Impact of Hypocalcemia on Dickkopf-Related Protein 1 and HMGB1 in Transfusion-Dependent Thalassemia Patients / Original study

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تأثير نقص الكالسيوم في الدم على بروتين ديكوبف المرتبط 1 و HMGB1 في مرضى الثلاسيميا المعتمدين على نقل الدم

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Abstract

Background. Transfusion-dependent thalassemia (TDT) is associated with various symptoms including anaemia and bone problems. There is a need for more biomarkers of calcium disorders in TDT patients. In the present study, DKK 1 and HMGB1 are studied in TDT patients with hypocalcemia (TDT+Hypocalcemia) and normocalcemia (TDT+Normocalcemia) as a function of inflammation.

Methods. TDT patients were classified according to the cut-off value (2.12 mM) of the adjusted serum calcium (Adj.T.Ca) into TDT+Normocalcemia (n=54) and TDT+Hypocalcemia (n=41) groups. Forty healthy controls have participated in the present study. DKK1 and HMGB1 were measured using the ELISA technique.

Results. Both patient groups have significantly higher DKK1, HMGB1, C-reactive protein, and ferritin than the control groups. The TDT+Hypocalcemia group has a significantly higher serum DKK1 level than the TDT+Normocalcemia. The results show CRP has a significant correlation with DKK1 and ferritin, in addition to a significant inverse correlation with splenomegaly, and adj.T.Ca. There is also a significant correlation between HMGB1 and DKK1. DKK1 also has a significant inverse correlation with sex, splenomegaly, and Adj.T.Ca. The results of receiver operating characteristics (ROC) revealed an increase in DKK1 more than the cut-off concentration (901.44 pg/ml) indicating that the TDT subjects may have hypocalcemia significantly ($p<0.001$) in a sensitivity of 73.2% and specificity of 72.2% (Youdin's J statistic=0.454). While HMGB1 and ferritin have no significant predictability of hypocalcemia.

Conclusion. DKK1 is an important factor in hypocalcemia in TDT patients and may act as a drug target for the treatment of bone disorders in

thalassemia patients.

Keywords: Thalassemia, hypocalcemia, DKK 1, calcium, and HMGB1.

Abbreviations

HMGB1: high mobility group box 1

TDT: Transfusion-dependent thalassemia

TDT+Hypocalcemia: TDT patients with hypocalcemia

TDT+Normocalcemia: TDT patients with normocalcemia

Adj.T.Ca: adjusted serum calcium into

DKK1: Dickkopf-related protein 1

PTH: parathyroid hormone

β-TM: Beta-thalassemia

RBC: Red blood cell

IO: Iron overload

TLR: Toll-like receptors

ELFA: enzyme-linked fluorescence immunoassay

CV: coefficients of variation

ELISA: enzyme-linked immunosorbent assay

CRP: C-Reactive Protein

ANOVA: Analysis of variance

GLM: general-linear model

Ln: natural logarithm

ROC: Receiver-operating characteristic

المستخلص

المقدمة: يرتبط الثلاسيميا المعتمد على نقل الدم (TDT) بأعراض مختلفة بما في ذلك فقر الدم ومشاكل العظام. هناك حاجة لمزيد من المؤشرات الحيوية لاضطرابات الكالسيوم في مرضى الثلاسيميا المعتمد على نقل الدم. في الدراسة الحالية، تمت دراسة DKK 1 و HMGB1 في مرضى الثلاسيميا المعتمد على نقل الدم الذين يعانون من نقص كالسيوم الدم (نقص كالسيوم (TDT+والمرضى الذين لديهم مستوى كالسيوم الدم الطبيعي) كالسيوم طبيعي (TDT+ كدالة للالتهاب.

طرق العمل: تم تصنيف مرضى الثلاسيميا المعتمد على نقل الدم وفقًا لقيمة القطع (2.12 مليمول) من الكالسيوم في المصل المعدل (Adj.T.Ca) إلى مجموعتين (نقص كالسيوم (TDT+وعددهم 54 ومجموعة تحوي 41 مريضا ولديهم كالسيوم طبيعي) كالسيوم طبيعي (TDT+كما شارك أربعون شخصًا سليمًا في الدراسة الحالية. تم قياس DKK1 و HMGB1 باستخدام تقنية الايلايزا.

النتائج: لوحظ ارتفاع معنوي في مستويات DKK1 و HMGB1 و بروتين سي التفاعلي و الفيريتينت في كلتا مجموعتي المرضى مقارنة مع مجموعات التحكم. أظهرت مجموعة (نقص كالسيوم (TDT+مستوى DKK1 في المصل أعلى بشكل ملحوظ من مجموعة) كالسيوم طبيعي (TDT+ أظهرت النتائج أن البروتين التفاعلي سي له ارتباط كبير مع DKK1 والفيريتين، بالإضافة إلى ارتباط عكسي كبير مع تضخم الطحال، و Adj.T.Ca. كما يوجد ارتباط كبير بين HMGB1 وDKK1. كما أن DKK1 له ارتباط عكسي كبير مع الجنس، وتضخم الطحال، وAdj.T.Ca. كشفت نتائج خصائص تشغيل المستقبل (ROC) عن زيادة في DKK1 أكثر من تركيز القطع (901.44 بيكو جرام/مل) مما يشير إلى أن الأشخاص الذين خضعوا لــ TDT قد يعانون من نقص كالسيوم الدم بشكل ملحوظ ($p<0.001$) بحساسية 73.2% وخصوصية 72.2% (Youdin's J=0.454). في حين أن HMGB1 والفيريتين ليس لديهما قابلية للتنبؤ بنقص كالسيوم الدم بشكل ملحوظ.

