Assessment of Complement Regulatory Proteins CD55 and CD59 on Erythrocytes in Beta-Thalassemia Major Patients

Asmaa M. Zahran[1], Asmaa S. Shaltout[2], Iman H. A. Hamada[3], Asmaa M. E. Mohammed[4], Amir M. Abo Elgheet[5], Khalid I. Elsayh[6]

[1]Clinical Pathology Department, South Egypt Cancer Institute, Assiut University, Assiut, Egypt, [2]Medical Microbiology & Immunology Department, Faculty of Medicine, Assiut University, Assiut, Egypt, [3]Clinical Pathology department, South Egypt Cancer Institute, Assiut University, Assiut, Egypt, [4]Pediatric Department, Faculty of Medicine, Aswan University, Aswan, Egypt, [5]Pediatric Department, Faculty of Medicine, Assiut University, Assiut, Egypt and [6]Pediatric Department, Faculty of Medicine, Assiut University, Assiut, Egypt.

This study intended to measure the expression of complement regulatory proteins CD55 and CD59 on RBCs membrane in patients with β-thalassemia (β- thal) major in addition to investigate if splenectomy affects their expression pattern. This was a case-control study, participants were allocated in three groups. The study group 1 consisted of β-thal patients who underwent splenectomy. The study group 2 consisted of β-thal patients without splenectomy. Group 3 consisted of apparently healthy volunteers as a control group. A significant decrease in CD55 expression in patients' group 1 (46.35±14.61) and group 2 (56.90±9.28) in comparison with group 3 (86.20±9.62) was observed. The percentage of CD55 expression was significantly lower in group 1 patients than group 2 (*P*=0.01). However, there was no difference in the percentage of CD59 marker expression between any of the patient's groups and the control group. In conclusion, CD55 under-expression on RBCs of β- thal patients may be considered one of the factors that cause hemolysis in those patients and this complement mediated hemolysis may be one of the underlying causes of organ damage. Additional deficiency of this receptor occurs with splenectomy.

Beta-Thalassemia (β-thal) is a genetic disease caused by mutations affecting the β-globin gene that lead to inappropriate hemoglobin (Hb) synthesis. Inhabitants of tropical and sub-tropical regions of Africa, Asia and the Mediterranean area are the most common affected populations [1, 2].

Several mechanisms responsible for cell lysis problem in β-thal patients have been reported. Complement system abnormality is an immunological disorder that may be a possible mechanism that aggravates the hemolysis. This mechanism has been supported by a decrease in C3 and C4 level previously described in those patients [3].

Clinical presentation of β -thal patients depends on their treatment which includes regular blood transfusions and iron chelation therapy that reduce the harmful effects of iron overload and decreases organs damage in those patients [4, 5].

In case of anemia if the bone marrow becomes insufficient to counteract it, the spleen works as an extra medullary organ for hematopoiesis and hypersplenism develops in those patients. Hypersplenism caused by extramedullary hematopoiesis necessitates splenectomy [6].

In Normal cells, hemolysis induced by activation of the complement system is prevented by complement regulatory

proteins in the cell membrane; the main proteins are CD55 or decay accelerating factor (DAF) and CD59 or membrane inhibitor of reactive lysis (MIRL).

CD55 prevents C3 and C5 cleavage by inhibiting the formation of new C3 and C5 convertases, additionally accelerating decay of the preformed enzymes [7].

CD59 protein interferes directly on the membrane attack complex (MAC) structure through binding C9, inhibiting its incorporation into the C5b-8 complex and preventing the terminal polymerization of the MAC [8].

Different immunological abnormalities affected by multi factors as splenectomy, iron overload, and frequent exposure to foreign allogenic antigens during the blood transfusion and liver damage after hepatitis. These factors can influence the clinical presentation of the patients from different geographical places [9].

Thus, this study aimed to assess the expression of CD55, and CD59 on RBCs of patients with β-thal major to give an idea on the role of these makers in inhibition of complement mediated hemolysis in those patients and whether splenectomy affects their expression pattern.

Material and Methods

Study type, settings, and duration

The present study was a prospective case-control study conducted in Assuit Pediatric University Hospital, Faculty of Medicine, Assuit University. The Assuit Medical School Ethical Review Board reviewed and approved the study protocol; approval number was (17300301). One of the parents of each participated children signed written informed consent before their children were included in the study.

