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Efficacy of Cancer Antigen 15-3, Trefoil Factor-3, and Human Epididymis Protein-4 in the Diagnosis of Breast Cancer

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ABSTRACT

Background: Breast cancer (BC) is a significant global health concern, and delayed or frequently inadequate diagnosis has led to fatalities in many women. Consequently, research is needed to evaluate novel biomarkers for BC detection and monitoring. The current study aimed to assess the effectiveness of *Cancer Antigen 15-3 (CA15-3)*, *Trefoil Factor-3 (TFF3)*, and *Human Epididymis Protein-4 (HE4)* in diagnosing and monitoring BC.

Methods: The present case-control study recruited 72 women with BC, who were categorized into pre-treatment (n=15) and post-treatment with chemotherapy involving anthracycline, cyclophosphamide, and docetaxel (n=57). Additionally, 15 healthy women served as controls. Serum levels of these biomarkers were measured at Al-Sadder Teaching Hospital in Iraq, using COBAS Integra 400 Plus for CA15-3 and enzyme-linked immunosorbent assay (ELISA) for TFF3 and HE4.

Results: Statistical analysis revealed significantly elevated CA15-3 and TFF3 levels in pre-treatment and post-treatment groups compared to controls (P<0.0001), with CA15-3 increasing from 10.27±2.89 U/mL (controls) to 63.3±19.24 U/mL (pre-treatment) and TFF3 from 4.73±0.97 pg/mL to 1811.0±155 pg/mL. The HE4 levels remained consistent across all groups (P=0.409).

Conclusion: These results support the use of *CA15-3* and *TFF3* as complementary biomarkers for BC management, particularly in tracking treatment response and disease recurrence.

INTRODUCTION

Breast cancer (BC) is a significant global health concern. It is the most frequently diagnosed cancer worldwide and the leading cause of cancer-related mortality in women.[1] In 2022, BC accounted for 2.3 million new cases and approximately 670,000 deaths globally, with an age-standardized incidence rate (ASIR) of 26.88 per 100,000. Significant epidemiological disparities were observed across Socio-Demographic Index (SDI) regions, with high-SDI areas exhibiting the highest ASIR (66.89 per 100,000) compared with low-SDI regions (6.99 per 100,000).[2,3] Projections for 2023–2024 indicate a continued rise in global incidence, with pronounced inequities between the developed and developing regions. In countries with a very high Human Development Index (HDI), lifetime BC risk reached 1 in 12 women, whereas low-HDI regions reported lower incidence (1 in 27 women) but disproportionately higher mortality (1 in 48 deaths), reflecting systemic gaps in healthcare access, early detection, and treatment.[4] *Cancer Antigen 15-3 (CA15-3)* was first identified in the early 1980s as part of a broader effort to identify markers that could aid in cancer diagnosis and monitoring. It is the product of the *Mucin 1 gene*, which encodes a mucin protein found in normal breast tissue. However, this protein

