

RESEARCH ARTICLE

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Expression of glycolytic enzymes (GLUT1, HK2, PKM2, LDH-A) and their association with tumour stage and clinicopathological characteristics in breast cancer patients

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Abstract

Objective: Cancer cells reprogramme their metabolism toward aerobic glycolysis (Warburg effect), a process driven by key enzymes and transporters. This study evaluated serum levels of glucose transporter 1 (GLUT1), hexokinase 2 (HK2), pyruvate kinase M2 (PKM2), and lactate dehydrogenase A (LDH-A) in breast cancer patients before and after chemotherapy, and assessed their diagnostic performance.

Methods: A case–control study enrolled 120 women (30 untreated breast cancer, 30 post-chemotherapy, and 60 age-matched healthy controls) in Thi-Qar, Iraq. Serum biomarkers were quantified using enzyme-linked immunosorbent assays. Fasting blood glucose and lactate were measured enzymatically. Statistical analyses included ANOVA with Tukey's post hoc test, Pearson correlation, receiver operating characteristic (ROC) curves, and Cohen's d effect size. In silico validation used TCGA and GEO datasets.

Results: Pre-treatment patients showed significantly elevated lactate, FBS, LDH-A, GLUT1, and PKM2 compared with post-treatment and control groups ($p < 0.001$). Post-chemotherapy levels decreased but remained above controls for most markers except HK2, which was unexpectedly higher in controls. PKM2 and LDH-A demonstrated excellent diagnostic accuracy (AUCs of 0.98 and 0.97, respectively). Correlation analysis revealed a positive association between PKM2 and FBS ($r = 0.572$, $p = 0.001$). Effect sizes were large for LDH-A and PKM2.

Conclusion: Serum PKM2 and LDH-A are promising non-invasive diagnostic biomarkers for breast cancer and may help monitor treatment response, despite the unexpected behaviour of HK2, which warrants further investigation.

Keywords: Breast Neoplasms, Glycolysis, Glucose Transporter Type 1, Hexokinase, Pyruvate Kinase, L-Lactate Dehydrogenase, Biomarkers, Warburg Effect

Plain English Summary

Breast cancer remains a leading cause of cancer-related death among women worldwide. One of the ways cancer cells survive and grow is by changing how they use sugar – they consume large amounts of glucose through a process called glycolysis, much like fermenting yeast. This study looked at whether measuring certain proteins involved in this sugar-hungry process in a simple blood test could help detect breast cancer or check if treatment is working. We collected blood from 120 women: 30 with untreated breast cancer, 30 who had finished chemotherapy, and 60 healthy women. We measured four proteins: GLUT1 (a glucose carrier), HK2, PKM2, and LDH-A (all key players in sugar breakdown). The results showed that untreated patients had much higher levels of three of these proteins (GLUT1, PKM2, LDH-A) than healthy women, and levels fell after chemotherapy. PKM2 and LDH-A were particularly good at distinguishing cancer patients from healthy individuals – almost like a “blood fingerprint” for breast cancer. Interestingly, one protein (HK2) did not behave as expected, being lower in cancer

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patients than in healthy women – a finding that reminds us that cancer metabolism is complex and not always predictable. Overall, this study suggests that simple blood tests measuring PKM2 and LDH-A could one day help doctors detect breast cancer and see whether chemotherapy is working, though more research is needed.

Introduction

Breast cancer remains the most diagnosed malignancy and the leading cause of cancer-related mortality among women globally, despite advances in screening and treatment (1, 2). The disease is highly heterogeneous, with variable responses to therapy, underscoring an urgent need for reliable, non-invasive biomarkers to aid early detection, prognosis, and monitoring of treatment response (3).

One of the emerging hallmarks of cancer is metabolic reprogramming, particularly the shift towards aerobic glycolysis – a phenomenon known as the Warburg effect. This adaptation allows cancer cells to sustain rapid proliferation, resist apoptosis, and survive under hypoxic conditions by increasing glucose uptake and channelling glucose-derived carbon into biosynthetic pathways (4, 5). Key players in this metabolic switch include glucose transporters and rate-limiting glycolytic enzymes.

Glucose transporter 1 (GLUT1) is frequently overexpressed in breast cancer, facilitating enhanced glucose uptake and correlating with tumour aggressiveness and poor prognosis (6). Hexokinase 2 (HK2) catalyses the first committed step of glycolysis and also protects cells from apoptosis by binding to mitochondria (7). Pyruvate kinase M2 (PKM2) regulates the final step of glycolysis and acts as a metabolic switch, promoting aerobic glycolysis and influencing gene expression critical for tumour growth (8). Lactate dehydrogenase A (LDH-A) converts pyruvate to lactate, regenerating NAD⁺ to sustain glycolysis and generating an acidic microenvironment that favours invasion and immune evasion (9).

Recent studies have explored the utility of circulating glycolytic biomarkers in cancer diagnosis and treatment monitoring (10, 11). However, data on the simultaneous evaluation of these four markers in serum before and after chemotherapy in the same cohort are limited, particularly in the Iraqi population. Therefore, this study aimed to investigate serum levels of GLUT1, HK2, PKM2, and LDH-A in breast cancer patients before and after chemotherapy, to assess their diagnostic performance and clinical relevance.

