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The assessment of cytokine-dependent hematopoietic cell linker and interleukin-3 levels in children with beta-thalassemia major

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Beta-thalassemia is caused by a lack of or failure to synthesize beta globin chains in hemoglobin resulting in an excess of alpha chains. Cytokine-dependent hematopoietic cell linker (CLNK) is an adapter protein which is involved in the regulation of immunoreceptor signaling. It was found to be associated with a tyrosine-phosphorylated polypeptide (p92) in response to immunoreceptor stimulation. In thalassemia, oxidative stress causes tyrosine phosphorylation of the cytoplasmic domain of band 3. Therefore, we aimed to see how serum CLNK and interleukin-3 correlated with serum ferritin and annual transfusion index in children with beta-thalassemia major (β-TM). This case-control study included 100 non-splenectomized, transfusion-dependent β-TM pediatric patients receiving oral deferasirox and 100 healthy controls. The study was approved by the Institutional Review Board (IRB) of the Menoufia Faculty of Medicine, the approval number is 19/4/2021.PEDI. All procedures were carried out in accordance with relevant guidelines and regulations. In both groups, serum ferritin, interleukin-3, hemoglobin and CLNK levels were measured. They were found to be significantly higher in the β-TM patients than in the controls (p 0.001). There was a negative correlation between serum CLNK and hemoglobin (r = –0.483, p < 0.001), and a positive correlation between serum CLNK and ferritin levels (r = 0.855, p < 0.001). There was a positive correlation between serum CLNK, ferritin, and annual transfusion index. Increased serum CLNK in transfusion-dependent β-TM patients was associated with elevated serum ferritin concentrations and high annual transfusion index. This could be explained by reciprocal effects between immune signaling system and immature erythrocytes which release signaling molecules, such as CLNK, in the blood.

Key words: *cytokine-dependent hematopoietic cell linker, interleukin-3, transfusion-dependent thalassemia major, iron overload, children*

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Beta-thalassemia, one of the most common hemoglobinopathy in children, is an inherited single-gene disorder [1, 2]. It is caused by a lack or absence of beta-globin chains in hemoglobin (Hb). Beta globin synthesis is controlled by one gene on each chromosome 11 [3]. Beta-thalassemia major (β-TM) is a blood disorder that is usually diagnosed in children during their first year of life and requires regular blood transfusions [4].

Iron overload is caused by frequent blood transfusions, which can lead to a variety of complications, including endocrine disorders (such as disorders of growth, pubertal delay, gonadal dysfunction, and diabetes mellitus), cardiovascular and liver diseases [5].

In β-TM patients, cyclic adenosine monophosphate (cAMP) levels are elevated in both red blood cells and nucleated erythroblasts. In nucleated erythroblasts, the transcription factor cAMP response element-binding protein (CREB) is phosphorylated, and the levels of phosphorylation correlate with gamma-globin gene (HBG) mRNA expression levels. In beta-thalassemia patients, the levels of cytokines such as erythropoietin, transforming growth factor-beta, and stem cell factor are elevated, and these cytokines induce HBG mRNA expression and CREB phosphorylation. In beta-thalassemia, the cAMP-dependent pathways, the activity of which is augmented by multiple cytokines, play a crucial role in regulating HBG expression [6].

By modulating tyrosine phosphorylation, the Src family of protein tyrosine kinases (SFKs) regulates RBC membrane cotransport, including K/Cl cotransport. SFKs are a class of non-receptor tyrosine kinases that play a role in hematopoietic cell function and control via the cell membrane [7].

The cytosolic adaptor protein Src homology 2 domain-containing leukocyte phosphoprotein of 76 kD (SLP-76) plays a role in the TCR-induced signaling cascade [8]. SLP-76-related molecule was termed cytokine-dependent hematopoietic cell linker (CLNK) [9].

