Original Article

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Association of Matricellular Protein, Growth Factor, and Transcription Factor with Molecular Subtypes of Breast Cancer: A Case Control Study

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ABSTRACT

Background: Breast cancer is a heterogeneous disease comprising molecular subtypes such as Luminal A, Luminal B, Human Epidermal Growth Factor 2 (HER-2)-Enriched, and Triple Negative, each with distinct clinical, molecular, and immunological features. Immunological markers play a critical role in modulating tumor behavior, angiogenesis, and immune evasion. **Aim**: This study aimed to identify key immunological markers that correlate with the molecular (intrinsic) subtypes of breast cancer and to explore their potential as prognostic factors. **Methods**: Serum samples from 180 breast cancer patients (56 untreated and 74 treated) were analyzed using Enzyme-Linked Immunosorbent Assay (ELISA) to determine the levels of four immunological biomarkers, including Cellular Communication Network Factor 6/WNT 1 Inducible Signaling Pathway Protein (CCN6/WISP3), Vascular Endothelial Growth Factor A (VEGF-A), Nuclear Factor Kappa B (NF-κB p65), and Cancer Antigen CA15-3. Demographic data and clinical parameters were collected using questionnaires. **Results**: Compared to the controls, patients with breast cancer exhibited significantly elevated levels of VEGF-A, NF-κB p65, and CA15-3, alongside decreased CCN6 levels ($P < 0.05$). At the molecular subtype level, HER2-enriched tumors showed the highest concentrations of CCN6 and VEGF-A, while elevated NF-κB p65 and CA15-3 levels were noted in Luminal B tumors, indicating the lowest CCN6 concentration in triple-negative tumors. **Conclusions**: This study demonstrates the roles of CCN6, VEGF, NF-κB p65, and CA15-3 as reliable biomarkers for distinguishing between molecular subtypes and suggests their potential as prognostic indicators.

Keywords: Breast cancer, molecular subtypes, CCN6, VEGF-A, NF-κB, P65, CA 15-3.

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INTRODUCTION

Breast cancer remains one of the most commonly diagnosed malignancies in women, accounting for approximately 11.6% of all newly diagnosed cancer cases worldwide. It affects more than 2.4 million women annually, underscoring its significant impact on global health[1]. Its incidence is increasing globally, particularly in industrialized countries, which account for nearly half of all cases of this disease. Breast cancer develops through

the interaction of multiple internal and external factors that influence its initiation and progression[2,3] .Breast cancer is categorized into four primary molecular subtypes: Luminal A, Luminal B, HER2-enriched, and triple-negative[4] .Based on the expression of estrogen and progesterone receptors, HER-2 status, and the Ki-67 proliferation index, as determined by immunohistochemical analysis, each subtype exhibits distinct behaviors, treatment responses, and prognoses[5].More widely recognized immunological markers, such as Cellular Communication Network Factor 6 (CCN6), Vascular Endothelial Growth Factor A (VEGF-A), Nuclear Factor Kappa B (NF-κB p65), and Cancer Antigen 15-3 (CA 15-3), are central to delineating tumor subtypes. Biomarkers play a role in regulating tumor interactions with the immune system, promoting the growth of blood vessels around cancer cells, facilitating cell movement into other regions of the body, and accelerating disease development. Recent studies aim to elucidate how elements of the CCN group shape breast cancer development and progression[6]. CCN6 is a member of the CCN matricellular proteins. WISP-3/CCN6[7] is the last member of the WNT-inducible signaling pathway subfamily, which comprises four distinct structural elements: an N-terminal signal sequence linked to an insulin-like growth factor binding protein (IGFBP) domain, a Von Willebrand Factor type C (vWC) segment, a thrombospondin type 1 (TSP-1) region, and a C-terminal cysteine knot (CT) repeat at its carboxy terminus[6] .Research shows CCN6 inhibits tumor growth, migration, and the spread of breast cancer; it is associated with Epithelial-Mesenchymal Transition (EMT) events that increase malignancy, particularly in Triple Negative Breast Cancer (TNBC) cases[8,9] .Vascular Endothelial Growth Factor A (VEGF-A) is a key member of the VEGF family and a major regulator of angiogenesis and vasculogenesis. It is strongly expressed across numerous cancer types and is closely associated with tumor growth and disease progression[10] .Nuclear factor kappa B p65 (NF-κB p65) is also a central player in communication between tumors and immune effector cells, activating multiple cancer-promoting pathways of cell growth, survival, epithelial–mesenchymal transition, invasion, angiogenesis, and metastasis[11] .Additionally, NF-κB activation contributes to resistance to endocrine and chemotherapeutic interventions, presenting[12] a formidable challenge in controlling advanced and metastatic cancer[13] .Cancer Antigen 15-3 (CA15-3) is a breast cancer cell-derived glycoprotein known to be a sensitive prognostic indicator[14] .A positive elevation of CA15-3 is useful for diagnosing breast cancer, predicting relapse, and evaluating therapeutic success and patient responsiveness[15] .Despite advances in detection and treatment, disease progression and therapeutic resistance remain major challenges. However, the variable response to targeted therapies highlights the need for new molecular biomarkers. CCN6 (WISP3) has been proposed as a tumor suppressor, and its downregulation may enhance NF-κB p65 and VEGF-A activity, promoting inflammation, angiogenesis, and metastasis. The limited data on CCN6 in breast cancer underscores the importance of investigating its potential diagnostic and prognostic value. Therefore, the current study aims to evaluate CCN6, NF-κB p65, and VEGF-A expression in relation to molecular subtypes and seeks to assess the potential diagnostic and prognostic value of these biomarkers, which may contribute to insights into their diagnostic and prognostic significance.

