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INVESTIGATING THE CHANGING LEVELS OF IMMUNE CHECKPOINT PROTEINS IN THE SERUM OF BREAST CANCER PATIENTS

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ABSTRACT

Breast cancer is the most frequent kind of invasive cancer in women. Biomarker monitoring and prognosis provide outstanding clinical information that may be employed to battle various cancers. This study aimed to examine a variety of biomarkers utilizing a multiplex bead array. BTLA, CD27, CD28, TIM-3, HVEM, CD40, LAG-3, TLR-2, PD-1, CD80, CD86, PDL-1, PDL-2, and ICOS are common biomarker checkpoint proteins found in a selective panel. This panel examined a research group of 59 patients with BC and 17 healthy people. The expression patterns of a handful of the fourteen biomarkers varied considerably between people with BC and healthy controls. With *P*-values of (p= 0.05), (p= 0.02), (p= 0.01), and (p= 0.02), general features of the two groups of malignant and non-malignant patients revealed significant correlations in parameters such as the age of first menstruation, pregnancy, menopausal status, and hormone replacement treatment. With *P*- values, there was a substantial rise in BTLA, HVEM, TLR-2, and PDL-1 serum levels, as well as a significant drop in CD86. Furthermore, these markers indicated a significant relationship with the clinic-pathological aspects of the patients. With a *P*-value of 0.0002, both BTLA and HVME showed a statistically significant connection with hormone receptor phenotypes. For ER status, BTLA, CD86, and PDL1 had equivalent significance values of (p= 0.0148), (p= 0.0166), and (p= 0.0001). BTLA was related to lymph node involvement (p=0.0397), whereas TLR2 was associated with HER2 status (p=0.0332). BTLA, HVEM, TLR-2 CD86, and PDL-1 may be valuable biomarkers for cancer monitoring and prognosis.

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Introduction

Breast cancer (BC) is the most common cancer in women globally, accounting for 11.7% of all cancer cases. It kills 685,000 women worldwide [1]. Saudi Arabia has the lowest BC frequency in the Middle East, yet BC has the highest fatality rate of all cancers [2]. Arab BC rates vary per region. Saudi Arabia had 22 BC cases per 100,000 women, the UAE 23, Kuwait, Jordan, and Qatar 40, and Bahrain 53 [3]. Saudi Arabia has a higher BC rate, particularly in younger women, than in western

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nations [4]. Saudi Arabian women are more likely than westerners to have advanced BC in young premenopausal women. Screening, mammography, clinical breast exams, and breast self-examinations have reduced BC mortality in many affluent countries [5].

BC prevention and early diagnosis reduce the risk of metastases and cancer. Despite frequent testing, terminal diagnosis reasons are still being studied [6]. Thus, improved cancer diagnostic and management strategies are needed to lower cancer mortality.

Immune checkpoints restrict immune responses depending on physical status [7]. They sustain self-tolerance. Cancer cells may "take over" immune checkpoint pathways to bypass immunological surveillance and survive the patient's T cells [8]. Inhibiting immunological checkpoints may limit tumor growth by preventing tumor cells from evading the immune system. Many immunological checkpoint pathways have been identified. The most researched and established pathways are CD80/86-CTLA-4 interactions and PD-1-PDL-1 tumor cell binding [9]. Both pathways inhibit T cell multiplication and function, allowing immunological evasion. B7 family inhibitory ligands such B7-H3 (CD276), TIM3 (HAVcr2), LAG3, CD244, BTLA (CD272), B7-H4 (VCTN1), VISTA, TIGIT, ADORA2, and IDO1 may constitute immunological checkpoints. Blocking several immune-checkpoint ligands or receptors boosts antitumor immunity in experimental animal cancer models. These trials used mice [10]. Many researchers believe tumor microenvironment cancer cells may evade host immunity. Cancer cells have many immunoinhibitory signalling proteins. This system's immune checkpoints include tumor-induced immune suppression (immune checkpoint) mediated by the programmed cell death protein 1 (PD-1) and its ligand, PDL-1. Mature T, B, macrophage, dendritic, and NK cells express PD-1, also known as CD279. The tumor cells' interaction with PD-1 and its programmed death-ligand 1 (PDL-1) ligand activates a signalling cascade that enhances T-cell responsiveness and antitumor activity. Another immunoglobulin superfamily member, LAG-3 (lymphocyte activation gene-3), was discovered in 1990. LAG-3 is expressed on activated CD8+, CD4+, and certain NK cells. It has a similar structure to CD4 and binds MHC-II better than CD4. LAG-3 activity is uncertain; however, chronically stimulated T cells may express more LAG-3. PD-1 and LAG-3 suppress activated CD8+ T cells in a surprising synergy. Albalawi *et al*. found a fundamental relationship with the vascular endothelial growth factor gene variant in Saudi BC patients. However, finding particular markers in BC patients' serum is debatable. Therefore, finding trends in BC biomarkers might reveal possible therapeutic targets and lead to a novel BC treatment [11].

Multiplex bead array is quicker and cheaper than standard immunoassays since it measures proteins/antibodies in low numbers. Additionally, it directly identifies biomarkers without pre-treatments. Multiplex bead-based immunoassay uses flow cytometry's fluorescently coded beads to collect antibodies accurately [12]. An integrated method for biomarkers and indicators to improve malignancy diagnosis has also been suggested [13].

BTLA, TIM-3, CD28, CD27, CD40, CD80, CD86, HVEM, LAG-3, TLR-2, PD-1, PDL-1, PDL-2, and ICOS were examined for their possible therapeutic uses in breast cancer early detection and targeted treatment. The present research also analyzed several analytes at high throughput.

