USING CYSTATIN C TO ASSESS KIDNEY DAMAGE IN BETA THALASSEMIC PATIENTS RESIDING IN SULAIMANI CITY

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ABSTRACT

Background

In beta-thalassemia, majority of patients usually have anemia and iron overload which affects the function of many organs such as the heart, liver, and kidneys. Many previous studies concentrated on heart and liver complications, but fewer researches have focused on kidney dysfunction.

Objectives

Our main aim was to study renal dysfunction in BTM patients in the Sulaimani thalassemia center, using both novel urinary dysfunction markers (cystatin C) and traditional urine biomarkers.

Methods

This study was a case-control study (101 thalassemic patients and 97 controls). We studied the hematological parameters of the patients; we also assessed kidney function using cystatin c, serum creatinine, and blood urea and albumin creatinine ratio. T-test was used to assess the difference between study and control groups.

Results

Our results for the thalassemic group were as follow; serum iron levels were 240.27 (std.= ±80.80) µg/dl, mean serum ferritin was 1559.32 (std= ±1066.02) ng/ml; mean haemoglobin levels were 7.99 (std=± 1.14). The mean blood urea was 26.13 mg/dl (std.= ±7.38), serum creatinine was 0.43 mg/dl (std.=±0.16). The mean urinary albumin creatinine ratio was 271.14 mg/g (std=±131.23); mean eGFR (creatinine) was 170.0 (std.=101.1); mean eGFR (cystatin c) was 76.7 ml/min/1.73 (std.=±39.5).

Conclusion

Our results showed the majority of BTM were anaemic and suffered from iron overload despite the use of iron chelating agents. Our kidney function tests showed that although traditional urinary markers doesn't show kidney damage, the novel biomarker cystatin C showed BTM might have early kidney damage.

Keywords: *Cystatin C; Beta thalassemia major; Kidney dysfunction; eGFR; Biomarker*

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INTRODUCTION

Thalassemia is an autosomal recessive genetic disorder caused by the defective synthesis of globin haemoglobin chains [1]. Haemoglobin is composed of 2 alpha-globin chains and 2 beta chains, defective synthesis of these globin chains is the basis of thalassemia classification, i.e., alpha and beta-thalassemia [1] .In beta-thalassemia major (BTM), hemoglobin beta-globin chains are mutated causing defective beta-globin chain synthesis, the defects might cause the incomplete formation of the beta-globin chains or total deletion of the globin chains [1].BTM is more common in the Mediterranean and Southeast Asian countries. Thalassemia is prevalent in Iraq and it is part of a belt from the coastal areas of the Caspian Sea to the Persian Gulf. 10 % of the global population either are a carrier of the defected gene or are thalassemic.

According to a study, the prevalence of thalassemia in Iraq was 37.1/100,000 and 73.9% of them had BTM [2].The prevalence of thalassemia in Iraq was higher compared to other Middle Eastern and neighboring countries (25/100,000 in 2004 in Iran [3], 14/100,000 in Lebanon [4] in 2006, and 90/100,000 in Bahrain [4]. Most BTM patients in the past decades died before reaching 20 years old. Regular administration of chelating agents doubled the life expectancy of BTM patients [5]. At the thalassemia center of Sulaimani city of Iraqi Kurdistan, there is a new generation of thalassemia patients who are living beyond the age of 20, yet there are no studies on the health status and the quality of life of these patients. BTM present in the first year of life, these patients typically have severe anemia, and therefore they require regular blood transfusions, which might cause iron overload mainly because the body does not have any mechanism to remove excess iron from the body. This cause various organ damage therefore, iron chelation therapy is required to regulate iron blood levels in transfusion-dependent patients [6].

Multiple blood transfusion improves the health of BTM patients and it also stops ineffective hematopoiesis complications, however, repeated transfusion might cause hemosiderosis in the liver, heart, and endocrine glands. In addition, iron deposition in various body organs might cause premature death; therefore, it is necessary to eliminate the excess iron from the body by using iron-chelating agents [7]. The main cause of death in BTM patients is still heart disease caused by iron deposition. Digestive problems such as nausea, abdomen pain, vomiting diarrhea, skin rashes, and increased liver enzymes are side effects of deferasirox, in particular, severe kidney dysfunction is observed in BTM patients on deferasirox [8].

BTM complications are multifactorial and they include free radical formation from oxidative stress, iron overload caused by multiple blood transfusions, hypoxia caused by anemia [9]. In BTM, renal damage is mainly linked to chronic anemia, iron overload, and regular use of some iron chelators [6]. Most importantly, traditional renal function markers such as blood urea, serum creatinine, proteinuria and do not detect kidney damage at early stages [10].

