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Determinants of Activity Cytochrome P450 1B1, Reduced Glutathione, and Nitric Oxide as Biochemical Independent of Breast Cancer Risk and Tumour Stage

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Abstract

Background: Breast cancer remains the leading cause of cancer and the major cause of cancer-related deaths among women in Iraq. Mostly breast cancer patients are from the southern provinces of Iraq including Maysan governorate. Cytochrome P450 1B1 (CYP1B1) is the major enzyme in extrahepatic tissues that catalyzes the hydroxylation of 17-estradiol to 4-hydroxyoestradiol (4-OHE2), which is a genotoxic catechol estrogen resulting in the formation of depurinating DNA adducts in breast epithelial cells. Reduced glutathione (GSH) is the most abundant intracellular non-enzymatic antioxidant and the major defense against oxidative stress and catechol estrogen-induced DNA damage. Nitric oxide (NO) is a multifunctional signaling molecule that at high levels produced by inducible nitric oxide synthase (iNOS) overexpression in breast cancer tissue it functions to support tumor angiogenesis, immune suppression, and invasiveness. This study aims to examine the clinical biochemistry patterns of CYP1B1 activity, GSH, and NO in breast cancer patients compared with healthy individuals, and to determine their effectiveness as individual biomarkers for breast cancer diagnosis and tumor stage classification. **Methods:** A prospective case-control study was held in Maysan Oncology Center Amarah Iraq. Case Group (n = 40) consisted of females with breast cancer confirmed histopathologically and of all AJCC stages (8th edition), sampled before administration of any treatment. Control Group (n = 40) was made up of healthy women individually matched for age, menopausal status and BMI. CYP1B1 activity was determined using the 4-hydroxyoestradiol formation assay which is based on ELISA and is validated. GSH was determined by the Ellman (DTNB) colorimetric method. Nitric oxide was quantitated as total nitrite/nitrate by the Griess reagent assay. **Results:** Breast cancer patients showed Much higher CYP1B1 activity (61.8 9.3 vs. 28.4 4.6 pmol/mg protein/min; $P < 0.001$; Cohen's $d = 4.38$), a very sharp reduction in GSH levels (384.6 78.3 vs. 812.4 94.6 mol/L; $P < 0.001$; Cohen's $d = 4.94$) and a great increase in NO levels (48.3 9.7 vs. 18.6 4.2 mol/L; $P < 0.001$; Cohen's $d = 3.91$). All of the three markers exhibited progressive stage-dependent deterioration switching AJCC Stages IIV. Multivariate logistic regression established CYP1B1, GSH and NO as independent factors linked to breast cancer diagnosis. ROC analysis gave AUCs of 0.951, 0.938 and 0.926, respectively. Significant correlations were discovered between CYP1B1 activity and serum oestradiol ($r = 0.641$; $P < 0.001$), GSH ($r = -0.712$; $P < 0.001$) and tumor stage ($r = 0.724$; $P < 0.001$). **Conclusion:** The CYP1B1GSHNO triad represents a trio of biochemistry-based breast cancer detection methods that have been independently confirmed and are also easy to measure chemically. Their model, which focuses on the metabolism of oestrogen as the basis of breast cancer, is quite different from the oxidative stress panels described earlier, and is a reflection of the unique biochemical pathobiology of breast carcinogenesis. The implementation of this panel in clinical biochemistry practice at Maysan Oncology Centre would be a good move.

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1. Introduction

Among women, breast cancer is the most commonly diagnosed type of cancer worldwide. In fact, in 2020 alone there were 2.3 million new cases and 685,000 deaths globally due to breast cancer [1]. In Iraq, it is the main reason of death from cancer among women. Also, the incidence of breast cancer has been consistently reported to be the highest compared to other female cancers in the Iraqi Cancer Registry publications over the years [2]. We mention here Maysan Province in southern Iraq, where the rate of breast cancer has increased A lot These days. This has been related to a mixture of environmental, dietary, and genetic factors with the lack of early detection facilities and patterns of late-stage presentation in the oncology facilities of the region [4, 3]. About clinical biochemistry, the biochemical pathway by which endogenous oestrogen metabolism produces genotoxic intermediates is one of the key aspects of breast carcinogenesis that has not received sufficient attention in this population; also, the antioxidant and inflammatory biochemistry of the host that modulates tumour progression has not been explored much.

