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PRECLINICAL STUDY

Differential oxidative status and immune characterization of the early and advanced stages of human breast cancer

C. Panis · V. J. Victorino · A. C. S. A. Herrera · L. F. Freitas · T. De Rossi · F. C. Campos · A. N. Colado Simão · D. S. Barbosa · P. Pinge-Filho · R. Cecchini · A. L. Cecchini

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Abstract Breast cancer is the malignant neoplasia with the highest incidence in women worldwide. Chronic oxidative stress and inflammation have been indicated as major mediators during carcinogenesis and cancer progression. Human studies have not considered the complexity of tumor biology during the stages of cancer advance, limiting their clinical application. The purpose of this study was to characterize systemic oxidative stress and immune response parameters in early (ED; TNM I and II) and advanced disease (AD; TNM III and IV) of patients diagnosed with infiltrative ductal carcinoma breast cancer. Oxidative stress parameters were evaluated by plasmatic lipoperoxidation, carbonyl content, thiobarbituric reactive substances (TBARS), nitric oxide levels (NO), total radical antioxidant parameter (TRAP), superoxide dismutase, and catalase activities and GSH levels. Immune evaluation was determined by TNF-α, IL-1β, IL-12, and IL-10 levels and leukocytes oxidative burst evaluation by chemiluminescence. Tissue damage analysis included heart (total CK and CKMB), liver (AST, ALT, GGT), and renal (creatinine, urea, and uric acid) plasmatic markers. C-reactive protein (CRP) and iron metabolism were also evaluated. Analysis of the results verified different oxidative stress statuses occur at distinct cancer stages. ED was characterized by reduction in catalase, 8-isoprostanes, and GSH levels, with enhanced lipid peroxidation and TBARS levels. AD exhibited more pronounced oxidative status, with reduction in catalase activity and TRAP, intense lipid peroxidation and high levels of NO, TBARs, and carbonyl content. ED patients presented a Th2 immune pattern, while AD exhibited Th1 status. CRP levels and ferritin were increased in both stages of disease. Leukocytes burst impairment was observed in both the groups. Plasma iron levels were significantly elevated in AD. The data obtained indicated that oxidative stress enhancement and immune response impairment may be necessary to ensure cancer progression to advanced stages and may result from both host and tumor inflammatory mediators.

Keywords Breast cancer · Immune response status · Oxidative stress · Inflammation

Abbreviations

AD	Advanced breast cancer patients
ED	Early disease breast cancer patients
TNM	Tumor node metastasis classification
SOD	Superoxide dismutase
GSH	Reduced glutathione
TCA	Trichloric acetic acid
TRAP	Total antioxidant capacity
ABAP	2,2′azobis
RLU	Relative light unities
NO	Nitric oxide
TBARS	Thiobarbituric reactive substances
MDA	Malondialdehyde

C. Panis · V. J. Victorino · A. C. S. A. Herrera · L. F. Freitas · T. De Rossi · F. C. Campos · R. Cecchini · A. L. Cecchini (✉)
Laboratório de Patologia e Radicais Livres, Departamento de Patologia Geral—Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná 86051-990, Brazil
e-mail: alcecchini@uel.br

A. N. C. Simão · D. S. Barbosa
Hospital Universitario, Departamento de Farmacia, Universidade Estadual de Londrina, Londrina, Paraná, Brazil

P. Pinge-Filho
Laboratório de Imunopatologia, Departamento de Patologia Geral—Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná, Brazil

DNPH	Dinitrophenylhydrazine
AUC	Area under the curve
LDL	Low density lipoprotein
CL	Chemiluminescence
TNF-α	Tumor necrosis factor alpha
ER	Estrogen receptors
PR	Progesterone receptors
HER-2	Human epidermal growth factor receptor
CAT	Catalase
GSH	Reduced glutathione
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GGT	Gamma glutamyl transpeptidase
CK	Creatine kinase
CKMB	Creatine kinase MB fraction
URCA	Uric acid levels
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-12	Interleukin 12
IL-10	Interleukin 10

Introduction

Breast cancer is the most frequent malignant neoplasia among women worldwide [1] and presents high rates of death in developing countries [2–4].

