



Study the Association between TNF- α Gene Expression with Antioxidant Defense and Certain Cytokine in Breast Cancer Patients

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Abstract

Breast cancer is the most prevalent malignancy among women around the globe, wherein chronic inflammation, oxidative stress, and impaired immunity play pivotal roles. To analyze TNF- α gene expression and its relationship with antioxidant status and some selected cytokines in breast cancer patients. This case-control study was conducted from July of 2025 to February of 2026 at the Zahra Teaching Hospital, Kut city, Wasit. Patients and methods: The sample consisted of 87 breast cancer patients & 80 age and sex matched healthy controls. We measured antioxidants and cytokines in serum samples using ELISA kits (SunLong, China). Total RNA was extracted with TRIzol method and assessed for its gene expression of TNF- α by RT-qPCR. The majority of breast cancer patients were aged >60 years (55.17%). Most patients had tumor size 2–5 cm (58.62%) and left-sided tumors (55.17%). ER-positive cases represented 64.37% of patients. Antioxidant markers were significantly reduced in patients, including GSH (2.15 ± 0.19 vs 13.82 ± 1.04), SOD (0.59 ± 0.13 vs 8.15 ± 0.61), and TAC (0.09 ± 0.02 vs 0.31 ± 0.07), $P=0.001$. Inflammatory cytokines were significantly elevated in patients, including TNF- α (84.62 ± 13.48 vs 17.41 ± 3.92), IFN- β (285.15 ± 32.93 vs 37.11 ± 6.47), and IL-1 α (52.12 ± 9.44 vs 21.39 ± 5.27), $P<0.001$. TNF- α gene expression was significantly upregulated in patients (fold change 2.69 vs 1.39, $P=0.001$). ROC analysis showed high diagnostic performance, with IFN- β (AUC=0.96), SOD (0.95), and TNF- α (0.94) showing the strongest discrimination. TNF- α gene expression and inflammatory/oxidative biomarkers may serve as potential diagnostic indicators in breast cancer.

Keywords: Breast cancer; TNF- α ; oxidative stress; cytokines; gene expression

Introduction

Breast cancer is the most commonly diagnosed cancer in females globally and remains one of the leading causes of cancer-related mortality, even in the face of remarkable progress in technology and therapeutics. Recent global estimates show that breast cancer now constitutes a significant fraction of all newly diagnosed cancer cases and cancer deaths among women, and thus with its increasing prominence in the worldwide public health agenda (Arnold *et al.*, 2022; Bray *et al.*, 2022). As a result, the disease burden is especially dire in developing countries, where a lack of healthcare resources, late diagnosis and unsatisfactory screening programs, contributes to worse clinical outcomes (Batan *et al.*, 2025). Breast cancer is the most frequent malignancy in females and is a marked public health issue in Iraq. For the last few decades, the incidence of breast cancer has been steadily increasing, and then applying tremendous medical, social, and economic pressure on breast cancer patients and providers (Alrawi, 2022; Ali and Muslim, 2025). Breast cancer also affects psychological health, quality of life and is associated with higher levels of anxiety (Sedighi *et al.*, 2023; Ali and Al-Qazaz 2025), depression, fatigue, and social functioning among patients during treatment and survivors (Sharma *et al.*, 2021; Álvarez *et al.*, 2023). Breast cancer is a multifactorial disease driven by a series of genetic alterations as well as chronic inflammation, immune dysregulation and oxidative stress. It has been shown that molecules participating in inflammation secreted from tumor cells and immune cells in the tumor microenvironment contribute to cancer initiation, progression and control the angiogenesis and metastasis of tumors (Sharma *et al.*, 2021). Among these mediators, tumor necrosis factor-alpha (TNF- α) is one of the most significant pro-inflammatory cytokines, which regulates numerous cellular processes involved in cell proliferation, apoptosis, immune responses, and inflammatory signal pathways (Abdulhameed and Mohammed, 2022). Aberrant expression of TNF- α is reported to be involved in several pathological processes and triggers tumor growth via activation of nuclear factor-kappa B (NF- κ B) and other oncogenic signals (Abdulhameed and Mohammed, 2022; Mohamed and Abbas, 2026). Oxidative Stress Another well-known mediator that acts on breast cancer pathogenesis. This phenomenon occurs when the production of reactive oxygen species (ROS) is not balanced by the ability of antioxidant defense systems to eliminate active species. While ROS play a positive role at physiological concentrations, the excessive production of ROS generates DNA damage, genomic instability, lipid peroxidation, and protein modifications, all of which may promote cancer and tumor progression. Multiple studies demonstrated the alteration of antioxidant defense and the improvement of oxidative damage in breast cancer patients suggesting the importance of the disturbance of the redox balance in breast cancer initiation and progression (Suzuki and Nishizawa, 2014; Sawczuk *et al.*, 2019). The interaction of inflammatory cytokines and oxidative stress is an integral part of cancer biology. For instance, TNF- α is a major driver of ROS generation and oxidative stress in cells as well as a powerful proinflammatory cytokine, and this activity contributes to a positive feed-forward loop of induction of genes that mediate these effects, thus promoting cell survival and oncogenesis. Other cytokines participate in breast tumorigenesis in addition to TNF- α , such as interleukin-6 (IL-6) and interleukin-10 (IL-10), and their relation with breast cancer has been investigated in terms of both disease immunity and prognosis (Ahmed *et al.*, 2016; Yousif, 2024).

