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EVALUATION OF PROLINE LEVEL AS A POTENTIAL BIOMARKER AND ITS CORRELATION WITH STANDARD MARKERCA15-3IN PATIENTS WITH BREAST TUMOR

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***Abstract*—**Iraqi women have had more cancers of the breast in recent years. To achieve successful treatment, delay dissemination, and reduce fatalities in breast cancer, the best way is to monitor protein levels in body fluids for early diagnosis and prognosis.The objective of the present research was to determine the diagnostic and prognostic significance of proline as a marker in individuals diagnosed with breast cancerTo examine the correlations between proline and the established biomarker CA 15-3, as well as clinicopathological characteristics. This research involved 30 newly diagnosed female with breast cancer before surgical operation and compare with their levels after one month of the surgical operation, and 28femalehad noncancerous breast tumors as control subjects. CA15-3was measuredby ELISA technique and proline level by HPLC technique.A notable variations in the average proline levels was observed between the categories of study participants; the benign group exhibited the highest level, then post-operative category, and finally the newly diagnosed category.Additionally, notable variations were observed in the average CA-15-3 levels with the newly diagnosed category having the highest level, then the post-operative category and then benign category.Proline concentration showed a significant positive correlation with CA15-3 in malignant and benign groups. Both of Proline & CA-15-3 were strong predictors by ROC, 100% & 90%for Proline, 93% &93%, for CA-15-3 respectively.

***Keywords*---**Breast cancer, Proline, CA 15-3, Tumor

Introduction:

Breast cancer (BC) is the most common malignancy in women worldwide, occurring at an incidence rate of around 12%. Consequently, despite the fact that most tumours of the breast are

noncancerous, a methodical & comprehensive method is necessary under all circumstances. Overall, methodology should following triple-assessment chain, which consists of pathology analysis, imaging using radiology & clinical examination. (Karim *et al*., 2020) (Quan *et al*.,2023)

In women, benign breast problems are extremely prevalent. Most breast alterations are benign. Benign breast disorders aren't life-threatening in contrast to malignant breast malignancies. However, certain factors are linked to a higher likelihood of developing BC later (Golshan M.,2021)BCis a heterogeneous illness&from main causes of mortality in female (Nolan et al., 2023). An older age, early appearance of monthly periods, delayed appearance of menopause, not having children, not to breastfeed, family history, dense tissue in the breasts, hormone therapy, and having previously received chest radiation treatment are all risk factors for breast cancer. Genetic variations linked to a higher likelihood of developing BC encompass mutations in the genes known as BC susceptibility 1 and 2 (BRCA1/2) (Trayes & Cokenakes., 2021).

The main constituent of the extracellular matrix (ECM) is collagen & has significant involvement in cellular gene expression, proliferation, differentiation, as well as tumorigenicity & invasiveness (Popova & Jücker .,2022) (Karamanos et al., 2021)
Therefore, any alterations in the metabolic processes of collagen have the potential to impact the movement and metabolic activities of the cells.The metalloproteinases in the ECM have a role in modifying the matrix & facilitating invasion of malignancies &typical cells through barriers of the tissue. even though extracellular collagenases start the collagen breakdown, prolidase completes the ultimate stage of collagen degradation (Eni-Aganga et al., 2021)(Syabakhash et al., 2020) Prolidase catalyzes the hydrolysis of iminodipeptides that have Hydroxyproline or Proline at the C-terminal (X-Pro), resulting in release of the proline component(Kabashima et al., 2023)
Recent attention has been drawn to proline metabolism due to its role in the metabolic reprogramming that takes place in cancer cells(Elia et al., 2018).
Furthermore, in the last few years, there has been an increase of evidence establishing a link between proline metabolism & critical regulatory routes, including: mTOR, ERK, JAK/STAT, & ROS-driven signaling(Sawicka et al., 2022)
One distinctive feature of metabolism ofProline is the process of cycling proline and P5C to ensure a balanced redox state throughout the cytosol & mitochondria. The complete understanding of proline production in cancer is still limited. The metabolic shift in cancer cells is heavily influenced by proline metabolism, making it a crucial aspect. Specific enzymes involved in proline metabolism have been identified as possible targets for cancer therapy(Bergers and Fendt 2021).

