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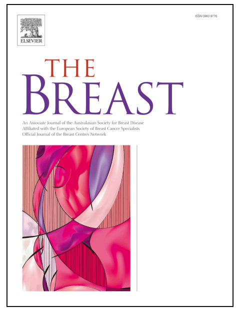


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Trace element concentrations in breast cancer patients

Noemí Cabré[a,*], Fedra Luciano-Mateo[a,*], Meritxell Arenas[b], Martí Nadal[c], Gerard Baiges-Gaya[a], Anna Hernández-Aguilera[a], Isabel Fort-Gallifa[a], Elisabet Rodríguez[a,b], Francesc Riu[c], Jordi Camps[a,†], Jorge Joven[a], José Luis Domingo[d]

[a] *Unitat de Recerca Biomèdica, Hospital Universitari Sant Joan, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus, Spain*

[b] *Department of Radiation Oncology, Hospital Universitari de Sant Joan, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus, Spain*

[c] *Department of Pathology, Hospital Universitari de Sant Joan, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus, Spain*

[d] *Laboratory of Toxicology and Environmental Health, School of Medicine, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus, Spain*

*These authors contributed equally to this work

[†]Corresponding author at: Unitat de Recerca Biomèdica, Hospital Universitari Sant Joan, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Carrer Sant Joan s/n, 43201 Reus, Spain

E-mail address: jcamps@grupsagessa.com (J. Camps)

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ABSTRACT

Alterations in the circulating levels of trace elements have been observed in breast cancer (BC) patients. However, the relationships between these alterations and the metabolic and clinical consequences of BC are unknown. The treatment-of-choice of BC is surgery followed by radiation therapy (RT). The present study was aimed at investigating: 1) the concentrations of several trace elements in BC patients, and their relationships with the intrinsic molecular subtypes of tumors; 2) the toxicological effect of RT. We studied 49 women with BC who were scheduled to receive RT following excision of the tumor. Plasma samples were obtained before and after the irradiation procedure. The control group was composed of 49 healthy women. Patients had significantly lower pre-RT concentrations of B, Cu, and Zn, and significantly higher concentrations of Sr than the control group. Irradiation was associated with a striking increase in plasma B concentrations, while Cu, Fe, Sr and Zn concentrations were not significantly different from pre-RT levels, albeit Sr and Zn showed non-significant trends towards increases. The plasma concentrations of B, Cu, Fe, Sr, and Zn were associated with the tumor expression of hormone receptors, epidermal growth factor receptor 2, Ki67 antigen, as well as dermatitis and asthenia, all of which represent the main toxicological responses to RT.

*Keywords:* Breast cancer; metals; radiation therapy; trace elements

*Abbreviations:* BC: breast cancer; Her: human epidermal growth factor receptor; ICP-MS: inductively coupled plasma mass spectrometry; PCA: principal component analysis; RT: radiation therapy; VIP: variable importance in projection

## Introduction

Breast cancer (BC) is the most frequent type of cancer in women worldwide [1]. BC burden is growing and, hence, a better knowledge of the metabolic alterations related to this disease is required to identify new tools for early detection, diagnosis and treatment of BC [2]. Essential trace elements have relevant functions in a large number of cellular processes, with multiple roles in the correct functioning of metabolic enzymes. Several studies observed alterations in the circulating levels of trace elements in BC [3-6], but the relationships between these alterations and the metabolic and clinical history of BC are largely unknown. Recent studies have evaluated the efficacy of metal-based drugs in the treatment of several cancers including BC, but toxicities and drug resistance have limited their implementation in clinical practice, with the exception of platinum-containing chemotherapy [7,8]. A major difficulty is that BC is a heterogeneous disease and the tumors are classified with respect to different intrinsic molecular subtypes. According to the St. Gallen 2011 and 2013 guidelines, a proxy for the molecular subtype classification based on immunohistochemical analysis and *in situ* hybridization of estrogen receptors, progesterone receptors, Ki67 and human epidermal growth factor receptor 2 (HER2) can be used to divide tumors into four or five main subtypes: luminal A-like, luminal B-like (HER2-positive or HER2-negative), HER2-positive (non-luminal) and triple-negative breast cancer [9,10].

The treatment-of-choice for BC is surgery, followed by loco-regional radiation therapy (RT). In addition, most patients receive adjuvant chemotherapy and/or hormone therapy. The rationale for this approach is to eradicate residual micrometastatic disease [11]. However, RT has important toxic effects, including dermatitis, asthenia and breast pain, that severely affect the patients' quality-of-life and, when they are severe can cause the temporary suspension of treatment [12]. The severity of these effects can be influenced by extrinsic factors such as radiation dose, volume, fraction size and administration technique, and by intrinsic factors such as genetic background, breast size and geometry, age, and tobacco use [13]. Some studies suggest that trace elements may play a role in these toxic reactions, but these aspects have been under-investigated [14].

The aim of the present study was to investigate the effects of a number of trace elements in BC patients and their relationships with the intrinsic molecular subtypes of tumors. The toxicological effect of RT was also evaluated in relation to these elements and the molecular subtypes.

