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NOVEL BIOCHEMICAL MARKERS FOR THE PREDICTION OF RENAL INJURY IN BETA-THALASSEMIA MAJOR PEDIATRIC PATIENTS

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Beta-thalassemia major is a severe inherited disorder characterized by inadequate production of hemoglobin beta chains, ineffective erythropoiesis, chronic hemolysis and necessitates lifelong transfusions, which lead to iron overload. The disease manifests itself in early childhood and persists throughout an individual's life with a high risk of developing renal impairment, which cannot be reliably determined using routine markers. The objective of this research was to apply biomarkers to the evaluation of renal injury in pediatric patients within the age range of 1–14 years diagnosed with beta-thalassemia major. In the case-control study, the blood samples obtained in the Genetic Hematology Center in Thi-Qar Iraq Province were used: 60 samples from healthy individuals and 60 samples from the patients with beta-thalassemia, subdivided into 1-7 and 8-14 years old groups. The levels of hemoglobin, ferritin, creatinine and potassium were estimated with standard tests, enzyme-linked immunoassay was used to determine the level of neutrophil gelatinase-associated lipocalin (NGAL) and beta-2-microglobulin (β2M) as novel markers of tubular and glomerular dysfunctions. The study revealed a statistically significant decrease in hemoglobin, serum potassium levels and an increase in ferritin, NGAL and β2M levels in the patients from both groups compared to controls and elevation of creatinine level in the 8–14-year-old group. It was concluded that NGAL and β2M levels may be considered indicators for the early diagnosis of renal injury in pediatric patients with beta-thalassemia, as these biomarkers exhibit elevated levels before an increase in creatinine is observed.

K e y w o r d s: beta-thalassemia major, renal injury, neutrophil gelatinase-associated lipocalin, beta-2-microglobulin, ferritin, creatinine.

Thalassemia is the most prevalent genetic disorder worldwide. In Iraq, between 2010 and 2015, the prevalence of thalassemia rose from 33.5/100,000 to 37.1/100,000. During the same period, the mean incidence rate decreased from 72.4/100,000 live births to 34.6/100,000 live births. Among all types of thalassemia, β-Thalassemia major (β-TM) accounted for 73.9% of cases [1]. In addition, the prevalence of the β-thalassemia trait was 6.94% (864 out of 12,448), and this is considered an important factor in the prevalence of the major type [2] Characterized by reduced synthesis of hemoglobin chains and various hereditary disorders [3]. Beta-thalassemia major is an extremely severe condition that necessitates lifelong transfusions and is associated with many complications [4]. It is characterized by inadequate production of beta chains in the body, resulting in ineffective erythropoiesis and chronic hemolysis. This anemia begins in early childhood and persists throughout an individual's life. Patients with β-TM rely on regular transfusions, which lead to iron accumulation, known as hemosiderosis, in various organs [5]. The patients require iron chelator therapy (ICT) to ensure their survival. Without appropriate chelation therapy, the life expectancy of individuals with transfusion-dependent thalassemia (TDT) is significantly reduced [6]. With therapy, survival rates have improved among thalassemia patients, yet previously undetected complications arise. Renal injury has become a significant concern for individuals with thalassemia [7]. Hypoxia, chronic anemia, ICT, and hemosiderosis have all been proposed as potential triggers for kidney damage in TDT patients. Chronic

anemia and hypoxia result in renal fibrosis and sclerosis, lowering systemic vascular resistance while increasing hyperfiltration, renal plasma flow, apoptosis, and tubular cell damage. Hypoxia and chronic anemia also lead to free radical production, lipid peroxidation, and oxidative stress [8]. Additionally, renal damage in thalassemic patients significantly contributes to the pathophysiology of iron overload. Furthermore, iron chelator toxicity can cause glomerular dysfunction [9].

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein produced by kidney tubule cells in response to harmful stimuli and is present in activated neutrophils. Consequently, NGAL is recognized as a marker for acute kidney injury (AKI) [10]. Multiple experimental and clinical studies have consistently demonstrated a substantial increase in the expression of urinary and serum NGAL in cases of AKI [11]. It is secreted by epithelial cells lining the loop of Henle, distal tubules, and proximal tubules [12].