الاستنتاج. يعد DKK1 عاملاً مهمًا في نقص كالسيوم الدم لدى مرضى الثلاسيميا وقد يعمل كهدف دوائي لعلاج اضطرابات العظام لدى مرضى الثلاسيميا.

الكلمات المفتاحية: الثلاسيميا ، نقص الكالسيوم ، بروتين مرتبط ديكوبف مرتبط 1، الكاليسيوم ، البروتين HMGB1

Introduction

Beta-thalassemia (β-TM) is a family of genetic diseases that manifests as anaemia and erythrocyte membrane disintegration due to a lack of the β-globin chain in haemoglobin A (Raguram *et al.*, 2022). This condition is associated with significant morbidity and death (Sanchez-Villalobos *et al.*, 2022). Among the world's most common hereditary diseases, thalassaemia is especially common in Mediterranean nations like Iraq (Al-Hakeim *et al.*, 2020). Red blood cell (RBC) depletion is addressed by continuously transfusing blood into these individuals (Shah *et al.*, 2019). Blood transfusions are necessary for β-TM patients throughout their lives in order to raise haemoglobin levels and reduce the negative impacts of poor erythropoiesis (Aydinok, 2020). Because of the high frequency of blood transfusions, patients with transfusion-dependent thalassaemia (TDT) are at increased risk for a variety of problems (Shah, Sayani *et al.*, 2019). Iron overload (IO) may be caused by chronic blood transfusions and can cause toxicity in several organs, such as the endocrine glands, heart, and liver (Daher *et al.*, 2017).

Hypoparathyroidism and hypovitaminosis D are symptoms that TDT patients may have due to the parathyroid gland being one of the glands impacted by iron excess. Consequently, hypocalcaemia, or low blood calcium levels, will occur in TDT patients due to impaired or absent parathyroid hormone (PTH) synthesis, which in turn reduces calcium absorption from the intestines (Ansaf *et al.*, 2024; Lertsuwan *et al.*, 2018a). Among those diagnosed with beta major thalassaemia, 22% had hypocalcaemia (Mirhosseini *et al.*, 2013a). Hypocalcaemia is recognised to cause bone abnormalities (Merchant & Gafni, 2024), but its impact on

biomarkers of other associated pathways is less well understood. Specifically, we aimed to compare the levels of Dickkopf-related protein 1 (DKK1) and high mobility group box 1 (HMGB1) in TDT patients who had hypocalcaemia and those who did not (Wulandari *et al.*, 2023; Yuan *et al.*, 2020). Active monocytes, natural killer cells, macrophages, and mature dendritic cells, may leak the nonhistone chromosomal binding protein HMGB1, whereas necrotic and injured cells can passively release it (Chi *et al.*, 2024; Yuan *et al.*, 2024). When the body detects an inflammatory stimulus, such as an injury or infection, it secretes HMGB1, a cytokine that promotes further inflammation by binding to complexes of the receptors for Toll-like receptors (TLR) 2 and 4 and advanced glycation end-products (RAGE) (Fan *et al.*, 2024; Ostrand-Rosenberg *et al.*, 2023).

The extracellular HMGB1 regulates inflammation and immunological responses via various receptors or direct absorption after its active secretion or passive release (Chen *et al.*, 2022). More so, HMGB1 may maintain the chronic inflammatory state linked to diabetes (Ngcobo & Sibiya, 2024), and interaction with TLR-4 increases insulin resistance by phosphorylating the peripheral insulin-receptor substrate (Ren *et al.*, 2023). After being secreted or released passively from cells, HMGB1 normally acts as a damage-associated molecular pattern molecule outside of cells, influencing immune responses and inflammation via different receptors or direct absorption (Chen, Kang *et al.*, 2022).

Inflammation is often accompanied by an increase in the levels of a pro-inflammatory glycoprotein called DKK1, which is released by several cell types including endothelial cells and platelets (Chae & Bothwell, 2019). Acute infections are associated with a spike in systemic DKK1 levels (Mazon *et al.*,

2018) and this protein may play a role in inflammatory reactions by stimulating the release of cytokines that promote inflammation (Jaschke *et al.*, 2022). Atherosclerosis (Al-Dujaili *et al.*, 2021), cardiovascular disorders (Garcia-Martín *et al.*, 2014), and other related pathologies are influenced by proteins whose expression DKK1 directly controls. There is a negative correlation between serum DKK1 and PTH and alkaline phosphatase concentrations and a positive correlation with serum calcium concentrations (Wang *et al.*, 2024).