Several studies support findings that reactive species (RS) are involved in the etiology and progression of breast cancer because certain markers of oxidative stress, including DNA adducts [5] and lipid peroxidation products, such as malondialdehyde [6, 7] and 8-isoprostanes [8], are frequently identified in breast cancer patients.

Some studies suggest that breast cancer leads to iron metabolism imbalance that results in plasma iron overload and additional oxidative stress [4, 9, 10]. In addition to this evidence, increases in C-reactive protein and iron-binding protein are associated with iron-driven inflammation in the breast environment, leading to the activation of an inflammatory process in premalignant cancer stages [11].

Chronic activation of the immune response results in the accumulation of regulatory T cells and activated B lymphocytes that secrete progrowth factors, such as interleukins 4 and 10, which enhance tumor tolerance and progression [12]. Once Th2 status is established, the burst capacity and immune functions of innate immune cells, such as macrophages and neutrophils, could be impaired.

Interestingly, most studies involving the characterization of oxidative stress and inflammatory markers in breast cancer are based on culture cells or experimental data. Human studies regarding this issue do not consider the possible differential biology of tumor stages and have frequently employed low sensitivity techniques and the evaluation of few parameters in the same patient. This study was developed to characterize the systemic oxidative and immune status in patients with breast cancer in early and advanced stages of disease.

Methods

Patient selection and study design

A total of 60 women were recruited at Londrina Cancer Institute from January 2009 and September 2010 and 30 healthy women volunteers composed the control group. This study was approved by the National Research Ethics Council (CAAE 0009.0.268.000-07), all practices were approved by the institutional board and all patients signed a term of free, informed consent. Patient characterization included age at diagnosis, weight, comorbidities, TNM classification, and hormonal status. Patients were divided in early breast cancer disease (ED; $n = 30$) and advanced breast cancer disease (AD; $n = 30$). The control group consisted in health women, aged 30 to 77 years old, with no previous history of any type of cancer, chemotherapy, hormonal or antioxidant therapy, or chronic diseases. The ED group consisted of women with malignant breast tumor in early stages of disease (TNM stages I and II), with no previous chemotherapy infusion. The AD group was formed of women with malignant breast tumor in advanced stages of disease (TNM stages IIIc and IV). Women were excluded if they were current smokers, presented hepatic, cardiac or renal dysfunction, obesity, use of drugs, hypertension, sedentarism, diabetes, and other eventual chronic conditions.

Sample acquisition and biochemical analysis of tissue damage markers

Whole blood was collected in EDTA tubes then divided for biochemical, antioxidant, and oxidative analyses. For the last two, the samples were centrifuged, the separated plasma was immediately frozen at −80°C and erythrocytes were immediately processed for enzymatic and GSH analysis. Hemograms were performed in an automatized Coulter. Biochemical analysis was performed by measurement of plasmatic levels of creatine-kinase MB fraction (CKMB), uric acid, urea, creatinine, aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), gamma-glutamyl-transpeptidase (GGT), total bilirubin (TBIL), ferritin, plasma iron, and C-reactive protein. Analyses were automatically performed in Dimension RxL® (Dade-Behring—Siemens Corp.).

Antioxidant parameters

Total radical antioxidant parameter (TRAP) of plasma was determined as previously described [13]. Erythrocytic catalase and SOD activities were determined as described by Aebi [14] and Marklund and Marklund [15]. Erythrytic GSH levels were measured as previously described [16].