Materials and Methods

Study Subjects and Samples

The Unit of Mammography, Department of Radiography, and King Abdulaziz University Hospital (KAUH), Jeddah, Saudi Arabia's Biomedical Ethics Research Committee authorized this study's enrolment and sample collection (HA-02-J-008). 59 BC patients and 17 non-malignant females. After consenting, a questionnaire was used to collect patient data, and anthropometric data were collected using standard methods. We also collected clinic-pathological characteristics with Jeddah's KAUH Pathology Department.

After blood clotting, serum was separated by centrifugation. Aliquoted serum sets were kept at -80°C. This research included blood samples from 76 patients—59 malignant and 17 non-malignant. The multiplex immunoassay required just thawing at four °C.

Multiplex Beads Array Immunoassay

Due to its broad range of multiplexed cancer marker assays, compatible with serum, plasma, tissues, cultured cells, and other biological samples, the beads-based MILLIPLEX® Human Immuno-Oncology Checkpoint Protein Premixed 17-plex Panel 1 - Immuno-Oncology Multiplex Assay was used in this study. Only 14 proteins were assayed: BTLA, CD27, CD28, TIM-3, HVEM, CD40, LAG-3, TLR-2, PD-1, CD80, CD86, PDL-1, PDL-2, and ICO Merck Millipore sold the kit.

Pipette analyte-conjugated beads into filter base microplate wells and incubate. Standards, controls, and undefined samples are pipetted. This first incubation binds analytes to the beads' capture antibodies. After washing, beads are incubated with analyte-specific biotinylated sensor antibodies. During this second incubation, analyte-specific biotinylated sensor antibodies identify their epitopes and attach them to the corresponding immobilized analytes. Streptavidin-RPE was added and incubated after biotinylated sensor antibodies were removed. This final incubation binds Streptavidin-RPE to biotinylated sensor antibodies on the beads' immune complexes to create a four-member solid-phase sandwich. After washing to remove unattached Streptavidin-RPE, the beads are examined using Luminex100/200TM. Calculate analyte concentrations by observing the beads' spectral properties and R-phycoerythrin (RPE) fluorescence. The Millipore multiplex test employed a 96-well filter plate (Merck KGaA, Darmstadt, Germany). Before adding 25 µL of standards, controls, or samples, each well was

washed with 200 µL of washing buffer and decanted. Control and patient samples were diluted before being dispersed in duplicate into the wells. The plates were left at room temperature for 16–18 hours after each well received 25 µL of the primary antibody-bead combination. After this, 25 µL of biotinylated detection antibodies and 25 µL of streptavidin-phycoerythrin were mixed and incubated at room temperature for one hour. A vacuum manifold-equipped washing buffer was used to double-wash after each process (Millipore Corp., Billerica, MA, USA). The final wash step resuspended the samples in 150 µL of Sheath Fluid PLUS before reading on a Luminex-200TM (Luminex Inc., Austin, TX, USA). The standard curve was constructed using a five-parameter logistic or spline curve-fitting technique to calculate the analyte concentration of each sample. Beadview also assessed the results (Upstate Biotechnology Inc., Lake Placid, NY, USA) [14].

Statistical Analysis

Statistical analysis was achieved using Statistical Package for Social Science (SPSS) IBM version 27.0 for Windows; Graph Pad Prism 7 was used alongside SPSS for graphics. One Way ANOVA was used to compare the mean difference between the variables in the case of parametric data. In contrast, the Kruskal Wallis test in the case of nonparametric data was applied to assess the association between variables. A two-tailed Student's t-test was used to find the differences between groups that were statistically significant. The results were expressed as mean ± standard error of the mean (SEM). *P*-value ≤0.05 was considered significant.

Results and Discussion

Characteristics of Subjects Participating in the Study

In this research, there were 76 female participants, 17 of whom did not have any detectable BC malignancy, whereas 59 were diagnosed with BC. Before the study, the individuals diagnosed with BC did not get any therapy. Comparison studies on patients' general characteristics between both groups of malignant and non-malignant patients showed no significant correlations in most parameters apart from the age of first menstruation, pregnancy, menopausal status, and hormone replacement. Therapy, with the *P*-value of (p= 0.05), (p= 0.02), (p= 0.01), and (p= 0.02), data collected from all subjects showed no significant differences in age (mean SEM) of the control non-malignant group (47.8±1.77) and the malignant group (51.81±1.59) **(Table 1)**.

Table 1. Comparison of the mean value of general characteristics parameters in control non-malignant and malignant patients

Parameters	Categories	Total N (%)	Non-malignant BC *n* (%)	Malignant BC *n* (%)	*p*-value
Number of patients, n (%)		76 (100)	17 (22.4)	59 (77.6)	
Age (years)	≤40	17 (22.4)	4 (23.5)	13 (76.5)	0.12
	41-60	47 (61.8)	13 (27.7)	34 (72.3)	
	>60	12 (15.8)	0 (0.0)	12 (100.0)	
BMI	Lean	10 (13.2)	2 (20.0)	8 (80.0)	0.91
	Overweight	28 (36.8)	7 (25.0)	21 (75.0)	
	Obese	38 (50.0)	8 (21.1)	30 (78.9)	
Marital status	Single	7 (9.2)	2 (28.6)	5 (71.4)	0.91
	Married	65 (85.5)	14 (21.5)	51 (78.5)	
	Divorce	4 (5.3)	1 (25.0)	3 (75.0)	
Age of first menstruation (years)	< 12	6 (7.9)	2 (33.3)	4 (66.7)	**0.05***
	12-15	63 (82.9)	12 (19.0)	51 (81.0)	
	> 15	5 (6.6)	1 (20.0)	4 (80.0)	
	No answer	2 (2.6)	2 (100.0)	0 (0.0)	
Age of pregnancy (years)	≤ 20	30 (39.5)	9 (30.0)	21 (70.0)	**0.02***
	21-30	31 (40.8)	3 (9.7)	28 (90.3)	
	> 30	5 (6.6)	0 (0.0)	5 (100.0)	
	No pregnancy	10 (13.2)	5 (50.0)	5 (50.0)	
Menopausal status	Premenopausal	34 (44.7)	9 (26.5)	25 (73.5)	**0.01***
	Postmenopausal	40 (52.6)	6 (15.0)	34 (85.0)	

