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Serum Free HIF-1A RNA Level Correlation with Carbonic Anhydrase CAIX Patterns in Different Grade and Treatment Status of Breast Cancer

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Abstract : Breast cancer is a complex disease that has many different molecular subtypes and the most Tumor cells use several adaptations, such as carbonic anhydrase (CAIX) and hypoxia-inducible factor 1-alpha (HIF-1α), to survive and proliferate in hypoxic environments. This study aims to investigate the role of Carbonic anhydrase 9 (CAIX) in breast cancer and correlation with HIF1A gene expression. Different assay used in this research included RT-qPCR (Quantitative reverse transcription polymerase chain reaction) for HIF1A expression and the ELISA (Enzyme-linked immunosorbent Assay) technique for measuring CAIX activity. The study included 100 newly diagnosed cases of BC divided in to four groups. There were twenty-five patients with low grade of Bc before treatment (LBT) and twenty-five patients with low grade of Bc after treatment (LAT). The remaining twenty-five patients with high grade of Bc before treatment (HBT) and twenty-five patients with high grade of Bc after treatment (HAT). There were fifteen health individuals in the control group. After sample collection, the blood sample divided in to two aliquots. One for RNA extraction, cDNA synthesis and serum sample for CAIX measurement. The results of this research with area under the curve (AUC), showed HIF-1α expression with (AUC= 0.77) in high grade before treatment and AUC=0.6) in high grade after treatment. Other types of samples display (AUC< 0.4) indicate low diagnostic accuracy. CA9 showed exhibited moderate diagnostic result with (AUC=0.6) in high grade after treatment, others showed AUC <0.5). Furthermore, increasing median BMI and age group showed moderate significance with HIF-1α and CA9 expression in all experimental groups. The correlation analysis between HIF-1α, CA9 and control group displayed no significance for all experimental groups ($p>0.05$).

Key words: Breast cancer, Hypoxia, HIF1A gene expression, CAIX, ELISA

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Introduction

Cancer, including breast cancer, is a complex genetic disease(1). In recent decades, the incidence of breast cancer has rose, and about 13% of women develop breast cancer in their lifetime which caused over 40,000 deaths per year (2). Breast cancer is the primary cause of cancer among women and the leading cancer-related female mortality in Iraq (3). It causes death in 1 in 3 patients, and ranked second among the causes of death in women, with globally 2.3 million new cases of breast cancer recorded in 2022.Tumoral hypoxia, also associated with aggressiveness, in many cancers including, breast cancer. But measuring hypoxia is not an easy task (4). As a common feature of the tumor microenvironment (TME) in most solid

tumors, hypoxia is described as an imbalance between insufficient O2 delivery and rapid tumor growth (5). The acidic conditions of the microenvironment of solid tumors such as breast cancer can lead to aggressiveness and resistance to therapy, and it is one of the factors significant in changing the gene expression (6). To adapt to hypoxic conditions, the tumor cells undergo several drastic alterations that are beneficial for their survival and proliferation. Among these changes, two gene products are critically essential, Carbonic Anhydrase (CAIX) and Hypoxia-Inducible Factor1-alpha (HIF-1α) (7).The master regulator HIF-1α regulates the expression of carbonic anhydrase (CAIX), which is an endogenously reliable marker for hypoxia(8). CAIX expression has been associated with poor prognosis in different solid tumors, although its role in breast cancer remains unclear. In addition, CA9 mediates the expression of HIF1A, and activity of HIF1A leads to upregulation of CA9-induced genes (9).Carbonic Anhydrase 9 (CAIX) belongs to a family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide into bicarbonate and protons (10). CA9 is contributory to acidosis adaptation and relevant to the malignant progression of cancer (11). The gene encoding CA9 in humans is localized on chromosome 9q34.1 and consists of 12 exons. CA IX is a tumor-associated, cell-surface glycoprotein that is induced by hypoxia, involved in adaptation to acidosis and implicated in cancer progression by means of its catalytic activity and/or non-catalytic functions (12).

Materials and methods

Samples and data collection

A short, structured questionnaire was used to gather the data, and each participant's name, age, height, weight, and family history of breast cancer or other cancers were entered on a data collection sheet created specifically for this study. This study was approved by the Council of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies at the University of Baghdad, and the study protocol was approved by the Ethics Committee of the Ministry of Health and Environment- Al-Anbar Directorate of Health (AO No. 1812). All the requirement of this research with samples collection were done in the Oncology Center in Anbar Province from July 2024 to January 2025.

