Current Research in Immunology 4 (2023) 100059

Contents lists available at ScienceDirect

Current Research in Immunology

journal homepage: www.sciencedirect.com/journal/current-research-in-immunology

Excess body weight significantly affects systemic and tumor inflammatory status and correlates to poor prognosis parameters in patients with breast cancer

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ARTICLE INFO

Keywords:
Obesity
Breast cancer
Systemic inflammation

ABSTRACT

Introduction: Obesity is a pro-inflammatory disease critical for developing breast cancer (BC), which impacts the profiles of systemic inflammatory mediators and determinants of different disease clinical outcomes remains little explored.

Methods: A total of 195 patients diagnosed with breast cancer were included. Aiming to exclude chemotherapy interference on circulating mediators, samples were collected at diagnosis, out of the treatment period. Patients were classified as normal weight (BMI up to 24.9 kg/m2) or overweight (BMI ≥25.0 kg/m2). Serum levels of IL-4, IL-12, hydroperoxides, and nitric oxide metabolites (NOx) were measured. Also, tumor expression of inducible nitric oxide synthase (iNOS), TGF-β1, $CD4^+$, and $CD8^+$ lymphocytes were evaluated.

Results: IL-4 levels were significantly increased in the overweight BC group ($p = 0.0329$), including patients with luminal B subtype ($p = 0.0443$), presence of lymph node metastases ($p = 0.0115$) and age of diagnosis below 50 years, ($p = 0.0488$). IL-12 levels were significantly increased in overweight BC patients with lymph node metastases ($p = 0.0115$). Hydroperoxides were increased in overweight BC patients ($p = 0.0437$), including those with tumors smaller than 2 cm ($p = 0.05$). NOx levels were also increased in overweight BC patients, including those with luminal B disorders ($p = 0.0443$), high-grade tumors ($p = 0.0351$) and lymph node metastases ($p = 0.0155$). The expression of iNOS ($p < 0.001$) and TCD4+ lymphocytes ($p = 0.0378$) was significantly investigated in tumor biopsies from overweight BC women.

Conclusions: These data provide a picture of the influence of excess body weight on inflammatory mediators' systemic and tumoral profiles, especially in patients displaying poor outcome BC.

1. Introduction

Breast cancer (BC) is a public health problem of high proportions worldwide. It is the most frequent cancer among women worldwide and the most common cause of cancer death in this population (The American Cancer Society, 2021; WHO, 2020). Among the various risk factors associated with BC development and prognosis, excess body weight has been pointed out as a determinant of poor disease prognosis. Increased mortality, worse survival, growth of larger tumors, greater lymph node invasion, metastasis development, increased recurrence, and lower response to treatment have been widely reported in such with obesity. (Barone et al..,2020; Biglia et al.., 2013; Chan et al.., 2014; Ewertz et al.., 2011; Hao, S. et al.., 2015; Kawai, M. et al.., 2012; Pang, Wei, Kartsonaki, 2022; Protani, Coory, Martin, 2010; Raman et al.., 2016; Vaysse, Muller, Fallone, 2019). The obesogenic state affects breast tumor cells and their surrounding microenvironment. Four mechanisms have been proposed to explain its role in the genesis and promotion of breast cancer, including 1) production of estrogen hormones through aromatization of androgen hormones, 2) insulin signaling and insulin-like growth factor 1 (IGF-1); 3) altered levels of adipokines, adiponectin,

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https://doi.org/10.1016/j.crimmu.2023.100059
Received 6 February 2023; Received in revised form 20 April 2023; Accepted 28 April 2023

leptin, and 4) chronic low-grade adipose systemic inflammation and oxidative stress (Avgerinos et al.., 2019). Therefore, adipose tissue has the potential to produce and sustain chronic inflammation, which seems to be the critical link between obesity and cancer (Crujeiras et al., 2013).

Although obesity consists of a pro-inflammatory disease and is considered a critical agent for BC development (Bhardwaj and Brown, 2021; Olefsky and Glass, 2010; Rech, D. et al.., 2021) the impact of the obesogenic state on the regulation of inflammatory responses triggered against BC, as well as its influence on the clinical determinants of the disease prognosis remains to be understood (Zhang et al.., 2020; Wang, Z. et al., 2019). Studies have not considered the influence of pro-inflammatory confounding conditions, such as its behavior under specific clinicopathological features determinant of BC outcome. Also, most of the studies analyze samples of patients under chemotherapic treatment or do not mention this in their design, which limits the interpretation of obtained data.