CA15-3is a kind of mucin that is part of a wide group of glycoproteins produced by the gene MUC1. A raised pre surgical CA15-3 concentration is immediately associated with the size of tumor present & serves as an independence prognostic factor for BC(Coppola et al., 2021) can be utilized alongside diagnostic imaging, history, & physical inspection to monitor patients throughout active therapy. The assessment of CA15-3is valuable in assessing the recurrence of a

BC & the effectiveness of treatment. However, it is not advised to utilize it as a standard practice for screening, diagnosing, staging, or monitoring BC due to insufficient accessible data. As to the 2007 ASCO guidelines (Clatot et al., 2020)

So, It is not considered a specific indicator in diagnosis for BC because its levels increase as well in benign illnesses(Coppola et al., 2021) thus, Enhancing the identification and prognosis of disease using unique biomarkers is crucial for advancing diagnosing& management of BC (Saadati et al., 2019) (Li, J. et al., 2020)
The aim of this investigation was to evaluate the significance diagnostic & prognostic of Proline &CA15-3 as biomarkers., in female with benign & malignant breast tumors to examine connections between proline and other biomarkers in serum of women with BC before surgical operation & compare with their levels after one month of the surgical operation.

Materials and Methods

Study subjects

Study of a case-control design was conducted on a set of 58 women who had breast tumors, including both malignant & benign cases. Female patients were enlisted from Al-Imamain Al-kadhimain Medical City & Baghdad Medical City from 10 January 2023 to 30 June 2023. The experimental aspect was performed in the Research Labsat College of Medicine/Al-Nahrain University's Department of Chemistry and Biochemistry, as well as in the labs of the Department of Environment & Water at the Ministry of Science &Technology.BC & Benign women can be classified into three distinct subcategories: individuals with obesity, having a BMI more than 30 kg/m2, individuals who are overweight, with a BMI rang of 25 to 30 kg/m2, and individuals who are either of typical weight or underweight, with a BMI that is below 25 kg/m2(Syabakhash et al., 2020)

The samples were collected then divided into 3 groups:
❋Benign group : which includes 28 serum samples as control group.
❋Malignant group : comprises 30 serum samples from individuals who have just been diagnosed with the initial stage BC.
❋Post operation group: Each participate will be followed up after 1 month of the operative surgery.

These are the criteria that were used for selection: {1}A consultant doctor diagnosed every individual with early stages, non-metastatic BC {2}There was no recurring or metastatic BC & any of neoadjuvant therapy. {3} Absence of the any of systemic. After explaining the study's goals and receiving permission from every participant, clinical and demographic information were getting by meeting and questionnaire.Institutional Review Board of Al-Nahrain University's College of Medicine approving the study.

Blood sample

Venous blood was positioned in a gel tube and maintained at 4 °C. To acquire serum samples, it was centrifuged at 3000 rpm for ten minutes within thirty minutes of receiving from subjects. Serum samples were kept in storage at -80°C.

Determination of Concentration of Human Proline (Pro)

a) Principle

The extraction of amino acids followed the protocol outlined by scientist Rasmus Dahl-Lassen (2018). A volume of 100 microliters was obtained from sample & transferred to a volumetric container holding ten milliliters. Subsequently, 2.5 milliliters of hydrochloric acid (1M) add to the vial at a temperature of 45°C. After being subjected to a 10-minute process, the sample is dehydrated using a rotating evaporator. Subsequently, fivemilliliters of Sodium Citrate with a pH of 2.2 is introduced to the sample. a plastic filter with 0.45 micrometer-pore-size is used to filter the material. and is then sent to the injection operation.

