM classification		
I stage	40.90%	None
II stage	56.06%	52.17%
III stage	None	47.83%
IV stage	3.03%	None

ER, estrogen receptors; PR, progesterone receptor; Her-2/neu, human epidermal growth factor receptor; TNM, tumour, node, metastasis classification.

human tumours, but it was not detected in normal blood vessels [9]. Other studies have demonstrated that estradiol induces APA expression in prostate cancer cells. These results are in accordance with Katsumata et al. [10], who demonstrated that APA expression was stimulated by progesterone in human choriocarcinoma cells. Another aminopeptidase implicated in the tumoural process is the cytosolic ASAP. This enzymatic activity is modified in breast tissues from women with breast cancer [11] and is significantly increased in head and neck squamous cell carcinoma tissues [12].

In this context, the aim of the present work is to fluorometrically analyse serum for APN, APB, APA and ASAP activities using their corresponding aminoacyl-β-naphthylamides as substrates in women with breast cancer treated or not with a neoadjuvant therapy of paclitaxel and anthracycline and in healthy women volunteers.

2. Material and methods

2.1. Experimental design

In the present study, we analysed 112 women diagnosed with ductal infiltrating carcinoma. Sixty-six women did not receive chemotherapy, whereas 46 of them were treated with a neoadjuvant therapy of paclitaxel and anthracycline. Also, 37 healthy women volunteers were included. The study was approved by the Hospital Ethics Committee, and all patients signed an informed consent form. Table 1 summarises the clinicopathological characteristics of the patients studied.

Blood samples were obtained and centrifuged for 10 min at 3000 × *g* to obtain the serum. Samples were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Angiotensinase aminopeptidase assays

ASAP was determined fluorimetrically using aspartyl-β-naphthylamide (AspNNap) as the substrate, according to the method previously described [13]. Briefly, 10 μl of each sample was incubated in triplicate for 30 min at 37 °C with 100 μl of the substrate solution: 100 μM AspNNap, 1.3 μM ethylenediaminetetraacetic acid (EDTA) and 2 mM $MnCl_2$ in 50 mM of phosphate buffer, pH 7.4.

APA activity was measured in the same way using glutamyl-β-naphthylamide (GluNNap) as the substrate, as previously described [14]. Ten microliters of each sample was incubated in triplicate for 30 min at 37 °C with 100 μl of the substrate solution: 100 μM GluNNap, 0.65 mM dithiothreitol (DTT) and 50 mM $CaCl_2$ in 50 mM of phosphate buffer, pH 7.4.

APN and APB were also measured fluorometrically using alanyl-β-naphthylamide (AlaNNap) or arginyl-β-naphthylamide (ArgNNap) as the substrate, as previously described [15]. Ten microliters of each supernatant were incubated for 30 min at 37 °C with 100 μM of the substrate solution: 100 μM AlaNNap or 100 μM ArgNNap and 0.65 mM dithiothreitol (DTT) in 50 mM of phosphate buffer, pH 7.4.

All the reactions were stopped by adding 100 μl of 0.1 M acetate buffer, pH 4.2.

The amount of β-naphthylamine released as the result of the enzymatic activities was measured fluorimetrically at 412 nm emission wavelength with and excitation wavelength of 345 nm. Proteins were quantified also in triplicate by the method of Bradford, using bovine serum albumin (BSA) as standard. Serum specific APN, APB, ASAP and APA activities were expressed as nanomoles of Ala-, Arg-, Asp- and Glu-β-naphthylamide hydrolysed per min per mg of protein, by using a standard curve prepared with the latter compound under corresponding assay conditions.

2.3. Statistical analysis

To analyze the differences between healthy and women diagnosed with ductal infiltrating carcinoma, we have used unpaired Newman–Keuls. All comparisons with *p*-values below 0.05 were considered significant.

3. Results

Table 1 shows the clinicolpathological characteristics of patients included in this study.

Fig. 1 illustrates the results obtained on specific serum APN, APB, APA and ASAP activities in women with breast cancer (BC), women with breast cancer treated with a neoadjuvant therapy of paclitaxel and anthracycline (BC + PCTX) and in healthy women volunteers.

