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Urinary biomarkers of early kidney injury in children with beta-thalassemia

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Beta-thalassemia is considered as one of the most common genetic disorders in the world caused by the reduced or absent synthesis of β-globin chain. The aim of this work was to evaluate renal function in children with β-thalassemia using predictive biomarkers such as urinary neutrophil gelatinase-associated lipocalin (NGAL) and N-acetyl-β-D-glycosaminidase (NAG). This prospective case–control study was carried out on 80 subjects aged between 3 and 17 years. The subjects were divided into two equal groups: thalassemia group which included 40 children with β-thalassemia major, and control group which included 40 age- and gender-matched healthy, non-anemic controls without any diseases that could potentially affect renal function, who underwent routine check-ups. All thalassemia patients received chelation therapy: 35 (87.5%) of them were treated with deferasirox and 5 (12.5%) patients received deferasirox + deferoxamine. The results of echocardiography, kidney function tests (serum creatinine, urea and estimated glomerular filtration rate as well as urinary albumin/creatinine ratio and urinary NGAL/urinary albumin–creatinine ratio were insignificantly different between the two groups. Abdominal ultrasound findings were significantly different between both groups. Total WBC counts, platelet counts, the results of liver function tests (total bilirubin, direct bilirubin, alanine transaminase and aspartate aminotransferase), urinary NAG/creatinine ratios, urinary NGAL/creatinine ratios and serum ferritin levels were significantly higher in the thalassemia group, while hemoglobin, urinary NAG/urinary albumin–creatinine ratios were significantly lower in this group. Urinary markers NGAL and NAG could be used as predictive markers of renal disease in β-thalassemia major patients. The study was initiated after obtaining approval from the Ethical Committee of Menoufia University Hospital. Informed written consent was obtained from the parents of the children involved in the study.

Key words: *urinary kidney injury, children, β-thalassemia*

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Beta-thalassemia (β-TM) is considered as one of the most common genetic disorders in the world caused by the reduction or absence of β-globin chain synthesis [1].

Approximately 1.5% of the global population are heterozygotes (carriers) of the β-TMs; there is a high incidence in populations from the Mediterranean basin, throughout the Middle East, the Indian subcontinent, Southeast Asia, and Melanesia to the Pacific Islands [2]. In Egypt, the oldest civilization in the Mediterranean region, thalassemia is the most prevalent hemoglobinopathy. The carrier rate of this disease varies between 5.3 and ≥ 9%. It was estimated that 1000/1.5 million live births per year suffer from thalassemia [3]. Individuals with β_0-TM mutations have a marked imbalance of globin chain synthesis which is considered as the underlying basis of their severe phenotype [4].

Recent studies have revealed that there are different tubular and glomerular abnormalities in patients with β-TM major and that renal dysfunction may occur in such patients without any clinical symptoms [5].

Long-term damage to the renal system has become evident with more studies targeting renal biomarkers and prevalence of different types of renal injuries in thalassemia [6].

So, there is an urgent need for early predictive biomarkers of renal impairment in β-TM major as early intervention can significantly improve the prognosis [7].

The urinary enzyme N-acetyl-β-D-glycosaminidase (NAG) is found in the lysosomes of proximal tubule epithelial cells. Increased NAG excretion in urine is caused exclusively by proximal tubular cell injury [8]. NAG has recently been recognized as a tubular injury marker related to renal failure. This biomarker is known for its high molecular weight that cannot be filtered by the glomerulus; thus, it is released in the tubular lumen as a consequence of proximal tubular damage [9]. The aim of this work was to assess renal function in children with β-TM using predictive biomarkers such as urinary, neutrophil gelatinase-associated lipocalin (NGAL) and NAG.

MATERIALS AND METHODS

In this prospective case–control study, we enrolled 80 subjects aged between 3 and 17 years: 40 patients diagnosed with β-TM based on the results of a complete

blood count and hemoglobin (Hb) electrophoresis (all of them were in stable condition and received regular packed red blood cell transfusions every 3–4 weeks and iron chelation therapy) and 40 age- and gender-matched healthy controls. Our study was conducted from February to December 2021.