Aiming to investigate the impact of excess body weight on the systemic and tumor inflammatory profiles of patients diagnosed with BC, we analyzed the profile of cytokines and oxidative stress mediators before chemotherapy in both tumor and blood samples. To understand the clinical meaning of such findings, we correlated them to clinicopathological features that are determinants of disease prognosis by comparing normal and overweight BC patients.

2. Methods

2.1. Study design

The Institutional Ethics Committee approved this proposal under CAAE 35524814.4.0000.0107, opinion nº 810.501. All patients included signed the informed consent terms. This study was carried out at Hospital do Cancer Francisco Beltrão (CEONC, Paraná-Brazil), screening patients attended from May 2015 to January 2022. A total of 605 women with suspicious BC were screened, and a total of 195 were diagnosed with unilateral infiltrating ductal carcinoma and entered the study. To determine the trophic-adipose status of patients, the body mass index (BMI) calculation was used (BMI = weight (kg)/height x height (m)). All patients with a BMI equal to or less than 24.9 kg/m2 were categorized as normalweight, and the remaining patients were included in the overweight group. Clinicopathological data were collected from medical records and included molecular BC subtype (analyzed individually or grouped as high aggressiveness – triple-negative + luminal B or low aggressiveness – other subtypes); tumor size (< 2 cm and ≥2 cm), histological tumor grade (low grade: 1 and 2, high grade: 3); age at diagnosis (< 50 years or ≥ 50 years) and risk stratification of death and recurrence according to Goldhirsch et al.., 2011. The schematic design of the study is showed in Fig. 1.

2.2. Measurement of the systemic inflammatory profile: cytokines and oxidative stress levels

Heparinized peripheral blood samples (10 mL) were collected, and plasma was obtained by centrifugation (4000 rpm for 5 min). Samples were frozen until analysis. Interleukin 4 (IL-4) and interleukin 12 (IL-12) were measured by using commercial kits based on the enzyme-linked immunoassay method, following manufacturers' instructions (e-Biosciences, USA). The limit of detection for the kit is 2 pg/mL. Hydroperoxides were measured by high-sensitivity chemiluminescence (QL), adapted from Gonzalez-Flecha et al., 1991 by Panis et al., (2012). Aliquots of 125 μL of plasma were added to 855 μL of monobasic phosphate