Oxidative parameters

Plasma lipoperoxidation [17] was evaluated in a Glomax luminometer (TD 20/20 Turner Designers) and nitrite levels were measured as an estimate of NO levels and determined as previous described by Panis et al. [18]. The TBARS assay estimates MDA levels [19], which was performed in a spectrophotometer (Shimadzu UV-1650PC). Carbonyl protein contents were determined as previously described by Panis et al. [20] and total proteins were also measured [21]. The plasma levels of free 8-iso-prostanes F_2 were quantified using a competitive immunoenzymatic kit (Cayman Chemical).

Cytokines levels

TNF-α, interleukin 1 (IL-1), IL-10, and IL-12 plasmatic levels were determined using a commercial antibody-specific RSG ELISA kit (eBiosciences, USA) and analyzed with a ELISA microplate reader at 490 nm. The results were calculated in pg/ml.

Leukocytes oxidative burst evaluation

Real-time kinetics of superoxide anion production stimulated by phorbol-myristate (PMA) was performed [22]. Chemiluminescence was monitored in a Glomax luminometer (TD 20/20 Turner Designers). The results were expressed in relative light units (RLU).

Statistical analysis

CL curves were compared using two-way analysis of variance (ANOVA). SOD, glutathione, carbonyl proteins, and total radical trapping antioxidant parameter (TRAP) were compared by the Student's unpaired *t* test. $P < 0.05$ was considered significant. All statistical analyses were performed using GRAPHPAD PRISM version 5.0 (GRAPHPAD Software, San Diego, CA), comparing all groups with controls.

Results

The study involves a population sample characterized by the clinicopathological parameters presented in Table 1.

Table 1 Clinicopathological characterization of patients

Age at diagnosis (years)	ED	AD
<40	2 (10%)	3 (10%)
41–49	1 (5%)	3 (10%)
50–59	12 (60%)	17 (56.67%)
>50	5 (25%)	7 (23.33%)
Median	54.2	56.7
Range	37–66	39–72
Histological type		
Ductal	20 (100%)	30 (100%)
Lobular	None	None
Mixed	None	None
Molecular receptors status		
ER	70%	75%
PR	60%	62%
HER-2/neu	58%	59%
TNM classification		
I stage	2 (10%)	None
II stage	18 (90%)	None
III stage	None	4 (13.33%)
IV stage	None	26 (86.67%)

ED early disease group, *AD* advanced disease group, *ER* estrogen receptors, *PR* progesterone receptors, *HER-2/neu* human epidermal growth factor receptor, *TNM* tumor, node, metastasis classification

Most of the ED patients were classified as TNM stage II (90%) and AD patients as TNM stage IV (86.67%). The entire population studied was diagnosed according to the histological type of breast cancer disease (100%), infiltrative ductal carcinoma.

Oxidative status, antioxidant parameters, and biochemical analyses are presented in Table 2. Catalase activity presented a decline in both ED (525.8 ± 17.14 abs/min/ml; $P < 0.05$) and AD (527.6 ± 11.96 abs/min/ml; $P < 0.05$) cancer groups, while GSH levels varied only in patients with ED (11.83 ± 1.87 nmols/l; $P < 0.05$). Renal parameters, represented by urea, creatinine, and uric acid levels, did not show any alteration in the ED group. Uric acid levels were significantly higher in the AD group (4.68 ± 0.22 mg/dl; $P < 0.05$). The hepatic profile, evaluated by AST, ALT, GGT, and total bilirubin levels, showed high levels in patients with advanced disease (61.06 ± 10.37, 53.03 ± 6.46 and 142.9 ± 30.86 U/L, $P < 0.05$, respectively). GGT also exhibited a discrete elevation in the ED group (28.1 ± 4.33 U/L, $P < 0.05$). The results of cardiac damage evaluation was reflected in increased CK-MB levels in AD patients (5.39 ± 1.39 U/L, $P < 0.05$). Plasma iron levels were significantly higher only in the AD group (125.1 ± 12.86 μg/dl, $P < 0.05$), while ferritin measurement demonstrated a significant increase in both the ED (147 ± 42.87 mg/dl; $P < 0.05$) and the AD (523.6 ± 155.6 mg/dl; $P < 0.05$) cancer groups.