Low levels of calcium and vitamin D are common in TDT patients who get frequent transfusions despite receiving chelation therapy (Li *et al.*, 2024). Some studies have shown that β-TM contains small amounts of calcium (Ananvutisombat *et al.*, 2024; Mirhosseini *et al.*, 2013b). Calcium and vitamin D levels are lower in thalassemic children compared to healthy children of the same age group, according to much research conducted around the globe (Ara *et al.*, 2023; Ridha *et al.*, 2022). Compared to healthy children, patients undergoing TDT had substantially lower levels of blood total calcium, greater levels of serum ferritin, and considerably lesser levels of serum inorganic phosphate (Urmi *et al.*, 2023). In children suffering from β-thalassemia, there is a significant reduction in blood calcium, vitamin D, and parathyroid hormone levels, which may lead to growth failure. These abnormalities might be caused by excessive iron and poor nutritional support, which brings attention to the need for therapeutic techniques (Meshram *et al.*, 2024). The primary aim of the current investigation is the study of DKK1 and HMGB1 levels in TDT patients with hypocalcemia (TDT+Hypocalcemia) and normocalcemia (TDT+Normocalcemia) as a function of inflammation. The secondary aim of the study is to ascertain the

diagnostic sensitivity and specificity of the observed biomarkers for predicting hypocalcemia in TDT patients.

Materials and Methods

Participants

Ninety-five Iraqi TDT children (41 males and 54 females) aged 7-12 years were collected at the Thalassemia Unit at Al-Zahra'a-Teaching Hospital, Najaf, Iraq. Diagnosis of thalassemia was performed according to the Diagnosis Code number D56.1 from the ICD-10-CM (2019). The diagnosis was based on the clinical symptoms, haematological parameters (primarily; Hb<7g/dl, and in blood smear there is microcytic-hypochromic RBCs with anisopoikilocytosis and increased reticulocyte percentage), and by haemoglobin HPLC which assayed using HPLC (VARIANTTM β-Thalassemia Program). The patients were classified according to the results of the adjusted serum calcium (Adj.T.Ca) into the TDT group with normal levels of the Adj.T.Ca (TDT+Normocalcemia) and TDT group with hypocalcemia (TDT+Hypocalcemia). Hypocalcemia is defined as the serum level of Adj.T.Ca below the cut-off value of 2.12 mmol/L (8 mg/dl) (Pepe *et al.*, 2020).

Blood transfusions were a fundamental component of the therapeutic regimen for each patient. To maintain haemoglobin levels over 90 g/L before transfusions, all patients received packed red blood cell transfusions every two to four weeks. Based on the ferritin level, patients received an iron-chelating therapy with an infusion of Desferal® (deferoxamine mesylate) at a dose of 25-50 mg/kg/day over eight hours daily. The recommended dose for

Exjade (Deferasirox) is twenty mg/kg/day of body weight each day. The control group included forty adolescents, with ages comparable to the patients (17 males and 23 females), all of whom seemed to be in fair health. The subjects had no indications of systemic or inflammatory illnesses, and none were anemic. Systemic illnesses such as diabetes mellitus, hypertension, or renal issues were excluded, along with patients or controls who had splenectomy. Both parents or other first-degree relatives of the patient were asked to sign an informed consent form. Approval number 214/2024 was given by the University of Kufa's IRB for the project.

Assays

The blood samples were collected in the morning hours. Iron in serum was measured by using Ferrozine method (Linear®, Spain). The VIDAS ferritin test, an automated equipment supplied by BioMérieux Co., France, measures serum ferritin levels using an enzyme-linked fluorescence immunoassay (ELFA). The ferritin intra-assay precision within-assay coefficients of variation (CV) was less than 5.70 per cent and the iron CV% was less than 2.19%. Commercial enzyme-linked immunosorbent assay (ELISA) sandwich kits were used to measure serum DKK1 and HMGB1 (Pars Biochem Co, Nanjing, China). We diluted samples as needed for those containing very intense biomarkers. The intra-assay CV% were less than 10%. The C-Reactive Protein (CRP) latex slide test is used for measuring CRP in human serum semi-quantitatively (Spinreact®, Barcelona, Spain). The titer of serum is equal to the positive control concentration times reciprocal of the highest dilution showing positive reactivity. The approximate formula for calculating the CRP concentration (in mg/l) in the patient sample is 6 multiplied by the CRP titer.

Biostatistical analysis

Analysis of variance (ANOVA) was used to evaluate variations in continuous variables among groups, whereas contingency table analysis (χ^2-test) was performed to examine comparison in the categorical variables. Relationships between variables were calculated using Pearson's correlation coefficients. We used multivariate general-linear model (GLM) analysis to elucidate the impacts of hypocalcemia across three groups: TDT+Normocalcemia, TDT+Hypocalcemia, and healthy controls while adjusting for confounders such as age, sex, weight, and height. To normalise the distribution of the detected biomarkers, which were evaluated using the Kolmogorov-Smirnov test, we used a natural logarithm (Ln) transformation to their data. Receiver-operating characteristic (ROC) analysis was used to assess the hypocalcemia effectiveness of the discovered biomarkers. The statistics, cut-off points, sensitivities, and specificities of Youdin are computed variables. The statistical analyses were conducted with SPSS software version 27.

Results

Comparison of the characteristics among groups

The comparison of the demographic and clinical characteristics of TDT patients with and without hypocalcemia and the control groups is shown in Table (1).

