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Evaluation of serum CCN6, VEGF-A, and NF-κB p65 and their clinicopathological correlations in breast cancer

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ABSTRACT

Introduction and aim. CCN6, a transcriptional regulator within the CCN family, influences breast tumorigenesis by interacting with key angiogenic (VEGF-A) and inflammatory (NF-κB p65) pathways. This study establishes simultaneous evaluation of these serum biomarkers and comparison with CA 15-3 across untreated and treated breast cancer cohorts. This study aimed to evaluate their serum level and clinicopathological associations to understand their role in tumor progression and potential diagnostic utility.

Materials and methods. Serum biomarkers were measured via ELISA in 130 breast cancer patients (56 untreated and 74 treated) and 50 controls. Demographic and clinical data were collected through questionnaire-based interviews and clinicopathological data from medical records.

Results. CCN6 had the greatest effect size (F=1.10), followed by NF-κB p65 (F=0.51) and VEGF-A (F=0.22). The diagnostic efficacy (AUC=0.995; p=0.0001), sensitivity (96%), and specificity (100%) of CCN6 were superior to those of CA15-3 (AUC=0.855) and NF-κB p65 (AUC=0.813). Logistic regression demonstrated that downregulated CCN6 was inversely associated with disease outcome (OR=0.440; p=0.0001), whereas elevated NF-κB p65 (OR=1.737) levels increased breast cancer risk.

Conclusion. CCN6 showed promising diagnostic performance and exhibits an inverse association with breast cancer, outperforming NF-κB p65 and CA 15-3; these findings underscore CCN6's potential as a preliminary non-invasive diagnostic biomarker.

Keywords. breast cancer, CA 15-3, CCN6, NF-κB p65, VEGF-A

Introduction

Cellular communication network factor 6 (CCN6) is a matricellular protein member of the CCN family, which comprises six proteins: CCN1 (CYR61), CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP1), CCN5 (WISP2), and CCN6 (WISP3).[1] These proteins regulate various biological processes, including cell proliferation, differentiation, adhesion, migration, angiogenesis, and tissue repair.[1,2] Particularly, it has been shown that CCN6 maintains the integrity of epithelial cells by preventing epithelial-mesenchymal transition (EMT) and restraining the invasive phenotype.[3] Since breast cancer is a highly prevalent malignancy,[4] recent studies have investigated how CCN6 might participate in its development and progression.[5,6] By downregulating the insulin growth factor-1 (IGF-1)/insulin growth factor-1 receptor (IGF-1R), Wnt/β-catenin signaling pathway, and phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) pathways, CCN6 preserves the epithelial phenotype and thereby inhibits EMT, which is one of the main mechanisms through which tumors invade and metastasize.[5] resulting in reduced proliferation, differentiation, and motility of cancer cells.[7] Consequently, CCN6 downregulation correlates with invasive tumor behavior and poor prognosis.[3]

Concurrent research has investigated vascular endothelial growth factor A (VEGF-A) and nuclear factor kappa B (NF-κB), two pivotal regulators of angiogenesis, inflammation, and cancer cell survival.[8,9] VEGF-A stimulates endothelial proliferation and vascularization by VEGFR-2-activated mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, and its expression is associated with advanced tumor stage and reduced patient survival.[10] NF-κB, particularly its p65 subunit, regulates the expression of pro-inflammatory, proliferation, and apoptosis-resistant genes. Persistent NF-κB activation has been observed in invasive forms of breast cancer, where NF-κB stimulates the expression of VEGF-A, B-cell lymphoma-2 (Bcl-2), and matrix metalloproteinase-9 (MMP-9) to facilitate angiogenesis and invasion.[11.] Conversely, the inhibition of NF-κB activity leads to a significant decrease in VEGF-A mRNA levels.[12]

Clinically, CA15-3 is a widely utilized serum tumor marker in the management of breast cancer due to its role in diagnosis and prognostication.[13] It is secreted from mucin 1 (MUC1) glycoprotein and serves as a measure of tumor burden and is useful for disease monitoring and recurrence detection. However, its decreased sensitivity in early-stage disease has prompted studies into its use in combination with other molecular markers to provide a more effective and comprehensive diagnostic profile.[14]

Despite advances in conventional breast cancer treatments, the molecular heterogeneity of malignancy necessitates the continuous evolution of therapeutic strategies. While the individual roles of these biomarkers are recognized, it remains unknown how their serum levels interact across different breast cancer clinical states or how a combinatorial panel performs relative to the standard marker. The single markers fail to capture the multi-faceted tumor microenvironment; therefore, the simultaneous evaluation of these four biomarkers represents a vital advance.

Aim

This study aims to determine the diagnostic potential of CCN6, VEGF-A, NF-κB p65, and CA 15-3 by comparing their levels across untreated and treated breast cancer patients with healthy controls and assess their correlation with critical

clinicopathological factors, which may contribute to a better understanding of the underlying molecular mechanism and improve early disease detection.

Material and methods

Study design

A cross-sectional study was conducted between October 2024 and June 2025, enrolling 180 women aged 25–80, as illustrated in Fig. 1. Of these, 130 were patients with breast cancer diagnosed by an oncologist at the Oncology Center of Al-Sadr Teaching Hospital in Basrah City, Southern Iraq.

Participants and eligibility

This study involved 180 subjects classified into patient and control groups. Patients were subdivided based on their treatment status into two cohorts:

- Untreated group: 56 newly diagnosed patients with breast cancer aged 25–80 years, 1–12 months post-diagnosis, who had yet to receive any treatment modality.
- Treated group: 74 patients with breast cancer, aged 30–75 years, either currently receiving treatment or 2–12 years post-treatment. Treatment protocols varied according to the cancer stage, lymph node involvement, organ metastasis, and tumor grade, encompassing radiotherapy; chemotherapy (with doxorubicin, cyclophosphamide, docetaxel, paclitaxel, and carboplatin); hormonal therapy (used for hormone-positive breast cancer with tamoxifen, letrozole, or anastrozole depending on menopausal status); and HER2-targeted therapy with trastuzumab if HER2 was positive, given with chemotherapy if indicated; the heterogeneity of these treatment modalities acknowledged a potential source of biological variability in biomarkers' expression. The AJCC Tumor-Node-Metastasis (TNM) classification is used for breast cancer staging.[15] This system standardizes breast cancer treatment and prognosis via stage.
- Control group: 50 healthy female volunteers (hospital staff, community) matched to the patients' cohort by age and geographical distributions, with no history of autoimmune, chronic, or genetic diseases or sickle cell anemia.

Inclusion criteria

- female patients, either newly diagnosed before treatment (untreated group) or previously/actively receiving treatment (treated group),
- patients with complete histological and laboratory data,
- patients providing written informed consent.

Exclusion criteria

- pregnant women,
- individuals with psychiatric or cognitive disorders,
- patients aged <25 years,

- patients refusing to provide written informed consent,
- patients with incomplete medical records or missing clinicopathological data.

Sample and effect size calculation

The total sample size required to conduct this study was calculated a priori using G*Power version 3.1.9.7 for a three-group comparison with a medium effect size (f=0.25), two-tailed testing (α=0.05), and power (1–β) of 0.80, resulting in a minimum requirement of approximately 159 total samples. To ensure robust statistical power, a total of 180 samples were collected.

Data collection

Demographics and clinical data (age, weight, Body Mass Index [BMI], family history, and menopausal status) were collected via questionnaire-based interviews. Clinicopathological data (disease stage, histological type, molecular subtype, tumor grade, lymphovascular invasion [LVI], KI-67% proliferation index, CA 15-3, ER, PR, and HER-2 status) were collected from the oncology center's medical records and electronic laboratory system. The health assessments were conducted according to STORBE guidelines. BMI was computed as the subject's weight in kilograms (kg) divided by height in square meters (m^2) and grouped according to the WHO standards into four groups: underweight (under 18.5 kg/m^2), normal weight (18.5–25 kg/m^2), overweight (25-30 kg/m^2), and obese (above 30 kg/m^2).[16] In the statistical analysis, these categories of BMI were used to establish any potential association between BMI and the serum concentrations of the biomarkers under investigation (CCN6, VEGF-A, NF-κB p65, and CA 15-3) among the study participants.

