Immune checkpoints as biomarkers in the diagnosis and progression of breast cancer

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Received 18 Feb 2024; Accepted 10 March 2024; Published 12 April 2024

Abstract

Breast cancer (BC) is the most common tumor happened to women. The identification of immune checkpoint molecules like programmed cell death protein 1 (PD-1), programmed cell death ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), is important in the field of BC. A case-control study was conducted on 60 BC patients and 30 healthy controls. This study aimed to evaluate the immune checkpoint as a biomarker for the diagnosis and progression of BC disease. The serum level of PD-1, PD-L1, and CTLA-4 was determined with an Enzyme-linked immunosorbent assay. Medium levels of PD-1, PD-L1, and CTLA-4 were significantly elevated in BC compared to controls. Its increase in breast cancer patients with metastasis may also indicate a relationship between it and the progression of the disease to metastasis. Receiver operating characteristic curve analysis revealed that PD-L1 was a significant predictor of BC and a good area under the curve was demonstrated. A relationship can be concluded between PD-1, PD-L1, and CTLA-4 levels and BC, as its significant increase was observed in patients, and its increase in BC patients with metastatic may indicate the existence of a relationship between it and the progression of the disease until it reaches the state of metastatic.

Keywords: breast cancer, immune biomarkers, PD-1, PD-L1, CTLA-4

Introduction

Over the past 20 years, the incidence of breast cancer has continued to increase globally, with a similar rate of increase in the Middle East and North Africa region according to 2016 statistics (Hashim *et al.*, 2018) [4]. Scientific advances in cancer treatment have led to many new treatment options, PD-L1 inhibitors. And PD-1. Because of this, spending on cancer care and breast cancer has increased alone. At the same time, the first immune checkpoint inhibitor approved by the US Food and Drug Administration is called ipilimumab, which specifically targets CTLA-4 to enhance the anti-tumor immune response. Therefore, CAR-T cells are an effective treatment in people with... In acute lymphoblastic leukemia, multiple myeloma, and breast cancer (Johnson *et al.*, 2022) [5], immunotherapy has transformed the care of cancer patients, providing good results across indications and survival. However, the rapid expansion and use of immuno-oncology treatment options may put healthcare budgets under pressure. However, it is very important to currently highlight the importance of early detection, as the 5-year survival rate for women with early-stage breast cancer exceeds 90%, and survival rates decrease significantly in the most advanced stages of cancer (Dougan *et al.*, 2021) [3], 63% of women with breast cancer in the United States of America were diagnosed at an early stage, while only 47% of women between the ages of 15 and 39 years were diagnosed specifically at early stages, and this may be due to Until the routine examination, which does not begin until the age of 40 years, In all subtypes and stages of breast cancer, survival rates are relatively lower in women under the age of 40. (Medina *et al.*, 2020) [8].

Since the major component of PD-1, known as CD274 or B7-H1, is known to belong to the B7 family and may be produced by cells other than immune cells, early identification and diagnosis of cancer are undoubtedly crucial for effective therapy (Medina *et al.*, 2020, Misir *et al*., 2022) [8, 9]. It has been shown in recent research that ICI-based cancer immunotherapy inhibits negative immune regulation to boost immune activity against cancer cells. The FDA has authorized more monoclonal antibody medications that inhibit the PD-1 or PD-L1 ligand, respectively. The medication is used either alone or in conjunction with other medications to treat a variety of malignant tumors, including skin, lung, lymphoma, esophageal, gastric, and liver cancers (Kubli *et al.*, 2021) [6]. Even with response indicators like PD-L1 expression, presently licensed ICIs only help a fraction of patients despite their exceptional clinical effectiveness. ICIs, especially single-agent ones, often cause initial resistance, and some responding patients gain resistance over time. Furthermore, excessive progression brought on by ICIs presents major clinical hurdles to immunotherapy, and immune-related adverse events (irAEs) caused by ICIs might result in many organ problems or excessive immune system activation (Pan *et al.*, 2020) [11]. As a result, establishing the biological features of Immune checkpoints' molecular and regulatory processes is essential for enhancing treatment choices and managing side effects.