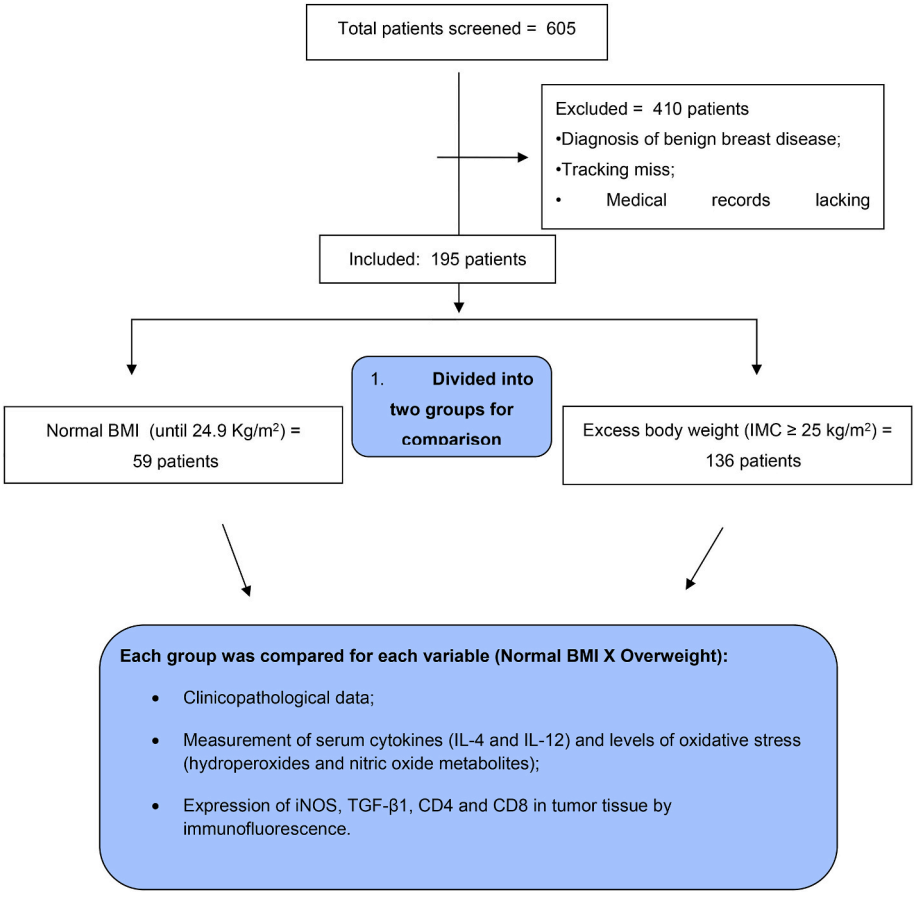

Fig. 1. Design of the study. The study was designed following the STROBE Guideline (https://www.equator-network.org/reporting-guidelines/strobe/). BMI = Body Mass Index.

buffer (10 mM, pH 7.4). The reaction was started by adding 20 μL of tert-butyl solution (3 mM) and monitored in a Glomax 20/20 luminometer (Promega, USA) to evaluate the photon emission profile. The results obtained by integrating the emission curve were expressed in relative light units (RLU). To measure nitric oxide metabolites (NOx), we used the cadmium-copper reaction and total nitrite detection by the Griess method, as described by Panis et al. Briefly, aliquots of plasma (60 μL) were deproteinized, and the supernatant was incubated with cadmium granules in a copper sulfate solution. After adding the Griess reagent, samples were read at 550 nm in a spectrophotometer. Results were expressed as μM.

2.3. Tumor expression of iNOS, TGF-β1, CD4 and CD8 lymphocytes by immunofluorescence

After deparaffinization, tumor samples were incubated with specific primary antibodies against iNOS (catalog number 610329), TGF-β1 (catalog number 562545), CD4 (catalog number 555344) and CD8 lymphocytes (catalog number 550372), followed by a secondary immunofluorescent antibody (Alexa Fluor 488 for iNOS and TGF-β1, and Texas Red for CD4 and CD8 + T lymphocytes). All antibodies were tested and standardized before their use. Sections were counterstained with 4, 6 diamidinophenylindole (DAPI) for nucleus labeling (Sigma Aldrich, St. Louis, MO, USA). Images were captured on a Motic BA410E fluorescence microscope coupled to a MOTICAM ProS5 Plus camera and Motic Images Plus 3.0 ML image acquisition and processing software. Slides were read using a DAPI excitation filter at 200 or × 400 magnification. The following adjustments have been preset: auto exposure, +20 gain, and zero offsets. The obtained images were stored in BMP format, with a 2048 × 1536 pixels resolution.

2.4. Data analysis

The clinical-pathological variables of the patients were categorized and tabulated in Microsoft Excel® spreadsheets. The frequencies of the categories of each variable were compared for patients belonging to both groups using the chi-square test for adherence, considering a 5% statistical significance. Data were analyzed using GraphPad Prism .9.0 and SPSS 25.0.0 software. Data distribution was tested using the Shapiro-Wilk test. Thus, variables with normal distribution were analyzed using parametric tests. When the assumption of normality was not met, non-parametric tests were used. Accordingly, Student's t-test or Mann-Whitney test was used to compare the two groups. In the results section, the data is shown as min-maximum. P values ≤ 0.05 were considered significant.

3. Results

The clinicopathological profile of the 195 patients divided according to the classification of normal BMI (up to 24.9 kg/m2) versus excess body weight (BMI ≥25.0 kg/m2) is presented in Table 1. The frequency of women with histological grade 3 was higher in the BC overweight patients compared to the normalweight ones (p = 0.0499).

Fig. 2 shows BC patients' systemic IL-4 according to BMI categories. IL-4 levels were increased in overweight BC patients compared to the normalweight BC patients (Fig. 2A, range: 10.88–29.71 pg/mL in normalweight patients and range:10.90–74.38 pg/mL in the overweight group, p = 0.0329). A significant increase in IL-4 was also observed in overweight BC patients carrying luminal B tumors (Fig. 2B, range: 11.84–74.38 pg/mL) compared to the normalweight BC patients (range: 14.39–26.20 pg/ml, p = 0.0443). Increased IL-4 was also observed in overweight BC patients with lymph nodal invasion (Fig. 2C, range: 5.14–74.38 pg/mL) compared to normalweight BC patients (range: 10.88–29.71 pg/mL, p = 0.0115). BC patients diagnosed before age 50 and overweight had higher levels of IL-4 (range: 16–51 pg/mL) than normalweight BC patients (Fig. 2D, range: 10–29 pg/mL, p = 0.0488).

