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Peripheral CD71+ erythroid cells as markers of ineffective erythropoiesis in β-thalassemia major

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Abstract:

BACKGROUND: Transferrin receptor 1, commonly referred to as cluster of differentiation 71 (CD71), is a crucial glycoprotein that mediates the cellular uptake of iron, avails as a substantial index of erythroid growth and iron metabolism, and typically exists in erythroid lineage cells within the bone marrow. We investigated the expression of CD71 in the peripheral blood of patients with transfusion-dependent beta-thalassemia major (βTM).

OBJECTIVES: This study examines the distinct role of CD71 in erythropoiesis, particularly in the context of βTM, and highlights its relevance in studying peripheral blood CD71 levels and their association with clinical and demographic criteria in Iraqi patients with βTM.

MATERIALS AND METHODS: This case–control study consisted of 60 βTM patients and 28 healthy controls (matched for age and sex), and they were evaluated. Samples of peripheral blood were examined by flow cytometry using anti-CD71 and anti-CD235a. Complete blood count, serum ferritin (SF), and liver function tests were assessed for all participants. Statistical associations with demographic, clinical, and laboratory parameters were examined.

RESULTS: The CD71 expression range was notably greater in patients (0.17%–18.20%) compared to controls (0.08%–0.19%), with a statistically significant difference ($P = 0.0001$). Thirty percent of patients exhibited <1% expression. Higher levels were found in splenectomized patients compared to splenomegaly patients, and in males compared to females. Positive correlations were identified with white blood cells, platelets, and liver transaminase enzymes. No correlations were found with hemoglobin, red blood cells, alkaline phosphatase, total serum bilirubin, or SF.

CONCLUSIONS: CD71 expression is a critical marker of ineffective erythropoiesis in βTM, affected by splenic status, gender, adherence to blood transfusion schedules, and correlates with various hematological and liver parameters. Incorporating CD71 into routine assessment may enhance the evaluation of disease activity and guide individualized prognosis and management in transfusion-dependent thalassemia patients.

Keywords:

Cluster of differentiation 71, ineffective erythropoiesis, splenectomy, beta-thalassemia major

Introduction

Cluster of differentiation 71 (CD71) is a type II glycoprotein transmembrane protein, with a weight of around 90 kDa, consisting of nearly 760 amino acids. It exists as a homodimer of approximately 180 kDa, held together by an intermolecular disulfide (S-S) bond located on the cell surface.[1]

CD71 is crucial for cellular iron assimilation in rapidly dividing cells, mainly during erythropoiesis. Its expression is markedly elevated in immature erythroid precursors such as erythroblasts and reticulocytes, whereas it is absent in fully differentiated erythrocytes. In healthy

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adults, CD71+ erythroid cells (CECs) are predominantly localized in the bone marrow (BM); however, elevated levels in the circulation have been reported under pathological conditions such as anemia, cancer, and ineffective erythropoiesis (IE).[2,3]

In patients with beta-thalassemia major (βTM), beta globin chains assembly is impaired, leading to IE, which results in long-standing anemia and compensatory hyperplasia of erythroid progenitors in the BM, which is often accompanied by elevation of CD71 expression, reflecting the increased iron demand and erythropoietic stress, worsening the risk of iron overload and related complications.[4-6]

The CD71 expression is widely used in flow cytometry panels to delineate and monitor erythroid differentiation and maturation in the BM. In contrast, few studies have evaluated CD71 levels in peripheral blood. Moreover, the effects of splenectomy status and demographic factors on CD71 expression are not well defined.[7-9]

Due to the limited available data on circulatory CD71 levels, this study aims to evaluate its expression among Iraqi patients with βTM by examining the relationship between CD71 levels and various clinical and demographic factors, seeking to assess its potential as a biomarker for erythropoietic activity and treatment efficacy in this unique genetic and environmental context.

Materials and Methods

Sixty βTM patient samples were collected before transfusion at the Ibn Al-Balady Thalassemia Center in Baghdad city. Sex was random. All patients were at least 19 years old. Twenty-eight sex- and age-matched controls were recruited from a Primary Health Care Center. This case–control study was conducted from April to June 2025, received approval from the ethics committee, and all participants submitted written informed consent.