Studies have shown that interleukins are messengers produced by some cells of the immune system, or T cells specifically. Giving interleukins can help treat metastatic melanoma, and may be useful in kidney cancer. Interleukins, which are produced by some white blood cells, may regulate checkpoints. Immunomodulators, function as gatekeepers during the body's immune response to prevent the immune system from

becoming overactive. They are a group of molecules produced in immune cells that can regulate the degree of immunological activation (Ohaegbulam *et al.*, 2015) [10]. Several point molecules have been found in the last several decades. immunoassays, such as T-cell immunoglobulin, CTLA-4, lymphocyte-activating gene 3 (LAG-3), PD-1/PD-L1, and so on, albeit the number of points Newly developed immunoassays are fantastic, but because of their intricate mechanism, FDA-approved ICIs are still very scarce. As is well known, a growing body of research in the field of molecular oncology has shown the intricate regulatory systems that restrict the expression of immunological checkpoints. Additionally, they bind non-coding RNAs (ncRNAs) directly. An abundant component of the human transcriptome involved in all hallmarks of cancer, significantly regulating the expression of immune checkpoints (Almouh *et al.*, 2022) [1].

Method

From August through September of 2023, research samples were gathered from Salah al-Din Oncology Centre located in Tikrit. Based on the medical staff's exams and the results of past breast cancer diagnoses, they included 60 blood samples from women. The goal of the research was to assess several immune characteristics in women in the Salah al-Din Governorate of Iraq who had been diagnosed with breast cancer. Based on prevalence, the afflicted women were split into two groups: Women with breast cancer were included in the first group (the prevalence group or the third and fourth stages), and the third and fourth stages contained 30 samples with ages ranging from 30 to 70. The second group (the non-proliferation group or the first and second stages) included women with breast cancer. In the first and second stages, their ages ranged between (37-78) and included 03 samples. 30 blood samples were collected from women who did not have breast cancer and had no family history of the disease and were in good health, their ages ranged between (30-74 years) as control samples. Information about affected women was collected through an information form that included much information related to the subject of the study.

We use ELISA technology for the determination of PD-1, PD-L1, and CTLA-4 concentration in blood serum, by using an analysis kit produced by the Chinese company Biotech Sunlong, according to the steps attached to it.

Results

Plots of known standard concentrations of Human PDL-1 are shown on a logarithmic scale (x-axis) and their corresponding reading is on a logarithmic scale (y-axis). By setting the OD of the sample. On the y-axis, the concentration of Human PDL-1 in the sample can be calculated. The dilution factor is multiplied to determine the original concentration Data were analyzed using GraphPad Prism version statistical software Table (1) shows the clinical-demographic characteristics of breast cancer patients, such as age, type of treatment, and family history of the disease after they were distributed into two groups: the spread group or stages three and four (n=30) and the non-spread group, which included patients with stages one and two (n=30). The results showed that there were no statistically significant differences in age between the two groups of patients without spread and patients with spread (p>0.05), where the median age was 53.5 years in the non-spread group versus 53 years in the spread group. Moreover, no significant differences were observed among patients in terms of their distribution into age groups of less than 50 years or 50 years and above, as well as their subjection to chemotherapy, biological, radiological, immunotherapy, or surgical interventions, in addition to family history of illness. To breast cancer (p>0.05).

Table 1: Clinical-demographic characteristics of patients participating in the study

Variable	Metastatic Cancer (n = 30)	Non-Metastatic Cancer (n = 30)	P
Age (years)	53 (63.25-48.75)	53.5 (44.25– 61)	0.791
Age groups			
<50 years	8 (26.7 %)	12 (40%)	0.412
≤50 years	22 (73.3 %)	18 (60%)	
Disease stage			
Phase I	–	6 (20%)	–
Phase II	–	24(%80)	
Phase III	16 (53.3%)	–	
Phase IV	14 (46.7%)	–	
Chemotherapy			
No	27 (90%)	30 (100%)	0.237
Yes	3 (10%)	0 (0%)	
Biological therapy			
Yes	10 (33.3%)	15 (50%)	0.295
No	20 (66.7%)	15 (50%)	
Radiation therapy			
Yes	9 (30%)	10 (33.3%)	0.999
No	21 (70%)	20 (66.7%)	
Immunotherapy			
Yes	23 (77 %)	28 (93%)	
No	7 (23 %)	2 (7%)	0.145
Surgery			
Yes	28(93%)	30 (100%)	
No	2 (7%)	0 (0%)	0.492
Family history			
Yes	9 (30%)	10 (33.3%)	0.999
No	21 (70%)	20 (66.7%)	

Age was expressed using median and interquartile range (median, IQR), and for the other categorical variables using frequency and percentage. The ages of patients in the two groups were compared using Student's t-test for independent samples and the rest of the variables using Fisher's exact test or chi-square test.

