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ORIGINAL RESEARCH ARTICLE

Glycolytic Enzyme Profile in Beta-Thalassemia Major: Coordinated Alterations in Hexokinase, Pyruvate Kinase, and Phosphofructokinase with Implications for Therapeutic Targeting

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ABSTRACT

Background: Mature erythrocytes depend exclusively on glycolysis for ATP production, making glycolytic enzymes critical for red blood cell survival. Beta-thalassemia major is characterized by oxidative stress and metabolic perturbations that may affect glycolytic enzyme function. **Objective:** To characterize the glycolytic enzyme profile (hexokinase [HK], pyruvate kinase [PK], phosphofructokinase [PFK]) and *PFKP* gene expression in beta-thalassemia major patients and to evaluate inter-enzyme correlations indicative of coordinated metabolic regulation. **Methods:** This case-control study included 42 patients with beta-thalassemia major and 30 healthy controls. Serum enzyme levels were measured by ELISA, and *PFKP* gene expression was assessed using RT-qPCR with the $2^{-}\Delta\Delta Ct$ method. **Results:** Patients demonstrated significantly elevated HK (327.71 ± 282.40 vs. 131.47 ± 158.67 pg/mL; $p = 0.0012$) and PFK (1.83 ± 1.52 vs. 0.51 ± 0.53 ng/mL; $p < 0.0001$). A very strong positive correlation existed between HK and PFK ($r = 0.874$, $p < 0.0001$), with moderate correlations among all enzymes. PFKP gene expression did not correlate with serum protein levels ($r = 0.006$, $p = 0.968$). **Conclusion:** Beta-thalassemia major exhibits coordinated elevation of glycolytic enzymes, with PFK showing the highest diagnostic potential. The discordance between gene expression and protein levels suggests post-transcriptional regulation. These findings support therapeutic strategies targeting glycolytic metabolism, including pyruvate kinase activators.

HIGHLIGHTS

• Hexokinase and phosphofructokinase are significantly elevated in patients with beta-thalassemia major.

• A very strong correlation between HK and PFK ($r = 0.874$) suggests coordinated regulation of glycolysis.

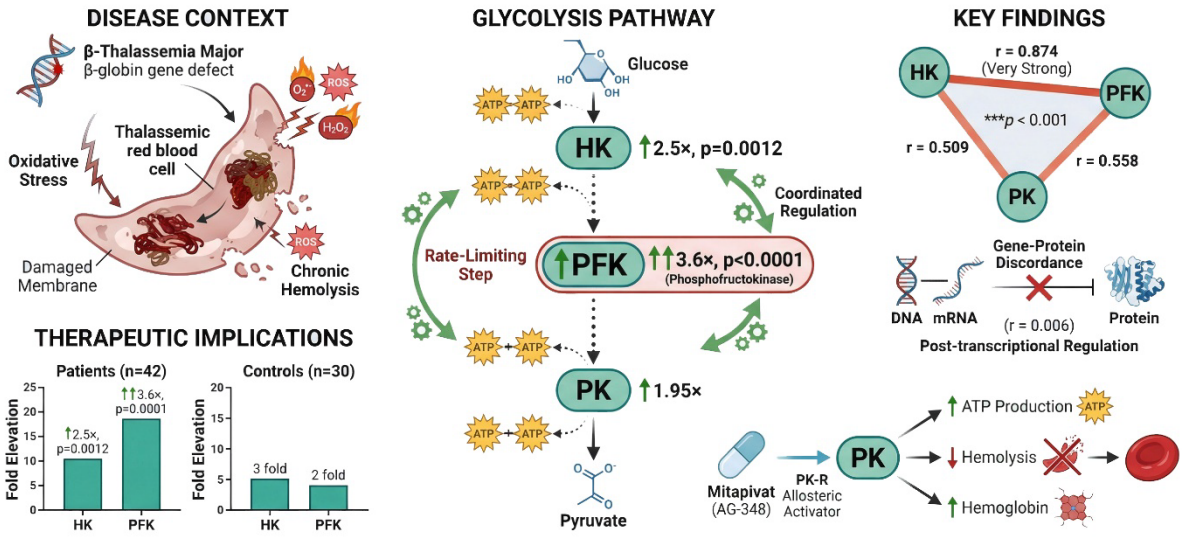
• All three glycolytic enzymes show significant intercorrelations ($r = 0.509–0.874$).

• *PFKP* gene expression does not correlate with serum PFK protein levels, indicating post-transcriptional control.

• Findings support the use of pyruvate kinase activators as potential therapeutic agents in thalassemia..