Globally, most studies focus on heart and liver complications of thalassemia. Although a large number of studies have been performed on different complications of thalassemia, little information is available on renal involvement. Kidney iron overload is highly expected in BTM patients but there is limited research on kidney dysfunction in pediatric beta thalassemic patients, especially patients using chelating agents. Beta thalassemia is prevalent in Iraq and especially in Sulaimani city [4]. Many patients use chelating agents such as deferasirox and deferoxamine to control iron overload, however, despite the prevalence use of chelating agents no one has investigated renal complications in BTM patients in Sulaimani city. BTM nephropathy is a serious condition, which needs careful monitoring because it is irreversible at advanced stages. Physicians can delay the progress of nephropathy by detecting renal damages as soon as possible [10].

A routine finding of renal failure includes increased creatinine and decreased creatinine clearance, proteinuria. Serum creatinine is the most common biomarker used to assess kidney function, however, creatinine is an insensitive biomarker and cannot detect small changes in renal function, in addition, serum creatinine is altered by many factors such as gender, race, lean muscle mass, and age. Notably, 50% of patients with abnormal GFR had normal creatinine levels [11].

Since serum creatinine is not a very reliable marker, we thought of using Cystatin C as a marker to detect eGFR. Cystatin C is a low molecular-weight cysteine protease inhibitor, which is secreted by all nucleated cells [12]. Cystatin C is a better marker than creatinine for estimating glomerular filtration rate, this is mainly because that cystatin C is not secreted by renal tubules or reabsorbed back to the serum cystatin C and unlike

creatinine, cystatin c is also unaffected by diet, sex, height and muscle mass [13].

The main goal of this study was to assess kidney dysfunction in BTM children residing in Sulaimani city of Iraqi Kurdistan by using both traditional and novel kidney dysfunction biomarkers.

SUBJECTS AND METHODS

This study was a case-control study consisting of 101 BTM patients and 97 matched healthy control. The age of the participants was between 11-18 years old who were registered patients at the thalassemia center at Sulaimani city from May 2019 to July 2019; healthy controls were collected from primary schools and high schools in Sulaimani city during the same period and they were matched for age and sex. The BTM patients were regularly attending the thalassemia center for treatment. Patients with diabetes mellitus or other chronic diseases, patients on diuretic therapy, patients on anti-epileptic drugs, patients with primary renal disease, liver disease; other hemoglobinopathies such as sickle cell anemia, hemolytic anemia were all excluded from this study. Informed written consent was obtained from the guardian of the enrolled children. The study was approved by the university Sulaimani ethical committee.

Enrolled children were subjected to:

- Thorough history taking with stress on age at first transfusion, type of chelator, duration of chelation therapy, compliance to chelation therapy and history of splenectomy,
- Clinical examination, including the anthropometric measures, pubertal stage by Tanner's classification, clinical evidence of complications of iron overload and skin hemosiderosis.

Biochemical analysis

- 4 mL blood samples were taken in plain test tubes without anticoagulant. The samples were allowed to clot for 30 minutes at room temperature, then centrifuged for 15 minutes at 1000× *g*. The serum was removed, aliquoted, and stored at ≤−20°C until assayed. The separated serum was used for the determination of hematological parameters including serum iron, hemoglobin, and ferritin. Kidney function test was also studied using blood urea, urine albumin creatinine ratio, serum creatinine, serum cystatin C, and eGFR using both creatinine and cystatin C.
- CystatinCwasanalyzedusingimmunoturbidimetric assay from Roche Tina-quant Cystatin C assay Switzerland (Cat.No.04975723 190); the normal range is 0.61-0.95 mg/L**.** Estimated glomerular filtration rate (eGFR) was measured using Schwartz formula for paediatric population 26: eGFR (mL/min/1.73 m^2)=height (cm)×constant/ serum creatinine (mg/dL), where height was expressed in "cm" and constants were 0.44 (for children <2 years) and 0.55 (for children ≥2 years). Renal dysfunction was defined as eGFR<90 mL/ min/1.73 m^2 .This Schwarts formula was used for both eGFRcreatinine and eGFRcystatin C
- Serum creatinine was estimated by colorimetric reaction (Jaffe reaction) using COBAS-ROCHE kits, normal range was 0.6 – 1.5 mg/dl
- Blood urea was measured using Roche-Cobas kits and the normal range was 10-50 mg/dl.
- The albumin creatinine ratio was estimated using a spot urine test from Roche; the normal range was less than 30, 30-300 considered microalbuminuria and > 300 mg was considered macroalbuminuria.
- Serum ferritin (µg/dL) was estimated by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA); the normal range was for Males: 22-322 ng/ml and Females 10-291 ng/ml.
- Iron was used by colorimetric assay using Roche/ Hitachi Cobas c systems, the normal range is for females: 35-145 µg/dl and for males: 60-160 µg/dl
- Hemoglobin was determined according to the colorimetric method (Cyanmetomoglobin) using biolabo kits, normal range was for males:13-17 g/ dl and females:11.5-15.5 g/dl