Immune variables in serum

PD-1

The levels of PD-1 and other serum variables in control and breast cancer groups were evaluated by enzyme-linked immunosorbent (ELISA) technique. The average concentration of PD-1 was equal to 1559±672.1 pg/ml in the control group compared to 2011±808.0 pg/ml in the group of breast cancer patients (overall). The independent samples t-test showed a clear, statistically significant difference in the means of PD-1 between the two study groups (Figure 1A). To explore the differences accurately, a one-way analysis of variance

(ANOVA) was employed, which revealed significant differences in the levels of PD-1 for the control group compared to the two groups of cancer patients in the case of spread and without spread, as it was significantly lower (p<0.05) in the control group compared to its levels. In the two groups of patients with metastatic and non-metastatic breast cancer, the concentrations were 1559±672.1, 1990±900.7, and 2031±718.6 pg/ml, respectively (Figure 1B). More precisely, a significant increase in the level of PD-1 was observed in patients with non-metastatic cancer compared to individuals in the control group (1606±680.0 vs. 2334±584, p<0.05), while there was no significant difference when distributing patients according to groups. age, less than 50 years or 50 years and older (Figure 1C, p>0.05).

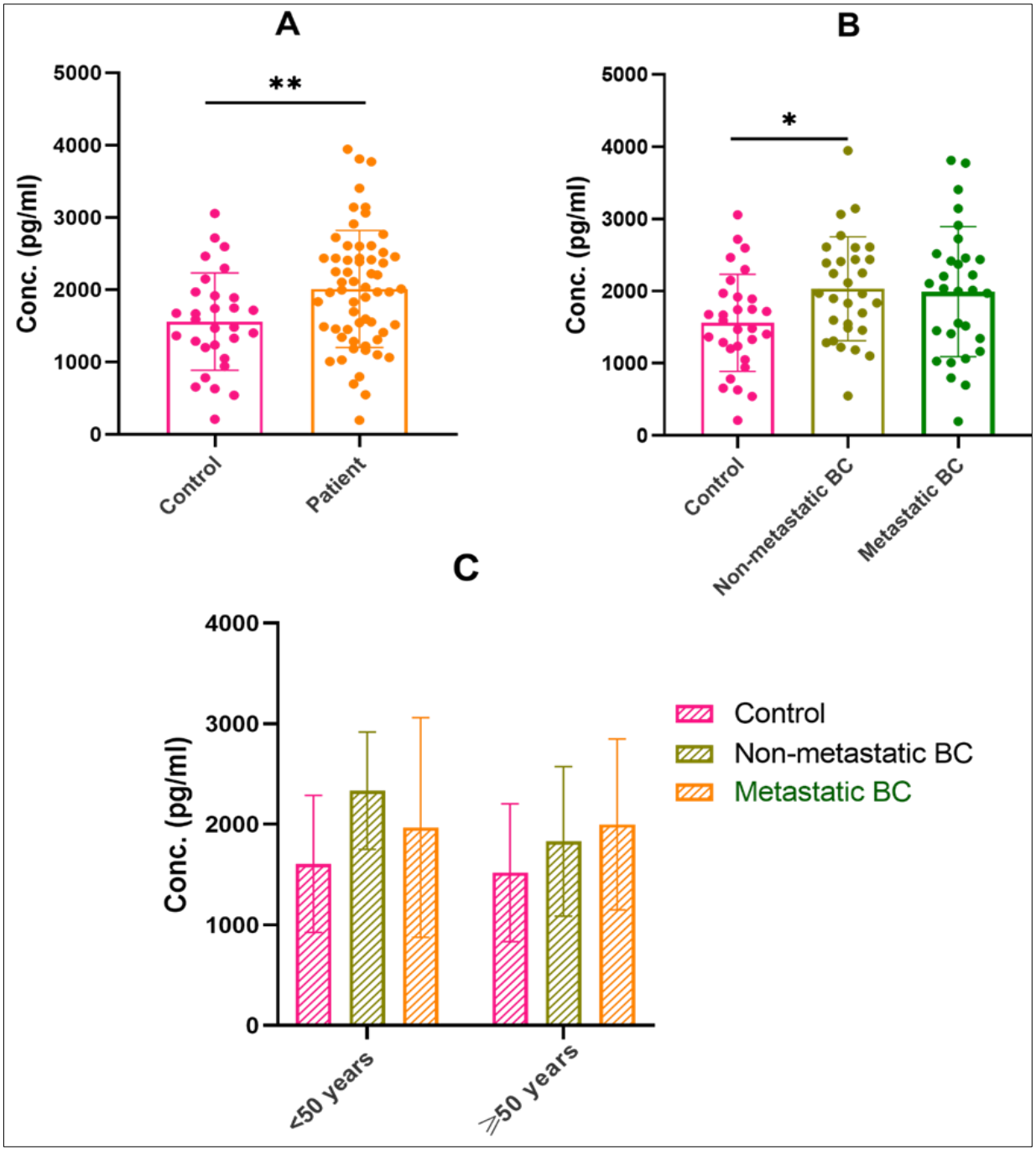

Figure 1: PD-1 level in the serum of study participants. A describes the difference in concentration between healthy people and patients in general; B describes the difference in concentration between healthy people and patients with metastatic and non-metastatic breast cancer, while C describes the serum PD-1 level between the groups based on different age groups (less than 50 years, and 50 years or more). The error bar (error bar) indicates 95%Cl in A and B and the standard deviation value in C. The sign (*) indicates the presence of a significant difference at the 0.05 significance level, and the sign (**) indicates the presence of a significant difference at the significance level. 0.01.

PD-L1

The results showed that the concentration of PD-L1 was significantly higher in the breast cancer group compared to the control group (Figure 2A), where the concentrations in the cancer patient group (overall) and the control group were 2818±1470 and 1964±1025 pg/ml, respectively, and the p-

value was equal to 0. .01. An additional analysis showed that PD-L1 levels differed between patients in the two breast cancer groups according to metastasis and the control group (Figure 2B), where the PD-L1 concentrations in breast cancer patients in the metastasis condition were: 2966±1605 pg/ml and in the non-metastasis condition: 2658±1331 pg/ml, while in the control group: 1964±1025 pg/ml. The results of the multiple comparison test (Dunn's test) showed that the expression of PD-L1 was significantly higher in patients with metastatic breast cancer compared to the control group with an error rate (p<0.05), and no significant difference was found between patients with non-metastatic breast cancer and the control group (p>0.05) or between the two patient groups (p>0.05). Similarly, no statistically significant difference was observed at the pre-specified significance level between the study participants according to their age after being divided into two groups (under 50 years and 50 years and older) (Figure 2C). Therefore, the results suggest an association between PD-L1 and breast cancer, as a significant increase was observed in patients. The results also suggest an association between PD-L1 and the progression of breast cancer to the metastatic state.