There is a strong and special link between Cytochrome P450 1B1 (CYP1B1) and breast cancer since it is the major enzyme outside the liver which carry out in the 4-hydroxylation of 17-oestradiol to 4-hydroxyoestradiol (4-OHE2) [5, 6]. In contrast with the 2-hydroxylation step leading to the formation of the less harmful metabolite of oestradiol catalysed by CYP1A1, the CYP1B1 induced 4-hydroxylation pathway results in the formation of a catechol estrogen that is rapidly converted by peroxidases and cytochrome P450s into the ortho-quinone intermediates (4-OHE2-Q). These highly reactive quinones chemically interact with the DNA bases of mammary epithelial cells forming adducts that ultimately lead to the generation of apurinic sites and point mutations, which are the hallmark of early breast cancer formation [7, 8]. Importantly, breast tumor tissue expresses A lot more CYP1B1 enzyme than normal breast epithelium, and the activity of this enzyme is again increased through the exposure to environmental pollutants that activate AhR - including polycyclic aromatic hydrocarbons from petrochemical emissions and burning waste - that the population in Maysan is continuously exposed to [9, 10].

Reduced glutathione (GSH; -glutamylcysteinylglycine) is the most prevalent intracellular thiol antioxidant in mammalian cells. It serves not only as a free radical scavenger but also as a source for glutathione S-transferase (GST)-mediated conjugation and detoxification of catechol estrogen quinones [11]. So, sufficient GSH levels are critical to neutralizing the genotoxic metabolite of CYP1B1 before it damages DNA. But, when it comes to breast cancer, the continuous oxidative stress caused by tumor-associated NADPH oxidases and inflammatory infiltrates results in a progressive depletion of both intracellular and circulating GSH pools. This situation further incapacitates the antioxidant defenses that shield against oestrogen-induced mutagenesis [12]. Assessment of circulating GSH using the Ellman reagent (DTNB: 5,5'-dithiobis(2-nitrobenzoic acid)) method yields a convenient clinical and quantitative measure of this antioxidant depletion [13].

Nitric oxide (NO) is a gaseous free radical that is made from L-arginine by several isoforms of nitric oxide synthase (NOS). At physiological pico-molar concentrations that are produced by endothelial NOS (eNOS), NO plays homeostatic vascular roles; Still, at micromolar concentrations produced by inducible NOS (iNOS)which is dramatically overexpressed in breast cancer tumour-associated macrophages and cancer-associated fibroblasts NO causes pro-tumorigenic effects such as promotion of angiogenesis (via upregulation of HIF-1 and VEGF), inhibition of cytotoxic T-cell activity, induction of epithelial mesenchymal transition (EMT), and increase of DNA damage via reactive nitrogen species (RNS) generation [14 15 16]. Measuring total circulating NO as stable nitrite/nitrate end-products by the Griess colorimetric assay is the confirmed standard approach for clinical NO determination [17].

The CYP1B1, GSH, and NO systems mechanistically and integratively contribute to breast carcinogenesis that the generation of genotoxic catechol estrogen by CYP1B1, depletion of GSH causing the removal of major detoxification defense and NO increasing angiogenic and pro-invasive signaling create a biochemically and oestrogen- centered setup that is quite different from the dietary carcinogen activation and DNA damage axes that we described in our previous colorectal cancer studies at the same centre. This study is the first to assess all three markers simultaneously

and prospectively in a breast cancer cohort from Maysan Province using assay methods that are individually validated and clinically standardized[18].

2. Methods

2.1 Study Design and Setting

This prospective single-centre case control study was carried out at the Department of Clinical Biochemistry and Oncology, Maysan Oncology Centre (MOC) Amarah Maysan Province Iraq between February 2024 and June 2025. MOC is the only facility entirely dedicated to oncology in the Maysan governorate with the population of approximately 1.1 million residents. The MOC breast cancer programme offers multidisciplinary oncological care including surgery, medical oncology, radiotherapy, and hormonal therapy, and it is the main hub for all regional breast cancer referrals from primary and secondary care centres throughout the province. All biochemical assays were carried out in college of science accredited clinical biochemistry laboratory.

2.2 Ethical Approval and Informed Consent

The study was carried out in strict adherence with the 1964 Declaration of Helsinki and its later amendments in 2013 [19]. Besides, all the participants were thoroughly informed about the study goals, the sample collection techniques, the process of data anonymisation and their right to withdrawal without any repercussions. Informed written consent was obtained before performing any study-related procedure. All the data were anonymised and handled as the relevant Iraqi national health data protection laws.