MATERIALS AND METHODS

This case-control retrospective study, conducted between October 2024 and June 2025, recruited a total of 180 women aged between 23 and 80 years. Of these, 130 were confirmed to have breast cancer after consultation with an oncologist during their visits to the Oncology Center of Al-Sadr Teaching Hospital in Basra City, south of Iraq. The study included 180 participants divided into three main groups: the untreated group comprised 56 newly diagnosed female breast cancer patients prior to any treatment (ranging from 1 to 12 months), regardless of stage or tumor grade. The treated group included 74 patients, some of whom were continuing treatment while others had completed their treatment (ranging from 2 to 15 years) with various protocols based on cancer stage, lymph node involvement, and tumor grade. These protocols encompassed radiotherapy, chemotherapy (using agents such as doxorubicin, cyclophosphamide, docetaxel, paclitaxel, and carboplatin), hormonal therapy for hormone-positive breast cancer (using tamoxifen, letrozole, or anastrozole depending on menopausal status), or HER2-targeted therapy with trastuzumab in combination with chemotherapy if HER2 was positive. The third group, the healthy controls, consisted of 50 female hospital staff and community volunteers aged 23-60 years without autoimmune, chronic, or genetic diseases or sickle cell anemia. Excluded patients included

males, pregnant women, patients with psychiatric or cognitive disorders, and those aged < 25 years (Fig. 1). Questionnaire-based interviews with participants were employed to collect anthropometric, pathological, and laboratory data from patients' files and the laboratory electronic system at the Oncology Center of Al-Sadr Teaching Hospital. The information encompassed the demographic and anthropometric data of participants: age, height, and weight to calculate the Body Mass Index (BMI) for the participants. The body mass index (BMI) of each participant was computed according to the World Health Organization (WHO) criteria by dividing weight (kilograms) by height (meters) squared. Participants were categorized by WHO into four groups: underweight (BMI < 18.5 kg/m²), normal (BMI 18.5-24.9 kg/m²), overweight (BMI 25-29.9 kg/m²), and obese (BMI ≥ 30 kg/m²)[16] . These BMI categories were used during the statistical analysis to classify participants and evaluate the possible association between BMI and serum levels of the studied biomarkers (CCN6, VEGF-A, and NF-κB p65) among study participants. Laboratory evaluations comprised a complete blood count (CBC), assessments of liver and kidney function, and CA15-3 tests. The serum concentrations of CCN6, VEGF-A, and NF-κB p65 in all female participants were measured using Enzyme-Linked Immunosorbent Assay (ELISA) kits, adhering to the manufacturers' guidelines (BT Lab, China, for CCN6; Elabscience, USA, for VEGF-A and NF-κB p65). The study was granted ethical approval by the Research Committee of the Training and Human Development Center, Basrah Health Department, Ministry of Health (resolution NO 629, October 20, 2024). Written informed consent was obtained from all participants. Data were processed using IBM SPSS Statistics version 31 (SPSS Inc., Chicago, IL, USA), and figures were prepared using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA). Normality of continuous variables was assessed by employing the Shapiro–Wilk and Kolmogorov–Smirnov tests, while Levene's test was employed to test equal variances across groups. Continuous data are presented as mean ± standard deviation (SD), and categorical variables are listed frequencies and by percentages. Independent samples t-tests were performed for normally distributed data for group comparisons, while Mann–Whitney U tests were used for non-normally distributed data for group comparisons. One-way Analysis of Variance (ANOVA), Kruskal–Wallis tests, Tukey's HSD, and Dunn's post hoc test were performed during the analysis of more than two groups if a significant difference was found. Spearman's rank order correlation coefficient was utilized to study associations between continuous and categorical variables. A p-value of less than 0.05 was considered statistically significant, and all tests were two-tailed.