The study was initiated after approval from the Ethical Committee Menoufia University Hospital. Informed written consent was obtained from the parents of the children involved in the study.

Exclusion criteria: any other types of anemia, concomitant diseases that could affect kidney functions or potentially lead to renal damage, such as diabetes, rheumatic heart disease, thyroid or hepatic diseases, sepsis, consumption of nephrotoxic drugs, medical history suggestive of recurrent urinary tract infections, family history of hereditary renal diseases, any active infection, renal stones, hydronephrosis, urinary reflux, the use of corticosteroids, trimethoprim, or cephalosporin in the week before the collection of blood samples.

All the study participants were subjected to complete history taking, general and abdominal examinations, anthropometric measurements, and renal function (creatinine, urea) testing.

All the patients received regular packed red blood cell transfusions every 3–4 weeks and chelation therapy with deferasirox at a dose of 20–40 mg/kg/day when their ferritin levels were above 1000 ng/mL, among them 5 patients in combination with deferoxamine. Splenectomy in children above 5 years of age was done when blood transfusion requirements increased to 200–250 mL/kg/year to achieve target Hb levels or in the presence of signs of hypersplenism.

Measurement of urinary biomarkers: Urine levels of NAG and NGAL were determined by the enzyme-linked immunosorbent assay (ELISA), estimated glomerular filtration rate (eGFR) was calculated using the modified Schwartz formula for children: eGFR (mL/min/1.73 m^2) = height (cm) × 0.413/serum creatinine (mg/dL).

The eGFR was considered normal if the value was ≥ 90 mL/min/1.73 m^2, and decreased if it was < 90 mL/min/1.73 m^2 [10].

ELISA technique (the double-antibody sandwich enzyme-linked immunosorbent assay): 3 mL of urine was collected, then centrifuged at 3000 rpm for 10 minutes and separated in 2 tubes and stored at –20°C for further measurement of NGAL and NAG levels. At the time of the assay, all frozen samples were thawed at room temperature.

NGAL and NAG assays: ELISA kits (Shanghai SunRed Biological Technology Co., Ltd,) were used to determine the levels of NGAL and NAG in randomized serum samples. To perform the assay, 50 µl of each standard was added to the standard wells and 40 µl of samples was added to the test wells. We added 50 µl of the prepared streptavidin–HRP solution to each of the standard and test wells; then 10 µl of NGAL–antibody was added to each well of the microtiter plates. The plates were incubated for 60 minutes at 37°C and then were washed five times with wash buffer. After washing, 50 µl of chromogen solution A and 50 µl of chromogen solution B were added into each well (color of the liquid changed into blue). The plates were incubated away from light for 10 min at 37°C. Then, 50 µl of stop solution was added into each well (the blue color changed into yellow immediately). The absorbance of each colored solution was measured at 450 nm wavelength using a microplate reader.

For NGAL and NAG: A standard curve was constructed and a linear regression equation was calculated using optical density (OD) values and corresponding concentration of standards. OD values of samples were applied to the regression equation to calculate the corresponding concentration of the samples.

Statistical analysis

Statistical analysis was performed using SPSS v28 (IBM©, Chicago IL, USA). The Shapiro–Wilks test and histograms were used to determine the normality of data distribution. Quantitative parametric data were presented as the mean and standard deviation (SD) and were analyzed by unpaired Student's t-test. Quantitative non-parametric data were presented as the median and interquartile range (IQR). Qualitative variables were presented as frequency and percentage (%) and analyzed using the Chi-square test or Fisher's exact test when appropriate. A two-tailed *p*-value < 0.05 was considered significant.

RESULTS

The mean age and male:female ratio were insignificantly different between both groups. Body weight, height, body mass index (BMI), and Hb counts were significantly lower in the thalassemia group. The results of liver function tests (total bilirubin, direct bilirubin, alanine transaminase (ALT) and aspartate aminotransferase (AST)), urinary NAG/creatinine ratios, urinary NGAL/creatinine ratios, total leucocyte count (TLC), platelet counts and serum ferritin levels were significantly higher in the thalassaemia group. Urinary NAG/urinary albumin–creatinine ratios were significantly lower in the thalassaemia group. Urinary NAG and NGAL levels, as well as urinary albumin /creatinine and urinary NGAL/urinary albumin–creatinine ratio were insignificantly different between the two groups. In the thalassemia group, 39 (97.5%) patients had

normal echo findings and 1 patient had a mitral valve prolapse. Abdominal ultrasound findings were significantly different between both groups (*table 1*).