Derivation method: One milliliter of samples after extraction was collected and 200 microliters of orthophthalein aldehyde (five percent) (OPA) was introduced into it. The sample was agitated for a duration of two minutes. Subsequently, a volume of 100 microlitres from the final mixture was extracted and introduced into the Amino acid analysis apparatus..

The examination was performed in the labs of the Department of Environment & Water at Ministry of Science &Technology utilizing a German-made high-performance liquid chromatography instrument (SYKAM model HPLC). The researcher adhered to the methodology described by Scriver CR (2001), employing a carrier phase comprising 5% formic acid and a mixture of methanol and acetonitrile in proportions: 20:60:20 (at a 1 ml/min flow rate). Amino acid was separated using a separation column (C18-NH, 25 cm * 4.6 mm), and it was identifiedby using detector of fluorescence at specific frequencies (Ex = 445 nm, Em = 465 nm). A Clarity 2015 program was utilized for the amino acid analysis.

Statistical analysis

The data that obtain could be analyzed using SPSS version Analysis of variance , Results are demonstrated as mean ± standard deviation. The Student's t-test or Mann-Whitney U-test was utilize to compare the groups binary in order to assess the variation in proline level between the three groups. Association between proline and CA15-3levels was evaluated by Pearson correlation analysis.

Result

A total of 58 patients underwent examinations over the duration of the trial. The study included the following categories of patients: 30 women with newly diagnosed breast cancer who had not undergone surgery; their levels were compared before and one month after the surgical operation; and 28 women with breast benign tumors who serving as the control group(Table 1).

Table 1: Clinical Features of the Patients Groups

Variables	Breast cancer (n=30)	Benign tumor(n=28)	p- value
Age, years **Mean±SD** **Range**	 38.3±7.27 20-46	 29.57±5.0 19-38	 <0.001
BMI, kg/m^2 **Mean±SD** **Range**	 26.5±2.33 23-30	 25.64±4.36 18-35	 0.350

BC:breast cancer, **BBT**:Benign Breast Tumor**BMI**:body mass index, **SD**:standard deviation,

The results indicated the presence of significant variations in the average age in different research groups. Average of women's age with (BC) was (38.3±7.27) years which was more than that of individuals with Benign Breast Tumors (BBT) was (29.57±5.0 years) with a highly significant difference. In contrast, the two groups were comparable in term of BMI with no significant difference(Table 1).

Assessment Levels of CA15-3 &Proline in women with BC & BBT:

Levels of Proline with CA-15-3 vary among the different study categories, as illustrated in Tab2, And Tab3.There was a substantial variance in the average proline level among the different research categories; The most elevated level was observed in the benign category, followed by the post-operative category, and finally the newly diagnosed category.There was a notable variance in the average **CA15-3** level between the different research categories. The highest level was observed in the newly diagnosed category, next by the post-operative category then the benign category.

Table 2: Levels of Proline and CA15-3 in women with BC and BBT

Variables	Breast cancer (n==30)	Benign tumor (n=28)	p- value
CA_{15-3}, pg/mL Mean±SD Range	 31.06±1.22 18.3-43.0	 15.66±3.02 9.98-21.7	 **<0.001**
Proline, pg/mL Mean±SD Range	 9.92±1.6 7.06-13.28	 24.83±1.74 20.44-27.58	 **<0.001**

BC: breast cancer, **BBT**: Benign Breast Tumor**BMI**: body mass index, **SD**: standard deviation,

Impact of Surgical Operation on levels of Proline and CA15-3

Level of CA15-3 decreased to 18.09±5.42 pg/ml, with a mean difference of (12.97±8.73pg/ml) with a remarkably significant difference. While level of Proline increased to become (17.12±2.39

pg/ml) after surgery with a mean difference (between pre and post-surgical operation) of (7.2±2.96 pg/ml) with a highly significant difference, (Tab 3).