Our results show a significant increase ($p < 0.01$) in serum APN (Fig. 1A) and APB (Fig. 1B) activities in the women with breast cancer (BC) when compared to the control women, and this increase was significantly higher ($p < 0.001$) in women treated with the neoadjuvant therapy. A similar increase ($p < 0.001$) was found in serum APA activity in BC + PCTX (Fig. 1C), although women with breast cancer without treatment did not show a significant decrease in APA activity. We also found a significant decrease ($p < 0.05$) (Fig. 1D) in serum ASAP activity in women with breast cancer, although ASAP activity increased significantly ($p < 0.001$) in treated women.

4. Discussion

A better understanding of the modifications of changes in the physiological systems happening during the development of breast cancer would improve diagnostic tools. This would be translated in turn into a higher survival index. Alteration of the proteolytic enzymes involved in the RAS is an example. Recently, we described changes in serum RAS-regulating AP activities in women with breast cancer, suggesting the predominant effect of Ang II in pre- and post-menopausal women with breast cancer [16]. In the present work, our results show a significant increase in serum APN

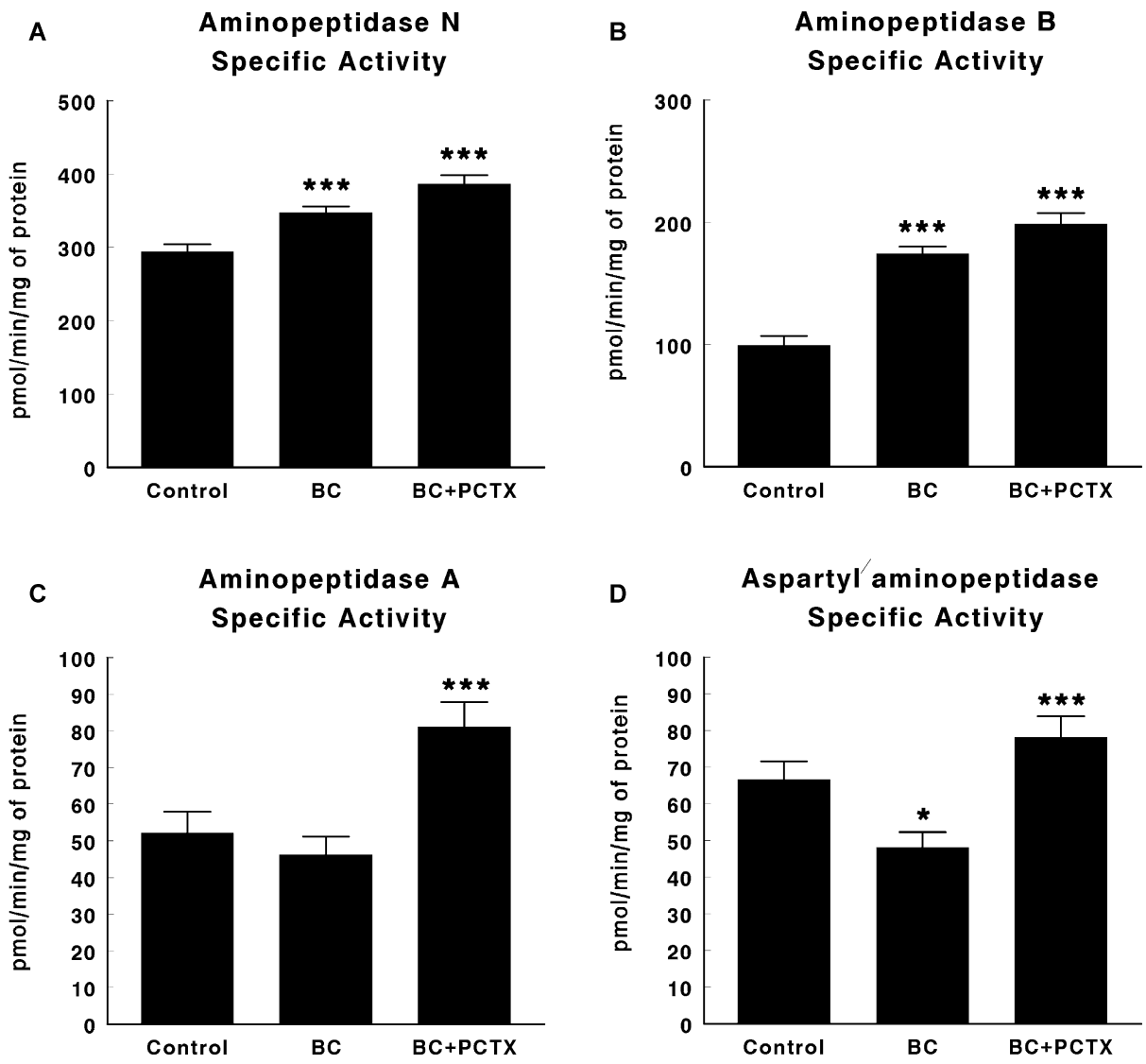

Fig. 1. Specific aminopeptidase N, aminopeptidase B, aminopeptidase A and aspartyl aminopeptidase activities in serum of control women, women with breast cancer (BC), and women with breast cancer treated with neoadjuvant therapy of paclitaxel and anthracyclines (BC + PCTX). Results are expressed in pmoles of their corresponding aminoacyl-β-naphthylamide hydrolysed per min and per mg of protein (mean ± SEM; $n = 37–56$; $^{*}p < 0.05$, $^{***}p < 0.001$).

and APB activities in women with breast cancer compared to those activities in control women, and this increase was higher still in women treated with neoadjuvant therapy. On the contrary, we also found a significant decrease in serum ASAP activity in women with breast cancer, although ASAP activity increased significantly in treated women. A similar increase with neoadjuvant treatment was found in serum APA