Clinical information about the βTM patient's diagnosis, which was done by either hemoglobin (Hb) electrophoresis or the High Performance Liquid Chromatography (HPLC) technique in the Thalassemia Center, was recorded in the patient's file. All the βTM patients were on regular transfusions every 2–4 weeks, with the last transfusion 1–2 weeks before samples were taken. Chelation therapy was administered, with doses based on serum ferritin (SF) levels. To keep the study's accuracy, exclusion criteria included anyone with co-inherited hemoglobinopathies (such as HbS or HbE), other hemolytic disorders, HIV, hepatitis B virus, hepatitis C virus, pregnant females, or those who did not agree to participate. Furthermore, exclusion of any participant with positive C-reactive protein and controls with high erythrocyte sedimentation rates, to avoid results affected by inflammation. The control group was healthy, with no known medical or genetic conditions.

Samples were collected from all participants using peripheral venous blood in EDTA anticoagulant (for hematological and flow cytometry analyses) and gel tubes (for biochemical analyses). Complete blood count (CBC) parameters were measured using a fully automated hematology analyzer (DxH 500 Beckman Coulter, USA). SF was measured using a chemiluminescent immunoassay on the Beckman Coulter Access 2 analyzer (USA) device. Liver function test (LFT) and total serum bilirubin (TSB) were performed by spectrophotometer using an AU 480 Beckman Coulter (USA) device. For some patients, results concerning SF and LFT were obtained from their medical records if these tests had been performed within 3 months prior to sample collection time.

Allophycocyanin (APC)-labeled anti-CD71 with FITC-labeled anti-CD235a, both markers were from BD Biosciences, Pharmingen, USA, were used for the analysis via flow cytometry on a fluorescence-activated cell sorter (FACS) Canto II (Becton Dickinson, USA) device in a private lab, as stated in the following steps:

1. 100 µL of fresh blood was added to a polystyrene round-bottom tube
2. Dilution of 100 µL of fresh blood with 2 mL of FACS buffer, which is composed of phosphate-buffered saline + 1% bovine serum albumin, was performed to remove plasma proteins that may occur due to hemolysis
3. Centrifuging by the cell wash centrifuge was done at 1500 rpm for 5 min for all samples
4. Removal of supernatants and gentle resuspension of each pellet in 100 µL of FACS buffer were performed, then mixed by the vortex mixer
5. Preparation of the antibody cocktail, which is composed of APC Antihuman CD71(transferrin receptor 1 marker) and its gating FITC Antihuman CD235a (red blood cell [RBC] marker), from each, 5 µL was added in a polystyrene round-bottom tube
6. Mixing gently of the combination of 100 µL of the sample, which was added to the antibody tube, and incubation was done for 20 min in the incubator away from light at room temperature
7. After incubation, another 2 mL of FACS buffer for washing is added to each sample to remove the unbound antibodies, and centrifugation at 1500 rpm for 5 min is done
8. Then, the removal of the supernatant and resuspension of the pellet with 1 mL of FACS buffer were performed
9. The samples were ready for reading by the flow cytometry device (BD FACS Canto II), and the data were obtained.

Ethics approval and agreement to participate

All participants provided informed written consent before joining the study. The study protocol was approved by The College of Medicine/Mustansiriyah University's Institutional Review Board, according to the administrative order 898, dated December 2, 2024, which examined and approved this research at each site's independent ethics committee/institutional review board.

Statistical analysis

The gathered data were encoded, submitted, presented, and analyzed using IBM SPSS 29 software (Statistical Package of Social Sciences, version 29, Chicago, IL, USA). Results were shown through basic descriptive statistics, including mean, frequency, percentage, range (from minimum to maximum values), and standard deviation. For inferential comparisons, hypothesis tests were applied to assess significance. Student's *t*-test was employed to compare two independent means. Analysis of variance was used for comparisons involving more than two means. Differences in qualitative data percentages were assessed using the Pearson Chi-square test (χ^2-test), with Yates' correction or Fisher's Exact test applied as needed. A statistic is considered significant when $P \leq 0.05$. The performance of the correlation coefficient (r) is as follows: values <0.3 indicate no correlation, values between 0.3 and 0.5 suggest a weak correlation, values between 0.5 and 0.7 indicate a moderate association, and values >0.7 represent a strong correlation.