Table 3: Impact of Surgical Operation on serum levels of Proline and CA15-3 in women with BC

Variables	Before surgery (n==30)	After surgery (n=30)	Mean Δdifference	p-value
CA_{15-3}, pg/mL				
Mean±SD	31.06±1.22	18.09±5.42	12.97±8.73	**<0.001**
Range	18.3-43.0	3.9-28.8	-4.0 to 34.1	
Proline, pg/mL				
Mean±SD	9.92±1.6	17.12±2.39	7.2±2.96	**<0.001**
Range	7.06-13.28	12.66-21.15	-0.17 to 12.05	

Correlation between serum biomarkers concentrations and with other parameters

In Women with BC Proline concentration showed a significant positive correlation with CA15-3 asdemonstrated in (table4), And no notable associations between proline and CA15-3levels and age or BMI,

Table 4 Pearson's correlation of proline and CA15-3 with age and BMI in patients with **BC**

Variable		CA15-3	Proline
Age	*r*	0.072	-0.133
	p	0.704	0.485
BMI	*r*	0.124	0.144
	p	0.514	0.448
Proline	*r*	**0.350**	
	p	**0.050**	

In Women with BBTCA15-3 had a significant positive correlation with proline as demonstrated in (table5), Alsoabsent of notable associations between serum proline and CA15-3levels to age or BMI.

Table 5: Pearson's correlation of proline and CA15-3 with age and BMI in patients with BBT

Variable		CA15-3	Proline
Age	*r*	0.006	-0.167
	p	0.976	0.396

BMI	*r*	0.303	0.063
	p	0.117	0.751
Proline	*r*	**0.469**	
	p	**0.012**	

Association of Proline and CA15-3 with clinical characteristics of patients with BC

Women with stage B2 TNM showed lower Proline level (8.92±1.73 pg/ml) and higher CA15-3 concentration than other stages with significant differences.Women with nodal involvement showed higher serum level of CA15-3 than those without such involvement (36.25±4.89 pg/ml vs. 29.17±6.34 pg/ml) with a significant difference. (table 6)

Table 6 : Association of Proline and CA15-3 with clinical characteristics of patients with BC

Variables	Proline	CA15-3
Family history		31.39±5.79
No	12.19±5.37	30.62±8.0
Yes	9.18±1.52	
p-value	0.061	0.763
TNM stage		
A1	15.0±6.9 [a]	25.15±4.31[a]
A2	9.77±0.74[a]	31.9±5.24[ab]
B2	8.92±1.73[b]	37.16±5.1[b]
p-value	**0.004**	**<0.001**
Tumor size (mm)		
<2	11.13±5.31	30.84±6.94
2-5	10.4±1.34	31.49±6.57
p-value	0.674	0.808
Nodal Status		
pN0	11.0±5.08	29.17±6.34
pN+	10.6±1.42	36.25±4.89
p-value	0.834	**0.008**
Receptors		
ER+/ PR+/ Her2-	10.56±4.43	31.34±5.31
ER+/ PR-/ Her2-	10.12±1.70	29.84±5.53
ER-/ PR-/Her2+	14.82±7.43	36.25±6.4
Triple negative	9.16±0.97	25.43±7.36
p-value	0.268	0.191

The diagnostic potential of biomarkers in identifying breast tumors:

Receiver Operating Characteristic (ROC) analysisutilized to identify diagnostic efficacy of proline and CA15-3 in distinguishing between (BC) and benign breast tumors (BBT). and The outcomes are presented in table 7., and figure 1.

Table 7: Diagnostic value of proline and CA15-3 in the context of discrimination between BC and BBT

Markers	AUC	Sensitivity	Specificity	Cut off value
Proline	0.943	100%	90%	15 pg/ml
CA15-3	**0.989**	**93%**	**93%**	**19.87 pg/ml**