activity, although we did not find significant decrease in APA activity in women with breast cancer with no treatment (Fig. 2). These results suggest that neoadjuvant therapy acts on angiotensinases, increasing Ang II catabolism and inhibiting tumour growth. In this way, the RAS might be involved in the regulation of tumour angiogenesis via Ang II. Ang II mediates its effects by binding to two different receptors, Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R) [3]. The activating effects of Ang II are mediated via G-protein-coupled AT1R [17,18] and opposed via AT2R [19]. AT1R is over-expressed in breast hyperplasia and ductal carcinoma *in situ* (DCIS). In microvascular endothelial cells, AT1R causes an increase in vascular endothelial growth factor (VEGF). In endothelial cells, AT1R also increases the expression of vascular endothelial growth factor-2 (VEGF2) receptor and angiopoietin-2, a major determinant of angiogenesis. In addition, AT1R is highly expressed in macrophages that surround the tumour and produce VEGF. The AT1R subtype also displays anti-apoptotic effects in microvascular endothelial cells via activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway [20]. The AT2R generally exerts antagonistic actions on AT1R, including the inhibition of the proliferation and angiogenesis and promotion of apoptosis [21], although its specific role in carcinogenesis has not been clearly explained due to conflicting results in various other studies.

In this sense, Ang-receptor blockers (ARBs) are a widely used drug class approved for treatment of hypertension, heart failure, diabetic nephropathy, and most recently cardiovascular risk reduction. Experimental studies implicate the RAS, particularly AT1R and AT2R, in the regulation of cell proliferation, angiogenesis and tumour progression [22].

Our results show that in women with breast cancer, the changes observed in RAS-regulated aminopeptidase activities lead to a putative decrease in Ang II.

In previous studies, we suggested that estradiol and progesterone influence RAS-regulated activities at different levels of the hypothalamus-pituitary-adrenal axis in female mice [23]. In this