The receiver operating characteristic (ROC) curve analysis was conducted to evaluate the diagnostic or screening efficacy of the studied parameters and to determine the optimal cutoff point that is recognized when both sensitivity and specificity are maximized. Area under the curve (AUC) was notably explained as follows:

0.9–1.0 = Excellent (Perfect), 0.8– 0.9 = Good, 0.7–0.8 = Fair, 0.6–0.7 = Poor, and <0.6 = Failed discrimination.

The values of sensitivity, specificity, the percentage of incorrect negatives, the percentage of incorrect positives, the probable value of a positive test, the probable value of a negative test, and the overall accuracy rate were calculated using standard formulas based on true positive, true negative, incorrect positive, and incorrect negative classifications.[10-12]

Results

A total of 60 patients with βTM and 28 healthy controls were included in this study. The baseline demographic and clinical characteristics of the participants are summarized in Table 1. The Mean age of the patient group was 23.7 years, while it was 25.5 years for the control group; there is no significant variance between the two study groups concerning age and gender. As for family consanguinity, it existed in 88.3% of the patient group, while none existed in the controls. Spleen status: 53.3% of patients had splenectomy, and 46.7% of patients had splenomegaly; in contrast, all the controls had normal spleen.

A distinct profile was demonstrated by the CBC indices of patients, with reduced Hb and hematocrit levels. RBC counts were diminished in approximately 96.7% of patients, whereas leukocytosis was observed in about 48.3% and thrombocytosis in 46.7%. SF concentrations were uniformly elevated across all cases. Each of these findings had a $P = 0.0001$ when compared to the control group, as detailed in Table 1.

LFTs provided a vivid picture: 55% of patients had elevated alkaline phosphatase (ALP), 23.3% had high alanine transaminase, 28.3% had high aspartate transaminase (AST), and a striking 80% showed increased TSB levels. $P = 0.0001$, 0.005, 00.2, and 0.0001, respectively, as presented in Table 2.

CD71% expression was significantly elevated in βTM patients compared to the controls, as shown in Figure 1.

There was a significant increase in CD71% among patients, with a Mean ± standard deviation of 3.8 ± 4.7, Range 0.17–18.20, and Median 1.5, when compared to those of the control group, noting that 30% of patients had CD71% values below 1%, with a $P = 0.0001$. All results are presented in Table 3, while Figure 2 shows representative dot plot examples for control and patient groups.

Demographics/Clinical Parameters of patient group [Table 4] revealed that 32 patients (19 ♂, 13 ♀) were splenectomized versus 28 (9 ♂, 19 ♀) non-splenectomized (splenomegaly), with a $P = 0.0001$.

High CD71% in males, with a Mean ± SD of 6.02 ± 5.74, versus females 1.95 ± 2.51, with a $P = 0.001$.

In splenectomized, CD71 expression was of Mean ± SD were 6.51 ± 5.21, Range (1.1–18.2), Median 4.05 vs. that of nonsplenectomized 0.81 ± 0.50, (0.1–2.0), 0.7, respectively, with a $P = 0.0001$. CD71% correlated positively with splenectomized patients and male gender, as mentioned, while no significant correlation was found with age and consanguinity, with $P > 0.05$ for each.

The control group showed no distinct association between CD71 expression and any of the demographic criteria, with a $P > 0.05$.