Study participants

Eligible participants were assigned to three groups. The study group 1 consisted of β-thal patients who underwent splenectomy. The study group 2 consisted of β-thal patients without splenectomy. Group 3 consisted of volunteer control. Patients attending the Hematology Unit outpatient's Clinic during their routine follow up visits were invited to participate in the study. Enrolled patients included previously diagnosed as β-thal patients by clinical and laboratory examinations, as well as patients received blood transfusion according to pretransfusion hemoglobin and blood availability. Personal history was taken from all patients. All patients were subjected to physical examination including height, weight and body mass index. Patients with diabetes mellitus, cardiac, renal, infectious, inflammatory, pulmonary diseases or received any immunosuppressive medications, were excluded from the study. The volunteer controls were matched for age and sex.

Sample taking

From each participant, 4ml, venous blood was obtained by clean venipuncture then divided into two sterile tubes containing K2-EDTA anticoagulant, each one contained 2ml. One blood aliquot was used for flow cytometry analysis, and the second was examined for hematological analysis. A CBC was carried out for all samples on an automatic hematology analyzer (Celltac E, Nihon Kohden Corporation, Tokyo, Japan).

Flow cytometry

The EDTA blood sample was collected from all participants in the study and processed within one hour. The blood sample was diluted with phosphate-buffered saline (PBS) to achieve a concentration of 10,000 RBC/µl. One hundred µl of the samples were stained with 10 µl of fluoroisothiocyanate (FITC)-conjugated CD55 and phycoerythrin (PE)-conjugated CD59 (Becton Dickinson (BD) Biosciences, San Jose, CA, USA). After incubation for 20 minutes at room temperature in the dark, washing with PBS was done. The cells were resuspended in PBS and analyzed by FACSCalibur flow cytometry with Cell Quest software (BD Biosciences, California, USA). Anti-human IgG isotype-matched negative control was used for each sample. Scatter histogram was used to define the erythrocytes (RBCs) population, which then gated for further analysis of the expression of CD55 and CD59 (Figure 1).

Statistical Analysis

Statistical package for social sciences (SPSS) version 20 was used for data analysis. All quantitative data were expressed as mean ± standard deviation (SD). Differences in the mean between the different groups of subjects were calculated using the independent t-

test. Pearson correlation was used to detect the relation between different quantitative variables, *P*-value <0.05 was considered significant.