1n this study, one hundred Iraqi female participates, all of them were confirmed as newly diagnosed as breast cancer patients aged between 30-70 years. The selected patients were grouped according to specific criteria in to four groups, each group included 25 patients. Twenty five patients with low grade of Bc before treatment (LBT) and twenty five patients with low grade of Bc after treatment (LAT). Other twenty five patients with high grade of Bc before treatment (HBT) and twenty five patients with high grade of Bc after treatment (HAT). The control group included fifteen health participants. The blood samples were taken before treatment and six weeks after treatment (chemotherapy only) for all patients who attended Al-Anbar oncology Center. After venous blood collection, a volume of 1.5 ml of blood was collected and transferred into an EDTA anticoagulant tube. After gentle mixing. 0.5 ml of whole blood directly placed in Trizol preservation for the extraction of RNA and kept at -70°C until the

molecular analysis. The remaining blood samples were centrifuged at (3000) rpm for 5 minutes to obtain (0.3ml) of the serum stored at (-70°C) till examination of CAIX levels by ELISA.

Molecular assays

RNA extraction protocol

The extraction of total RNA including microRNA was carried out from the sample following the protocol of TRIzol™ Reagent; 0.2 mL of chloroform was added to the aqueous phase containing RNA; 0.5 mL of isopropanol was added for RNA precipitated as a white gel-like pellet; and 0.5 mL of 70% ethanol was used for RNA washing. In the end, the supernatant was discarded and the pellet was resuspended in 50µl of nuclease free water and the RNA kept in -70°C until RT-PCR reaction

Measurement of serum CAIX concentration

The Sandwich-ELISA kit (Sunlong Biotech, China) was utilized to evaluate the serum CAIX levels in the samples using an ELISA technique. The procedure was carried out in compliance with the instructions.

Reverse transcription

The complementary DNA (cDNA) synthesis was carried out using a reverse transcriptase kit (ELK Biotechnology, Chine). The reverse transcription master mix was placed in a 0.2 ml PCR tube, and the template RNA and cDNA primer were added and gently mixed. For reverse transcription, the thermal cycle settings were 25°C for 5 minutes every cycle, 42°C for 60 minutes, and 70°C for 1 minute.

Quantitative real-time polymerase chain reaction (RT-q PCR)

Following the manufacturer's instructions, the SYBR Green PCR Kit (Bioer LineGene, China) was used to perform Quantitative Real-Time Polymerase Chain Reaction (RT-q PCR). The reaction was carried out using a Real-Time PCR equipment and had a total volume of 20 µL. It contained 10 µL of SYBR Green Master Mix, 1 µL of specific primers forward and reverse, 3 µL of cDNA, and 6 µL of RNase-free water.

The following were the cycling conditions: 95˚C for 1 min 1 cycle and 45 cycles of 95˚C for 20 seconds and 60˚C for 30 seconds. HIF1A expression levels were presented in terms of fold change normalized by reference gene GAPDH using the formula **2^– ΔΔCT.** The primers set adopted in the current study listed in the (Table 1).

Table (1): HIF1A and GAPDH primers

Primer Name		***Sequence 5´- 3´***
HIF1A	Forward	GTCTCGAGATGCAGCCAGAT
	Reverse	CCTCACACGCAAATAGCTGA
GAPDH (Universal)	Forward	TGCCACCCAGAAGACTGTGG
	Reverse	TTCAGCTCAGGGATGACCTT

***Primers were designed by the researcher in current study**

Statistical analysis

The Statistical Analysis was performed using IBM SPSS Statistics 26.0. Program to detect the effect of difference factors in study parameters. Categorical data were expressed as numbers and percentages, whereas the nonparametric were expressed as the median and interquartile range (IQR). Receiver operating curve (ROC) analysis was employed to calculate the area under the curve (AUC).