Keywords: Beta-thalassemia major; Glycolytic enzymes; Hexokinase; Pyruvate kinase; Phosphofructokinase; PFKP gene expression; Erythrocyte metabolism; Pyruvate kinase activators; Biomarkers s

GRAPHICAL ABSTRACT



1. Introduction

Mature human erythrocytes are unique among cells in their complete dependence on anaerobic glycolysis for energy production. Lacking nuclei and mitochondria, red blood cells cannot perform oxidative phosphorylation and must rely solely on the Embden-Meyerhof pathway to generate adenosine triphosphate (ATP). This metabolic pathway, comprising ten enzymatic reactions, is essential for maintaining erythrocyte membrane integrity, ion gradients, and the deformability required for microcirculatory passage [1,2]. Consequently, defects in glycolytic enzymes have profound implications for red blood cell survival and function.

Three enzymes serve as critical regulatory checkpoints in erythrocyte glycolysis: hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK). Hexokinase catalyzes the first committed step, phosphorylating glucose to glucose-6-phosphate, thereby trapping glucose within the cell. Phosphofructokinase is the rate-limiting enzyme, converting fructose-6-phosphate to fructose-1,6-bisphosphate and serving as the principal control point for glycolytic flux. Pyruvate kinase catalyzes the final ATP-generating step, converting phosphoenolpyruvate to pyruvate [3,4]. Genetic deficiencies in any of these enzymes result in hereditary hemolytic anemias, underscoring their critical importance.

Beta-thalassemia major, characterized by absent or severely reduced beta-globin chain synthesis, creates a hostile intracellular environment marked by oxidative stress from excess alpha-globin precipitation and iron-catalyzed reactive oxygen species generation [5,6]. This oxidative milieu has been shown to damage glycolytic enzymes by oxidizing critical sulfhydryl groups, potentially compromising their catalytic function. However, the coordinated changes in glycolytic enzyme levels and their interrelationships in beta-thalassemia remain incompletely characterized [7,8].

Recent therapeutic advances have highlighted the potential of targeting glycolytic metabolism in hemolytic anemias. Mitapivat, a first-in-class allosteric activator of pyruvate kinase-R, has demonstrated

efficacy in reducing hemolysis and improving anemia in both pyruvate kinase deficiency and thalassemia syndromes [9,10]. Understanding the glycolytic enzyme profile in beta-thalassemia may inform the application of such metabolic therapies. This study aimed to comprehensively characterize serum levels of HK, PK, and PFK, alongside PFKP gene expression, and to evaluate their interrelationships in Iraqi patients with beta-thalassemia major.

2. Materials and methods

2.1. Study design and participants

This case-control study was conducted at the Center for Genetic Blood Diseases (Thalassemia Center), Al-Diwaniyah Health Directorate, Iraq, between October 2024 and February 2025. The study enrolled 42 patients with confirmed beta-thalassemia major and 30 age-matched healthy controls (age range: 6–35 years). Diagnosis was established by hemoglobin electrophoresis, which demonstrated elevated HbF (defined as HbF >90% of total hemoglobin) with reduced or absent HbA (<10%), in accordance with Thalassemia International Federation guidelines. Exclusion criteria included other thalassemia variants (alpha-thalassemia, thalassemia intermedia), systemic comorbidities (diabetes mellitus, hepatic or cardiac failure), chronic infections, malignancies, and acute illness at the time of sampling. Ethical approval was obtained from the institutional review board, and written informed consent was acquired from all participants or their legal guardians.

Table 1 summarizes the demographic and baseline clinical characteristics of the study population. The mean age of patients was 15.8 ± 8.4 years compared to 17.2 ± 7.9 years in controls ($p = 0.47$). The patient group comprised 23 males (54.8%) and 19 females (45.2%), while the control group included 16 males (53.3%) and 14 females (46.7%), with no significant difference in sex distribution between groups ($p = 0.91$). Age and sex matching between groups was confirmed using independent t-test and chi-square test, respectively. The study population included both pediatric (<18 years; n = 28 patients, n = 18 controls) and adult (≥18 years; n = 14 patients, n = 12 controls) participants [11,12]. Given the known physiological differences between age groups, this represents a methodological limitation that should be considered when interpreting the results; however, subgroup analysis was not performed due to limited sample size in each stratum.