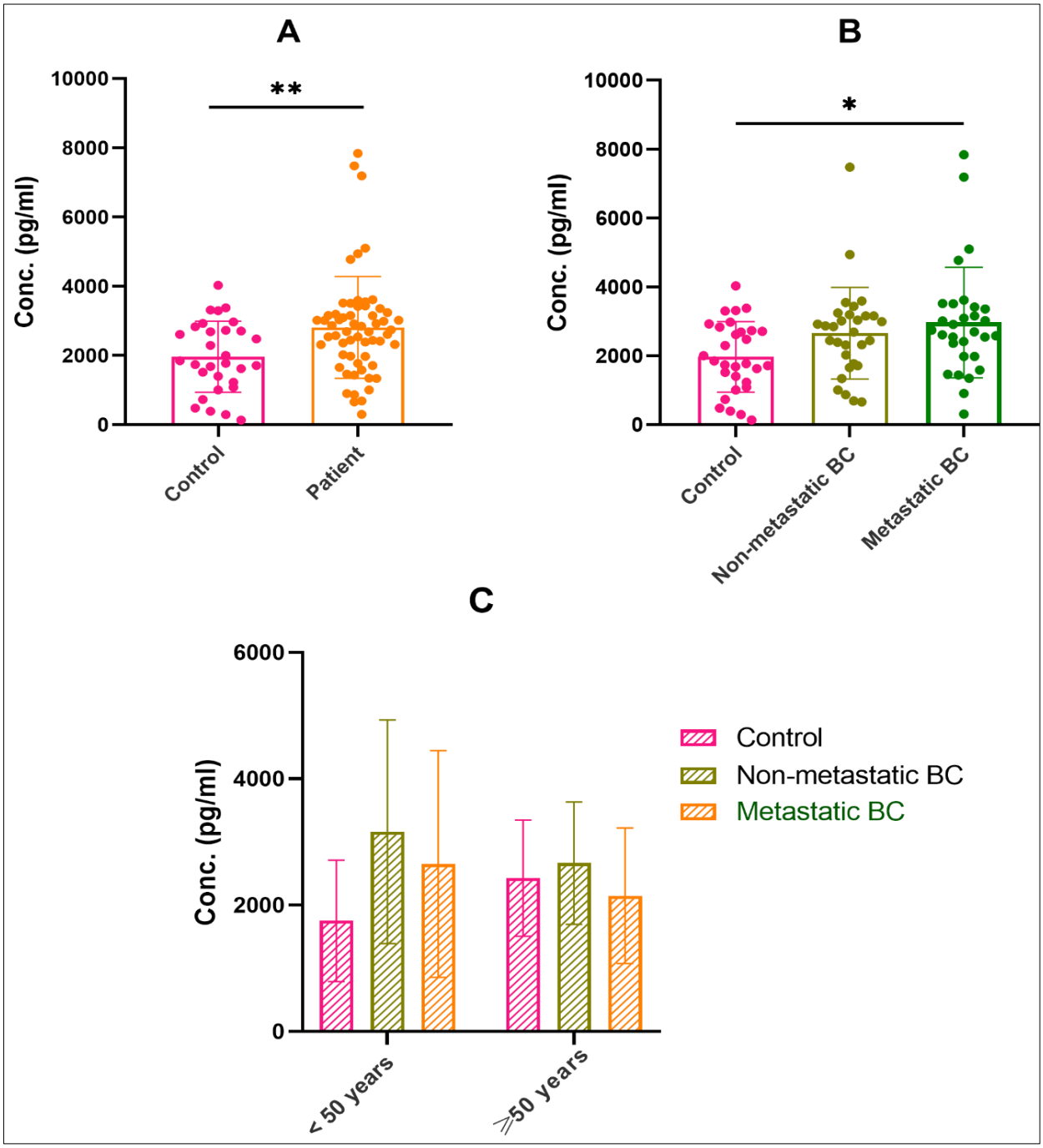

Figure 2: PD-L1 level in the serum of study participants. A describes the difference in concentration between healthy people and patients in general, while B describes the difference in concentration between healthy people and patients with metastatic and non-metastatic breast cancer, and C describes the serum PD-L1 level between the groups based on different age groups (less than 50 years, and 50 years or more). The error bar (error bar) indicates 95% Cl in A and B and the value of the standard deviation in C. The sign (*) indicates the presence of a significant difference at the 0.05 level of significance, and the sign (**) indicates the presence of a significant difference at the significance level. 0.01.

CTLA-4

The CTLA-4 concentration was significantly higher in breast cancer patients 329.9±189 pg/ml compared to controls at 247.8±82.79 pg according to Mann-Whitney's U test at the significance level ($p<0.05$) (Figure 3A). To find the differences between the means of the groups according to prevalence, the Kruskal-Wallis test was performed, which revealed a statistically significant difference in CTLA-4 expression between the breast cancer groups and the control group at the significance level ($p<0.05$). Multiple comparison tests showed that CTLA-4 expression was significantly higher in the breast cancer with metastasis group ($p<0.05$) when compared with the control group, where the mean rank was 52.95 compared to the control group with a mean rank of 36.62 (Figure 3B). There was no significant difference in CTLA-4 expression between the non-metastatic breast cancer group (mean rank = 46.93) and the control group in the post hoc analysis, nor between the breast cancer groups with and without metastasis, although the average ranks showed a gradual increase in CTLA-4 levels are proportional to the severity of the disease. Furthermore, no statistically significant differences were observed at the predetermined significance level between the study participants according to age after dividing them into two groups (less than 50 years old and 50 years old and above) (Figure 3C).

Therefore, it can be concluded that there is a relationship between CTLA-4 and breast cancer, as its significant elevation was observed in patients. Additionally, its elevation in breast cancer patients with dissemination may indicate a relationship between CTLA-4 and disease progression and metastasis.

