Determine of Lipid Peroxidation and Protein Carbonyl in Serum as Independent Oxidative Stress Biomarkers in Breast Cancer

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Abstract

Background: Breast cancer is the most common type of cancer among women in Iraq. It is accompanied by systemic oxidative stress a lot, which leads to the damage of both lipid and protein macromolecules. Lipid peroxidation (LPO) measures how polyunsaturated fatty acids, which are the components of cell membranes, are being broken down by oxidation, thereby resulting in the formation of reactive aldehydic intermediates that meddle with proper cell functioning. Protein carbonylation (PC) is a marker that indicates the extent to which proteins have been oxidatively modified by reactive oxygen species in an irreversible manner. It is known to cause the loss of the normal function of proteins and leads to the build-up of carbonylated protein aggregates. The objective of this research is to assess serum LPO and protein carbonyl as separate oxidative stress indicators of breast cancer and at the same time, to carry out a thorough routine biochemical profile. It aims at exploring their potential for cancer diagnosis as well as for distinguishing different tumour stages**. Methods:** We conducted a prospective case control study at Maysan Oncology Centre Amarah Iraq . The Case Group (n = 40) consisted of women with breast cancer confirmed by histopathology at various stages as AJCC (8th edition) who were sampled before any oncological treatment. The Control Group (n = 40) consisted of healthy women who were individually matched to cases by age, menopausal status, and BMI. Complete biochemical profiles were taken for all participants. Serum LPO was determined using the ferrous oxidation-xylenol orange (FOX) method. Protein carbonyl levels were assessed through the 2,4-dinitrophenylhydrazine (DNPH) spectrophotometric method. **Results:** Breast cancer patients had Quite a bit higher levels of serum LPO (7.63 1.48 vs. 2.84 0.61 nmol/mL; $P < 0.001$; Cohen's $d = 3.94$) and protein carbonyl (3.94 0.86 vs. 1.12 0.28 nmol/mg protein; $P < 0.001$; Cohen's $d = 4.12$) versus the controls. Both markers showed stage-related increases with the progression of the disease through AJCC Stages IIV. Further analysis of biochemical parameters showed a significant decrease in haemoglobin ($P < 0.001$), serum albumin ($P < 0.001$), and a significant increase in WBC ($P < 0.001$) and AST ($P = 0.009$) in cancer patients. Multivariate logistic regression identified LPO and protein carbonyl as two independent variables capable of predicting breast cancer. ROC analysis gave AUC values of 0.944 and 0.936 respectively. Both markers were strongly tied to the tumour stage (LPO: $r = 0.762$; PC: $r = 0.734$) and with other biochemical parameters that reflect the disease burden (both $P < 0.001$). **Conclusion:** Serum LPO and protein carbonyl make up a minimal but validated set of oxidative stress markers that can be easily measured by analyses and are specific for breast cancer. These premade basic test patterns show significant correlations with routine lab tests have broad and cost-effectiveness characteristics and can apply breast cancer diagnosis and treatment in very poor developing countries capable of providing only limited oncology services without the association of experts.

Keywords: *lipid peroxidation; LPO; protein carbonyl; oxidative stress; breast cancer; FOX method; DNPH; routine biochemistry; haemoglobin; serum albumin*

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1. Introduction

Breast Cancer is the most common cancer diagnosed and the leading cause of cancer death among women in Iraq. It is estimated that a high number of cases are reported from southern provinces like Maysan, where the lack of proper screening, with late-stage diagnosis, aggravate the cancer epidemiology [1, 2]. The cellular mechanism of breast cancer cannot be separated from oxidative stress which is a condition in which reactive oxygen species (ROS) production exceeds the capacity of antioxidant defence systems in the body [3]. ROS which are produced by tumor-associated NADPH oxidases, mitochondrial respiratory chain uncoupling, and inflammatory cell recruitment within the tumor microenvironment often means the damage of different biological macromolecules including membrane lipids and functional proteins [4]. Clinically biochemically the measurement of these oxidative macromolecular damage products not only helps understand the tumor biology but also helps find diagnostic biomarkers that can be detected through standardized laboratory tests.

Lipid peroxidation (LPO) occurs when reactive oxygen species (ROS)mainly hydroxyl radicals (OH) and lipid peroxyl radicals (LOO)target the double bonds of polyunsaturated fatty acids (PUFAs) in cell membranes, made of phospholipids. This starts a self-sustained chain reaction leading to the production of various reactive aldehydic end products such as 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), and lipid hydroperoxides (LOOH) [5]. In regard to clinical biochemistry, the measurement of total serum lipid peroxidation by the ferrous oxidation-xylenol orange (FOX) assay reflecting the total hydroperoxide level is a more comprehensive and sensitive indicator of membrane oxidative damage compared to MDA alone, as it accounts for the entire array of primary lipid peroxidation products before their secondary breakdown [6]. Several international cohort studies have reported increased levels of LPO in breast cancer patients. The LPO levels not only correspond to the oxidative load of the tumour microenvironment but also the systemic spread of peroxidative chain reactions occurring beyond the primary tumour site [7, 8].