CLNK can be detected in a variety of cell types, including T cells, natural killer cells, and mast cells, and its expression is influenced by cytokines such as

interleukin-2 (IL-2) and interleukin-3 (IL-3). CLNK is involved in the stimulation of immunoreceptors via a tyrosine-phosphorylated polypeptide (p92) [10]. The tyrosine phosphorylation of cytoplasmic domain of band 3 that occurs in thalassemia causes major alterations in erythrocyte shape and leads to the release of microparticles and local red cell membrane instability in hemolytic anemia [11].

Aim of the study – to evaluate the levels of CLNK and IL-3 in children with β-TM and the association of CLNK concentrations with serum ferritin levels and increased frequency of blood transfusions.

MATERIALS AND METHODS

Study design and ethical statement

The study involved 100 β-TM children and 100 healthy controls and took place between August 2019 and March 2020. They were recruited from the Hematology Unit and the Outpatient Clinic of the Department of Pediatrics, Menoufia University. Before being included in the study, all participants and their parents/legal guardians provided their written informed consent. We took a thorough medical history and performed a complete physical examination of all the patients. The study was approved by the Institutional Review Board (IRB) of the Menoufia Faculty of Medicine, the approval number is 19/4/2021.PEDI. All procedures were carried out in accordance with relevant guidelines and regulations.

Inclusion criteria

Children with β-TM as determined by Hb electrophoresis, transfusion-dependent, treated with iron chelation therapy (oral deferasirox), not requiring splenectomy. All patients had their C-reactive protein levels checked to rule out any inflammation.

Exclusion criteria

Patients with systemic conditions such as diabetes, heart disease, or hepatic problems.

Methods

Five milliliters of whole blood was collected from each β-TM patient (before blood transfusion) and healthy control and divided into two aliquots. One aliquot was transferred to an EDTA tube for hematological analysis, while the other was transferred to a plain tube and centrifuged for 10 minutes at 3000 rpm to separate serum. Serum was stored at 2 to 8°C for up to 48 hours or frozen until used for CLNK and IL-3 testing.

CLNK concentration was measured in the patients' serum using a solid-phase enzyme-linked immunosorbent assay (Bioassay Technology Laboratory, Cat. No E4818Hu, China) as directed by the manufacturer. First, we added 50 μL of standard reagent (provided in the kits) to the standard well. Next, we added 40 μL of each sample to sample wells and then added 10 μL of anti-CLNK antibody to sample wells. After that, 50 μL of streptavidin-HRP was added. Following a 60-minute incubation period at 37°C and five automated washing cycles, we added 50 μL of substrate solution A and 50 μL of substrate solution B to each well and incubated for 10 minutes in the dark. Finally, a stop solution was added to each well, and the optical density was determined using a 450 nm microplate reader. Curve and regression analysis were used to calculate the results with computer-based tools.

Double antibody sandwich ELISA kits were used to assess human IL-3 in serum (Sunred Biological Technology, Catalog No. 201-12-0094, China).

Fifty microliter of each standard was combined with 50 μL of streptavidin-HRP solution in the standard wells. In the sample wells, 40 μL of a sample was mixed with 50 μL of Streptavidin-HRP, and then 10 μL of IL-3 antibody was added to each ELISA plate. After 1 hour of incubation at 37°C, the plates were washed five times with a wash buffer.

After that, 50 μL of chromogen solution A and 50 μL of chromogen solution B were added to each well (the liquid turned blue) and incubated for 10 minutes at 37°C in the dark. Last but not least, 50 μL of stop solution was added (the blue color turned yellow in an instant). The plates were scanned using a microplate reader set to a wavelength suitable for the color reaction (the optical density was measured at 450 nm). The correlation between color intensity and sample IL-3 levels was found to be positive.

Serum ferritin levels were determined using an automated analytical technique (Cobas e604 Roche Company).

Sample size calculation

The sample size was calculated to achieve 95% confidence intervaland 80% power. We used an unpaired t-test and a (two-sided) α of 0.05. Based on a previous study [12], the mean CLNK in the group of β-thalassemia patients was 6.24 ng/mL while that in healthy controls was 1.95; SD was 10.81, with a group size ratio of 1/1 (100 participants in each group).