Sample collection

Five milliliters of whole blood were collected via venipuncture from breast cancer patients during their scheduled morning visits to the oncology center following an overnight fast, either pre-treatment or before chemotherapy/hormonal therapy cycles, and from healthy controls. Two milliliters of blood were drawn into purple ethylenediaminetetraacetic acid (EDTA) tubes for complete blood count analysis of hematological parameters. The remaining 3 mL, collected in yellow-colored gel separator tubes, was permitted to coagulate at ambient temperature and then centrifuged at 4100 rpm for 4 minutes and stored in a deep freezer (-80°C). The samples underwent only a single freeze-thaw cycle before biomarker quantification and routine laboratory tests assessment.

Routine laboratory tests assessment

Complete blood count test

Hematological parameters were quantified using 2 milliliters of blood collected in EDTA-anticoagulant tubes. A Sysmex automated hematology analyzer (Sysmex, Kobe, Japan) was utilized to execute complete blood counts and quantify white blood cells (WBCs), neutrophils, monocytes, lymphocytes, red blood cell (RBC) counts, and hemoglobin levels.

Enzyme-linked immunosorbent assay (ELISA)

Serum concentrations of CCN6, VEGF-A, and NF-κB p65 were quantified using commercially available ELISA kits, following the manufacturer's instructions. Specifically, CCN6 was measured using the BT Lab Human CCN6 ELISA kit (Cat. No. E6409Hu; range: 0.05–30 ng/mL, sensitivity: 0.018 ng/mL). VEGF-A levels were measured using an Elabscience Human VEGF-A ELISA kit (Cat. No. E-EL-H0111; range: 31.25–2000 pg/mL, sensitivity: 18.75 pg/mL). NF-κB p65 levels were measured using an Elabscience Human NF-κB p65 ELISA kit (Cat. No. E-EL-H1388; range: 0.16–10 ng/mL; sensitivity: 0.09 ng/mL). For quality controls, all standards and samples were tested in duplicates. An ELISA washer (Thermo Scientific, USA) was employed for washing, and absorbance was recorded at 450 nm using an ELISA microplate reader (Thermo Scientific, USA). The concentrations were calculated from the standard curves. The inter- and intra-assay coefficient of variation (CV) for CCN6 ELISA were <8% and <10%, respectively; for NF-κB p65, (6.09–8.5%) and (6.55–6.83%), respectively; and for VEGF-A, (6.61–8.27%) and (4.92–6.95%), respectively. Laboratory blinding was enforced during the biomarker assessment to alleviate the information bias.

Statistical analysis

IBM SPSS Statistics, version 31 (SPSS Inc., Chicago, IL, USA), was used for data analysis, while graphs were plotted using GraphPad Prism, version 8.0.1 (GraphPad Software, San Diego, CA, USA). Continuous variables are presented as mean±standard deviation (SD). Categorical variables were presented as frequencies and percentages extracted from the chi-square and Fisher's exact tests. Shapiro-Wilk and Kolmogorov-Smirnov tests were used for the normality test. The Kruskal–Wallis test with Dunn's post-hoc test was used for the three-group comparison. Spearman's correlation analysis was performed to assess associations. Bonferroni correction was applied following multiple comparisons and correlations to adjust the significance threshold and minimize the risk of type 1 errors. Diagnostic performance of the serum biomarkers was evaluated by ROC curve analysis, and the AUC, optimal cut-offs, sensitivity, specificity, and accuracy were calculated. The cutoff points were assessed by the Youden index. Univariate binary logistic regression was used to identify independent risk factors of breast cancer, followed by multivariate binary logistic regression. To avoid the confounding influence of the therapeutic interventions, the previously mentioned analyses were purposefully restricted to the untreated group versus healthy controls. Statistical significance was set at $p<0.05$.

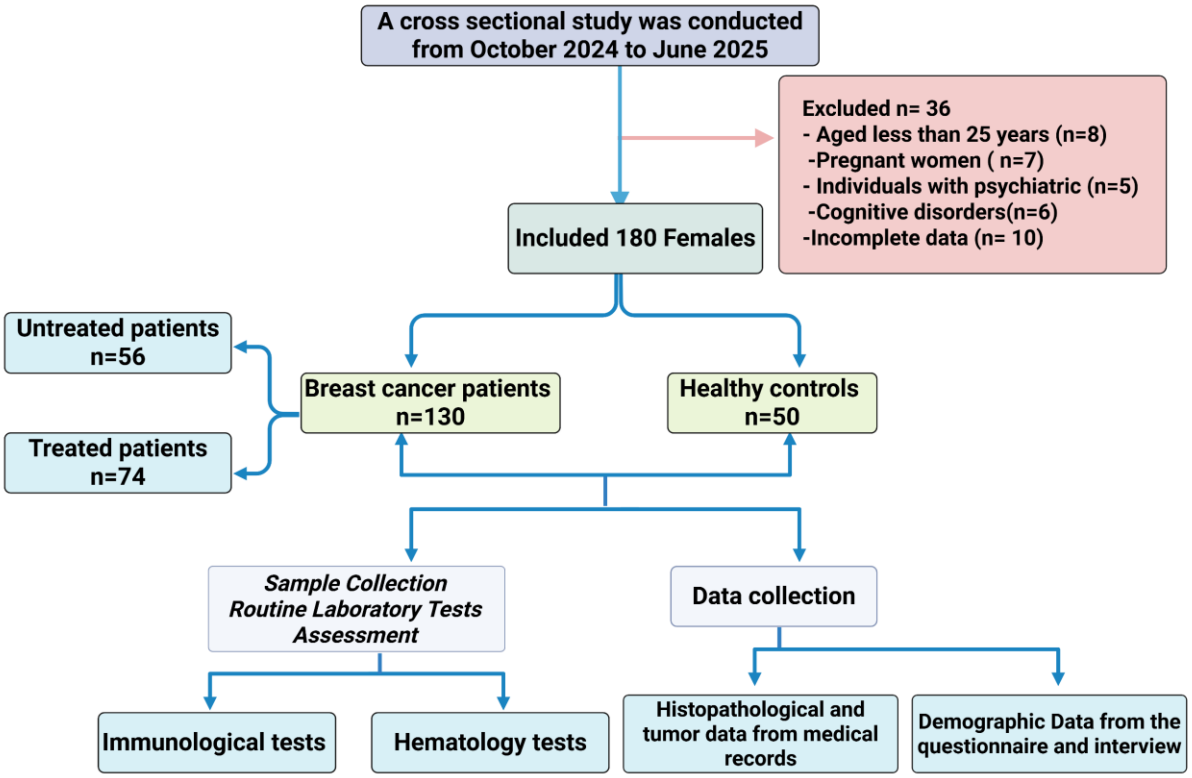

Fig. 1. Flow diagram of participant selection and enrollment in the study

Results

Patients' demographic and histopathologic characteristics

The study cohort comprised 180 participants, categorized into three groups: untreated patients (n=56), treated patients (n=74), and healthy controls (n=50). Patients' demographic and histopathologic characteristics are detailed in Table 1. The analysis confirmed that age, BMI, and family history were statistically balanced across the patient groups ($p>0.05$). The majority of patients in both groups were aged between 40–55 years, overweight (BMI>25 kg/m²), and had a negative family history. However, a significant disparity was observed in the menopausal status ($p=0.0001$), with peri-menopausal status being the predominant status in the treated group (55.4%), whereas patients were more evenly distributed between pre- (39.3%) and post-menopausal (35.7%) statuses in the untreated group.