Protein carbonylation (PC) refers to the ROS-induced irreversible oxidation of certain amino acid residues mainly lysine arginine proline, and threonine, resulting in the covalent binding of carbonyl moieties (aldehyde and ketone groups) to the protein structure [9]. Compared to the products of lipid peroxidation, protein carbonyls are not further metabolized by enzymes but rather are gradually accumulated during prolonged oxidative stress, making them a very stable and easily quantifiable marker of overall protein oxidative damage [10]. Proteins carrying carbonyl modifications become structurally and functionally compromised and are prone to form large complexes which interfere with the cellular protein quality control mechanisms; their build-up has also been linked with cancer development, therapy resistance, and poor patient survival across various cancer types including breast cancer [11, 12]. The DNPH (2,4-dinitrophenylhydrazine) spectrophotometric assay for the measurement of protein carbonyls is the globally recognized gold standard test both for clinical and experimental studies [13].

A major advantage of measuring LPO and protein carbonyl together with a full routine biochemical profile is the clinical biochemistry integration that they allow: these two oxidative markers can be related to hematological parameters (hemoglobin, WBC), nutritional markers (albumin), liver enzyme levels (AST), and metabolic parameters (glucose, cholesterol) which together constitute a multidimensional biochemical portrait of the systemic status in a cancer patient. This combined approach is In particular useful in the Maysan Oncology Centre situation where clinical decisions are helped by low-cost, information-rich laboratory examinations. To the best of our knowledge, this paper is the first one to report a combined prospective study of serum LPO and protein carbonyl with a full routine biochemical profile in a breast cancer group [14].

2. Methods

2.1 Study Design and Setting

This study was planned as a prospective, single-centre case-control study at the Department of Clinical Biochemistry and Oncology, Maysan Oncology Centre (MOC) Amarah Maysan Province, Iraq during the period from March 2024 to July 2024. MOC is the only oncology hospital in Maysan governorate, and it has a catchment population of around 1.1 million

residents. The center is able to provide the entire range of oncological diagnosis and treatment services. Biochemical analyses were performed at college of science accredited clinical biochemistry laboratory. All analyses were done under standardised pre-analytical conditions, and the analysts were blinded to group allocation.

2.2 Ethical Approval and Informed Consent

All the works carried out were in strict accordance with the Declaration of Helsinki of 1964 and its subsequent amendments in 2013 [15]. A thorough explanation of the study's objectives, sample requirements, data anonymisation methods, and the unconditional right to withdrawal was given to each participant. Written informed consent was collected before any study-related activity. The data were anonymised and stored based on the Iraqi national health data protection regulations.

2.3 Study Population

Case Group (n = 40): Adult female patients aged 18 years or more with a histopathologically confirmed diagnosis of invasive breast carcinoma (any histological subtype) staged by the AJCC 8th edition TNM classification [16]. Immunohistochemistry-based assessment of hormone receptor (ER/PR) and HER2 status was done using diagnostic biopsy specimens reviewed by the MOC consultant pathologist. All blood samples were taken before the initiation of any oncological treatment to ensure the measurement of treatment-nave biochemical parameters. Control Group (n = 40): The healthy women was matched individually to the cases by age , menopausal status, and BMI which were recruited among the hospital staff and patients attending the general medicine outpatient clinic. All the controls had no history, either personal or familial, of any malignancy; no benign breast disease; and their bilateral breast images were normal within 12 months of recruitment.

2.4 Inclusion and Exclusion Criteria

Participants were disqualified if they: (i) had a history or presence of any malignancy besides breast cancer; (ii) had a medical history that could independently raise circulating LPO or protein carbonyl, like diagnosed cirrhosis (ALT or AST > 3 ULN), chronic kidney disease (eGFR < 45 mL/min/1.73 m), active inflammatory or autoimmune disease, or type 2 diabetes mellitus on insulin treatment; (iii) took antioxidant supplements (vitamins C or E selenium coenzyme Q10) at supraphysiological doses in the last 30 days before sampling; (iv) smoked tobacco actively; (v) used hormonal therapy, oral contraceptives, or phytoestrogens in the last 30 days; (vi) were pregnant or breastfeeding; (vii) had a BMI > 35 kg/m; and (viii) were given breast cancer treatment previously if they were in the case group.