	No answer	2 (2.6)	2 (100.0)	0 (0.0)	
Number of children	None	6 (7.9)	3 (50.0)	3 (50.0)	0.15
	3 or less	31 (40.8)	4 (12.9)	27 (87.1)	
	More than 4	37 (48.7)	10 (27.0)	27 (73.0)	
	No answer	2 (2.6)	0 (0.0)	2 (100.0)	
Hormone replacement therapy	Yes	2 (2.6)	0 (0.0)	2 (100.0)	**0.02***
	No	72 (94.7)	15 (20.8)	57 (79.2)	
	No answer	2 (2.6)	2 (100.0)	0 (0.0)	
Breast feeding	Never	14 (18.4)	3 (21.4)	11 (78.6)	0.17
	Yes	59 (77.6)	12 (20.3)	47 (79.7)	
	No answer	3 (3.9)	2 (66.7)	1 (33.3)	
Family history of BC	Yes	14 (18.4)	3 (21.4)	11 (78.6)	0.39
	No	58 (76.3)	12 (20.7)	46 (79.3)	
	No answer	4 (5.3)	2 (50.0)	2 (50.0)	
Family history of other cancers	Yes	11 (14.5)	1 (9.1)	10 (90.9)	0.24
	No	61 (80.3)	14 (23.0)	47 (77.0)	
	No answer	4 (5.3)	2 (50.0)	2 (50.0)	
Polycystic fibrosis status	Yes	8 (10.5)	0 (0.0)	8 (100.0)	0.06
	No	65 (85.5)	15 (23.1)	50 (76.9)	
	No answer	3 (3.9)	2 (66.7)	1 (33.3)	
Diabetes mellitus status	Yes	63 (82.9)	15 (23.8)	48 (76.2)	0.51
	No	13 (17.1)	2 (15.4)	11 (84.6)	
Physical activities Performance	Yes	24 (31.6)	5 (20.8)	19 (79.2)	0.83
	No	52 (68.4)	12 (23.1)	40 (76.9)	
Smoking	Yes	3 (3.9)	0 (0.0)	3 (100.0)	0.34
	No	73 (96.1)	17 (23.3)	56 (76.7)	

* *P*-value is ≤ 0.05.

Distribution of Clinic-Pathological Characteristics of the Malignant Group

The clinic-pathological characteristics of the individuals with malignant tumors, as seen in **(Table 2)**. Hormone receptor phenotype was found to be Luminal in 45 patients (76.3% of the group's populations), followed by HER2-enriched in 9 (15.3%), and triple-negative in 4 (6.8%). Furthermore, the majority of patients had a positive ER status, which was 44 (74.6%), whereas the majority of patients had a negative HER2 status, which was 35 (59.3%) and 23 (39.0%). Finally, lymph node involvement was determined to be primarily negative for 24 of the group's populations (40.7%), positive for 15 of the populations (25.4%), and unknown for 20 of the populations (33.9%).

Table 2. Distribution of clinic-pathological characteristics of the malignant group

Parameters	**Categories**	**Malignant BC, *n* (%)**
Number of patients, n (%)		59 (100)
Hormone receptor phenotype	Luminal	45 (76.3)
	HER2-enriched	9 (15.3)
	Triple negative	4 (6.8)
	Unknown	1 (1.7)
ER status	ER-	14 (23.7)
	ER+	44 (74.6)
	Unknown	1 (1.7)
PR status	PR-	22 (37.3)

	PR+	36 (61.0)
	Unknown	1 (1.7)
HER2 status	HER2-	35 (59.3)
	HER2+	23 (39.0)
	Unknown	1 (1.7)
Lymph node involvement	Negative	24 (40.7)
	Positive	15 (25.4)
	Unknown	20 (33.9)
Size of tumor (cm)	< 2	32 (54.2)
	5-2	21 (35.6)
	> 5	3 (5.1)
	Unknown	3 (5.1)
Tumor grade	I	7 (11.9)
	II	34 (57.6)
	III	17 (28.8)
	Unknown	1 (1.7)
Histotype	DCIS	46 (78.0)
	LCIS	5 (8.5)
	Mixture of ductal and lobular	2 (3.4)
	Mammary	5 (8.5)
	Unknown	1 (1.7)
Vascular invasion	Negative	39 (66.1)
	Positive	9 (15.3)
	Unknown	11 (18.6)
Margin	Negative	35 (59.3)
	Positive	1 (4.2)
	Unknown	23 (39.0)

The Serum Levels of the Studied Markers in Malignant Versus Non-Malignant Patients Using the Multiplex Immunoassay

The multiplex immunoassay was used to compare the levels of the examined indicators in the serum of individuals with malignant and non-malignant diseases. Among these 14 proteins, which were tested, the levels of serum BTLA, CD27, CD28, CD40, CD80, CD86, TIM-3, HVEM, LAG-3, TLR-2, PD-1, PDL-1, and PDL-2 were shown to be substantially different in malignant BC patients associated with control participants. In malignant samples, significant increases in levels of BTLA, HVEM, TLR2, and PDL-1 were found, with *P*-values of (p= 0.0250), (p= 0.0332), (p= 0.0499), and (p= 0.434), respectively. On the other hand, the CD86 serum level was shown to be considerably lower in malignant samples as compared to non-malignant samples (p= 0.0437). Despite this, two of the markers, TIM-3 and LAG-3, were very close to being significant at (p= 0.0713) and (p= 0.0776), correspondingly, whereas the remainder of the markers did not exhibit any significant alterations **(Table 3)**.