The treated patients presented with more aggressive disease features characterized by a higher frequency of grade III tumors (63.5%), while grade II was more prevalent in the untreated group (57.1%), $p=0.010$. Furthermore, tumor invasiveness varied significantly ($p=0.032$), though invasive disease was predominant in both groups (100% of treated and 92.9% of untreated patients). Histological subtypes showed significant variation ($p=0.008$), with invasive ductal carcinoma, no specific type (IDC NST), being the prevalent subtype across both groups. Other clinicopathological characteristics, including LVI, disease stage, hormone receptor status (ER, PR), HER-2 status, and molecular subtypes, did not differ

significantly between the untreated and treated cohorts (p>0.05). Stage II accounted for the highest proportion of cases in both groups. Immunohistochemical profiling indicated that estrogen and progesterone receptor positivity was predominant in both groups, whereas HER-2 receptor negativity was more frequent. Consequently, luminal B was the most prevalent subtype in the untreated group (39.3%), compared to the treated group, which represents equal proportions of both luminal A and B (36.5% each).

Surgical interventions differed significantly between the two groups (p=0.0001), with most untreated patients enrolled before the surgery. Nearly all the treated patients underwent surgical resection, accounting for 97.3% of the cases. Similarly, the treatment protocol distributions were highly significant (p=0.0001), as all the untreated patients were enrolled prior to the initiation of therapeutic interventions, while the majority of treated patients (79.7%) had received a combination therapy consisting of chemotherapy, radiation, and hormonal therapy; alternative combination types were presented less frequently within the group.

Table 1. Demographic, anthropometric, and histopathological characteristics of breast cancer patients*

Characters	**Untreated patients n=56**	**Treated patients n=74**	**p**
Age, n (%)			
<40	13 (23.2)	9 (12.2)	0.130
40–55	23 (41.1)	42 (56.8)	
>55	20 (35.7)	23 (31.1)	
Mean ± SD	49.8±11.6	52±9.69	0.347
Body mass index, n (%)			
Under weight	10 (17.9)	16 (21.6)	0.235
Normal weight	15 (26.8)	28 (37.8)	
Overweight	31 (55.4)	30 (40.5)	
Obese	10 (17.9)	16 (21.6)	
Family history, n (%)			
Positive	10 (17.9)	13 (17.6)	0.966
Negative	46 (82.1)	61 (82.4)	
Menopausal status, n (%)			
Pre-menopausal	22 (39.3)	9 (12.2)	0.0001
Perimenopausal	14 (25)	41 (55.4)	
Post-menopausal	20 (35.7)	24 (32.4)	
Disease stage, n (%)			
Stage 0 (DCIS)	2 (3.6)	0	0.313

Stage I	8 (14.3)	10 (14.9)	
Stage II	26 (46.4)	27 (36.5)	
Stage III	12 (21.4)	20 (27)	
Stage IV	8 (14.3)	16 (21.6)	
Tumor grade, n (%)			
I	2 (3.6)	0	0.010
II	32 (57.1)	27 (36.5)	
III	22 (39.3)	47 (63.5)	
Lymphovascular invasion, n (%)			
Present	18 (32.1)	13 (17.6)	0.063
Not identified	38 (67.9)	61 (82.4)	
Tumor invasiveness, n (%)			
Noninvasive	4 (7.1)	0	0.032
Invasive	52 (92.9)	74 (100)	
Histological type, n (%)			
Ductal carcinoma in situ	4 (7.1)	0	0.008
Invasive ductal carcinoma	45 (80.4)	70 (94.6)	
Invasive lobular carcinoma	6 (10.7)	2 (2.7)	
Mixed invasive carcinoma	0	2 (2.7)	
Invasive metaplastic carcinoma	1 (1.8)	0	
Hormone receptor, n (%)			
Estrogen receptor status			
Positive	40 (71.4)	54 (73)	0.846
Negative	16 (28.6)	20 (27)	
Progesterone receptor status, n (%)			
Positive	38 (67.9)	52 (70.3)	0.848
Negative	18 (32.1)	22 (29.7)	
HER-2 status, n (%)			
Positive	10 (17.9)	14 (18.9)	0.986
Negative	46 (82.1)	60 (81.1)	
Molecular subtype, n (%)			
Luminal A	18 (32.1)	27 (36.5)	0.556
Luminal B	22 (39.3)	27 (36.5)	
HER-2 enriched	6 (10.7)	12 (16.2)	

Triple negative	10 (17.9)	8 (10.8)	
Surgical intervention, n (%)			
Yes	12 (21.4)	72(97.3)	0.0001
No	44 (78.6)	2(2.7)	
Treatment protocol, n (%)			
No treatment	56 (100)	0	0.0001
Chemotherapy+hormonal	0	1 (1.4)	
Chemotherapy+radiation	0	12 (16.2)	
Chemotherapy+radiation+ hormonal therapy	0	59 (79.7)	
Radiation+hormonal therapy	0	2(2.7)	

* Differences were considered statistically significant at a two-tailed p-value of $p \leq 0.05$; statistical analysis was conducted using the chi-square test with variables represented as frequency and percentage (n, %); DCIS – Ductal carcinoma in situ; n – number; % – percentage; SD – standard deviation

Comparative analysis of demographic, hematological, and biochemical characteristics

Table 2 exhibited no significant differences in age (p=0.107) and BMI (p=0.117) among the three groups. The average age was (49.8±11.6) years for the untreated group, (52±9.69 years) for the treated, and (48.6±10.9 years) for the healthy control. The untreated group had a slightly elevated BMI compared with treated and healthy controls (30.5±5.6 vs. 30.1±5.85 and 28.6±3.42 kg/m²). Significant physiological and hematological alterations were observed; healthy controls demonstrated significantly higher RBCs (4.53±0.38 x 10^{12}/L) and HB (126±10.3 g/L) compared to both patient groups (p=0.0001).

Untreated patients revealed significantly elevated WBC and neutrophil counts (7.16±1.63x10^9/L and 4.53±1.08 x 10^9/L, p=0.0001) relative to the treated and control groups. Furthermore, significant variations were observed in the neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) across all groups (p=0.0001), with healthy controls exhibiting the highest LMR (5.57±2.54) and lowest NLR (1.79±0.96) ratios. No significant differences were observed in the platelet counts between the groups.

Table 2. Comparative analysis of demographic data, hematological, and biochemical markers among untreated patients, treated patients, and healthy controls

Parameter	Total patients n = 130		Healthy control (n=50)	p
	Untreated (n=56)	Treated (n=74)		

Age (year)	49.8±11.6	52±9.69	48.6±10.9	0.107
BMI (kg/m²)	30.5±5.6	30.1±5.85	28.6±3.42	0.117
HB (g/L)	117.82±23.66	117.0±14.6	126.0±10.3	0.0001
RBC × 10^{12}/L	4.3±0.78	4.17±0.6	4.53±0.4	0.0001
HCT (L/L)	0.35±0.07	0.35±0.04	0.37±0.024	0.014
MCV (fL)	79.68±13.23	84.13±12.65	83.61±4.28	0.071
MCHC (g/L)	327.57±12.63	331.09±15.87	333.26±16.95	0.051
WBC × 10^9/L	7.16±1.63	5.98±2.19	6.04±1.57	0.0001
NEU ×10^9/L	4.53±1.08	3.55±1.76	3.37±1.27	0.0001
LYM ×10^9/L	1.98±0.72	1.68±0.68	2.07±0.64	0.009
MONO ×10^9/L	0.47±0.19	0.62±0.38	0.42±0.16	0.006
LMR	4.78±2.79	3.55±2.34	5.57±2.54	0.0001
NLR	2.51±0.89	2.51±1.07	1.79±0.96	0.0001
PLT × 10^9/L	270.60±69.02	263.77±108.47	268.06±54.17	0.153

* Data are represented as mean ± SD. Differences were considered statistically significant at a two-tailed p-value of $p \leq 0.05$; the differences in means between the groups were assessed using the Kruskal-Wallis test, Bonferroni correction-adjusted p-value ($p<0.002$), WBC – white blood cells, MCV – mean corpuscular volume, MCHC – mean corpuscular hemoglobin concentration, NEU – neutrophils, LYM – lymphocytes, MONO – monocytes, LMR – lymphocytes to monocytes ratio, NLR – neutrophils to lymphocytes ratio

To evaluate the impact of the treatment and remission time on the parameter level, the treated group was stratified into three subgroups depending on the time since treatment. The comparative analysis revealed no significant variations in the biomarker levels between patients in active treatment and those with short- and long-term remission times ($p>0.1$). The correlation analysis further confirmed that the elapsed time since treatment did not significantly impact the biomarker levels ($rho<0.200$, $p>0.1$).