Few studies have evaluated the association between gene expression of TNF- α , antioxidant defense system and some cytokines in patients with breast cancer in the same person and among Iraqi women. Hence the present study was conducted to analyze TNF- α gene expression and to observe its association with antioxidant defense and some selected cytokines in breast cancer patients for a better understanding of the role of TNF- α in the pathogenesis of breast cancer.

Materials and methods

Study Design and Participants

This is a case-control study that was performed from July 2025 to February 2026. The study consisted of 87 women who were diagnosed with breast cancer and who used to visit the Zahra Teaching Hospital, Wasit, Iraq. Diagnosis of breast cancer was made based on clinical examination, mammography, ultrasonography, and histopathological analysis of biopsy samples. The control group was composed of 80 apparently healthy women without history of malignancy, auto-immunities or chronic inflammatory diseases. Controls were matched for age to the patient group and were recruited within the same study period.

Blood Sample Collection

5mL venous blood was drawn from each participant into sterile disposable syringes under aseptic conditions. Blood samples were immediately placed into gel clot activator tubes for clotting at room temperature. The samples were then centrifuged at 3000 rpm for 15 minutes to get serum. The serum was separated, apportioned equally into four, sterile Eppendorf tubes and stored at -20°C (or -80°C where available) until cytokine and biochemical analyses were carried out. In the case of gene expression analysis, we immediately mixed 250 μL of whole blood with 750 μL of TRIzol reagent for proper RNA stabilization and extraction. Samples were mildly homogenized and then colocated at -80°C until RNA isolation and molecular analysis of TNF- α gene expression.

Antioxidant and Cytokine Assays

Oxidative stress biomarkers and inflammatory cytokines assessment was performed using serum samples. Evaluation of the internal antioxidant status by the assessment of reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and total antioxidant capacity (TAC) The concentrations of inflammatory cytokines (IFN- α , IFN- γ , TNF- α , and IL-1 α) from plasma were examined as biomarkers of systemic inflammation and local immune response in breast cancer patients. About commercial enzyme-linked immunosorbent assay (ELISA) kits provided by SunLong Biotech Co., Ltd. (China) were used to detect all antioxidant enzymes and cytokines were measured. In general, for each assay, the wells received sample (10 μL) and sample diluent (40 μL), and then three HRP-conjugate reagents (HPR) (50 μL) were added. Normal B14-C4C plates were incubated following the manufacturer's protocol for antigen-antibody binding. Wells were washed to remove unbound components, followed by the addition of Chromogen Solution A and Chromogen Solution B; the enzymatic reaction was developed in the dark, with color formation proportional to analyte concentration. Stop solution was then added to stop the reaction, and the optical density was measured at 450 nm using ELISA microplate reader.

Molecular Analysis of TNF- α Gene Expression

RNA Extraction and Quantification

For gene expression analysis, 0.25 mL of whole blood was collected from each participant and immediately mixed with 0.75 mL of TRIzol reagent for RNA stabilization. Total RNA was extracted using an RNA purification kit (Promega, USA) according to the manufacturer's instructions. The concentration and purity of RNA were determined using a NanoDrop spectrophotometer (Bioneer, Korea) as described by (Mohammed, 2018). Only high-quality RNA samples were included in subsequent analyses.

cDNA Synthesis and qRT-PCR

Gene expression analysis of TNF- α was performed using One-Step RT-qPCR according to (Mohammed, 2018). Primers were designed based on NCBI database sequences and supplied by Macrogen (Korea). β -Globin was used as the housekeeping gene for normalization. The primer sequences used in this study are presented in **Table 1**.