Questionnaire

Pre-tested structured questionnaires were administered to respondents as they reported to the thalassemia center. The questionnaire was used to gather socioeconomic information, which included age of children, gender, level of education, weight (kg), height, place of resident, social status, duration of the disease**,** education level of fathert, education level of the mother, occupation of father**,** occupation of mother, age of the father, age of mother, monthly income, number of

children in the family, number of parity in family, order of sibling, splenectomy, socioeconomic status, type of chelation therapy which included exjade (deferasirox) tablet - deferoxamine IV, duration of chelation therapy, frequency of transfusion, blood pressure, dietary pattern and lifestyle was also collected.

food frequency questionnaire was recorded as follows: the subjects described their intake as consuming certain foods "never", "a few times per year", "once per month", "2-3 times per month", "once per week", "2 times per week", "3-4 times per week", "5-6 times per week", or "every day" (Goldberg, E. K. at el, 2018**)**

Statistical methods

SPSS version 16 software (Spaces Inc, Chicago, IL, USA) was used for statistical analysis in this study. All results were presented as mean and SD values, Student's *t*-test was used to compare means. A $P<0.05$ was considered significant throughout all the studies.

RESULTS

Characteristic of the study participants

Table 1 shows participant characteristics. The study was conducted on 198 children aged (11–18 years). The participants were divided into 2 groups: group I included 101 beta thalassemic patients with a mean age (14.63±2.30). Group II included 97 healthy children; age and sex-matched with the control group, with a mean age (14.32±2.31) p-value between the two groups was insignificant. Except for BMI, no significant differences were found between the patients and controls in terms of age, gender, and anthropometric measurements (p>0.05; Table 1).

The BMI difference between the thalassemia group and the control group was highly significant (thalassemia group 18.18 ± 4.25, healthy controls 20.71 ± 4.04; P < 0.001). The age of diagnosis of thalassemia patients was (8.39 ± 3.14 months); the mean frequency of transfusion was (19.69 ± 5.32 days).

There was a significant difference in the socioeconomic status between the control and thalassemia patients, p-value < 0.001. A total of 74 thalassemic patients were from rural areas and 27 from urban areas and a total of 43 participants in the control group were from rural areas, while the number of participants from urban areas was 54; p-values were < 0.001. This indicates that the difference in place of residency between the thalassemic group and the control group was highly significant.

There was a significant difference between the education levels of the parents of thalassemic patients and the control group. Consanguinity in the thalassemia group was 47 positive and 54 negative. However, in the control group, consanguinity was 13 positive and 84 negatives (P <0.001).

Hematological characteristics of BTM patients and control group.

The mean ferritin levels in the thalassemia group were 1559.32 (std= ±1066.02) ng/ml, while in the control group the mean ferritin level was 53.9 (std.= ±44.41) ng/ml.

Mean hemoglobin levels were 7.99 (std= ±1.14) for the thalassemic group and 13.3 (std=±2.07) for the control group. Mean serum iron in the thalassemic group was 240.27 (std.= ±80.80) µg/dl; for the control group, the mean serum iron was 83.57 (std.= ±27.42) ng/ml. The differences in the hematological parameters (serum ferritin, hemoglobin, and serum iron) between the thalassemic and the control groups were statistically significantly different (p-value <0.0001).

Renal function tests of BTM patient and control group.

In thalassemic patients, the mean cystatin C levels were 1.00 mg/l (std= ±0.20) and in the control group the mean cystatin C levels were 0.85 mg/l (std=±0.12), p-value between the two groups was <0.001.

The mean bold urea was 26.13 mg/dl (std.= ±7.38) in the thalassemia group and in the control group the mean blood urea was 24.77 (std.=7.31mg/dl); p-value was 0.20, which is non-significant.

Mean serum creatinine was 0.43 mg/dl (std.=±0.16) in thalassemia patients and in the control group the serum creatinine was 0.56 mg/dl (std.=±0.15) in the control group with a p-value between the two groups was <0.001, which is highly significant.