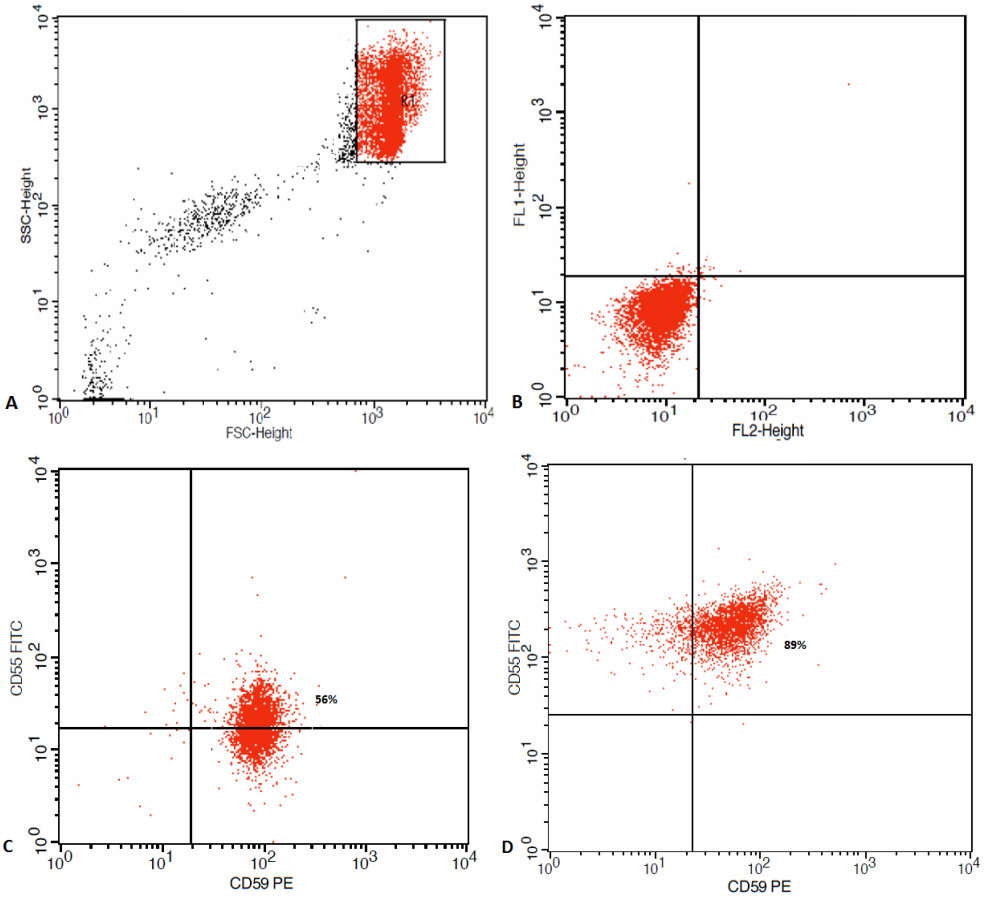

Figure 1. Flow cytometric detection of erythrocytes expression of CD55 and CD59 in β-thalassemia patients

A: Scatter histogram was used to define the erythrocytes (RBCs) population (R1), B: Erythrocytes expression of isotype controls of both CD55 and CD59, C: The expression of CD55 and CD59 on erythrocytes in one patient, D: The expression of CD55 and CD59 on erythrocytes in normal control.

Results

A total of 62 children participated in the study. All were assigned to the three groups; 17 in group 1, 20 in group 2 and 25 in group 3. The age of study participants ranged from 1 to 16 years. Table 1 shows the demographic and clinical characteristics of β–thal patients.

Hematological parameters were significantly different in patients than those in the controls. Hemoglobin was significantly lower in groups 1 and 2 than in group 3 but serum ferritin was significantly higher in groups 1 and 2 than group 3 as shown in Table 2.

Table 1. Demographic and clinical characteristics of β-thalassemia major patients.

Variables	Results
gender:	
_ Number of males	22
_ Number of females	15
Splenectomy:	
_ Number of splenectomized patients	17
_ Number of patients without splenectomy	20
Mean age in years ±SD:	8.70± 4.41
Mean body mass index in Kg/m^2±SD:	15.74± 2.18
Mean weight in Kg ±SD:	21.56± 8.81
Mean height in cm ±SD :	114.59±23.63

Table 2. Hematological parameters, CD55 and CD59 expression in β-thalassemia patients with splenectomy and patients without splenectomy compared to control subjects.

Parameter	Patients with splenectomy Mean ± SD Group 1	Patients without splenectomy Mean ± SD Group 2	Control subjects Mean ± SD Group 3	*P* value (1)	*P* value (2)	*P* value (3)
Hemoglobin (g/dl)	6.10±1.00	5.61±0.90	12.08±0.73	NS	0.000*	0.000*
Serum ferritin (ng/ml)	1272.58±416.69	1237.64±456.12	96.48±44.87	NS	0.000*	0.000*
CD55	46.35±14.61	56.90±9.28	86.20±9.62	0.01*	0.000*	0.000*
CD59	81.76±13.08	82.10±11.6	83.84±9.56	NS	NS	NS

P value (1): significance of difference between patients with splenectomy and patients without splenectomy

P value (2): significance of difference between patients with splenectomy and controls

P value (3): significance of difference between patients without splenectomy and controls

* *P* > 0.05 is significant (NS).

The mean serum ferritin level in patients' samples was 1253.69 ± 432.76 ng/ml. Further, according to the age; patients less than six years had mean serum ferritin of 918.04± 635.56 ng/ml. Patients from six to ten years had mean serum ferritin of 1369.11±130.49ng/ml, while patients more than ten years had mean ferritin value of 1450.71±81.65ng/ml.

Accordingly, a significant moderate positive correlation was observed between age and serum ferritin level (r=0.589, *P*=0.000), as shown in Figure 2.