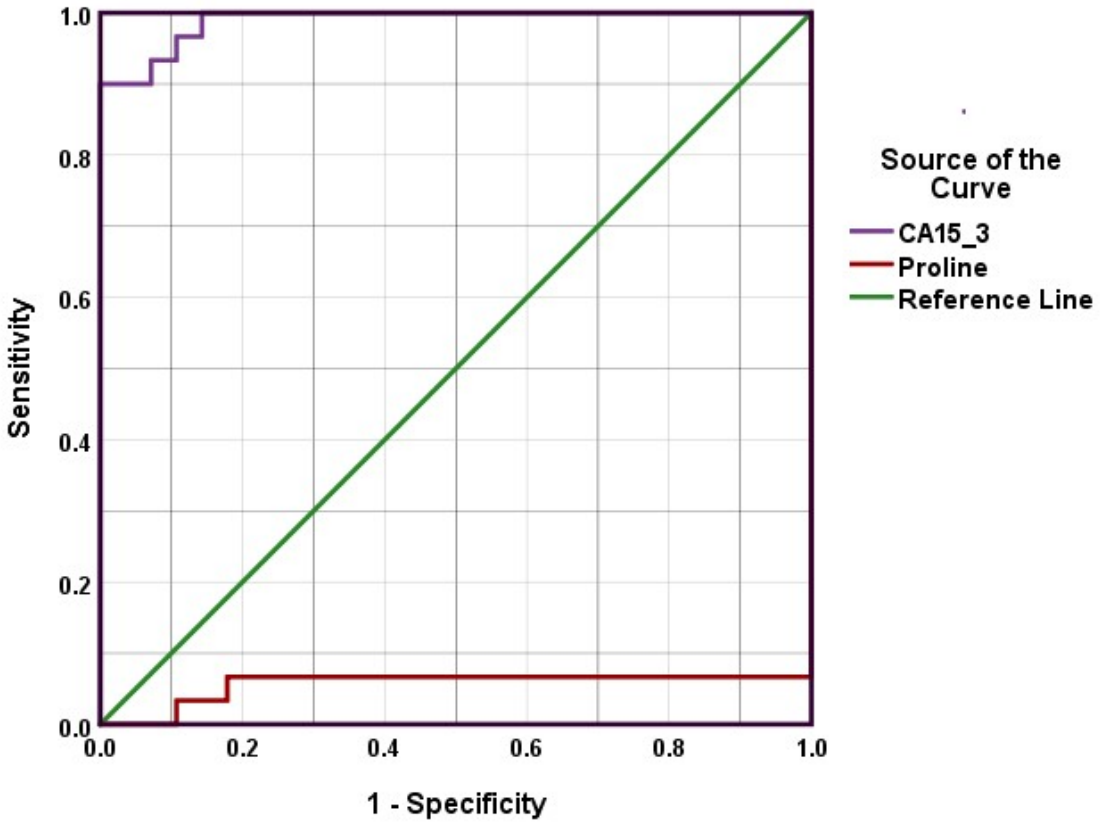

Figure 1: Receiver operating characteristic curve for proline, and CA15-3 in the context of discrimination between BC and BBT

For the combination of CA15-3 and proline, The (AUC) was 1.0, with a 95% confidence interval (CI) of 1.0-1.0, and a p-value of less than 0.001. The test exhibited a sensitivity and specificity of 100% for both parameters. at the defined cut off value(Fig2).