**Participants and methods**

*Participants*

All procedures were approved by the Ethics Committee of our Hospital (Institutional Review Board), and written informed consent was obtained from all participants. We studied 49 women with BC recruited from among those attending the Department of Radiation Oncology of the *Hospital Universitari de Sant Joan* following surgical extirpation of the tumor. All of them had a Karnofsky Index >70 and were classified as 0 or 1 on the Eastern Cooperative Oncology Group scale [15]. The exclusion criteria included having previously received RT at the same anatomical site, or to be pregnant or breastfeeding. In the present study, 8 patients received adjuvant chemotherapy, 21 received adjuvant hormone therapy, 15 received both treatments, and 5 received the RT alone. The adjuvant chemotherapy treatment duration was for 4 to 5 months, and concluded 1 to 2 months before starting RT. The adjuvant hormone treatment commenced 1 to 2 months post-surgery and, usually, was administered simultaneously with RT. Thirty patients received normofractionated RT (50 Gy at 2 Gy/day on the affected breast and 16 Gy at 2 Gy/day on the tumor bed for 5 days/week). Nineteen patients received hypofractionated RT (40 Gy at 2.67 Gy/day for 5 days/week ) [16,17]. Some patients received irradiation of regional lymph nodes, according to risk factor status [18,19]. During RT, a weekly acute toxicity assessment was performed using the criteria of the Radiation Therapy Oncology Group and the European Organization for Research and Treatment of Cancer [20].

The control group was composed of 49 healthy women participating in a population-based study conducted in our geographical area. They were ostensibly healthy individuals with no clinical or analytical evidence of infectious diseases, renal insufficiency, hepatic damage, neoplasia, oligophrenia, or dementia. A detailed description of this population has been published recently [21].

Blood samples were obtained after an overnight fast. EDTA-plasma samples from patients and controls were randomly chosen from our collections in the Biological Sample Bank of our Institution.

*Trace element analyses*

The concentrations of trace elements were determined by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer Sciex-Elan 6000, Wellesley, MA, USA). Prior to analysis, an aliquot (1.2 mL) of each sample was digested with 1 mL of $HNO_3$ (65% Suprapur, Merck, Darmstadt, Germany) in a Milestone Start D Microwave Digestion System (Sorisole, Italy) for 35 min at 190ºC. After cooling, the digested mixture was made up to 10 mL with ultrapure water and stored at -20ºC until batched elemental analyses. The levels of the following 26 elements were analyzed in each extract: Al, As, B, Ba, Be, Bi, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Sb, Sc, Se, Sm, Sn, Sr, Ti, Tl, V, and Zn. A detailed description of the method has been published previously [22,23]. The percentage recoveries for the different trace elements ranged between 74 and 142%. The detection limits of the assays (mg/L) were as follows: Al: 0.25; As: 0.01; B: 0.1; Ba: 0.005; Be: 0.005; Bi: 0.0025; Cd: 0.0025; Co: 0.005; Cr: 0.005; Cu: 0.005; Fe: 0.5; Hg: 0.01; Mn: 0.005; Mo: 0.005; Ni: 0.01; Pb: 0.005; Sb: 0.01; Sc: 0.005; Se: 0.1; Sm: 0.005; Sn: 0.005; Sr: 0.01; Ti: 0.05; Tl: 0.0025; V: 0.05; Zn: 0.1.

*Histological analyses*

The expressions of the estrogen and progesterone receptors, Her2, and Ki67 antigens were analyzed on pre-surgical biopsy materials by immunohistochemistry, using an automated Autostainer Link 48 (Dako, Agilent Technologies, Santa Clara, CA, USA). Technical details on the procedures used are presented in Supplementary Table 1.

*Statistical analyses*

All statistical analyses were performed by GraphPad Prism software 6.00, SPSS 22.0 package (IBM Corp. Armonk, NY, USA) and MetaboAnalist 3.0. Differences between groups were assessed by the Student's *t*-test and correlations between variables were determined by Pearson's test. We also used principal component analysis (PCA), and the variable importance in projection (VIP) score to evaluate the relative magnitude of observed changes [24]. Linear regression analyses were fitted to evaluate the variables that were independently associated with selected trace element concentrations. Differences were considered statistically significant when the *p* value was <0.05. Results are expressed as median and interquartile range.

**Results**

*Clinical characteristics of patients*

The main clinical and biochemical characteristics of the BC patients and their tumors are shown in Table 1. Patients attending the Department of Radiation Oncology have already had their tumors operated upon. The incidence of smoking and alcohol intake was lower than in the control women, but with a similar incidence of arterial hypertension and diabetes. In the majority of cases the tumors were classified as luminal A or B, were positive for estrogen and progesterone receptors, relatively small in size, and had not developed metastases.

*Alterations in the plasma concentrations of trace elements*

The plasma concentrations of Al, As, Ba, Be, Bi, Cd, Co, Cr, Hg, Mn, Mo, Ni, Pb, Sb, Sc, Se, Sm, Sn, Ti, Tl, and V were below the detection limit of the assay in a significant proportion of patients and controls (≥ 50%) and, consequently, were excluded from further statistical analysis. Results of the remaining 5 elements are depicted in Fig. 1. BC patients showed significantly lower pre-RT concentrations of B, Cu, and Zn, and significantly higher concentrations of Sr than women in the control group. These differences were maintained when adjusted for smoking habit and alcohol intake (Supplementary Table 2). Patients and controls had similar plasma Fe concentrations. Irradiation was associated with a striking increase in plasma B concentrations, while Cu, Fe, Sr, and Zn concentrations did not show any significant difference with respect to the pre-irradiation levels; albeit Sr and Zn showed a non-significant trend towards an increase.

