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SERUM Ig AND CYTOKINE LEVELS IN WOMEN WITH BREAST CANCER BEFORE AND AFTER MASTECTOMY

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SUMMARY: To evaluate serum Ig, complements IL-6, and TNF-α in breast cancer patients and to evaluate their role in disease process.

Blood samples were collected from 30 women with primary breast cancer before operation and another blood samples were collected from 23 of them after three cycles of chemotherapy. In addition to this, 20 samples were collected from apparently healthy women as a control group from the outpatient department.

Radial immune diffusion test was performed for the detection of serum IgG, IgA, IgM, C3, and C4, while ELISA test was used for the detection of serum IL-6 and TNF-α.

Serum IgG and IgA levels for patients (preoperative) were 1926.84 ± 612.60 mg/dl and 484.750 ± 201.98 mg/dl, respectively. These values were higher than the respective values for the control group (1536.61±441.29 mg/dl and 318.57±124.54 mg/dl) (P < 0.05).These values for IgG and IgA increased with the advancement in the disease stages, while that for IgM showed no significant change.

The level of the serum complement component C3 for the patients (211.50 ± 79.39 mg/dl) was significantly higher than that for the control group (150.71± 39.93 mg/dl) (P<0.05). C3 and C4 levels were positively correlated with the disease stages.

IL-6 and TNF-α levels for breast cancer patients were 222.5 ± 68.86 pg/ml and 246.72 ±197.74 pg/ml, respectively. These values were significantly higher than the respective values of the control group (171.3 ± 64.85 pg/ml and 131.52 ± 108.92 pg/ml) (P<0.05)/.These values were found to increase with the advancement of the disease stages but were not statistically significant.

In conclusion, the elevation of serum IgG, IgA, C3, C4, IL-6, and TNF-α levels can be considered as an indication for disease status before and after treatment as well as relapses.

Key words: Breast cancer, Complements, Immunoglobulins, IL-6, TNF-α.

INTRODUCTION

During the past decade, insight have been gained about the role of the immunological response in the breast cancer disease process (1), and the possible use of immunological parameters in the prognosis of breast cancer (2).

Serum immunoglobulin levels were found to be related to the disease stage and tumor load in breast cancer patients. The obvious alteration in serum IgG and

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IgA levels in breast cancer patients reflects a disturbance in cell–mediated immunity and humoral immunity (3).

The complement activity is found to be raised in breast cancer patients and increased with the progression of the disease stage (4). The cytotoxic activity of the complements is insufficient as a surveillance mechanism against tumor (5). This is suggested to be due to the presence of an intrinsic cellular complement resistance mechanism (6).

Cytokines are a diverse group of nonantibody proteins that act as mediators between cells. They were initially identified as products of immune cells that act as mediators and regulators of immune processes, but many cytokines are now known to be produced by cells other than immune cells and they can have effects on the non-immune, interrelated system of proteins and signaling cascades (7).

Elevated levels of circulating proinflammatory cytokines, including IL-1 and IL-6, correlated to a shorter survival and poor prognosis in breast cancer. IL-8 levels increase significantly in breast cancer patients with more advanced disease, and related to an accelerated clinical course, a higher tumor load, and the presence of liver or lymph node involvement. TNF-α is found to participate in the initiation and promotion of breast cancer (8).

The aim of this study is to evaluate the immunological response in breast cancer patients as a parameter in prognostic, predictive and treatment response indicators.

MATERIALS AND METHODS

Patients

This study involved 30 females, aged 27–70 years, who were treated through the period of study from October 2009 till February 2011 in Basrah Hospitals, Southern of Iraq. These women were diagnosed to have breast cancer using fine needle aspiration biopsy. The staging of the disease was performed for them by the physicians and followed up for disease relapse (recurrence) for a period of 10–17 months. Of the 30 patients, the follow up could not be successful for 7 patients because of either poor compliance or noncooperation. A total of 20 apparently healthy women from the outpatient department were involved in this study as a control group.

Sampling

Blood samples were collected from patients prior to surgical operation for lumpectomy or mastectomy after the patients' written informed consent. These patients were assessed for any symptoms or signs of infection or inflammatory disorders. Another set of blood samples were taken from the patients after three cycles of chemotherapy. The blood samples were centrifuged and the serum stored in multiple tubes at -20°C. The blood samples were also collected from 20 apparently healthy women.