Baseline hematological parameters in thalassemia patients showed characteristic findings: hemoglobin concentration 7.2 ± 1.1 g/dL (range: 5.1–9.8 g/dL), red blood cell count 3.1 ± 0.6 × 10^{12}/L, mean corpuscular volume (MCV) 68.4 ± 8.2 fL, mean corpuscular hemoglobin (MCH) 21.8 ± 3.1 pg, and hematocrit 22.6 ± 4.2%. Control subjects demonstrated normal hematological values: hemoglobin 13.8 ± 1.4 g/dL, RBC count 4.8 ± 0.5 × 10^{12}/L, MCV 86.2 ± 5.4 fL, MCH 28.4 ± 2.1 pg, and hematocrit 41.2 ± 3.8%. All hematological parameters differed significantly between groups ($p < 0.001$).

Regarding transfusion profile, all patients were transfusion-dependent and received regular packed red blood cell transfusions at intervals of 3–4 weeks. The mean pre-transfusion hemoglobin concentration was 7.2 ± 1.1 g/dL, and all samples were collected immediately prior to scheduled transfusion to minimize the confounding effect of recently transfused cells on metabolic measurements. The mean transfusion interval was 25.4 ± 5.2 days. Serum ferritin levels were markedly elevated in patients (1,847 ± 1,124 ng/mL; range: 486–4,892 ng/mL), indicating significant iron overload [13,14]. All patients (42/42, 100%) were receiving iron chelation therapy: 31 patients (73.8%) were on deferasirox monotherapy, 8 patients (19.0%) on deferoxamine, and 3 patients (7.1%) on combination therapy. Chelation therapy adherence was self-reported and not independently verified, which should be considered a limitation of the study.

Table 1. Demographic and baseline clinical characteristics of study participants

Parameter	Patients (n=42)	Controls (n=30)	p-value
Age (years), mean ± SD	15.8 ± 8.4	17.2 ± 7.9	0.47
Sex (Male/Female), n (%)	23/19 (54.8/45.2)	16/14 (53.3/46.7)	0.91
Hemoglobin (g/dL)	7.2 ± 1.1	13.8 ± 1.4	<0.001
RBC count ($\times 10^{12}$/L)	3.1 ± 0.6	4.8 ± 0.5	<0.001
MCV (fL)	68.4 ± 8.2	86.2 ± 5.4	<0.001
MCH (pg)	21.8 ± 3.1	28.4 ± 2.1	<0.001
Hematocrit (%)	22.6 ± 4.2	41.2 ± 3.8	<0.001
Serum Ferritin (ng/mL)	1847 ± 1124	N/A	—
Transfusion interval (days)	25.4 ± 5.2	N/A	—

Values are expressed as mean ± SD or n (%). N/A: Not applicable. Statistical significance: $p < 0.05$.

2.2. Blood sample collection and processing

Venous blood samples (6 mL) were collected between 8:00 AM and 2:00 PM via venipuncture. For thalassemia patients, samples were obtained prior to scheduled transfusion. Blood was distributed as follows: 2 mL into K_3-EDTA tubes for RNA extraction, and 4 mL into serum separator tubes. Serum samples were obtained after clotting (15 minutes at room temperature), followed by centrifugation (3600 rpm, 6 minutes). Aliquots were stored at −20°C pending analysis. All laboratory procedures were performed at the Biochemistry Research Laboratory, College of Science, University of Baghdad.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Serum concentrations of hexokinase, pyruvate kinase, and phosphofructokinase were quantified using commercial sandwich ELISA kits (Bioassay Technology Laboratory, China) according to manufacturer protocols. The assay principle involves capture of target proteins by pre-coated antibodies, detection with biotinylated secondary antibodies, and colorimetric quantification using HRP-streptavidin conjugate with TMB substrate. Standard curves were generated for each analyte (**Table 2**), and optical density was measured at 450 nm within 10 minutes of the addition of the stop solution. All samples were analyzed in duplicate, and concentrations were extrapolated from standard curves.

Table 2. ELISA standard curve parameters for glycolytic enzymes

Enzyme	Standard Range	R^2 Value	Units
Hexokinase (HK)	75 - 900	0.970	pg/mL
Pyruvate Kinase (PK)	40 - 480	0.990	pg/mL
Phosphofructokinase (PFK)	0.3 - 3.6	0.995	ng/mL

2.4. RNA Extraction, cDNA Synthesis, and RT-qPCR

Total RNA was isolated from whole blood using TRIzol™ reagent (Invitrogen, USA) following the manufacturer's protocol. RNA concentration and purity were assessed using Qubit 4 Fluorometer (Invitrogen). Complementary DNA synthesis was performed using EasyScript® First-Strand cDNA Synthesis SuperMix (Transgen, China) with the thermocycler program: priming (25°C, 10 min), reverse transcription (50°C, 60 min), enzyme inactivation (80°C, 5 min), and hold (12°C).