The 3-D score plot of the PCA analysis showed that the pre-RT values of trace elements in patients were very different to those of the control women, while the post-RT values were closer to those of the control women than to the pre-RT values (Fig. 2A). Figure 2B shows the results of the VIP score. This score measures a variable’s degree of alteration associated with the disease i.e. higher VIP scores are considered more relevant in classification. The current analysis identified B as the element that presented the greatest changes in relation to RT.

Patients who had received adjuvant hormone therapy had significantly higher plasma B concentrations than those who did not, and patients who had received chemotherapy had significantly higher Cu concentrations than those who did not. We did not observe any other significant association between trace element concentrations and hormone therapy or chemotherapy (Supplementary Tables 1 to 4).

**Table 1.** Clinical characteristics and selected biochemical variables in the control group and in breast cancer patients

| | | Control group n = 49 | Patients n = 49 | *P*-value |
|---|---|---|---|---|
| **Demographic and clinical characteristics** | | | | |
| Age; years | | 46 (43-60) | 57 (50-65) | 0.728 |
| Smoking habit; n (% yes *vs.* no) | | 10 (20.4) | 2 (4.1) | <0.001 |
| Alcohol intake; n (% some *vs.* none) | | 10 (20.4) | 2 (4.1) | <0.001 |
| Surgical procedure | | | | |
| | Lumpectomy | N.A. | 39 (79.6) | |
| | Mastectomy | N.A. | 10 (20.4) | |
| Tumor size; TNM system | | | | |
| | 1 | N.A. | 23 (46.9) | |
| | 2 | N.A. | 21 (42.9) | |
| | 3 | N.A. | 4 (8.2) | |
| | 4 | N.A. | 1 (2.0) | |
| Nodes; TNM system | | | | |

| | | | |
|---|---|---|---|
| | N0 | N.A. | 31 (63.3) |
| | N1 | N.A. | 15 (30.6) |
| | N2 | N.A. | 3 (6.1) |
| | N3 | N.A. | 0 |
| Metastases; TNM system | | | |
| | M0 | N.A. | 46 (93.9) |
| | M1 | N.A. | 3 (6.1) |
| Estrogen receptors | | | |
| | Negative | N.A. | 10 (20.4) |
| | Positive | N.A. | 39 (79.6) |
| Progesterone receptors | | | |
| | Negative | N.A. | 18 (36.7) |
| | Positive | N.A. | 31 (63.3) |
| Ki67 antigen | | | |
| | <15% | N.A. | 23 (46.9) |
| | 15-50% | N.A. | 20 (40.9) |
| | >50% | N.A. | 6 (12.2) |
| HER2 receptors | | | |
| | Negative | N.A. | 35 (71.4) |
| | Positive [a] | N.A. | 14 (28.6) |
| Tumor intrinsic molecular subtypes | | | |
| | Luminal A | N.A. | 16 (32.7) |
| | Luminal B | N.A. | 15 (30.6) |
| | HER2 positive | N.A. | 13 (26.5) |
| | Triple negative | N.A. | 5 (10.2) |
| **Adjuvant therapy** | | | |
| | Adjuvant chemotherapy [c] | N.A. | 8 (16.3) |
| | Adjuvant hormone therapy [d] | N.A. | 21 (42.9) |
| | Chemotherapy + hormone therapy | N.A. | 15 (30.6) |
| | None | N.A. | 5 (10.2) |
| **Toxic effects of radiation therapy** | | | |
| Dermatitis | | | |
| | G1 | N.A. | 15 (30.6) |
| | G2 | N.A. | 31 (63.3) |
| | G3 | N.A. | 3 (6.1) |
| Breast pain | | | |
| | No | N.A. | 6 (12.2) |
| | Yes | N.A. | 43 (87.8) |
| Asthenia | | | |
| | No | N.A. | 20 (40.8) |
| | Yes | N.A. | 29 (59.2) |
| Other toxicities [b] | | | |
| | No | N.A. | 20 (40.8) |
| | Yes | N.A. | 29 (59.2) |

[a] Patients receiving anti-HER2 treatment (trastuzumab); [b] Abscess, arthromyalgia, edema, folliculitis, hyperpigmentation, mastitis or neurotoxicity; [c] Anthracyclines, 1 patient; taxanes, 5 patients; anthracyclines + taxanes, 17 patients; [d] Tamoxifen, 13 patients; aromatase inhibitors, 21 patients; tamoxifen + aromatase inhibitors, 2 patients. Values are presented as absolute numbers and percentages or as medians and interquartile ranges: HDL: high-density lipoprotein; LDL: low-density lipoprotein; N.A.: not applicable; TNM: tumor, node, metastases