Comparative analysis of serum immunological biomarkers between patients and healthy controls

The serum levels of CCN6, VEGF-A, and NF-κB p65 demonstrated significant variations (Table 3). The control group exhibited significantly elevated CCN6 levels (19.92±6.92 µg/L) in comparison with both untreated (2.04±0.17 µg/L) and treated groups (3.58±1.22 µg/L), P=0.0001, showing a very large effect size (Cohen's f=1.10) and 100% statistical power. Conversely, VEGF-A levels were significantly elevated in the untreated group (157.28±83.55 ng/L), Figure 2, compared to both the treated and control groups, P=0.013, with a small effect size (Cohen's f=0.22 and 74% power. Similarly, NF-κB p65 levels demonstrated significant elevation in the untreated group (6.85±1.86 µg/L) relative to the other groups (P=0.0001), yielding a large effect size (Cohen's f=0.51) and 99% power.

Table 3. Comparative analysis of serum immunological biomarkers among untreated, treated patients and healthy controls*

Parameter	Total patients, n = 130		Healthy control (n=50)	p	ε^2	(F)	Power
	Untreated (n=56)	Treated (n=74)					
CCN6 (µg/L)	↓ 2.04±0.17	↑ 3.58±1.22	19.92±6.92	0.0001	0.55	1.10	100%
VEGF-A (ng/L)	↑ 157.28±83.55	↓ 136.09±60.37	143.56±30.29	0.013	0.04	0.22	74%
NF-κB p65 (µg/L)	↑ 6.85±1.86	↓ 5.68±1.32	2.78±0.90	0.0001	0.20	0.51	99%

* Data are represented as mean±SD; differences were considered statistically significant at a two-tailed p-value of p≤0.05, the differences in means between the groups were assessed using the Kruskal-Wallis test
Bonferroni correction (adjusted p<0.016), H – Kruskal-Wallis statistic, F – Cohen's effect size, CCN6 – human WNT1-inducible-signaling pathway protein 3, VEGF-A – vascular endothelial growth factor A, NF-κB p65 – nuclear factor NF-kappa-B p65 subunit

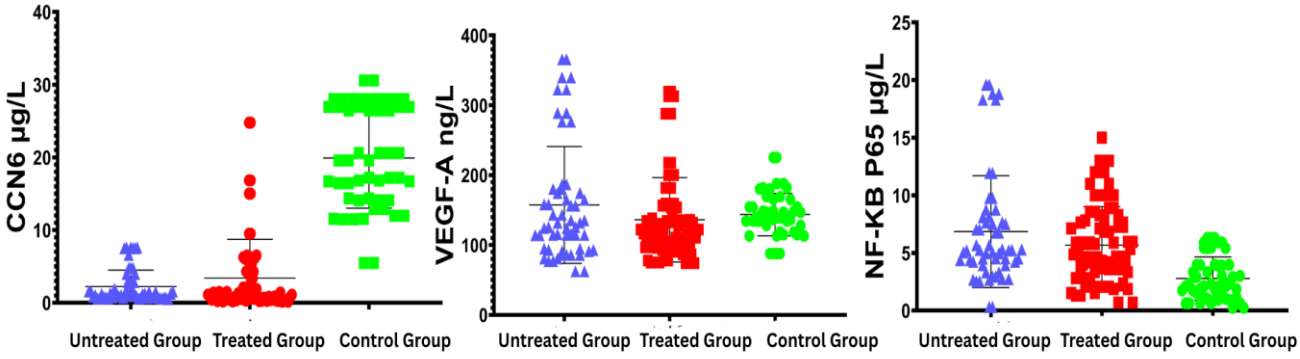

Fig. 2. Scatter plot for the comparative distribution of serum immunological biomarkers among untreated, treated, and healthy controls

Association serum levels of CCN6, NF-κB P65, VEGF-A, and CA15 with disease stages

In the untreated patients, disease stages significantly correlated with circulating biomarker levels (Fig. 3 and 4). Following the application of the Bonferroni correction (adjusted P=0.008), CCN6 levels were not significantly varied across stages in the untreated (p=0.040), peaking in stage IV (3.73±0.51 µg/L). In the treated group, the variation was also non-significant

(p=0.037), and the highest levels were in stage II (5.34±1.06 µg/L). CA15-3 levels showed significant stage-dependent upregulation in the untreated and treated groups (P=0.0001 and P=0.002, respectively), reaching maximum levels in advanced-stage disease (IV) (122.49±23.39 U/mL and 50.94±10.9 U/mL, respectively). VEGF-A and NF-κBp65 concentrations increased with advancing disease stages in the untreated group, but these variations were not statistically significant in the untreated (p=0.347 and p=0.087, respectively) and the treated group (p=0.0.25 and p=0.181, respectively).