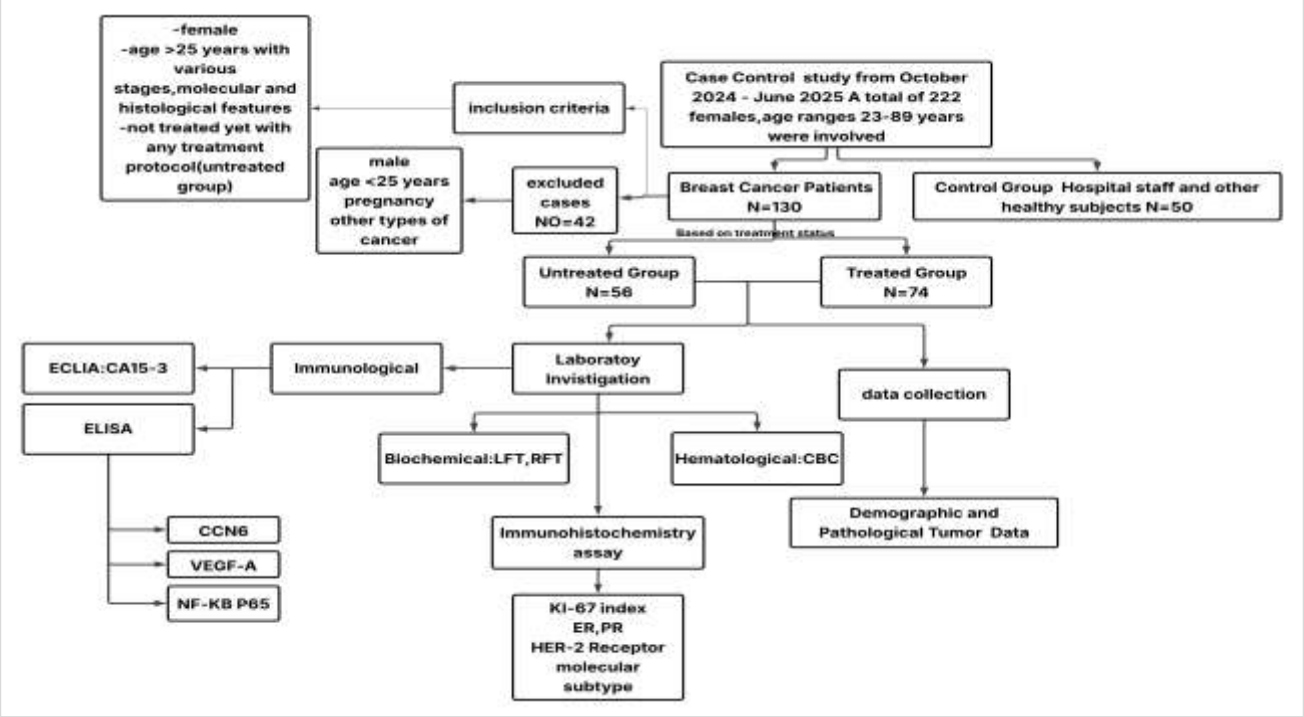

Figure 1: Flowchart of the study

RESULTS

Comparative analysis of demographic, hematological, biochemical, and serum immune markers in female breast cancer patients versus healthy controls: Overall, of the 180 participants (130 breast cancer patients and 50 healthy women as the control group, as shown in Table (1)), mean differences were significant (P≤0.05) between the breast cancer group and the control group. Breast cancer patients had higher age and BMI (51.8±11.8 and 30.55±5.8, respectively) than healthy controls. Hematological parameters, such as hemoglobin (HB), Red Blood Cells (RBCs) count, and hematocrit (HCT), were significantly lower in breast cancer patients. Inflammatory markers, such as neutrophil count, monocyte count, and neutrophil-to-lymphocyte ratio (NLR), were elevated in patients, whereas lymphocyte count and lymphocyte-to-monocyte ratio (LMR) were decreased. NF-κB p65 levels were notably elevated in breast cancer patients, with serum levels of VEGF-A and NF-κB p65 measuring 145.22±71.76 and 6.19±4.07, respectively, compared to healthy individuals, who had levels of 143.5±30.29 and 2.78±1.87, respectively (P=0.01, 0.0001). Conversely, CCN6 serum levels were significantly higher in healthy controls (19.92 ± 6.92) than in those with breast cancer (2.92±0.24, P=0.0001). The association of serum level biomarkers with different molecular subtypes in the untreated and treated groups: Figures 2 and 3 depict the association of serum level biomarkers with different molecular subtypes in the untreated and treated groups, respectively. In the untreated group, the HER-2-enriched subtype showed the highest mean CCN6 level (3.76±2.01), while the triple-negative subtype demonstrated the lowest (1.20±0.13). VEGF-A concentration was highest in the HER-2-enriched subtype (201±98) and lowest in the Luminal B subtype (150±45). The Luminal B subtype presented the highest NF-κB p65 levels (8.08±4.16), while HER-2-enriched had the lowest (5.05±1.48). CA 15-3 levels were higher in the Luminal B subtype (77.91±23) than in other subtypes (Figure 2). In the treated group (Figure 2), the Luminal B subtype showed the highest mean CCN6 level (4.29±0.86). VEGF-A and CA 15-3 levels were elevated in the HER-2-enriched subtype (163.3±77.9 and 52.7±9.2, respectively). NF-κB p65 levels were significantly higher (P<0.05) in the Luminal A subtype than in the other subtypes (2.84±2.07). Association between Ki-67 expression and molecular subtypes in the untreated and treated groups: There was a significant difference in Ki-67 expression among the molecular subtypes (P<0.05), as illustrated in Figure 4. The highest expression in the untreated group was in the Luminal B subtype (46.18±10.06), while the Luminal A subtype showed the lowest expression (8.7±4.45). Similarly, in the treated group, the Luminal A subtype had the lowest Ki-67 expression (11.5±4.7), while the triple-negative subtype was associated with the highest Ki-67 proliferation index (51.3±16.15). Association of serum CCN6, NF-κB p65, VEGF-A, and CA15-3 levels with Ki-67 expression in the untreated group: The relationship between Ki-67 expression and serum biomarker concentrations is presented in Table (2). For CCN6, the mean levels exhibited a slight increase with elevated Ki-67 expression. VEGF-A demonstrated a nonlinear relationship with a significant difference (P=0.0483) among the Ki-67% expression groups (the lowest mean concentration (112±38.06) was observed in the intermediate 15-30% Ki-67% expression group, while higher levels were observed in the high and low Ki-67% expression groups (169.6±58.44 and 179.2±75, respectively). NF-κB p65 and CA 15-3 levels did not vary significantly among the different Ki-67% groups. Correlation analysis between immunological biomarkers, histological characteristics, and age in patients with breast cancer: The analysis revealed significant correlations across several parameters (Table 3). CCN6 showed a negative correlation with the Luminal B subtype (rho=-0.270, P=0.044), while it positively correlated with the HER-2-enriched subtype and HER-2 status (rho=0.307, P=0.021 and rho=0.398, P=0.002, respectively). VEGF-A levels were positively correlated with triple-negative breast cancer (rho=0.2056, P=0.019). Additionally, NF-κB p65 exhibited significant positive correlations with estrogen receptor (rho=0.233, P=0.008), progesterone receptor (rho=0.336, P=0.0001), Luminal A subtype (rho=0.219, P=0.012), and triple negative subtype (rho=0.175, P=0.047). CA15-3 was positively correlated with HER-2 status (rho=236, P=0.007). Ki-67% was significantly correlated with multiple histopathological features, including a negative correlation with estrogen and progesterone receptors (rho=-0.287, P=0.001 and rho=-0.257, P=0.004, respectively). It also had a positive correlation with HER-2 status (rho=0.224, P=0.012). In terms of molecular subtypes, Ki-67% showed a significant negative correlation with the Luminal A subtype (rho=-0.619, P=0.0001) and a positive correlation with subtypes with poor prognosis, including Luminal B and Triple-Negative