The mean number of blood transfusions per year in the thalassemia group was 11.95 ± 0.22. Thirty-five (87.5%) thalassemia patients received deferasirox and 5 (12.5%) patients received a combination of deferasirox and deferoxamine (*table 2*).

Urinary NAG/creatinine ratio can significantly predict β-TM ($p < 0.001$ and AUC = 0.707) at the cut-off value > 0.3 with 67.50% sensitivity, 60.00% specificity, 62.8% positive predictive value (PPV) and 64.9% negative predictive value (NPV). Urinary NGAL/creatinine ratio can significantly predict β-TM (p = 0.002 and AUC = 0.684) at the cut-off value of > 3.5 with 62.50% sensitivity, 50.00% specificity, 55.6% PPV and 57.1% NPV (*figure*).

DISCUSSION

Recently, several tubular damage markers, including kidney injury molecule-1, NGAL, NAG, heart fatty acid-binding protein, and cystatin C, have gained considerable attention because of their clinical implications as sensitive and specific biomarkers for predicting the development and progression of early stage Diabetic kidney disease [11].

In the present study, the number of blood transfusions per year in the thalassemia group was 11.95 ± 0.22. A total of 87.5% of the patients received Jadenu and 12.5% of the patients received Jadenu + Desferal. A. Hagag et al. [12] reported that 41.3% of their patients received Jadenu and 26.8% of the patients received combined therapy (Jadenu + Deferiprone).

In our study, the results of echocardiograms were insignificantly different between both groups (p-value < 0.001). Abdominal ultrasound findings were significantly different between the cases and controls.

Aziz et al. [13] reported that their β-TM patients developed hepatosplenomegaly significantly more often than the controls, which is compatible with our results. Thirty percent of their patients had splenomegaly and 63.3% of the patients had hepatomegaly.

Table 1
Baseline characteristics of the cases and controls; the results of echo and abdominal ultrasound, complete blood count and serum ferritin tests; the results of liver and kidney function tests and urinary novel early biomarkers–creatinine ratios in the studied groups

Parameters		Thalassemia group (n = 40)	Control group (n = 40)	p-value
Age, years		10.5 ± 3.3	11.1 ± 3.24	0.415
Sex	Male	24 (60%)	26 (65%)	0.817
	Female	16 (40%)	14 (35%)	
Weight, kg		31.8 ± 11.74	52.7 ± 20.2	< 0.001*
Height, m		1.3 ± 0.12	1.5 ± 0.17	< 0.001*
BMI, kg/m^2		17.2 ± 3.38	23.5 ± 6.33	< 0.001*
Echo	Normal	39 (97.5%)	40 (100%)	1
	Mitral valves prolapse	1 (2.5%)	0	
Abdominal ultrasound	Normal	2 (5%)	40 (100%)	< 0.001*
	Splenomegaly	25 (62.5%)	0	
	Hepatosplenomegaly	11 (27.5%)	0	
	Mild splenomegaly	1 (2.5%)	0	
	Mild hepatosplenomegaly	1 (2.5%)	0	
TLC, × 10^3/μl		8.55 (7.2–13.3)	6.3 (5.3–7.2)	0.001*
Hb, mg/dL		7.3 ± 1.09	12.2 ± 1.72	< 0.001*
Platelets, × 10^3/μl		479.9 ± 300.1	262.5 ± 60.5	< 0.001*
Serum ferritin, ng/mL		2392 (1219.2–3528.7)	120 (90–136.2)	< 0.001*
Total bilirubin, mg/dL		1.9 ± 0.89	0.8 ± 0.14	< 0.001*
Direct bilirubin, mg/dL		0.4 (0.3–0.5)	0.1 (0.1–0.1)	0.002*
ALT, U/L		51.7 ± 35.39	28 ± 4.09	< 0.001*
AST, U/L		52.6 ± 30.84	30.9 ± 5.53	< 0.001*
Serum creatinine,mg/dL		0.50 ± 0.06	0.52 ± 0.07	0.114
Urea, mg/dL		27.3 ± 8.94	29.4 ± 5.37	0.199
eGFR		119.6 ± 14.07	117.6 ± 10.94	0.482
Urinary NAG, U/L		27.5 ± 15.28	28.7 ± 5.43	0.652
Urinary NGAL, U/L		318.5 ± 132.36	332.8 ± 93.62	0.577
Urinary NAG/creatinine		0.53 (0.27–0.73)	0.30 (0.20–0.44)	**0.006***
Urinary NGAL/creatinine		6.1 (3.4–9.8)	3.5 (2.2–5.2)	**0.013***
Urinary albumin/creatinine		28 (17.7–55.5)	25.5 (9.5–77.2)	0.497
Urinary NAG/urinary albumin–creatinine ratio		0.92 (0.57–1.65)	1.21 (0.43–3.41)	**0.016***
Urinary NGAL/urinary albumin–creatinine ratio		10.64 (5.34–22.43)	12.39 (5.53–38.48)	0.110