Table 2 Oxidative status, antioxidant parameters, and biochemical analysis

	Control	ED group	AD group
SOD (U/L)	1835 ± 81.15	1194 ± 76.94	1531 ± 127.3
GSH (nmols/l)	17.07 ± 1.37	11.83 ± 1.87*	17.16 ± 1.56
CAT (abs/min/ml)	562.6 ± 9.83	525.8 ± 17.14*	527.6 ± 11.96*
Urea (mg/dl)	29 ± 1.37	32.25 ± 1.98	29.58 ± 1.69
Creatinine (mg/dl)	0.92 ± 0.02	0.89 ± 0.08	0.86 ± 0.04
Uric acid (mg/dl)	3.7 ± 0.21	4.14 ± 0.23	4.68 ± 0.22*
AST (U/L)	26.16 ± 1.37	30.1 ± 2.76	61.06 ± 10.37*
ALT (U/L)	33.62 ± 1.68	36 ± 2.39	53.03 ± 6.46*
Bilirubin (mg/dl)	0.55 ± 0.027	0.43 ± 0.089	0.50 ± 0.063
GGT (U/L)	17.83 ± 2.07	28.1 ± 4.33*	142.9 ± 30.86*
CK (U/L)	71.82 ± 6.02	44.43 ± 8.28	67.87 ± 8.99
CKMB (U/L)	1.3 ± 0.34	1.78 ± 0.81	5.39 ± 1.39*
Plasma iron (μg/dl)	89.17 ± 9.5	112.3 ± 19.1	125.1 ± 12.86*
Ferritin (mg/dl)	50 ± 3.16	147 ± 42.87*	523.6 ± 155.6*

Results are represented as mean standard errors of the mean. *SOD* superoxide dismutase activity, *GSH* reduced glutathione; *CAT* catalase activity; *TRAP* total radical antioxidant parameter; *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma glutamyl transferase, *CK* total creatine kinase, *CKMB* MB fraction of creatine kinase, *LDH* lactate dehydrogenase, *CRP* C reactive protein; *CTR* control group; *ED* early disease group (patients with breast cancer, TNM I and II); *AD* advanced disease group (patients with breast cancer, TNM IIIc and IV). * indicate statistical difference when related to control ($P < 0.05$)

Plasmatic lipoperoxidation (Fig. 1a) showed an increase in both the cancer groups, AD (33928 ± 6557 URL) and ED (16494 ± 322 URL), compared to the control group (11423 ± 1251 URL). NO levels (Fig. 1b) were significantly increased (21.21 ± 1.78 μM) in the AD group. The MDA results (Fig. 2a) were elevated in both the ED (155.8 ± 30.46 nmols/l) and the AD (166.7 ± 19.86 nmols/l) groups compared to controls (101.6 ± 7.06 nM), while a significant decrease in 8-isoprostanes levels (Fig. 2b) was observed in ED patients (143.2 ± 0.46 pg/ml) compared to controls (144.7 ± 0.29 pg/ml). Carbonyl content (Fig. 2c) was significantly increased in AD patients (9.92 ± 0.5 nmols/ml/mg protein) compared to the control group (8.55 ± 0.47 nmols/ml/mg protein). Figure 3a shows the TRAP of all the three groups analyzed, though no significant difference occurred between them, but when TRAP was expressed in relation to uric acid levels (Fig. 3b), a significant reduction was observed in AD patients (66.72 ± 6.2 nM trolox/mg × dl^{-1}) in relation to controls (113.6 ± 12.02 nM trolox/mg × dl^{-1}). Reduced leukocytes oxidative burst (Fig. 4a) was observed in ED ($3.76 \times 10^6 \pm 1.26 \times 10^6$) and AD ($4.16 \times 10^6 \pm 1.5 \times 10^6$) patients compared to the control group ($9.75 \times 10^6 \pm 1.2 \times 10^6$). Figure 4b shows that C-reactive protein levels were increased in both the ED (5.62 ± 2.24 mg/dl) and the AD (6.51 ± 1.31 mg/dl) groups compared to the control group (0.66 ± 0.18 mg/dl).