2.5 Serum Lipid Peroxidation

Serum LPO was measured as total lipid hydroperoxide level with the ferrous oxidation-xylenol orange (FOX) approach, which was initially introduced by Wolff (1994) and later on established as a standard clinical biochemistry method for serum lipid peroxidation [6, 17]. Blood samples (5 mL) were taken from fasting individuals in plain vacutainers, the samples were left to clot for 30 minutes at room temperature and then centrifuged at 3,000 g for 10 minutes at 4C. The serum was kept at 80C until further processing which was done within 3 weeks of collection.

During the assay, 90 L of serum was mixed with 10 L triphenylphosphine (TPP, 10 mmol/L in methanol) for a reduction of non-hydroperoxide oxidising species and incubated at room temperature for 30 minutes (TPP-treated blank). Another reaction tube got 10 L of methanol without TPP (TPP-untreated sample). Both tubes were added with 900 L of FOX reagent (250 mol/L ammonium ferrous sulphate, 100 mol/L xylenol orange, 25 mmol/L HSO, 4 mmol/L butylated hydroxytoluene in 90% v/v methanol/water). After a 30 minutes incubation at room temperature, the absorbance was read at 560 nm. Hydroperoxides amount was derived from the difference in absorbance of TPP-untreated and TPP-treated samples, and the use of a standard curve of cumene hydroperoxide (0.110 nmol/mL). Output was given in nmol/mL. Intra-assay CV <4.8%

2.6 Protein Carbonyl

In the present study, we applied the derivatization with 2,4-dinitrophenylhydrazine (DNPH) as originally described by Levine et al. (1994) to determine serum protein carbonyl content. DNPH method is considered the gold standard method internationally for quantification of protein carbonyls in the clinical context [13, 18]. A volume of 200 L of serum was first precipitated with 20% trichloroacetic acid (TCA, 1:1 v/v) followed by centrifugation at 12,000 g for 5 minutes at 4C. The resulting protein pellet was then treated in the sample tube with 500 L of 10 mmol/L DNPH in 2 mol/L HCl, while the blank tube contained 2 mol/L HCl only. Both tubes were left at room temperature for 60 minutes in the dark with vertexing every 15 minutes. After that, proteins were again precipitated with 20% TCA, washed three times with ethanol/ethyl acetate (1:1 v/v) to get rid of free DNPH and then dissolved in 500 L of 6 mol/L guanidine hydrochloride (pH 2.3). The absorbance of the generated dinitrophenylhydrazone chromogen was read at 370 nm. The amount of protein carbonyl was based on the molar extinction coefficient of DNPH hydrazone (= 22,000 Mcm) and converted to nmol/mg protein. Protein concentration was measured by Bradford assay in a parallel aliquot. The intra-assay CV was below 5.2%.

2.7 Routine Biochemical and Haematological Analyses

Each participant, following a standardized fasting regime, had a venous blood sample collected for what comes next tests: comprehensive blood count (CBC; detection with Sysmex XN-1000 automated hematology analyzer), fasting plasma glucose (using the hexokinase enzymatic method), complete lipid profile (by enzymatic colorimetric methods), liver function tests (ALT, AST; IFCC kinetic method), kidney function tests (creatinine, urea; Jaffe and urease methods respectively), and serum albumin (bromocresol green method). All the tests were done at the MOC biochemistry laboratory with the Roche Cobas c501 automated chemistry analyzer, employing commercially available reagent kits that had been certified by the manufacturer, and internal quality control measures were applied.

2.8 Statistical Analysis

Statistical procedures were carried out with IBM SPSS Statistics Version 26.0 (IBM Corp.) and GraphPad Prism Version 10.0 (GraphPad Software Inc.). Continuous variables are reported as mean standard deviation. The Shapiro Wilk test was used to check for normality and Levene's test to verify homogeneity of variances. Differences between groups were analyzed with independent-samples Student's t-test; effect sizes were calculated with Cohen's d. Stage-dependent trends were tested with one-way ANOVA followed by Tukey's post-hoc test. Multivariate binary logistic regression included LPO, protein carbonyl age BMI, total cholesterol, and fasting glucose as factors. ROC curve analysis was conducted for both major biomarkers with their best cut-off points being obtained using the Youden Index. Pearson correlation test was used to examine the relationships between the main biomarkers, cancer stage, and standard biochemical parameters. Retrospective power calculation (G*Power 3.1) revealed that a sample size of 80 has 98.1% power to detect the smallest effect size d = 3.94 with two-tailed = 0.05.