*Note. Data are presented as mean ± SD or median (IQR). * – significant if p-value < 0.05.*

TLC and platelet counts were significantly higher in the thalassaemia group than in the control group, while Hb levels were significantly lower in the thalassaemia group.

Our findings agree with Pallewar et al. [14] who reported that Hb was significantly lower in cases than in controls. Their results differed from ours in that TLC levels and platelet counts were significantly decreased in their cases as compared with the controls (p = 0.001and p = 0.043, respectively).

In contrast, Aziz et al. [13] found that Hb levels were significantly lower in thalassemia patients.

In the present study, the results of liver function tests (total bilirubin, direct bilirubin, ALT and AST) were significantly higher in the thalassemia group than in the control group.

In line with our results, Pallewar et al. [14] reported that serum bilirubin, AST, ALT, and alkaline phosphatase levels were raised in cases compared with controls.

Similarly, Aziz et al. [13] observed a significant increase in the levels of liver enzymes (ALT and AST) in β-TM patients compared with controls. Galeotti et al. [15], reported that deferasirox minimum plasma concentrations were significantly correlated with hepatic toxicities and that deferasirox pharmacokinetics were significantly influenced by many factors such as lean body mass (bioavailability and absorption constant), body weight (volume of distribution) and serum creatinine (clearance).

On the contrary, Cappellini et al. [16] reported that deferasirox therapy decreased iron overload and improved liver enzymes.

In addition, Al Hafidh and Younis [17] and Sengsuk et al. [18] demonstrated that elevated ALT after deferasirox therapy was short-lived and lasted for 4 weeks in 95.5% of patients.

In the current study, the results of kidney function tests (serum creatinine, urea and eGFR) were insignificantly different between the two groups.

Karaman et al. [19] conducted a study with 37 patients with β-TM major and 37 healthy controls to assess the level of NGAL suggested to be used as a marker in recent studies for the detection of early renal damage before regular blood transfusion in patients with β-TM. They found that there was no significant difference between groups regarding urea levels (p = 0.878). However, their findings regarding creatinine level differed from our results: they found that creatinine level in the control group was significantly higher than in the study group (p < 0.001).

Similarly, Shoemark et al. [20] reported that patients with β-TM major and controls had similar mean serum urea, creatinine and eGFR levels (p > 0.05 for all). In contrast to our results, Mohkam et al. [21] reported that the levels of serum urea, creatinine and uric acid were higher in patients with β-TM.