Results

This study aimed to analyze the expression of a panel of biomarkers, including HIF-1A, and CA9, across different breast cancer subtypes (LBT, LAT, HBT, and HAT) besides to identify potential associations between these biomarkers and specific cancer subtypes in relation with age and Body Mass Index (BMI). This study involved 115 women were enrolled in the study, includes 100 women with newly diagnosed of breast cancer. The RT-q PCR results for HIF1A analyzed by the relative quantification of gene expression levels (folding changes) based on the (Ct) values. Study findings reveal distinct patterns of expression in relation to age and BMI and highlights potential links between these proteins, aging, obesity, and cancer stage-specific factors. Biomarkers in relation to age, BMI, and cancer subtype, suggesting complex regulatory mechanisms and potential roles in the biological characteristics of each subtype.

Distributional Disparities and Biological Insights

The analysis of the dataset reveals notable distributional disparities across several variables. Upon analysis of Body Mass Index (BMI) and age across five different sample groups, including a control group and four cancer-related groups (HAT, HBT, LAT, and LBT), it was that revealed that the control group exhibits the lowest median BMI and the narrowest interquartile range, indicating both a lower average BMI and less variability within this group compared to the cancer-related groups. The cancer-related groups generally show higher median BMIs and wider interquartile ranges, suggesting higher average BMIs and greater variability. Furthermore, assessing the age distributions for the same sample groups revealed that the control group demonstrates a lower median age and a narrower interquartile range, indicating that the control individuals were generally younger and had a smaller age range. The cancer-related groups, in contrast, show noticeably higher median ages and wider interquartile ranges, suggesting that these individuals were older and had a broader age range (Figure1).

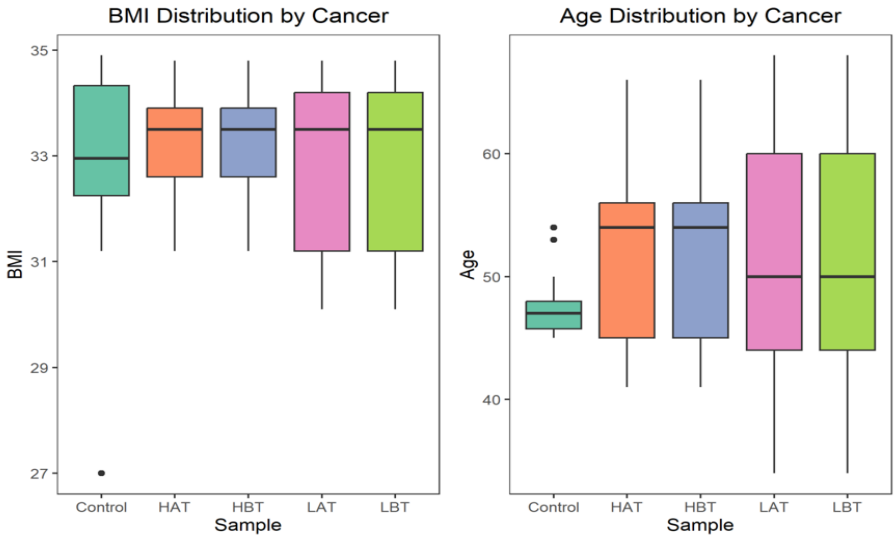

Figure (1): Comparative analysis of BMI and Age distributions across cancer and control groups

Biomarker Expression Analysis across Age and Cancer Grades

The study categorized samples into two age groups: "adult" (age < 50) and "old" (age > 50). The result for CA9, showed a notable increase in expression with age in most samples, particularly pronounced in HBT and LAT, suggests a potential elevation in CA9-related activity with age, possibly reflecting changes in the tissue microenvironment or an increased stress response in these specific sample types. While control samples maintained consistently low levels across age groups. This age-related increase in CA9 levels, especially within HBT and LAT samples. The control group demonstrated a lower median age and a narrower interquartile range, indicating that the control individuals were generally younger and had a smaller age range. The cancer-related groups, in contrast, showed noticeably higher median ages and wider interquartile ranges, suggesting that these individuals were older and had a broader age range (Figure 2).