Quantitative real-time PCR was performed on a Bioer LineGene 4800 system (Japan) using Luna Universal qPCR Master Mix with SYBR Green detection. *PFKP* (phosphofructokinase, platelet type) was the target gene, with *GAPDH* serving as the endogenous reference. Primer sequences are presented in **Table 3**. Reaction conditions included an initial denaturation step (95°C, 5 min), followed by 40 cycles of denaturation

(95°C, 20 sec), annealing (56.3°C, 30 sec), and extension (72°C, 30 sec), with melting curve analysis for specificity verification. Relative gene expression was calculated using the $2^{-}\Delta\Delta Ct$ method (Livak method).

Table 3. RT-qPCR primer sequences

Gene	Primer Sequence (5'-3')	Amplicon
PFKP (Forward) *PFKP* (Reverse)	GACTCAGGATGTGCAGAAGGC CCCCACGTTGATGACAGCTAC	169 bp
GAPDH (Forward) *GAPDH* (Reverse)	ATCACCATCTTCCAGGAGCGA CAGAGGGGGCAGAGATGATGA	157 bp

2.5. Statistical analysis

Statistical analyses were performed using SPSS version 26 (IBM Corporation) and Microsoft Excel 2016. Continuous variables were tested for normality using the Kolmogorov-Smirnov test and expressed as mean ± standard deviation (SD). Between-group comparisons were performed using an independent-samples t-test. Pearson's correlation coefficient was employed to assess relationships between continuous variables. Correlation strength was interpreted as: weak ($|r| < 0.3$), moderate ($0.3 \leq |r| < 0.7$), and strong ($|r| \geq 0.7$). Statistical significance was defined as $p \leq 0.05$, with $p \leq 0.01$ considered highly significant.

3. Results and discussion

3.1. Glycolytic enzyme profile in beta-thalassemia major

The glycolytic enzyme profile demonstrated significant alterations in beta-thalassemia major patients compared to healthy controls (**Table 4**; **Figures 1–3**). Hexokinase levels were significantly elevated in patients (327.71 ± 282.40 pg/mL) compared to controls (131.47 ± 158.67 pg/mL; $p = 0.0012$), representing a 2.5-fold increase. The concentration range was notably wider in patients (25.18–848.71 pg/mL) versus controls (4.59–246.94 pg/mL), indicating substantial inter-patient variability. Phosphofructokinase exhibited the most pronounced elevation, with patient levels (1.83 ± 1.52 ng/mL) approximately 3.6-fold higher than controls (0.51 ± 0.53 ng/mL; $p < 0.0001$). This was the most statistically significant finding among the parameters studied, suggesting that PFK may be a sensitive marker of metabolic perturbation in thalassemia. Pyruvate kinase showed a trend toward elevation in patients (227.00 ± 288.48 pg/mL) compared to controls (116.30 ± 158.38 pg/mL), though this difference did not reach statistical significance ($p = 0.0643$). The marginally significant result may reflect the smaller effect size or the high variability observed within both groups.

Table 4. Serum glycolytic enzyme levels in beta-thalassemia patients and controls

Parameter	Control (n=30)	Patients (n=42)	Fold Δ	p-value
HK (pg/mL)	131.47 ± 158.67 *(4.59-246.94)*	327.71 ± 282.40 *(25.18-848.71)*	2.49×	0.0012**
PK (pg/mL)	116.30 ± 158.38 *(27.05-224.19)*	227.00 ± 288.48 *(27.05-950.86)*	1.95×	0.0643
PFK (ng/mL)	0.51 ± 0.53 *(0.12-0.77)*	1.83 ± 1.52 *(0.16-4.65)*	3.59×	<0.0001****

*Data expressed as Mean ± SD (Range); Fold Δ = fold change vs. control; **p ≤ 0.01; ****p ≤ 0.0001*