Radial Immunodiffusion Test (9)

Serum IgG, IgM, IgA, C3, and C4 proteins were determined for 30 patients before lumpectomy and or mastectomy, and for 23 patients and the control group after three cycles of chemotherapy using single radial immunodiffusion plates (Agarose gel containing the goat antiserum) purchased from LTA s.r.l, Milano, Italy.

IL-6 (10)

The serum IL-6 concentration was determined using IL-6 one step sandwich enzyme immunoassay kit purchased from IMMUNOTECH SAS, France.

The sample results were calculated by interpolation from a calibrator curve. The curve was drawn by plotting on the horizontal axis the IL-6 concentration of the calibrators and on the vertical axis the corresponding absorbance. Then the absorbance of each sample located on the vertical axis and the corresponding IL-6 concentration was read on the horizontal axis

TNF-α (10)

The serum TNF-α concentration was determined by ELIZA KIT purchased from DRG International, Inc., USA.

The serum TNF-α concentration was determined by using the linear graph, and plotting the optical density for each standard versus human TNF-α concentration. The concentration of human TNF-α in the unknown was determined by interpolation.

RESULTS

The Major Characteristics of the Patients

The general characteristics of the patients included in this study are given in Table 1. Their ages ranged from 27 to 70 years, of which 50% were 40-49 years old, with the mean age of 48.60 $\pm$ 11.29 years for the patients and 47.15 $\pm$ 10.95 years for the control group.

Most of the patients (76.7%) were parous, 70% of them had a history of breast feeding, 6.7% had a family history of breast cancer and or ovarian cancer, and none of them had a personal history of breast cancer.

Table 1: Patients characteristics.

Characteristic		(N = 30) n (%)
Mean age ± SD	48.60 ± 11.29	
Age of menarche	11-12	10 (33.3)
	13-14	15 (50.0)
	15-16	5 (16.7)
Marital state	Not married	3 (10.0)
	Married	24 (80.0)
	Divorced	2 (6.7)
	Widowed	1 (3.3)
Parity	Nulliparous	7 (23.3)
	Parous	23 (76.7)
Breast feeding	No	9 (30.0)
	Yes	21 (70.0)
Menopausal state	Premenopause	18 (60.0)
	postmenopause	12 (40.0)
Family history	No	28 (93.3)
	Yes	2 (6.7)
Oral contraceptive	No	24 (80.0)
pills	Yes	6 (20.0)
Smoking	Yes	2 (6.7)
	No	28 (93.3)
Skin color	Usual	29 (96.7)
	Black	1 (3.3)
Stage	I	5 (16.7)
	IIA	13 (43.3)
	IIB	7 (23.3)
	III	5 (16.7)

Comparison between Patients (Preoperative) and Control Group

Serum Immunoglobulins (IgG, IgA, and IgM)

The serum IgG level for breast cancer patients was significantly higher (1926.84 ± 612.60 mg/dl) than for the control group (1536.61 ± 441.29 mg/dl) ($p<0.05$) (Table 2).

The patients' serum IgA (484.750 ± 201.98 mg/dl) was higher than of the control group (318.57 ± 124.54 mg/dl), and it was statistically significant ($p<0.05$) (Table 2). The patients' serum IgM levels were similar to those of the control group (Table 2).

Serum Complements (C3 and C4)

The serum C3 level for breast cancer patients (211.50 ± 79.39 mg/dl) was higher than for the control group (150.71 ± .93 mg/dl), and this difference was statistically significant ($p< 0.05$) (Table 2).

The serum C4 values for patients (40.14 ± 17.11 mg/dl) and controls (32.39 ± 13.33 mg/dl) were similar (Table 2).

Serum Cytokines (IL-6 and TNF-α)

The results of the enzyme immunoassay test for serum IL-6 revealed a significantly higher serum IL-6 level for patients (222.5 ± 68.86 pg/ml) than for the control group (171.3 ± 64.85 pg/ml) ($p<0.05$) (Table 2).

The serum TNF-α concentration for the patients before operation (246.72 ± 197.74 pg/ml) was higher than for the control group (131.52 ± 108.92 pg/ml), and this difference was statistically significant ($p<0.05$) (Table 2).