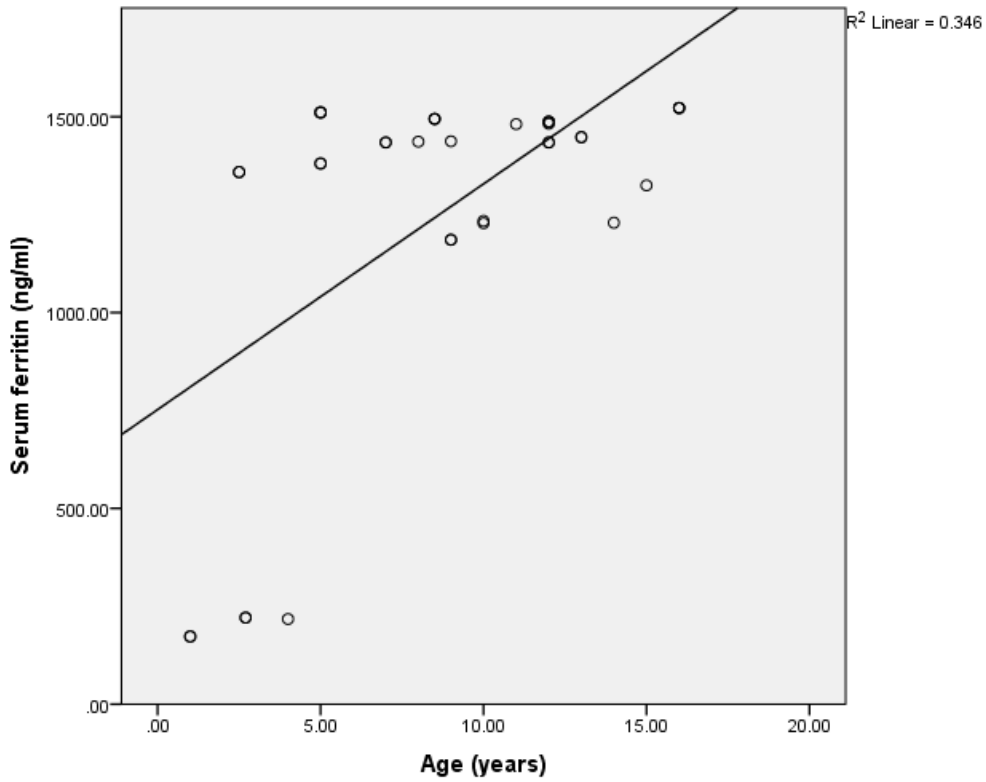

Figure 2. Correlation between serum ferritin level and age of β -thalassemia patients

Flow cytometry analysis of CD55 expression on the RBCs of β-thal patients showed a significant decrease in expression in patients' group 1 and group 2 in comparison with group 3, as shown in Table 2 and Figure 3. Interestingly CD55 expression was significantly lower in patients of group 1 than group 2 as shown in Table 2 and Figure 3. Additionally, a significant mild positive correlation was observed between CD55 and hemoglobin level in all patients (r=0.405, *P*=0.013) as shown in Figure 4. Further investigation of CD59 expression showed no difference in the percentage of the expression marker between any patients group and the controls.

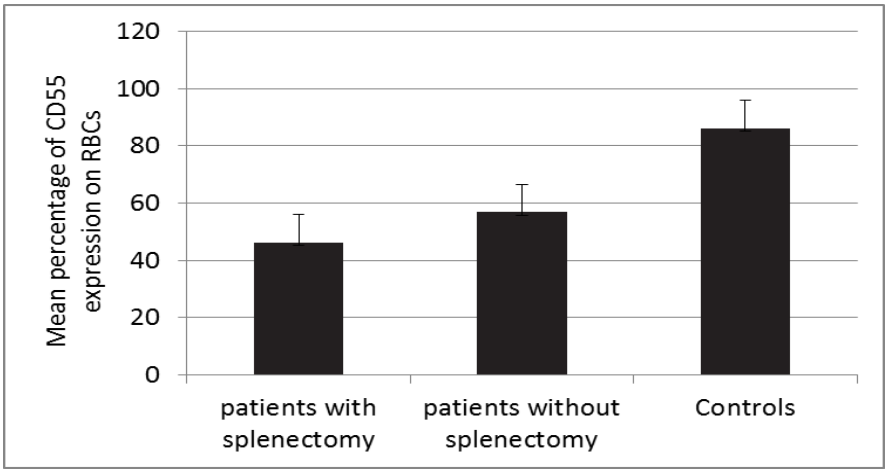

Figure 3. Mean percentage of CD55 expression on RBCs in different groups.