Table 1: Demographic and clinical characteristics of the transfusion-dependent thalassemia (TDT) patients with and without hypocalcemia, and the control groups

Parameters	Controls [A] n=40	TDT+ Normocalcemia[B] n=54	TDT+ Hypocalcemia[C] n=41	F	p
Age year				0.26	0.768
Sex male/female				0.42	0.810
Rural/Urban				1.52	0.469
Family history No/				100.42	<0.001
Yes				0.06	0.939
Weight kg				0.13	0.883
Height cm				MWUT	0.701
Duration of Dis.	9.73±1.92	9.56±1.73	9.83±1.95	MWUT	0.226
Years	18/22	24/30	17/24	MWUT	0.315
Age of onset,	13/27	16/38	17/24	MWUT	0.392
months	40/0[B, C]	7/47 [A]	2/39 [A]	2.25	0.325
No. of transfusion	26.42±9.68	27.01±8.19	26.58±7.53	13.335	0.001
Days after last	126.05±16.19	127.11±12.29	125.71±15.14	126.02	<0.001
transfusion	-	8.8(7-10.9)	9(7.45-10.85)	23.79	<0.001
Splenectomy No/	-	8(5.75-12)	6(4.5-9)	12.98	0.002
Yes	-	144(96-219)	164(121.5-240)	63.38	<0.001
Splenomegaly	-	23(15-30)	20(15-29)	5.21	0.074
No/Yes	40/0	51/3	38/3		
Folic acid No/Yes	40/0[B, C]	41/13[A]	29/12[A]		
Vitamin B12 No/	40/0[B, C]	2/52[A]	0/41[A]		
Yes	40/0[B, C]	30/24[A]	26/15[A]		
Desferral No/Yes	40/0[B, C]	43/11[A, C]	29/12[A, B]		
Exjade No/Yes	40/0[B, C]	15/39 [A, C]	9/32[A, B]		
1 Alpha No/Yes	40/0	48/6	39/2		

[A, B, C]: pairwise comparison. Results are expressed as mean ± standard deviation for normally distributed data. Binomial data were expressed as ratios and analyzed by Chi-squared test. p: probability value.

Table 2: Comparison of the Biomarkers levels among the transfusion-dependent

thalassemia (TDT) patients with and without hypocalcemia, and the control groups

Parameters	Controls [A] n=40	TDT+ Normocalcemia[B] n=54	TDT+ Hypocalcemia[C] n=41	F	p
PCV %	40.98±4.16[B,C]	23.11±3.51[A]	24.29±3.42[A]	312.58	<0.001
Hemoglobin g/dl	13.85±1.35[B,C]	8.08±1.13[A]	8.46±1.11[A]	312.92	<0.001
Albumin g/dl	4.47±0.6[B]	3.98±0.51[A,C]	4.61±0.51[B]	17.74	<0.001
T.Mg mM	1.27±0.30[B,C]	1.05±0.24[A]	1.11±0.31[A]	7.89	0.001
I.Mg mM	0.88±0.2[B,C]	0.73±0.16[A]	0.77±0.2[A]	7.89	0.001
Adj.T.Ca mM	2.37±0.14[B,C]	2.24±0.08[A,C]	1.99±0.1[A,B]	132.49	<0.001
Adj.I.Ca mM	1.31±0.04[B,C]	1.28±0.02[A,C]	1.21±0.03[A,B]	130.72	<0.001
T.(Ca/Mg)	1.97±0.51[B]	2.22±0.42[A,C]	1.91±0.46[B]	6.18	0.003
Ionized (Ca/Mg)	1.57±0.37[B]	1.81±0.32[A,C]	1.66±0.37[B]	5.82	0.004
Iron µg/dl	116.91±20.56[B,C]	211.05±58.34[A]	208.33±56.35[A]	49.61	<0.001
CRP mg/l	4.01(2.03-4.99)[B,C]	7.98(5.61-9.48)[A]	8.22(6-14.6)[A]	KWT	<0.001
Ferritin ng/ml	84.00(64.25-124.75)[B,C]	2505.15(1497.35-3242.13)[A]	2449.40(1741.05-3486.45)[A]	KWT	<0.001

[A, B, C]: pairwise comparison. PCV: packed cell volume, T.Mg: total magnesium, I.Mg: ionized magnesium, Adj.T.Ca: adjusted total calcium, Adj.I.Ca: adjusted ionized calcium, CRP: C-reactive protein.

There is no significant difference in the age, sex ratio, residency, weight, height, disease duration, age of onset, splenectomy, number of transfusions, 1-Alpha taken, and days after the last transfusion have no significant difference between TDT patients with and without hypocalcemia. TDT patient groups have a substantial increase in the family history, splenomegaly, and drugs taken (folic acid, vitamin B12, Desferral, and Exjade) compared with the control groups with no significant difference between TDT groups.

Comparison of the DKK1 and HMGB1 among groups

Serum HMGB1 of the TDT patients with and without hypocalcemia and the control groups are presented in Figure (1).