Table 1
Frequency (n) and percentage (%) of clinicopathological variables considering BMI divided into normal and excess body weight (overweight and obesity) groups.

		Normal BMI	Excessive BMI	p-value
Molecular Subtype	Luminal B	20 (33,90)	43 (31,62)	0,7544
	Luminal – HER	1 (1,69)	9 (6,62)	0,1522
	HER2	8 (13,56)	16 (11,76)	0,7260
	triple negative	10 (16,95)	27 (19,85)	0,6347
Molecular subtype aggressiveness	Too much Subtypes	49 (83,05)	10[a] (80,15)	0,6347
	triple negative	10 (16,95)	27 (19,85)	0,6347
Tumor size (in cm)	< 2	23 (38,98)	37 (27,21)	0,1017
	≥ 2	36 (61,02)	99 (72,79)	0,1017
Histological grade	Grade 1	20 (33,90)	39 (28,68)	0,4659
	Grade 2	31 (52,54)	63 46,32)	0,4247
	Grade 3	8 (13,56)	34 (25,00)	**0,0499**
Grouped histological grade	Grade 1 ou 2	51 (86,44)	102 (75,00)	**0,0499**
	Grade 3	8 (13,56)	34 (25,00)	**0,0499**
Metastasis	No	29 (49,15)	83 (61,03)	0,1233
	Yea	30 (50,85)	53 38,97)	0,1233
Risk stratification	Low	3 (5,08)	6 (4,41)	0,8370
	Intermediary	29[a] (49,15)	71 (52,21)	0,6952
	High	27[a] (45,76)	59 (43,38)	0,7584
Family history	Not	28 (47,46)	66 (48,53)	0,8906
	Yes	31 (52,54)	70 (51,47)	0,8906
Age range at diagnosis (in years)	30–34	3 (5,08)	3 (2,21)	0,2849
	35–39	3 (5,08)	12 (8,82)	0,3681
	40–44	12 (20,34)	16 (11,76)	0,1168
	45–49	5 (8,47)	18 (13,24)	0,3437
	50–54	4 (6,78)	20 (14,71)	0,1217

For the other clinical-pathological parameters, no significant variations of IL-4 were observed (p < 0.05).

IL-12 (Fig. 3) was significantly augmented in overweight BC patients with lymph node metastases (Fig. 3B, range: 10.88–18.83 pg/mL for normalweight and 5.16–74.38 pg/mL overweight group, p = 0.0115). For the other clinical-pathological parameters, no significant variations of IL-12 were observed (p < 0.05).

The oxidative stress profile (Fig. 4) was assessed by measuring the systemic hydroperoxides and NOx levels. Increased hydroperoxide was found in overweight BC patients concerning the normalweight BC group (Fig. 4A, range: 382282–18865527 RLU, and range: 307457–3451196 RLU, respectively, p = 0.0437). Also, overweight BC patients with tumors smaller than 2 cm showed higher hydroperoxides when compared to normalweight BC patients with the same tumor size profile (Fig. 4B, range: 441312–2455082 URL and range:475943–6220347 URL, respectively, p = 0.05). No significant variations of hydroperoxides were observed for the other clinical-pathological parameters (p < 0.05).

There was an increase in NOx levels in overweight BC patients compared to normalweight BC patients (Fig. 5A). Also, patients with luminal B tumors had higher levels of NOx (Fig. 5B, range: 14.39–26.20 μM in eutrophic patients and range: 11.84–74.38 μM in overweight, p = 0.0443). Augmented NOx was also observed in overweight patients with high-grade tumors (Fig. 5C, range: 26.76–31.98 μM) compared to