subtypes (rho=0.470, P=0.0001 and rho=0.207, P=0.018, respectively). Correlation analysis between parameters and age revealed weak, non-significant negative associations: CCN6 (rho = -0.183, p = 0.353), VEGF-A (rho = -0.074, p = 0.707), NF-κB p65 (rho = -0.166, p = 0.400), and CA 15-3 (rho = -0.026, p = 0.894). None were statistically significant.

Table 1: Comparative analysis of demographic, hematological, biochemical, and serum immunological biomarkers in female breast cancer patients versus healthy controls.

Parameter	Female with Breast Cancer N=130	Healthy Control N=50	P-Value
Age (years)	51.8±11.8	32.9±9.23	0.0001
BMI (kg/m²)	30.55±5.8	26.23±3.7	0.0001
HB (g/dL)	11.7±1.9	12.6±1.03	0.001
RBC x 10^6 /µl	4.24±0.68	4.53±0.38	0.003
HCT (%)	35.5±5.55	37.8±2.42	0.005
MCV (fL)	82.2±13.04	83.61±4.28	0.901
MCHC (g/dL)	32.9±1.46	33.32±1.69	0.020
WBC x 10^3/µl	6.49±2.05	6.04±1.57	0.132
NEU x10^3/µl	3.98±1.58	3.37±1.27	0.028
LYM x10^3/µl	1.81±0.71	2.06±0.64	0.016
MONO x 10^3/µl	0.55±0.32	0.42±0.16	0.004
LMR	4.08±3.64	5.6±2.54	0.0001
NLR	2.51±1.66	1.8±0.96	0.001
PLT x 10^3/µl	266.71±93.28	268.1±54.17	0.504
ALP (U/L)	105.43±29.97	69.34±23.6	0.0001
AST (U/L)	41.65±19.98	20.22±9.74	0.061
ALT (U/L)	38.82±18.71	18.46±10.54	0.062
S. Cr (mg/dL)	0.80±0.66	0.59±0.30	0.646
Urea (mg/dL)	24.84±9.94	23.68±5.87	0.145
CCN6 (ng/mL)	2.92±0.24	19.92±6.92	0.0001
VEGF-A (ng/mL)	145.22±71.76	143.5±30.29	0.01
NF-κB P65 (ng/mL)	6.19±4.07	2.78±1.87	0.0001

BMI: Body Mass Index, 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, ALP: Alkaline Phosphatase Enzyme, ALT: Alanine Transferase Enzyme, AST: Aspartate Aminotransferase, S.Cr: Serum Creatinine, 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.

Table 2: Association of serum levels of CCN6, NF-κB p65, VEGF-A, and CA15-3 with Ki-67 expression in the untreated group.

Ki-67 expression	CCN6	VEGF-A	NF-κB p65	CA15-3
< 15%	1.949±0.95	109.2±75.1	7.2±5.4	42.63±25.51
15-30%	1.96±1.45	112±38.06	7.65±5.02	20.82±8.04
>30%	2.22±1.386	169.6±58.44	6.5±4.65	60.38±43.62
P-value	0.95	0.0483	0.636	0.14

Statistical significance at P ≤ 0.05.
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, CA15-3.: Cancer Antigen 15-3. KI-67: Kiel (city) 67: number of the original clone.