Beta-2-microglobulin (β2M) plays an essential role in the major histocompatibility antigen. Serum levels of immunoglobulin G, hepcidin, and albumin are all influenced by their interaction with the neonatal Fc receptor [13]. Therefore, it is involved in numerous disease processes, such as glomerulonephritis, iron overload, and AKI. This protein is filtered through the glomerulus, with 99.9% of it being reabsorbed by the proximal tubule. Consequently, β2M serves as a valuable tool for assessing the functionality of both the renal glomerulus and tubules [14]. Several tests, such as NGAL and β2M, have been selected as novel biomarkers for the prediction of renal damage in pediatric patients prior to the manifestation of evident symptoms.

Materials and Methods

Study design. A case-control study performed at The Genetic Hematology Center in Thi-Qar Province is presently gathering samples from patients diagnosed with β-TM. A pediatric consultant specializing in blood disorders primarily supports the study. The study population consists of 60 pediatric patients, with an equal distribution of 30 males and 30 females aged between 1 and 14 years. Additionally, 60 healthy controls, comprising 29 males and 31 females, have been included. The sample collection occurred between July and December 2022.

Exclusion criteria. Exclusion criteria for the study include the co-inheritance of β-TM with other hemoglobinopathies, renal failure, chronic diseases, hepatitis, splenectomized patients, and patients who received blood transfusions within the past ten days.

Sample collection. Sample collection involved conducting a venous puncture on each β-TM patient to obtain 5 ml of peripheral blood. Two portions were taken from the total blood sample: one milliliter of blood was collected and placed into an EDTA tube for a complete blood count, primarily focusing on platelet (PLT) count and hemoglobin (Hb) levels, while the remaining 4 ml were placed in a gel tube to allow coagulation at room temperature. Subsequently, the tubes were centrifuged at 3000 rpm for 15 min to separate the serum. The extracted serum was then carefully transferred into multiple Eppendorf tubes and stored at (-80°C) to facilitate further analysis, including ferritin (FER), creatinine, potassium (K), NGAL, and β2M.

Methodology. The quantitative enzyme-linked fluorescence immunoassay measurement of serum ferritin levels and β2M uses the VIDAS device developed in France. The kit allows for room-temperature assembly and includes a strip and SPR for each sample, a control, and a calibrator, and the instrument performs all steps automatically. Hemoglobin levels and platelet counts are estimated using the Sysmex XP-300, an automated hematology analyzer from Germany. About 50 µl of anticoagulant-treated blood is aspirated and needs solutions that are stromatolyser and diluent to perform a complete blood count. ELISA kits from SUNLONG Company in China are employed to measure NGAL levels. This sandwich-ELISA kit employs a micro-ELISA strip plate that is pre-coated with an NGAL-specific antibody. After several washings and incubations to determine the NGAL concentration, the optical density at 450 nm is measured. The determination of serum creatinine is carried out using the fully automated COBAS C 111 instrument produced by Roche in Germany. It is important to note that the performance of applications that have not been verified by Roche is not guaranteed. Therefore, it is the responsibility of the user to define acceptable performance. Serum potassium from a spectrum company in Egypt is tested using a spectrophotometer. The turbidimetric tetraphenylborate (TPB) method from Spectrum Company is utilized for the following assay principle: Under alkaline pH conditions, TPB and K^+ ions interact to produce a turbid emulsion. This turbidity can be measured quantitatively using a photometer set to 578 nm. The absorbance values

directly correlate with the concentration of K in the sample, indicating an increased quantity of K.

Statistical analysis. Statistical analysis was performed using the Statistical Package for Social Sciences version 22. Parametric variables were analyzed using an independent sample *t*-test, while non-parametric variables were evaluated using a Mann-Whitney U test. Pearson's correlation coefficient analysis assessed the correlation between dependent variables. A significance level ($P \leq 0.05$) was considered statistically significant.

Results

In Table 1, the demographic distribution of patients based on age and gender is presented. The study included 30 female and 30 male pediatric patients ranging in age from 1 to 14 years.

In Table 2, the comparison of patients and controls exhibited a significantly decreased level of Hb, Cr, and K compared to the controls. The patients exhibited a significantly higher increase in PLT, FER, NGAL, and β2M in patients than the control.