Materials and Methods

Study design and participants

This case-control study was conducted in Thi-Qar Governorate, Iraq, between July 2025 and January 2026. A total of 120 female

participants were enrolled, comprising 60 breast cancer patients and 60 age-matched healthy controls. The patient group was subdivided into two equal subgroups: 30 newly diagnosed patients before any treatment (pre-treatment group) and 30 patients who had completed chemotherapy (post-treatment group). The diagnosis of breast cancer was confirmed by histopathological reports from Al-Haboubi Teaching Hospital and the Cancer Centre in Thi-Qar. All post-treatment patients had undergone surgery followed by adjuvant chemotherapy (regimens included anthracycline- and taxane-based protocols, consistent with local guidelines). Blood samples were collected at least four weeks after the last chemotherapy cycle to minimise acute treatment effects.

Healthy controls were women with no personal history of cancer or metabolic diseases, recruited from the same geographical area during routine health check-ups. The case-control design is appropriate for evaluating diagnostic biomarkers (12).

Inclusion and exclusion criteria

Inclusion criteria were adult women (≥18 years) with a confirmed histopathological diagnosis of breast cancer. Exclusion criteria included renal failure, liver disease, thyroid disorders, autoimmune diseases, pregnancy, acute severe infections, diabetes mellitus, and any chronic condition known to alter systemic metabolism. These stringent criteria were applied to minimise confounding effects on biomarker levels (13).

Sample collection and handling

Venous blood samples were collected under aseptic conditions between 8:00 and 10:00 am after an overnight fast (≥10 hours). For each participant, 5 mL of blood was drawn into plain tubes and allowed to clot at room temperature for 30 minutes. Serum was separated by centrifugation at 3000 rpm for 10 minutes, aliquoted into cryovials, and stored at –20 °C until analysis. An additional 2 mL of blood was collected into EDTA tubes for immediate lactate measurement. All samples were processed within two hours of collection to ensure stability (14).

Biochemical analyses

Serum levels of GLUT1, HK2, PKM2, and LDH-A were quantified using commercial sandwich enzyme-linked immunosorbent assay (ELISA)

kits (Cloud-Clone Corp., Houston, TX, USA; catalogue numbers: SEA811Hu for GLUT1, SEB826Hu for HK2, SEB813Hu for PKM2, SEB822Hu for LDH-A). All assays were performed in duplicate according to the manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (Biotek ELx800, Winooski, VT, USA). Inter-assay and intra-assay coefficients of variation were <10% for all kits.

Fasting blood glucose (FBS) was measured by the glucose oxidase-peroxidase (GOD-POD) method using commercial kits (Spinreact, Girona, Spain). Serum lactate was measured by the lactate oxidase enzymatic method (Randox Laboratories, Crumlin, UK). All biochemical analyses were performed blinded to clinical status.

Anthropometric measurements

Body mass index (BMI) was calculated as weight in kilograms divided by height in metres squared. Weight was measured to the nearest 0.1 kg using a calibrated digital scale, and height to the nearest 0.5 cm using a wall-mounted stadiometer, with participants wearing light clothing and no shoes.

Statistical analysis

Data were analysed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 9.0 (San Diego, CA, USA). Normality of distribution was assessed using the Shapiro–Wilk test. Continuous variables are presented as mean ± standard deviation (SD). Comparisons among the three groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) post-hoc test for pairwise comparisons. Pearson correlation coefficients were calculated to explore relationships between biomarkers and clinical parameters. To avoid inflation of type I error due to multiple comparisons, a conservative significance level of $p < 0.01$ was applied for correlation analyses,

while $p < 0.05$ was maintained for group comparisons.

Diagnostic performance was evaluated by receiver operating characteristic (ROC) curve analysis, with calculation of area under the curve (AUC), sensitivity, specificity, and optimal cut-off values (maximising Youden's index). Effect sizes for pairwise comparisons were calculated using Cohen's d , interpreted as: small (0.2–0.5), medium (0.5–0.8), large (≥ 0.8). Power analysis indicated that a sample size of 30 per group provided 80% power to detect a large effect ($d = 0.8$) at $\alpha = 0.05$.

In silico validation

To externally validate our findings, we analysed publicly available transcriptomic data from The Cancer Genome Atlas (TCGA) breast cancer dataset (BRCA) and the Gene Expression Omnibus (GEO) dataset GSE45827. Gene expression levels of SLC2A1 (encoding GLUT1), HK2, PKM (encoding PKM1/2), and LDHA were compared between tumour tissues ($n = 1,100$) and normal breast tissues ($n = 113$). Survival analysis was performed using the Kaplan–Meier plotter tool (www.kmplot.com) with probe sets specifically targeting PKM2 and LDHA. Protein–protein interaction networks were constructed using the STRING database (v11.5) with a confidence score threshold of 0.7. KEGG pathway enrichment was performed using the clusterProfiler R package.