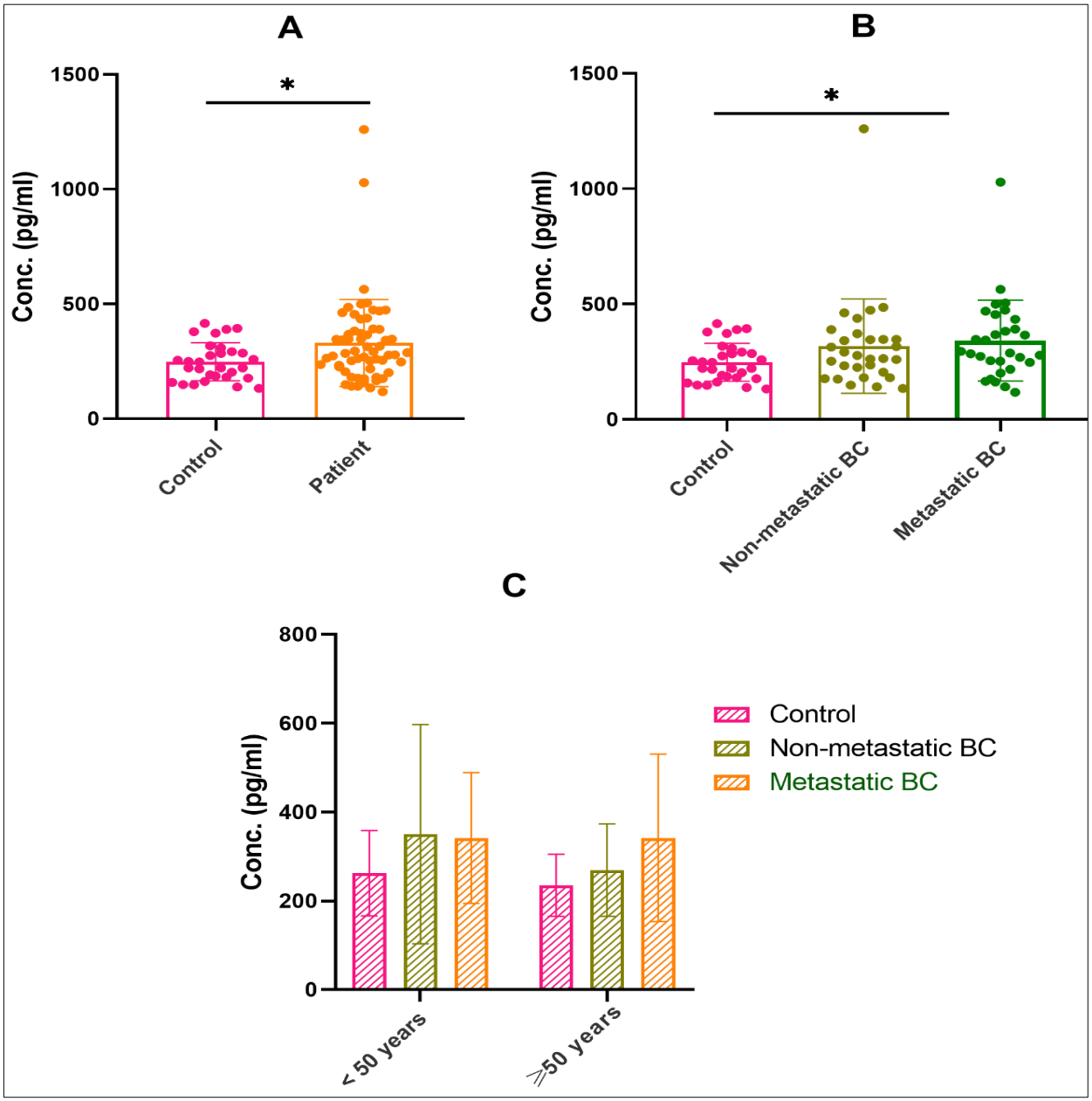

Figure 3: CTLA-4 level in the serum of study participants. A describes the difference in concentration between healthy people and patients in general, while B describes the difference in concentration between healthy people and patients with metastatic and non-metastatic breast cancer, and C describes the serum CTLA-4 level between the groups based on different age groups (less than 50 years, and 50 years or more). The error bar (error bar) indicates 95% Cl in A and B the standard deviation value in C, and the sign (*) indicates a significant difference at the significance level of 0.05.

Serum immune variables according to treatment

Figure 4 shows the level of immune markers in the serum of breast cancer patients who underwent chemotherapy and those who did not. The comparison between metastasis and non-metastasis was not performed due to the small sample size and the lack of sufficient data. The results showed that when comparing the means of PD-1 (Figure 4A), PD-L1 (Figure 4B), and CTLA-4 (Figure 4C) for patients who received treatment and those who did not, there was a difference in the concentrations of these markers. However, this difference was not statistically significant except in the case of PD-1 and CTLA-4.