The mean urinary albumin creatinine ratio in thalassemic patients was 271.14 mg/g (std=±131.23); in the control group the mean albumin creatinine ratio was 103.2 mg/g (std= 106.71), the difference between the two groups was statistically significant (P<0.001).

The mean eGFR (creatinine) in thalassemic patients was 170.0 (std.= ±101.1) and in the control group, the mean was 121.4 (std.=±26.9) mL/min/1.73, the difference

between the two groups was highly significant (P <0.001).

Mean eGFR (cystatin c) for the thalassemic group was 76.7 ml/min/1.73 (std.=±39.5), for the control group the mean eGFR was 84.4 ml/min/1.7 (std.=±14.6) p-value was 0

In this study, we constructed a food frequency questionnaire to assess the quality of nutrition in beta-thalassemia major patients in Sulaimani. In summary, our questionnaire revealed that patients at the Sulaimani thalassemia center are usually given nutritional support and they are routinely advised by doctors to follow a diet created specifically for beta-thalassemia patients. Each patient was given a leaflet containing information about what food to eat and what type of food to avoid. Our questionnaire revealed that patients at the thalassemia center don't have optimal dietary style. They mostly don't consume national foods such as vegetables and fruits, but mostly rely on carbohydrate-rich foods such as rice, bread, however, our questionnaire showed that BTM patients adhere to the doctors' guidelines on restricting certain food types such as meat, fish, and iron-rich vegetables such as spinach and iron-rich beans.

Table 1. Socio-demographic characteristic of studied groups.

Socio-demographic characteristics	BTM patients (n= 101)	Controls (n= 97)	P value
Age (Mean ± SD)	14.63 ± 2.30	14.32 ± 2.31	0.35
Age of father (Mean ± SD)	49.17 ± 4.87	48.49 ± 3.56	0.27
Age of mother (Mean ± SD)	44.86 ± 4.94	44.37 ± 3.69	0.43
BMI (Mean ± SD)	18.18 ± 4.25	20.71 ± 4.04	< 0.001
Gender			
Male	47	47	0.79
Female	54	50	
Socioeconomic status			
Low	40	14	< 0.001
Medium	57	78	
High	4	5	
Place of resident			
Rural	74	43	< 0.001
Urban	27	54	
Education level of father			
No school	31	24	
Primary	13	2	
Secondary	12	4	
Graduated	43	67	
Education level of mother			
No school	52	34	< 0.001
Primary	7	1	
Secondary	12	3	
Graduated	30	59	
Consanguinity			
Yes	47	13	< 0.001
No	54	84	

Table 2. Haematological characteristic of BTM patient and control group.

Renal function test	Thalassemia group		Control group		p-value
	Mean	std.	Mean	std.	
Serum ferritin(ng/ml)	1559.32	1066.02	53.92	44.41	<0.001
Hb(g/dl)	7.99	1.14	13.30	2.07	<0.001
Serum iron(µg/dl)	240.27	80.80	83.57	27.42	<0.001

Table 3. Renal function tests of BTM patient and control group.

Renal function	Thalassemia patients		Control group		P-value
	Mean	Std. Deviation	Mean	Std. Deviation	
Cystatin c (mg/L)	1.00	0.20	0.85	0.12	< 0.001
Blood urea (mg/dl)	26.13	7.38	24.77	7.31	0.20
Creatinine (mg/dl)	0.43	0.16	0.56	0.15	< 0.001
Albumin/creatinine (mg/dl)	271.14	131.23	103.20	106.71	< 0.001
eGFR (creatinine) mL/min/1.73	170.0	101.1	121.4	26.9	< 0.001
eGFR (Cystatin C) mL/min/1.73	76.7	39.5	84.4	14.6	0.07

DISCUSSION

Blood transfusion and chelation therapy caused increased BTM patients' life expectancy and improved quality of life compared with previous last decades. However, these patients have developed other side effects resulting from treatment with both chelation therapies and blood transfusion causing iron overload, which mostly affects kidney function [13]. Although many studies focus on the side effects of treatments on the liver and heart, less research is performed on the effects of chelation therapy on kidney function. In addition, most previous studies are cross-sectional and focused on renal failure at the advanced stage of the disease. Few studies focus on the emergence of kidney dysfunction at an early stage, this is important to prevent further kidney complication development [10].