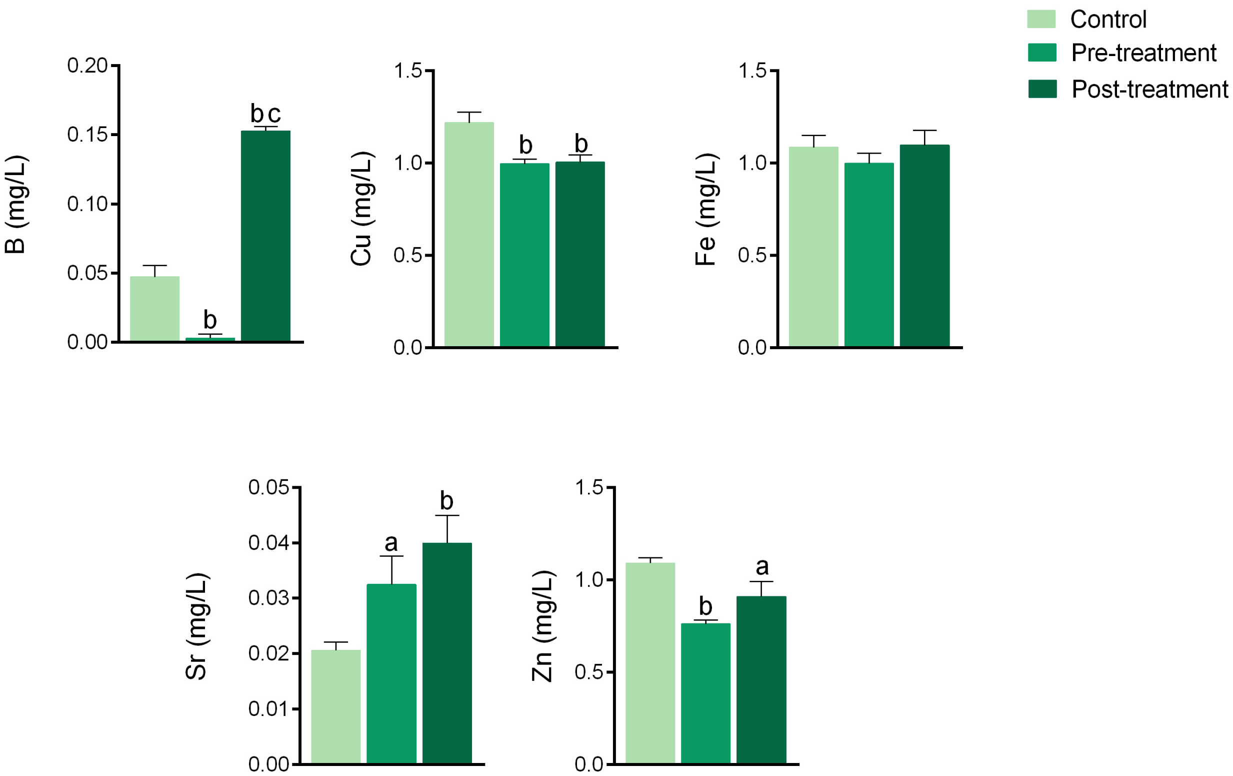


**Fig. 1** Plasma concentrations of trace elements in the control group and breast cancer patients pre- and post-radiation therapy. a=$p < 0.05$ and b=$p < 0.01$, with respect to the control group; c=$p < 0.001$, with respect to pre-radiation therapy

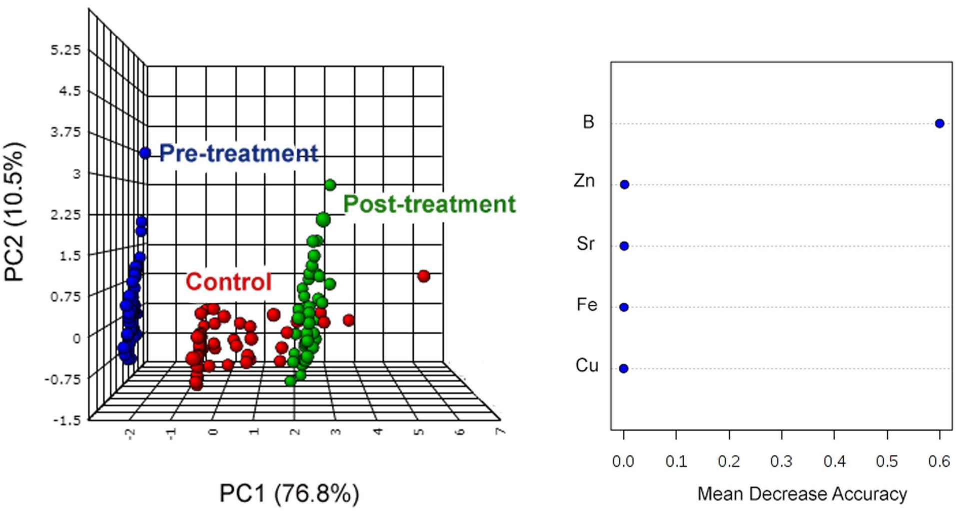


**Fig. 2** (A) 3-D score plot of the principal component analysis (PCA) for the selected trace elements measured. The PCA constructs a linear transformation that chooses a new coordinate system for the original data-set in which the largest variance of the data-set is captured on the first axis (PC1) and the second largest variance is the second axis (PC2). (B) Variable importance in projection (VIP) score identifies the level of importance of variables in classifying the data. The plot shows

each variable on the y-axis, and its importance on the x-axis. The variables are ranked top-to-bottom as most- to least-important. Therefore, the most important variable is at the top and an estimate of its importance is given by the position of the dot on the x-axis. The more the accuracy model created decreases (due to the exclusion or permutation) of a single variable, the more important that variable is deemed. Hence, a variable with a large mean decrease in accuracy is more important for classification of the data. The current analysis identified B as the most relevant trace element associated with alterations observed in breast cancer patients pre- and post-radiation therapy.

*Associations between trace elements and the molecular characteristics of tumors*

Pre-RT, patients with tumors negative for estrogen or progesterone receptors had significantly higher concentrations of Sr. Triple negative patients had significantly lower concentrations of Cu. Patients with tumors negative for HER2 receptors had significantly lower concentrations of Sr. Patients with Ki67 expression >50% had higher concentration of Fe (Fig. 3). Post-RT, patients with tumors negative for progesterone receptors had significantly lower concentrations of Cu. Patients with tumors negative for estrogen receptors had significantly lower concentrations of B. Patients with Ki67 expression >50% had significantly lower concentrations of Fe, Zn, and B (Fig. 4).