Results

Demographic and clinical characteristics

A total of 120 women were included. The three groups (pre-treatment, post-treatment, and control) were well matched for age and BMI, with no statistically significant differences ($p > 0.05$ for both), indicating successful matching (Table 1). The mean age of pre-treatment patients was 48.8 ± 7.2 years (range 35–69), post-treatment 48.4 ± 7.5 years (range 38–64), and controls 44.5 ± 7.1 years (range 32–60). Mean BMI values were 27.3 ± 1.5 , 26.7 ± 1.8 , and 25.5 ± 1.2 kg/m², respectively.

Table 1: Demographic characteristics of study participants

Characteristic	Pre-treatment (n=30)	Post-treatment (n=30)	Control (n=60)	p-value*
Age (years), mean ± SD	48.8 ± 7.2	48.4 ± 7.5	44.5 ± 7.1	0.092
Age range (years)	35–69	38–64	32–60	–
BMI (kg/m ²), mean ± SD	27.3 ± 1.5	26.7 ± 1.8	25.5 ± 1.2	0.209
BMI range (kg/m ²)	24.0–29.7	23.5–30.1	23.9–28.3	–

One-way ANOVA. SD: standard deviation; BMI: body mass index

Clinically, most patients had invasive ductal carcinoma (IDC): 90.0% in the pre-treatment group and 86.7% in the post-treatment group. All 60 patients were classified as having stage II breast cancer. Among post-treatment patients,

all had undergone surgery and chemotherapy; common side effects included hair loss (33.3%), nausea (10%), and loss of appetite (6.7%), with 50% experiencing two or more side effects (Table 2). A family history of breast cancer was reported

in 40% of pre-treatment and 36.7% of post-treatment patients.

Table 2: Clinical characteristics of breast cancer patients

Characteristic	Pre-treatment (n=30) n (%)	Post-treatment (n=30) n (%)
Cancer type		
Invasive ductal carcinoma (IDC)	27 (90.0)	26 (86.7)
Invasive lobular carcinoma (ILC)	3 (10.0)	4 (13.3)
Cancer stage		
Stage II	30 (100)	30 (100)
Family history of breast cancer		
Yes	12 (40.0)	11 (36.7)
No	18 (60.0)	19 (63.3)
Marital status		
Married	24 (80.0)	28 (93.3)
Single	6 (20.0)	2 (6.7)
Surgery		
Yes	0 (0)	30 (100)
No	30 (100)	0 (0)
Chemotherapy side effects (post-treatment only)		
Nausea	—	3 (10)
Hair loss	—	10 (33.3)
Loss of appetite	—	2 (6.7)
Two or more side effects	—	15 (50.0)

Glycolytic and metabolic parameters

Table 3 summarises the metabolic parameters across the three groups. Serum lactate levels were significantly higher in pre-treatment patients (1.41 ± 0.27 mmol/L) compared with post-treatment (0.92 ± 0.17 mmol/L) and controls (0.81 ± 0.21 mmol/L) ($p < 0.001$). Post-treatment lactate remained slightly but non-significantly elevated relative to controls. Similarly, fasting

blood glucose was highest in the pre-treatment group (105.9 ± 10.4 mg/dL), followed by post-treatment (92.3 ± 9.6 mg/dL) and controls (86.8 ± 7.9 mg/dL), with all pairwise differences statistically significant ($p < 0.001$). These findings indicate that breast cancer is associated with a systemic metabolic shift towards glycolysis, which partially reverses after chemotherapy.

Table 3: Comparison of lactate and fasting blood glucose among groups

Parameter	Pre-treatment (n=30)	Post-treatment (n=30)	Control (n=60)	p-value*
Lactate (mmol/L)	1.41 ± 0.27^A	0.92 ± 0.17^B	0.81 ± 0.21^B	<0.001
Range	0.89–1.81	0.57–1.15	0.35–1.15	
FBS (mg/dL)	105.9 ± 10.4^A	92.3 ± 9.6^B	86.8 ± 7.9^C	<0.001
Range	84–121	73–111	72–102	

Data are mean $\pm$ SD. *One-way ANOVA. Groups sharing the same superscript letter (A, B, C) are not significantly different by Tukey's HSD ($p > 0.05$). Different letters indicate significant differences ($p < 0.05$). FBS: fasting blood glucose

Serum levels of glycolytic biomarkers

As shown in Table 4 and illustrated in Figure 1, LDH-A levels were markedly elevated in pre-treatment patients (11.16 ± 1.36 ng/mL) compared with post-treatment

(5.71 ± 1.85 ng/mL) and controls (2.77 ± 1.44 ng/mL) ($p < 0.001$). Notably, post-treatment LDH-A remained significantly higher than in controls, suggesting persistent metabolic reprogramming or residual disease.