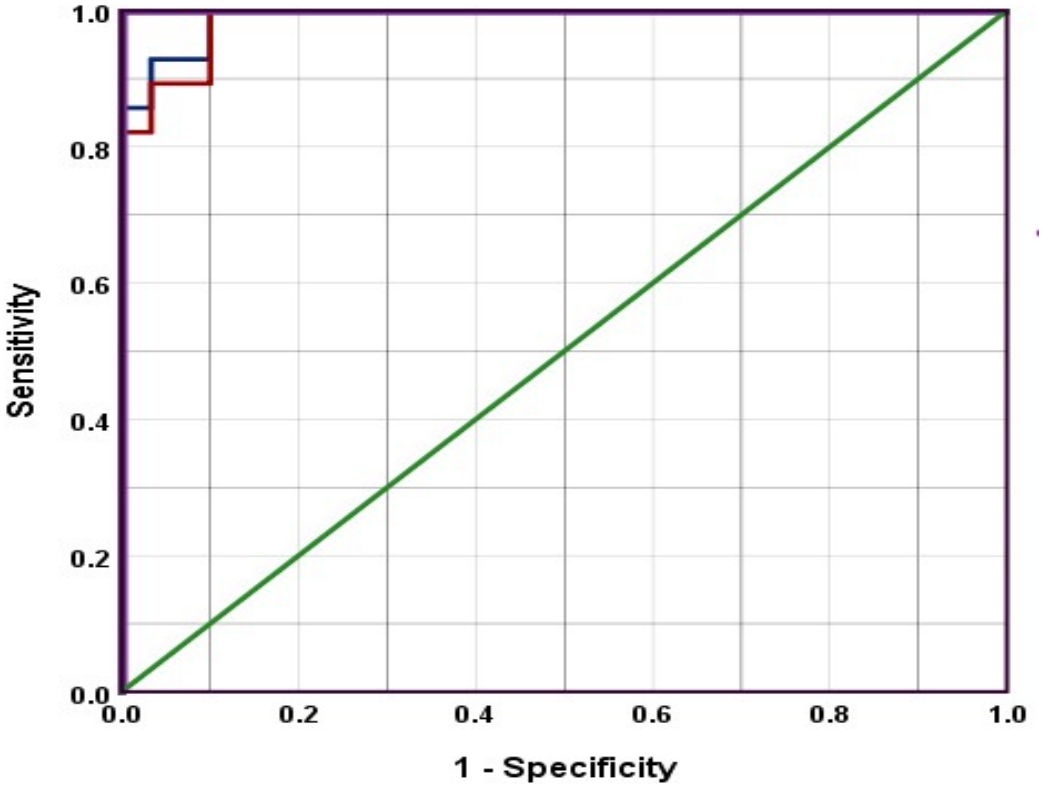

Figure 2: Receiver operating characteristic curve for a combination of proline with CA15-3 in distinguishing between BC and BBT.

Discussion:

Currently, breast cancer holds the distinction of being the most often detected form of cancer worldwide. Based on the statistics of latest cancer burden globally, Approximately 2.26 million new cases of BC in 2020 year. This type of cancer is the primary cause of deaths among females worldwide(Wilkinson, L., & Gathani, T., 2022).

Therefore, a substantial requirement for the identification of dependable and innovative markers to assist in the timely identification of BC, improve the accuracy of prognosis, permit precise prediction of illness manner, and help the advancement of focused therapy strategies (Golestan et al., 2024).

BC diagnosis, prediction, and treatment depend on biomarkers. Many biomarkers, such as (CEA), CA 15-3, and CA 27–29, lack the sensitivity and specificity to identify BC. These tests

are suggested for surveillanceof disease progression and therapy efficacy, especially in metastatic BC patients(Hing et al., 2020)(Hussein et al., 2022)

The current investigation demonstrated a substantial variance in the average proline concentration among the investigated categories. The greatest amount was observed in the benign category, next by the postoperative category, and finally newly diagnosed category, with **strong** predictors, when it is performed using a receiver operating characteristics (ROC) analysis. The same finding also obtained from previous studies (Azmy *et al*, 2023) (Liang *et al*, 2015). The research performed by Azmy *et al* 2023, found thatAmino acids, which are necessary for the development& tumor cells maintenance of, are required by all living cells. Due to their accelerated rate of division in comparison to normal cells, tumor cells exhibit a significantly elevated requirement for amino acids.

Due to the fact that proline can be utilized as both a precursor for protein synthesis and energy source, proline biosynthesis increases respond to raised demand for this amino acid. (Pietkiewicz et al., 2021)that's disagreement with Research done by Li et al. 2020, Demonstrated elevated proline levels in plasma & serum samples obtained from BC patients in comparison to healthy controls.