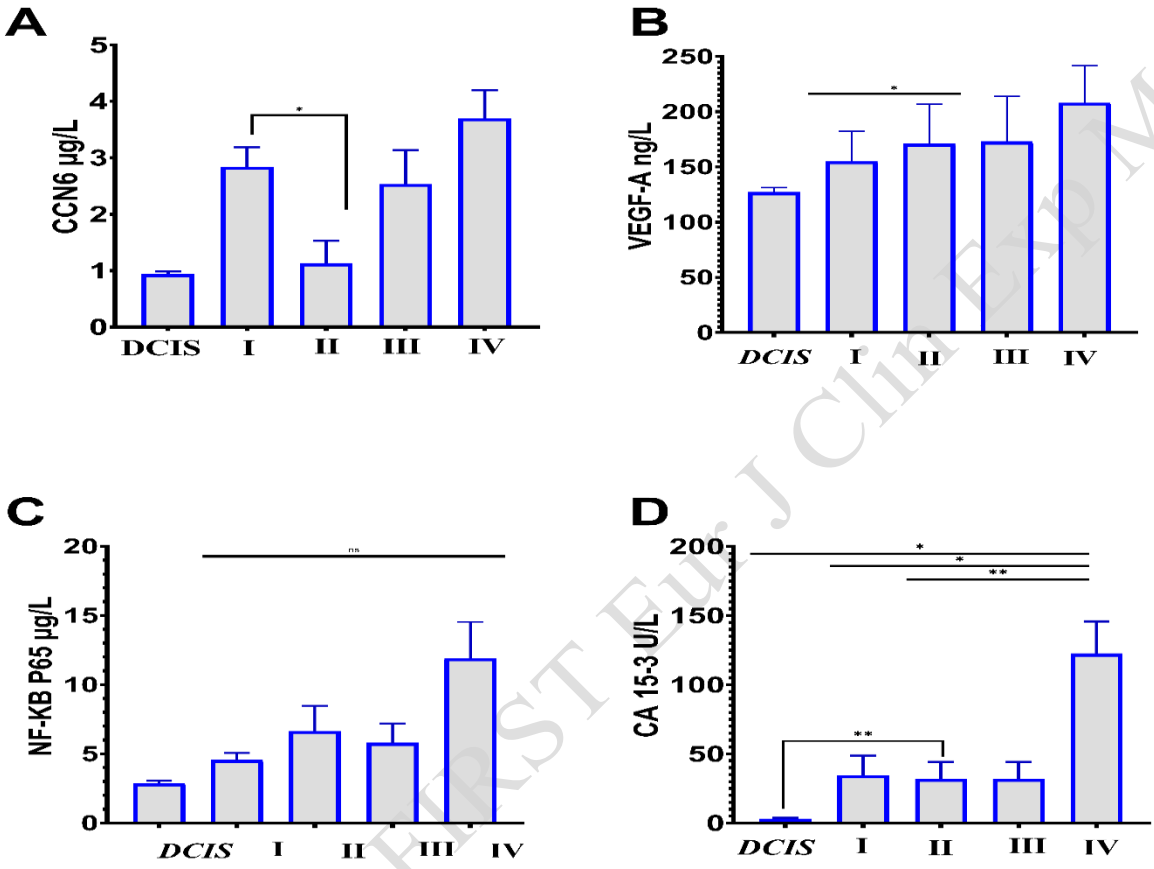

Fig. 3. Association of serum levels of CCN6, NF-κB P65, VEGF-A, and CA15 with disease stages in the untreated group (*– p<0.05, ** – p<0.01, ns: non-significant>0.05), the differences in means between the groups were assessed using the Kruskal-Wallis test

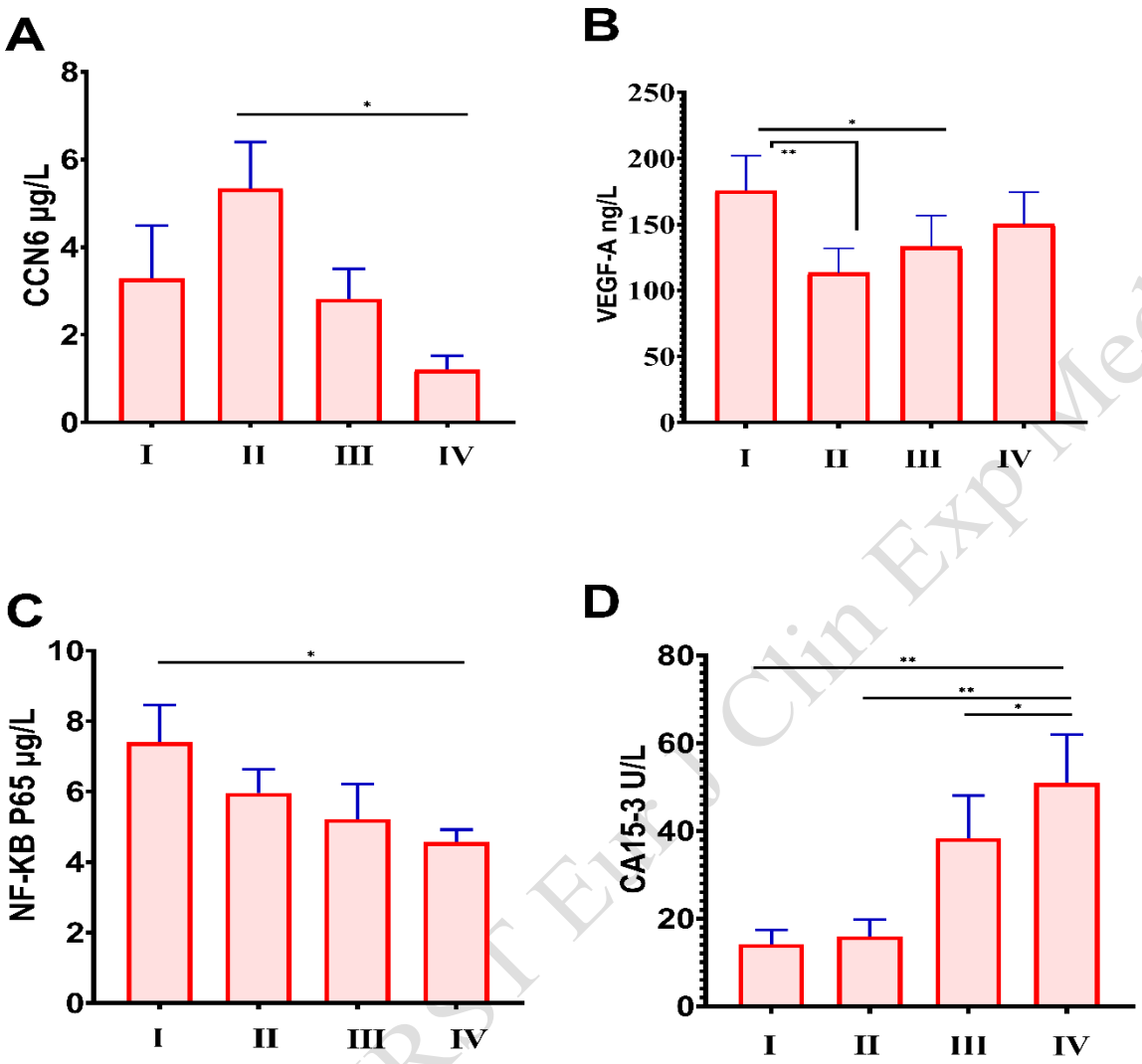

Fig. 4. Association of serum levels of CCN6, NFKB-p65, VEGF-A, and CA15 with disease stages in the treated groups (*– $p<0.05$, **– $p<0.01$, ns: non-significant >0.05); the differences in means between the groups were assessed using the Kruskal-Wallis test

Note: The following tests were performed utilizing the untreated patient cohort.

Assessment of Spearman correlation

The correlation analysis of immunological biomarkers within the untreated group is presented in Table 4. Following the application of the Bonferroni correction (adjusted threshold $p<0.0125$), there was a significant negative correlation between CCN6 and several markers of disease progression, including NF-κB P65 (rho=-0.316, p=0.0001) and CA15-3 (rho=-0.499, p=0.0001). No significant relationship could be found between the levels of CCN6 and those of VEGF-A and KI-67. The level of CA15-3 was positively correlated with NF-κB P65 (rho=0.231, p=0.002) and VEGF-A (rho=0.420, p=0.001). Furthermore, a synergistic correlation was observed between NF-κB P65 and VEGF-A (rho=-0.194, p=0.009).

Table 4. Spearman correlation analysis among serum immunological biomarkers*

* $^{\Delta}$ – Correlation is significant at the 0.01 level (2-tailed)

Variables	Spearman correlation (r)	p	95% CI		Interpretation
			Lower	Upper	
CCN6 vs NF-κB p65	-0.316 ↓	0.0001$^{\Delta}$	-0.445	-0.174	Significant moderate negative correlation
CCN6 vs. VEGF-A	-0.073↓	0.593	-0.337	0.201	No correlation/No significant association
CCN6 vs. CA15-3	-0.499 ↓	0.0001$^{\Delta}$	-0.582	-0.069	Significant moderate negative correlation
CCN6 vs. Ki-67%	-0.214 ↓	0.014	-0.377	-0.039	Significant weak negative correlation
CA15-3 vs. VEGF-A	+0.420 ↑	0.001$^{\Delta}$	0.169	0.620	Significant moderate positive correlation
VEGF-A vs NF-κB p65	-0.194 ↓	0.009$^{\Delta}$	-0.335	-0.045	Significant weak negative correlation
NF-κB p65 vs. CA15-3	+0.231 ↑	0.002$^{\Delta}$	0.083	0.368	Significant moderate positive correlation

Further analysis was conducted with demographic and clinicopathological features with Bonferroni correction $p<0.01$. As shown in Table 5, downregulated CCN6 was negatively associated with tumor grade. In contrast, the

elevation of NF-κBP65 was positively associated with tumor grade (rho=0.181, p=0.009). Furthermore, CA 15-3 levels correlated positively with tumor stage (rho=0.343, p=0.0001). Finally, the correlations analysis between the immunological parameters and both age and BMI revealed weak and negative associations, which were not statistically significant.