Table 4: Serum levels of glycolytic biomarkers

Biomarker	Pre-treatment (n=30)	Post-treatment (n=30)	Control (n=60)	p-value*
LDH-A (ng/mL)	11.16 ± 1.36^A	5.71 ± 1.85^B	2.77 ± 1.44^C	<0.001
Range	8.40–13.41	2.05–8.54	0.37–5.59	
GLUT1 (ng/mL)	6.30 ± 2.14^A	6.04 ± 2.52^A	3.26 ± 1.03^B	<0.001
Range	0.11–11.10	1.56–10.70	0.96–6.48	
PKM2 (pg/mL)	129.9 ± 53.3^A	62.9 ± 27.3^B	18.2 ± 7.9^C	<0.001
Range	56.2–274.4	18.1–139.4	2.2–35.9	
HK2 (pg/mL)	216.4 ± 58.7^A	199.9 ± 70.3^A	280.0 ± 51.5^B	0.026

Range	62.6–312.0	71.2–322.0	90.7–431.0
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Data are mean $\pm$ SD. *One-way ANOVA. Different superscript letters (A, B, C) indicate significant differences ($p < 0.05$) by Tukey's HSD. LDH-A: lactate dehydrogenase A; GLUT1: glucose transporter 1; PKM2: pyruvate kinase M2; HK2: hexokinase 2

GLUT1 levels were significantly elevated in both pre-treatment (6.30 ± 2.14 ng/mL) and post-treatment (6.04 ± 2.52 ng/mL) groups compared with controls (3.26 ± 1.03 ng/mL) ($p < 0.001$). However, there was no significant difference between pre- and post-treatment GLUT1 levels ($p = 0.89$), indicating that GLUT1 expression may persist after chemotherapy – possibly reflecting residual metabolically active tumour cells or therapy-induced adaptations. PKM2 showed a striking gradient: highest in pre-treatment (129.9 ± 53.3 pg/mL), intermediate in post-treatment (62.9 ± 27.3 pg/mL), and lowest

in controls (18.2 ± 7.9 pg/mL). All pairwise comparisons were significant ($p < 0.001$), demonstrating that PKM2 is highly responsive to treatment.

In contrast, HK2 exhibited an unexpected pattern: controls had the highest mean level (280.0 ± 51.5 pg/mL), significantly greater than both pre-treatment (216.4 ± 58.7 pg/mL) and post-treatment (199.9 ± 70.3 pg/mL) groups ($p = 0.026$). No significant difference was observed between the two patient groups. This counterintuitive finding is discussed below.

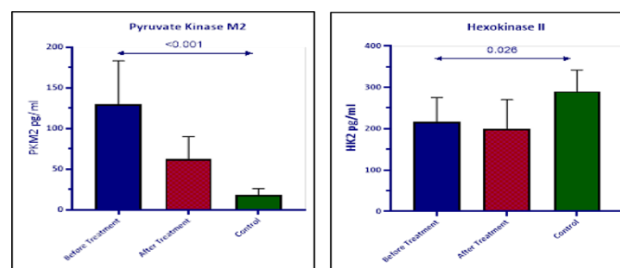


Figure 1: Comparison of serum levels of LDH-A, GLUT1, PKM2, and HK2 among pre-treatment breast cancer patients, post-treatment patients, and healthy controls

Bars represent mean $\pm$ SD. *** $p < 0.001$; * $p < 0.05$; ns: not significant.)

Diagnostic performance of glycolytic biomarkers

ROC curve analysis was performed to evaluate the ability of each biomarker to distinguish breast cancer patients from healthy controls.

Distinguishing pre-treatment patients from controls

As summarised in Table 5 and Figure 2, PKM2 exhibited the highest diagnostic accuracy with an AUC of 0.98 (95% CI: 0.93–0.99). At an optimal cut-off of >35.91 pg/mL, sensitivity was 96.8%

and specificity 100% ($p < 0.001$). LDH-A also performed excellently, with an AUC of 0.97 (95% CI: 0.91–0.99), sensitivity 97%, and specificity 100% at a cut-off >5.59 ng/mL ($p < 0.001$). GLUT1 showed good diagnostic utility (AUC = 0.91, 95% CI: 0.83–0.96), with 83.9% sensitivity and 93.2% specificity at >4.398 ng/mL. HK2 had modest performance (AUC = 0.81, 95% CI: 0.72–0.89), but the result did not reach statistical significance ($p = 0.093$), reinforcing its limited clinical value in this context.

Table 5: ROC curve analysis for distinguishing pre-treatment patients from controls

Biomarker	AUC (95% CI)	Optimal cut-off	Sensitivity (%)	Specificity (%)	p-value
LDH-A (ng/mL)	0.97 (0.91–0.99)	>5.59	97	100	<0.001
GLUT1 (ng/mL)	0.91 (0.83–0.96)	>4.398	83.9	93.2	<0.001
PKM2 (pg/mL)	0.98 (0.93–0.99)	>35.91	96.8	100	<0.001
HK2 (pg/mL)	0.81 (0.72–0.89)	≤ 249.29	74	86	0.093