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structure differs in cancerous tissues, with enhanced levels of *CA15-3* in the blood.[5] *CA15-3* is not employed for the initial diagnosis; however, it is frequently used to evaluate treatment response, recurrence, and disease progression. Araz *et al.* found that elevated levels of *CA15-3* can be a sign of disease progression or recurrence in BC patients.[6] Research has considered the combined use of *CA15-3* with other tumor markers, including *carcinoembryonic antigen (CEA)*, to increase diagnostic accuracy and prognostic value, suggesting that combined marker assays may show a higher predictive capability.[7] *CA15-3* levels can also be increased in other conditions such as lung, ovarian, and liver cancer. Furthermore, in benign conditions, including liver and breast disease, and even some autoimmune disorders, progress in liquid biopsy technologies has enabled more sensitive detection of tumor markers.[8] Trefoil factors (TFF) are components of mucus barriers that can be found in the exocrine body fluids, including gastric juice, saliva, breast milk, urine, and tears.[9] Trefoil factor 3 (TFF3) belongs to the trefoil factor family of small secreted proteins, which play important roles in mucosal protection, repair, and cell proliferation regulation. *TFF3* shows the highest expression level in the gastrointestinal tract, especially in the epithelial cells of the intestine, where its activity is essential for the maintenance of the mucosal structure.[10] *TFF3* stabilizes the mucus layer, promotes epithelial cell migration, and aids in healing damaged tissues. It can stimulate the proliferation of intestinal epithelial cells, contributing to tissue regeneration after injury. *TFF3* has been linked to gastrointestinal diseases such as inflammatory bowel disease, with altered expression observed in patients, suggesting its involvement in inflammatory response and tissue repair mechanisms.[11] Also, the protective role of *TFF3* in mucosal healing may be relevant in ulcerative conditions, such as Peptic Ulcers. Its overexpression in gastric cancers correlates with tumor progression and poor prognosis.[12,13] Elevated *TFF3* levels are associated with aggressive cancer and metastasis. *TFF3* has been implicated in promoting tumor cell proliferation and survival in BC.[14] *TFF3* expression levels in tumor tissues and body fluids have been investigated as potential biomarkers for diagnosis and prognosis, offering insights into disease progression and therapeutic responses.[15] The presence of *TFF3* has been observed in invasive BC, with some cases exhibiting reduced expression and others showing elevated expression.[16] Researchers have explored strategies to target *TFF3* in cancer therapy, considering its role in tumor growth and metastasis. This involves monoclonal antibodies or small-molecule inhibitors that disrupt *TFF3* signaling.[17] *Human Epididymal Protein 4* (*HE4*) is primarily expressed in the epithelial cells of the reproductive tract, particularly in the epididymis, as well as in various other tissues. It is encoded by the *WAP Four-Disulfide Core Domain 2* (*WFDC2*) gene, located on chromosome 20. *HE4* is a member of the whey acidic protein (WAP) family and is involved in various biological processes, including immune responses and epithelial differentiation.[18] *HE4* was first identified in the context of male fertility; however, it has gained prominence in cancer research, particularly in ovarian cancer. Its expression levels were found to be significantly elevated in patients with ovarian tumors. The discovery of *HE4* as a potential biomarker for ovarian cancer was pivotal, leading to its inclusion in diagnostic protocols along with other markers, such as *Cancer Antigen 125* (*CA-125*).[19] *HE4* has also been studied in the context of endometrial, lung, and BC. Elevated *HE4* levels have been correlated with disease progression and poor prognosis in these malignancies.[20] Building on these findings, this study aimed to assess the effectiveness of *CA15-3*, *TFF3*, and *HE4* in detecting and monitoring BC.

METHODS

This was a case-control study. Samples were collected at the Al-Sadder Teaching Hospital in Al-Basrah Governorate, Oncology and Hematology Center, Tumor LAB Department, Basrah, Iraq, between December 2023 and May 2024. BC staging was contingent upon physician assessment using the Tumor-Node-Metastasis (TNM) classification system as per the American Joint Committee on Cancer (AJCC) guidelines. This framework stratifies disease progression through the systematic evaluation of three parameters: primary tumor dimensions (T), regional lymph node involvement (N), and distant metastatic spread (M). Staging was determined at the time of the initial diagnosis via multimodal diagnostic approaches, including clinical evaluation, imaging modalities (e.g., mammography and ultrasonography), and histopathological analysis of biopsy specimens. Overall, 72 women diagnosed with BC were subdivided into pre-treatment (n=15) and post-treatment (n=57, stages 1–3) groups.. Also, 15 healthy women were included in the control group. The control group was sourced from healthy women selected from a general population similar in characteristics (age, gender, ethnicity, socioeconomic status, and lifestyle factors). CA15-3 levels were quantified as per the COBAS Integra 400 Plus protocol (Roche Diagnostics, Switzerland). The levels of the other two biomarkers in serum were evaluated using enzyme-linked immunosorbent assay (ELISA) and specific commercial kits (TFF3 and

HE4) (Elabscience, USA), following the manufacturer's instructions.

Inclusion criteria required women to have a confirmed BC diagnosis. The patients with chronic diseases other than BC, cardiovascular disease, infections, and endocrine disorders were excluded from the study.

The participants in this study (patients and controls) provided five millilitres of blood, which was then transferred to sterilized test tubes and allowed to coagulate at room temperature for 30 minutes. The blood samples were centrifuged at 3000 rpm for 15 minutes. Subsequently, the sera were separated and stored at −20 °C until further use.

Statistical analysis

The collected data were analyzed using SPSS version 26 (IBM Corp.). Categorical variables were coded and summarized as frequencies and percentages, while continuous variables were expressed as means ± standard deviations. The Kolmogorov test was used to examine the normality of continuous variables. For non-normally distributed variables or ordinal data, differences across the five study groups were evaluated using the Kruskal-Wallis test. Pairwise comparisons were conducted for variables that showed statistically significant differences. Corresponding post-hoc tests were used to examine between-group differences. Statistical significance was set at $P<0.05$.