Table 5. Spearman correlation analysis between immunological biomarkers, pathological and histological features, and age*

Variables	Spearman Correlation (r)	p	95% CI		Interpretation
			Lower	Upper	
CCN6 vs. age	-0.081 ↓	0.55	-0.320	0.179	No correlation/No significant association
CCN6 vs BMI	0.205 ↑	0.13	-0.065	0.467	Weak positive, non-significant correlation
CCN6 vs. tumor grade	-0.173 ↓	0.049	-0.339	0.005	No correlation/No significant association
VEGF-A vs. age	-0.136 ↓	0.31	-0.438	0.166	Weak negative, non-significant correlation
VEGF-A vs. BMI	0.049 ↓	0.72	-0.245	0.323	No correlation/No significant association
VEGF-A vs. tumor grade	0.179 ↑	0.042	0.002	0.345	No correlation/No significant association
NF-κB p65 vs. age	-0.155 ↓	0.26	-0.411	0.118	Weak negative, non-significant correlation
NF-κB p65 vs. BMI	-0.088 ↓	0.52	-0.347	0.175	No correlation/No significant association
NF-κB p65 vs. tumor grade	0.181 ↑	0.009Δ	0.036	0.375	Significant weak positive correlation
CA15-3 vs. age	-0.051 ↓	0.71	-0.33	0.233	No correlation/No significant association

CA15-3 vs. BMI	-0.013 ↓	0.93	-0.28	0.282	No correlation/No significant association
CA15-3 vs. tumor stage	0.343 ↑	0.0001$^{\Delta}$	0.176	0.490	Significant moderate positive correlation

* $^{\Delta}$ – Correlation is significant at the 0.01 level (2-tailed)

Receiver operating characteristic of biomarkers' diagnostic performance

The receiver operating characteristic (ROC) curve technique was employed to evaluate the diagnostic potential of serum biomarkers. As illustrated in the individual ROC curves (Fig. 5 and Table 6), CCN6 exhibited the highest value (AUC=0.995, p=0.0001). With a cutoff of 9.5 µg/L (Table 7), CCN6 achieved 96% sensitivity and 100% specificity, with 98.1% TAR and 1.9% TMR, outperforming the traditional conventional biomarker CA 15-3 (AUC=0.855) and NF-κBP65 (AUC=0.813). At cut-off of 18.32 U/mL, CA15-3 had a sensitivity of 69.2%, a specificity of 90%, a 79.4% TAR, and a misclassification rate (TMR) of 20.6%. While NF-κB P65 (cut-off: 4.25 µg/L) demonstrated moderate levels of sensitivity and specificity at 75% and 80%, respectively, it resulted in a total agreement rate of 77.4% and a misclassification rate of 22.6%. VEGF-A has the lowest AUC of 0.558; with a cut-off value of 126.8 ng/L, VEGF-A exhibited low sensitivity (50%) but moderate specificity (78%), resulting in an undesirable TAR (63.21%). Internal validation by bootstrapping was conducted to assess the stability of the model; the bootstrap-corrected AUC of CCN6 remains consistent at 0.995 (95% CI: 0.990–1.00), minimizing the likelihood of overfitting.

Table 6. Receiver operating characteristic analysis for biomarkers' diagnostic performance*

Diagnostic Biomarkers	**AUC**	**Std. Error**	**p**	**95% Confidence interval**	
				Lower bond	**Upper bound**
CCN6 (µg/L)	0.995[a]	0.007	0.0001**	0.982	1
VEGF-A (ng/L)	0.558	0.056	0.321	0.444	0.672
NF-κBP65 (µg/L)	0.813[c]	0.041	0.0001**	0.732	0.894
CA15-3 (U/mL)	0.855[b]	0.038	0.0001**	0.795	0.935

* The null hypothesis true area is 0.5, ** – the p was significant at 0.01, [a] – highest AUC value, [b, c] – moderate AUC value, AUC – area under the curve

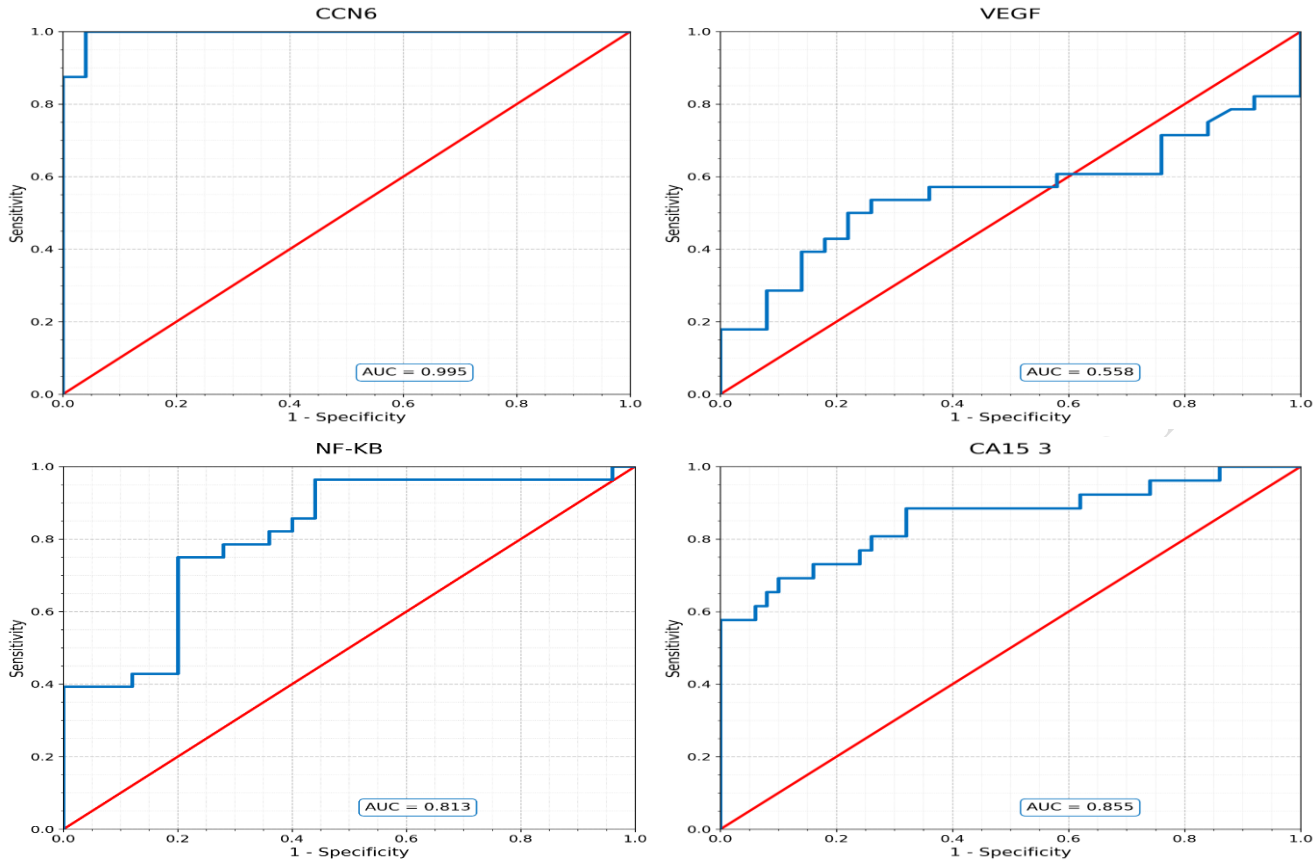

Fig. 5. ROC curve analysis for biomarkers' diagnostic performance

Table 7. Sensitivity, specificity, positive predictive value, negative predictive value, total agreement rate, and total misclassification rate of serum biomarkers for the diagnosis of breast cancer patients*