PCR Reaction Mixture

The composition of the qRT-PCR reaction mixture (final volume 10 μ L) is summarized in **Table 2**, following the manufacturer's protocol (Promega, USA).

Thermal Cycling Conditions

The RT-qPCR amplification program was performed using a Real-Time PCR system (Molecular Systems, Australia), and the detailed thermal cycling conditions are presented in **Table 3**.

Primer Sequences

Table 1. Primer sequences used for qRT-PCR

Gene	Primer	Sequence (5'–3')	Reference
TNF- α	F	CCCAGGCAGTCAGATCATCTTC	(Enayati et al. 2015)
TNF- α	R	GTTTCAGGAGGCTGGCATGA	
β -Globin	F	ACACAACCTGTGTTCACCTAGC	
β -Globin	R	CAACTTCATCCACGTTCCACC	

Table 2. Composition of qRT-PCR reaction mixture (10 μ L total volume)

Component	Volume (μ L)
qPCR Master Mix (Promega, USA)	5.0
RT Mix	0.25
MgCl ₂	0.25
Forward Primer	0.5
Reverse Primer	0.5
Nuclease-free Water	2.5
RNA Template	1.0
Total Volume	10 μ L

Table 3. RT-qPCR amplification program

Step	Temperature (°C)	Time	Cycles
Reverse Transcription	37	15 min	1
Initial Denaturation	95	5 min	1
Denaturation	95	20 sec	40
Annealing	55–65	20 sec	
Extension	72	20 sec	

Relative expression levels of TNF- α were calculated using the $\Delta\Delta C_t$ method after normalization with β -globin as the housekeeping gene.

Statistical Analysis

Data were analyzed with SPSS ver. 18 software and expressed as mean $\pm$ SD. Differences between the variables were assessed using independent t-test, while the Pearson correlation was performed to establish associations. The diagnostic performance of TNF- α gene expression and biochemical markers for RA was assessed by ROC curve analysis with AUC, sensitivity, and specificity calculated. Statistical significance was set at $p < 0.05$.

Results and discussion

The age distribution of breast cancer patients showed that out of 87 cases, 1 patient (1.15%) was in the age group 20–30 years, 4 patients (4.60%) were in the age group 31–40 years, and 11 patients (12.64%) were in the age group 41–50 years. In addition, 23 patients (26.44%) were in the age group 51–60 years, while the majority of patients, 48 (55.17%), were aged above 60 years, Table 4.

Table 4. Age distribution of breast cancer patients

Age group (years)	No.	Percentage (%)
20–30	1	1.15%
31–40	4	4.60%
41–50	11	12.64%
51–60	23	26.44%
>60	48	55.17%
Total	87	100%

Regarding tumor diameter, among breast cancer patients ($n = 87$), 27 patients (31.03%) had tumors with a diameter < 2 cm. The majority of patients, 51 (58.62%), had tumor sizes ranging between 2–5 cm, while 9 patients (10.34%) had tumors larger than 5 cm, Table 5.

Table 5. Tumor diameter distribution in breast cancer patients

Tumor diameter (cm)	No.	Percentage (%)
< 2 cm	27	31.03%
2–5 cm	51	58.62%
> 5 cm	9	10.34%
Total	87	100%

Regarding tumor laterality, out of 87 breast cancer patients, 48 cases (55.17%) had tumors located in the left breast, while 39 cases (44.83%) were found in the right breast.

Table 6. Distribution of breast cancer patients according to tumor side

Tumor side	No.	Percentage (%)
Left	48	55.17%
Right	39	44.83%
Total	87	100%

Regarding estrogen receptor (ER) expression, 56 out of 87 breast cancer patients (64.37%) showed positive ER expression, while 31 patients (35.63%) were ER-negative, Table 7.