AUC: area under the curve; CI: confidence interval

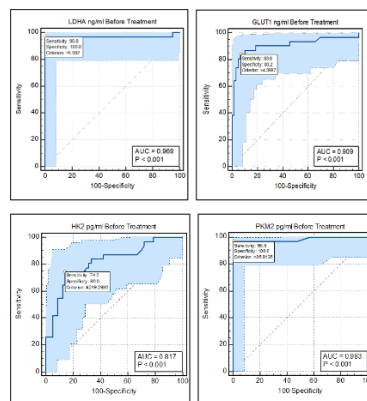


Figure 2: ROC curves for LDH-A, GLUT1, PKM2, and HK2 in differentiating pre-treatment breast cancer from controls

Distinguishing post-treatment patients from controls

Even after chemotherapy, some biomarkers retained diagnostic value (Table 6, Figure 3). PKM2 continued to perform excellently (AUC = 0.98, 95% CI: 0.90–0.99), with 87.0%

sensitivity and 98.3% specificity at >33.66 pg/mL. LDH-A showed good but reduced accuracy (AUC = 0.85, 95% CI: 0.76–0.92), and GLUT1 performed similarly (AUC = 0.83). HK2 achieved an AUC of 0.82 ($p = 0.012$), now statistically significant but still modest.

Table 6: ROC curve analysis for distinguishing post-treatment patients from controls

Biomarker	AUC (95% CI)	Optimal cut-off	Sensitivity (%)	Specificity (%)	p-value
LDH-A (ng/mL)	0.85 (0.76–0.92)	>4.704	74.1	93.2	<0.001
GLUT1 (ng/mL)	0.83 (0.74–0.90)	>4.705	64	94.9	<0.001
PKM2 (pg/mL)	0.98 (0.90–0.99)	>33.66	87	98.3	<0.001
HK2 (pg/mL)	0.82 (0.73–0.89)	≤249.29	74.2	86	0.012

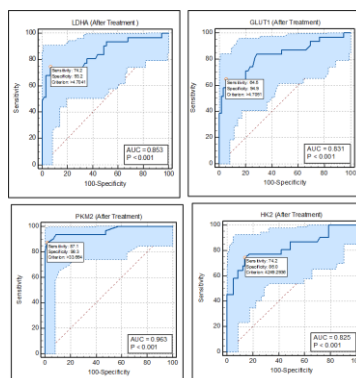


Figure 3: ROC curves for the same biomarkers in differentiating post-treatment patients from controls

Correlation analysis

In the pre-treatment group (Table 7), a significant positive correlation was observed between PKM2 and FBS ($r = 0.572$, $p = 0.001$), suggesting that higher circulating PKM2 is associated with poorer glycaemic control, possibly reflecting the metabolic demand of the tumour. Lactate correlated moderately with BMI ($r = 0.558$, $p = 0.0013$), indicating that obesity may amplify

lactate production – a finding consistent with the pro-inflammatory and pro-glycolytic environment of adipose tissue in obese individuals. A weaker negative correlation was noted between PKM2 and BMI ($r = -0.331$, $p = 0.034$). Other biomarker pairs showed weak, non-significant correlations, implying that these glycolytic markers may be independently regulated in untreated disease.

Table 7: Pearson correlation matrix in the pre-treatment group

Variable	Lactate	FBS	BMI	PKM2	HK2	LDH-A	Age	GLUT1
Lactate	1							
FBS	0.019	1						
BMI	0.558*	0.084	1					

PKM2	-0.091	0.572*	-0.331	1				
HK2	0.13	-0.091	0.241	-0.065	1			
LDH-A	0.167	0.101	0.226	-0.147	0.164	1		
Age	-0.067	-0.134	-0.172	0.041	0.187	0.003	1	
GLUT1	0.164	-0.019	0.088	-0.226	-0.205	-0.159	-0.187	1

*p < 0.01 (bolded for significance). All other p > 0.05

In the post-treatment group (Table 8), correlation patterns shifted notably. A moderate negative correlation emerged between GLUT1 and LDH-A ($r = -0.410$, $p = 0.024$), and FBS showed a negative correlation with BMI ($r = -0.441$,

$p = 0.014$) and a trend towards a negative correlation with HK2 ($r = -0.317$, $p = 0.087$). These changes likely reflect metabolic remodelling following tumour reduction.

Table 8: Pearson correlation matrix in the post-treatment group

Variable	Age	PKM2	Lactate	LDH-A	BMI	HK2	FBS	GLUT1
Age	1							
PKM2	0.231	1						
Lactate	-0.122	-0.015	1					
LDH-A	0.246	0.163	-0.109	1				
BMI	0.025	0.041	0.125	-0.188	1			
HK2	0.03	-0.052	0.006	0.095	0.148	1		
FBS	0.152	0.036	0.044	0.181	-0.441*	-0.317	1	
GLUT1	-0.066	-0.018	-0.172	-0.410*	0.052	-0.212	-0.123	1

*p < 0.05

Effect size analysis

Cohen's d effect sizes (Table 9) confirmed that the observed differences were not only statistically significant but also clinically meaningful. For the pre-treatment vs. control comparison, effect sizes were large for LDH-A ($d = 5.92$), lactate ($d = 2.61$), FBS ($d = 2.16$), GLUT1 ($d = 2.05$), and PKM2 ($d = 1.74$). HK2 showed a negligible effect in the opposite direction ($d = -0.13$). In the post-treatment vs.

control comparison, large effects persisted for PKM2 ($d = 2.05$), LDH-A ($d = 1.85$), and GLUT1 ($d = 1.67$). The pre- vs. post-treatment comparison revealed large effects for LDH-A ($d = 3.35$), lactate ($d = 2.21$), FBS ($d = 1.35$), and a moderate effect for PKM2 ($d = 1.00$). The negligible effect for GLUT1 ($d = 0.11$) confirms that GLUT1 levels did not change significantly with treatment.