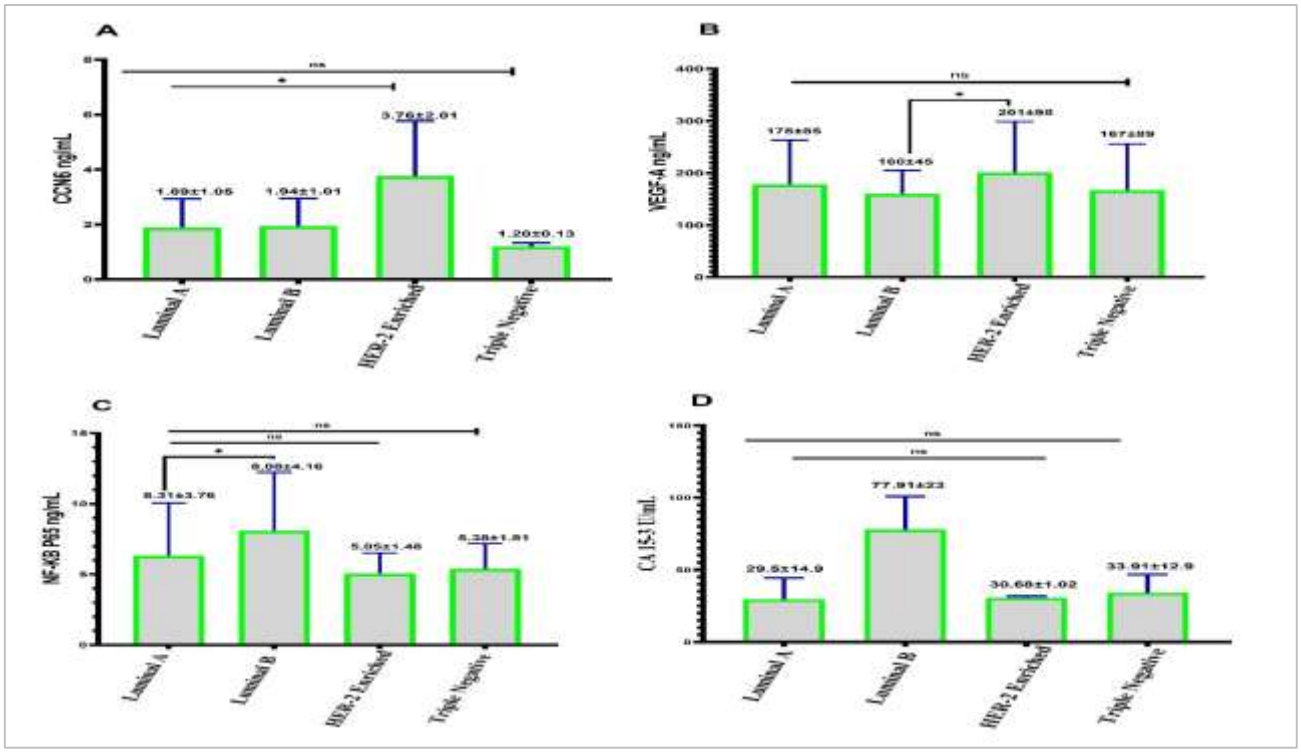

Figure 2: Association of serum levels of CCN6, NF-κB p65, VEGF-A, and CA15-3 with molecular subtypes in the untreated group.
A: CCN6, B: VEGF-A, C: NF-κB P65, D: CA 15-3
CCN6: Human WNT1-inducible-signaling pathway protein 3,VEGF-A: Vascular Endothelial Growth Factor A, NF-κB p65:Nuclear factor NF-kappa-B p65 subunit, CA15-3:Cancer Antigen 15-3.

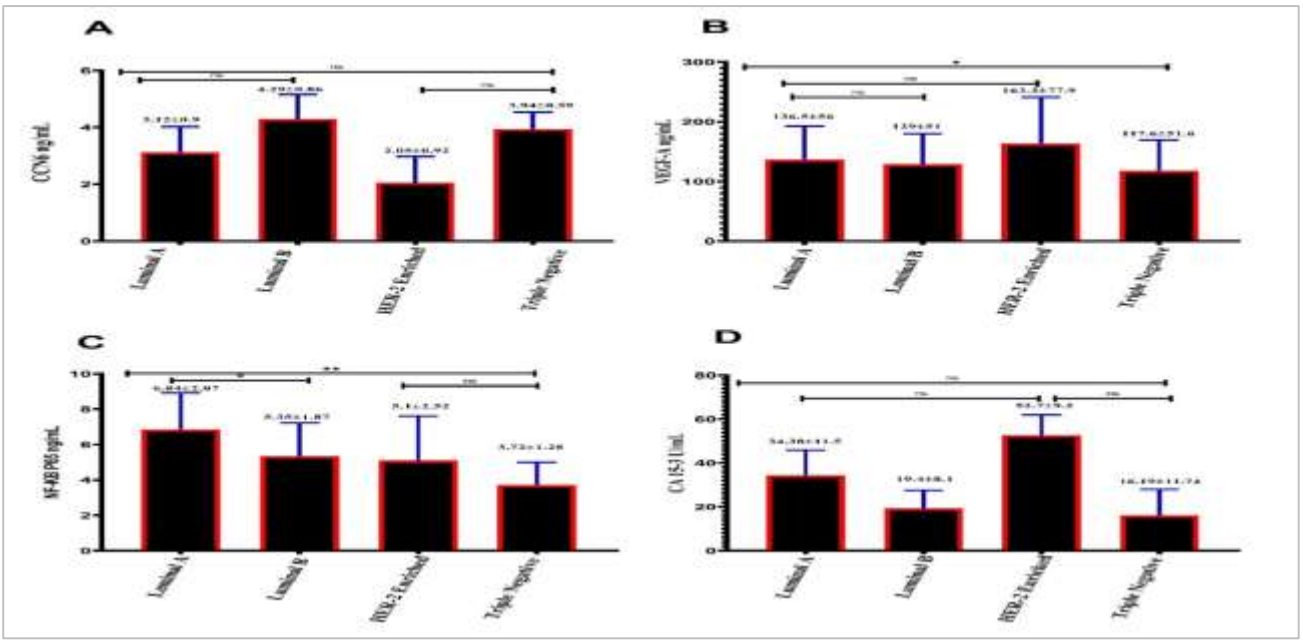

Figure 3: Association of serum levels of CCN6, NF-κB p65, VEGF-A, and CA15-3 with molecular subtypes in the treated group. A: CCN6, B: VEGF-A, C: NF-κB P65, D: CA 15-3
CCN6: Human WNT1-inducible-signaling pathway protein 3,VEGF-A: Vascular Endothelial Growth Factor A, NF-κB p65:Nuclear factor NF-kappa-B p65 subunit, CA15-3:Cancer Antigen 15-3.