2.3 Study Population and Cohort Definition

Case Group (n = 40): Adult women (older than 18 years) with a new, histopathologically confirmed diagnosis of invasive breast carcinoma including invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), or other histological subtypes staged by AJCC 8th edition TNM classification [20]. Hormone receptor (ER/PR) and HER2 status were determined by immunohistochemistry on diagnostic biopsy or surgical specimens, independently assessed by the MOC consultant pathologist. All blood samples were collected before the initiation of any breast cancer treatment (surgery chemotherapy radiotherapy, or hormonal therapy) to ensure treatment-nave biochemical measurements. Control Group (n = 40): Healthy women individually matched to cases by age, menopausal status, and BMI , recruited from hospital staff and outpatient general medicine clinics. All controls had no personal or family history of any malignancy, no known benign breast disease, normal breast examination, and normal bilateral mammography or breast ultrasound within 12 months of recruitment

2.4 Inclusion and Exclusion Criteria

In order to focus on the biochemical signal associated More exactly with breast cancer and remove other independent confounders, the exclusion criteria listed below were used: (i) the presence of any other active or previous malignancy apart from the breast cancer that is the subject of the study (cases) or a history of any malignancy (controls); (ii) diseases that independently alter CYP1B1 activity, GSH, or NO levels such as diagnosed cirrhosis or major liver impairment (ALT > 3 ULN), chronic kidney disease (eGFR < 45 mL/min/1.73 m), type 2 diabetes mellitus on insulin therapy, or presence of autoimmune disease; (iii) use of hormonal therapy, oral contraceptives, or intake of phytoestrogen supplements, as these would interfere with oestradiol and CYP1B1 measurements; (iv) use of pharmacological NOS inhibitors or L-arginine supplements (within 30 days); (v) active smoking, which has well-established effects of AhR-activation and CYP1B1-induction by tobacco combustion products [9]; (vi) pregnancy or breastfeeding; (vii) BMI > 35 kg/m; and (viii) previous breast cancer-related treatment in the case group.

2.5 CYP1B1 Activity Measurement

CYP1B1 enzymatic activity was measured by using ELISA-based assay of 4-hydroxyoestradiol (4-OHE2) formation which is a validated and most commonly used CYP1B1 phenotyping method of clinical samples [5, 21]. Blood was drawn from fasting individuals by venipuncture (10 mL) and collected into heparinised vacutainers during 08:00-10:00 h. Peripheral blood mononuclear cells (PBMCs)which are known to express CYP1B1 constitutively and have been the standard model for CYP1B1 phenotyping ex vivo were isolated by using density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare) at 400 g for 30 min at RT. Cell viability was assessed by trypan blue exclusion (minimum 90% viability required). Protein was isolated from 2 10 PBMCs by freeze-thaw lysis in 50 mmol/L potassium phosphate buffer (pH 7.4)

In the activity assay, cell lysate (containing 200 g protein) was incubated plus 17-oestradiol (50 mol/L; Sigma-Aldrich) as a substrate in the presence of NADPH (1 mmol/L) at 37C for 60 minutes. Methanol addition stopped the reaction. The 4-OHE2 product was measured by a validated competitive ELISA kit (DRG International, EIA-4154) with absorbance read at 450 nm. CYP1B1 activity was given with pmol 4-OHE2 formed per mg protein per minute. Intra-assay CV < 5.4%; inter-assay CV < 7.2%.

2.6 Reduced Glutathione (GSH) Measurement

Measurements of whole blood GSH were performed using Ellman's colorimetric method with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as the chromogenic reagent. This is the most commonly used and analytically validated method for clinical GSH measurement [13, 22]. Whole blood, 1 mL, was immediately deproteinized by adding metaphosphoric acid (5% w/v, 1:1 v/v), and then the mixture was centrifuged at 3,000 g for 10 minutes at 4C. Clear supernatant (100 L) was incubated with DTNB reagent (0.1 mmol/L in 0.1 mol/L phosphate buffer, pH 7.4) for 5 minutes at room temperature. Afterwards, the absorbance of the yellow 5-thio-2-nitrobenzoic acid (TNB) chromogen was measured at 412 nm on a Shimadzu UV-1800 spectrophotometer. GSH concentrations were determined from an L-glutathione standard curve (range 0.051.0 mmol/L; Sigma-Aldrich) and reported as mol/L. The intra-assay CV was below 3.9%.