The other investigations indicated a reduction in proline contents by (Wang et al., 2016) (Jasbi et al., 2019)potentially as a result of enhanced proline metabolism in cancer cells.Amino acids are a crucial category of metabolites. Many studies have emphasized their potential as biomarkers(Synakiewicz et al., 2021)They have a crucial function in numerous biological pathways, including the production of diverse components including nucleic acids, proteins, enzymes, etc. They also function as a source for energy and help keep redox equilibrium. (Wei et al., 2021).As tumors develop, the demand for free amino acids for the production of DNA and proteins in cells raise, lead to a decrease in the amount of these amino acids in the bloodstream. Conversely, the elevated levels of amino acids in tumors may suggest rapid growth rate.

The current research has identified several amino acids that may be suggestive of cancer, however the data is inconclusive. These discrepancies may arise from the utilization of distinct biological matrices of study, the application of diverse analytical techniques, or variations in patient populations among investigations. In addition, diagnostic procedures sometimes rely on biomarker panels composed of many chemicals instead of a single marker. Therefore, it appears that marker research should not concentrate on a single compound, but instead discover alterations in metabolic pathways that involve numerous substances typically connected by diverse interactions(Pietkiewicz et al., 2021)

The current study demonstrated a notable variance in the average CA15-3 among the various study categories. The greatest amount was observed in the newly diagnosed category, then by the postoperative category, and finally benign category. These findings were determined to be excellent indicators when assessed using (ROC) analysis, That's in agreement with (Hameed et

al., 2022) research, which revealed a substantial difference in the serum levels of primary BC (50±48.21U/ml) compared to benign breast tumors (19.28±6.57U/ml) vs control women (15.11±1.39U/ml). also study done by Zhang et al., 2013 , revealed that when the pathological staging increased, the levels of serum CA15-3, CEA, & CA125 in the observation group also increased. This increase was statistically significant when compared to other groups. Also reveal a significant difference in levels of serum CA15-3, CA125, and CEA were significantly higher among individuals with lymph node metastasis compared to those without lymph node metastasis.

Another investigation discovered that CA15-3levels drop following breast surgery. These results indicate that CA15-3plays a significant prognostic effect in BC. Although preoperative CA15-3results may be within the normal range, but postoperative CA15-3 results are necessary to identify progression of disease, recurrence or metastasis(Khan et al., 2016)

The current investigation shown a notable association between the level of CA15.3 and the Clinicopathological features; tumor sizes, nodal state, but not correlate with, hormone receptors & HER2, This is in line with a study performed by (Li et al., 2020) demonstrated in study including 10,836 women that CA-15.3 is correlate with tumor sizes, stages, nodal state& PR, but not correlate with grade, ER and HER2.

The present studies disagreement with previous study (Elfagieh et al., 2012) that demonstrated that CA15.3 not correlates with stages, tumor size, nodal state, grade & menopause status. There are contradictory findings in the scientific research, with the majority of studies finding no association between CA 15.3 & the status of several hormone receptors (Molina et al., 2010) (Yerushalmi et al., 2012). Nevertheless, several researches have established association (Choudhury and Agarwala, 2018; Daniele et al., 2013).

Approximately twenty percent of metastatic BC cases do not show increased levels of CA15-3and hence cannot be monitored using this tumor marker(Yerushalmi et al., 2012). Until now, there has been insufficient information to support the utilize of CA-15.3 in place of traditional follow-up (Clatot et al., 2020).This result is corroborating by numerous researches in the scientific literature, included those conducted by (Kumar et al., 2016). A study indicated that most often utilized blood marker for detect and monitor the activity of BC is CA-15.3.(Hussein et al., 2022)

The test involving the combination of CA15-3 and proline apppear a sensitivity and specificity of 100%. This suggests that the inclusion of proline in conjunction with CA15-3biomarkers enhances the sensitivity of the biomarker. The findings of this investigation indicate that utilizing a combination of proline and CA15-3may offer significant advantages, as these two tests seem to offer complementary information.

Ethical Clearance: The research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq.

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