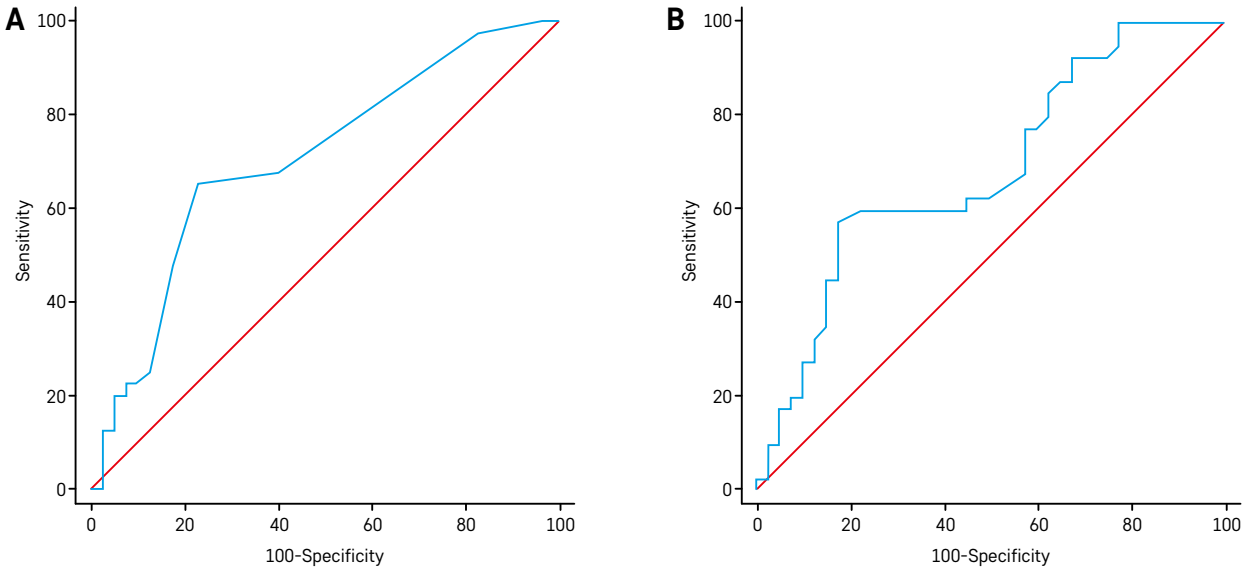
In our study, urinary NAG/creatinine ratio, urinary NGAL/creatinine ratio and serum ferritin levels were significantly higher in the thalassemia group while urinary NAG/urinary albumin-creatinine ratio was significantly lower in the thalassemia group.

Table 2
The number of blood transfusions per year and chelation therapy in the thalassemia group (n = 40)

Parameters		Value
Number of blood transfusions per year		11.95 ± 0.22
Chelation therapy	deferasirox	35 (87.5%)
	deferasirox + deferoxamine	5 (12.5%)

Note. Data are presented as mean ± SD or frequency (percentage).

Figure
ROC curve of urinary NAG/creatinine (A); ROC curve of urinary NGAL/creatinine (B)



Our results are in line with Karaman et al. [19] who reported that NGAL levels were significantly higher in the β-TM group than in the control group (p = 0.019).

Furthermore, Shoemark et al. [20] suggested that urinary NGAL levels could be a reliable indicator of kidney damage in patients with β-TM and that urinary NGAL levels could be used as a biomarker for screening of renal dysfunction, thus identifying patients who are likely to have impaired kidney function in the future.

In accordance with our results, Schrezenmeier et al. [22] found that the urinary albumin/creatinine ratio and NAG levels were significantly increased in participants with renal disease. Ahmadzadeh et al. [23] found that the mean UNAG activity was significantly elevated in β-TM major group compared with the controls.

In our study, urinary albumin/creatinine ratio and urinary NGAL/urinary albumin- creatinine ratio were insignificantly different between both groups.

Karaman et al. [19] found that before an increase in urea and creatinine levels due to iron accumulation in the kidneys, there was an increase in urine NGAL used as a biomarker of early renal involvement.

Study limitations: The sample size was relatively small. The study was carried out in a single center which limited the ability to infer a causal relation between urinary NGAL and NAG levels and renal damage in β-TM major patients.

CONCLUSIONS

Urinary biomarkers NGAL and NAG could be used to detect renal disease in β-TM major patients. A close watch for early renal damage should be kept in thalassemia patients receiving regular erythrocyte transfusions and chelation therapy to increase their quality of life.

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

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