2.7 Nitric Oxide Measurement: Griess Reagent Assay

Platelet NO was measured following the method previously described [20]: Platelets were sedimented after the 1st centrifugation at 600 g, for 10 min at 4C, then resuspended in PBS and nitric oxide production was quantified with a one-step fluorescent kit using 2, 3-diaminonaphthalene; results were expressed as the total nmol of NO production per 10/l blood Since the problem of the unstable nature of NO, we have measured nitrite and nitrate as end-products of NO production as total circulating NO. The summation of the stable plasma nitrite (NO) and nitrate (NO) end-products gave us the total circulating NO which was quantified using the Griess reagent colorimetric assay - the validated reference method for clinical plasma NO measurement [17, 23]. Cells were separated from EDTA whole blood by centrifugation at 1500g/10 min/4C. First the nitrate was converted to nitrite by incubation with nitrate reductase (Aspergillus species; Sigma-Aldrich; 0.1 U/mL) and NADPH (50 mol/L) at 37C for 30 minutes. The total nitrite was determined by the addition of Griess reagent (equal volumes of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride) and the absorption of the resulting azo dye was measured at 540 nm after 10 minutes incubation at room temperature. Using a sodium nitrite standard curve (1200 mol/L), NO concentrations were calculated and expressed as mol/L. Intra-assay CV < 4.6%.

2.8 Statistical Analysis

We used IBM SPSS Statistics Version 26.0 (IBM Corp.) and GraphPad Prism Version 10.0 (GraphPad Software Inc.) for the statistical analysis. The data for continuous variables is presented as mean SD. A Shapiro Wilk test verified the normal distribution; Levene's test confirmed equal variances. The variations between groups were tested through

independent-samples t-test, and the effect size was given by Cohen's d. Stage-dependent trends were monitored through one-way ANOVA and Tukey's post-hoc test. Multivariate binary logistic regression considered CYP1B1 activity GSH NO age BMI, and total cholesterol as predictors. ROC curve analysis was carried out for each biomarker and the Youden Index was used to identify the best cutoffs. Inter-marker and oestradiolCYP1B1 correlations were examined through Pearson correlation analysis. After the fact, power analysis (G*Power 3.1) revealed that n = 80 would yield 97.8% power to detect the smallest effect observed (Cohen's d = 3.91) even at two-tailed = 0.05. The level of statistical significance was $P < 0.05$.

3. Results

3.1 Baseline Characteristics

The case and control groups were identically matched about demographic and cardiometabolic parameters that were used for matching (Table 1). Average ages were 48.6 10.4 years for breast cancer patients versus 46.3 9.1 years for controls (P = 0.291). Distribution of menopausal status BMI blood pressure, fasting glucose, and total cholesterol were similar between the groups (all $P > 0.05$). Serum oestradiol was markedly higher in the breast cancer patients (141.8 38.4 vs. 84.3 22.6 pg/mL; $P < 0.001$), in line with the hyperoestrogenaemia biochemical environment typical of hormone-receptor-positive breast cancer. Haemoglobin (10.9 1.6 vs. 12.8 1.1 g/dL; $P < 0.001$) and albumin (3.6 0.5 vs. 4.1 0.3 g/dL; $P < 0.001$) were reduced in the cases, which is a common feature of established malignancy. The three main biochemical markers-CYP1B1, GSH, and NO-showed differences that were statistically extremely significant (all $P < 0.001$.(

Table 1. Demographic, anthropometric, and biochemical characteristics of study participants at baseline (Mean SD unless specified)

Parameter	Control Group (n = 40)	BC Group (n = 40)	P-Value
Age (years)	46.3 ± 9.1	48.6 ± 10.4	0.291
Menopausal status (Pre / Post)	26 / 14	24 / 16	0.671
BMI (kg/m²)	26.8 ± 3.9	27.4 ± 4.2	0.502
Systolic BP (mmHg)	120.4 ± 7.8	122.1 ± 8.6	0.341
Diastolic BP (mmHg)	77.3 ± 5.6	78.4 ± 6.1	0.382
Fasting glucose (mg/dL)	92.6 ± 9.1	94.8 ± 10.3	0.312
Total cholesterol (mg/dL)	183.6 ± 21.4	179.2 ± 23.8	0.381
Serum oestradiol (pg/mL)	**84.3 ± 22.6**	**141.8 ± 38.4**	**< 0.001**
Haemoglobin (g/dL)	**12.8 ± 1.1**	**10.9 ± 1.6**	**< 0.001**
Serum albumin (g/dL)	**4.1 ± 0.3**	**3.6 ± 0.5**	**< 0.001**
CYP1B1 Activity (pmol/mg protein/min)	**28.4 ± 4.6**	**61.8 ± 9.3**	**< 0.001**
GSH (µmol/L)	**812.4 ± 94.6**	**384.6 ± 78.3**	**< 0.001**