Table 1. A Comparison Between the Studied Biomarkers in All 5 Groups

Parameter	Control (n=15)	Pretreatment (n=15)	Stage 1 (n=18)	Stage 2 (n=32)	Stage 3 (n=7)	P value*
Age, years	44.60±13.94^{A}	49.27±6.2^{A}	49.28±7.48^{A}	48.75±7.02^{A}	51.14±5.01^{A}	0.682
CA15.3, U/mL	10.27±2.89^{A}	63.3±19.24^{B}	20.17±2.92^{C}	21.19±6.53^{C}	37.77±20.1^{D}	0.0001**
TFF3, pg/mL	4.73±0.97^{A}	1811.0±155^{B}	2012.27±351.03^{B}	2014.5±313^{B}	1914.28±234.28^{B}	0.0001**
HE4, ng/mL	1.24±0.37	1.27±0.42	1.41 ± 0.53	1.13±0.44	1.22±0.49	0.409

*Kruskal-Wallis test; **Significant at 0.05 level. Capital letters A, B, and C indicate the level of significance following Tukey's multiple comparisons test; similar letters indicate no significant differences, whereas different letters indicate significant differences. *CA15-3, Cancer Antigen 15-3; HE4, Human Epididymis Protein-4; TFF3, Trefoil Factor-3.*

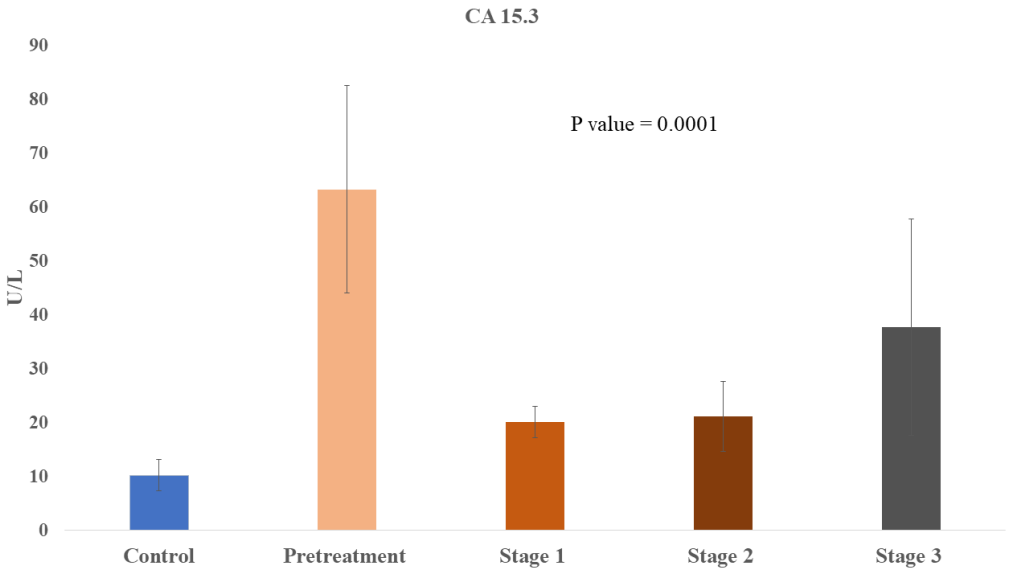

Figure 1. Level of *Cancer Antigen 15-3* (*CA15-3*) in Serum of the Participants in the Study Groups

RESULTS

The current case-control study recruited 72 women diagnosed with BC, who were categorized into 2 groups: the pre-therapy group (Group 2) with 15 participants and the post-chemotherapy group with 57 patients: 18 women at Stage 1 (Group 3), 32 women at Stage 2 (Group 4), and 7 women at Stage 3 (Group 5). Furthermore, a control group of 15 healthy women (Group 1) was recruited. Table 1 shows the age range of the participants in different study groups, i.e., the control group (40.6±13.9), the pre-treatment group (49.27±6.2), and three chemotherapy groups (Stage 1: 49.28±7.48, Stage 2: 48.75±7.02, Stage 3: 51.14±5.01).

Measurement of the studied biomarkers

Serum *CA15-3* levels were significantly higher in the pre-treatment group compared to controls ($P<0.0001$). A stage-dependent elevation was also observed, with notably increased levels in Stage 1, 2,

and 3 patients compared to the controls (P<0.0001). TFF3 levels increased significantly in the pre-treatment and Stage 1, 2, and 3 groups compared to the control group (P<0.0001), whereas HE4 levels remained consistent across the groups, as shown in Table 1 and Figures 1, 2, and 3.