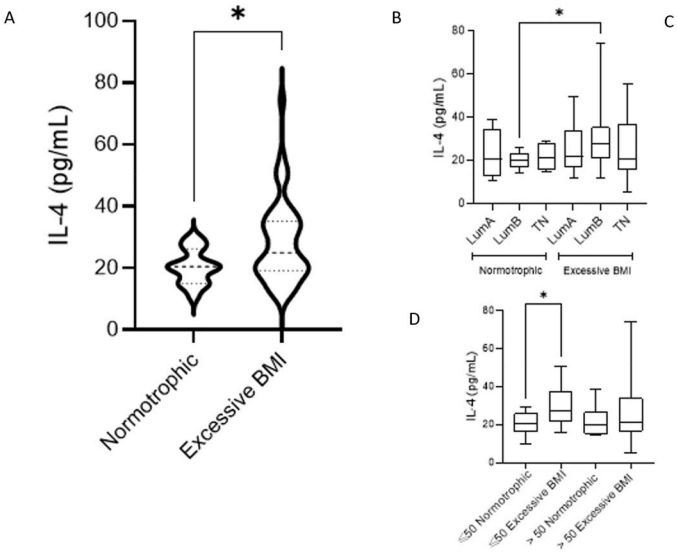

Fig. 2. Plasma levels of interleukin 4 (IL-4) in patients with breast cancer distributed according to BMI categories and clinicopathological criteria In A, overall IL-4 levels according to BMI categorization as eutrophic or overweight. In B, distribution of levels according to molecular subtype. In C, comparison of IL-4 levels according to the presence of metastases in lymph nodes (LN) and in D according to age at diagnosis (under or equal and over 50 years). LumA = luminal A, LumB = luminal B, TN = triple negative. * indicates statistical difference.

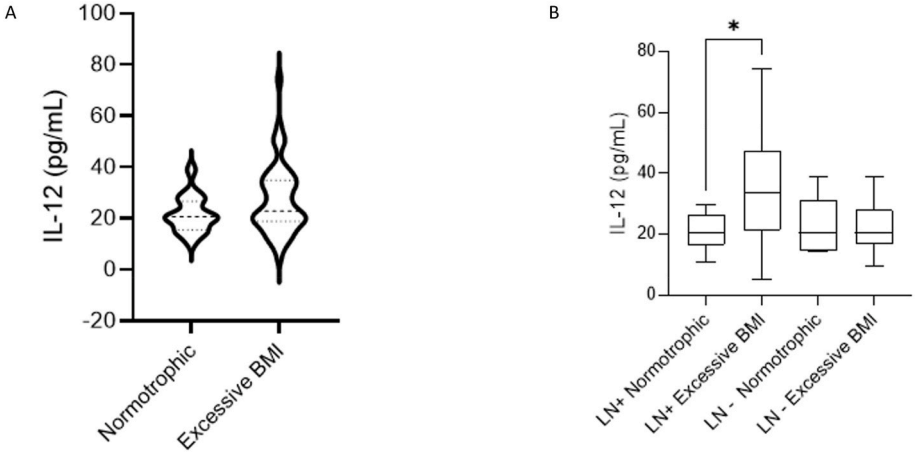

Fig. 3. Plasma levels of interleukin 12 (IL-12) in patients with breast cancer distributed according to BMI categories and clinicopathological parameters. In A, overall IL-4 levels according to BMI categorization as eutrophic or overweight. In B, distribution of levels according to the presence of metastases in lymph nodes (LN). * indicates statistical difference.

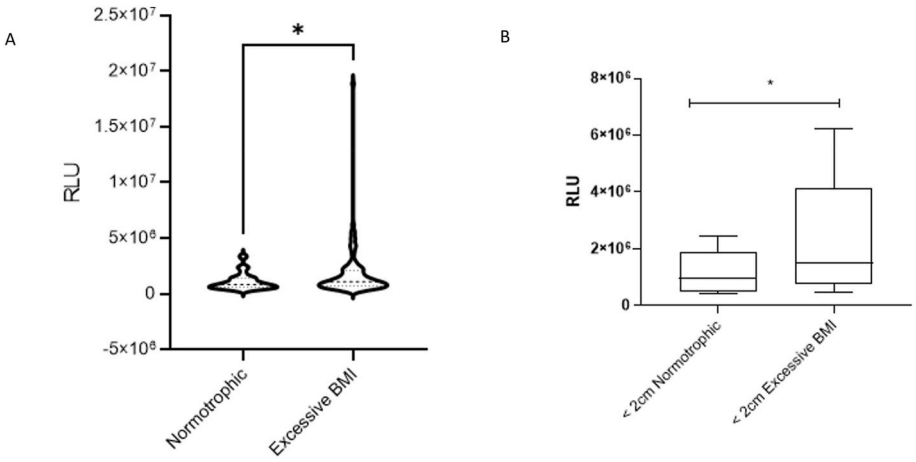

Fig. 4. Plasma levels of hydroperoxides in patients with breast cancer distributed according to BMI categories and clinicopathological parameters. In A, overall IL-4 levels according to BMI categorization as eutrophic or overweight. In B, distribution of levels according to tumor size. RLU = integral of the area under the curve measured in relative units of light. * indicates statistical difference.