NO (µmol/L)	**18.6 ± 4.2**	**48.3 ± 9.7**	**< 0.001**

3.2 Clinical and Histopathological Profile

Invasive ductal carcinoma (IDC) accounted for most of the cases, constituting 80% of the histology subtype, while the upper outer quadrant was the most regular site of the tumor (45%) (Table 2). Stage II was the most common with 40%, then Stage III with 32.5%, Stage I with 15%, and finally Stage IV with 12.5%. ER+/PR+ tumours made up 60% of the cases, which fits with most luminal subtypes in the Iraqi breast cancer population. TNBC comprised 17.5% of cases. Lymph node involvement was seen in 52.5% and distant metastasis in 12.5%.

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

Clinical Variable	Category	n (%)
Tumour anatomical site	Upper outer quadrant	18 (45.0%)
	Upper inner quadrant	9 (22.5%)
	Lower outer quadrant	7 (17.5%)
	Lower inner quadrant / central	6 (15.0%)
Tumour stage (AJCC 8th ed.)	Stage I	6 (15.0%)
	Stage II	16 (40.0%)
	Stage III	13 (32.5%)
	Stage IV	5 (12.5%)
Histological type	Invasive ductal carcinoma (IDC)	32 (80.0%)
	Invasive lobular carcinoma (ILC)	5 (12.5%)
	Other (mucinous, medullary)	3 (7.5%)
Histological grade	Grade 1 (well differentiated)	6 (15.0%)
	Grade 2 (moderately differentiated)	22 (55.0%)
	Grade 3 (poorly differentiated)	12 (30.0%)
Hormone receptor status	ER+/PR+	24 (60.0%)
	HER2+	9 (22.5%)
	Triple negative (TNBC)	7 (17.5%)
Lymph node involvement	Present	21 (52.5%)
Distant metastasis	Absent	35 (87.5%)
	Present	5 (12.5%)

3.3 Primary Biochemical Outcomes: CYP1B1, GSH, and NO

Each of the three main biomarkers showed very highly significant and large-scale differences between breast cancer patients and healthy individuals. CYP1B1 enzyme activity was 117.6% higher in breast cancer patients than in controls (61.8 9.3 vs. 28.4 4.6 pmol/mg protein/min; t = 21.34; df = 78; P < 0.001; Cohen's d = 4.38), which is a reflection of the continuous and constitutive overexpression of this enzyme both in the tumor and tumor-adjacent breast tissues leading to its release in the circulation via immune cell fractions. GSH levels were downregulated by 52.6% in case patients compared to controls (384.6 78.3 vs. 812.4 94.6 mol/L; t = 24.16; df = 78; P < 0.001; Cohen's d = 4.94), So confirming the profound depletion of this intracellular antioxidant and substrate for detoxifying catechol estrogens. NO levels were 159.7% higher in breast cancer patients (48.3 9.7 vs. 18.6 4.2 mol/L; t = 18.84; df = 78; P < 0.001; Cohen's d = 3.91), in line with the elevated iNOS expression observed in breast cancer-associated macrophages and the subsequent generation of a pro-angiogenic inflammatory microenvironment.

3.4 Stage-Dependent Biochemical Gradient

Progression for all the three markers in a stage-specific manner that was statistically significant and consistent was shown across AJCC Stages IIV (Table 3; one-way ANOVA: CYP1B1 F = 36.42, P < 0.001; GSH F = 29.18, P < 0.001; NO F = 31.64, P < 0.001; Tukey's post-hoc all P < 0.05 for adjacent stages). The level of CYP1B1 increased from 42.3 5.8 pmol/mg protein/min at Stage I to 82.7 11.2 at Stage IV. GSH decreased from 534.2 68.4 mol/L at Stage I to 214.3 48.2 mol/L at Stage IV. NO went up from 28.4 5.1 mol/L at Stage I to 72.6 10.8 mol/L at Stage IV. This simultaneous progressive deteriorationCYP1B1 going up whilst GSH dropping and NO rising biochemically describes the growing genotoxic, antioxidant depletion, and pro-inflammatory features of breast carcinogenesis.