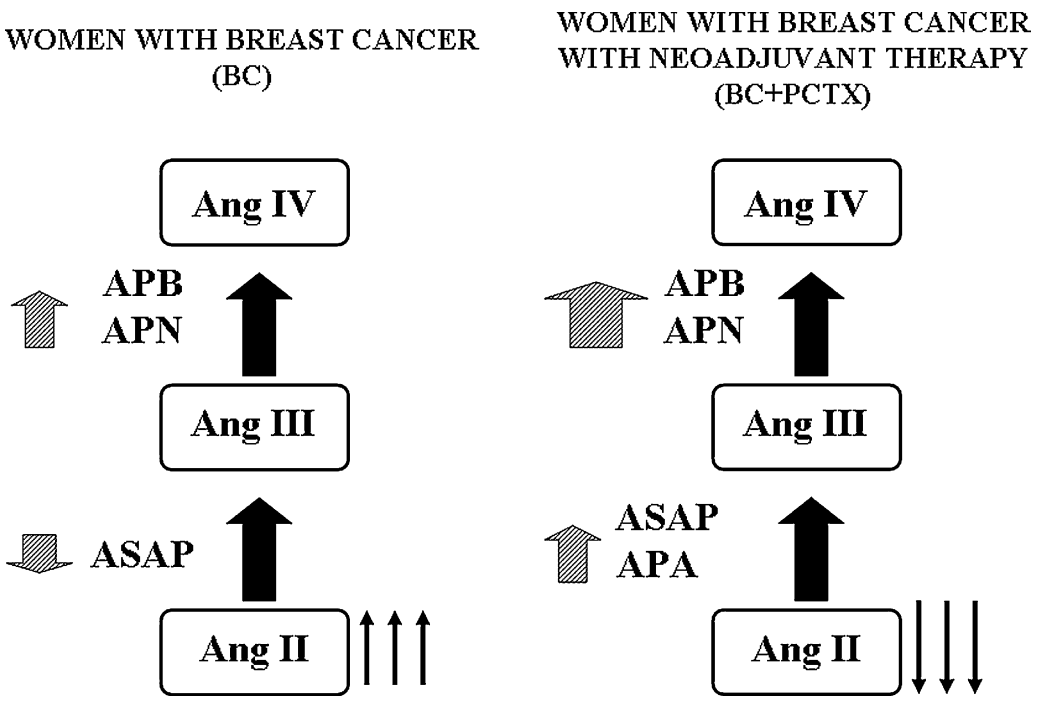

Fig. 2. Scheme with the results obtained for angiotensinase activities in serum of control women, women with breast cancer (BC), and women with breast cancer treated with neoadjuvant therapy of paclitaxel and anthracyclines (BC + PCTX).

sense, E2 is able to induce APA expression in prostate cancer cells. A recent report indicates that APA was up-regulated and enzymatically active in blood vessels of human tumours but was not detected in normal blood vessels [9]. As APA mainly plays a role in the metabolism from Ang-II to Ang-III, it is possible that APA may be implicated in the neovascularisation phase of tumour development. In addition, APA expression was found on dysplastic cells and was increased in pre-cancerous lesions and invasive cervical cancer [24]. These data suggest that APA may play a regulatory role in neoplasic transformation and disease progression in various cancers [25]. Furthermore, alterations in peptide activities involved in RAS by abnormal expression or catalytic functions of cell-surface peptidases may contribute to neoplastic transformation or progression.

5. Conclusion

Present results show changes in serum angiotensinase activities in women with breast cancer and in women with breast cancer treated with a neoadjuvant therapy of paclitaxel and anthracycline. In the present work, we show in non-treated patients a decrease in angiotensinase activity, which support the putative increase of Ang II levels, indicating that the tumour process would favour the development of the disease. In women treated with a neoadjuvant therapy, we described an increase in ASAP and APA activities, supporting the idea that Ang II catabolism increases. The resulting decrease in Ang II level could lead to an inhibition of tumour growth. Also, the increases found in APN and APB activities support a role for Ang IV. Therefore, considerable attention should be focused on the development of RAS blockade therapy as a new strategy for breast cancer treatment.

Contributors

Dr M.J. Ramirez-Expósito, Dr. M.P. Carrera-González and Dr. M.D. Mayas analyzed fluorometrically serum angiotensinase activities in women with breast cancer and in women with breast cancer treated with a neoadjuvant therapy of paclitaxel and anthracycline. As well as Dr. M.P. Carrera and Dr. M.J. Ramirez-Expósito participated in the development of this paper.

Dr. B. Dueñas and Dr. J. Martinez-Ferrol provided the samples of this study. All patients signed an informed consent form.

Dr. J.M. Martinez-Martos took part in all the tasks necessary for this work.

Competing interest

The authors declare that there are no conflicts of interest.

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