A case-control study of Metallothionein-1 expression in breast cancer and breast fibroadenoma

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The overexpression of Metallothionein-1 (MT-1) may play an important role in breast cancer; however, few studies have compared MT-1 expression between breast cancer and fibroadenoma. A cross-sectional controlled study was performed in 66 premenopausal women, aged 20–49 years, who had been histologically diagnosed with breast fibroadenoma or breast cancer. The patients were divided into two groups: group A, control (fibroadenoma, \( n = 36 \)) and group B, study (breast cancer, \( n = 30 \)). Immunohistochemistry was performed on tissue samples of fibroadenoma and breast cancer patients to evaluate the expression of metallothionein using an anti-MT-1 polyclonal antibody (rabbit polyclonal anti-metallothionein-Catalog Number biorbyt-orb11042) at a dilution of 1:100. The data were analyzed using NOVA (\( p < 0.05 \)). Microscopic analysis showed a higher concentration of anti-MT-1-stained nuclei in breast cancer tissues than in fibroadenoma tissues. The mean proportion of cells with anti-MT-1-stained nuclei was 26.93% and 9.10%, respectively, in the study and control groups (\( p < 0.001 \)). Histological grade 3 tumors showed a significantly higher MT-1 expression than histological grade 1 (\( p < 0.05 \)), while breast tumors negative for estrogen-, progesterone- and HER2- receptors had a significantly higher MT-1 expression than positive breast tumors positive for these parameters (\( p < 0.05 \)). MT-1 protein in women of reproductive age was significantly higher in breast cancer than in fibroadenoma in this study. Furthermore, there was higher MT-1 immunoreactivity in more aggressive tumors.

Breast cancer is the most common malignancy that affects women in western countries and is the leading cause of cancer death among women worldwide1,2. In Brazil, 59,700 new breast cancer cases were estimated for the year 2018, with an estimated risk of 56.33 cases every 100 thousand women3. Although physical examination and mammography are important to ensure an early diagnosis of the disease and to reduce mortality, breast cancer is still frequently diagnosed at an advanced stage in Brazil. As a result, mortality rates are high even with current therapeutic strategies4.

Nevertheless, it has been suggested that more adequate therapeutic and prognostic strategies in breast cancer can be developed using protein biomarkers, such as metallothionein. This protein has the advantage of not only interfering with cell apoptosis and proliferation but also undergoing alterations well before the occurrence of clinical tumor alterations5 and thus may better guide treatment and prognostic strategies6–7. Metallothionein (MT) is a protein that has been widely studied as a prognostic marker for breast cancer, since it promotes apoptosis, proliferation and differentiation of malignant tumor cells, making them more resistant to treatment8–10. Studies suggest that overexpression of metallothionein is associated with more aggressive breast tumors and more advanced-stage disease11–14.

Some studies have shown that increased expression of MT is associated with a higher proliferative potential in various types of cancer and a more rapid disease progression, as well as cellular resistance to chemotherapy and

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have compared MT-1 expression between fibroadenoma and breast cancer. Significant levels were set at p values ≤ 0.05. To compare more than two means between normal and homogenous data, we used Student's t-test and ANOVA. The data was tested with the Kolmogorov-Smirnov test. The Levene test was used to verify data homogeneity. To determine metallothionein expression, we counted nuclei of stained cells under a microscope with a magnification of 400X. At least 500 cells of the breast epithelium were counted on each slide, in random fields, starting in the area of highest MT-1 concentration in the cell nucleus, using Processing Software and Image Analysis-Image Lab® (SOFTIUM Informatica Ltda, São Paulo, Brazil) for image analysis.

Immunohistochemistry for MT-1. Breast tissue samples fixed in buffered formalin were cut into 3-μm-thick sections. Sections were deparaffinized in xylol for 5 minutes, dehydrated in absolute ethanol, washed in buffered saline solution at pH 7.4 for 5 minutes and then treated for 5 minutes with 3% hydrogen peroxide (H2O2) in buffered solution to block endogenous peroxidase activity. For antigen retrieval, the slides were placed in racks containing 0.21% citric acid (pH 6.0) and heated in a microwave oven for 15 minutes at maximum power. The slides were cooled, and phosphate-buffered saline was added for a cooling period of 20 minutes. Tissue samples were incubated overnight at 4–8 °C with monoclonal antibody metallothionein. A polyclonal antibody against MT-1 (rabbit polyclonal anti-metallothionein – Catalog Number biornbyt-orb11042) was used at a dilution of 1:100.

To determine metallothionein expression, we counted nuclei of stained cells under a microscope with a magnification of 400X. At least 500 cells of the breast epithelium were counted on each slide, in random fields, starting in the area of highest MT-1 concentration in the cell nucleus, using Processing Software and Image Analysis-Image Lab® (SOFTIUM Informatica Ltda, São Paulo, Brazil).