Table 3. Serum levels of the studied markers in malignant BC patients and control non-malignant using the multiplex immunoassay technology

Parameter	**Control Non-malignant (17)**	**Malignant (59)**	***P*-value**
N (%)	17 (21.3)	59 (78.7)	-
BTLA (pg/ml)	79.00 ± 7.811	118.0 ± 9.007	0.0250*
CD27 (pg/ml)	334.1 ± 49.16	2158 ± 236.9	0.3525
CD28 (pg/ml)	357.5± 57.37	439.8± 44.48	0.2133
TIM3 (pg/ml)	2463 ± 245.9	3677 ± 353.2	0.0713

HVEM (pg/ml)	3019 ± 522.5	4210 ± 248.4	0.0332*
CD40 (pg/ml)	592.8 ± 62.47	798.9 ± 51.91	0.0533
LAG3 (pg/ml)	29405 ± 4329	43549 ± 4087	0.0776
TLR2 (pg/ml)	435.8 ± 61.05	661.9 ± 60.75	0.0499*
PD-1 (pg/ml)	195.0 ± 31.98	196.5 ± 17.81	0.9690
CD80 (pg/ml)	18.07 ± 3.697	23.41 ± 2.729	0.3390
CD86 (pg/ml)	127.7 ± 13.03	93.10 ± 8.214	0.0437*
PDL1 (pg/ml)	20.12 ± 3.139	34.06 ± 3.820	0.0434*
PDL2 (pg/ml)	12341 ± 1570	12962 ± 900.5	0.7439
ICOS (pg/ml)	218.1 ± 34.28	253.4 ± 32.51	0.5687

(N=number of samples), Data presented as Mean ± SEM, * *P*-value is ≤ 0.05.

Tumor cells create tumor markers in response to carcinogenesis. Therapeutic biomarkers include CEA and CA15-3. Further study is needed to address numerous breast cancer monitoring, prognosis, and diagnostic issues. Many biomarkers were examined using a precision high-throughput multiplex bead array. Fourteen immune checkpoint proteins—BTLA, CD27, CD28, TIM-3, HVEM, CD40, LAG-3, TLR2, PD-1, CD80, CD86, PDL1, PDL2, and ICOS—were examined in serum. This study found substantial differences between malignant and non-malignant BTLA, HVEM, TLR2, CD86, and PDL-1 serum levels. Decreasing CD86 levels in malignant individuals (p=0.0437); rising levels of the other markers (p=0.0250; p=0.0332; p=0.0499; and p=0.0434). The remaining markers, TIM-3, CD40, and LAG3, have *P*-values of 0.0713, 0.0533, and 0.0776, respectively.

The Receiver Operating Characteristic (ROC) curve compares the true positive rate (TPR) and false positive rate (FPR). This graphic illustrates data diagnostics. The real positive rate (TP/ (TP + FN)) is the ratio of positive observations to those projected to be positive. The false positive rate (FP/ (TN + FP)) is the percentage of negative observations misinterpreted as positive. In diagnostic testing, the "true positive rate" is the percentage of patients accurately diagnosed with the ailment.

ROC is a discrete classifier that only returns the anticipated class. The score threshold may be modified to create a curve for probabilistic classifiers, which assign a probability or score to an instance's class membership. These probabilistic classifiers provide a probability or score for each event, indicating its class. Probabilistic classifiers assess instances based on their class membership. To score discrete classifiers, "analyse" their instance statistics. For example, a decision tree classifies a leaf node by its fraction of total occurrences. This improves tree correctness. Summarizing each classifier's findings into a single metric might make it easier to compare their effectiveness.

AUC is one method used to calculate the area under the ROC curve. The two-sample Wilcoxon rank-sum statistic evaluates the chance that a randomly picked positive occurrence will be scored higher than a randomly selected negative instance. It is analogous to the chance that a positive case picked randomly would be evaluated higher than a bad one.

It refers to the potential that a favourably selected example is rated higher than a negatively chosen one. High-AUC classifiers may have low AUCs in some areas. In practice, the AUC works well as a proxy for prediction accuracy. Curves were created utilizing blood levels of BTLA, HVEM, TLR2, CD86, and PDL-1 in malignant breast cancer patients and non-malignant controls to investigate the clinical sensitivity and specificity of these putative biomarkers and their value.

ROC curves indicated that blood levels of BTLA, HVEM, TLR2, CD86, and PDL1 substantially differentiate between malignant and non-malignant breast cancer, with AUC values of 0.665, 0.682, and 0.664, respectively.

CTLA4 had a 0.69 AUC (p= 0.063). HVEM may be a genetic biomarker of aggressive breast cancer **(Table 4)**. BTLA, HVEM, TLR2, PDL1, and CD86, markers of significant changes, were examined to evaluate how closely they correlated with patients' general and clinic-pathological features **(Figure 1)**.

Table 4. The 5 Biomarkers candidates that were analysed to identify their significance when evaluated between malignant and non-malignant samples

Parameter	n	AUC	95% CI	ROC p-value
BTLA	69	0.6645	0.5308 to 0.7982	0.0473
HVEM	70	0.6824	0.5108 to 0.8541	0.0312
TLR2	66	0.6644	0.5132 to 0.8155	0.0491
CD86	67	0.6910	0.5599 to 0.8221	0.0250
PDL-1	56	0.6837	0.5380 to 0.8293	0.0249