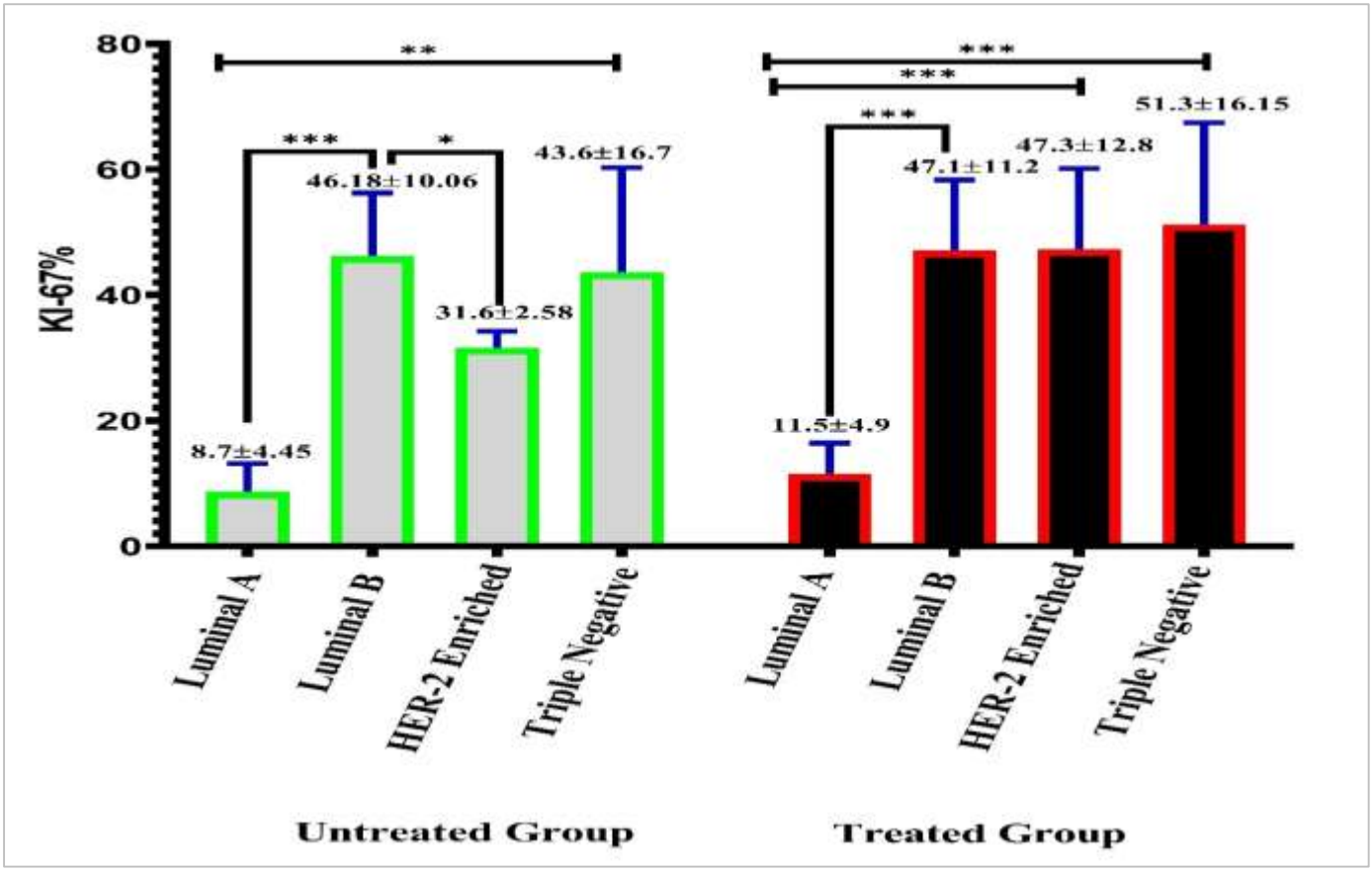

Figure 4: Association of Ki-67 expression with molecular subtypes in the untreated group.