Statistical analysis

The obtained data were analyzed using the IBM SPSS Statistics version 22.0 (SPSS Inc., Chicago, IL, USA). A chi-square test was used to examine the relationship between qualitative variables. For quantitative data, the two groups were compared using either Student's t-test or Mann–Whitney U test (a

non-parametric t-test) as appropriate. Pearson's correlation coefficient or Spearman's Rho method (as appropriate) was used to examine the correlation between numerical variables. Multiple linear regression was carried out to investigate the relationship between the dependent variable (CLNK) and independent (other laboratory variables) variables. A *p*-value < 0.05 was considered significant.

RESULTS

The mean age of the β-TM patients was 8.8 (SD – 4.2) years, with males accounting for 58% (*n* = 58) of the total number of patients, while the mean age of the healthy controls was 7.9 (SD – 3.7) years, with females making up 51 percent (*n* = 51) of the group. There was no significant difference in age or gender between the two groups, but there was a significant difference in Hb, mean corpuscular volume (MCV), and mean corpuscular Hb (MCH) between the groups, with much lower values in the β-TM patients compared with the controls ($p < 0.001$). In the β-TM patients, serum ferritin, IL-3, and CLNK levels were significantly higher than in the controls ($p < 0.001$), as shown in *table 1*.

There was a significant negative correlation between serum CLNK and Hb levels (r = –0.483, $p < 0.001$), and a significant positive correlation between serum CLNK and serum ferritin and IL-3 (r = 0.855, $p < 0.001$ and r = 0.664, $p < 0.001$, respectively). The two groups did not differ significantly in terms of age, MCV, MCH, white blood cells, and platelets (*table 2*). High CLNK levels correlated with significantly decreased Hb and HCT levels (r = –0.918, $p < 0.001$ and r = –0.334, $p < 0.001$, respectively) and increased serum ferritin and IL-3 concentrations and number of transfusions per year ($p < 0.001$) (*table 3*). As shown in *figure*, there was a significant positive correlation between serum ferritin and transfusion index per year (r = 0.538, $p < 0.001$ and r = 0.847, $p < 0.001$, respectively).

Table 2
A correlation between CLNK levels and other parameters in the thalassemia major patients

Parameters	CLNK, ng/mL	
	r	*p*-value
Age, year	–0.151	0.13
Hb, g/dL	–0.483	< 0.001*
MCV, fL	–0.027	0.79
MCH, pg	0.092	0.36
PLT, 10^3/mcL	0.091	0.37
WBC, × 10^9/L	–0.028	0.78
Ferritin, μg/L	0.855	< 0.001*
IL-3, pg/mL	0.664	< 0.001*

Table 3
Multiple linear regression analysis to detect predictable factors for CLNK among the thalassemia major patients

Predictors	β	r	*p*-value
Hb, g/dL	–0.493	0.332*	< 0.001*
MCV, fL	–0.021	0.037	0.15
MCH, pg	0.039	0.316	0.24
Ferritin, μg/L	0.001	0.502*	< 0.001*
Transfusion index, mL/kg/year	0.058	0.580*	< 0.001*
IL-3, pg/mL	0.646	0.503*	< 0.001*