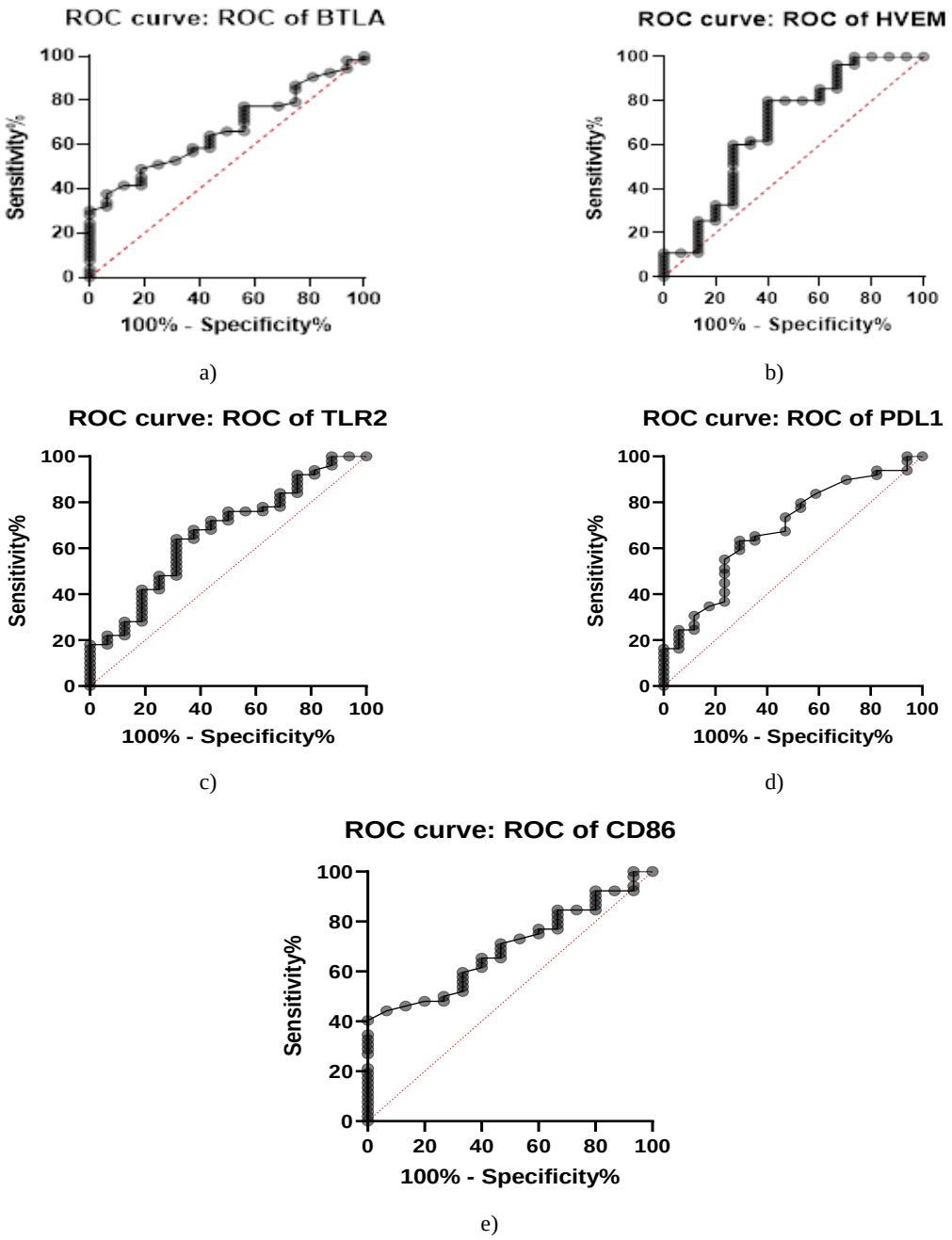


Figure 1. Receiver Operating Characteristic Curve (ROC) for a) **BTLA**, AUC (0.6645) *P*-value (0.0473); b) **HVEM**, AUC (0.6824) *P*-value (0.0312); c) **TLR2**, AUC 0.6644 *P*-value (0.0491); d) **PDL1**, AUC (0.6837) *P*-value (0.0249)' and e) **CD86**, AUC (0.6910) *P*-value (0.0250); Suggesting their role as a potential biomarker in BC patients.

Association of Malignant Patient's Serum Levels BTLA, HVEM, TLR2, PDL1, and CD86 with General Characteristics' Using Multiplex Immunoassay

This research examined serum markers and overall patient characteristics **(Figure 2)** demonstrate that many subcategories were associated with malignant serum levels of various markers and general characteristics categories. However, other parameter comparisons were insignificant **(Figure 3)**.

Marital status, family history of BC, and other malignancies were the primary variables linked to BTLA protein, with *P*-values of 0.0211, 0.0115, and 0.0474, respectively. These investigations found higher BTLA levels in married women and individuals with a family history of cancer. Additionally, married women have a greater cancer risk **(Figure 2)**.

Family history of BC and history of other malignancies were the main factors associated with TLR2 serum level, with *P*-values of 0.0022 and 0.0023, respectively. These indicators are significantly correlated with TLR2 serum levels. This shows that those with a documented family history of BC and other malignancies had higher TLR2 levels **(Figure 2)**. Hormone replacement therapy, a family history of BC, and a history of other malignancies also differed in PDL-1 protein levels, with *P*-values of 0.0281, 0.0141, and 0.0041, respectively. HVME and CD86 blood levels did not correlate with any of the other parameters in this study **(Table 1)**.