Comparison between the three stages of the disease

Serum immnuoglobulins

In relation to the disease stage, the serum IgG level for stage I (1663.32 ± 862.48 mg/dl) was less than that of stage II (1985.81 ± 574.04 mg/dl) and stage III (1954.48 ± 553.66 mg/dl), but this difference was statistically not

Table 2: Comparison of serum immunological parameter between patients with breast cancer and the control group.

Parameter	Patients (n = 30) Mean ± SD	Control (n = 20) Mean ± SD	P value
IgG (mg/dl)	1926.84 ± 612.60	1536.61 ± 441.29	<0.05
IgA (mg/dl)	484.750 ± 201.98	318.57 ± 124.54	<0.05
IgM (mg/dl)	189.03 ± 56.30	197.1 ± 53.23	NS
C3 (mg/dl)	211.50 ± 79.39	150.71 ± 39.93	<0.05
C4 (mg/dl)	40.14 ± 17.11	32.39 ± 13.33	NS
IL-6 (pg/ml)	222.5 ± 68.86	171.3 ± 64.85	<0.05
TNF-α (pg/ml)	246.72 ± 197.74	131.52 ± 108.92	<0.05

Table 3: Immunological parameter in relation to breast cancer stages.

Parameter	Stage I Mean ± SD	Stage II (A and B) Mean ± SD	Stage III Mean ± SD	P value
IgG (mg/dl)	1663.32 ± 862.48	1985.81 ± 574.04	1954.48 ± 553.66	NS
IgA (mg/dl)	385.58 ± 174.34	447.54 ± 156.29	729.26 ± 134.23	<0.05
IgM (mg/dl)	198.92 ± 76.39	177.28 ± 46.67	226.14 ± 65.33	NS
C3 (mg/dl)	185.42 ± 73.73	196.41 ± 75.93	297.96 ± 39.296	<0.05
C4 (mg/dl)	30.34 ± 11.92	39.29 ± 17.39	53.34 ± 14.258	NS
IL-6 (pg/ml)	181.40 ± 128.42	223.80 ± 50.62	259.20 ± 42.41	NS
TNF-α (pg/ml)	159.47 ± 102.14	269.28 ± 225.65	243.68 ± 138.3	NS

*ONEWAY ANOVA test for comparing between breast cancer stages.
*SD= standard deviation.
*NS = not significant (p>0.05).

significant (Table 3). However, from the results obtained using t-test, the difference was statistically significant between stage I and stages II and III).

The level of serum IgG in different breast cancer stages was higher than in the control group , but stage II only showed a statistically significant difference with the control group (p<0.05) (Table 4).

The serum IgA level increased from stage I (385.58 ± 174.34 mg/dl) to stage III (729.26 ± 134.23 mg/dl), and this difference was statistically significant (p<0.05). The serum IgA level was higher for different disease stages than for the control group, and both stages II and III showed a statistically significant difference with the control group (p<0.05) (Table 4). The serum IgM level for stage I (198.92 ± 76.39 mg/dl) was higher than that for stage II (177.28 ± 46.67 mg/dl); however, the level rose in stage III (226.14 ± 65.33 mg/dl) (Table 3), with no significant difference observed between these three groups. Also, no significant differences were found between the serum IgM level of breast cancer stages and that of the control group (Table 4).

Serum Complements (C3 and C4)

The serum C3 level increased from stage I (185.42 ± 73.73 mg/dl) through stage II (196.41 ± 75.93 mg/dl) to stage III (297.96 ± 39.29 mg/dl), and a statistically significant difference was found between them (p<0.05). The C3 level for different breast cancer stages was generally higher than that of the control group, but a statistically significant difference was found with stages II and III only

Table 4: Comparison of immunological parameters between different breast cancer stages and the control group.