Table 7. Estrogen receptor (ER) expression in breast cancer patients

ER expression	No.	Percentage (%)
Positive	56	64.37%
Negative	31	35.63%
Total	87	100%

Table 8 showed a significant ($P \leq 0.05$) reduction in antioxidant defense markers in breast cancer patients compared with controls. The levels of GSH, GPx, SOD, CAT, and TAC were markedly decreased in patients (2.15 ± 0.19 $\mu\text{g/mL}$, 6.58 ± 0.41 pmol/mL , 0.59 ± 0.13 ng/mL , 0.14 ± 0.07 ng/mL , and 0.09 ± 0.02 $\mu\text{mol/mL}$, respectively) compared to controls (13.82 ± 1.04 $\mu\text{g/mL}$, 29.46 ± 2.27 pmol/mL , 8.15 ± 0.61 ng/mL , 3.28 ± 0.19 ng/mL , and 0.31 ± 0.07 $\mu\text{mol/mL}$, respectively).

Table 8. Antioxidant status in breast cancer patients and controls

Parameter	Control (Mean $\pm$ SD)	Patients (Mean $\pm$ SD)	P-value
GSH ($\mu\text{g/mL}$)	13.82 ± 1.04	2.15 ± 0.19	0.001
GPx (pmol/mL)	29.46 ± 2.27	6.58 ± 0.41	0.001
SOD (ng/mL)	8.15 ± 0.61	0.59 ± 0.13	0.001
CAT (ng/mL)	3.28 ± 0.19	0.14 ± 0.07	0.001
TAC ($\mu\text{mol/mL}$)	0.31 ± 0.07	0.09 ± 0.02	0.001

The results demonstrated a significant increase in all measured inflammatory cytokines in breast cancer patients compared with controls. IL-1 α , IFN- α , and IFN- γ were significantly increased in patients (52.12 ± 9.44 pg/mL , 65.19 ± 8.35 pg/mL , and 285.15 ± 32.93 pg/mL , respectively) compared to controls (21.39 ± 5.27 pg/mL , 12.73 ± 3.26 pg/mL , and 37.11 ± 6.47 pg/mL , respectively). All differences were highly statistically significant ($P < 0.001$). TNF- α levels were markedly elevated in patients (84.62 ± 13.48 pg/mL) compared to controls (17.41 ± 3.92 pg/mL), Table 9.

Table 9. Serum cytokine levels in breast cancer patients and controls

Cytokine	Control (Mean $\pm$ SD)	Patients (Mean $\pm$ SD)	P-value
IL-1 α (pg/mL)	21.39 $\pm$ 5.27	52.12 $\pm$ 9.44	0.001
IFN- α (pg/mL)	12.73 $\pm$ 3.26	65.19 $\pm$ 8.35	0.001
IFN- γ (pg/mL)	37.11 $\pm$ 6.47	285.15 $\pm$ 32.93	0.001
TNF- α (pg/mL)	17.41 $\pm$ 3.92	84.62 $\pm$ 13.48	0.001

TNF- α gene expression analysis showed higher expression in breast cancer patients compared with controls. The mean fold change was 2.69 in patients versus 1.39 in controls, indicating upregulation of TNF- α in breast cancer cases, Table 10 and Figure 1.

Table 10. Relative expression levels of TNF- α in BC patients and controls

Group	TNF- α	β -Globin	Δ CT	$\Delta\Delta$ CT	2- $\Delta\Delta$ Ct	Folding	Mean	P value
Control	31.5	25.1	6.4	0.675	0.626332219	0.626332219	1.393037	0.001
	31.2	25.2	6	0.275	0.826450318	0.826450318		
	31.1	25.2	5.9	0.175	0.885767519	0.885767519		
	30.3	25.2	5.1	-0.625	1.542210825	1.542210825		
Patients	29.9	25.8	4.1	-1.625	3.084421651	3.084421651	2.69448	
	30.3	25.2	5.1	-0.625	1.542210825	1.542210825		
	29.1	25.7	3.4	-2.325	5.010657754	5.010657754		
	30.8	25.6	5.2	-0.525	1.43893358	1.43893358		
	29.7	25.5	4.2	-1.525	2.87786716	2.87786716		
	29.3	25.3	4	-1.725	3.305801273	3.305801273		
	29.5	24.9	4.6	-1.125	2.181015465	2.181015465		
	28.41	25.1	3.31	-2.415	5.333194708	5.333194708		
	30.4	25.6	4.8	-0.925	1.898684242	1.898684242		
	29.5	25.2	4.3	-1.425	2.685145006	2.685145006		
	31.2	24.9	6.3	0.575	0.671286251	0.671286251		