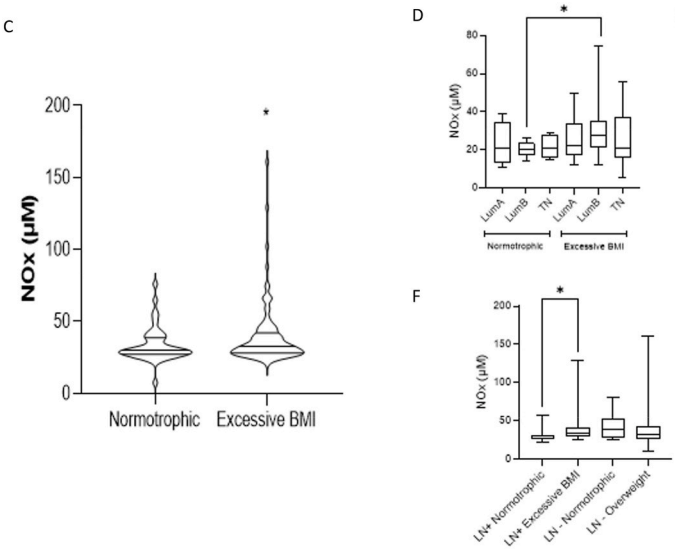

Fig. 5. Plasma levels of nitric oxide (NOx) metabolites in patients with breast cancer distributed according to BMI categories and clinicopathological parameters. In A, general NOx levels according to BMI categorization as eutrophic or overweight. In B, distribution of levels according to molecular subtype. In C, comparison of NOx levels according to histological grade. In D, NOx levels according to the presence of metpastases in the lymph nodes (LN). LumA = luminal A, LumB = luminal B, TN = triple negative. * indicates statistical difference.

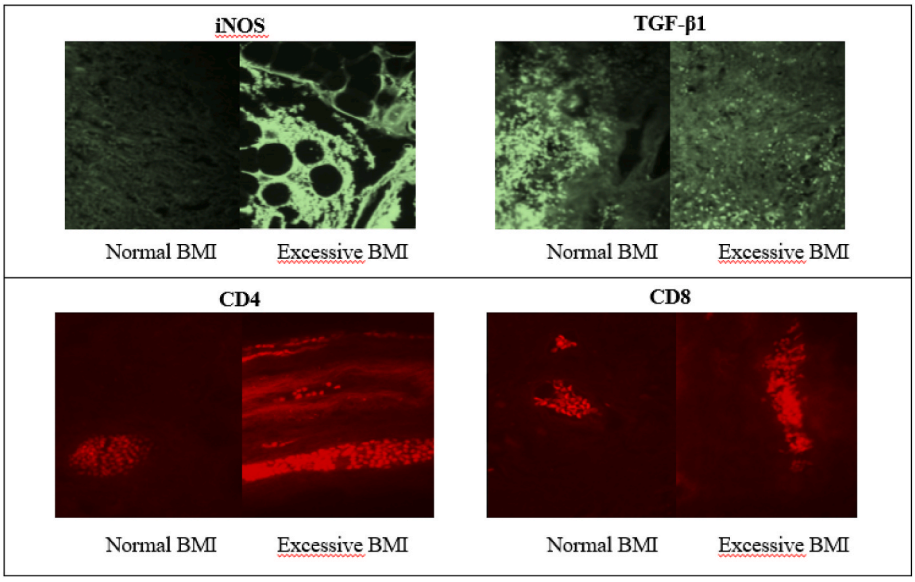

Fig. 6. - iNOS, TGF-β1, CD4 + T lymphocytes and CD8 + T lymphocytes expression profile in tumor biopsies from normal and excess body weight breast cancer patients. iNOs = inducible nitric oxide synthase, TGF-β1 = transforming growth factor beta 1, CD4 = $CD4^+$ T lymphocytes, CD8 = $CD8^+$ T lumphocytes, BMI = body mass index.

eutrophic patients (range: 23.28–102.2 µM, p = 0.0351). The same behavior was observed in the presence of lymph nodal metastases (Fig. 5D, range: 22.20–56.76 µM for the eutrophic group and range: 25.24–129.2 µM for the overweight group, p = 0.0155). For the other clinicopathological parameters, no significant NOx variations were observed (p < 0.05) (see Fig. 6).