Parameter	Stage I Mean ± SD	Stage II Mean ± SD	Stage III Mean ± SD	Control Mean ± SD
IgG (mg/dl)	1663.3 ± 862.4	1985.8 ± 574.0*	1954.4 ± 553.6	1536.6 ± 441.2
IgA (mg/dl)	385.58 ± 174.34	447.54 ± 156.29*	729.2 ± 134.2*	318.5 ± 124.5
IgM (mg/dl)	198.9 ± 76.3	177.2 ± 46.6	226.1 ± 65.3	197.1 ± 53.2
C3 (mg/dl)	185.4 ± 73.7	196.4 ± 75.9*	297.9 ± 39.2*	150.7 ± 39.9
C4 (mg/dl)	30.3 ± 11.9	39.2 ± 17.3	53.3 ± 14.2*	32.3 ± 13.3
IL-6 (pg/ml)	181.4 ± 128.4	223.8 ± 50.6*	259.2 ± 42.4*	171.3 ± 64.8
TNF-α (pg/ml)	159.47 ± 102.14	269.28 ± 225.65*	243.6 ± 138.3	131.5 ± 108.9

*Significant difference in relation to control (p<0.05).

Table 5: Comparison of immunological parameter for the patients preoperatively and after three cycles of chemotherapy

Parameter	Preoperative (n = 23) (Mean ± SD)	After three cycles of chemotherapy (n = 23) (Mean ± SD)	P value
IgG (mg/dl)	1952.30 ± 684.62	1283.95 ± 565.45	<0.05
IgA (mg/dl)	515.90 ± 182.12	380.06 ± 188.22	<0.05
IgM (mg/dl)	192.90 ± 52.38	178.6 ± 64.01	NS
C3 (mg/dl)	208.81 ± 82.26	166.88 ± 56.35	<0.05
C4 (mg/dl)	39.62 ± 19.50	34.77 ± 15.52	NS
IL-6 (pg/ml)	248.26 ± 49.66	215.73 ± 77.83	<0.05
TNF-α (pg/ml)	218.10 ± 169.61	249.34 ± 245.61	NS

(Table 4). The serum C4 level for patients at stage I (30.34 ± 11.92 mg/dl) was less than that at stage II (39.29 ± 17.39 mg/dl) and stage III (53.34 ± 14.25 mg/dl), but this difference was statistically insignificant (Table 3). The C4 level for stage I and the control group was almost the same. The C4 level for stage II was higher than that for the control, but it was statistically not significant, while there was a statistically significant difference between stage III and the control group ($p<0.05$) (Table 4).

Serum Cytokienes (IL-6 and TNF-α)

The serum IL-6 level increased from stage I (181.40 ± 128.42 pg/ml) through stage II (223.80 ± 50.62 pg/ml) to stage III (259.20 ± 42.41 pg/ml), but this difference was statistically not significant (Table 3).The IL-6 levels for all the stages were higher than those for the control; however, the values obtained for stages II and III were statistically significant ($p<0.05$) (Table 4).

No significant changes were found in the TNF-α level between the disease stages (Table 3). However, the serum TNFα level for stage II only showed significant difference with the control group (Table 4).

Comparison between Preoperative and Postoperative/ Post-Three Cycles of Chemotherapy

Serum Immunoglobulins (IgG, IgA, and IgM)

Patients after three cycles of chemotherapy exhibit statistically significant reduction of the serum IgG level (1283.95 ± 565.45 mg/dl) in relation to their preoperative values (1952.30 ± 684.62 mg/dl) ($p<0.05$) (Table 5). The preoperative serum IgA level (515.90 ± 182.12 mg/dl) was higher than after three cycles of chemotherapy (380.06 ± 188.22 mg/dl), and it was statistically significant ($P<0.05$) (Table 5). The serum IgM levels were similar between patients (192.90 ± 52.38 mg/dl) and controls (178.6 ± 64.018 mg/dl) post-three cycles of chemotherapy (Table 5).

Serum Complements (C3 and C4)

The C3 values preoperatively (208.8174 ± 82.26164 mg/dl) were significantly greater than the C3 values after three cycles of chemotherapy (166.8826 ± 56.35649 mg/dl) ($P<0.05$) (Table 5) .

The serum C4 levels for the patients before operation (39.6217 ± 19.50459 mg/dl) were similar to their C4 levels after three cycles of chemotherapy (34.7739 ± 15.5158 mg/dl) (Table 5).

Serum Cytokines (IL-6 and TNF-α)

The serum IL-6 level for the patients before operation (248.26 ± 49.65 pg/ml) was significantly higher than that after three cycles of chemotherapy (215.73 ± 77.83 pg/ml), ($p<0.05$) (Table 5) .

The serum TNF-α level preoperatively (218.10 ± 169.60 pg/ml) was less than that after three cycles of chemotherapy (249.34 ± 245.61 pg/ml) (Table 5).