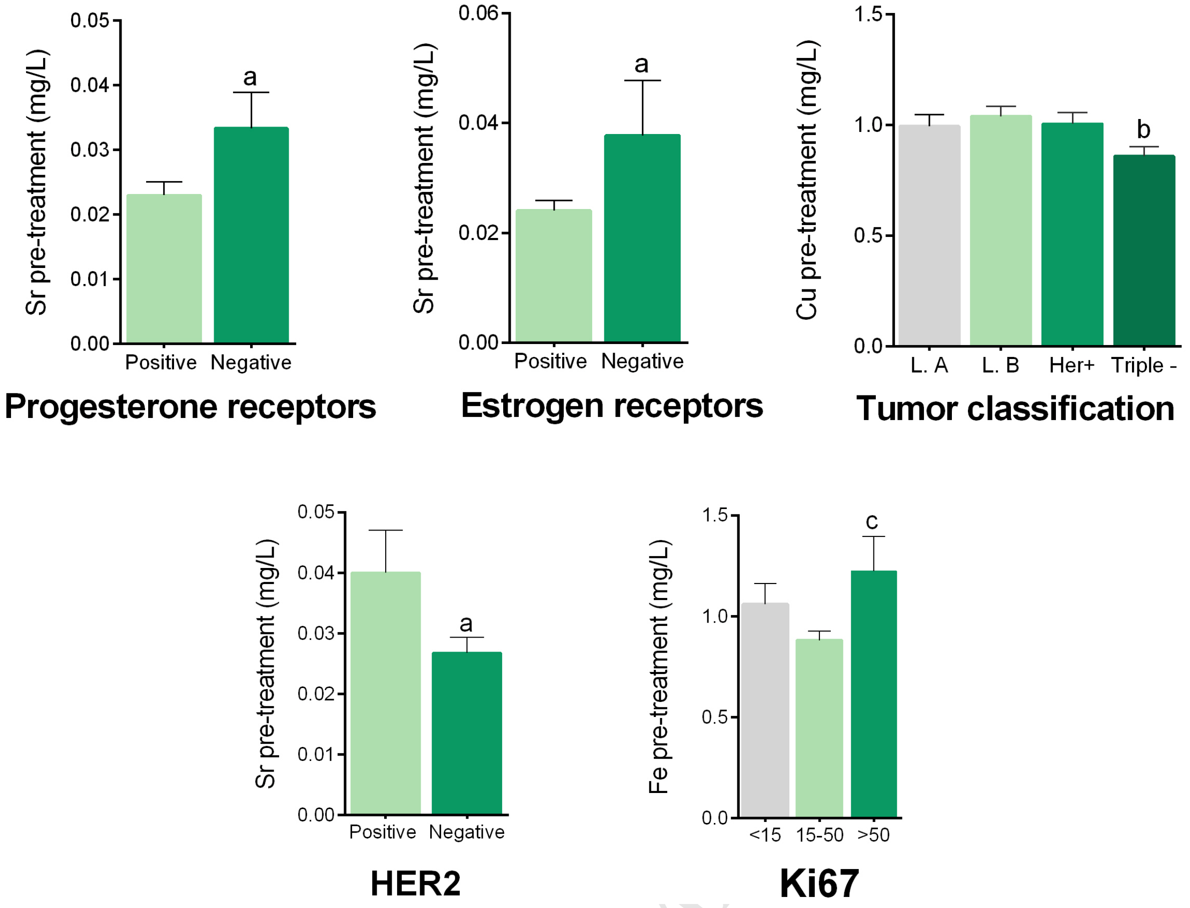


**Fig. 3** Plasma concentrations of trace elements pre-radiation therapy, and the molecular characteristics of breast tumors; a=$p < 0.05$, with respect to positive; b=$p < 0.05$, with respect to luminal B (L. B); c=$p < 0.05$, with respect to patients with Ki67 antigen expression <15%