Table 1: Comparison of demographic criteria, hematology profiles, and serum ferritin levels between the patient and control groups

	β-thalassemia, *n* (%)	Controls, *n* (%)	*P*
Age, mean±SD (range)	23.7±3.9 (19–33)	25.5±4.3 (19–33)	0.062
Gender			
Male	28 (46.7)	9 (32.1)	0.199
Female	32 (53.3)	19 (67.9)	
Family consanguinity			
Yes	53 (88.3)	-	-
No	7 (11.7)	28 (100.0)	
Spleen status			
Splenectomy	32 (53.3)	-	-
Splenomegaly	28 (46.7)	-	
Normal	-	28 (100.0)	
CBC results			
Hb (g/dL)			
Normal (13–16 male; 11–15 female)	-	23 (82.1)	0.0001*
Low	60 (100.0)	5 (17.9)	
HCT (%)			
Normal (38–48 male; 35–45 female)	-	27 (96.4)	0.0001*
Low	60 (100.0)	1 (3.6)	
RBC ($\times10^6$/μL)			
Normal (4.3–5.6 male; 3.9–5.1 female)	2 (3.3)	28 (100.0)	0.0001*
Low	58 (96.7)	-	
WBC ($\times10^3$/μL)			
Normal (3.7–10.6)	31 (51.7)	28 (100.0)	0.0001*
High	29 (48.3)	-	
PLTs ($\times10^3$/μL)			
Normal (150–450)	32 (53.3)	28 (100.0)	0.0001*
High	28 (46.7)	-	
SF (μg/L)			
Normal (15–300 male; 15–150 female)	-	28 (100.0)	0.0001*
High	60 (100.0)	-	
Mean±SD (range)	3454.1±1939.6 (684.0–6988.0)	59.63±48.71 (16.3–149.1)	0.0001#

*Distinct difference between percentages by applying the Pearson Chi-square test (two-test) at the 0.05 level, #Statistically distinct difference between two independent group means was assessed by utilizing the Student's *t*-test at a 0.05 probability, The accepted SF level is <1000 μg/L for the thalassemia patients. CBC=Complete blood count, SD=Standard deviation, Hb=Hemoglobin, HCT=Hematocrit, RBC=Red blood cell, WBC=White blood cell, SF=Serum ferritin, PLTs=Platelets

Table 2: Comparative percentage of liver function test values between the patient and control groups

LFT	Thalassemia, *n* (%)	Controls, *n* (%)	*P*
ALP (U/L)			
Normal (30–120)	27 (45.0)	28 (100.0)	0.0001*
High	33 (55.0)	-	
ALT (U/L)			
Normal (10–50)	46 (76.7)	28 (100.0)	0.005*
High	14 (23.3)	-	
AST (U/L)			
Normal (10–50)	43 (71.7)	28 (100.0)	0.002*
High	17 (28.3)	-	
TSB (μmol/L)			
Normal (5–21)	12 (20.0)	28 (100.0)	0.0001*
High	48 (80.0)	-	

*Statistically distinct variation in proportions was determined using the Pearson Chi-square test at a 0.05 significance threshold. ALP=Alkaline phosphatase, ALT=Alanine transaminase, AST=Aspartate transaminase, TSB=Total serum bilirubin, LFT=Liver function test

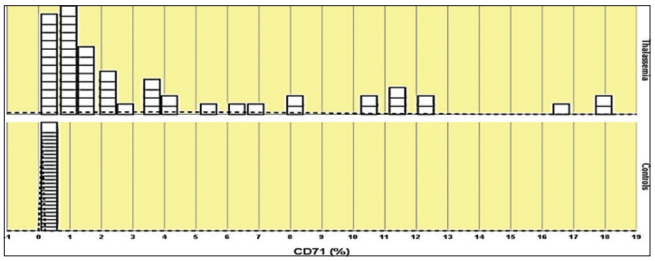

Figure 1: Stacked histogram clarifies the comparative distribution of cluster of differentiation 71% values measured by flow cytometry in both the patient and control groups

CD71% Correlations with CBC, LFT, and SF [Table 5] reveal a moderate strength correlation with platelets (PLTs) and AST, and a strongly significant association with white blood cells (WBCs), with a $P = 0.0001$. As for RBCs, ALP, and ALT, there was a feeble correlation, with $P = 0.009$, 0.018, and 0.001, respectively.