For PD-1, it was statistically significantly higher in patients who underwent chemotherapy compared to those who did not receive any doses (1125±364 vs. 2058±799.3 pg/ml, p=0.05). As for PD-L1, its levels were elevated in the serum of patients in the treated group compared to the other group, but this elevation was not statistically significant (2195±743 vs. 2845±1495, p=0.413). However, CTLA-4 levels were significantly decreased at a significance level of 0.05 in the serum of patients who underwent treatment compared to those who did not (440.6±61.34 vs. 324.1±192.2, p=0.045).

overall, these results suggest that chemotherapy alters the immune landscape in breast cancer by enhancing the suppressive PD-1/PD-L1 pathway, while potentially alleviation some anti-tumor immune brakes such as CTLA-4. This highlights the complex interplay between cytotoxic therapy and immune activation state in breast cancer.

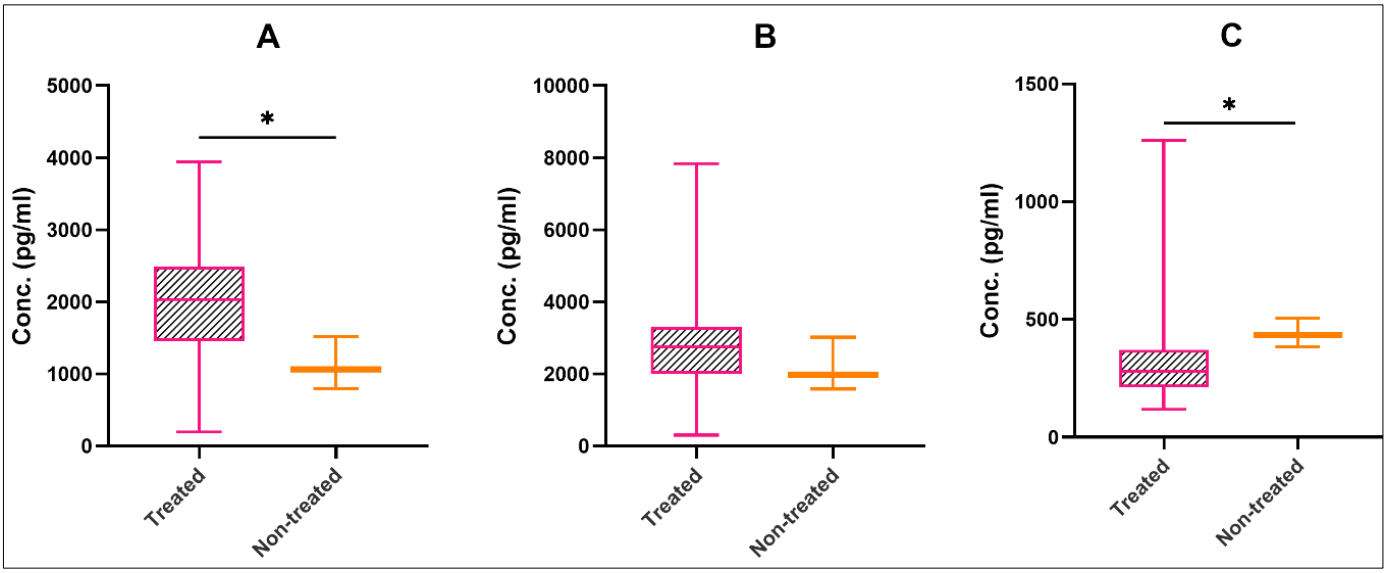

Fig 4: Level of immune variables in the serum of breast cancer patients who underwent chemotherapy and those who did not undergo any treatment. A describes PD-1 levels, B describes PD-L1 concentrations, and C describes serum CTLA-4 levels between groups. The sign (*) indicates a significant difference at the significance level of 0.05.

Discussion

Age groups most affected by breast cancer

It is clear that the sample population included somewhat advanced age groups, which were close to or average age of 50 years (Table 1), and was limited to 44 years to 63 years, whether the disease was widespread or not alike, and certainly this may be due to this. The extent to which this age group of women is affected by breast cancer, regardless of the nature of the infection or the cause of breast cancer, is consistent with the study (Sharma *et al.*, 2021) [14], which confirmed the same principle in terms of the age groups, which is most affected by breast cancer.