Table 3. Stage-dependent biochemical profiles: CYP1B1, GSH, and NO across AJCC tumour stages in breast cancer patients (* vs. controls, all P < 0.001)

Marker	Stage I (n=6)	Stage II (n=16)	Stage III (n=13)	Stage IV (n=5)
CYP1B1 (pmol/mg protein/min)	42.3 ± 5.8	58.6 ± 7.4	68.4 ± 8.9	82.7 ± 11.2
GSH (µmol/L)	534.2 ± 68.4	412.8 ± 61.3	318.6 ± 54.7	214.3 ± 48.2
NO (µmol/L)	28.4 ± 5.1	44.8 ± 7.6	54.3 ± 8.4	72.6 ± 10.8
P-value (vs. controls)*	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

3.5 ROC Curve Analysis

Each of the three markers showed excellent ability in distinguishing diagnosis (Table 4). CYP1B1 activity was the best performer with an AUC of 0.951 (95% CI: 0.9160.986; P < 0.001; cut-off 38.6 pmol/mg protein/min; sensitivity 90.0%; specificity 92.5%). GSH registered an AUC of 0.938 (95% CI: 0.8980.978; P < 0.001; cut-off 614.8 mol/L; sensitivity 87.5%; specificity 92.5%). NO obtained an AUC of 0.926 (95% CI: 0.8820.970; P < 0.001; cut-off 28.4 mol/L; sensitivity 87.5%; specificity 90.0%). A very tight AUC range (0.9260.951) indicates that all three markers have similar and complementing diagnostic potential.

Table 4. ROC curve analysis: diagnostic performance of CYP1B1 activity, GSH, and NO for breast cancer detection

Biomarker	AUC	P-Value	Cut-off	95% CI Lower	95% CI Upper	Sensitivity	Specificity
CYP1B1 Activity	**0.951**	**< 0.001**	**38.6 pmol/mg/min**	**0.916**	**0.986**	**90.0%**	**92.5%**
GSH	**0.938**	**< 0.001**	**614.8 µmol/L**	**0.898**	**0.978**	**87.5%**	**92.5%**
NO	**0.926**	**< 0.001**	**28.4 µmol/L**	**0.882**	**0.970**	**87.5%**	**90.0%**

3.6 Multivariate Logistic Regression

The multivariate binary logistic regression verified that all three primary biomarkers independently predicted the presence of breast cancer even when adjusted for age, BMI, and total cholesterol (See Table 5). The activities of CYP1B1 (Wald = 23.41; P < 0.001), GSH (Wald = 16.84; P < 0.001), and NO (Wald = 20.97; P < 0.001) were each independently significant, demonstrating that these markers are non-redundantly complementary. Age, BMI, and cholesterol were not significant (all P > 0.05.(

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

Predictor Variable	β	S.E.	Wald	df	P-Value
CYP1B1 Activity (pmol/mg protein/min)	**0.184**	**0.038**	**23.41**	**1**	**< 0.001**
GSH (µmol/L)	**−0.012**	**0.003**	**16.84**	**1**	**< 0.001**
NO (µmol/L)	**0.142**	**0.031**	**20.97**	**1**	**< 0.001**
Age (years)	0.021	0.043	0.239	1	0.625
BMI (kg/m²)	0.034	0.088	0.149	1	0.699
Total cholesterol (mg/dL)	−0.003	0.016	0.035	1	0.851

3.7 Pearson Correlation Analysis

A Pearson correlation analysis performed on the entire cohort (n=80) disclosed a well-linked inter-marker network on the mechanistic level (see Table 6). Activity of CYP1B1 was very strongly anti-correlated with the level of GSH (r = -0.712; P < 0.001), supporting that the more CYP1B1-induced catechol estrogen production is generating, the less GSH is left for conjugation and detoxification activities. CYP1B1 was linked to NO positively (r = 0.698; P < 0.001), which agrees with In reality pro-inflammatory stimuli and AhR-activating stimuli promote both CYP1B1 overexpression and NO production via iNOS. GSH was also negatively correlated with NO (r = -0.681; P < 0.001), which corresponds to the oxidation of GSH by RNS that is brought about by excess NO. Most Especially, a positive association was observed between CYP1B1 and serum oestradiol (r = 0.641; P < 0.001), supporting the presence of the oestrogen-CYP1B1 axis as a biochemical basis of the panel. The correlations of all three markers with tumour stage were highly significant (CYP1B1: r = 0.724; GSH: r = -0.703; NO: r = 0.716; all P < 0.001). CYP1B1 activity was not correlated A lot with age (r = 0.094; P = 0.408) or BMI (r = 0.071; P = 0.532.(

Table 6. Pearson correlation analysis: inter-marker associations, oestradiol, and tumour stage correlations (full cohort, n = 80)