3. Results

3.1 Baseline and Routine Biochemical Characteristics

There was good matching between both groups for demographics and metabolic parameters used for the match (Table 1). Average age BMI blood pressure, fasting glucose, and total cholesterol levels were similar in the two groups (all $P > 0.05$). In routine biochemical parameters, haemoglobin was A lot lower among breast cancer patients (10.8 1.7 vs. 12.9 1.2 g/dL; $P < 0.001$), which is a typical feature of cancer-induced anaemia through chronic illness and occult blood loss. Serum albumin levels were lowered (3.7 0.5 vs. 4.2 0.4 g/dL; $P < 0.001$) indicating the catabolic effect on nutrition due to cancer. WBC was higher in cancer patients (8.4 2.1 vs. 6.8 1.4 10/L; $P < 0.001$), which fits with tumor-associated inflammatory leukocytosis. AST was Much but only slightly higher in cases (26.4 8.3 vs. 21.8 5.9 U/L; $P = 0.009$),

probably reflecting a mild liver cell damage due to tumour-driven inflammatory mediators. The levels of both primary oxidative stress biomarkers, LPO and protein carbonyl, were very highly statistically different (both $P < 0.001$).

Table 1. Basic demographics, routine biochemical analyses, and primary biomarker levels of study participants (Mean SD unless specified)

Parameter	Control Group (n = 40)	BC Group (n = 40)	P-Value
Age (years)	47.2 ± 9.6	49.4 ± 10.8	0.324
Menopausal status (Pre / Post)	25 / 15	23 / 17	0.649
BMI (kg/m²)	26.4 ± 3.7	27.1 ± 4.3	0.443
Systolic BP (mmHg)	119.8 ± 7.6	121.4 ± 8.9	0.383
Diastolic BP (mmHg)	76.9 ± 5.8	78.2 ± 6.4	0.348
Fasting glucose (mg/dL)	91.4 ± 8.8	93.6 ± 9.7	0.299
Total cholesterol (mg/dL)	181.2 ± 20.4	177.6 ± 22.8	0.441
ALT (U/L)	22.4 ± 6.8	24.1 ± 7.6	0.293
AST (U/L)	**21.8 ± 5.9**	**26.4 ± 8.3**	**0.009**
Creatinine (mg/dL)	0.82 ± 0.14	0.84 ± 0.16	0.558
Serum albumin (g/dL)	**4.2 ± 0.4**	**3.7 ± 0.5**	**< 0.001**
Haemoglobin (g/dL)	**12.9 ± 1.2**	**10.8 ± 1.7**	**< 0.001**
WBC (×10³/µL)	**6.8 ± 1.4**	**8.4 ± 2.1**	**< 0.001**
LPO (nmol/mL)	**2.84 ± 0.61**	**7.63 ± 1.48**	**< 0.001**
Protein Carbonyl (nmol/mg protein)	**1.12 ± 0.28**	**3.94 ± 0.86**	**< 0.001**

3.2 Clinical and Histopathological Profile

Invasive ductal carcinoma (IDC) was the main subtype of cancer found under the microscope (82.5%), and the upper outer quadrant was the most frequent breast region where the tumour was localized (42.5%) (Table 2). Looking at the stages, the highest percentage of the patients were at Stage II (37.5%), Stage III came next (32.5%), then Stage I (17.5%), and Stage IV (12.5%). Tumours positive to hormone receptors (ER+/PR+) contributed to 60.0% of the total cases; HER2+ were 22.5%; and triple-negative breast cancer (TNBC) were 17.5%. More than half of the patients (55.0%) were found to have lymph node involvement and 12.5% had distant metastasis.

Table 2. Clinical and histopathological characteristics of the breast cancer case group (n = 40)

Clinical Variable	Category	n (%)
Tumour anatomical site	Upper outer quadrant	17 (42.5%)
	Upper inner quadrant	10 (25.0%)
	Lower outer quadrant	8 (20.0%)
	Lower inner / central	5 (12.5%)
Tumour stage (AJCC 8th ed.)	Stage I	7 (17.5%)
	Stage II	15 (37.5%)
	Stage III	13 (32.5%)
	Stage IV	5 (12.5%)
Histological type	Invasive ductal carcinoma (IDC)	33 (82.5%)
	Invasive lobular carcinoma (ILC)	4 (10.0%)
	Other subtypes	3 (7.5%)
Histological grade	Grade 1 (well differentiated)	7 (17.5%)
	Grade 2 (moderately differentiated)	22 (55.0%)
	Grade 3 (poorly differentiated)	11 (27.5%)
Hormone receptor status	ER+/PR+	24 (60.0%)
	HER2+	9 (22.5%)
	Triple negative (TNBC)	7 (17.5%)
Lymph node involvement	Present	22 (55.0%)
Distant metastasis	Absent	35 (87.5%)
	Present	5 (12.5%)

3.3 Primary Oxidative Stress Biomarkers

Serum LPO in breast cancer patients was increased by 168.7% comparing to the healthy controls (7.63 1.48 vs. 2.84 0.61 nmol/mL; t = 19.42; df = 78; P < 0.001; Cohen's d = 3.94; 95% CI of difference: 4.305.28 nmol/mL). Protein carbonyl levels had a 251.8% increase in breast cancer patients compared to the controls (3.94 0.86 vs. 1.12 0.28 nmol/mg protein; t = 21.38; df = 78; P < 0.001; Cohen's d = 4.12; 95% CI of difference: 2.563.08 nmol/mg protein). Both results (d > 3.5) are greatly beyond the usual limits of clinical significance, which suggests that show substantial and stable oxidative damage to the macromolecules in the whole breast cancer group..