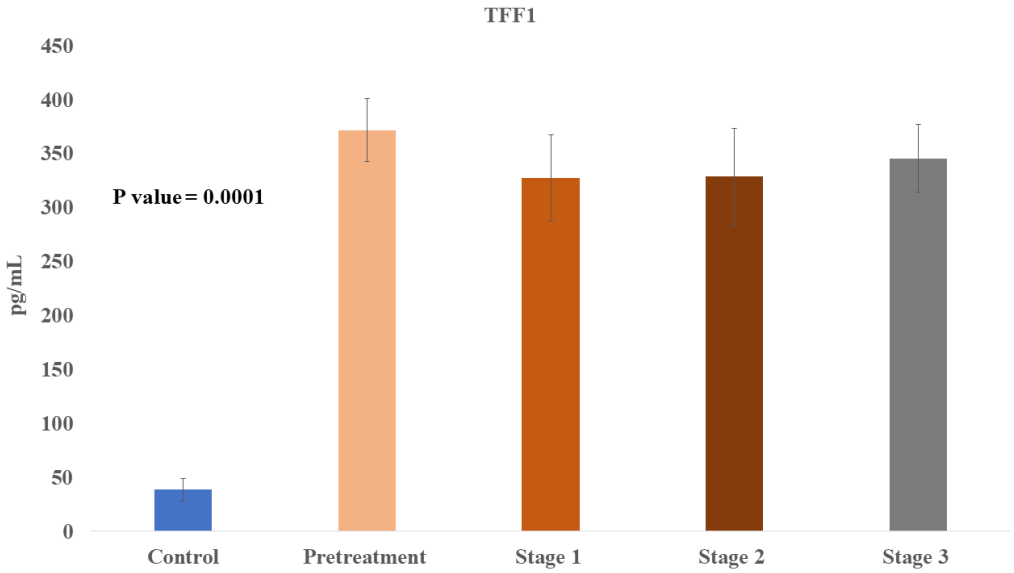

Figure 2. Level of *Trefoil Factor-3* (*TFF3*) in Serum of the Participants in the Study Groups

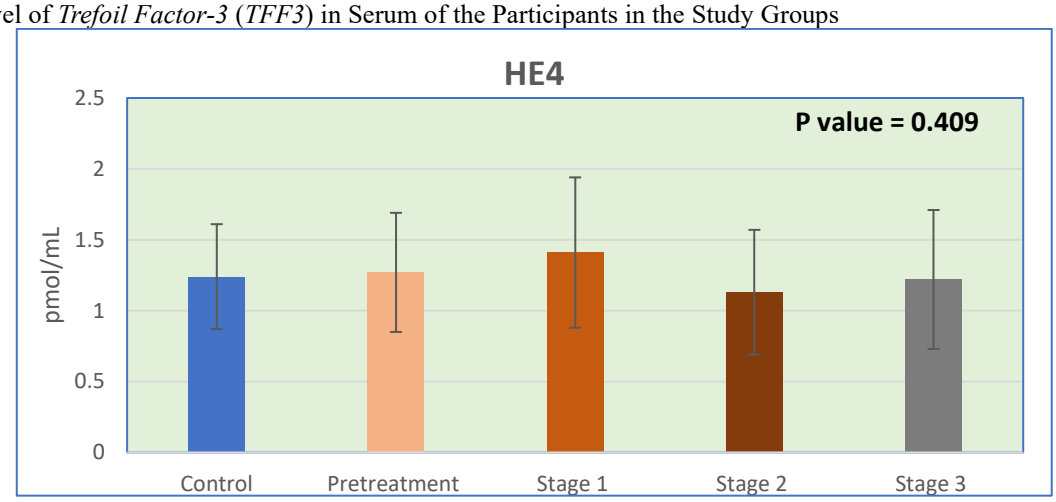

Figure 3. Level of *Human Epididymis Protein-4* (*HE4*) in Serum of the Participants in the Study Groups