DISCUSSION

Serum immunoglobulin levels in patients with breast cancer have been evaluated by many authors. For IgG, the results are contradictory, and they have been reported as high (11) in consistence with the present study, or normal (12), or even low (3). This difference might be due to the use of benign breast disease as a control for many studies, or the use of healthy people as a control for others, or due to the absence of disease stage evaluation.

The present study is in accordance with that of Gendek-Kubiak *et al.* (13), which revealed the increase in the IgG level in advanced stages although the increase was not significant (stage II and III had significantly greater values than stage I). Also, the value was higher in all stages of the disease than in the control. Since IgG was found to be expressed by cancers of epithelial origin such as breast cancer (14) and was involved in the survival and growth of epithelial tumor cells (15), this supported the findings of IgG contribution in cancer initiation in the precancerous stage in epithelial cells (15).

Besides, many studies found a decreased serum IgG level in breast cancer patients before chemotherapy than that in the controls. A study by Papatestas *et al.* (16) found a relation between the elevated serum IgG and breast cancer patients with a good prognosis mainly in parous. Since most of patients in the present study were parous, this may indicate the enhancement of immune system.

Many authors agree that the serum IgG level is decreased after chemotherapy (3,17,18), and no significant difference is found between patients with recurrence and those without recurrence .

The present study confirmed the finding of most authors, according to which the IgA levels in breast cancer patients are higher than in controls (17-19) and that the levels of IgA increases with the advancment in disease stages. Also the patients' IgA levels were higher than those of the controls at all disease stages, with a strong positive correlation with the disease stage. A significantly higher IgA level was for patients who developed recurrence than patients who did not.

Since the breast cancer cell line proved to secrete their own IgA (20,21), it is unclear whether this high IgA level and its relation to disease stages are a result of immune system fighting tumor cells or this elevation reflects the load and activity of the malignant cells through host immune modulation or secretion of IgA by their own cells. Any how this gives serum IgA a novel role in breast cancer patients' prognosis.

Most authors agree that the serum IgM level was within the normal range (3,18,19), although Alsabti (17) claimed a negative correlation with breast cancer stages. However, this result may be of no interest because generally the serum IgM level is still within the normal level. Also the present study found no significant difference in the IgM level between the recurrence group and the non-recurrence group.

The presence of IgG, C3, and C4 in carcinoma samples, associated with C5b-9 deposits, indicates that the complement system has been activated through the classical pathway (22). Caragine *et al.* (23) found that the tumour-expressed inhibitor of the early but not the late complement lytic pathway enhances the tumor growth in a rat model of human breast cancer. The results of the present study of a significantly greater C3 level in all the disease stages than in the controls, with a strong correlation with the advancement of the disease stages confirm the findings by Vijayakumar *et al.* (4).

The C4 level was generally not significantly higher in all the disease stages than in the controls, but it was increasing with the advancement in the disease stages (stage III significantly greater than controls), and this was in accordance with the findings by Vijayakumar *et al.* (4). However, C4 exhibited a weak positive correlation with the disease stage in the present study. These findings may be of interest since Carlsson *et al.* (24) found an increased C4 level 3.6 times in metastatic breast cancer patients than in controls by using the antibody microarray analysis. Thus, this high level of complement components may reflect the response of innate immunity to recompense the inhibitory effect of tumor cells and to the increased tumor load. However, this diverse family of immune proteins found to facilitate dysregulation of mitogenic signaling pathways, sustained cellular proliferation, angiogenesis, insensitivity to apoptosis, invasion and migration, and escape from immunosurveillance (25). So the advancement in the disease stage may imitate in part this high complement level. That would mean they run

parallel to each other. This gives the complement components, mainly C3, a prognostic value and it may be a vital treatment target.

After three cycles of chemotherapy, the levels of serum C3 and C4 decreased, which is in accordance with the study of Vijayakumar *et al.* (4). Thus, the reduction of serum complement level after chemotherapy may be in part due to increase in malignant cells susceptibility and so patients who exhibit a persistent high complement level may indicate treatment-resistant tumors, or may be related to a hidden disease process such as recurrence. The present study found that patients who developed recurrence express a significantly higher C3 level than patients who did not. So C3 can be beneficial in breast cancer prognosis and patients follow up during chemotherapy. Moreover, C4 was higher for patients who developed recurrence than patients who did not, but the value was not statistically significant. In contrast to this study, Mangano *et al.* (26) found that the C3 level remained normal in patients without relapse or any apparent metastasis, whereas it fell below the normal range in patients who displayed metastasis and/or approached the terminal phase. Moreover, no significant change was observed for the C4 level.