For tumor tissue analyses (Fig. 5 and Table 2), iNOS and $CD4^+$ T lymphocytes had augmented expression in biopsies from BC patients with overweight compared to eutrophic women (iNOS: 66% vs. 90%, p < 0.001 and $CD4^+$ T lymphocytes: 20% vs. 34%, p = 0.0378, respectively).

Table 2
iNOS, TGF-β1, CD4 + **T lymphocytes** and CD8 + **T lymphocytes** profiles in tumor biopsies from normal and excess body weight breast cancer patients.

Marker	Normal BMI	Excessive BMI	Fisher's P value
iNOS	66%	90%	<0.001*
TGF-β1	68%	70%	>0.999
CD4 + T lymphocytes	20%	34%	0.0378*
$CD8^+$ T lymphocytes	25%	32%	0.2146

4. Discussion

In this study, we characterized some systemic and tumoral inflammatory mediators present in BC patients under poor prognosis conditions. Increased cytokines and oxidative stress markers were found in both systemic and tumor tissue samples of overweight BC patients compared to normalweight BC women out of the chemotherapic regimen. Our findings suggest that excessive BMI propitiates a disbalance in specific cytokines and oxidative stress markers, significantly changing tumor expression under poor prognosis conditions. The connection between obesity and BC is attributed to the sustained low-grade chronic systemic inflammation induced by excessive fat (Crujeiras et al..,2013) and the metabolic alterations caused by the adipose tissue

that modify the global immune responses and affect the cells of the tumor microenvironment (Wang et al., 2019.). Thus, excessive BMI may affect the systemic production of immune-related mediators and the tumor expression of such molecules to some extent.

The polarization patterns of effector $CD4^+$ T cells into Th1 - anti-tumor or Th2 - pro-tumor responses represent a crucial factor for tumor promotion in cancer (Basu et al..,2021). In this sense, we investigated two antagonistic cytokines, IL-4 and IL-12, which are keys in the differentiation of effector $CD4^+$ T cells, and also produced by adipose tissue cells (Basu, A. et al..,2021; Mclaughlin et al., 2017). In BC, the Th2 pattern is associated with developing tumors with worse prognosis and survival. (Paccagnella et al.., 2022; Abeen, et al., 2018). Even though reduced IL-12 has been reported in BC patients (Tzang et al.., 2020), we did not observe this change when considering the BMI categorization. This finding suggests that excessive BMI may change the dynamics of cytokine production already known for BC. Overweight BC patients clearly showed a pattern of high production of the Th2 cytokine IL-4. Increased circulating IL-4 was found in overweight BC patients bearing tumors with aggressive features as luminal B molecular subtypes, as well as in those with clinical characteristics of poor prognosis as lymph node metastasis and early age at diagnosis. It suggests that BC patients with excessive BMI incline the pro-tumor pattern and immune escape, which is a determinant for poor clinical outcomes. However, there are no studies in the literature that analyzed the dosages of standard Th1 and Th2 cytokines in the BC in the presence of excess weight. In this context, we further examined the cellular profile of tumor infiltrates for CD4 and CD8 lymphocytes. Breast tumor immune infiltrates are associated with the response to treatment and disease outcomes (Savas et al.., 2016). Th1 CD4 T cells are induced by IL-12 and promote the differentiation and expansion of CD8 T cells that are cytotoxic against tumor cells. In contrast, IL-4 polarizes CD4 T cells towards the Th2 pro-tumor pattern (Ostrand-Rosenberg, 2008). Experimental data indicate that the obesogenic environment significantly increases the percentage of intratumoral $CD4^+$ cells and reduces CD8 counting in BC (Elisia, 2020; Núñez-Ruiz A et al..,2022). Each increase of 10 units (kg/m2) in BMI in humans results in a 1.6% increase in $CD4^+$ infiltration in the BC tissue (Asad, Damicis, Heng, 2022), but no data about cytokines profile is available in this context. We found significantly increased intratumoral $CD4^+$ T cells in BC women with excessive BMI, concomitantly to increased circulating levels of IL-4, which suggests a Th2 profile that may be associated with poor prognosis disease.