Statistical analysis. Statistical analyses for this study were conducted using the software R, version 3.2.2. Data were expressed as frequencies, percentages, measures of central tendency and dispersion. The normality of the data was tested with the Kolmogorov-Smirnov test. The Levene test was used to verify data homogeneity. To compare more than two means between normal and homogenous data, we used Student's t-test and ANOVA. Significant levels were set at p values ≤ 0.05.

Results
Under optical microscopy, breast cancer cells had a higher concentration of nuclei stained with anti-MT-1 than fibroadenomas (Fig. 1). The characteristics of both groups were similar, except for age and waist circumference (Table 1).

Quantitative analysis showed mean percentages of nuclei stained with Metallothionein-1 per 500 breast epithelial cells in women at 9.10 ± 5.90 and 26.93 ± 15.87 in the control and study groups, respectively (Table 2).

Furthermore, Metallothionein-1 expression was statistically significant in histological grade 3 than in grade 1 tumors p < 0.05 (Fig. 2). MT-1 expression was statistically significant in breast cancers negative for HER2-, estrogen- and progesterone-receptors in comparison to tumors that were positive for these hormone receptors (p < 0.05), (Table 3).

Discussion
Metallothioneins participate in carcinogenesis by mechanisms promoting the development of tumor cells that are more resistant to chemotherapy or radiotherapy. Elevated levels of this protein, with its antioxidant effect, can protect cancer cells against damage from free radicals. This protein has antiapoptotic and pro-proliferative effects, which support uncontrolled cellular growth in breast cancer.
There is accumulating evidence that metallothionein is an immunohistochemical biomarker due to its elevated expression in myoepithelial cells of invasive breast carcinoma\(^1\). However, very few studies have attempted to elucidate the behavior of this protein in fibroadenoma, a benign tumor that does not increase the risk of developing breast cancer\(^1\), and this characteristic makes this condition an ideal control to determine the effect of metallothionein expression on prognosis in breast cancer.

In the current study, overexpression of Metallothionein-1 was observed in cells of breast cancer tissues relative to that in the cells of fibroadenoma tissues. Based on our literature search, only El Sharkawy and Farrag\(^4\) have investigated metallothionein expression in human breast fibroadenomas. According to those authors, higher MT-1 expression is related to more aggressive tumor behavior in ductal breast carcinoma. Other authors have
provided additional confirmation that a higher nuclear expression of MT-1 is more frequently observed in carcinomas than in benign tumors.21

Furthermore, it is noteworthy that a significant difference was found between the mean age of breast cancer patients and breast fibroadenoma patients. Nevertheless, fibroadenomas are known to be more common in younger women, while breast cancer occurs more frequently in older women. Waist circumference was larger in women with cancer, consistent with the literature, since premenopausal women with excess visceral fat have a higher risk of developing triple-negative breast cancer, which has a relatively worse prognosis.23

Agresti et al.24 observed that overweight premenopausal patients were at higher risk of developing triple-negative breast cancer than menopausal women. These findings suggest that obesity may play a role in the biological mechanisms underlying more aggressive types of breast cancer and the higher expression of metallothionein.25

Based on some authors, elevated MT expression blocks cellular apoptosis by sequestering zinc ions that stabilize p53, a gene that acts as a tumor suppressor by inducing apoptosis. Thus, MT enables the maintenance and integrity of the genome. Recent studies indicate a strong relationship between p53 and MT, where overexpression of metallothionein is consistently associated with the presence of mutant p53, and in breast cancer, this relationship has been associated with a smaller number of apoptotic cells and a worse prognosis.26

Histologic tumor grade is associated with the immunoreactivity of metallothionein in breast cancer in addition to its anti-apoptotic effects. This discovery is consistent with previous observations in invasive breast carcinomas and in situ tumors reported by other researchers.27–29 The current study showed a significant difference in MT-1 expression according to histologic tumor grade. Grade 3 tumors have a higher positivity for MT-1 protein, as well as HER2-overexpressing and negative hormonal receptors breast cancer. The association between these variables is relevant, indicating that metallothionein has a role in the differentiation, proliferation and progression of breast cancers. Proliferation is an important guide for the prognosis and therapy of malignant tumors.28,29

Therefore, studies have indicated that the expression of MT proteins in ductal breast cancer may represent an unfavorable prognostic index, since its highest expression is related to malignant cells with a higher histologic grade. Nevertheless, there is a need for further studies to obtain a better understanding of the behavior of metallothionein in tumorigenesis and to define the clinical significance of its expression in malignant and benign breast tumors.

**Ethical approval.** The internal review board of the Federal University of Piauí approved this protocol. Informed consent was obtained from all individual participants included in the study. All the procedures performed in this study complied with current Brazilian laws and were in accordance with the ethical standards of the institutional and national research committees, as well as the 1964 Helsinki declaration and its later amendments.

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Author Contributions
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Additional Information

Competing Interests: The authors declare no competing interests.

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