DISCUSSION

BC is the predominant type of cancer affecting women worldwide. It is a diverse collection of diseases that vary in their characteristics. Identifying reliable prognostic markers is crucial for determining the likelihood of recurrence and selecting appropriate treatment for patients with specific pathological and clinical characteristics.[21] The present findings align with those of Li *et al.* and Hing *et al.*, who reported that significant elevations in *CA15-3* concentrations function as prognostic biomarkers for the early detection of metastatic progression in BC. Elevated *CA15-3* levels have been positively correlated with adverse clinicopathological characteristics, including advanced tumor stage, higher histological grade, and metastatic involvement. The observed increase in *CA15-3* concentrations in advanced-stage malignancies may reflect the underlying pathophysiological mechanisms associated with increased tumor burden and metastatic spread. This likely reflects increased shedding of tumor-associated antigens into the systemic circulation during neoplastic progression and metastatic evolution.[22,23]

A study by Fu *et al.* showed that increased levels of *CA15-3* in the blood at the time of diagnosis were linked to more advanced stages of BC, larger tumor size, and the presence of cancer cells in the axillary lymph nodes of the armpit.[24] Serum indicators were evaluated sequentially in multiple studies to determine their use in the early identification of the disease and tracking treatment response.[25] *CA15-3* can be particularly useful for monitoring the response to treatment and detecting recurrence, as elevated levels often correlate with disease progression. Our study findings are consistent with the results reported in Taghizadeh *et al.*,[26] which

found elevated *CA15-3* levels exhibited a robust connection with the advanced stages of cancer. Lian *et al.* reported similar results, emphasizing the significance of tumor markers.[27] According to previous studies, serum *CA15-3* can be used as an indicator of advanced disease and metastasis.[28–30] Both oncologists and surgeons are recommended to examine *CA15-3* levels, as this might assist in determining the necessity of an intensive treatment plan.[31] The diagnostic utility of *CA15-3* as a BC biomarker has been contested owing to limitations in specificity and sensitivity, as evidenced by conflicting study findings such as those reported by Coppola *et al.* Discrepancies arise from nonspecific elevations in non-malignant conditions (e.g., benign breast lesions) and non-breast malignancies, increasing false-positive rates and reducing clinical validity. These cross-reactive elevations undermine its reliability as an independent diagnostic tool, necessitating complementary biomarkers or multimodal approaches to improve diagnostic accuracy.[32] There is also some debate regarding *CA15-3* consistency as a stage-specific marker. A study by Kabir *et al.* noted that while *CA15-3* levels generally increase with cancer stage, this correlation is not uniform across all patients. Factors such as individual tumor biology, treatment history, and other patient-specific variables can influence *CA15-3* levels, leading to variability in its utility for staging. Elevated *CA15-3* levels can occur in benign conditions, and not all patients with BC show elevated *CA15-3* levels. This complicates its use as a diagnostic tool.[33]

This study demonstrates that *TFF3* exhibits a diagnostic performance for BC comparable to the established biomarker *CA15-3*, supported by statistically significant findings, consistent with prior observations by Abdelrazek *et al.* Elevated serum *TFF3* levels positively correlated with advanced clinical stage and poor prognostic indicators, suggesting the utility of *TFF3* as a biomarker for disease progression. Mechanistically, *TFF3* dysregulation may promote oncogenic signaling pathways, enhancing tumor proliferation and metastatic potential, which aligns with its association with aggressive disease phenotypes. These findings underscore *TFF3*'s dual diagnostic and prognostic relevance, highlighting its potential clinical value in BC management.[34] Another study reported statistically significant TFF3 expression differences ($P < 0.05$) between metastatic and non-metastatic breast cancer cases, demonstrating its potential as a discriminative biomarker with clinically relevant sensitivity and specificity.[35] Furthermore, Wu *et al.* identified *TFF3* as a highly responsive biomarker for predicting the effectiveness of endocrine therapy in BC cells.[36] Also, Wang *et al.* found that *TFF3* might facilitate tumor progression through its effects on cell adhesion and migration.[37] Elevated *TFF3* levels can alter cellular interactions and contribute to the metastasis of cancer cells. Higher *TFF3* levels in the pre-treatment and staged groups could mean that the disease is more advanced or aggressive. This dual role in both physiological maintenance and pathological progression underscores *TFF3*'s clinical relevance in BC diagnostics and prognostication.[37] *TFF3*'s role in cellular protection and repair may contribute to its elevated expression in cancerous tissues. Its overexpression could reflect the tumor's attempt to repair damaged tissues or to support cancer cell survival. Elevated TFF3 expression correlates with advanced breast cancer stage ($p<0.01$), suggesting a potential role in tumor progression. This observation is supported by multiple cohort studies demonstrating significant associations between high TFF3 levels and poor prognostic outcomes. Studies have suggested that *TFF3* acts as a separate risk factor, contributing to the occurrence of lymph vascular invasion and spread of cancer cells to the lymph nodes.[38] Some researchers have reported results that are inconsistent with our findings; for example, a study by Shen *et al.* found no significant difference in *TFF3* levels in BC patients and healthy controls, suggesting that *TFF3* may not always be a reliable biomarker for BC. Some researchers attribute this variability to variations in study designs, patient populations, or methodological approaches.[39] Yan *et al.* founds that factors unrelated to BC, such as inflammation or other benign conditions, could influence *TFF3* levels. This implies that elevated *TFF3* levels might not be specific to BC, which challenges the use of *TFF3* as a standalone biomarker.[40] Technical issues in measuring *TFF3* levels, such as assay sensitivity and specificity, may also lead to inconsistent findings. According to Zhang *et al.,* discrepancies in *TFF3* measurements across different laboratories and techniques can affect the interpretation of its role in cancer.[41]