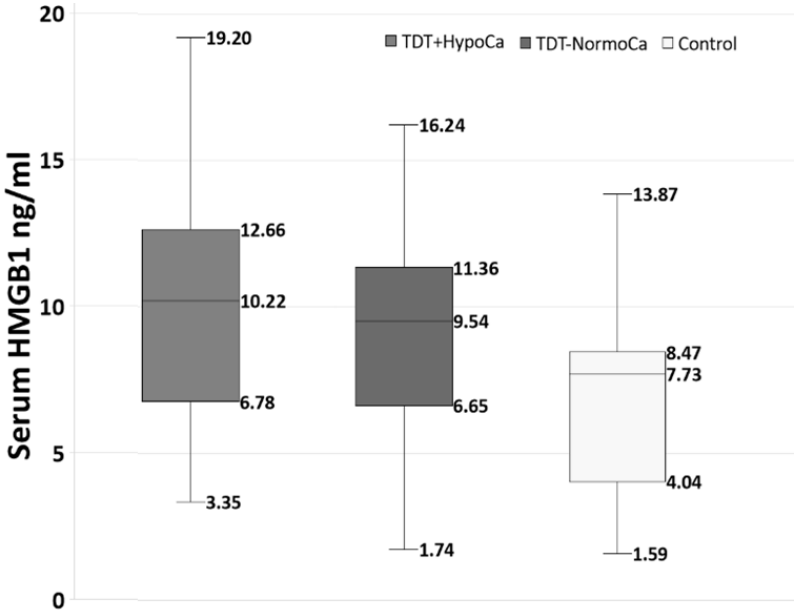

Figure 1. Serum HMGB1 of the transfusion-dependent thalassemia (TDT) patients with and without hypocalcemia, and the control groups. The medians, interquartiles, and the ranges of data are presented in this Figure too.

The results showed that both TDT+Normocalcemia group (9.54(6.65-11.36) ng/ml) and TDT+Hypocalcemia group (10.22(6.78-12.66) ng/ml) have a a significantly higher HMGB1 ($p<0.001$) than the control group (7.73(4.04-8.47) ng/ml). While there is no significant difference between TDT groups.

Figure (2) illustrates the serum DKK1 levels in TDT patients with and without hypocalcemia alongside the control groups.

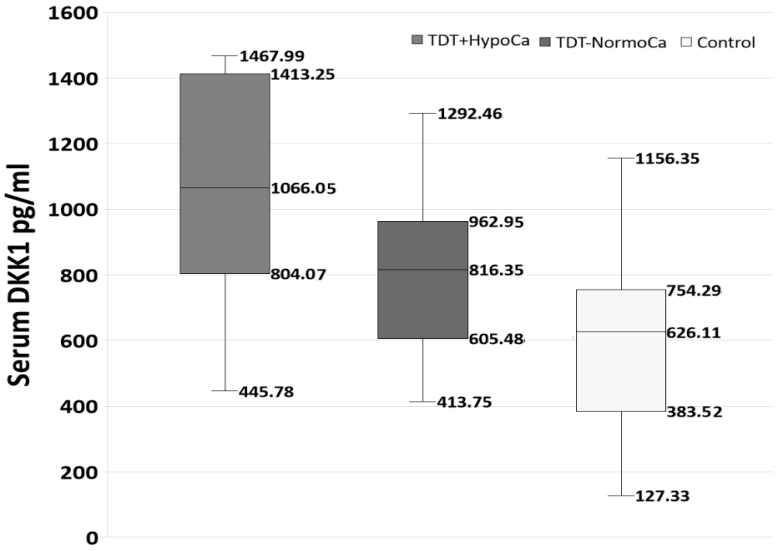

Figure 2. Serum DKK1 of the transfusion-dependent thalassemia (TDT) patients with and without hypocalcemia, and the control groups. The medians, interquartiles, and ranges of data are presented in this Figure too.

The findings indicated that both the TDT+Normocalcemia group (816.34 (603.77-962.95) pg/ml) and the TDT+Hypocalcemia group (1066.05 (804.07-1413.25) pg/ml) exhibited significantly elevated DKK1 levels ($p<0.001$) compared to the control group (626.11 (383.52-754.29) pg/ml). Also, the TDT+Hypocalcemia group has a significantly higher ($p<0.001$) serum DKK1 than the TDT+Normocalcemia group.

Comparison of the routinely measured biomarkers among groups

Table (2) shows the comparison of the routinely measured biomarkers among groups. Hemoglobin, PCV, total and ionized magnesium, have significant decreases in both TDT groups in comparison with the control group. While, iron, CRP, and ferritin levels are higher in both TDT groups compared with the control group. While no significant difference between TDT+Normocalcemia and the TDT+Hypocalcemia groups. TDT+Normocalcemia

has significantly higher albumin and total and ionized Ca/Mg ratios than the TDT+Hypocalcemia and the control groups. As expected from the classification of groups, the control group has a significantly higher Adj.T.Ca and Adj.I.Ca than both TDT groups with the the lowest level in the TDT+Hypocalcemia group.

Results of multivariate generalized linear model (GLM) analysis

Table (3) shows the results of multivariate GLM analysis which examined the association between the measured biomarkers and diagnosis (control versus TDT+Normocalcemia and TDT+Hypocalcemia) while adjusting for covariates (age, sex, residency, weight, and height).

Table 3: Results of multivariate GLM analysis with the biomarkers as dependent variables and diagnosis as explanatory variables while adjusting for extraneous variables.