Table 3: Assessment of spearman correlation between immunological biomarkers, molecular subtypes, and age**.**

Parameters	Spearman's rho	Significance	95% CI (2-tailed)	
			Lower	Upper
CCN6-Luminal B	-0.270	0.044	-0.504	0.0001
CCN6-HER-2 Enriched	0.307	0.021	0.040	0.533
VEGF-A-TNBC	0.205	0.019	0.029	0.368
NF-κB p65-Luminal A	0.219	0.012	0.044	0.381
NF-κB p65-TNBC	0.175	0.047	0.003	0.341
Ki-67%-Luminal A	-0.619	0.0001	-0.717	-0.426
Ki-67%-Luminal B	0.470	0.0001	0.319	0.597
Ki-67%-TNBC	0.207	0.018	0.031	0.371
ALP - CCN6	-0.494	0.008	0.137	0.737
ALP - NF-κB p65	-0.381	0.018	-0.630	-0.060
CCN6-Age	-0.183	0.353	-0.529	0.216
VEGF-A-Age	-0.074	0.707	-0.445	0.318
NF-κB p65-Age	-0.166	0.400	-0.516	0.232
CA 15-3-Age	-0.026	0.894	-0.405	0.360

Significant correlation at the 0.05 level (two-tailed).
At the 0.01 level (two-tailed), the correlations were strongly significant.
BMI: Body Mass Index, CCN6: Cellular Communication Network Factor 6, NF-κB p65: Nuclear factor NF-kappa-B p65 subunit, CA15-3: Cancer Antigen, HER: Human Epidermal Growth Factor, Ki-67: Kiel (city) 67 (number of original clones), TNBC: Triple-negative breast cancer.

DISCUSSION

This study is significant due to its emphasis on the relationship between the molecular subtypes of breast cancer and variations in the concentrations of CCN6, VEGF-A, and NF-κB p65. These biomarkers are crucial for understanding tumor biology and the mechanisms of progression. The reduction in CCN6 and elevation of VEGF-A and NF-κB p65 are linked to more aggressive subtypes, such as triple-negative and HER2-enriched breast cancer, highlighting their roles in promoting angiogenesis, inflammatory stimulation, and cancer cell metastasis. As a result, these biomarkers serve as essential diagnostic and prognostic instruments that help identify patients with a poor outlook and facilitate the development of targeted treatment approaches, ultimately improving clinical outcomes for individuals with breast cancer. A significant outcome of this study is that breast cancer patients exhibited significantly lower CCN6 levels compared to healthy controls, but significantly higher serum VEGF-A and NF-κB p65 levels. The elevated NF-κB p65 and VEGF-A levels align with previous studies [17],suggesting a pathophysiological mechanism where NF-κB p65 increases VEGF-A levels by translocating to the nucleus and activating VEGF-A gene transcription. Therefore, inhibiting NF-κB p65 pharmacologically or genetically reduces VEGF-A levels[18].Targeted therapies against VEGF-A and NF-κB have been developed for various diseases, including breast cancer[19,20]. The present data show that breast cancer patients, particularly postmenopausal women, tend to be older and have higher body mass indices (BMIs) compared to healthy controls, suggesting that obesity constitutes a substantial risk factor for breast cancer. This outcome aligns with the result of earlier study[21]. This association is linked to factors such as chronic inflammation and hormonal changes related to increased body fat, suggesting that a high BMI is a poor prognostic indicator. Conversely[22], the present data showed significantly lower levels of HB, RBC count, HCT, MCHC, and LMR, potentially indicative of cancer-associated bone marrow suppression and anemia[23].While neutrophil, monocyte counts, and NLR were consistent with some previous research[24], the findings regarding WBC counts differed. These hematological shifts are likely linked to tumor-secreted inflammatory cytokines, which can induce leukocytosis and anemia[25].The study results were surprising, as the serum liver parameters (AST and ALT) were consistent with recent clinical observations[26], where elevated ALP, rather than transaminases, correlates with higher cancer prevalence and poorer outcomes[26] .Increased ALP levels may result from Wnt/β-catenin pathway activation in liver and bone metastases, where β-catenin translocation stimulates ALP gene transcription[27] .Our study builds upon the findings of Jiang et al. and assumes a similar explanation. ALP and CCN6 are key biomarkers in breast cancer. Elevated ALP levels typically indicate bone or liver metastases resulting from disease progression. Conversely, CCN6, a tumor suppressor, exhibits decreased expression, correlating with increased tumor aggressiveness and metastasis, particularly in aggressive subtypes like Triple-Negative Breast Cancer (TNBC), raising the risk of early bone and liver metastasis. Thus, low CCN6 signals increased risk of metastasis, whereas high ALP levels indicate existing metastases, especially in the bone and liver, reflecting their indirect relationship with breast cancer progression. This is supported by our study's findings of an inverse relationship between ALP, CCN6, and NF-κB p65, where NF-κB p65 activation promotes bone metastasis via bone resorption, which is clinically reflected by elevated ALP levels. Renal function parameters (S.Cr,Urea) showed no significant differences, consistent with the findings of a previous study[28]. Furthermore, our results demonstrated elevated VEGF-A levels when the Ki-67 proliferation index exceeded 30%, consistent with Sharma et al. (2025), who identified a notable link between the genetic expression of VEGF-A and Ki-67 in breast cancer[29]. Similarly, Smolanka et al. (2023) reported 1.6-fold higher VEGF-A levels in inflammatory breast cancer patients with Ki-67 >25%[30] .This elevation may stem from hypoxia-inducible factor 1-alpha (HIF-1α) activation, which enhances VEGF-A transcription and promotes angiogenesis to support rapid tumor proliferation[31] .The lack of association between Ki-67 expression and NF-κB p65 serum level aligns with Al-Mutairi & Habashy (2022), whose histological study found no correlation between NF-κB p65 level and Ki-67 expression[32] .While many patients exhibit elevated CA15-3 levels with high Ki-67 expression, a direct correlation between the two is not consistently significant[33] .The analysis revealed that Ki-67 proliferation expression varied significantly among the four molecular subtypes, with Luminal B and triple-negative breast cancers revealing higher expression and positive correlation, whereas Luminal A showed the