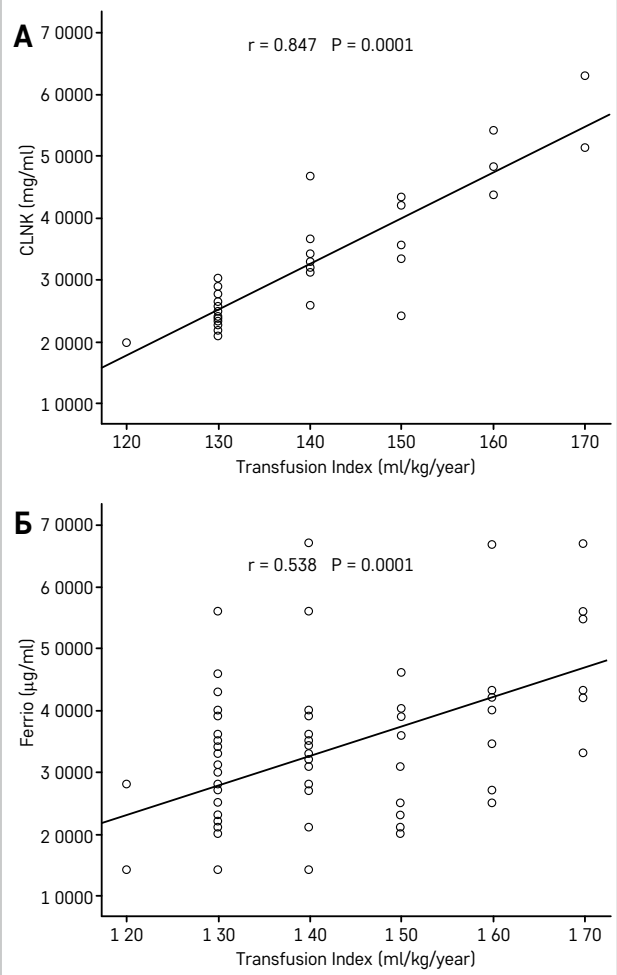
*Note. A dependent variable was CLNK. * – significant difference.*

Table 1
Demographic and laboratory characteristics of the groups

Indicator		Mean (SD)	Median (range)	Test of significance	*p*-value
Age, year	β-TM patients	8.8 (4.2)	8 (2–17)	Mann–Whitney test = 0.72	0.47
	Controls	7.9 (3.7)	8 (2–12)		
Sex		Male	Female		
	β-TM patients	58 (58.0%)	42 (42.0%)	χ^2 test = 1.63	0.20
	Controls	49 (49.0%)	51 (51.0%)		
Hb, g/dL	β-TM patients	6.7 (0.66)	6.8 (4.8–7.9)	T-test = 50.83	< 0.001*
	Controls	12.1 (0.81)	11.8 (11–14)		
MCV, fL	β-TM patients	69.9 (4.6)	70 (60–78)	T-test = 14.20	< 0.001*
	Controls	78.6 (3.9)	78 (74–90)		
MCH, pg	β-TM patients	23.5 (1.9)	23.5 (20–29.4)	T-test = 13.21	< 0.001*
	Controls	27.1 (1.9)	27 (24–32)		
PLT, 10^3/mcL	β-TM patients	375.8 (133.7)	356 (223–895)	Mann–Whitney test = 1.09	0.28
	Controls	359.7 (68.3)	402 (223–423)		
WBC, × 10^9/L	β-TM patients	10.3 (3.5)	9.5 (4.7–22)	Mann–Whitney test = 1.06	0.29
	Controls	9.4 (2.7)	8.7 (5.5–12.8)		
Ferritin, μg/L	β-TM patients	3250.5 (1169.1)	3100 (1400–6700)	Mann–Whitney test = 12.23	< 0.001*
	Controls	43.3 (13.04)	37 (27–70)		
CLNK, ng/mL	β-TM patients	3.3(1.1)	3.02 (1.9–6.3)	T-test = 16.77	< 0.001*
	Controls	1.4(0.22)	1.5 (0.92–1.7)		
IL-3, pg/mL	β-TM patients	18.2 ± 7.8	20 (0–22.3)	T-test = 14.8	< 0.001*
	Controls	0.8 ± 0.3	0.9 (0–2.1)		

*Note. In this table and in table 2: PLT – platelet; WBC – white blood cells; * – significant difference.*

Figure
Correlations between serum CLNK and transfusion index per year (A) and between serum ferritin and transfusion index per year (B)