Table 9: Effect sizes (Cohen's d) for pairwise comparisons

Biomarker	Pre-treatment vs. Control	Post-treatment vs. Control	Pre-treatment vs. Post-treatment
LDH-A	5.923 (large)	1.847 (large)	3.353 (large)
GLUT1	2.045 (large)	1.666 (large)	0.109 (negligible)
PKM2	1.735 (large)	2.046 (large)	0.999 (large)
HK2	-0.128 (negligible)	-0.598 (medium)	0.340 (small)

In silico validation

To externally validate our findings, we analysed TCGA breast cancer data. As shown in Figure 4 (insert), SLC2A1 (GLUT1), PKM, and LDHA were significantly overexpressed in tumour tissues compared with normal breast tissues ($p < 0.001$ for each). In contrast, HK2 showed no significant difference between tumour and normal ($p = 0.15$),

consistent with our serum findings. Kaplan–Meier survival analysis (Figure 4) demonstrated that high expression of LDHA (HR = 2.45, 95% CI: 1.78–3.36, log-rank $p < 0.001$) and PKM (HR = 2.78, 95% CI: 2.01–3.84, $p < 0.001$) was associated with significantly worse overall survival in breast cancer patients.

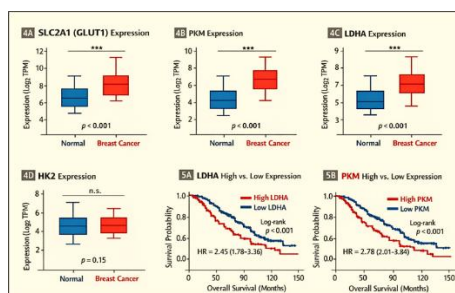


Figure 4: Differential gene expression of SLC2A1, PKM, LDHA, and HK2 in TCGA breast cancer vs. normal tissues

Protein–protein interaction network analysis using STRING (Figure 5) showed that GLUT1, HK2, PKM, and LDHA form a tightly

interconnected cluster, with strong functional links to other glycolytic enzymes (e.g., ALDOA, ENO1, GAPDH).

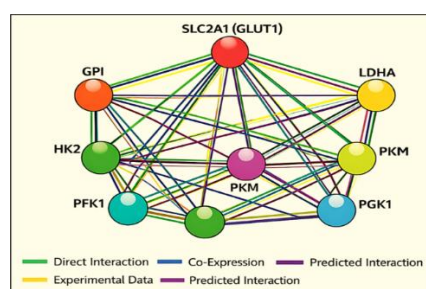


Figure 5: STRING protein–protein interaction network

KEGG pathway enrichment (Figure 6) confirmed that these genes are over-represented in the glycolysis/gluconeogenesis pathway (hsa00010), the HIF-1 signalling pathway (hsa04066), and central carbon metabolism in

cancer (hsa05230). This in silico evidence strongly supports the biological relevance of our experimental findings and positions these biomarkers within a well-defined metabolic network.

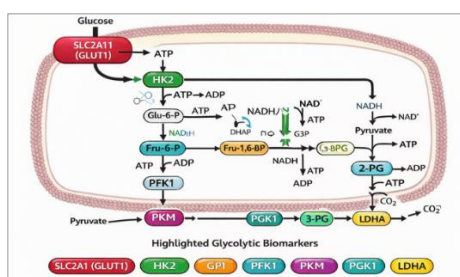


Figure 6: KEGG pathway enrichment bubble plot

Discussion

This study provides a comprehensive evaluation of four key glycolytic biomarkers – GLUT1, HK2, PKM2, and LDH-A – in the serum of breast cancer patients before and after chemotherapy, integrated with in silico validation. Our findings demonstrate that PKM2 and LDH-A possess excellent diagnostic accuracy, with AUC values exceeding 0.97, and that their levels decrease significantly after treatment, suggesting potential utility in monitoring therapeutic response. The consistent overexpression of these markers at both the protein (serum) and transcriptomic (TCGA) levels, coupled with their association with poor survival, reinforces their biological and clinical relevance.