Diagnostic biomarkers	Optimal cut-off	Sensitivity %	Specificity %	TAR%	TMR%
CCN6 (µg/L)	9.5	96	100	98.1	1.9
VEGF-A (ng/L)	126.8	50	78	63.21	36.79
NF-κB p65 (µg/L)	4.25	75	80	77.4	22.6
CA-153 (U/mL)	18.32	69.2	90	79.4	20.6

* TAR – total agreement rate, TMR – total misclassification rate

Binary logistic regression model

Individual univariate logistic regression models were evaluated for each biomarker to examine their association with breast cancer presence. As shown in Table 8, part 1, the model revealed that CCN6 demonstrated the strongest association with the outcome, explaining 95.4% of the variance (χ^2=130, p<0.001, R^2_N=0.954) with a low information criterion (AIC=20.9, BIC=26.2). The multivariate binary logistic regression model was conducted to assess the

predictive power of the biomarkers. Both NF-κB P65 and CA15-3 also demonstrated significant model fit ($R^2N = 0.407$ and $R^2N = 0.534$, respectively). Conversely, VEGF-A failed to establish a significant individual fit ($R^2N = 0.016$).

The multivariate model was subsequently conducted to evaluate the combined association of the biomarkers, which excluded CA 15-3, as shown in Table 8, Part 2. The overall multivariate model demonstrated excellent data fit, explaining 95.4% of the variance in the outcome (R^2_{McF}=0.907, R^2_N=0.954). The overall test confirmed that the combined model was highly significant (χ^2=133, df=3, p<0.001). The multivariable model yielded superior information criteria scores (AIC=21.6, BIC=32.3) compared to the individual model test. Within this final model, CCN6 maintained its significant inverse association with the outcome (B=-0.834, adjusted OR=0.434, 95% CI=0.243–0.755, p=0.005). In contrast, both NF-κB p65 and VEGF-A results were not significant in the multivariate setup (p>0.2).

Table 8. Binary logistic regression analysis and model fit diagnostics of tumor-related factors associated with breast cancer status*

Part 1. Models fit measures

	Univariate model fit				Overall model test		
Predictor	**AIC**	**BIC**	**R^2_{McF}**	**R^2_N**	**χ^2**	**df**	**p**
CCN6 (µg/L)	20.9	26.2	0.88	0.954	130	1	0.0001
VEGF-A (ng/L)	149	155	0.0084	0.016	1.24	1	0.266
NF-κB p65 (µg/L)	112	117	0.236	0.407	38.5	1	0.0001
CA-153 (U/mL)	96.4	102	0.370	0.534	54.2	1	0.0001
Overall multivariate model fit*							
	21.6	32.3	0.907	0.954	133	3	0.0001

Part 2. Model coefficients

Predictor	**Univariate coefficients**			**Multivariate coefficients**		
	B	**Adjusted OR (95% CI)**	**p**	**B**	**OR (95% CI)**	**p**
CCN6 (µg/L)	-0.821	0.440 (0.276–0.702)	0.0001	-0.834	0.434 (0.243–0.755)	0.005
VEGF-A (ng/L)	0.0034	1.003 (0.997–1.01)	0.276	0.0068	1.007 (0.990–1.024)	0.427
NF-κB p65 (µg/L)	0.552	1.737 (1.336–2.21)	0.0001	0.37	1.454 (0.743–2.244)	0.274
CA-153 (U/mL)	0.175	1.19 (1.001–1.29)	0.0001	-------	-------	
Constant				3.505	33.294 (0.167–6654)	0.195

* CA 15-3 was excluded from the multivariate model. Note: models estimated using a sample size of 106 (56 untreated patients and 50 healthy controls), AIC – Akaike Information Criterion, BIC – Bayesian Information Criterion, R^2McF – McFadden's R-squared, R^2N – Negelkerke R-squared, χ^2 – Chi-squared, df – degree of freedom, B – log odds coefficients, OR – odds ratio, CI – confidence interval

Discussion

Breast cancer is known to be the most prevalent form of cancer and one of the leading causes of cancer-related mortality among women. This necessitates the identification of precise biomarkers that could help in early detection and serve as potential targets for restoration therapy. While the traditional biomarkers, like CA15-3, remain clinical staples, their limited sensitivity and specificity in early-stage disease emphasize the necessity of a more comprehensive molecular panel. This study provides a multifaceted evaluation of CCN6, VEGF-A, NF-κBp65, and CA 15-3, illustrating their significant roles in the systemic landscape of breast cancer and their potential as promising diagnostic tools.

A pivotal finding of this study is the drastic downregulation of CCN6 in breast cancer patients, which exhibited a remarkably large effect size. As this matricellular protein is essential for maintaining epithelial integrity, its loss is associated with epithelial-to-mesenchymal transition (EMT) and tumor invasion.[17] Our data align with the significant negative correlation between CCN6 expression and aggressive features, such as tumor grade, the Ki-67 proliferation index, and CA 15-3 levels. Additionally, the significant inverse correlation between CCN6 and the pro-tumorigenic factor NF-κBp65 suggests that reduced CCN6 levels may be associated with increased activation of the NF-κB pathway, potentially reflecting the antagonistic regulatory role of CCN6 in modulating inflammatory and proliferative pathways.[18]

The biomarker profiles in the treated patients were not expected to normalize to healthy control baselines. The persistence of inflammatory (NF-κB p65) and tumor burden (CA 15-3) markers may reflect the complex interplay of the pharmacological and microenvironmental factors rather than active disease. Systemic chemotherapy has been shown to induce off-target cytotoxicity and systemic inflammation, which may sustain NF-κB activation. Furthermore, the massive therapy-induced cellular apoptosis and tissue remodeling may continuously shed the intracellular signaling proteins into the bloodstream.[19]

The most compelling finding of this study is the exceptional diagnostic performance of CCN6. These findings suggest that CCN6 may serve as a robust diagnostic biomarker, but they should be interpreted critically. The diagnostic accuracy may be subject to overestimation due to single-center design and the inherent characteristics of a cross-sectional case-control cohort, which can produce artificial disease prevalence and potential overfitting. These findings are considered preliminary and require rigorous validation in larger multicenter external cohorts. Consequently, the large negative regression coefficient suggests that further large-scale multicenter prospective histological studies are needed to understand the molecular role of CCN6 in the tumor microenvironment.