Type	Dependent Variables	Explanatory Variables	F	P	Partial η^2
Multivariate	PCV, Hb, CRP, Albumin, T.Mg, I.Mg, Adj.T.Ca, Adj.I.Ca, T.Ca/Mg, IonizedCa/Mg, Iron, Ferritin, HMGB1, and DKK1	Diagnosis	50.914	<0.001	0.851
		Sex	0.759	0.701	0.078
		Residency	1.090	0.374	0.109
		Age	1.027	0.431	0.103
		Weight	0.937	0.517	0.095
		Height	1.073	0.389	0.107
Between-subjects effect	Diagnosis	Adj.T.Ca	236.547	<0.001	0.649
		Adj.I.Ca	224.288	<0.001	0.637
		Hb	159.740	<0.001	0.556
		PCV	159.676	<0.001	0.555
		Ferritin	96.639	<0.001	0.430
		CRP	58.791	<0.001	0.315
		Iron	57.407	<0.001	0.310
		DKK1	44.404	<0.001	0.258
		HMGB1	13.762	<0.001	0.097
		T. & I.Mg	7.336	0.008	0.054
		IonizedCa/Mg	1.319	0.253	0.010
		Albumin	1.227	0.270	0.009
		T.Ca/Mg	0.227	0.635	0.002

The findings indicate that the diagnosis of TDT and hypocalcemia in a patient has a significant effect on the levels of the measured biomarkers ($F=50.914$, $p<0.001$) with the highest size effect (partial $\eta^2=0.851$). The cofounders (age, sex, residency, weight, and height) have no significant impact ($p>0.05$) on the biomarker levels. Based on these data, it appears that having TDT or being a patient is the main factor impacting the serum levels of the biomarkers. While other confounding variables included in the investigation do not significantly affect changes in the biomarker levels. The between-subject effects tests demonstrated that the inclusion of the disease had a substantial impact on multiple parameters. Out of all the factors examined, the diagnosis can explain 64.9% and 63.7% of the variation in adjusted total and ionized calcium, respectively. Other biomarkers that were significantly affected were haemoglobin (partial $\eta^2=0.556$), PCV (partial $\eta^2=0.555$), ferritin (partial $\eta^2=0.430$), CRP (partial $\eta2=0.315$), iron (partial $\eta^2=0.310$), and DKK1 (partial $\eta^2=0.258$), HMGB1 (partial $\eta^2=0.097$), total and ionized magnesium (both partial $\eta^2=0.054$).

Correlation between the biomarkers and other parameters

The correlation coefficients and their significance between the measured biomarkers and all parameters are presented in Table (4).

Table 4: Correlation matrix of the measured biomarkers with demographic, clinical, and biochemical parameters in transfusion-dependent thalassemia

Parameters	LnCRP	LnHMGB1	LnDKK1	LnFerritin
Sex	0.006	-0.196	-0.254*	0.020
Splenomegaly	-0.331**	-0.018	-0.156	-0.057
Adj.T.Ca	-0.260*	-0.133	-0.355**	-0.119
Adj.I.Ca	-0.260*	-0.138	-0.352**	-0.116
LnCRP	1	0.045	0.209*	0.399**
LnHMGB1	0.045	1	0.472**	-0.015
LnDKK1	0.209*	0.472**	1	0.069
LnFerritin	0.399**	-0.015	0.069	1
Ln(No.of transfusion)	0.169	0.000	0.025	0.245*
Ln(Days after transfusion)	-0.125	0.137	0.085	-0.205*

***. Correlation is significant at the 0.05 level. **. Correlation is significant at the 0.01 level. The following parameters are not mentioned in the correlation matrix because they have no significant correlation with any of the measured biomarkers: family history, splenectomy, age, weight, height, PCV, haemoglobin, albumin, T.Ca/Mg, IonizedCa/Mg, iron, T. & I.Mg, T.Ca/Mg, IonizedCa/Mg, duration of disease, and age of onset,**

The following parameters are not mentioned in the table because they have no significant correlation with the measured biomarkers (LnHMGB1, LnFerritin, LnCRP, and LnDKK1): family history, splenectomy, haemoglobin, age, weight, height, PCV, albumin, T.Ca/Mg, IonizedCa/Mg, iron, T. & I.Mg, T.Ca/Mg, IonizedCa/Mg, duration of disease, and age of onset. The results show LnCRP has a significant correlation with DKK1 and ferritin, in addition to a significant inverse correlation with splenomegaly, and total and ionized adj.Ca. There is also a significant correlation between HMGB1 and DKK1. DKK1 also has a significant inverse correlation with sex, splenomegaly, and Adj.T.Ca. Serum ferritin has a significant correlation with the number of transfusions and a significant inverse correlation with the number of days after transfusion.

Receiver operating characteristic analysis for prediction of hypocalcemia

A study of receiver operating characteristics (ROC) was conducted to ascertain the diagnostic sensitivity and specificity of the observed biomarkers for predicting hypocalcemia in TDT patients. Figure (3) displays the ROC curves of the study.