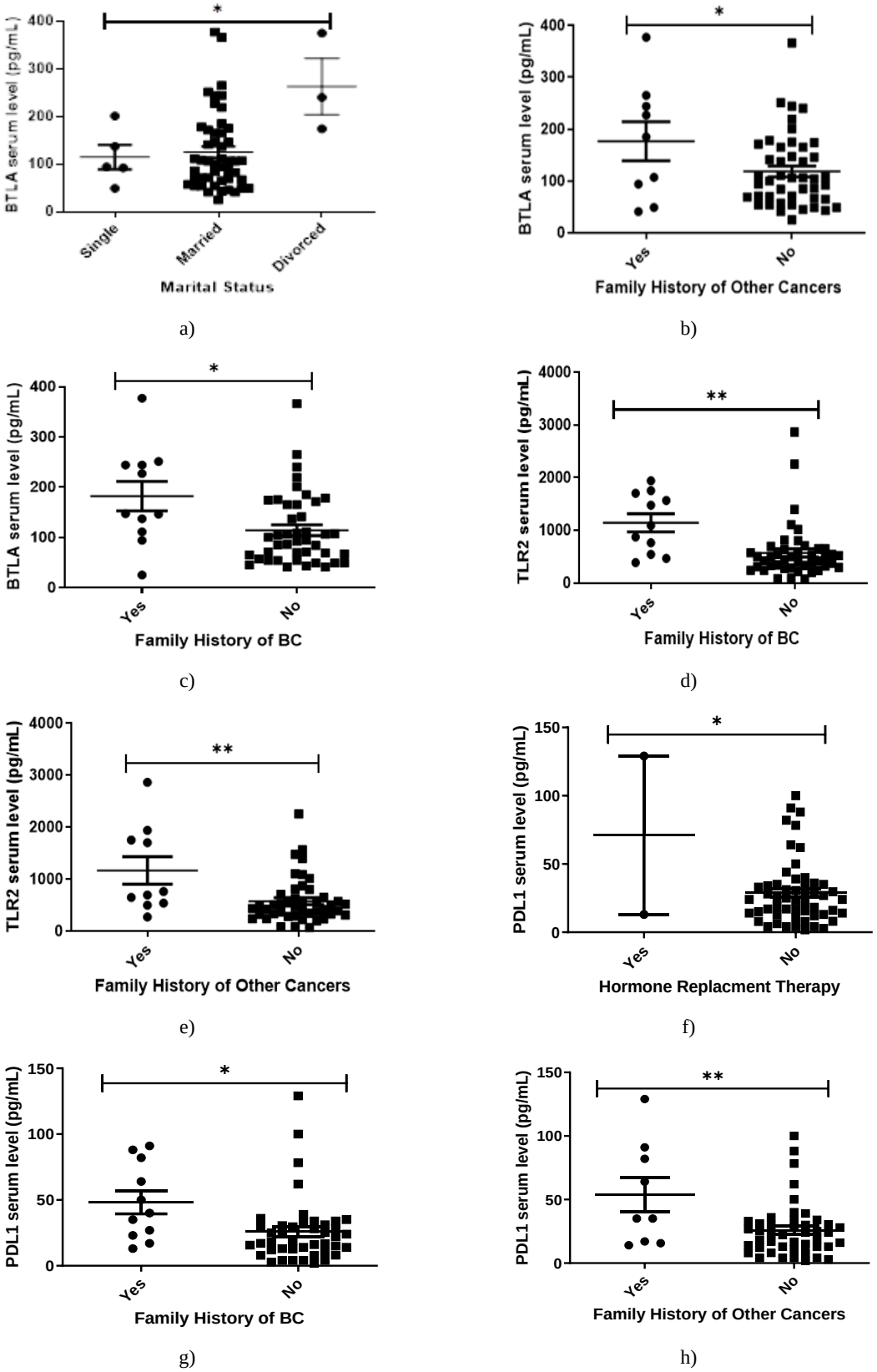
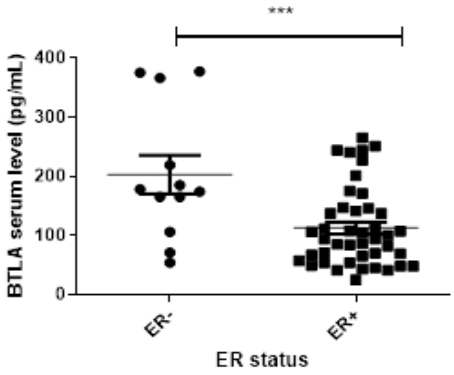


Figure 2. Association between malignant serum levels of BC patients with general characteristics that showed significant differences for BTLA. a); The Upregulation of BTLA levels in marital status for both married and divorced patients. b); family history of other cancers and c); a family history of BC. For TLR2. d); The Up regulations TLR2 for both family history of other cancers and e); family history of BC. For PDL1. f); The Upregulation of PDL1 in hormonal replacement therapy. g); As well as for both a family history of other cancers and h); a family history of BC.
* P-value ≤ 0.05 is considered significant.

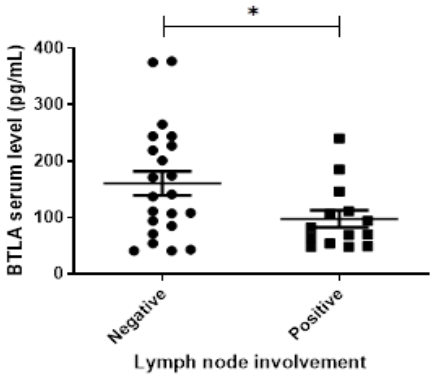
Investigating the correlation of patient's serum levels using multiplex immunoassay technology with clinic-pathological characteristics of malignant patients

Serum samples from patients with substantial differences between malignant and non-malignant were studied and compared to the study's clinic-pathological features. Cancer and non-cancer patients provided these samples. The investigation identified relationships between certain protein levels and clinic-pathological categories, whereas others were inconsequential.

The BTLA marker is correlated with hormone receptor phenotypes, starting with the HER2-enriched and ending with the luminal, with a *P*-value of 0.0002. ER- followed by ER+ was significant at 0.0148. In conclusion, negative lymph involvement increased BTLA expression (p=0.0397). Other metrics showed no significant variations between the individual categories. As P-values across subgroups, PR status was 0.1562, HER2 status was 0.0780, tumor size (cm) and grade were 0.6604 and 0.6604, respectively, histotype and vascular invasion were 0.2837 and 0.9591, respectively **(Figure 3)**. BTLA, as a diagnostic and predictive biomarker for ovarian cancer, is lacking, especially for assessing protein levels in peritoneal fluid. BTLA levels in tumor tissue have been linked to poor prognoses for ovarian and breast cancer patients. Another study [15], found that ovarian cancer patients had significantly higher median protein concentrations (BTLA, CD27, CD28, and CD80) in their blood. Recent research linked BTLA to lower immune checkpoint inhibitor patient survival. This information may help select candidates for cancer immunotherapy [16].