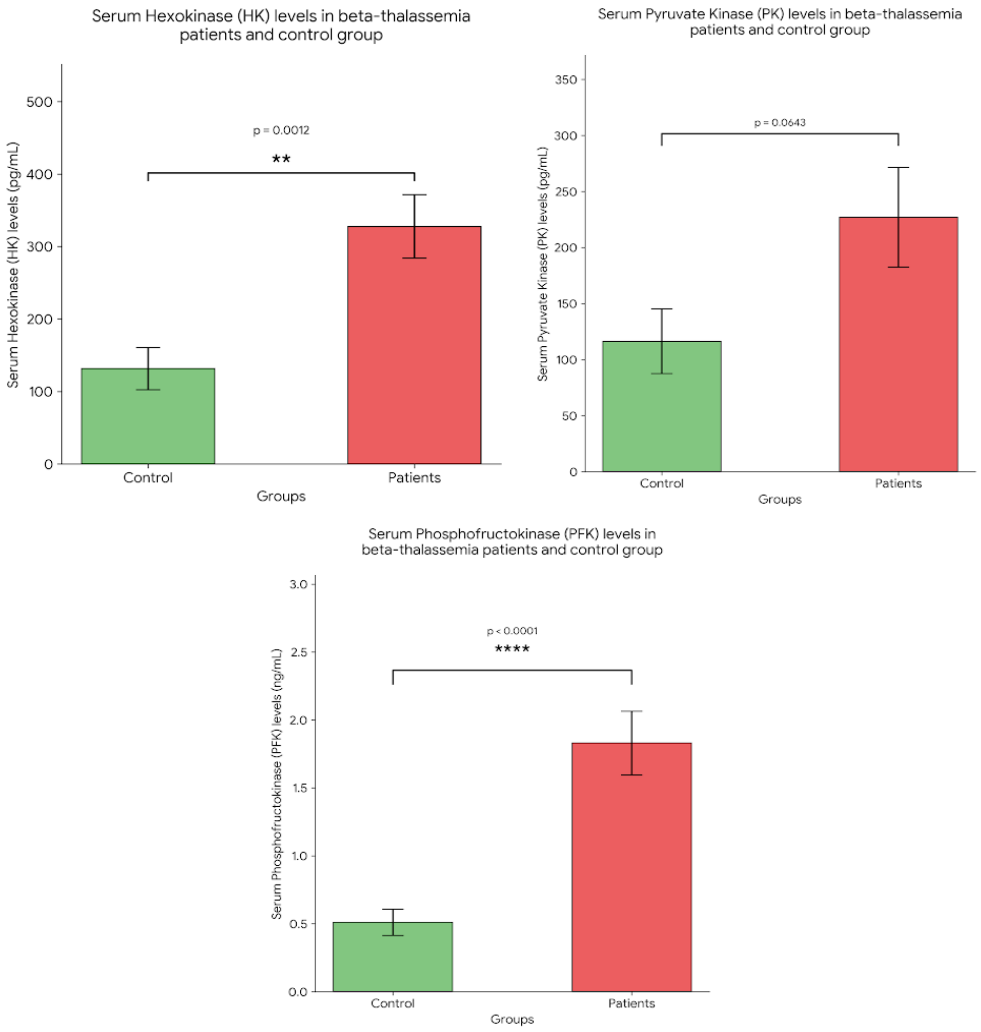


Figure 1. Serum glycolytic enzyme levels. (A) Hexokinase, (B) Pyruvate Kinase, (C) Phosphofructokinase. Data expressed as mean ± SEM. **p ≤ 0.01; ****p ≤ 0.0001 vs. control.

The elevation of glycolytic enzymes in beta-thalassemia patients likely reflects multiple pathophysiological mechanisms. First, accelerated erythropoiesis results in an increased proportion of young erythrocytes and reticulocytes, which contain substantially higher glycolytic enzyme activities than mature red cells—approximately 10-fold higher for hexokinase [15,16]. Second, chronic hemolysis leads to continuous release of intracellular enzymes into the circulation as damaged erythrocytes are cleared by the reticuloendothelial system [17].

Third, metabolic adaptation to the oxidative stress environment may drive compensatory upregulation of glycolytic capacity. The increased metabolic demands imposed by oxidative damage to membranes and proteins necessitate greater ATP production for cellular repair mechanisms [18,19]. Finally, hypoxia—resulting from chronic anemia—activates hypoxia-inducible factor 1-alpha (HIF-1α), which transcriptionally upregulates multiple glycolytic enzymes as part of the cellular adaptation to low oxygen conditions [20].

3.2. Coordinated regulation: inter-enzyme correlations

A striking finding of this study was the strong positive correlations observed among the three glycolytic enzymes (**Table 5**; **Figure 2**). The correlation between hexokinase and phosphofructokinase was particularly robust ($r = 0.874$, $p < 0.0001$), representing a very strong positive association. Moderate positive correlations were observed between hexokinase and pyruvate kinase ($r = 0.509$, $p = 0.0006$) and between pyruvate kinase and phosphofructokinase ($r = 0.558$, $p = 0.0001$).