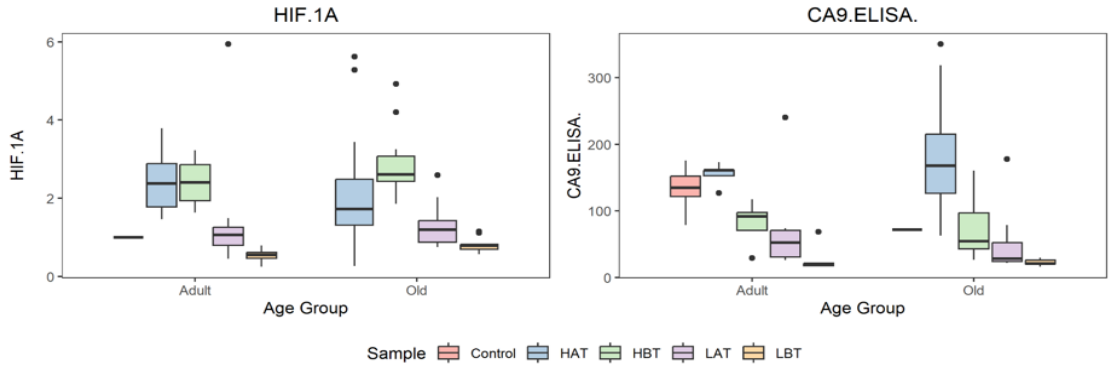

Figure (2): This figure is part of a series of figures (2-3) analyzing biomarker expression across age and cancer grades. It is grouped with Figure 3

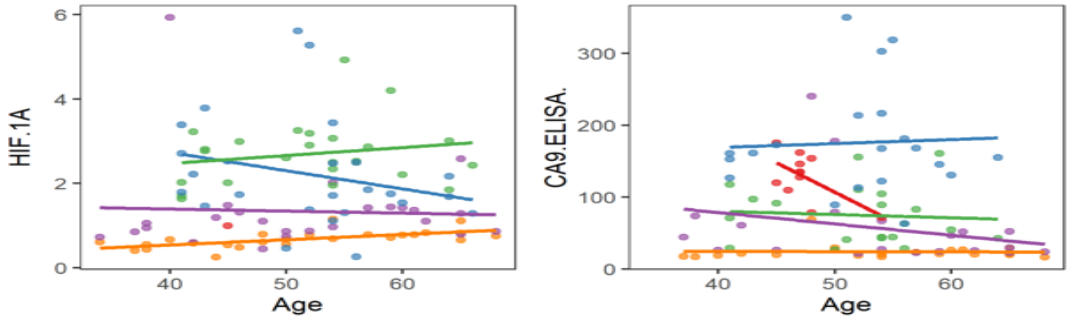

Figure (3): This figure is part of a series of figures (2-3) analyzing biomarker expression across age and cancer grades. Panel A shows the expression of HIF-1A and Panel B shows CA9

Biomarker Expression Analysis Across BMI and Cancer Grades

This study examined biomarker expression levels which included two proteins (HIF-1A, CA9) in individuals classified by Body Mass Index (BMI) as either "Overweight" or "Obese," with further stratification by sample type: Control, HAT, HBT, LAT, and LBT. HIF-1A exhibited higher expression in the obese group, most notably in HBT and LAT samples. Control samples consistently displayed low expression levels. This suggests a potential link between obesity and increased HIF-1A activity, possibly indicative of elevated cellular stress or hypoxia (Figure 4-5, Panel A). CA9 showed a significant

increase in expression in the obese group, particularly in HBT and LAT samples. Control samples maintained low levels across both BMI categories. This further supports the hypothesis of increased hypoxia-related activity in obesity, particularly within specific sample types (Figure 4-5, Panel B).

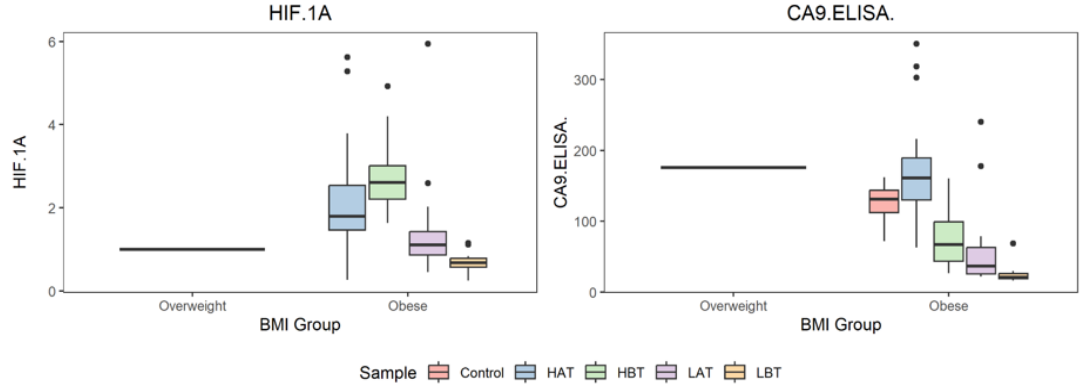

Figure (4): This figure is part of a series of figures (4-5) analyzing biomarker expression across age and cancer grades. It is grouped with Figure 5