In Table 3, presents the categorization of patients based on their ferritin levels, which exhibit considerable variation and are divided into two groups. The first group comprises 30 patients (50%) with ferritin levels ≤2000 mg/dl, while the second group consists of 30 patients (50%) with ferritin levels >2000 mg/dl. The results indicate no statistically significant differences in Hb, platelet count, creatinine, potassium, and NGAL levels between the two groups. However, there is a statistically significant increase in β2-microglobulin levels among patients with ferritin levels >2000 mg/dl ($P \leq 0.05$).

In Table 4, presents the classification of patient groups according to age, reflecting the duration of disease. The first group is 1–7 years old, and the second group is 8–14 years old. The results show a statistically significant increase in creatinine, NGAL, and β2M.

In Table 5, displays the correlation among all biomarkers studied, including age. The results reveal a positive correlation between age and creatinine, NGAL, and β2M. Additionally, there is a positive correlation observed between NGAL and β2M.

In Table 6, the reference range of Hb and PLT in children is from the reference book [15] and there is no study to calculate the exact value of normal HB and PLT. While FER, B2M, creatinine, and K are according to the kit. The normal range of the NGAL test was calculated according to the control group.

Table 1. Demographic characteristics, including age and sex of the study groups

Variable	Control (n = 60)	Patient (n = 60)	P Value
Age, years	8.97 ± 3.40	9.25 ± 3.53	0.71
Sex			
Male	30	29	0.88
Female	30	31	0.88

Discussion

Despite the advancements in managing individuals with β-thalassemia and improving survival rates, these advancements have also brought about unrecognized complications, including various renal abnormalities .[16] Chronic anemia, iron excess, and ICT are prevalent among almost all patients [6]. Several studies have investigated renal involvement in individuals with βTM [17, 18]. Our study aimed to

Table 2. The study parameters in groups of the patients and the control (mean ± SD)

Parameters	Control (n = 60)	Patient (n = 60)
HB level, g/dl	13.13 ± 1.04	7.46 ± 1.14*
Platelet, $\times 10^3$ cell/μl	292.13 ± 72.61	412.13 ± 187.82*
Ferritin, ng/ml	93.18 ± 27.17	2490.62 ± 1542.35*
S. creatinine, mg/dl	0.60 ± 0.15	0.36 ± 0.10*
S. potassium, mmol/l	4.24 ± 0.33	3.95 ± 0.30*
S. NGAL, pg/ml	39.65 ± 14.05	51.85 ± 20.00*
S. β2-microglobulin, pg/ml	1.24 ± 0.37	1.70 ± 0.51*

Note. NGAL – Neutrophil gelatinase-associated lipocalin. *Significant with P-value ≤ 0.05

Table 3. Distribution of the study parameters in the patients group based on ferritin levels (n = 30)

Parameters	mean ± SD	According to ferritin
HB level, g/dl	7.457 ± 1.03	≤2000
	7.463 ± 1.24	>2000
Platelet, $\times 10^3$ cell/µl	407.50 ± 145.62	≤2000
	416.77 ± 224.76	>2000
Ferritin, ng/ml	1261.00 ± 509.94	≤2000
	3720.23 ± 1204.77*	>2000
S. creatinine, mg/dl	0.35 ± 0.11	≤2000
	0.37 ± 0.07	>2000
S. potassium, mmol/l	4.00 ± 0.31	≤2000
	3.90 ± 0.27	>2000
S. NGAL, pg/ml	49.58 ± 19.30	≤2000
	54.11 ± 20.71	>2000
S. β2-microglobulin, mg/l	1.57 ± 0.49	≤2000
	1.83 ± 0.48*	>2000

Note. NGAL – Neutrophil gelatinase-associated lipocalin. *Significant with *P*-value ≤ 0.05

Table 4. Distribution of the study parameters according to age groups, (mean ± SD)

Parameters	1-7 years (*n* = 24)	8-14 years (*n* = 36)
Hemoglobin, g/dl	7.57 ± 0.94	7.38 ± 1.25
Platelet, $\times 10^3$ cell/µl	372.71 ± 113.52	438.42 ± 221.85
Ferritin, ng/ml	2230.63 ± 1510.99	2663.94 ± 1559.64
S. creatinine, mg/dl	0.31 ± 0.09	0.39 ± 0.08*
S. potassium, mmol/l	3.95 ± 0.31	3.944 ± 0.28
S. NGAL, pg/ml	41.39 ± 13.80	55.48 ± 23.75*
S. β2-microglobulin, mg/l	1.42 ± 0.48	1.97 ± 0.50*