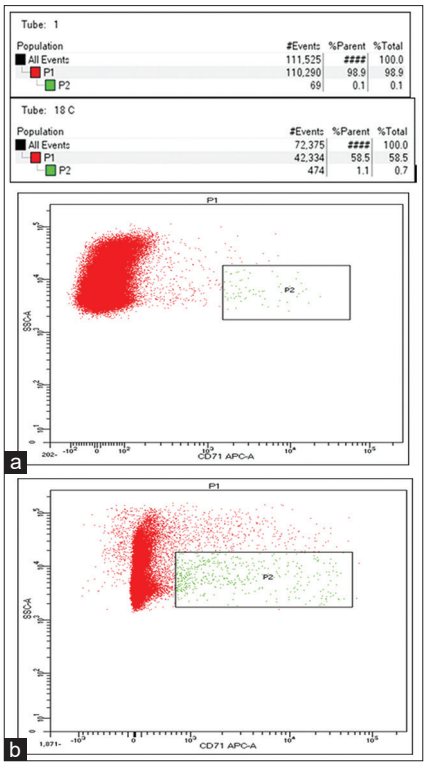

Figure 2: Flow Cytometry dot plots, where the X-axis represents cluster of differentiation 71 (CD71)-APC, and the Y-axis represents CD235a-FITC. The Area inside the rectangle represents CD71 expression in the lower right quadrant, specifically in immature erythroid precursors, which exhibit CD71 expression. (a) In control, CD71 expression is 0.1. (b) In a β-thalassemia patient, high CD71 (=1.1%) expression and low CD235a suggest no or a low number of mature red blood cell. CD71 = Cluster of differentiation 71

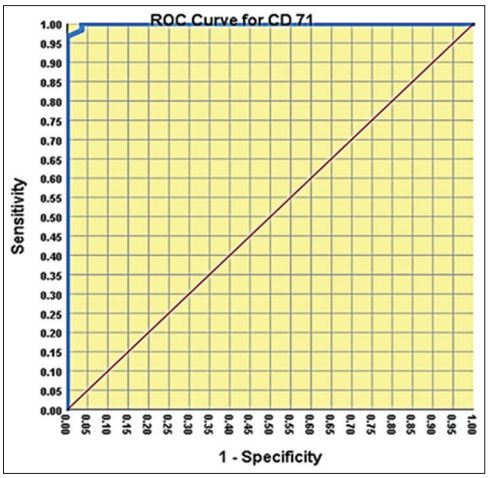

Figure 3: A receiver operating characteristic curve represents the area under the curve (AUC) of cluster of differentiation 71 (CD71). The red line represents the reference line, the Y-axis represents sensitivity, while the X-axis represents specificity. The blue line along the Y-axis represents the AUC of CD71. ROC = Receiver operating characteristic, CD71 = Cluster of differentiation 71

Table 3: Comparison of peripheral blood cluster of differentiation 71 expression measured by flow cytometry in both the patient and control groups

Markers	Thalassemia, *n* (%)	Controls, *n* (%)	*P*
CD71 (%)			
<1	18 (30.0)	28 (100.0)	0.0001*
≥1	42 (70.0)	-	
Mean±SD	3.854±4.759	0.096±0.020	0.0001#
(range) median	(0.17–18.20) 1.55	(0.08–0.19) 0.10	

*Statistically significant variation in proportions was determined by applying the Pearson Chi-square test at a 0.05 significance threshold. #Statistically distinct difference between two independent group means was assessed by using the Student's *t*-test at a 0.05 probability level. SD=Standard deviation, CD71=Cluster of differentiation 71

The cutoff percentage for CECs was established at 0.18%, resulting in a specificity of 96.4% and a sensitivity of 98.3%. A statistical analysis of the ROC curve was applied on our dataset [Figure 3], with the AUC illustrated in Table 6, to establish how the marker works perfectly at a range of 0.99–1.0 level, ensuring discrimination of utmost accuracy between patients and controls. The cutoff value is colored in yellow, as illustrated in Table 7.