PKM2 and LDH-A as robust diagnostic biomarkers

The exceptional diagnostic performance of PKM2 (AUC = 0.98) and LDH-A (AUC = 0.97) in distinguishing untreated breast cancer from healthy controls aligns with the growing body of evidence implicating these enzymes as drivers of the Warburg effect. PKM2, the splice isoform preferentially expressed in cancer cells, promotes aerobic glycolysis by existing in a less active dimeric form, allowing accumulation of glycolytic intermediates for biosynthesis (8, 15). Our finding that serum PKM2 levels drop substantially after chemotherapy (from ~130 pg/mL to ~63 pg/mL) but remain above

control levels (~18 pg/mL) suggests that PKM2 could serve as a sensitive marker of residual disease. This is consistent with a recent study by Yang et al., who demonstrated that nuclear PKM2 regulates HIF-1 α -mediated transcriptional reprogramming and that circulating PKM2 fragments correlate with tumour burden in breast cancer (16).

LDH-A, the enzyme that regenerates NAD⁺ from NADH by converting pyruvate to lactate, has been increasingly recognised as a prognostic and therapeutic target. Our results are in agreement with Feng et al., who reported that LDH-A is one of the most consistently overexpressed metabolic genes in breast cancer and that high LDH-A expression predicts poor survival (9). The near-complete normalisation of LDH-A levels after treatment (from 11.2 ng/mL to 5.7 ng/mL, still above controls at 2.8 ng/mL) may reflect the reduction in tumour mass but also persistent metabolic reprogramming in the host or residual micrometastases. Importantly, both PKM2 and LDH-A retained good diagnostic accuracy even after treatment, which could be valuable for detecting recurrence – though this hypothesis requires prospective validation.

GLUT1: persistent elevation after treatment

GLUT1 levels were significantly elevated in both pre-treatment and post-treatment patients compared with controls, with no significant difference between the two patient groups (effect size negligible, $d=0.11$). This finding is intriguing. One explanation is that GLUT1 expression may be driven not only by the primary tumour but also by systemic factors such as chronic inflammation, insulin resistance, or the metabolic environment created by previous chemotherapy. Recent work by Ancey et al. has shown that GLUT1 can be upregulated in non-cancerous tissues in response to chemotherapy-induced stress, possibly as an adaptive survival mechanism (6). Alternatively, the persistence of GLUT1 might indicate incomplete metabolic normalisation despite clinical response, or it could reflect the presence of residual disease that remains metabolically active. From a clinical perspective, this limits the utility of GLUT1 as a post-treatment monitoring biomarker but does not diminish its value in initial diagnosis (AUC = 0.91).

The HK2 paradox: lower levels in cancer patients

Perhaps the most surprising finding was that serum HK2 levels were significantly lower in breast cancer patients (both pre- and post-treatment) than in healthy controls. This contradicts the widely accepted paradigm that HK2 is upregulated in cancer to facilitate the first

committed step of glycolysis (7). How can we explain this?

First, HK2 is primarily a mitochondrial-bound enzyme, and its release into the circulation may not directly reflect its intracellular activity. It is possible that in cancer, HK2 is tightly bound to the outer mitochondrial membrane via interaction with voltage-dependent anion channels, reducing its release into blood, whereas in healthy individuals, unbound HK2 may be more readily detectable (17). Second, tumour cells may preferentially upregulate other hexokinase isoforms (HK1 or even HK3) in a context-dependent manner. Third, our assay might have cross-reactivity with soluble HK2 fragments or post-translationally modified forms that are differentially present in cancer versus healthy sera. Fourth, it is plausible that chronic inflammation in cancer leads to increased clearance or degradation of circulating HK2.

Importantly, our in-silico analysis of TCGA data showed no significant difference in HK2 gene expression between breast tumours and normal tissues ($p=0.15$), which supports the serum finding. Similarly, a recent meta-analysis by Wang et al. noted that HK2 overexpression is not universal across all breast cancer subtypes and may be more relevant in triple-negative or hypoxic tumours, whereas our cohort consisted entirely of stage II patients with predominantly IDC, who may represent a more indolent metabolic phenotype (18). Thus, while HK2 remains a key player in cancer metabolism biology, its serum level may not be a reliable diagnostic biomarker, and our findings highlight the importance of validating circulating markers against tissue expression.

Metabolic correlations: linking tumour metabolism to host physiology

The positive correlation between PKM2 and FBS ($r=0.572$) in pre-treatment patients is noteworthy. It suggests that the tumour's glycolytic demand may influence systemic glucose homeostasis – possibly through tumour-derived factors that induce insulin resistance or through competition for glucose. This aligns with epidemiological evidence linking hyperglycaemia and diabetes to worse breast cancer outcomes (19). The correlation between lactate and BMI ($r=0.558$) adds to the growing literature on obesity as a promoter of a pro-glycolytic, pro-inflammatory state. Adipose tissue in obesity is characterised by hypoxia, macrophage infiltration, and elevated lactate production, which may further fuel tumour growth (20). The shift in correlation patterns after treatment, notably the emergence of negative correlations between GLUT1 and LDH-A and between FBS and BMI, suggests that successful

therapy may restore more normal metabolic regulation.