Note. NGAL – Neutrophil gelatinase-associated lipocalin. *Significant with *P*-value ≤ 0.05

investigate renal injury by measuring various blood biomarkers. Specifically, we examined NGAL and β2M as markers for glomerular and tubular injury, respectively. Additionally, we assessed routine biomarkers such as Hb concentration and PLT count as hematological markers, FER level as an indicator of iron overload, and creatinine level and potassium as standard renal tests.

Hematological complications in β-thalassemia include anemia, measured by Hb concentration [19], and hypercoagulability, assessed by an estimated PLT count [20]. Our study reveals a statistically significant decrease in Hb levels ($P \leq 0.05$), as presented in Table 2. These findings are consistent with previous studies reporting similar results [21-23]. The primary cause of this condition is the excessive premature death of red blood cell precursors in the bone marrow due to apoptosis. This imbalance between the chains and oxidative membrane damage leads to ineffective erythropoiesis or hemolysis [19]. Furthermore, Table 2 depicts a significant increase in PLT count in the patient group compared to the control group ($P \leq 0.05$). Numerous studies have corroborated these findings, supporting the observed results [24-26]. The serum creatinine test is commonly employed to assess glomerular function [27]. Our study observed a significant decrease ($P \leq 0.05$) in patients' creatinine levels. The study in Iraq showed a significant decrease in creatinine [28]. It also aligns with the findings reported by Cetinkaya et al., which

Table 5. The correlation between all biomarkers for the patient group

Category		Hb	PLT	FER	Cr	K	NGAL	β2M
Age	R	-0.054	0.120	0.095	0.414	0.122	0.229	0.220
	P value	0.682	0.360	0.375	0.001	0.354	0.030	0.037
Hb	R		0.212	0.052	-0.242	-0.224	-0.111	-0.133
	P value		0.105	0.695	0.280	0.058	0.397	0.313
PLT	R			0.033	0.141	0.062	-0.219	0.078
	P value			0.800	0.284	0.636	0.093	0.555
FER	R				0.149	0.082	0.065	0.166
	P value				0.255	0.528	0.622	0.204
Cr	R					-0.121	0.023	-0.112
	P value					0.357	0.860	0.395
K	R						0-.134	0-.112
	P value						0.306	0.930
NGAL	R							0.434
	P value							0.010

Table 6. The reference range of the study parameters

Variable	Reference range	unit	According to
Hemoglobin	11.0-15.5	g/dl	According to the reference
Platelet	170-450	$\times 10^3$ cell/µl	
Ferritin	Men: 70 to 435 Female: 10 to 160	ng/ml	According to The kit
S.creatinine	0.17 – 0.87	mg/dl	According to The kit
S.potassium	3.6 - 5.5	mmol/l	According to The kit
S.NGAL	24.2-56.5	pg/ml	According to the result of control group
S.β2-microglobulin	0.81 to 2.19	mg/l	According to The kit

also demonstrated a significant decrease in creatinine levels [29]. Moreover, multiple studies specifically focusing on βTM consistently report similar results [12, 30]. It should be noted that the growth retardation observed in patients with βTM could be attributed to a lack of muscle mass, which may affect the creatinine value. Some studies did not find a significant correlation between creatinine and βTM [31, 32]. However, a study conducted by Mahmoud et al. demonstrated a significant increase in creatinine levels in patients compared to controls [22].

Ferritin levels are widely utilized to assess iron overload in thalassemia patients due to their positive correlation with iron overload [33]. In our study, FER levels exhibited a significant increase in patients, consistent with several prior studies [21, 22]. Recurrent blood transfusions are the primary therapeutic option for maintaining Hb levels below 7 g/dl, contributing to the elevation of FER levels [34]. Alternatively, renal damage may be associated with a disorder in iron metabolism, which may be attributed to the increased production of erythroferrone by erythroid precursors. This, in turn, leads to augmented hepatic hepcidin production, promoting increased iron absorption. Excessive free iron is known to act as a catalyst for lipid peroxidation, and the deposition of hemosiderin in renal tubules can cause tubular necrosis and interstitial fibrosis [26].