Discussion

The anemia and hemolysis in β-thalassemia lead to BM hyperplasia, alongside expanded erythropoiesis, particularly in the spleen and liver, as compensatory responses, resulting in hepatomegaly and distinct splenomegaly, which worsen the underlying anemia.[13,14] This condition is characterized by increased erythropoietin (EPO) and erythroferrone, suppressed hepcidin, and exacerbated iron overload, with high SF levels (the maximum value of SF accepted in thalassemia patients should be <1000 µg/L), which is further amplified by transfusions and hemolysis.[15-17]

Leukocytosis and thrombocytosis are exhibited in some patients, particularly those who have undergone splenectomy, as Bhutta *et al*.'s study mentioned in 2025.[18] Chronic Transfusion treatment and hemolysis will saturate the transferrin, exceed toxic nontransferrin-bound iron, generate reactive oxygen species, facilitate tissue injury,[19] and lead to an irregular increase in LFT, which aligns with hemolysis and hepatic iron deposition. The 2022 study by Faiq *et al*. supported this conclusion, emphasizing the importance of routine monitoring of LFT as part of the clinical evaluation of hepatic function in individuals with β-thalassemia.[20]

The notably elevated expression of CECs in βTM patients can be attributed to IE and extramedullary hematopoiesis (EMH). The strong correlation between CEC levels and splenectomized thalassemic patients may be attributed to the high number of erythroid lineage cells found in their peripheral blood. The 30% of patients who had CECs <1% are likely due to regular transfusions

Table 4: Association of peripheral cluster of differentiation 71 expression levels with demographic/clinical parameters in both the patient and control groups

Demographic/clinical criteria	CD71 (%)			
	Thalassemia		Controls	
	n	Mean±SD	*n*	Mean±SD
Age (years)				
<20	6	2.800±4.235	2	0.095±0.007
20–24	35	3.905±5.278	10	0.094±0.008
25–29	12	3.391±3.745	10	0.089±0.009
≥30	7	5.300±4.489	6	0.095±0.008
P		0.796		0.455
Gender				
Male	28	6.029±5.746	9	0.096±0.007
Female	32	1.952±2.517	19	0.091±0.009
P		0.001#		0.193
Family consanguinity				
Yes	53	3.964±4.843	-	-
No	7	3.029±4.302	28	0.093±0.008
P		0.629		-
Spleen status				
Splenectomy, mean±SD (range) median	32	6.519±5.213 (1.1–18.2) 4.05	-	-
Splenomegaly, mean±SD (range) median	28	0.810±0.503 (0.1–2.0) 0.7	-	-
Normal	-	-	28	0.093±0.008
P		0.0001*		-

*Statistically distinct difference between two independent group means was assessed via using the Student's *t*-test at a 0.05 probability level, #Statistically distinct variation via applying ANOVA among multiple independent group means was evaluated at a 0.05 significance level. ANOVA=Analysis of variance, SD=Standard deviation, CD71=Cluster of differentiation 71

suppressing marrow activity or from ongoing IE and splenic clearance of defective RBCs.[13,18] These observations are corroborated by a study conducted in Turkey by Görgülügil *et al*. in 2024.[9]

Although the two studies agree on the general concept of CECs, they differ in several aspects. For instance, there is a difference in the reported metrics: the patient group range of CECs expression is followed by the Median, and then the cutoff value. They identified a Range, Median, and cutoff value of 0.0%–15.0%, 0.6%, and 1%, respectively. In contrast, our study presented a Range of 0.17%–18.2%, a Median of 1.5%, and a cutoff value of 0.18%, using the ROC and AUC analyses, as it serves as a crucial marker for distinguishing patients requiring more intensive management.

Furthermore, while they identified a positive correlation between CECs and WBCs, our study also found a positive correlation with PLTs. These results, when aligned side by side, allow for more precise comparison and understanding of the differences in findings.