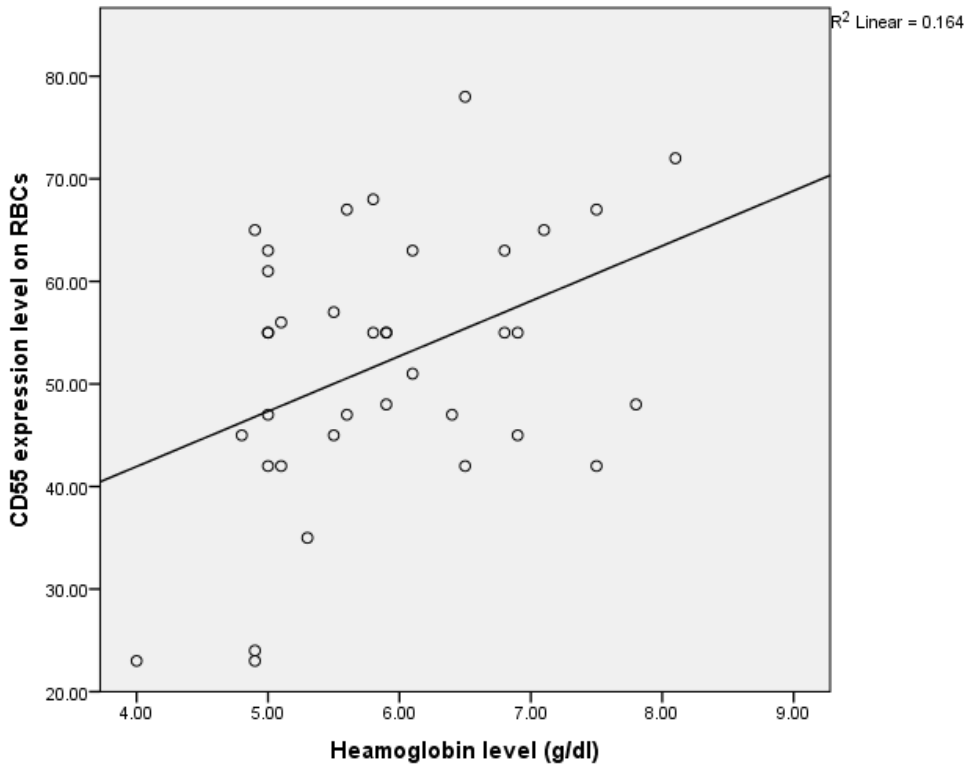

Figure 4. Correlation between CD55 expression and hemoglobin level in β -thalassemia patients

Discussion

In this study, we demonstrated decreased expression of complement regulatory protein on RBCs surface of β - thal patients. Accordingly, this may be one of the causes of hemolysis in these patients. RBCs destruction in β-thal caused mostly by changes occurring in the cell membrane that cause rigidity and exposure of its inner surface, which can activate the complements deposition on RBCs [10]. Hemolysis also occurs when the excess unbound alpha-globin chains precipitate on the cell membrane leading to oxidative reactions and cell lysis [11]. Furthermore, auto-antibodies against the red cell membrane will sensitize RBCs and make them ready to be lysed via the reticuloendothelial system [12].

In the current study hemoglobin level that characterizes the hypochromic microcytic anemia of the group 1 and 2 differed significantly from those of the control subjects. Our data agreed with other regional studies carried out by Ragab *et al.* [13] and Elsayh *et al*. [14] they reported that hemoglobin level was 5.7±1.16 g/dl and 5.946 ± 0.14, respectively. However, this was inconsistent with a study carried out in Germany by Cario *et al.* [15] who described the median baseline hemoglobin concentrations was 10.0 g/dl this difference might be related to that the average transfusion interval which was 3 weeks in their study. In our study, the frequency of blood transfusion was irregular due to low rate of blood donation. In Germany if blood transfusion is covered by health insurance, it would be free of charge which may explain the decreased hemoglobin level in our study sample.

The mean serum ferritin values in our study were lower compared with other studies. For example; a study by Ragab *et al.* [13] reported high mean serum ferritin levels (1928.66±1543.732 ng/ml), this difference may be due to the large sample size in their study with a wide range of serum ferritin from 96 to 9849 ng/ml. Another study in North America by Cunningham *et al.* [16] reported higher mean serum ferritin level (1696 ng/ml] which may be attributed to that the median age of the patients in their study that was 20 years as the level of serum ferritin increase with age.