Subdividing patients based on their FER levels allows a better understanding of the correlation

between excessive iron in the body and renal complications in thalassemia. In our study, only β2M demonstrated a statistically significant increase ($P \leq 0.05$) in the subgroup with FER >2000. This finding is consistent with a previous study conducted by ElAlfya and Elsherif, which reported a significant increase in β2M levels when subgrouped based on FER levels [35]. Another study by Elbedewy et al. found statistical significance in Hb and creatinine between patients and controls when divided into two groups according to FER levels. It is worth noting that the observed symptoms of the renal disorder in patients aged 18–29 years may be directly influenced by iron damage due to advanced age. Additionally, the study relatively small sample size of 40 patients may have impacted the results [31]. Electrolyte levels are tightly regulated by several hormones and the kidneys. As the primary organs responsible for electrolyte retention and elimination, the kidneys maintain a constant state of balance to ensure appropriate levels of electrolytes are maintained [36]. Our study's results indicate a significant decrease in serum potassium, although it remains within the normal range ($P \leq 0.05$). These findings confirm previous research, including studies on thalassemia patients [30, 32]. The decrease in serum potassium levels could be attributed to the fact that most potassium is reabsorbed in the loop of Henle and proximal tubule. In these regions, iron that enters proximal tubular cells with the transferrin is released from transferrin within lysosomes, resulting in free reactive iron. This reactive iron has the potential to trigger the generation of reactive oxygen species (ROS) and cause cellular damage. Consequently, there may be a defect in reabsorption, leading to increased urinary potassium excretion [32]. It is important to note that our study contradicts the findings, which reported a significant increase in serum potassium levels [37, 38]. The elevation of serum potassium levels observed in those studies may be attributed to factors such as hemolysis of transfused blood or could be influenced by a small number of patients in the studies.

Two receptors, lipocalin-2 (24p3r) and megalin, mediate the binding and action of NGAL on its target cells. The 24p3r receptor is involved in protein endocytosis through NGAL uptake in the cell and is specifically expressed in the distal nephron [39]. Nephron damage leads to the rapid excretion of NGAL, a biomarker that can be used to diagnose renal injury at various clinical levels since it is secreted directly from renal tubular epithelial cells [40]. Therefore, it was selected for use in my study. Mean NGAL levels were significantly higher in the case study group (51.84 ± 19.98) compared to the control group (39.64 ± 14.05) ($P < 0.05$). Similar results were found in previous studies regarding a single blood NGAL measurement [41], as well as in a urine sample [42-44] in patients with βTM. Roudkenar et al. previously demonstrated that iron overload causes ROS production, which in turn stimulates NGAL expression [45]. Additionally, elevated levels of NGAL in both serum and urine may be attributed to various underlying factors associated with renal injuries, such as chronic hypoxia, toxicity from iron chelation treatment, or tubular damage caused by iron deposition. The exact cause of this increase remains unclear, but it suggests a potential link to these factors [8]. Badeli et al. found no statistically significant association between renal damage and NGAL levels in the three groups of thalassemia patients who had received deferoxamine therapy [46].

β2-microglobulin is a low molecular weight protein (11.8 kDa) that is normally freely filtered at the glomerulus but almost completely reabsorbed and degraded by the proximal renal tubules. Therefore, an elevation of β2M serves as a sensitive and reliable early marker of proximal tubular dysfunction [29, 47], making it a suitable choice for this research. Serum β2M levels were significantly higher in the patient groups compared to the control group ($P < 0.05$). This finding is consistent with previous studies focusing on thalassemia as the primary subject [44, 48, 49]. The increase in β2M levels may be attributed to factors commonly associated with kidney damage, such as chronic anemia, hypoxia, and iron overload [32]. However, a study by Kaçar et al. that reported non-significant β2M results does not align with the findings of my own research [50]. It is possible that the lack of significance in their study could be due to a small sample size or limited exploration of the impact of bone marrow transplantation on renal function. For instance, Sumboonnanonda et al. conducted a study in 2009, which demonstrated that successful hematopoietic stem cell transplantation resulted in improved renal tubule function among patients with βTM who underwent the procedure [51].