Table 5. Correlation matrix among glycolytic enzymes in beta-thalassemia patients (n=42)

Enzyme Pair	r value	p-value	Interpretation
HK vs PFK	0.874	<0.0001***	Very Strong (+)
HK vs PK	0.509	0.0006***	Moderate (+)
PK vs PFK	0.558	0.0001***	Moderate (+)

*r: Pearson correlation coefficient; ***p < 0.001; (+): positive correlation*

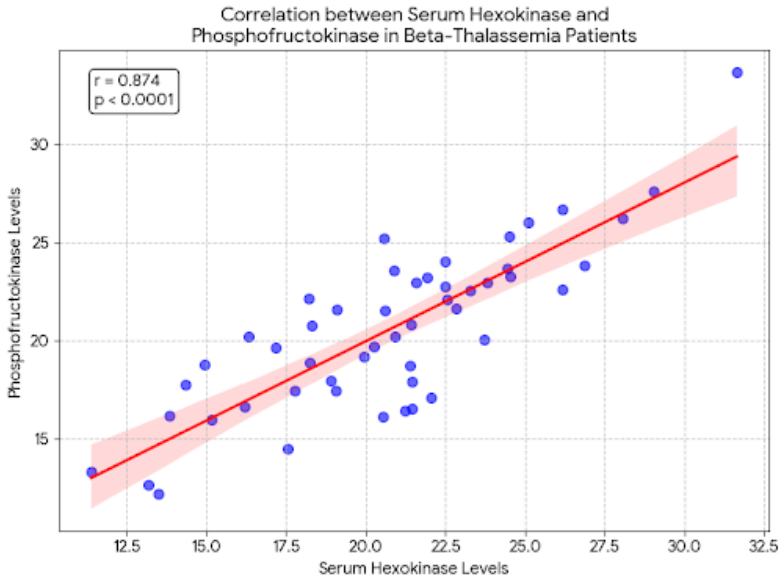


Figure 2. Scatter plot demonstrating the very strong positive correlation between serum hexokinase and phosphofructokinase levels in beta-thalassemia patients (r = 0.874, p < 0.0001). Dashed line indicates linear regression.

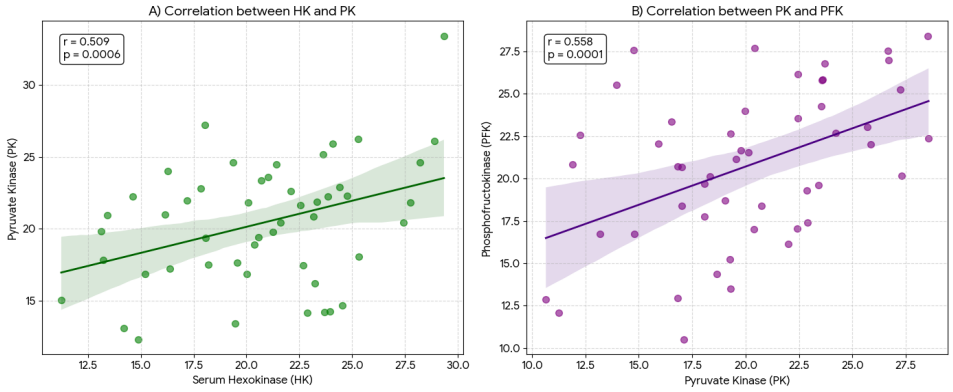


Figure 3. Scatter plots showing correlations among glycolytic enzymes. (A) HK vs PK (r = 0.509), (B) PK vs PFK (r = 0.558). All correlations are statistically significant (p < 0.001).

The particularly strong correlation between HK and PFK (r = 0.874) is mechanistically significant. These enzymes catalyze the two major irreversible, committed steps of glycolysis—glucose phosphorylation and the rate-limiting conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, respectively. Their coordinated elevation suggests either common transcriptional regulation (potentially via HIF-1α or other metabolic master regulators) or proportional release from erythrocytes during hemolysis [21,22]. From a physiological perspective, coordinated regulation of HK and PFK would ensure that increased glucose entry into the pathway is matched by increased flux through the rate-limiting step, preventing accumulation of potentially inhibitory intermediates. This metabolic coordination may represent an adaptive response to the increased energy demands in thalassemic erythrocytes [23].

3.3. PFKP Gene Expression: Discordance with Protein Levels

RT-qPCR analysis of *PFKP* gene expression revealed notable discordance with serum protein measurements (**Table 6**; **Figure 4**). While the mean fold change was numerically higher in patients (51.01 ± 145.76) compared to controls (16.49 ± 43.36), this difference was not statistically significant (p = 0.219). Both groups exhibited substantial gene expression variability, as reflected in the high standard deviations.