a)



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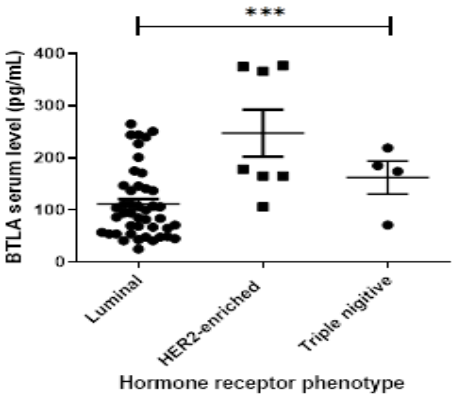
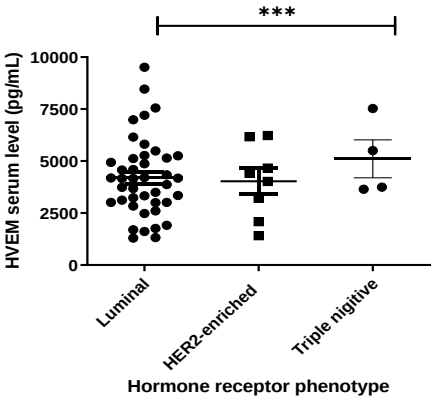
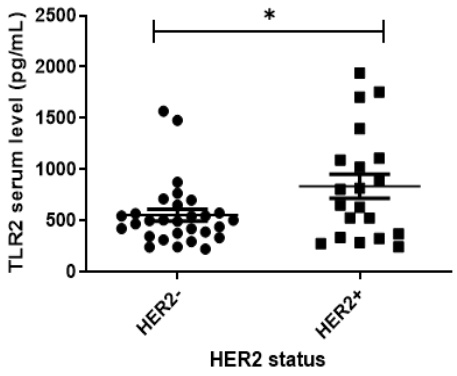


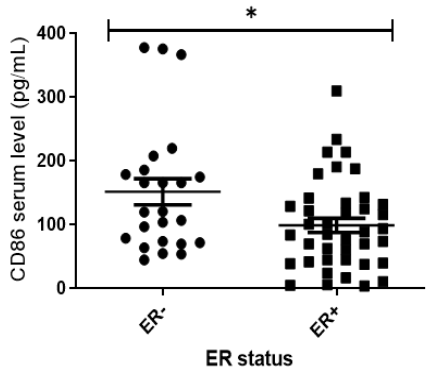
c)



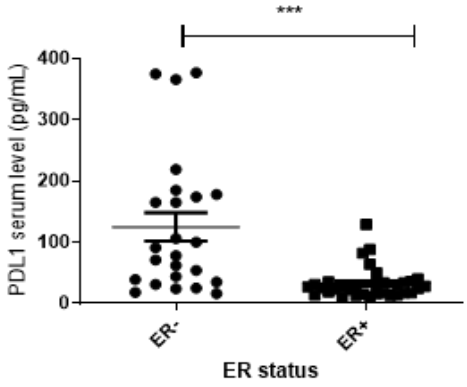
d)



e)



f)



g)

Figure 3. Comparison between malignant samples and clinic-pathological characteristics for BTLA. a); BTLA showed highly significant elevation for hormone receptor phenotype, particularly for HER2- enriched followed by the triple-negative phenotype, b); highly significant elevation for ER- status followed by ER+, c); and finally significant elevation for negative lymph node involvement. For HVME and TLR2. d); HVEM showed significant elevation for hormonal receptor phenotype with the highest for triple-negative followed by the luminal phenotype. e); TLR2 showed highly significant elevation for HER2+ statues. Both CD86 and PDL1 are significantly associated with ER- status. f); CD86 showed significance association and g), PDL1 showed, highly significance association with ER status.
* *P*-value ≤ 0.05, and *** *P*-value ≤ 0.0005 are considered as significant.

HVEM showed a strong correlation with hormone receptor phenotype, with greater expression in luminal phenotype followed by HER2-enriched phenotype and a *P*-value of 0.0002. Other metrics showed no significant variations between the individual categories. HER2 status, lymph node involvement, tumor size (cm), tumor grade, histotype, and vascular invasion were at *p*= 0.3649, 0.0808, 0.5124, 0.6604, 0.2837, and 0.1230, respectively. Hormone receptor phenotypic subgroups had 0.7876. ER, and PR status had P-values of 0.3622 **(Figure 3d)**. Several studies have indicated that the HVEM-BTLA signalling pathway modulates immunology in autoimmune disorders, cancer, transplantation, infection, and other diseases [17]. Recent research on the HVEM-BTLA signalling pathways has illuminated BTLA's significance and mechanism, and BTLA-targeted anticancer drugs are being developed [18].

HER2+ individuals expressed more TLR2 (*p*= 0.0232). Although most metrics did not indicate significant variations across distinct categories, the hormone receptor phenotype *P*-value was 0.0881, and the ER and PR status p-values were 0.2387 and 0.6471, respectively. Lymph node involvement was 0.3784, tumor size in centimetres was 0.4116, and tumor grade was 0.7816 **(Figure 3e)**. Recently, breast cancer tissue expressed more TLR2 than normal tissue. Tumor size, HER2 status, and subtype influenced this expression. HER2-positive BC patients had a worse prognosis due to TLR2 overexpression. Endocrine resistance prevents hormone treatment from working. TLR2 predicted endocrine resistance better in breast cancer patients with HER2 than other hormone receptors [19].

CD86 expression increased for ER^- and ER^+ at *p*= 0.0166. Other parameters did not differ across distinctive groupings. Hormone receptor phenotype, PR status, HER2 status, lymph node involvement, tumor size (cm), tumor grade, histotype, and vascular invasion all had p-values across groupings **(Figure 3f)**. CD86 dramatically improved TNBC's overall survival. High mRNA expression was associated with worse overall survival in luminal B and A breast cancer patients [20].