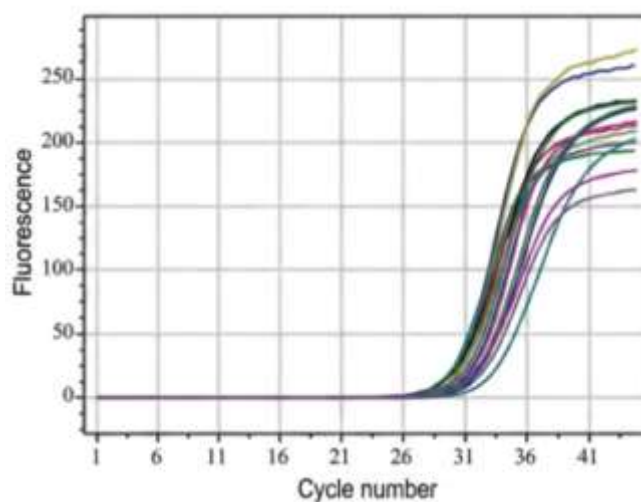


Figure 1. Relative expression levels of TNF- α in BC patients and controls

Receiver Operating Characteristic (ROC) curve analysis demonstrated excellent diagnostic performance of the studied biomarkers in differentiating breast cancer patients from healthy controls. IFN- β showed the highest diagnostic accuracy with an AUC of 0.96, followed by SOD (AUC = 0.95) and TNF- α (AUC = 0.94). TNF- α gene expression also exhibited strong discriminative ability (AUC = 0.91), indicating its potential as a reliable molecular marker. Antioxidant markers including GPx, GSH, CAT, and TAC showed good to moderate diagnostic performance with AUC values ranging from 0.85 to 0.93. Similarly, inflammatory cytokines such as IFN- α and IL-1 α demonstrated good diagnostic value with AUC values of 0.90 and 0.86, respectively, Table 11.

Table 11. Receiver Operating Characteristic (ROC) analysis of studied biomarkers

Marker	AUC	Cut-off value	Sensitivity (%)	Specificity (%)	P-value
IFN- γ	0.96	120.5	92.3	90.0	0.001
TNF- α (serum)	0.94	55.8	88.5	87.2	0.001
TNF- α gene expression	0.91	2.05	84.6	82.1	0.001
IFN- α	0.90	38.6	83.1	80.5	0.001
IL-1 α	0.86	30.2	79.5	77.0	0.001
GPx	0.93	12.4	86.7	85.3	0.001
SOD	0.95	2.10	90.1	88.4	0.001
GSH	0.92	6.8	87.6	86.0	0.001
CAT	0.88	1.05	80.4	78.9	0.001
TAC	0.85	0.14	78.2	76.5	0.001

In this regard, the current study revealed that the most of breast cancer patients were older women with the mean of 57.26 ± 12.74 . This result corresponds with a number of regional and local studies indicating that the prevalence of breast cancer is more common in women aged 50 years or older, which are similar to means of age in Iraq and neighboring populations (Abood, 2018; Fadhil *et al.*, 2019). This dominance of older age groups might be due to cumulative exposure to risk factors like hormonal changes, obesity, physical inactivity, and delayed diagnosis due to scarcity of screening programs in developing countries (Alwan *et al.*, 2018; Shwana *et al.*, 2018). Concerning tumor size, median size was 3.95 ± 1.76 cm (2–5 cm for the majority of them). This is consistent with studies showing that intermediate tumor diameters are most prevalent at diagnosis (Livingston *et al.*, 2019) (Liu *et al.*, 2021). Aside from globalization of cancer such as environmental factors, which do not seem to be the main driving force for trends in Asia, much higher proportions of larger than 2 cm tumors also mean later presentation, which can be due to poor medical awareness, inadequate screening programs and socioeconomic and demographic barriers that are delaying early diagnosis (Liu *et al.*, 2021). With regard to tumor laterality tumors were slightly more frequent at the left breast than at the right breast. This finding partially agrees with previous studies that also reported a subtle left-side predominance, although the difference between the two sides was not statistically significant (Ntekim *et al.*, 2019). The most likely explanation for the variation in tumor