AD patients presented high levels of TNF-α (23.85 ± 6.22 pg/ml), compared to the control group (9.47 ± 1.55 pg/ml) (Fig. 5a) and IL-1β for AD patients was increased (13.35 ± 3.74 pg/ml) compared to the control group (5.72 ± 1.74 pg/ml) (Fig. 5b). ED patients presented lower levels of TNF-α (2.57 ± 0.76 pg/ml) compared to controls (9.47 ± 1.55 pg/ml) (Fig. 5a) and a decrease in IL-12 levels (29.79 ± 1.33 pg/ml) compared to controls (33.4 ± 0.89 pg/ml). No alterations were observed in IL-10 levels among the groups analyzed (Fig. 5d).

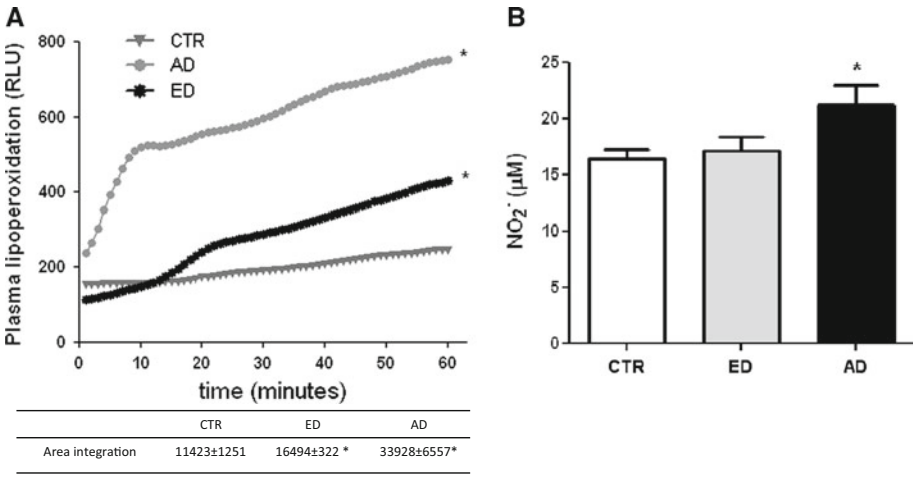
Discussion

For the first time, this study provides a profile of oxidative stress and immune response parameters in two groups of women diagnosed for breast cancer tumors during the different stages of progression of the disease. Systemic oxidative damage, cytokine levels, and leukocyte burst were analyzed.

Several reports present evidence that breast cancer patients exhibit enhanced levels of lipid peroxidation [4, 6, 23], while some evidence indicates a reduction in lipid peroxidation metabolites as a potential regulatory mechanism against breast cancer [8]. Experimental and clinical data regarding lipidic metabolite generation in other types of cancers also show conflicting results [24–27].

The antioxidant status in breast cancer patients demonstrated an increase in antioxidant defenses, including GSH levels, previously suggested as a response to enhanced lipoperoxidation [23, 28–30].