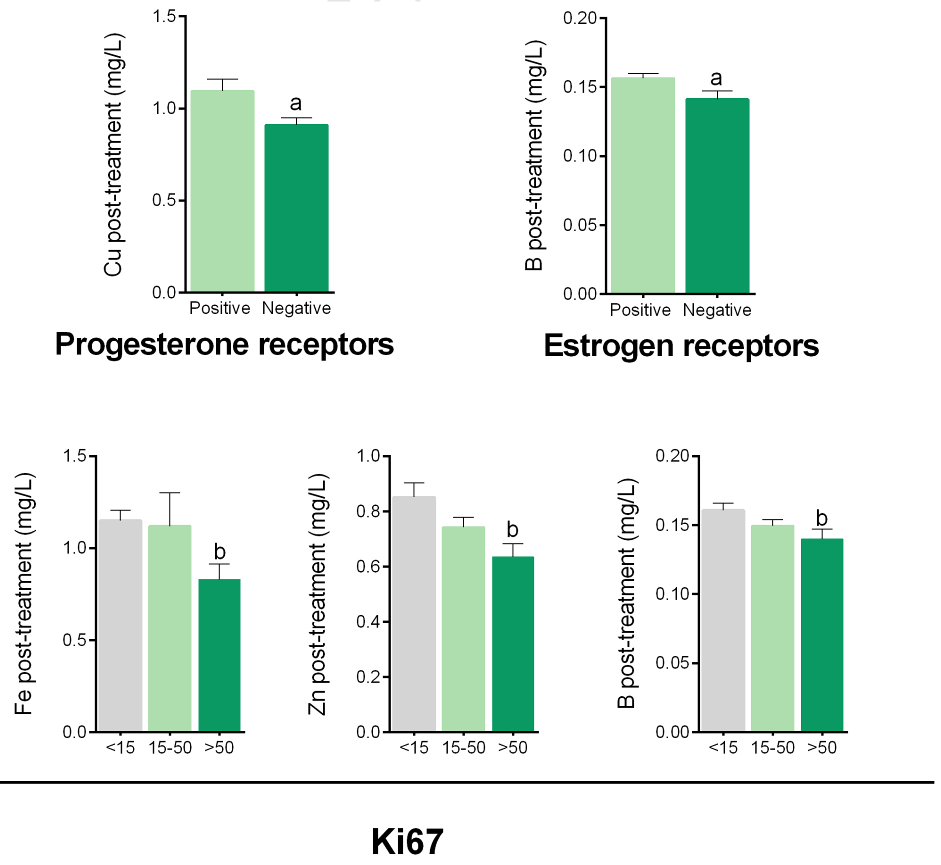

**Fig. 4** Plasma concentrations of trace elements post-radiation therapy, and the molecular characteristics of breast tumors; a=p < 0.05, with respect to positive; b= p < 0.05, with respect to patients with Ki67 antigen expression <15%

*Associations between trace elements and the toxicological effects of RT*

Cu concentrations pre-RT were slightly, but significantly, higher in patients with asthenia and lower in patients with breast pain (Fig. 5). Zn concentrations post-RT were lower in patients with severe dermatitis (Grades G2 and G3) compared with patients with mild dermatitis (Grade 1). Breast pain was associated with lower Fe and Zn concentrations post-RT (Fig. 6).

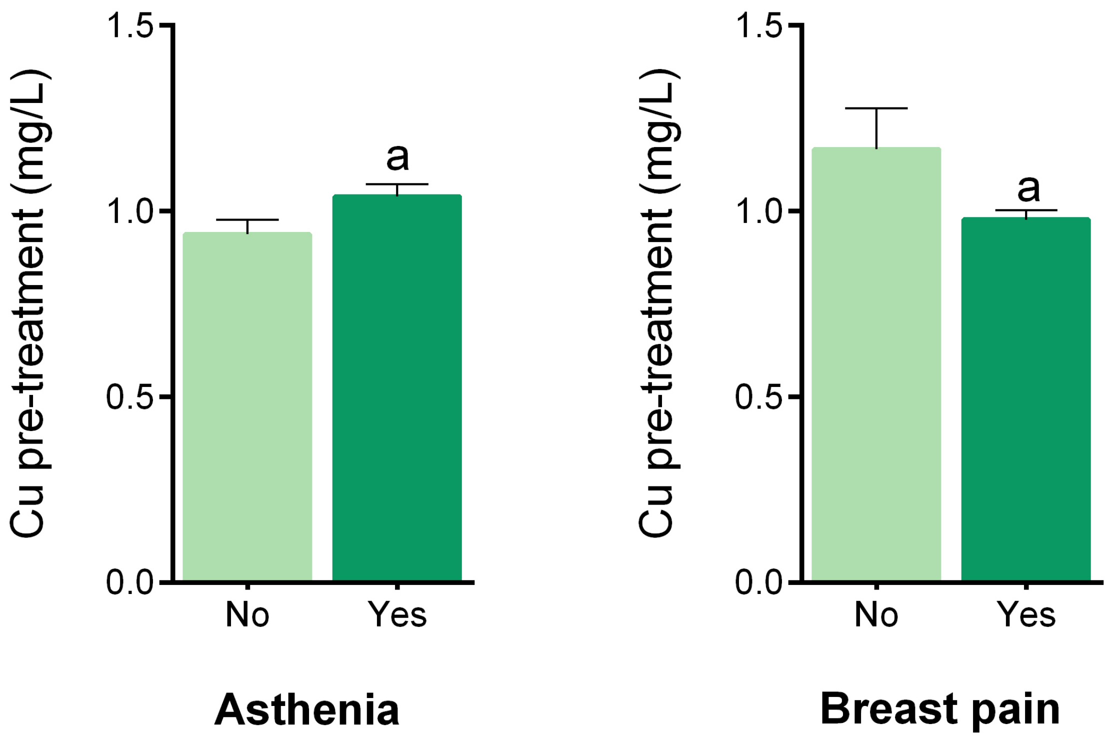


**Fig. 5** Plasma concentrations of trace elements pre-radiation therapy, and the toxicological response; a=$p$ < 0.05, with respect to patients without asthenia or breast pain.