Table 6. PFKP gene expression analysis and correlation with serum PFK protein

Parameter	Control (n=30)	Patients (n=42)	p-value
PFKP Ct	25.80 ± 1.43	27.39 ± 2.08	-
GAPDH Ct	22.00 ± 3.28	23.27 ± 4.47	-
Fold Change ($2^{-}\Delta\Delta Ct$)	16.49 ± 43.36	51.01 ± 145.76	0.219 (NS)
PFKP FC vs PFK protein	r = 0.006, p = 0.968	No correlation	

Ct: cycle threshold; FC: Fold Change; NS: non-significant

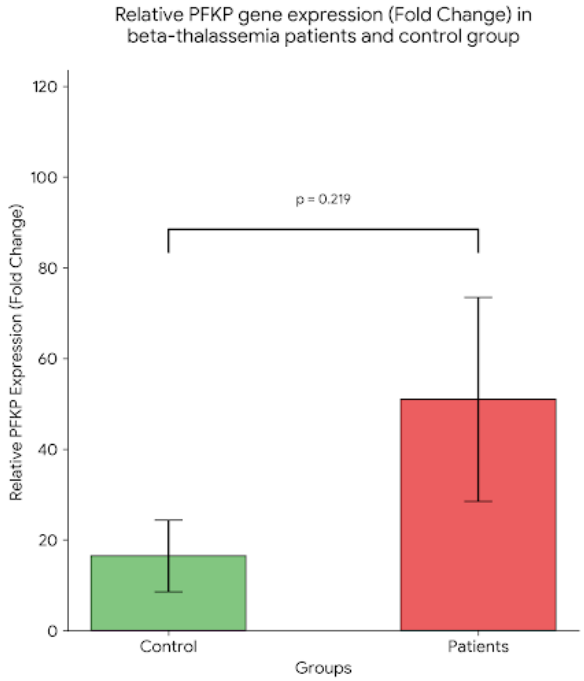


Figure 4. PFKP gene expression (Fold Change) in beta-thalassemia patients and controls. Despite numerical increase, the difference was not statistically significant (p = 0.219). Data expressed as mean ± SEM.

The most striking observation was the complete lack of correlation between PFKP gene expression and serum PFK protein levels (r = 0.006, p = 0.968). This discordance has important implications for understanding metabolic regulation in beta-thalassemia and suggests that serum enzyme levels are predominantly determined by post-transcriptional mechanisms rather than transcriptional upregulation [24,25].

Several factors may account for this mRNA-protein discordance: (1) Serum enzyme levels primarily reflect release from lysed or damaged cells rather than de novo synthesis; (2) Protein stability and degradation rates may differ substantially from mRNA half-life; (3) Peripheral blood mRNA does not represent expression in erythroid precursors, the cells most relevant to enzyme production; (4) Post-translational modifications and allosteric regulation affect protein detection independently of expression levels [26,27].

3.4. Therapeutic Implications: Pyruvate Kinase Activators

The glycolytic enzyme profile characterized in this study is directly relevant to emerging therapeutic strategies for beta-thalassemia. Pyruvate kinase activators, particularly mitapivat (AG-348), represent a novel approach to improving erythrocyte metabolism and reducing hemolysis. Mitapivat is a first-in-class oral allosteric activator that increases PK-R affinity for its substrate phosphoenolpyruvate, thereby enhancing ATP production even under conditions of metabolic stress [28,29].

Clinical trials have demonstrated that mitapivat increases hemoglobin levels and reduces transfusion requirements in patients with thalassemia. The ENERGIZE and ENERGIZE-T trials showed meaningful improvements in anemia and reductions in hemolysis markers. The mechanism involves enhanced glycolytic flux, leading to improved ATP availability, better maintenance of ion gradients and membrane integrity, and reduced phosphatidylserine exposure, which triggers macrophage-mediated clearance [30,31].

Our findings of coordinated elevation of glycolytic enzymes, particularly the strong HK-PFK correlation, suggest that the glycolytic pathway remains functionally intact in thalassemia despite the oxidative stress environment. This preserved metabolic machinery may be amenable to pharmacological activation through PK activators, which could amplify the compensatory metabolic response already evident in these patients [32].