Correlation Pair	Pearson r	P-Value
CYP1B1 Activity vs. GSH	**−0.712**	**< 0.001**
CYP1B1 Activity vs. NO	**0.698**	**< 0.001**
GSH vs. NO	**−0.681**	**< 0.001**
CYP1B1 Activity vs. Tumour Stage	**0.724**	**< 0.001**
GSH vs. Tumour Stage	**−0.703**	**< 0.001**
NO vs. Tumour Stage	**0.716**	**< 0.001**
CYP1B1 Activity vs. Serum Oestradiol	**0.641**	**< 0.001**
CYP1B1 Activity vs. Age	0.094	0.408
CYP1B1 Activity vs. BMI	0.071	0.532

4. Discussion

4.1 Principal Findings and Scientific Contribution

This prospective case-control study is a pioneer in measuring CYP1B1 enzyme activity and GSH and NO in a biochemical panel in breast cancer patients from Maysan Province, Iraq. The three major findings are: (i) the levels of the three markers are Greatly and Quite a bit different in breast cancer patients when compared to healthy control subjects, and the effect sizes are very large (Cohen's d range: 3.91-4.94); (ii) in multivariate analysis each marker alone can identify breast cancer; and (iii) all three markers follow the progression of tumour stage through AJCC Stages I-IV, which indicates their potential as diagnosis and staging biomarkers. The ROC performance of the panel (AUC 0.926-0.951) outperforms that of any single marker reported in previous breast cancer clinical biochemistry studies from Arabic-speaking populations [24, 25].

The oestrogen-CYP1B1 axis shows a strong link between CYP1B1 activity Serum oestradiol (r = 0. 641, and P < 0, plus 001), proving its biological basis. So this pathway offers a clear mechanism tied to breast cancer development. The CYP1B1 - GSH - NO system focuses To be exact on estrogen-related biology, not general oxidative stress. Usually, this precision makes the findings more relevant to breast cancer biology than broad metabolic markers.

4.2 Mechanistic Framework: The Oestrogen–CYP1B1–GSH–NO Axis

The series of mechanisms through which CYP1B1 overexpression results in breast cancer can be detailed like this. Firstly, 17-oestradiol (E2) is taken up by breast epithelial cells where CYP1B1 catalyses its hydroxylation at the C4 position, resulting in 4-hydroxyoestradiol (4-OHE2) [5, 6]. 4-OHE2 is then oxidised to the highly reactive 4-OHE2-ortho-quinone by cytochrome P450 peroxidase activity. These quinones predominantly form covalent bond with N3 of adenine and N7 of guanine in DNA, leading to the formation of depurinating adducts (4-OHE2-1-N3Ade; 4-OHE2-1-N7Gua) that produce the basic sites and, That means, the oncogenic point mutations in the breast epithelial cell genomes - Mainly in TP53 and KRAS - if not repaired [7, 8]. The current study's finding of a strong positive correlation between CYP1B1 activity and serum oestradiol (r = 0.641) offers a direct biochemical proof of substrate-driven CYP1B1 activation in the breast cancer group.

GSH is the major intracellular substrate for glutathione S-transferase (GST)-mediated conjugation and detoxification of both 4-OHE2 and its quinone intermediates to form stable, excretable 4-OHE2-GSH conjugates [11, 12]. In situations where CYP1B1 over-function results in the generation of estrogen quinones at a level exceeding that of the available GSH, quinones that are not conjugated become accumulated and then interact with DNA. A very strong inverse association has been found between CYP1B1 and GSH ($r = 0.712$; $P < 0.001$) which biochemically corroborates this competition: with an increase in CYP1B1-induced production of catechol estrogens, the amount of GSH available for detoxification is reduced and exhausted, thereby leading to a biochemical tipping point after which DNA damage is accumulated. And, the NO-mediated reactive nitrogen species (RNS)mainly peroxynitrite (ONOO), which is generated from the reaction of NO with superoxide directly oxidise GSH to glutathione disulphide (GSSG), Because of this further reducing the antioxidant pool [14].

The rise in NO levels in the blood of breast cancer patients is a result of an iNOS overexpression in tumor-associated macrophages, mast cells, and cancer-associated fibroblasts within breast tumor microenvironment [15, 16]. At the micromolar concentrations observed in the present case group (48.3 9.7 mol/L), NO could, in fact, be tumor promoting through several mechanisms: (i) prolongation of HIF-1 half-life even under normoxia, which in turn induces VEGF-dependent angiogenesis; (ii) p53 tumor suppressor protein being nitrated by peroxynitrite, This way losing its ability to induce apoptosis; (iii) S-nitrosylation of caspases 3 and 9, leading to their apoptosis inhibiting effect; and (iv) activation of matrix metalloproteinase (MMP)-2 and MMP-9 that help in degrading basement membrane and opening up metastatic invasion [15 16 26]. The stage-wise increase of NO (28.4 mol/L at Stage I to 72.6 at Stage IV) is in agreement with the notion that the iNOS-expressing tumor microenvironment constantly enlarges with the progression of the disease.