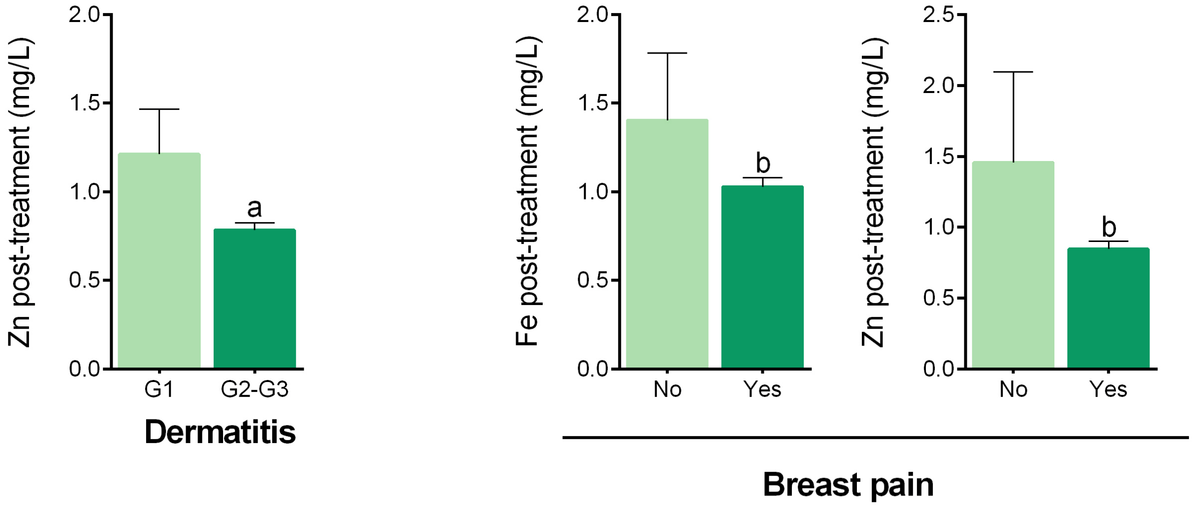


**Fig. 6** Plasma concentrations of trace elements post-radiation therapy, and the toxicological response; a=$p < 0.05$, with respect to G1; G1: Faint erythema or desquamation; G2: Moderate-to-brisk erythema or patchy, moist desquamation confined to skin folds and creases, moderate swelling; G3: Confluent, moist desquamation >1.5 cm diameter, which is not confined to the skin folds; Pitting edema (severe swelling); b=$p < 0.05$, with respect to patients without breast pain.

## Discussion

In this study, we found significant decreases in B, Cu, and Zn, and a significant increase in Sr concentrations in BC patients pre-RT compared with women in the control group. RT was associated with a significant increase in B and with non-significant trends towards increases in Sr and Zn concentrations, relative to pre-RT levels.

Alterations in B concentrations were the most important of all the trace elements analyzed. The concentrations of B were close to, or below, the limit of detection of the assay in BC patients pre-RT but were increased to three times the average of normal values post-RT. Why RT influences B concentrations in such a drastic way is an evident question, the answer to which may be convoluted since only meager information is available on the metabolism and physiological function of B in humans. B is ingested in the diet, essentially through the plants of the *Dicotyledoneae* class, and studies suggest that is present in circulation associated with unknown specific transporters. Organoboron compounds are widely distributed in the organism, and some critical biomolecules have a high affinity for this trace element, including S-adenosyl-methionine and nicotinamide adenine dinucleotide [25]. Experimental studies of B deprivation in animals have shown that this trace element is indispensable for cell replication, and its deficiency is associated with bone and central nervous diseases, and cancer [26]. In our study, patients with the lowest B levels post-RT were those with the highest expression of Ki67 antigen (a marker of tumor proliferation). The clinical relevance of these findings needs a perspective. For example, studies suggest that B has anti-inflammation properties [27-30]. A clinical trial in healthy volunteers found significant decreases in serum C-reactive protein and tumor necrosis factor-$\alpha$ after B supplementation [27]. Further, growing evidence suggests that B possesses anti-carcinogenic properties. B-rich diets are associated with significantly lower risk for prostate and cervical cancer, and to decreased risk of lung cancer in women with the smoking habit. Recently, natural and synthetic B-containing compounds have been used increasingly as anti-cancer agents, particularly in inoperable cancers and those with high malignancy. B-containing compounds interfere with the physiology and reproduction of cancer cells through several different mechanisms, including inhibition of serine proteases, inhibition of angiogenesis, mRNA splicing and cell division, receptor binding mimicry, and induction of apoptosis [31-33]. A recent study found that radiation-induced skin reactions were significantly lower in BC patients using a topical B-based gel [34]. This effect is probably local since, in the current study, we did not observe any significant association between plasma B concentrations and dermatitis in our patients.

Several authors have reported circulating levels of Zn to be decreased in BC patients [35-37]. The present results confirm these observations. Decreased serum Zn concentrations are associated with increased intra-tumor levels of this metal, and this has pathophysiological implications [36,37]. The intracellular levels of Zn are regulated through the coordinated expression of Zn transporters, which modulate Zn influx and efflux [38,39]. For example, LIV-1 is an estrogen-regulated protein that plays a role in cancer growth and metastasis [40,41]. Intracellular Zn activates Zn-dependent metalloproteinases which catalyze the breakdown of the extracellular matrix, and are involved in tumor proliferation, invasion, angiogenesis and metastasis [36,37,39]. In the present study, the levels of Zn showed a tendency towards an increase post-RT, while the patients who presented the lowest levels were those with tumors expressing higher levels of the Ki67 antigen. Patients with lower concentrations of Zn also had more severe toxic reactions to RT, including more extensive dermatitis and chest pain. This observation of low levels of Zn in the circulation (probably associated with higher intratumoral levels) could result from more avid uptake of Zn from the circulation by the tumor tissue. Taking into account the detrimental effects of this phenomenon, the option of nutritional supplementation with Zn in these patients should be undertaken with caution.