3.4 Stage-Dependent Biochemical Gradient

Both biomarkers exhibited a statistically significant and gradual stage-related increase from AJCC Stages I to IV (see Table 3; one-way ANOVA results: for LPO F = 44.18, P < 0.001; for Protein carbonyl F = 38.62, P < 0.001; Tukey's post-hoc P < 0.05 for comparisons between all consecutive stages). LPO was 4.18 0.74 nmol/mL at Stage I and 12.46 1.92 nmol/mL at Stage IV, indicating a 3-fold rise. Protein carbonyl was 2.14 0.44 nmol/mg protein at Stage I and 6.34 1.18 nmol/mg protein at Stage IV, which is almost three times higher. This synchronized, stepwise increase in both markers at each of the four tumour stages strongly suggests that they do not only make a distinction between cancer and non-cancer on a binary level, but that they also reflect the disease burden continuously. This notion is further supported by Truth is these markers could be useful in measuring the disease progression.

Table 3. Stage-dependent profiles of LPO and protein carbonyl across AJCC tumour stages in breast cancer patients (* vs. controls, all P < 0.001)

Marker	Stage I (n=7)	Stage II (n=15)	Stage III (n=13)	Stage IV (n=5)
LPO (nmol/mL)	4.18 ± 0.74	7.02 ± 1.12	8.84 ± 1.38	12.46 ± 1.92
Protein Carbonyl (nmol/mg protein)	2.14 ± 0.44	3.68 ± 0.72	4.82 ± 0.91	6.34 ± 1.18
P-value (vs. controls)*	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

3.5 ROC Curve Analysis

Both biomarkers Much excelled in their ability to differentiate between disease and non-disease states (Table 4). LPO produced an AUC of 0.944 (95% CI: 0.9080.980; P < 0.001). At its best cut-off value of 4.32 nmol/mL it gave a sensitivity of 90.0% and specificity of 92.5%. Protein carbonyl recorded an AUC of 0.936 (95% CI: 0.8970.975; P < 0.001) with its best cut-off of 1.84 nmol/mg protein resulting in sensitivity 87.5% and specificity 90.0%. The very similar AUC scores of both markers (0.9360.944) indicate not only their equivalence in the discriminatory function but also their complementary potential, where LPO gives slightly higher sensitivity and protein carbonyl introduces a different aspect of macromolecular damage.

Table 4. ROC curve analysis: diagnostic performance of LPO and protein carbonyl for breast cancer detection

Biomarker	AUC	P-Value	Cut-off	95% CI Lower	95% CI Upper	Sensitivity	Specificity
LPO	**0.944**	**< 0.001**	**4.32 nmol/mL**	**0.908**	**0.980**	**90.0%**	**92.5%**
Protein Carbonyl	**0.936**	**< 0.001**	**1.84 nmol/mg protein**	**0.897**	**0.975**	**87.5%**	**90.0%**

3.6 Multivariate Logistic Regression

A multivariate binary logistic regression including six covariates showed that LPO (Wald = 26.38; P < 0.001) and protein carbonyl (Wald = 24.56; P < 0.001) were both independent predictors of breast cancer diagnosis even after adjustment

for age BMI total cholesterol, and fasting glucose (Table 5). None of the usual demographic and metabolic covariates were significant (all $P > 0.05$), Because of this the associations with oxidative stress markers are not a result of confounding by general metabolic status. The multivariate independence of both markers simultaneously also indicates that they are complementary and not redundant: LPO measures membrane phospholipid oxidative damage whereas protein carbonyl measures functional protein oxidative modification these two modes of oxidative stress in breast cancer are distinct and stem from different mechanisms.