The study found no significant differences in serum *HE4* levels across BC stages or chemotherapy statuses. These findings are consistent with the multivariate analysis conducted by Baba *et al.,* who reported no positive association between *HE4* levels and histological grade or clinical stage in BC patients.[42] This finding supports the assumption that *HE4* lacks utility as a stage-specific biomarker in BC. Corroborating this observation, Zhu *et al.* found no substantial variation in *HE4* levels between healthy controls and BC patients, including those at different disease stages.[43] Collectively, these data suggest that *HE4* exhibits limited sensitivity for early-stage detection and inadequate discriminatory capacity to

stratify BC progression, underscoring its limited diagnostic applicability in staging or early diagnosis. The relatively stable levels of *HE4* across different stages and pre-treatments may reflect its low sensitivity and specificity for BC. In other words, it may not be useful in detecting subtle changes that occur with disease progression, or it may not be as effective in distinguishing between different stages. Our findings contrast with prior reports of significant associations between circulating HE4 levels and prognostic factors in breast cancer (e.g., tumor size, nodal status). While these discrepancies may reflect methodological differences, the observed HE4 patterns could suggest context-dependent roles in tumor biology, potentially including pro-tumorigenic functions during disease progression.[44]

Limitations and future studies

TFF3 is a BC biomarker that lacks specificity because increased expression can result from non-neoplastic physiological variations or co-morbidities. This diagnostic ambiguity and conflicting evidence underscore the necessity for rigorous validation of *TFF3* efficacy. Interestingly, *HE4* showed context-dependent variability owing to tumor heterogeneity, molecular subtypes, and synergistic interactions with surrogate markers. Together, these limitations emphasize the multifactorial complexity of biomarker behavior and indicate the need for further research using standardized methods, larger multicenter cohorts, and stratified analyses to define the interactions of biomarkers with tumor biology and other confounders. Future research should focus on longitudinal studies to monitor biomarker changes during treatment, multi-marker panels to improve the accuracy of diagnosis, and studies on the molecular mechanisms of *TFF3*'s dual roles in mucosal repair and carcinogenesis. A key limitation of this study was the difficulty in recruiting an adequate number of controls, which is always a problem in case-control studies because of financial limitations and time constraints on recruitment. Therefore, we recommend that in future studies, the size of the control group should be proportional to the patients sample size to ensure robust statistical conclusions. Overall, this study contributes to the evolving paradigm of biomarker-driven strategies for breast cancer diagnosis and personalized therapeutic decision-making.

CONCLUSION

The results revealed that serum levels of *CA15-3* and *TFF3* were markedly elevated in BC pre- and post-chemotherapy compared to healthy control subjects, highlighting their promising application as diagnostic biomarkers. Most importantly, *CA15-3* levels were associated with disease progression, consistent with its well-established signaling for tumor mass and metastasis. In line with this, *TFF3* upregulation correlated with advanced clinical stages, showing its role in tumor aggressiveness or in shaping metastatic pathways. Conversely, the limited diagnostic significance in BC stage or monitoring was confirmed by the insignificant changes in *HE4* between the study groups.

ETHICAL CONSIDERATIONS

Ethical approval was obtained from the ethical and research committee of the Department of Medical Laboratory Technology, College of Health and Medical Technology, Southern Technical University, Basrah, Iraq (No. 803 on 19/11/2023).

DATA AVAILABILITY

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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