3.5. Summary of findings

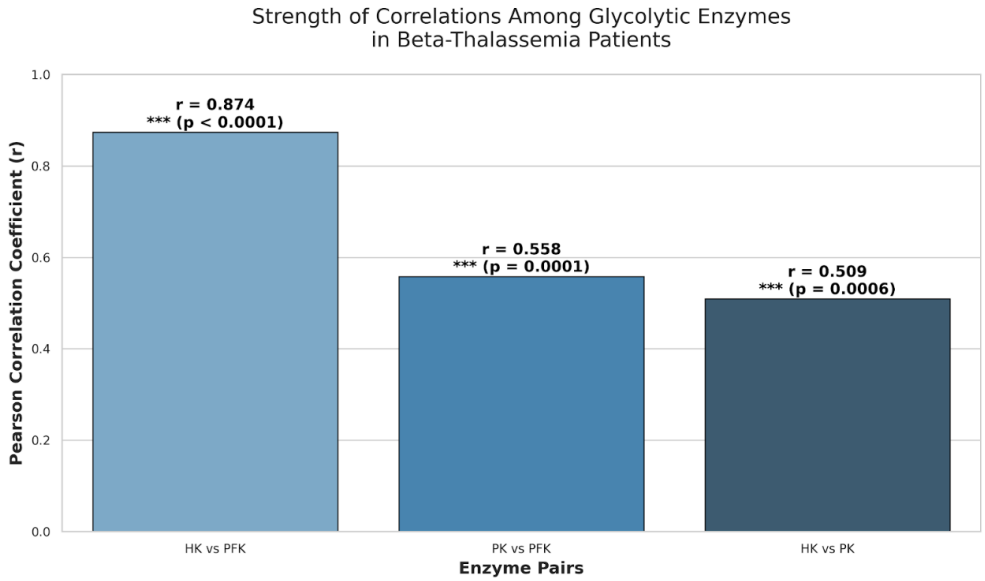


Figure 5. Correlation heatmap summarizing relationships among glycolytic enzymes and PFKP gene expression in beta-thalassemia patients. Strong correlations (darker colors) are observed among HK, PK, and PFK, while PFKP fold change shows no significant correlation with enzyme protein levels.

Table 7. Summary of key findings

Finding	Result	Clinical Implication
PFK elevation	3.6× increase (p<0.0001)	Potential diagnostic biomarker
HK-PFK correlation	r = 0.874 (p<0.0001)	Coordinated glycolytic regulation
Gene-protein discordance	r = 0.006 (NS)	Post-transcriptional regulation
Preserved glycolytic axis	All enzymes elevated/correlated	Supports PK activator therapy

3.6. Study limitations

Several methodological limitations should be acknowledged when interpreting the findings of this study. First, the cross-sectional design precludes assessment of temporal changes in enzyme activity relative to

disease progression or treatment modifications. Second, the study population included both pediatric and adult participants without age stratification analysis due to sample size constraints; given the known physiological and metabolic differences between these groups [33,34], this heterogeneity may have influenced the observed enzyme patterns. Third, several potential confounding factors were not systematically controlled, including disease duration, genotype severity classification, splenectomy status, and nutritional status, all of which may independently affect erythrocyte metabolism and enzyme activity [35]. Fourth, while iron status was assessed via serum ferritin, comprehensive iron studies (transferrin saturation, liver iron concentration) were not performed, and the potential confounding effect of variable chelation therapy adherence on enzyme activity remains uncertain [36,37]. Fifth, the reliance on self-reported chelation adherence rather than objective measures represents an additional limitation. Finally, the clinicobiochemical correlation between glycolytic enzyme alterations and clinical outcomes warrants further investigation; while significant correlations between enzyme levels and hematological parameters were observed, the clinical utility of these findings for disease monitoring, treatment response prediction, or prognostic stratification remains to be established in prospective longitudinal studies. Future studies with larger sample sizes should incorporate age-stratified subgroup analyses and multivariate regression models to better control for confounding variables and establish independent predictors of enzyme dysregulation in beta-thalassemia major[38,39].

4. Conclusion

This study provides a comprehensive characterization of the glycolytic enzyme profile in Iraqi patients with beta-thalassemia major. The key findings include: (1) significant elevation of hexokinase and phosphofructokinase, with PFK demonstrating the highest statistical significance ($p < 0.0001$); (2) very strong positive correlation between HK and PFK ($r = 0.874$), indicating coordinated metabolic regulation; (3) significant inter-correlations among all three glycolytic enzymes; and (4) discordance between PFKP gene expression and serum protein levels, suggesting predominant post-transcriptional control of circulating enzyme concentrations. These findings have important clinical implications. Phosphofructokinase may serve as a sensitive biomarker for metabolic perturbation and disease monitoring in beta-thalassemia. The coordinated elevation of glycolytic enzymes suggests preserved metabolic machinery that may be amenable to therapeutic modulation. The emerging class of pyruvate kinase activators, which enhance glycolytic flux and improve erythrocyte survival, represents a promising therapeutic avenue supported by these metabolic observations.

Conflict of interest

The authors declare no conflict of interest

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