laterality observed between these studies is a random distribution as well (Abdou *et al.*,2022). In breast cancer, the hormone receptor status is a recognized prognostic factor as well as a predictor of response to hormonal therapy (Li *et al.*,2020). In this study cohort, 64.37% were ER-positive, compared to 35.63% of patients being ER-negative. This is in line with earlier studies which demonstrate ER positivity rates of 50% to 65% (Zhang *et al.*,2019; Ligorio *et al.*, 2021). Nonetheless, ER positivity higher rates have been recorded in developed regions, including up to 80% ER positivity with breast cancer in the United States (Hanker *et al.*,2020). The relatively smaller proportion of ER-positive cases in this study may be related to more aggressive tumor biology or later presentation as reduced hormone receptor expression is associated with higher tumor grade and poor prognosis (Sohail *et al.*,2020). GSH levels in breast cancer patients were significantly lower in our study, as also supported by Saifullah *et al.* (2009) that demonstrated decreased tissue activity of reduced glutathione in breast tumors of Iraqi women, indicating depletion of non-enzymatic antioxidant stores probably due to chronic oxidative stress in these women. In cohesion, the activity of GPx was markedly decreased in patients as supported by Abbas *et al.* (2025), observed both lower GPx levels in treatment breast cancer patients, pointing to impaired enzymatic detoxification of peroxides. Moreover, patients had decreased levels of SOD, which was in agreement with studies conducted by Gusti *et al.* (2021) noted that changes in antioxidant defense systems related to SOD indicate reactive oxide imbalance and tumor advancement, indicating reduction in the ability to neutralize superoxide radicals. In accordance with the work of Jain *et al.*, (2023) who asserting that the reduced activity of enzymatic antioxidants in breast cancer promotes hydrogen peroxide accumulation and cellular damage. In addition, TAC showed a highly decreased level in patients, these observations are in agreement with Babakr and Nour Eldein (2025), they found that TAC decreased in breast cancer patients which means a generalized depression of the systemic antioxidant defense mechanisms. In our study, the levels of IL-1 α in patients were higher than those in patients, which was also consistent with the results of Cui *et al* (1982), reported that IL-1 α is strongly associated with questions of breast cancer and tumor-associated inflammation. This elevation may represent its involvement in regulating the tumor immune microenvironment and facilitating tumor progression. in present study, IFN- α levels were likewise significantly higher in patients (65.19 $\pm$ 8.35 pg/mL) than those in controls (12.73 $\pm$ 3.26 pg/mL) that consistent with the results of Lapenta *et al.*, (2024) showed that IFN- α could augment anti-tumor immunity via dendritic cell activation. This elevation may reflect activation of an immune response against tumor cells. In a parallel manner, the IFN- γ levels were also significantly elevated in patients (285.15 $\pm$ 32.93 pg/mL) compared to controls (37.11 $\pm$ 6.47 pg/mL), consistent with the findings made by García-Tuñón *et al.* (2007), which highlighted a role of interferon-gamma (IFN- γ) signaling in breast cancer biology and immune modulation. Such an increase could represent an arbitrary marker of activation of the immune in the tumor microenvironment. The levels of TNF- α were significantly increased in patients (84.62 $\pm$ 13.48 pg/mL) than controls (17.41 $\pm$ 3.92 pg/mL), which was in accordance with Qodir *et al.* (2025), demonstrating a robust association of TNF- α in breast cancer development and progression. Such elevation indicates a crucial role of TNF- α in the inflammatory signaling and tumor promotion. In addition, TNF- α gene expression analysis with upregulation was observed (mean fold change = 2.69 in breast cancer; mean fold change = 1.39 in controls), thus suggesting transcriptional activation of TNF- α to be more active in the cancer cases as compared to the controls.

This may cause oncogenic/inflammatory repetitive signal and increased tumor aggression. The significance of the biomarkers under investigation in the context of a diagnostic approach was confirmed in ROC analysis: thus, the maximum discriminative power was demonstrated by IFN- γ (AUC = 0.96) and SOD (AUC = 0.95), followed by TNF- α (AUC = 0.94), and finally, TNF- α gene expression (AUC = 0.91).

Conclusions

Breast cancer occurs in association with dysregulation of oxidative stress/antioxidant balance as well as increased inflammatory activity. This TNF- α is strongly associated between the breast cancer initiation and progression inflammatory processes. Purpose: Markers of inflammation and oxidative stress are potential candidates as markers of response and follow up in breast cancer as it affects individual prognosis.

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Author's contributions

All authors contributed equally to the study's conception and design, data collection, laboratory analysis, interpretation of results, and manuscript preparation. All authors reviewed and approved the final version of the manuscript

Ethics statement

This study was conducted following approval from the Research Ethics Committee at the University of Wasit. Written and verbal informed consent was obtained from all participants before their enrollment in the study. Consent for publication was also obtained from both the participants and the researchers.

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