VEGF-A is an important pro-angiogenic factor, which promotes vascular growth and permeability, facilitating tumor expansion and metastases.[9] In the present study, serum VEGF-A levels were elevated in the untreated group and exhibited

a recent correlation with tumor grade, suggesting its potential role as a disease-promoting factor, consistent with a previous study.[20] Furthermore, the positive correlation between VEGF-A and CA15-3 may reflect a concurrent advancement of VEGF-A-mediated neovascularization and tumor burden (reflected by CA15-3) during tumor progression.[21]This is practically relevant under hypoxic conditions, where high VEGF-A expression may promote tumor cell survival and correlate well with the microvessel density and adverse clinical outcomes.[22] However, despite its biological importance, our findings underscore the limited sensitivity and diagnostic utility of VEGF-A due to its non-specific elevation in various inflammatory and non-malignant conditions. In opposition to our findings, a previous study by Zajkowska et al. identified VEGF-A as a highly promising candidate for cancer diagnosis (particularly in the early stages).[23]

NF-κB is a master transcriptional factor in inflammation and cell survival, facilitating tumor growth by promoting cell proliferation, angiogenesis, and resistance to apoptosis.[8] The elevation of NF-κBp65 in untreated patients may reflect its association with tumor survival and metastatic potential. This is further evidenced by the positive association between NF-κBp65 and tumor grade, corroborating the notion that its activation is associated with increased disease severity.[24] Furthermore, the high odds ratios (OR) for NF-κBp65 suggest a central role in the inflammatory and angiogenic signaling pathways essential for tumor initiation and progression. Its modest diagnostic accuracy and strong association with cancer risk (OR) suggest it may act as a risk-promoting factor in carcinogenesis. These findings align with a recent study by Barnes et al., who reported the association of NF-κB with tumor grade and its high diagnostic accuracy by breast cancer molecular subtypes.[25]

Moderate diagnostic performance and weaker risk association (OR) were observed for CA15-3, consistent with Sekacheva et al.'s study[26] who mentioned that while CA15-3 lacks sufficient sensitivity for early diagnosis, it remains useful in monitoring disease relapses and treatment response. Suggesting that CA15-3 mirrors the tumor burden, rather than the early molecular events of carcinogenesis. Despite the heterogeneity of the treated group (2-12 years post-treatment). The treated subgroup analysis yielded no significant variations of CCN6, VEGF-A, and NF-κB p65 between the recent and long-term remissions. This lack of significant association indicates that these biomarkers may reach a sustained biological plateau following therapeutic interventions.

Notably, differences in the menopausal status between treated and untreated patients were identified; the treated group consisted primarily of peri-menopausal women, while the untreated group had a more even distribution of pre-menopausal and post-menopausal women. These findings suggest a potential interaction between metabolic and hormonal elements in the progression of breast cancer. Consequently, breast cancer is more common in aggressive presentations in younger patients, leading to worse outcomes and aggressive subtypes, including triple-negative breast cancer.[27]

The frequency of poor (grade III) and moderate (grade II) differentiation is consistent with clinical guidelines showing low 10-year survival for high-grade tumors compared to low-grade tumors.[28] The prognosis depends on the treatment modality. Multimodal treatment, which includes surgery, chemotherapy, radiotherapy, and hormonal therapy, remains the gold standard, leading to significant improvements in patients' overall survival. Studies nowadays are focused on the molecular basis of breast cancer so that novel diagnostic and treatment approaches can be applied to achieve better patient control and conquer drug resistance-related mechanisms.[29]

The analysis revealed reduced levels of RBCs, Hb, and HCT in breast cancer patients, which are consistent with anemia of chronic disease or myelosuppression due to chemotherapy. This finding confirms the study by Muthanna et al.[30] The elevated WBCs and neutrophils with low lymphocytes and LMR are indicative of systemic inflammation associated with active diseases. A previous study found that low NLR is associated with a good pathological response after neoadjuvant chemotherapy.[31] The stage-wise analysis of biomarkers in untreated and treated patients provides additional nuance. Serum CCN6 levels in untreated patients exhibited stage-dependent variability, peaking in stage IV. This re-emergence in advanced metastatic disease may represent a compensatory cellular response to vigorous extracellular matrix remodeling or a systemic release during tumor-associated necrosis. Concurrently, progressive elevation of VEGF-A, NF-κBp65, and CA 15-3 implies increased angiogenic activity and inflammatory activity, as well as tumor proliferation. An increase in VEGF-A is associated with its established role in facilitating neovascularization that is required for metastatic spread.[32] Its upregulation during the different stages of tumor progression suggests that it might thus be an intriguing candidate for tumor progression monitoring or anti-angiogenic treatment response assessment. These findings are consistent with previous reports by Al-Rubaye et al., who reported that VEGF-A levels were significantly high in advanced-stage breast cancer.[33]

The stepwise increase in CA 15-3 levels with advancing stage aligns with its well-known function as a marker for tumor burden and metastasis, corroborating the observation of a recent study by Li et al., where rising CA 15-3 concentrations characterize advanced and metastatic breast cancer, which is associated with poor prognosis.[34] This is further evidenced by the significant positive correlation of CA 15-3 levels with tumor stage.[34] Treated patients had a decline in serum CCN6, VEGF-A, and NF-κB P65. Their stage-specific expression suggests a biological regulation of angiogenic and inflammatory pathways. Specifically, pharmacological inhibition of NF-κB P65 may reduce VEGF expression, thereby potentially inhibiting pathological vascularization.[8,35] However, serum levels of CA 15-3 remained elevated, which coincides with Kim et al.'s previous study, who observed that this marker may remain elevated after treatment due to residual tumor activity.[36] Collectively, our findings underscore a significant association between the immunological biomarkers, reflecting a molecular profile where CCN6 depletion coincides with NF-κB and VEGF-A elevation, suggesting a coordinated expression pattern linked to inflammation and angiogenesis. Therefore, their tracking as a multi-biomarker panel could enhance disease staging accuracy and even improve prognostic determination in breast cancer treatment.

The lack of significant correlations of age and BMI with the biomarkers suggests that immunological and angiogenic alterations observed are disease-driven rather than age or weight-dependent. It has also been established that VEGF-A and CCN proteins are more influenced by disease biology.[22,37] NF-kB has been related to inflammatory pathways associated with aging, but primarily chronic diseases, rather than normal aging.[38] Reports indicate that CA 15-3 levels do not depend on age or reproductive factors among normal women, as well as patients with breast cancer.[39]

This study possesses several strengths, including its novel focus on CCN6, a poorly investigated member of the CCN protein family that could serve as a promising novel biomarker for breast cancer. The simultaneous study of CCN6 with VEGF-A, NF-κB p65, and the classic marker CA15-3 offers a comprehensive molecular and clinical perspective.

Study limitations

Nevertheless, the study has several limitations. First, the moderate sample size and single-center execution, combined with the absence of an external validation set, restrict the generalizability of the results. Second, the lack of benign breast disease control groups limits the ability to fully evaluate biomarker specificity. Third, our study design precludes the evaluation of long-term clinical outcomes, such as survival and recurrence rates, and prevents the calculation of real-world predictive values (PPV, NPV). Finally, the heterogeneity of the treated group may introduce a confounding variable when evaluating the post-treatment biomarker levels. We suggest subsequent multicenter prospective validation studies involving larger populations to corroborate and extend these findings.

Conclusion

This study highlights the roles of the CCN6, NF-κB p65, VEGF-A, and CA15-3 proteins as potential noninvasive diagnostic tools. The sensitivity and specificity of CCN6 are superior to those of other biomarkers for distinguishing patients with breast cancer from healthy controls, whereas the oncogenic role of NF-κBp65 in chronic inflammation has been elucidated. The inverse relationship between CCN6 expression and disease outcome aligns with the existing rationale that therapeutic restoration of CCN6 expression could inhibit epithelial–mesenchymal transition (EMT)-induced invasion. However, these findings do not imply immediate clinical applicability. Rigorous validation in larger multicenter prospective studies is warranted to translate this outcome into clinical practice.

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Declarations

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Author contributions

Conceptualization, M.M. and E.T.; Methodology, M.M. and E.T.; Software, M.M.; Validation, M.M. and E.T.; Formal Analysis, M.M. and E.T.; Investigation, M.M. and E.T.; Resources, M.M. and E.T.; Data Curation, M.M.; Writing – Original Draft Preparation, M.M., E.T., and M.A.; Writing – Review & Editing, M.M., E.T., and M.A.; Visualization, M.M.; Supervision, E.T.; Project Administration, E.T.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The dataset analyzed during the current study is available from the corresponding author on reasonable request.

Ethics approval

The study protocol received approval from the Research Committee of the Training and Human Development Center, Basra Health Department, Ministry of Health (Resolution No. 629, October 8, 2024).

Use of AI and AI-assisted technologies in the writing process

No AI or AI-assisted tool was used in the preparation of the manuscript.

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