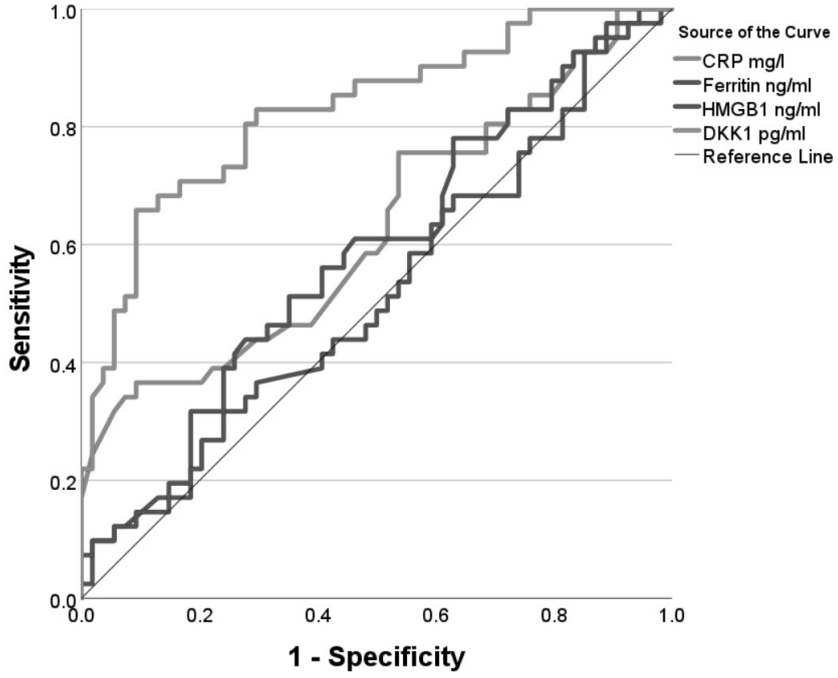

Figure 3. Receiver operating characteristic curves (ROC) of serum CRP, ferritin, HMGB1, and DKK1 for differentiation between TDT patients with and without hypocalcemia.

Table (5) presents the coordinates of the ROC values and the concentration cut-off that yields optimal sensitivities and specificities.

Table 5: Receiver operating characteristic-area under curve (ROC-AUC) analysis of the measured biomarkers for diagnosis of hypocalcemia in TDT patients.

Variable	Cut-off	Sensitivity %	Specificity %	Youdin's J statistic	AUC(95% CI)	P
DKK1 pg/ml	901.44	73.2	72.2	0.454	0.83(0.75-0.91)	<0.001
CRP mg/l	7.90	53.4	52.6	0.060	0.63(0.51-0.74)	0.034
HMGB1 ng/ml	9.71	56.1	55.6	0.117	0.56(0.44-0.68)	0.340
Ferritin ng/ml	2457.65	51.2	50.0	0.012	0.55(0.43-0.66)	0.452

Table (5) data reveal that an elevation in DKK1 over the threshold value (901.44 pg/ml) suggests that the participants may substantially exhibit hypocalcemia ($p<0.001$), with a sensitivity of 73.2% and specificity of 72.2% (Youden's J statistic=0.454). The findings indicated that a CRP rise beyond the cut-off value (7.90 mg/l) suggests the participants may have hypocalcemia, exhibiting low sensitivity (56.1%) and specificity (58.6%), along with a minimal Youden's J statistic (0.117). HMGB1 and ferritin have no significant predictive value for hypocalcemia ($p>0.05$).

Discussion

The primary findings of this research include increased levels of DKK1 and HMGB1 in TDT patients with hypocalcaemia compared to those without hypocalcaemia. This may be attributable to the influence of these markers on intracellular or plasma calcium concentrations. Prior research indicates that calcium levels are reduced in people with TDT relative to healthy individuals (Karim *et al.*, 2016).

Individuals with beta-thalassemia had reduced blood levels of vitamin D and calcium (Bashir *et al.*, 2023). In Iraqi persons with low ferritin levels

(about 500 ng/l), there is no significant change in blood calcium and albumin concentrations between the control group and thalassaemia patients (Al-Hakeim *et al.*, 2017). Nevertheless, our results, along with those of other studies, indicate that blood calcium levels decrease in people with elevated ferritin levels (Al-Dujaili *et al.*, 2019; Al-Hakeim & Alhillawi, 2018; Ridha *et al.*, 2024; Ridha *et al.*, 2018; Moustafa *et al.*, 2023). Individuals with raised ferritin levels have a higher prevalence of low calcium levels compared to those with normal ferritin levels (Iskandar *et al.*, 2020). In TDT patients, increased ferritin levels diminish calcium levels (Iskandar *et al.*, 2020). Insufficient blood calcium levels may signify poor bone mineral density, especially in areas with prevalent hypovitaminosis D (Ali *et al.*, 2023). Reduced parathyroid hormone levels correlate with declining calcium levels in these people (Ansaf *et al.*, 2024). Potential reasons for this variation across studies include the compensatory mechanisms of the body that keep calcium levels within a normal range despite possible underlying problems. Occasionally, these mechanisms may partially mitigate the hypocalcaemia effects, preventing the emergence of unconcealed symptoms (Lertsuwan *et al.*, 2018b). TDT patients had elevated blood HMGB1 levels in comparison to healthy people. Individuals with an intact spleen had lower levels of HMGB1 than those who underwent splenectomy (Chirico *et al.*, 2015). Patients with thalassemia had significantly elevated HMGB1 levels compared to healthy controls (Ghalwash *et al.*, 2024).