We observed that plasma Sr concentrations were increased in BC patients, and that RT was associated with a further increase in this parameter. There is a paucity of published data on Sr status in BC, but an early study reported that urinary Sr levels were higher in BC patients than in healthy individuals; this association being particularly strong in Her2-positive patients [42]. Indeed, our study found higher Sr concentrations pre-RT in Her2-positive BC patients. In addition, evidence exists that increased levels of Sr are pro-inflammatory and pro-atherogenic. For example, Sr ranelate is an osteogenic drug employed in the treatment of osteoporosis, but has important side effects such as venous thrombosis, pulmonary thromboembolism, and coronary heart disease [43]. Radiation has been reported to accelerate the atherosclerosis process, resulting in early onset of coronary artery disease [44]. Perhaps the increase in plasma Sr concentrations induced by RT is involved in the biochemical alterations underlying this complication. This hypothesis is of considerable clinical interest and warrants further investigation.

One of the aims of the present study was to investigate the relationships between plasma trace element concentrations and the molecular characteristics of tumors, since the latter are an estimate of the aggressiveness of the cancer and/or the difficulty of effective treatment. Also of interest are the relationships of trace elements with the toxicological response to RT. Patients positive for estrogen receptors had lower plasma B post-RT and higher plasma Sr pre-RT concentrations, while patients positive for progesterone receptors had higher Cu post-RT and lower Sr pre-RT than patients with hormone-negative tumors. One possible explanation is that these metals act as metalloestrogens, or endocrine disruptors. Metalloestrogens are metals that display hormonal functionality similar to estrogens, and affect estrogen receptor signaling [8]. Metalloestrogens increase uterine weight (including hypertrophy of the endometrial lining), enhance mammary tissue density *in vivo*, and activate and/or increase the expression of estrogen and progesterone receptors *in vitro* [45-47]. As such, metalloestrogens have been considered potential links to BC [48]. Metalloestrogen functions of Al and Cd have been better characterized, while such functions are less clear for Cu and Zn [8]. We observed associations between several trace elements and other features of the most aggressive and/or difficult-to-treat tumors. For example, patients with triple negative tumors had lower plasma Cu pre-RT, patients with Her2-positive tumors had higher Sr pre-RT, and patients with tumors with a Ki67 antigen expression >50% had lower B, Fe, and Zn than patients with a Ki67 antigen expression <50%.

The present study has several limitations. The studied group was small and heterogeneous in terms of systemic treatment schemes and RT regimes, and the immunohistochemical characteristics of the tumors differed from those found in the general BC population. We had a high percentage of HER2 positive patients, and the fraction of patients having received chemotherapy was relatively low. In addition, when patients attended the Department of Radiation Oncology they already had undergone surgery, so we could not investigate the effect of

RT on gross tumor rather than in suspected microscopic disease. Due to these considerations, our results should be considered preliminary. Of note, however, is that there is a large increase in the plasma concentration of B post-RT, and that the circulating levels of several trace elements are associated not only with the pathological characteristics of the tumors but with post-RT toxicity as well. To the best of our knowledge of the literature, these data are novel, and open to new lines of research into the relationships between trace elements and BC.

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**Conflict of interest:** None

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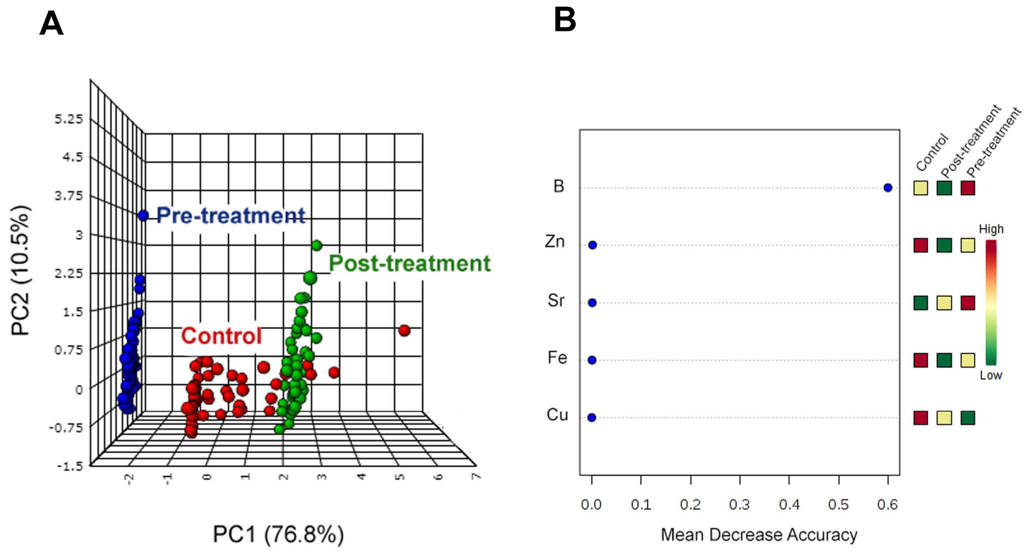
A
Pre-treatment
Post-treatment
Control
PC2 (10.5%)
PC1 (76.8%)
B
Control
Post-treatment
Pre-treatment
B
Zn
Sr
Fe
Cu
High
Low
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Mean Decrease Accuracy

**Highlights**

- Breast cancer patients have alterations in plasma trace element concentrations
- Radiotherapy was associated with an increased plasma boron concentration
- Trace elements were associated with the molecular characteristics of tumors
- Trace elements are related to the toxicological response to radiation therapy