Patients with ED exhibited a reduction in IL-12 levels, a key mediator of antitumor response and necessary for



	CTR	ED	AD
Area integration	11423±1251	16494±322 *	33928±6557*

Fig. 1 Plasmatic lipoperoxidation and NO levels. **a** Emission profile of lipoperoxidation in plasma. **b** Nitrite plasmatic levels measured as estimative of NO levels. Means were evaluated by Student's unpaired *t* test. *CTR* control group, *white bars*; *ED* early disease group (patients with breast cancer, TNM I and II), *gray bars*; *AD* advanced disease group (patients with breast cancer, TNM IIIc and IV), *black bars*. * Indicates statistical difference when related to control ($P < 0.05$)

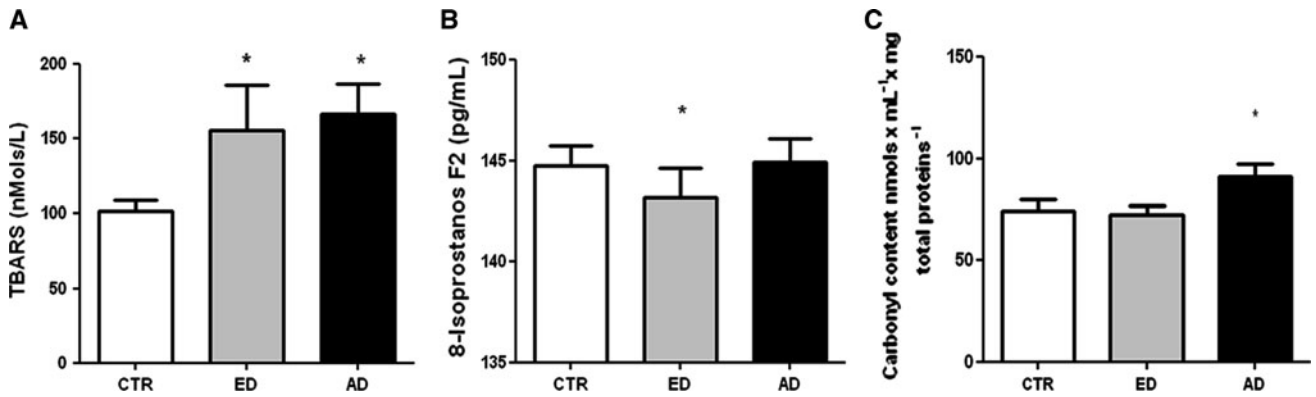


Fig. 2 TBARS, 8-isoprostanes, and carbonyl proteins levels. **a** MDA plasmatic levels, **b** 8-isoprostanes levels, and **c** carbonyl content were estimated as indicative of oxidative products formation during oxidative stress. Means were evaluated by Student's unpaired *t* test. *CTR* control group, *white bars*; *ED* early disease group (patients with breast cancer, TNM I and II), *gray bars*; *AD* advanced disease group (patients with breast cancer, TNM IIIc and IV), *black bars*. * Indicates statistical difference when related to control ($P < 0.05$)

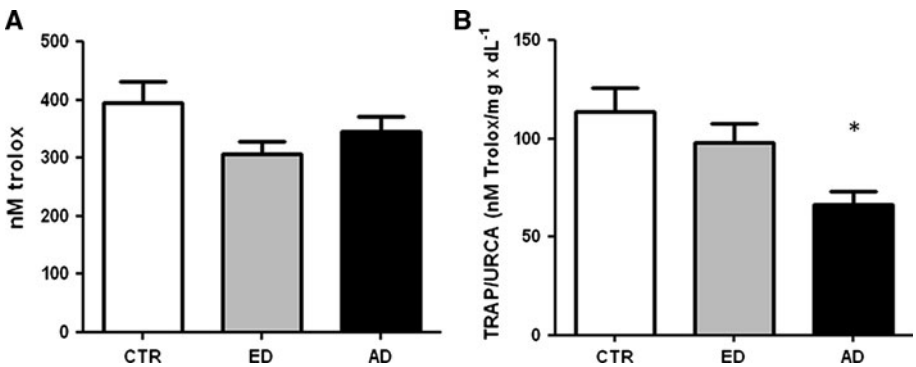


Fig. 3 Total radical antioxidant parameter in plasma. **a** TRAP was measured as estimative of total antioxidant capacity of plasma. **b** TRAP–uric acid (URCA) ratio. Means were evaluated by Student's unpaired *t* test. *CTR* control group, *white bars*; *ED* early disease group (patients with breast cancer, TNM I and II), *gray bars*; *AD* advanced disease group (patients with breast cancer, TNM IIIc and IV), *black bars*. * Indicate statistical difference when related to control ($P < 0.05$)