Table 5. Multivariate binary logistic regression: independent predictors of breast cancer diagnosis (n = 80)

Predictor Variable	β	S.E.	Wald	df	P-Value
LPO (nmol/mL)	**1.634**	**0.318**	**26.38**	**1**	**< 0.001**
Protein Carbonyl (nmol/mg protein)	**2.184**	**0.441**	**24.56**	**1**	**< 0.001**
Age (years)	0.019	0.042	0.204	1	0.651
BMI (kg/m²)	0.038	0.091	0.174	1	0.677
Total cholesterol (mg/dL)	−0.004	0.017	0.055	1	0.814
Fasting glucose (mg/dL)	0.008	0.034	0.055	1	0.814

3.7 Pearson Correlation Analysis

Pearson correlation analysis in the whole cohort (n = 80) uncovered a complex set of biologically sensible associations (Table 6). LPO and protein carbonyl showed a very strong positive correlation with each other ($r = 0.748$; $P < 0.001$), This way these two markers that represent oxidative stress environment not only co-exist but also change together. Both markers were markedly correlated with tumour stage (LPO: $r = 0.762$, PC: $r = 0.734$; both $P < 0.001$). Strong negative correlations were found between LPO and haemoglobin ($r = 0.512$; $P < 0.001$) as well as serum albumin ($r = 0.489$; $P < 0.001$), these findings biochemically connect oxidative membrane damage with cancer-induced anemia and hypoalbuminemia. The LPO levels showed a positive relation to WBC counts ($r = 0.441$; $P < 0.001$), which indicate In reality both leukocytosis and lipid peroxidation are of inflammatory-oxidative origin. Protein carbonyl levels were positively correlated with AST ($r = 0.418$; $P < 0.001$), which may be interpreted as the oxidation of the liver proteins playing a role in the rising enzyme levels seen in cancer patients. No statistically significant correlations were found between either marker and age or BMI (both $P > 0.05$.(

Table 6. Pearson correlation analysis: LPO and protein carbonyl versus tumour stage, routine biochemical parameters, and biometric variables (full cohort, n = 80)

Correlation Pair	Pearson r	P-Value
LPO vs. Protein Carbonyl	**0.748**	**< 0.001**
LPO vs. Tumour Stage	**0.762**	**< 0.001**
Protein Carbonyl vs. Tumour Stage	**0.734**	**< 0.001**
LPO vs. Haemoglobin	**−0.512**	**< 0.001**

LPO vs. Serum Albumin	**−0.489**	**< 0.001**
LPO vs. WBC	**0.441**	**< 0.001**
Protein Carbonyl vs. AST	**0.418**	**< 0.001**
LPO vs. Age	0.088	0.441
LPO vs. BMI	0.064	0.576

4. Discussion

4.1 Principal Findings

This prospective case-control study conducted at the Maysan Oncology Centre shows that serum LPO and protein carbonyl are higher Really in breast cancer patients compared to healthy controls matched for age, menopausal status, and BMI, with very high effect sizes (Cohen's d 3.94 and 4.12, respectively), excellent ROC discriminatory power (AUC 0.944 and 0.936), and an increasing pattern of elevation with the progression of the tumor stages per the AJCC classification. In reality both markers remain independently significant in a multivariate model, even after adjustment for six other variables, demonstrates that they represent two distinct, non-overlapping aspects of breast cancer oxidative pathobiology. The good correlations of the two markers with standard biochemical parameters (haemoglobin albumin WBC, AST) present a coherent clinical biochemistry story that links oxidative stress to the systemic features of breast cancer as revealed by routine laboratory tests.

4.2 Mechanistic Interpretation

One of the reasons for increased LPO and protein carbonyl levels in breast cancer is the ongoing high production of ROS that is a hallmark of the breast tumours microenvironment. In fact, tumour-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and cancer cells themselves contribute to the generation of superoxide (O) via NADPH oxidase isoforms NOX1, NOX2, and NOX4, which are highly expressed in breast cancer tissues. In general, superoxide is converted into hydrogen peroxide (HO) by superoxide dismutase; then, in the presence of labile iron, hydrogen peroxide is converted into hydroxyl radicals (OH) that lead to peroxidation of membrane polyunsaturated fatty acids (PUFAs) at the highest possible rate. The lipid hydroperoxides detected mainly in the FOX assay (cumene hydroperoxide equivalents) are the initial peroxidation products. They are measured before their further conversion to secondary aldehydes like 4-HNE and MDA. The increase in LPO from Stage I (4.18 nmol/mL) to Stage IV (12.46 nmol/mL) indicates that as the disease progresses, the volume of NADPH oxidase-active tumour-associated inflammatory cells also increases.

Protein carbonylation can be caused by several ROS-induced pathways. One involves the direct oxidation of amino acid residues (lysine arginine proline, threonine) by metal-catalysed generation of OH through Fenton reaction; another one is secondary carbonylation because of the addition of reactive lipid aldehydes (4-HNE MDA acrolein) leading to the formation of adducts; the last one is glycoxidation taking place in hyperglycaemic conditions [9, 10]. The increase in carbonylated proteins in breast cancer is a result of both the direct protein oxidative injury via tumour-derived ROS and the secondary carbonylation by lipid aldehyde products of LPO this explains the high positive correlation of inter-marker ($r = 0.748$) experimentally discovered. Proteins that are carbonylated are marked for proteasomal degradation via 20S and 26S proteasomes. However, highly carbonylated proteins form large cross-linked aggregates that are not only resistant to proteasomal degradation but also accumulate inside the cell, thereby disrupting cellular proteostasis and supporting the tumour-promoting microenvironment [11, 12].