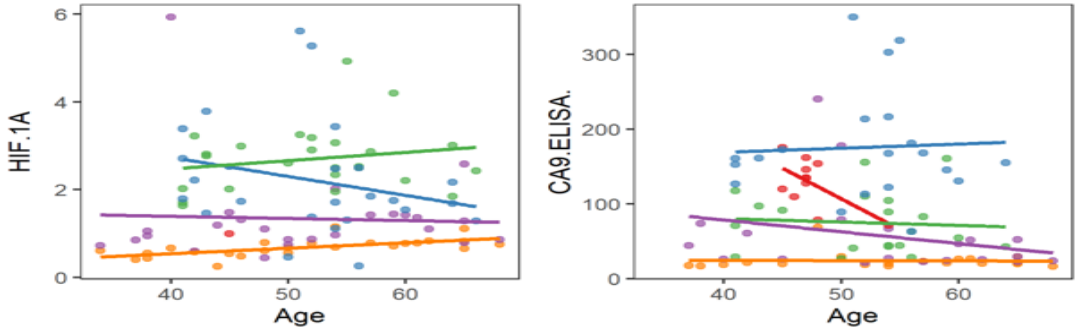

Figure (5): This figure is part of a series of figures (4-5) analyzing biomarker expression across age and cancer grades. Panel A shows the expression of HIF-1A and Panel B shows CA9 expression

HIF1A and CAIX Expression

According to HIF1A and CAIX results, the analysis revealed varying levels of performance (AUC) across different measurements and groups. Within the LBT group, HIF.1A (0.151), and CA9 (0.074) all had AUC values below 0.7. In the LAT group, all measurements HIF.1A (0.37), and CA9 (0.278), exhibited AUC values below 0.7. For the HBT group, only HIF.1A achieved an AUC greater than 0.7, with a value of 0.774. Whereas CA9 (0.344) fell below the 0.7 threshold. Finally, in the HAT group, HIF.1A (0.647) and CA9 had AUC values below 0.7, as shown in (table 2).

Table (2): AUC Analysis of Biomarkers with BC Groups vs. Control comparisons

Group*	Measurement	AUC
LBT	HIF.1A	0.151
	CAIX	0.074
LAT	HIF.1A	0.37
	CAIX	0.278
HBT	HIF.1A	0.774
	CAIX	0.344
HAT	HIF.1A	0.647
	CAIX	0.721

Group* LBT (Low grade before treatment) LAT (Low grade after treatment) HBT (high grade before treatment) HAT (high grade after treatment)

Hypoxia-inducible factor 1-alpha (HIF-1α) exhibited moderate diagnostic potential in the HBT vs. Control and HAT vs. Control comparisons (AUC > 0.60). This suggests that HIF1A may be a useful diagnostic marker for conditions associated with HBT samples, potentially reflecting its role in cellular stress response. CA9, a protein often associated with tumor hypoxia, displayed a complex expression pattern, with HBT samples exhibiting a notable increase with age, while LAT samples showed a less pronounced increase. Other sample types either showed a decrease or remained stable, with Control samples consistently displaying low levels. This suggests a potential age-related increase in CA9 expression in specific sample types, possibly related to changes in the tissue microenvironment or stress response.

Associating expression levels of HIF.1A and CA9 across different sample types: control, HAT, HBT, LAT, and LBT as shown in figure 6. This figure consists of two box plots: (A) panel comparing expression levels of HIF-1A with sample types. Y-axis (value): expression level of HIF.1A. The results showed HIF-1A was higher in HBT compared to LAT and LBT. Statistically significant differences: HBT vs. LAT and LBT ($p < 0.001$), Control vs. HBT ($p < 0.01$), Control vs. HAT: ns (not significant). For (B) panel which comparing expression levels of CA9 with sample types. Y-axis (value) CA9 protein levels measured by ELISA. CA9 levels were significantly elevated in Control and HAT, lower in LAT and LBT. Statistically significant differences: Control vs. LAT and LBT: ($p < 0.05$ to $p < 0.0001$). Control vs. HBT: ns (not significant). HAT vs. LBT: ($p < 0.05$).