Variations may arise from differences in patient cohorts, such as age, disease severity, transfusion frequency, and splenectomy status. Methodological discrepancies, including gating strategies, antibody protocols, and statistical methods, may also contribute to these differences. In addition, analytical approaches diversities, such as percentile-based comparisons versus ROC analysis or the use of Spearman's versus Pearson's correlation, as well as the difference in time of collecting samples, genetic factors, and regional background, may lead to different results.

A study in 2003 was performed by Kossiva *et al*. in Athens,[21] which measured CD71expression on erythroblast surfaces in the peripheral blood and BM of thalassemia intermedia patients using flow cytometry. They found reduced CD71% levels, attributing the result to erythrocyte membrane dysfunction. Their findings, however, cannot be generalized to CEC levels. The sample included only six splenectomized thalassemia intermedia patients, compared to the larger β-thalassemia population in our study.

The higher CEC expression in the 19 splenectomized male patients compared with the 13 females is possibly due to hormonal effects. Testosterone may trigger erythropoiesis, while estrogen may inhibit it.[22] Furthermore, EPO level tends to be higher in males, especially after splenectomy, leading to more extensive EMH and marrow expansion. EPO is almost absent in nonsplenectomized patients. This finding aligns with the results of Hapgood *et al*.[23]

Conclusions

This study highlights the importance of peripheral CECs as a sensitive marker for IE in transfusion-dependent βTM patients.

Table 5: Correlation between cluster of differentiation 71 and serum ferritin with both hematological and biochemical parameters in the patient group

Thalassemia	SF (µg/L)	CD71 (%)
Hb (g/dL)		
r	0.088	0.296
P	0.506	0.022
RBC (×10⁶/µL)		
r	0.036	0.336*
P	0.787	0.009
WBC (×10³/µL)		
r	0.137	0.819**
P	0.297	0.0001
PLTs (×10³/µL)		
r	−0.038	0.611**
P	0.776	0.0001
SF (µg/L)		
r	-	0.093
P	-	0.482
ALP (U/L)		
r	−0.144	0.303*
P	0.271	0.018
ALT (U/L)		
r	0.224	0.419*
P	0.085	0.001
AST (U/L)		
r	0.152	0.642**
P	0.246	0.0001
TSB (µmol/L)		
r	−0.201	0.286
P	0.124	0.027
CD71 (%)		
r	0.093	-
P	0.482	-

*Correlation is distinct at the 0.05 level, **Correlation is significant at the 0.01 level. Hb=Hemoglobin, RBC=Red blood cell, WBC=White blood cell, ALP=Alkaline phosphatase, ALT=Alanine transaminase, AST=Aspartate transaminase, TSB=Total serum bilirubin, CD71=Cluster of differentiation 71, SF=Serum ferritin, PLTs=Platelets

Table 6: Diagnostic performance of cluster of differentiation 71% as a biomarker in the β-thalassemia patient group: Receiver operating characteristic curve analysis

Test result variables	AUC	SE	*P*	95% CI	
				Lower bound	Upper bound
CD71%	0.999	0.001	0.0001#	0.997	1.000

#Statistically significant result at *P* value < 0.05AUC=Area under the curve, SE=Standard error, CI=Confidence interval, CD71=Cluster of differentiation 71

Table 7: Performance metrics of cluster of differentiation 71% at various diagnostic cutoffs in the β-thalassemia patient group

Test result variables	Positive if greater than or equal to	Sensitivity (%)	Specificity (%)
CD71 (%)	0.1350	100	96.4
	0.1800*	98.3*	96.4*
	0.195	96.7	100

*Statistically significant at P value < 0.05CD71=Cluster of differentiation 71

The strong correlation of the CD71 biomarker with clinical and laboratory indices, especially among splenectomized and male patients, highlights the capability to use CD71 as a practical procedure to monitor the disease. Incorporating CD71 assessment into routine clinical evaluation may improve the accuracy of prognosis and management strategies for individuals living with βTM. Further research with a larger, more diverse population, a multi-center design, and longitudinal monitoring of CD71 changes across transfusion cycles (these were limitations in our study due to financial constraints) could refine these findings and support the broader use of CD71 in clinical practice.

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Conflicts of interest

There are no conflicts of interest.

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