In the present study, we observed a significant moderate positive correlation between age and serum ferritin, probably occurred as a consequence of an excessive number of blood transfusions. This is consistent with data reported by Mishra and Tiwari [17]. Also, patients less than six years, had mean serum ferritin 918.04 ± 635.56 ng/ml. This is consistent with data of Ragab *et al.* [13] who reported that patients (mean age 5.8 ± 4.3 years) had serum ferritin below 1000 ng/ml. Conversely, Bandyopadhyay *et al.* [18] reported a higher serum ferritin level in young age children than in our study. They found that in 1-5 years age group, the average serum ferritin was 1750 ng/ml this might be attributed to poor compliance of chelation therapy in their study.

Complement regulatory protein deficiency was initially observed on erythrocytes membrane of paroxysmal nocturnal hemoglobinuria, it is a disorder in which the inability of glycosyl-phosphatidylinositol (GPI)-anchor synthesis, due to the PIG-A gene (phosphatidyl inositol glycan A) mutation, leading to an abnormal biosynthesis of GPI anchor [19]. Complement regulatory proteins, like CD55, CD59 and other proteins are considered as GPI-anchored proteins [20, 21].

Complement regulatory proteins are bounded with red blood cells surfaces to protect them from uncontrolled complement lysis [22, 23]. There are studies of complement regulatory proteins levels in diseases having a hemolytic course. Richaud-Patin *et al.* [24] reported reduced expression of CD55 and CD59 in erythrocytes of patients with lupus who presented secondary autoimmune hemolytic anemia.

In this study, the diminished CD55 expression on the RBCs of β-thal patients below the level in the control group indicated that activation of the complement cascade is less regulated, and hemolysis occurs. These data are consistent with those of Kurtoğllu *et al.* [25] and Obaid *et al.* [26].

CD55 and CD59 both are glycoproteins anchored to cell surface by GPI. Decreased CD55 expression in erythrocytes of β-thal patients without decrease in CD59 expression which anchored by the same anchor revealed that the decrease in CD55 expression is not due to mutation affect the synthesis of GPI anchor. However, more than 300 gene mutations were defined in β-thal patients in the literatures [27]. Decreased CD55 expression may be attributed to a genetic defect which affects the expression of the CD55 gene [28].

In this study indifference in CD59 expression between both patients and controls was found. Our results coincide with the study of Kurtoğllu *et al.* [25] and Obaid *et al.*, 2015 [26]. The latter study attributed the indifference in CD59 expression between patients and controls by that, the deficiency of CD55 receptor could be compensated by another receptor CD59 to prevent or alleviate cell lysis. Moreover may be no genetic mutation affect CD59 expression unlike previously described in CD55.

Massive splenomegaly develops early in the course of β-thal due to increased erythrocyte destruction and the presence of splenic extramedullary hematopoiesis [29]. In this study, we investigated how splenectomy affects the level of expression of complement regulatory proteins CD55 and CD59 on erythrocytes in β-thal patients.

The significant decreased levels of CD55 expression in patients with splenectomy as compared to non-splenectomized patients is consistant with Kurtoğllu *et al.* [25]. They demonstrated that splenectomy increases both the erythrocyte mitochondrial DNA and membrane damage in β-thal patients and this damage may be the cause of decreased CD55 expression.

In addition to that increase interval between transfusions in splenectomized patients give less amounts of circulating healthy erythrocytes compared to patients with a spleen who have a higher amounts of circulating healthy erythrocytes due to more blood transfusions however, this second explanation opposed by Obaid *et al.* [26].They found increase interval between transfusions may permit improvement and up-regulation in CD55 expression on erythrocytes.

Significant positive mild correlation was observed between CD55 expression and hemoglobin level in all patients, so this observations indicate that a decrease in RBCs destruction accompanied by increasing CD55 expression.

In conclusion, complement-mediated hemolysis by CD55 under-expression on RBCs of thalassemia patients is considered one of hemolysis causes in these patients. Splenectomy enhances the deficiency of this receptor. CD59 expression on RBCs was not affected. There is a need for further genetic studies investigating possible reasons for the low level of CD55 in β-thal patients.

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