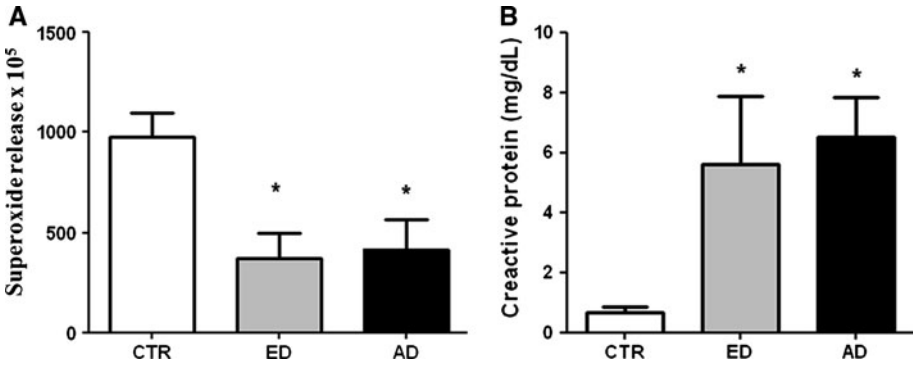


Fig. 4 Superoxide anion release and C reactive protein levels. **a** Area under curve integration as indicative of real-time superoxide anion release in whole blood during PMA challenge. **b** C reactive protein levels as indicative of inflammatory activity. Means were evaluated by Student's unpaired *t* test. *CTR* control group, *white bars*; *ED* early disease group (patients with breast cancer, TNM I and II), *gray bars*; *AD* advanced disease group (patients with breast cancer, TNM IIIc and IV), *black bars*. * Indicate statistical difference when related to control ($P < 0.05$)