PD-1

The PD-1 protein is considered an important diagnostic factor in identifying and diagnosing breast cancer, and the t-test for independent samples showed a statistically significant difference in the means of PD-1 between the two study groups (Figure 1A), as well as for the group Control compared to the two groups of cancer patients in the case of spread and without spread. More precisely, a significant increase in the level of PD-1 was observed in patients with non-spread cancer compared to individuals in the control group. This may be due to the increase in the rate of PD-1 protein at the beginning of the infection. Cancer is treated by the immune system until it reaches a stage of stability in the serum in the late stages of infection. It should also be noted that there was no significant difference when distributing patients according to age groups, less than 50 years or 50 years and older (Figure 1B, C1 It is very clear that there are significant differences between the concentration of PD-1 protein in blood serum, and this is very important, firstly from a diagnostic standpoint and secondly from a therapeutic standpoint as well. It is known that an increase in this protein gives positive indications of response to treatment. According to this study, the body can respond to immunotherapy to a greater extent when the concentration of this protein in the serum is high, and this is very consistent with (Shahenaz *et al.*, 2024) [12], as this study indicated that the expression of PD-L1 and IRF-1, along with infiltration CD8 is a powerful biomarker by which to identify BC patients with the highest odds of achieving an excellent response to immunotherapy, especially when emergency status and initial diagnosis are taken into account. It is known that ER expression levels are high in these cases. Therefore, the results

indicate a close relationship between PD-1 and breast cancer, as a significant increase was observed in patients.

PD-L1

As for the PD-L1, it was found that there is a close relationship with the development of the disease from the early stages to the late stages of the infection. This is done by knowing that the concentration of this protein gradually increases and does not stabilize at a certain stage, and this is very clear in Figure (3). The reason may be due to the sensitivity of this protein to the formed cancer cells and its concentration increases with the increase in the number of cancer cells, which forms a direct relationship according to this study. This is confirmed by some studies, such as the study (Anand *et al.*, 2019) [2].

CTLA-4

The high percentage of CTLA-4, specifically in the blood serum, is similar to the high concentrations of PD-L1 in terms of form and content, with a difference in function specifically. According to Figure (3), a relationship between CTLA-4 and breast cancer was developed, as it was observed a significant increase in patients and breast cancer patients. This may indicate the existence of a relationship between it and the development of the disease until it reaches the state of spread. The reason is due to the sensitivity of this type of cell and more specifically the cancer cells formed and its concentration increases with the increase in the number of cancer cells, which may form a direct relationship. According to this study, we notice, according to the same figure, an increase in the concentration of toxic cells CTLA-4 under the age of 50. This could be due to the activity of the immune system at ages under 50 years, which is confirmed by some studies, such as the study done by Anand *et al.*, (2019) [2], and for the same reasons mentioned previously.

Immune changes in blood serum

We note the level of immune variables in the serum of breast cancer patients who underwent chemotherapy and those who were not exposed to any treatment. So, the levels of PD-1 increased. This may indicate the sensitivity of this protein to chemotherapy and may stimulate the immune system to increase the production of this protein with increasing doses (Figure 4: A, B, C). The reason is that ER expression is directly related to PD-1 expression was inversely affected by PD-L1 expression, suggesting an antagonistic effect of ER expression on CD274 regulation. As for PD-L1, its levels increased in the serum of patients in the group that underwent treatment compared to the other group, but this increase was not significant. As for the levels of CTLA-4, they decreased statistically compared to before and after chemotherapy, and this may be due to the toxic relationship between the cells. The chemical dose may lead to teratogenesis of the proteins involved in the formation of CTLA-4 cytotoxic T cells, and this is in line with the results of a previous study conducted by (Shuai *et al.* 2020) [15], who reported that a high degree of PD-L1 immune expression is linked to the receptors at the therapeutic dose, which may also be related to estrogen.

Conclusion

There are significant differences in the concentration of the protein PD-1 in blood serum and a lower incidence of breast cancer. Besides, there is a close relationship with the development of the disease from the early stages to the late stages of infection with a high concentration of PD-L1. in blood serum. It was also observed that there is a relationship between CTLA-4 and breast cancer. Moreover, a significant increase was observed in breast cancer patients with spread, indicating that there is a relationship between it and the development of the disease until it reaches the state of spread. In general, these results indicate a limited effect. Treatment is based mostly on the cases that have been studied. This may be due to many factors, including the type and dose of treatment, the period of treatment, and the aggressiveness of the tumor.

Declaration

Availability of data and material:

All data in this manuscript are available**.**

Ethics approval and consent to participate

The Ethics Committee at the Center (Tikrit University) approved the study.

Competing interests

The authors declare that they have no competing interests

Funding

There is no fund received for this work.

Acknowledgments

We would like to express great thanks to Tikrit University, for its assistance and guidance during the study period, and to Salah al-Din Oncology Centre located in Tikrit for the support.

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