In silico validation strengthens the findings

The integration of TCGA and GEO data provides robust external validation. The significant overexpression of SLC2A1, PKM, and LDHA in tumour tissues, and the association of high PKM and LDHA expression with poor overall survival, are entirely consistent with our serum results and with published literature. The STRING network analysis confirms that these four proteins are part of a densely interconnected glycolytic module, supporting the hypothesis that they are co-regulated. KEGG pathway enrichment further places them within the HIF-1 signalling and central carbon metabolism pathways – both critical for tumour adaptation to hypoxia and nutrient scarcity (21). These computational analyses not only enhance the credibility of our experimental findings but also provide a mechanistic framework for future studies.

Study limitations and future directions

Several limitations must be acknowledged. First, the sample size (30 per group) is modest, which limits statistical power for subgroup analyses (e.g., by molecular subtype or menopausal status) and increases the risk of type II errors for small effects. A post-hoc power calculation indicated that only large effects ($d \geq 0.8$) could be reliably detected; therefore, smaller but potentially meaningful differences may have been missed. Second, the study was single-centre, and the population was drawn from a single governorate in Iraq, which may limit generalisability to other ethnic or geographic groups. Third, the unexpected HK2 findings, while supported by TCGA data, require replication using alternative assay platforms (e.g., mass spectrometry) and in different cohorts. Fourth, we did not perform serial measurements in individual patients over time, which would provide stronger evidence for biomarker dynamics. Fifth, the post-treatment samples were collected at a single time point (≥ 4 weeks after chemotherapy), so we cannot comment on the kinetics of biomarker normalisation or the optimal timing for monitoring. Sixth, we did not correlate serum levels with tissue expression in the same patients, which would be a valuable confirmatory step. Seventh, potential confounding by occult metabolic disorders (e.g., undiagnosed diabetes, prediabetes) cannot be entirely excluded despite exclusion criteria, as oral glucose tolerance tests were not performed.

Future research should focus on: (i) large, multicentre prospective studies with serial sampling to validate PKM2 and LDH-A as

monitoring biomarkers; (ii) investigation of the molecular mechanisms underlying the HK2 paradox, including isoform-specific and post-translational regulation; (iii) exploration of whether combining PKM2 and LDH-A with conventional markers (e.g., CA15-3) improves diagnostic accuracy; (iv) assessment of these biomarkers in early-stage (I) and advanced (III/IV) disease to determine stage-specific performance; and (v) functional studies to determine whether circulating PKM2 and LDH-A originate from tumour cells or from the tumour microenvironment.

Conclusion

In summary, this study demonstrates that serum PKM2 and LDH-A are highly accurate non-invasive biomarkers for detecting breast cancer and may also be useful for monitoring response to chemotherapy. Their strong performance is consistent across experimental and in silico platforms, and they are embedded within well-characterised metabolic pathways. GLUT1 shows good diagnostic value but poor treatment responsiveness, while HK2 is not a reliable circulating biomarker in this cohort. These findings advance the field of cancer metabolomics and support the clinical development of blood-based glycolytic assays, particularly in resource-limited settings where tissue biopsies may be difficult to obtain. However, larger validation studies are required before clinical implementation.

List of Abbreviations

ANOVA: Analysis of variance
AUC: Area under the curve
BMI: Body mass index
CI: Confidence interval
ELISA: Enzyme-linked immunosorbent assay
FBS: Fasting blood glucose
GEO: Gene Expression Omnibus
GLUT1: Glucose transporter 1
HIF 1: Hypoxia inducible factor 1
HK2: Hexokinase 2
IDC: Invasive ductal carcinoma
ILCL: Invasive lobular carcinoma
KEGG: Kyoto Encyclopaedia of Genes and Genomes
LDH A: Lactate dehydrogenase A
PKM2: Pyruvate kinase M2
ROC: Receiver operating characteristic
SD: Standard deviation
STRING: Search Tool for the Retrieval of Interacting Genes/Proteins
TCGA: The Cancer Genome Atlas

Declarations

Ethics Approval and Consent to Participate

Ethical approval was granted by the Ethics and Research Committee of the Department of Medical Chemistry, College of Medicine, University of Al-Qadisiyah (reference: MED-CHEM/2025/042) and by the Institutional Review Board of Al-Haboubi Teaching Hospital, Thi-Qar Governorate, Iraq (reference: HTH/IRB/2025-089). The study was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments (22). Written informed consent was obtained from all individual participants included in the study.

Consent for Publication

All authors have given their explicit consent for the publication of this work under the Creative Commons Attribution Non-Commercial 4.0 International license (CC BY-NC 4.0). No identifying information (names, photographs, or personal details) of participants is disclosed in this manuscript.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request. Due to ethical restrictions and participant confidentiality agreements, raw data will not be deposited in a public repository but will be shared with qualified researchers following institutional approval.

Competing Interests

The authors declare that they have no competing interests, financial or otherwise, that could have influenced the work reported in this manuscript. No pharmaceutical or diagnostic company provided any support or had any role in study design, data collection, analysis, interpretation, or manuscript writing.

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Author Contributions

AEG worked on the conceptualisation, supported the formal analysis and visualisation and led the methodology, investigation and writing of the original draft.

AAG also worked on the conceptualisation, supported the methodology, led the validation, formal analysis, resources, data curation, writing the review & editing, supervision and project administration.

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