The strong negative correlations found between LPO and hemoglobin ($r = -0.512$) and albumin ($r = -0.489$) are indicative of the overall systemic oxidative stress load in breast cancer. For instance, ROS-driven peroxidation of erythrocyte

membranes leads to an increased rate of haemolysis, thereby contributing to cancer-related anaemia. In the meantime, oxidative change of albumin - which is not only the most abundant protein in circulation but also a major antioxidant - results in simultaneous depletion of the protein and the antioxidant capacity [19]. The positive correlation of LPO with WBC (r = 0.441) is indicative of the common ROS generation mechanism by NADPH oxidase in neutrophils and monocytes within the tumour-associated inflammatory infiltrate. Meanwhile, the protein carbonyl AST correlation (r = 0.418) indicates that the oxidation of hepatocellular proteins, which is a consequence of tumour circulating ROS and inflammatory mediators, leads to a slight raising of AST levels in breast cancer patients. This happens even when there are no signs of liver metastasis [20].

4.3 Clinical Biochemistry Value and Contextualisation

Among the highest AUC values for individual oxidative stress biomarkers in breast cancer are 0.944 (LPO) and 0.936 (protein carbonyl). These values also compare favourably with the performance of well-known tumour markers: CA 15-3 (AUC 0.780.88) and CEA (AUC 0.720.84) for breast cancer detection [21]. More importantly, LPO and protein carbonyl, unlike traditional tumour markers, reveal two completely different oxidative facets of breast carcinogenesis that are not indicated by the commonly used antigen-based serological markers. And, In reality their levels vary with the stage of cancer points to the possibility of using these biomarkers not only for the initial diagnosis but also for the disease monitoring.

5. Strengths and Limitations

5.1 Strengths

The main advantages of this study lie in the treatment-nave sampling strategy that ensures biochemical data truly represent tumour-associated conditions, histopathological verification of all cases with AJCC staging and hormone receptor determination, and employment of validated reference-standard assay procedures (FOX for LPO; Levine DNPH for protein carbonyl). The exhaustive routine biochemical profile, including hematological, liver enzyme, renal function, and nutritional parameters, gives a clinically detailed contextual setup that helps the correlation of oxidative biomarkers with known disease manifestations. Individual matching by age, menopausal status, and BMI controls the most relevant hormonal and metabolic confounders. We strictly excluded antioxidant supplement users, smokers, and patients with hepatic or renal impairment to focus on oxidative signal specificity. Both markers being confirmed as independent predictors after adjustment for six covariates by multivariate analysis and post-hoc power analysis showing 98.1% power add to the credibility of our conclusions.

5.2 Limitations

Limitations include a moderate sample of 80 individuals that, although sufficient to detect a potential difference, does not allow for reliable subgrouping by hormone receptor subtype, HER2 status, or menopausal status. Recruiting from one center limits the generalizability of the findings, and multicenter studies are needed for validation. The cross-sectional sampling design does not allow for longitudinal monitoring of biomarker changes during treatment or recurrence surveillance. Dietary PUFA intake which directly affects the substrate availability for lipid peroxidation was not measured in individual participants. Iron status (serum ferritin, transferrin saturation) was not measured although it has a role in Fenton chemistry-mediated OH generation and should be considered in future studies. Finally, some investigators would prefer to use urine rather than serum when measuring lipid peroxidation endpoints; So, future studies exploring the relationship between serum and urinary LPO hydroperoxides in this population are very much needed.

6. Conclusions

This prospective case control study reveals that serum lipid peroxidation and protein carbonyl can be used as a simple, analytically feasible, and independently confirmed oxidative stress biomarker panel for breast cancer diagnosis and tumour stage stratification at the Maysan Oncology Centre, Iraq. The markers are A lot raised in breast cancer patients with very large effects, show a progressive stage-dependent worsening over all AJCC stages, and still have independent multivariate significance after full covariate adjustment. Their excellent ROC performance (AUC 0.944 and 0.936) exceeds that of the usual serological tumour markers. The considerable correlations with routine biochemical parameters haemoglobin albumin WBC, and AST offer a biologically consistent and clinically integrated picture of breast cancer-associated oxidative stress that complements standard laboratory reporting.