4.3 CYP1B1 in the Maysan Context

The exceptionally higher CYP1B1 enzyme activity in breast cancer patients from Maysan (61.8 9.3 pmol/mg protein/min) takes explanation by the environmental context. CYP1B1 is one of the strongest targets of the aryl hydrocarbon receptor (AhR) which is activated by polycyclic aromatic hydrocarbons (PAHs) from multiple environmental pollution sources in Maysan Province, including emissions from petrochemical facilities, gas flaring in oil-fields, and seasonal burning of biomass [9, 10]. Continuous activation of AhR by these environmental PAHs results in the expression of CYP1B1 in mammary epithelial cells and blood immune cells, leading to a steady increase of CYP1B1 activity over the baseline found in populations less exposed to PAHs. Such environmental increase of CYP1B1-driven oestrogen genotoxicity pathway may partly explain the high breast cancer rates in the southern Iraqi provinces [4] and is a scientifically worthful evidence of the molecular link between Maysan's industrial and environmental exposures and the breast cancer risk in the region.

5. Strengths And Limitations

5.1 Strengths

The main advantages are: (i) treatment-nave sampling which ensured that the measurements depict the breast cancer biochemical state and do not reflect any changes brought about by therapeutic drugs; (ii) histopathological identification of all cases supported with AJCC staging and hormone receptor/HER2 profiling; (iii) a set of individually validated, "gold standard" assay methods were used for determining the levels of all three markers; (iv) individual matching on age, menopausal status, and BMI, which controlled for the hormonal and metabolic confounders that are the most relevant to estrogen-driven carcinogenesis; (v) strict exclusion of hormonal medications, phytoestrogens, and NOS-modifying substances; (vi) the major CYP1B1estradiol correlation offering a mechanistic validation of the estrogen axis; (vii) the multivariate independence of each one of the three markers demonstrating their non-redundant complementarity; and (viii) an adequate post-hoc power (97.8%) for the observed effects.

5.2 Limitations

One of the limitations is the modest sample size (n = 80) that does not allow for the reliable subgroup analysis based on hormone receptor subtype, HER2 status, or menopausal status. Besides, the single-centre recruitment limits the immediate generalisability of the results. Also, the lack of serial longitudinal sampling makes it impossible to assess biomarker kinetics during disease progression or treatment. No data on the genetic polymorphisms of CYP1B1 (CYP1B1*2, *3 alleles influencing enzyme kinetics) were obtained during the clinical selection. Measuring for urinary catechol estrogen metabolites, a direct indicator of CYP1B1 mediated oestrogen metabolism in vivo was not performed together with serum CYP1B1 phenotyping. Finally, the exposure to AhR-activating environmental PAH was not measured individually which did not allow the formal analysis of environmentCYP1B1 interaction.

6. Conclusions

This prospective case control research shows that the clinical biochemistry trio of CYP1B1 enzymatic activity, reduced glutathione, and nitric oxide represents a mechanistically unified, independently confirmed, and easily measurable biomarker set for breast cancer diagnosis and tumour stage gradation in Maysan Province, Iraq. The panel depicts the entire estrogen-genotoxicity axis of breast carcinogenesis from CYP1B1-mediated catechol estrogen production through GSH-mediated detoxification failure to NO-driven pro-angiogenic and pro-invasive signaling with every component showing separate significance in multivariate analysis, remarkable ROC performance (AUC 0.9260.951), and gradual stage-dependent worsening across AJCC Stages IIV. The identified cutoff valuesCYP1B1 > 38.6 pmol/mg protein/min, GSH < 614.8 mol/L, and NO > 28.4 mol/Loffer feasible, cost-efficient standards for breast cancer risk classification in a resource-limited oncology environment of Maysan. The highly significant association of CYP1B1 activity with serum oestradiol supports the mechanistic basis of the panel and points to the particular importance of the estrogenCYP1B1 axis in breast cancer patients from Maysan, where exposure to environmental AhR activators could bring an elevation of this pathway beyond normal levels.

Declarations

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Data Availability Statement: De-identified participant individual data can be shared subject to a reasonable written request to the corresponding author and upon obtaining the approval of the MOC Institutional Review Board and the Maysan Health Directorate.

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