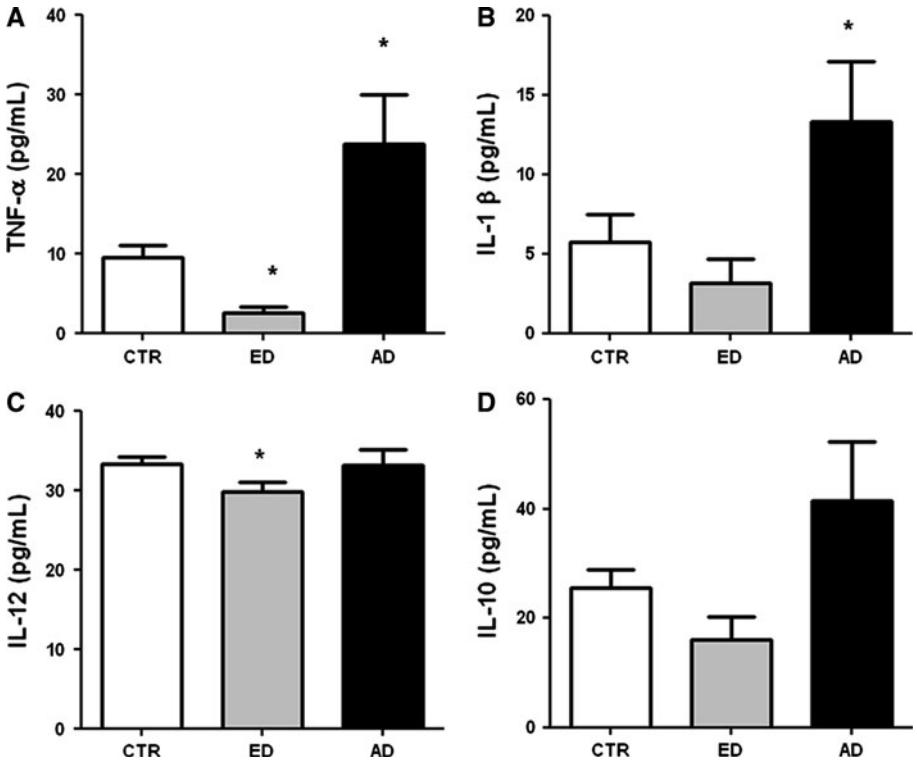


Fig. 5 Cytokines profile. Pro-inflammatory cytokines TNF-α (**a**), IL-1β (**b**), and IL-12 (**c**), as well the anti-inflammatory IL-10 (**d**) plasmatic levels were measured as indicators of immunological Th1/Th2 status. Means were evaluated by Student's unpaired *t* test. *CTR* control group, *white bars*; *ED* early disease group (patients with breast cancer, TNM I and II), *gray bars*; *AD* advanced disease group (patients with breast cancer, TNM IIIc and IV), *black bars*. * Indicate statistical difference when related to control ($P < 0.05$)

natural killer cell activation to achieve tumor cells lysis [12, 31–33].

AD patients showed enhanced lipoperoxidation, increased carbonyl protein content, high levels of NO and iron overload, as well as a reduction in catalase activity. These findings indicate that AD displays specific free radical oxidation involved in the propagation of inflammation consistent with cancer progression [34].

Significantly high levels of carbonyl proteins have been reported in the plasma of breast cancer patients [35] and within the breast microenvironment [36], suggesting that our findings could be associated with systemic oxidative damage to proteins and specific breast-derived carbonyl content. In addition to pro-oxidative markers, increased levels of C-reactive protein, TNF-α and IL-1β levels were also observed in the AD group, adding to the proinflammatory status. Data concerning the source of the inflammatory mediators and their role in human tumor development remain unclear [37–40]. Despite clinical data supporting the idea that systemic Th1 responses inhibit human tumor growth and predict improved patient outcome [41], a fairly consist body of evidence demonstrates that

TNF-α [42], IL-1β [43] and NO [44, 55] are mediators for tumor cells in favor of cancer invasiveness and progression.

Numerous studies corroborate our findings concerning the impairment of immunity in breast cancer patients [45–49]; however, these reports did not evaluated the difference among patients, grouping according to disease stages, as performed in this study.

Furthermore, AD patients exhibited high levels of plasma iron. Iron overload is extremely toxic because it catalyzes Fenton's reaction, providing the formation of hydroxyl radicals with lipoperoxidation [50] and tissue dysfunction by oxidative damage [51–53].

Similar to other studies, ferritin elevation was also detected in both stages of cancer disease. Normally considered a response to iron overload, it is frequently associated with poor prognosis in several human cancers as a consequence of Th1 cytokine chronic stimulation and liver damage, since the main source of this protein are hepatocytes [10, 11, 54]. Thus, our data showed that in AD, a sustained proinflammatory status occurs that could be amplified by plasma iron overload, promoting more inflammation and oxidative injury.

Some parameters were similar between AD and ED patients, such as MDA, catalase activity, GTT, and leukocytes burst impairment, suggesting that the maintenance of these parameters could be necessary to ensure disease progression. Together, these findings suggest that cancer metabolic changes in AD markedly enhance inflammation and oxidative stress, which exhibit a great potential to cause the systemic injury observed in this study.

In conclusion, the results obtained here contribute to the literature concerning human breast cancer and, for the first time, reveal a wide metabolic profile and the existence of differential inflammatory status between early and advanced stages of human breast cancer and add to the characterization of the immune response during host and tumor interactions. The study also provides new perspective regarding understanding the biochemical cancer behavior during advanced disease and highlights the need to develop clinical strategies for early detection and to contain cancer progression before it evolves to the life-threatening stages.

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Conflict of interest The authors declare there are no conflicts of interest.

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