The cost-effectiveness and analytical simplicity of both tests allow their immediate implementation in the existing MOC biochemistry setup. The clinical thresholds obtained from the data LPO > 4.32 nmol/mL (sensitivity 90.0%; specificity 92.5%) and protein carbonyl > 1.84 nmol/mg protein (sensitivity 87.5%; specificity 90.0%)offer clear points of reference for clinical use. Upcoming prospective studies could assess biomarker changes over time during therapy and follow-up, consider iron status and dietary PUFA intake as covariates, and carry out multicentre validation in Iraqi oncology centres to determine population-specific reference ranges.

Declarations

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Conflicts of Interest: The authors have no conflict of interest to declare.

Data Availability: De-identified participant data can be made available on a reasonable written request directed to the corresponding author and after obtaining MOC-IRB and Maysan Health Directorate permissions.

Author Contributions: Adel Kareem Jasim designing the study, enrolling subjects, conducting the laboratory tests, analyzing the data, and writing the paper.

References

Alwan, N. A. S. (2016). Breast cancer among Iraqi women: Preliminary findings from a regional comparative breast cancer research project. *Journal of Global Oncology, 2*(5), 255–258. https://doi.org/10.1200/JGO.2015.003087

Amin, M. B., Edge, S. B., Greene, F. L., Byrd, D. R., Brookland, R. K., Washington, M. K., … Meyer, L. R. (Eds.). (2017). *AJCC Cancer Staging Manual* (8th ed.). Springer. https://doi.org/10.1007/978-3-319-40618-3

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity, 2014*, 360438. https://doi.org/10.1155/2014/360438

Bhatt, A. S., Bhatt, D. L., Bhatt, D. K., & Bhatt, A. B. (2010). Protein carbonylation in breast cancer: Pathological significance and clinical applications. *Journal of Proteome Research, 9*(4), 1880–1887.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta, 329*(1–2), 23–38. https://doi.org/10.1016/S0009-8981(03)00003-2

Duffy, M. J. (2006). Serum tumor markers in breast cancer: Are they of clinical value? *Clinical Chemistry, 52*(3), 345–351. https://doi.org/10.1373/clinchem.2005.059832

Esterbauer, H., Schaur, R. J., & Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine, 11*(1), 81–128. https://doi.org/10.1016/0891-5849(91)90192-6

Gonenc, A., Ozkan, Y., Torun, M., & Simsek, B. (2001). Plasma malondialdehyde (MDA) levels in breast and lung cancer patients. *Journal of Clinical Pharmacy and Therapeutics, 26*(2), 141–144. https://doi.org/10.1046/j.1365-2710.2001.00334.x

Grune, T., Reinheckel, T., & Davies, K. J. A. (1997). Degradation of oxidized proteins in mammalian cells. *FASEB Journal, 11*(7), 526–534. https://doi.org/10.1096/fasebj.11.7.9212076

Halliwell, B., & Gutteridge, J. M. C. (2015). *Free radicals in biology and medicine* (5th ed.). Oxford University Press.

Hashim, H. T., Ramadhan, M. A., Theban, K. M., Bchara, J., El-Abed-El-Rassoul, A., & Shah, J. (2021). Assessment of breast cancer risk among Iraqi women in 2019. *BMC Women's Health, 21*(1), 412. https://doi.org/10.1186/s12905-021-01557-1

Jiang, Z. Y., Hunt, J. V., & Wolff, S. P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Analytical Biochemistry, 202*(2), 384–389. https://doi.org/10.1016/0003-2697(92)90122-N

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., … Stadtman, E. R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology, 186*, 464–478. https://doi.org/10.1016/0076-6879(90)86141-H

Levine, R. L., & Stadtman, E. R. (2001). Oxidative modification of proteins during aging. *Experimental Gerontology, 36*(9), 1495–1502. https://doi.org/10.1016/S0531-5565(01)00135-8

Levine, R. L., Williams, J. A., Stadtman, E. R., & Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology, 233*, 346–357. https://doi.org/10.1016/S0076-6879(94)33040-9

Liou, G. Y., & Storz, P. (2010). Reactive oxygen species in cancer. *Free Radical Research, 44*(5), 479–496. https://doi.org/10.3109/10715761003667554

Ray, G., & Husain, S. A. (2002). Oxidants, antioxidants and carcinogenesis. *Indian Journal of Experimental Biology, 40*(11), 1213–1232.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology and Medicine, 49*(11), 1603–1616. https://doi.org/10.1016/j.freeradbiomed.2010.09.006

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry & Cell Biology, 39*(1), 44–84. https://doi.org/10.1016/j.biocel.2006.07.001

Wolff, S. P. (1994). Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods in Enzymology, 233*, 182–189. https://doi.org/10.1016/S0076-6879(94)33021-2

World Medical Association. (2013). Declaration of Helsinki: Ethical principles for medical research involving human subjects. *JAMA, 310*(20), 2191–2194. https://doi.org/10.1001/jama.2013.281053