Furthermore, we showed that BC-overweight women had more oxidative stress than normal weight, characterizing a pro-oxidative environment. Previous studies demonstrate high ROS levels in patients with estrogen-positive BC and obesity (Madeddu C. et al.., 2014) and suggest that the redox imbalance can be due to enhanced fatty acid oxidation (Dai, 2022). The lipolysis mechanism may be considered here, especially by adipokines such as leptin (Fruhbeck et al.., 2001) and adiponectin (Fruhbeck et al.., 2001b; Pulido et al.., 2011). Adipokines are known to induce oxidative changes in BC since the leptin derived from mammary tissue adipocytes enhances the oxidative activity of BC stem cells (Wang et al.., 2019) and is affected by the reduced production of adiponectin reported in BC patients (Panis et al.., 2014). Other factors not evaluated in our work can further affect the adipose tissue functioning and the inflammatory status of these patients. For example, metabolic hormones such as insulin are pivotal in adipose tissue dysfunction, modulating the profile of secreted adipokines and inflammatory mediators production (Rodríguez et al.., 2012; Frühbeck, 2005; Sabater et al.., 2010; Moreno-Navarrete et al.., 2013).

We also observed differences related to NOx production that implicated this mediator in poor prognosis BC. NO axis is dichotomous in BC, where low levels mediate protective responses, and high levels mediate tumor proliferation (Chang et al., 2015). Furthermore, high iNOs functioning increases NO in the tumor environment, enhancing tumor proliferation (Somasundaram, et al.., 2020). We observed augmented NOx production in BC patients with excess weight with more aggressive tumors such as the luminal B subtype, histological high-grade, and presence of lymph node metastasis, as well as the elevation of iNOs expression in tumor tissue samples from the excess BMI group.

Our study has limitations. Considering the impact of BMI on metabolic status, measuring glucose, leptin, and insulin levels, aiming to get information about patients' diabetes mellitus and metabolic syndrome profiles would be very useful. Also, the evaluation of cytokines production over time was not assessed since, after diagnosis, most of the patients underwent chemotherapy, which strongly affects the evaluated markers (Panis et al.., 2012; Campos et al.., 2014; Lemos et L., 2015; Panis et al.., 2017; Broto et al., 2021; Garbim et al.., 2022). The modest sample size and lack of long-term follow-up to assess the impact of excess BMI on characteristics such as disease-free survival, overall survival, and disease recurrence are also limitations. Despite this, the main strength of our data is the evaluation of patients without the main confounding factor that biased several BC studies, namely, chemotherapy. Most BC studies do not consider this issue, which usually collects and analyzes samples regardless of the presence of treatment. Furthermore, the combined assessment of systemic and tissue markers related to the immune-inflammatory response is a strength in the context of excess body weight not previously addressed.

5. Conclusion

Our data demonstrated that excessive BMI impacts the systemic and tumoral profile of cytokines and oxidative stress in breast cancer and seems to be a determinant for poor prognosis outcomes.

Ethics approval and consent to participate

All ethical issues were considered in the study and are reported accordingly in the methods section.

Ethics statement

The Institutional Ethics Committee approved this proposal under CAAE 35524814.4.0000.0107, opinion nº 810.501. All patients included signed the informed consent terms.

Funding

Fundação Araucária/Programa de Pesquisa Para o SUS – PPSUS (Chamada Pública 01/2016), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Grants 402364/2021-0 and 305335/2021-9).

Author agreement

All authors agreed to the manuscript submission.

Availability of data and material

All data will be available under a reasonable request.

CRediT authorship contribution statement

Adma Poliana de Borba Cecílio da Silva: Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. The first draft of the manuscript was written by, all authors read and approved the final manuscript. **Hellen dos Santos Jaques:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Marina Ferronato:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization,

and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Fernanda Mara Alves:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Matheus Iago Colleto:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Mariane Okamoto Ferreira:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Julia Fernandes Orrutéa:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Mariane Mezzoni:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Ruan Gabriel Soares da Silva:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Daniel Rech:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. all authors read and approved the final manuscript. **Carolina Panis:** Data curation, Formal analysis, All authors contributed to the study, Conceptualization, and design, material preparation, data collection and analysis. The first draft of the manuscript was written by, all authors read and approved the final manuscript.

Declaration of competing interest

The authors have no conflict to declare.

Data availability

Data will be made available on request.

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