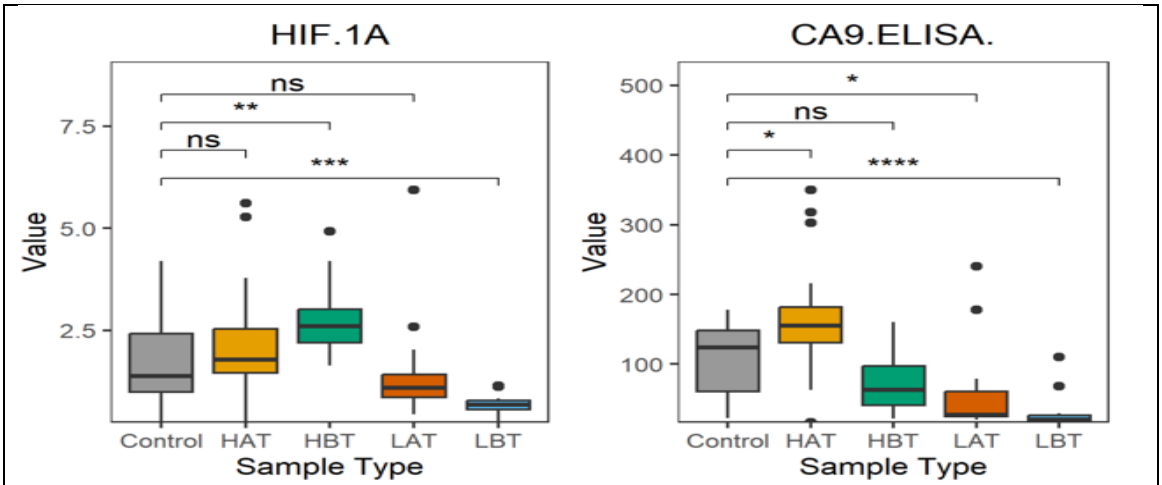

Figure (6): Expression levels for the biomarkers HIF1A and CA9 were distributed throughout different types of breast cancer samples

The expression levels measured for control group with HIF1A and CA9 biomarkers, the control condition does not show a significant correlation with the experimental groups (LBT, LAT, HBT, HAT) all p-values > 0.05.

Furthermore, high grade before treatment (HBT) samples showed increased levels of HIF-1A and CA9, which may indicate a connection between hypoxia and this particular subtype. This is because hypoxia

activates a complex cell signaling network in cancer cells, including the HIF. Others do not appear to be major direct players in these expression changes (p-values > 0.05). Furthermore, the correlations between these biomarkers were significantly altered under experimental conditions, highlighting the dynamic nature of their interactions.

The results correlation analyses for multiple comparisons HIF1A vs. CA9 showed the following results , LBT group (p= 0.37) , LAT group (p= 0.23), HBT group (p= 0.74) and HAT group (p= 0.2). There was no significant correlations with HIF1A between control group and all other all experimental groups (p>0.05)(Figure 7).