Patients with hypocalcemia have higher C-reactive protein levels relative to the TDT and control groups, offering further evidence of inflammation. An elevation in DKK1 and HMGB1 may indicate the presence of an inflammatory disorder. The translocation and release of HMGB1 during

infection (Zhang *et al.*, 2008) and inflammation (Tian *et al.*, 2020) are facilitated by calcium-mediated signal transduction. Upon release, extracellular HMGB1 interacts with the RAGE receptor on macrophages, subsequently activating the stimulator of the interferon response pathway to generate pro-inflammatory cytokines, including TNF and IL-6 (Fang *et al.*, 2021). The activation of Ca^{2+}/dependent protein kinases, namely Ca^{2+}/calmodulin-dependent protein kinase kinase (Li *et al.*, 2020), governs the phosphorylation and release of HMGB1. Calcium ions function as a universal second messenger by participating in many physiological processes via their allosteric regulatory effects on intracellular enzymes or proteins (Zhao *et al.*, 2017). Cellular demise or significant injury may result from disruption of intracellular calcium signalling (Criddle, 2016).

Table (3) indicates that the levels of the analysed biomarkers are unaltered by the confounding variables. Consequently, thalassaemia and its sequelae, which include increased ferritin, anaemia, and inflammation, account for the alterations in the levels of the evaluated biomarkers. The correlation matrix in Table 4 indicates that DKK1 exhibits a substantial association with CRP and HMGB1, and most notably, it has a significant negative correlation with Adj.T.Ca. These results underscored the importance of inflammation and calcium in the levels of DKK1 and HMGB1. Furthermore, elevated concentrations of the master inflammatory protein (HMGB1) and the pro-inflammatory glycoprotein (DKK1) may impair the blood-brain barrier (BBB) and contribute to neurodegenerative processes (Al-Dujaili *et al.*, 2020; Menet *et al.*, 2020). The second is an intrinsic inhibitor of the canonical Wnt pathway that may influence neuro-repair mechanisms and may lead to excitatory synaptic disintegration (Al-Dujaili *et al.*, 2020; Menet

et al., 2020). The inflammatory state and the positive connection with hsCRP may explain the increase in HMGB1 (Wang *et al.*, 2015; Yan *et al.*, 2009). HMGB1 may be released into the extracellular environment and may act as proinflammatory mediators (Czura & Tracey, 2003). HMGB1 may associate with toll-like receptors to activate NF-κB, consequently influencing total immune-inflammatory responses (Yang *et al.*, 2005) by promoting the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα (Park *et al.*, 2003). Upon the binding of HMGB1 to RAGE, the production of IL-6 may occur, hence modulating inflammation (Nativel *et al.*, 2013). The ROC analysis findings in Figure 3 and Table 4 indicated that the elevation of DKK1, followed by an increase in CRP, serves as the most effective predictor for hypocalcaemia in TDT patients. DKK1 serum levels were elevated in individuals with TDT (Voskaridou *et al.*, 2009). At baseline, all patients had elevated blood levels of DKK1 in comparison to controls (Voskaridou *et al.*, 2008). The rise of DKK1 may contribute to osteoblast dysfunction in thalassaemia and indicate a potential target for developing new treatments for bone loss in thalassaemia patients (Voskaridou *et al.*, 2008). DKK1 may have a significant role in certain essential facets of bone biology (Daoussis *et al.* 2010). DKK1 is involved in the aetiology of osteoporosis and may serve as a serological marker for assessing bone mass in adults (Butler *et al.*, 2011). DKK1 does not correlate with illness and inflammation (Giordano *et al.*, 2022). Patients with TDT exhibited elevated blood DKK1 levels and heightened bone turnover (Voskaridou *et al.*, 2012). DKK1 has an inverse correlation with whole-body BMD, indicating its potential use as a bone biomarker in this patient demographic (Wiromrat *et al.*, 2023). HMGB1, whether actively or passively released into the extracellular compartment, serves as a damage-

associated molecular pattern molecule, influencing immunological and inflammatory responses via RAGE and toll-like receptors (Chen *et al.*, 2022). HMGB1 functions as a pro-inflammatory cytokine upon extracellular release, initiating inflammation and immunological responses (Wei *et al.*, 2022). HMGB1 stimulates NF-κB to enhance the expression of pro-inflammatory factors (Lotze & Tracey, 2005). HMGB1 has a role in the pathophysiology of several immune-mediated and inflammatory diseases (Chen *et al.*, 2022). Elevated serum ferritin levels are posited to be associated with diminished Vitamin D levels, perhaps resulting from impaired calcium metabolism and increased iron absorption (Pishgahi *et al.*, 2020), which adversely impacts calcium absorption and bone metabolism (Amelia *et al.*, 2020).

Conclusion

Serum levels of DKK1, HMGB1, C-reactive protein, and ferritin are high in both TDT patient cohorts relative to the control groups. Only serum DKK1 exhibits a substantial elevation in the TDT+Hypocalcemia group compared to the TDT+Normocalcemia group. The results, along with elevated CRP levels, an inflammatory biomarker, indicate the involvement of DKK1 and inflammation in the hypocalcemic condition of TDT patients. Serum DKK1 serves as a predictor for hypocalcaemia in TDT patients, demonstrating adequate sensitivity and specificity. Consequently, DKK1 protein may serve as a therapeutic target for the bone issues of thalassaemia patients.

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