DISCUSSION

Beta-thalassemia is an inherited disorder characterized by reduced or absent β-globin production and an excess of unpaired α-globins [13]. Unpaired α-globins precipitate and autoxidize, releasing large amounts of ROS, free heme, and iron [14]. These molecules cause lipid and protein oxidation, which leads to band 3 clusterization and phosphatidylserine exposure, resulting in eryptosis of RBCs, which are then removed by macrophages [15]. Several studies have shown that an increased number of activated macrophages in the bone marrow of β-thalassemia patients indicates enhanced eryptosis, which is defined as suicidal RBC death that occurs following RBC injury [16]. CLNK is a cytokine inducible (IL-2 and IL-3) adaptor protein produced in hematopoietic cells that belongs to the SLP-76 family of adaptor proteins [17].

In β-TM RBCs have short half-lives and are prone to hemolysis, which results in the release of soluble receptors and signaling molecules including CLNK. Increased levels of several pro-inflammatory cytokines may be detected in β-TM and might contribute to erythropoiesis deficiencies as a result of chronic illness anemia [13]. In our study, blood CLNK levels in β-TM patients were found to be significantly higher than in the controls. There was a significant negative correlation between serum CLNK and Hb. On the other hand, we identified a significant positive correlation between serum CLNK and serum ferritin levels. Increased CLNK levels resulted in a significant drop in Hb. Quite the opposite, high CLNK levels were associated with an increase in serum ferritin and transfusion index per year. We also observed a significant positive correlation between serum ferritin, serum CLNK, and the annual transfusion index. Al-Hakeim et al. [12] found that serum CLNK was significantly associated with the number of transfusions and serum ferritin levels in patients with transfusion-dependent thalassemia. They also reported significant correlations between CLNK and Hb, PCV%, and serum ferritin. A positive correlation between CLNK and ferritin or the number of transfusions was explained by the dependence of CLNK on iron overload status. Karunaratna et al. [18] also reported a correlation between blood transfusion frequency and iron burden in the body, as well as higher serum ferritin levels. In beta thalassemia patients, the interaction between CLNK and iron overload biomarkers may be explained by the formation microvesicles in RBCs.

Bailey et al. [6] showed that the cAMP-dependent pathway, the activity of which is controlled by multiple cytokines, plays a role in g regulating HBG expression in β-TM. In thalassemia and sickle cell disease, there is a significant increase in microvesicle levels [19]. The formation, storage, and clustering of RBC microvesicles is associated with the phosphorylation of band 3 in β-TM patients [20]. The involvement of various signaling pathways in RBC vesiculation is supported by a large number of signaling proteins in microvesicles obtained from the plasma of healthy donors [21].

Macrovesicles are generated to remove oxidized Hb and damaged membrane constituents. Damage to Hb together with altered phosphorylation of membrane proteins, such as band 3, lead to a weakening of the binding between the cytoskeleton and the lipid bilayer, resulting in increased microparticle shedding [12]. These RBC-derived microvesicles play a role in thrombosis, inflammation, and autoimmune reactions [22].

In our study, children with β-TM had markedly increased levels of IL-3, and there was a significant positive correlation between CLNK and IL-3 levels. According to Kutukculer et al. [23], children with β-TM have higher levels of IL-3 than healthy controls. Oppenheim et al. [24] also reported a significant increase in IL-3 in β-TM patients. This could be explained by the role of IL-3 as a multi-colony stimulating factor in hematopoietic cell proliferation, particularly in stimulating erythroid colony formation [24].

CONCLUSION

Serum CLNK levels are increased in transfusion-dependent beta thalassemia major patients, and these increased CLNK levels are associated with elevated serum ferritin levels and increased number of transfusions per year. This could be related to the production of signaling molecules such as CLNK in the bloodstream by an immunological signaling pathway and immature erythrocytes.

Further research into cytokines affecting hematopoiesis and their implications in erythropoietic defects and immune responses in beta thalassemia major patients with partial functional immunodeficiency is needed.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

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