lowest expression and negative correlation. This finding agrees with previous studies indicating that Luminal B and Triple-Negative tumors have higher proliferation rates and worse outcomes than Luminal A tumors[34].Interestingly, the results revealed that CCN6 expression was significantly reduced in triple-negative breast cancer in the untreated group. The existing literature suggests that CCN6 acts as a tumor suppressor and is frequently absent in aggressive breast cancer types, especially triple-negative breast cancer. Functionally, CCN6 loss promotes Wnt/β-catenin activation, a key pathway in Epithelial-Mesenchymal Transition (EMT), facilitating tumor invasion and metastasis[35]. CCN6 overexpression in the treated group may have resulted from treatment-induced downregulation of the Wnt/β-catenin pathway, reducing EMT and preventing further breast cancer cell invasion. Elevated serum CCN6 levels and a positive correlation with HER2-enriched breast cancer likely reflect its tumor-suppressive role. Increased serum CCN6 levels in the treated group compared to those in the untreated group suggest a restoration of its tumor-suppressive function. These changes were more pronounced in the luminal and triple-negative breast cancer subtypes, supporting the treatment-resistant nature of certain molecular subtypes. VEGF-A serum levels were elevated in HER2-enriched breast cancer, consistent with the findings of previous studies. For example, a 2022 study found a significant correlation between VEGF-A and HER2 status in late-stage breast cancer ($P=0.036$)[36] .Similarly, inflammatory breast cancer showed 1.5-fold higher VEGF expression in HER2-positive tumors ($rho=0.36$, $P<0.05$)[30].This association is attributed to HER2 signaling pathway activation of HIF-1α, which upregulates VEGF-A transcription, promoting angiogenesis and contributing to faster tumor growth and poorer prognosis in the HER2 subtype[37] .In untreated patients, NF-κB p65 serum levels were elevated in the Luminal B subtype, aligning with its increased proliferation and inflammatory characteristics. Conversely, in treated patients, Luminal A tumors exhibited higher NF-κB p65 levels, which were positively correlated with serum levels of the protein. This contrasts with a recent study in Ghana[38], which demonstrated the highest NF-κB p65 expression in triple-negative breast cancer, the most common subtype among West African women[39]. Elevated NF-κB p65 levels may reflect endocrine therapy-induced activation, potentially promoting anti-apoptotic signaling by enhancing the transcription of pro-survival genes, thereby enabling tumor cells to survive following endocrine therapy. Consistent with Zhao et al. (2021), untreated Luminal B tumors exhibited elevated CA15-3 levels, although not significantly[40]. This aligns with a 2022 study associating Luminal B with higher CA15-3[41]. In the treated group, CA15-3 levels were higher in the HER2-enriched subtype. As CA15-3 is derived from mucin 1 *(MUC1)*, its elevation in HER2-enriched tumors may indicate drug resistance. Hosseinzadeh et al. (2022) suggest that MUC1 overexpression triggers compensatory signaling in HER2-targeted cells, promoting resistance to trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC)[42]. Previous research indicates that VEGF-A and CCN family proteins are more strongly influenced by disease biology than by chronological aging[43,44] .Investigations regarding NF-κB have similarly implicated it in age-related inflammatory and senescence pathways, but primarily in the context of chronic disease and tissue remodeling rather than normal aging[45] .In our study, no significant correlation was observed between age and any of the biomarkers, CCN6, VEGF-A, NF-κB p65, supporting the interpretation that observed differences between groups reflect tumor biology rather than age. Furthermore, as shown in our data, the lack of association between age and CA 15-3 is consistent with previous reports indicating that levels of CA 15-3 are independent of age and reproductive factors in both healthy women and breast cancer patients[46]. The study's strengths lie in a well-characterized diagnostic sample and the inclusion of a validation control group. Limitations include the small sample size for certain subtypes, such as triple-negative breast cancer, and the lack of assessment of other CCN family members. The use of serum instead of tissue, along with geographical limitations, necessitates further research into intracellular signaling pathways and WNT/inducible signaling protein (WISP) genetic variations in larger patient cohorts. Longitudinal analyses are recommended for future studies.

CONCLUSIONS

These findings provide novel insights into the potential of CCN6, VEGF, NF-κB p65, and CA15-3 as biomarkers for distinguishing between healthy individuals and patients with breast cancer, characterizing molecular subtypes, and predicting tumor aggressiveness and treatment response. Breast cancer patients had lower

serum CCN6 but higher VEGF-A and NF-κB p65 levels than healthy controls, with NF-κB p65 increasing VEGF-A levels. Elevated VEGF-A levels correlated with a high Ki-67 proliferation index, particularly in the Luminal B and triple-negative subtypes, whereas CCN6 levels were reduced in untreated triple-negative breast cancer, suggesting its tumor-suppressive role. HER-2-enriched breast cancer showed higher VEGF-A and CCN6 levels, and NF-κB p65 was elevated in luminal subtypes, potentially reflecting treatment-induced activation. These changes, which were more pronounced in luminal and triple-negative subtypes, support their treatment-resistant nature. These biomarkers could serve as prognostic and early diagnostic indicators, addressing delays associated with traditional diagnostic methods and emphasizing their clinical importance in breast cancer management.

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