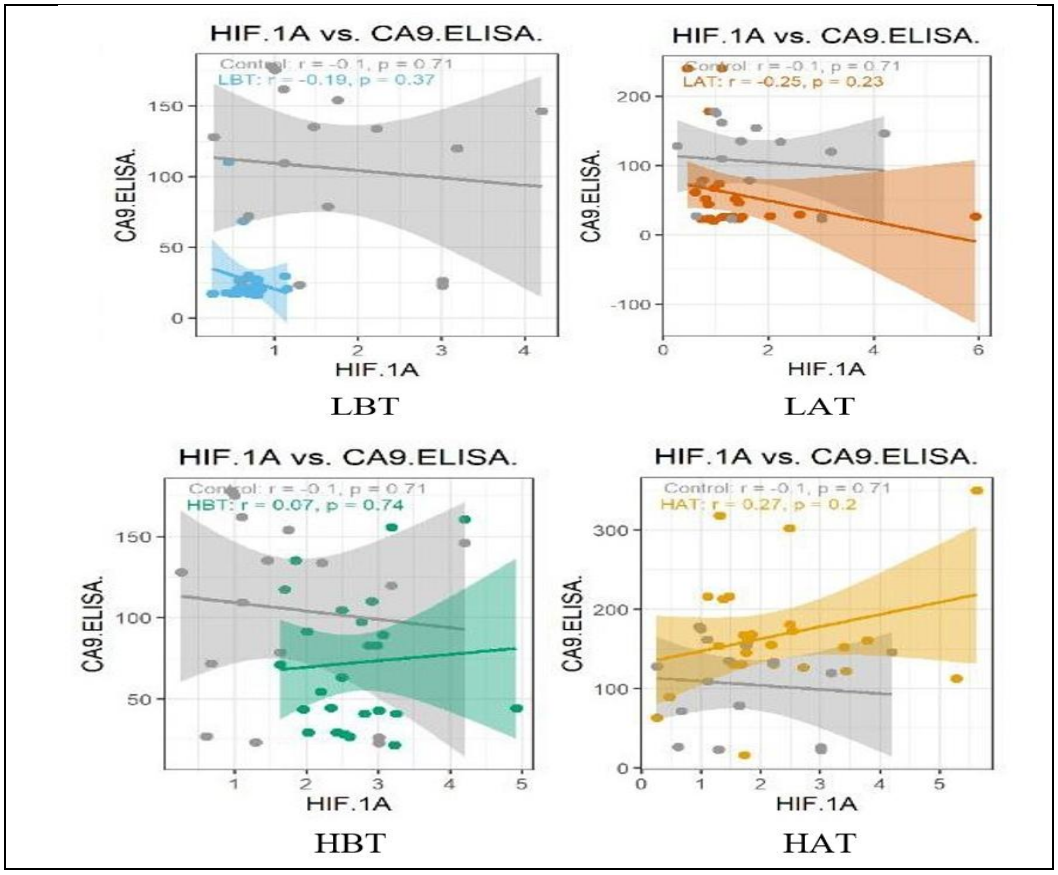

Figure (7): Correlation analysis of HIF1A vs. CA9. ELISA among different subtype of breast cancer

Discussion

According to age, BMI, and cancer subtype, results showed different patterns of expression for HIF1A and CA9 as biomarkers, pointing to intricate regulatory processes and possible roles in the biological traits of each subtype. The finding that the cancer-related groups' median BMIs were higher than those of the control group raises the possibility of a link between high BMI and cancer. The intricate connection between BMI and breast cancer subtypes has been the subject of numerous investigations. The current study's age-related BC was comparable to that of several Iraqi research, including (13, 14), which established that an elevated risk for BC was linked to age, particularly ≥50 years. However, this result that similar to Iraqi study by (Abbas & Aziz, 2022) mention that 70% of women with BC were over 50 years. According to another study by

(Al-khafaji et al., 2023), which split the patients into two age groups (≤50 and ≥50 years) based on age related to BC, the youngest age group had a 4% risk for BC-associated percentage, which was a highly significant difference (15). According to these findings (16,17), the risk of breast cancer can occur at any age, but it increases in middle age and beyond the age of fifty. As people age, their cells have more opportunity to create defects or mutate, which can lead to cancer or advanced age. (18) .In this study, HIF1A expression showed moderate significant with (AUC > 0.60) in high grade cancer before and after treatment, this indicate that HIF1A gene good diagnostic marker (19). Carbonic anhydrase CA9 in this research showed poor significant expression in major subtype of breast cancer, this agreed with (Hayes et al., 2001)) and his collages, they found that CA9 showed an independent poor prognostic biomarker for distant metastases and survival in breast cancer (20). Study found no statistically significant difference in CA9 levels with age, others had reported a correlation between CA9 expression and older age, both in general and specifically in sporadic male breast cancers (21).

Conclusion

The current study found that the expression level of HIF1A in breast cancer patients with high grade before and after treatment was higher than other types (low grade), this indicates its role in the aggressiveness of cancer of beast compare with other subtypes. Furthermore, control group showed no significance correlation with the experimental groups.CA9 on the other hand display lowest diagnostic accuracy across all comparisons, this suggested that CA9 is not a suitable diagnostic marker for BC patients in this study.

Approval

This study was approved by the Council of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies at the University of Baghdad, and the study protocol was approved by the Ethics Committee of the Ministry of Health and Environment- Al-Anbar Directorate of Health (AO No. 1812).

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