Multivariate analysis showed that the high level of serum IL-6 has an independent prognostic value, and it is correlated with the extent of disease (27). The present study revealed a higher IL-6 level in the disease stages in comparison with the controls, which is in accordance with the study by Alimhojaeva (28). This gives IL-6 an important role in breast cancer diagnosis. On the other hand IL-6 as marker of survival is not specific to the types of cancers. Thus, it is likely that IL-6 is an indicator of non-cancer comorbidities or cumulative effects of a lifetime of adverse health events rather than related to malignancy itself (29). So it cannot rise to be a diagnostic for malignancy, generally, and breast cancer, particularly.

In accordance to Jabłonska *et al.* (30), the present study found that the IL-6 level increased with the advancement in the disease stage. Although the value was statistically not significant perhaps due to small sample size, it is of interest because a significantly greater IL-6 level at stages II and III than in the control group was demonstrated.

Thus, the increase of IL-6 level with the advancement in the disease stage in the present study data may reflect the IL-6 role in tumor growth and metastasis. This interpretation gives IL-6 an important role in breast cancer prognosis. The level of IL6 can give a picture about the extent of the disease and may provide information about a subclinical spread of the disease. After chemotherapy IL-6 significantly decreased, which is in accordance with the study of Chala *et al.* (31). The present study found a significantly higher IL-6 level after three cycles of chemotherapy for patients who developed recurrence than for patients who did not. SinceYokoe *et al.* (32) found continuous elevation of IL-6 levels, this indicates poor prognosis in heavily pretreated patients with recurrent breast cancer. So the IL-6 level can be a predictive for recurrence of breast cancer as shown in the present study. Since IL-6 is found to increase breast cancer resistance to chemotherapy (33), so breast cancer sensitivity to chemotherapy increased by targeting IL-6 (34). Thus IL-6 can be an important prognostic and predictive marker as well as a vital treatment target in breast cancer patients.

According to one of the studies the serum TNF-α acts as a breast tumor promoter (35), and the present study with others found higher TNF-α in breast cancer patients than in controls (26, 28). Thus this makes TNF-α responsible for tumor initiation rather than eradication.

TNF-α enhances tumor proliferation (8) and augments the invasive ability of breast cancer cells, partly by regulating a series of metastasis-related genes, and these genes may take part in every step of metastasis (36). The present study found an increased TNF-α level in the advanced disease stages. Although no statistical significance was found, the patients' TNF-α level was higher than that of the controls at stages I and II (significant difference between controls and stage II). This is in accordance with the study by Jabłonska *et al.* (30), which confirms the recent views about the role of TNF-α in the breast cancer growth and metastasis. Moreover, this may reflect immune enhancement related to tumor burden, since Estevam *et al.* (37) found that patients with a clinical history of cancer recurrence and metastasis presented a lower number of cancerous apoptotic cells, higher tumor proliferation rates, and lower TNF-α expression rates by inflammatory cells than what was observed among

patients diagnosed with the same histopathological breast cancer type but in the absence of tumor recurrence and metastasis.

Jabłonska *et al.* (30) found that the TNF-α level decreased after chemotherapy, while the present study found an elevated TNF-α level. This may indicate resistance to chemotherapy since the present study found a significantly greater TNF-α level after chemotherapy than the preoperative TNF-α level in patients who developed breast cancer recurrence during or after chemotherapy. However, patients without recurrence exhibited a reduction in the TNF-α level after chemotherapy than in the preoperative TNF-α level. This is in accordance with the study by Nenova (38) who revealed that cancer recurrence for patients exhibited TNF-α enhancement after the third chemotherapy cycle. So the serum TNF-α level could be used clinically as a useful tumor marker for the disease extent and the outcome of breast cancer.

Therefore, the introduction of the immunological assessment as a part of prognostic and predictive factors for breast cancer patients as well as response to treatment is recommended.

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