Moreover, PDL-1 was significant for ER^- and ER^+ at *p*= <0.0001. However, other parameters did not differ across distinctive groupings. For example, hormone receptor phenotype, PR status, HER2 status, lymph node involvement, tumor size (cm), tumor grade, histotype, and vascular invasion all had p-values across groupings **(Figure 3g)**.

Hormone replacement treatment, family history of BC, and history of other malignancies had *P*-values of 0.0281, 0.0141, and 0.0041, respectively. As expected, serum levels of PDL-1 protein demonstrated significance between malignant and non-malignant tumors, showing an increase in PDL-1 levels in patients with a positive hormone replacement status and a documented family history of cancer. PDL-1 only showed significance in ER status, with ER- exhibiting significance first, followed by ER+ with a P-value of 0.0001. PDL-1, a novel predictive biomarker for immune checkpoint inhibitors in cancer, is not ideal. PDL-1 levels might be interpreted differently depending on tumor type and a checkpoint inhibitor. Cancer immunotherapy, companion diagnostics, and prediction biomarkers are advancing rapidly. PDL-1 expression improves anti-HER2 targeted treatment responsiveness in HER2+ BC patients. Because immunotherapy has used the PD-1/PDL-1 checkpoint pathway to treat various cancers. In compartmentalized tumors, immune cells expressed these proteins, whereas tumor cells expressed PDL-1. PD-1, PDL-1, and LAG-3 were also linked to TNBC tumor architecture [21].

Serum TIM-3 protein levels were substantially different between malignant and non-malignant tumors while considering patient characteristics. Statistically, malignant and non-malignant serum HVME protein levels differed by 0.0332. Compared to clinic-pathological traits, HER2 status was significant at p= 0.0332, with HER2+ displaying a more vital expression. Advanced cancer patients with higher baseline serum TIM-3 had better clinical outcomes. Plasma TIM-3 levels may grow. Another inhibitor molecule, TIM-3, may work differently depending on the immunological group it interacts with. Leukocytes

and mononuclear cells from peripheral blood showed that ovarian cancer patients had higher T cell TIM-3 expression than healthy persons. TIM-3 increased higher in T-cells during ovarian cancer recurrence, suggesting that it may be a biomarker for early diagnosis and detection [22]. Osteosarcoma occurred when peripheral T cells overexpressed TIM-3. The stage of the tumor and the number of metastases increased TIM-3 expression, which was linked to a worse chance of survival. These findings support TIM-3 as an osteosarcoma diagnostic and prognostic marker. In hepatocellular cancer, nearby cytokines induce TIM-3 expression [23]. TIM-3 and other T cell exhaustion indicators, including PD-1 and CTLA-4, are lower in the peripheral blood and tumor microenvironment. Therefore, immunological checkpoints may struggle to identify cancer owing to TIM-3 expression variability. In addition, TIM-3 only increased survival in non-ER patients [24].

Serum CD40 protein levels nearly distinguished malignant from non-malignant situations when combined with patient characteristics. Polycystic fibrosis increased CD40 levels at p= 0.0472. These indicators did not correlate with the clinic-pathological features of the study patients. A transcriptional splice variant of CD40 was found to be highly expressed in hormone receptor–positive breast carcinoma cytoplasm. Triple-negative breast cancer-surface membranes showed this variation [25].

Two criteria, hormone replacement treatment (0.0111) and a family history of previous malignancies (0.0015), were significant when linked with patients' characteristics. Serum LAG3 protein levels showed a virtually significant status between malignant and non-malignant at p=0.0776. However, this significance was not statistically significant. LAG3 levels increased in hormone replacement therapy patients with a family history of cancer. LAG3 was significant for hormone receptor phenotypes starting with the HER2-enriched phenotype and ending with the Triple negative phenotype, each with a *P*-value of 0.0002. LAG3 expression was linked to positive and negative lymph involvement with a *P*-value of 0.0366. Immunosuppression by LAG-3 promotes tumor escape. LAG-3 on immune cells penetrating a tumor is related to a worse prognosis and progression. Renal cell carcinoma, stomach cancer, bladder cancer, colon cancer, chronic lymphocytic leukemia, acute myeloid leukemia, follicular lymphoma, and hepatocellular carcinoma have verified this conclusion independently. Surprisingly, LAG-3 expression on tumor-infiltrating immune cells was associated with a favourable prognosis for cancer patients who had received treatment. LAG-3 may indicate activated T cells. LAG-3 expression was associated with better overall survival, particularly in early-stage patients, in a recent meta-analysis of diverse tumor types. The research found this. LAG-3 expression was linked to several cancers [26].

Conclusion

This research used a high throughput multiplex bead array since it is a susceptible and specific multiplex bead-based test for detecting human antibodies. Developing a platform that is both straightforward and capable of high throughput will make it easier to conduct practical and routine tests. In addition, the method known as multiplex bead-based assay gives the capacity to analyse many analyses concurrently, which might save time and make better use of costly resources. This technique uses a multiplex profiling approach that can identify up to one hundred different analytes in a sample with a very modest size volume. The limited number of participants and participants in the control group were two of the limitations of this research. However, the present work highlighted the possibility of early breast cancer detection with high sensitivity. Furthermore, it proved the utility of the antibody-bead array strategy in detecting signatures distinct for primary breast cancer that has not spread to other body parts. However, before array-based technology may be utilized frequently for early breast cancer diagnosis, more validation is necessary.

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Conflict of interest: None

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Ethics statement: The studies involving human participants were reviewed and approved by the Unit of Biomedical Ethics, Research Committee (Document number: HA-02-J-008), King Abdulaziz University Hospital, King Abdulaziz University, Jeddah, Saudi Arabia. The patients/participants provided their written informed consent to participate in this study.

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