On average, the probability of developing renal dysfunction was higher in elderly patients and individuals who had undergone prolonged iron chelation therapy and blood transfusions. This study demon-

strated an elevation of creatinine, β2M, and NGAL in the group of 8–14-year-olds. Our findings were supported by a study conducted in Iraq by Shaalan et al., which involved three age groups. The study revealed a notable rise in creatinine, while no significant alterations were observed in K [32]. Moreover, the investigation conducted by Younus et al. in Iraq revealed noteworthy age-related elevations in creatinine among thalassemia patients [52]. The study shows urinary β2M increases with age [53]. In their study, Mohammed et al. found that there was no significant variation in NGAL with age. This lack of variation could potentially be attributed to the inclusion of a group known as thalassemia intermedia in the case study [41].

Table 5 demonstrates that as patients age, the levels of creatinine, NGAL, and β2M increase. The findings of a study by Karaman et al. support the positive association between creatinine and age, as well as between NGAL and β2M in βTM, as identified in previous research [12]. This observation potentially suggests an escalation in damage as age advances. The correlation between β2M and age has been previously investigated [53]. However, Annayev et al. reported no correlation between β2M and age [54]. Similarly, no correlation was found between any biomarker and FER, which aligns with a study that demonstrated no correlation between NGAL and indices of iron overload [55]. Another previous study indicated no correlation between β2M and iron overload, possibly due to the fact that FER serves as an indicator for iron overload and is influenced by several factors, including regular or irregular iron chelation and the type of chelation used [6, 56]. However, FER levels can also be affected by other factors, such as inflammation, infection, and liver disease [57]. It is worth noting that ferritin levels were not found to be predictive of renal function impairment [58].

Conclusion. In conclusion, it is crucial to acknowledge that pediatric patients face a high risk of developing renal impairment, which cannot be reliably determined using routine markers. However, the measurement of NGAL and β2M provides a dependable and informative indicator for early injury detection, as these biomarkers exhibit elevated levels before an increase in creatinine is observed. Furthermore, the positive correlation observed between creatinine, NGAL, and β2M levels with age suggests a progressive increase in renal damage as patients grow older.

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НОВІ БІОХІМІЧНІ МАРКЕРИ ПРОГНОЗУВАННЯ УРАЖЕННЯ НИРОК У ДІТЕЙ З БЕТА-ТАЛАСЕМІЄЮ

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Бета-таласемія – важке спадкове захворювання, що характеризується недостатнім утворенням бета-ланцюгів гемоглобіну, неефективним еритропоезом, хронічним гемолізом і потребує пожиттєвих переливань крові, які призводять до перевантаження організму залізом. Захворювання проявляється в ранньому дитинстві і триває протягом усього життя з високим ризиком розвитку ниркової недостатності, яку неможливо достовірно виявити за допомогою традиційних маркерів. Метою цього дослідження було вивчення ефективності біомаркерів для оцінки ураження нирок у дітей віком від 1 до 14 років із діагнозом бета-таласемія. У дослідженні "випадок-контроль" аналізували зразки крові з Центру генетичної гематології (провінція Ті-Кар, Ірак): 60 зразків від здорових осіб та 60 – від пацієнтів з бета-таласемією, яких було розподілене на групи віком 1-7 та 8-14 років. Рівні гемоглобіну, феритину, креатиніну та калію визначали за допомогою стандартних тестів, а рівень нейтрофільного желатиназо-асоційованого ліпокаліну (NGAL) та бета-2-мікроглобуліну (β2M), як нових маркерів канальцевої та гломерулярної дисфункцій, оцінювали за допомогою імуноензимного аналізу. Було виявлено статистично значуще зниження рівня гемоглобіну, сироваткового калію та підвищення рівня феритину, NGAL і β2M у пацієнтів обох груп порівняно з кон-

трольною групою, а також підвищення рівня креатиніну у групі 8-14 років. Зроблено висновок, що рівні NGAL і β2M можуть бути показниками для раннього виявлення ураження нирок у педіатричних пацієнтів із бета-таласемією, оскільки рівні цих біомаркерів зростають до того як підвищується рівень креатиніну.

Ключові слова: велика бета-таласемія, ураження нирок, нейтрофільний желатиназоасоційований ліпокалін, бета-2-мікроглобулін, феритин, креатинін.

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