Most researchers used creatinine to determine kidney function, however, creatinine has many limitations. Firstly, because creatinine synthesis and excretion are dependent on different factors such as age, sex, dietary intake, and muscle mass, certain diseases affecting muscle mass also affect serum creatinine levels. Secondly, about 10-40% of creatinine are filtered by tubular secretion into the urine, this hides a large initial decline in GFR [14]. Finally, serum creatinine levels do not represent real-time variations in GFR that accompany acute kidney injury, serum creatinine rather needs time to rise before being detected as abnormal [15]. Hence, these factors cause considerable delay in the detection of acute kidney injury, which is crucial in kidney disease. Therefore, in this study, we aimed to investigate renal abnormalities using novel kidney function biomarker cystatin C in BTM patients and we also highlighted the status of kidney function in pediatric BTM patients in Sulaimani city, in particular, we observed patients using chelating agents such as deferoxamine and deferasirox.

Our study was in agreement with previous studies regarding hematological parameters of BTM patients, as expected BTM patients had severe anemia and iron overload [2]. Notably, and despite the prolonged use of iron chelating agents such as deferasirox and deferoxamine, BTM patients still suffer from iron overload, these results are in agreement with many previous studies in which they showed iron overload persisted despite the use of chelating agents [13].

In this study, we investigated kidney function in BTM patients using both novel and common kidney function biomarkers. BUN and creatinine levels were within the normal range for both beta thalassemic patients and control groups. However, there was a significant difference between thalassemic and control group,

there are many studies similar to our results in which both BUN and serum creatinine were within the normal range in BTM patients [16]. Şen V *et al* suggested that although chronic anemia and iron overload are common in BTM patients, kidney dysfunction tests in these BTM patients did not reach clinical detectable abnormal ranges for BUN, creatinine, and eGFR creatinine.

Interestingly, many studies showed that serum creatinine levels in BTM patients are contradictory, for example, Mohkam et al [17] showed higher than normal serum creatinine levels in BTM patients, while other studies and similar to our results showed normal serum creatinine levels in BTM patients [18].

In this study, both control and BTM patients had microalbuminuria. Albuminuria might result from chronic anemia, prostaglandin secretion, and prolonged hyperfiltration [19]. However, we used a spot test to measure macroalbuminuria, which is a screening test that might cause false results [24]. Our results, contrary to many studies which showed decreased eGFR levels in BTM [20]. Nevertheless, our results were in agreement with several studies also showing normal eGFR creatinine levels [21]. Serum Cystatin c levels were higher than normal in our study. Also, our result was similar to a study in which they showed that 36% of patients had higher than normal cystatin C serum levels [12]. Our patients had no complaints regarding their kidneys and none of our study participants at the thalassemia center was referred to kidney analysis, however, our results indicated early kidney dysfunctions as it is shown by the eGFR cystatin and macroalbuminuria. These results indicated that kidney dysfunction might be subclinical and kidney complications might manifest when patients get older. This study further suggests that BTM patients need to be monitored for their kidney function at least annually to avoid further complications.

Regarding our questionnaire, it should be noted gathering data for the questionnaire in Sulaimani city is problematic, self-reporting data are often regarded as unreliable, as most participants tend to exaggerate or underestimate the amount of food they consume. These false responses might be caused by recall bias, or sometimes social desirability bias [22]. When researchers ask the participants about private or sensitive topics such as social status, drug use, food intake, participants tend to reply with perfect lifestyle answers, because participants feel ashamed of being called uneducated or having poor social status. Other bias simply arises from participants being careless and not recalling the amount or type of food they consume because of lack of concentration or memorizing what they consume [22]. Whatever the cause of the bias, whether it is a recall or social desirability, we conclude that questionnaires are difficult to conduct and unreliable, nutritionist at Sulaimani university need to collaborate with hospitals to create a culture-specific questionnaire to accommodate the need for accurate nutritional data, and the questionnaire should be tailor-made for Kurdish population.

This study has few limitations, for instance, limited sample size, studies with larger sample sizes are necessary to get more accurate results (statistical significance) and to confirm renal safety of iron chelating agents in patients with BTM. Another limitation is that we used a case-control study, the most commonly cited disadvantage of a case-control study is the retrospective nature of a case-control study [23]. However, this study is a preliminary study in which it investigated renal dysfunction in BTM by evaluating kidney biomarkers in these thalassemic patients. In conclusion, this study highlights that renal dysfunction is subclinical in pediatric and young adult BTM patients taking oral chelating agents and this is might be due to the young age of the participants.

Despite normal blood urea and serum creatinine levels, the albumin creatinine ratio test show a low level of kidney damage, in addition, our cystatin C levels highlight the beginning of kidney damage in our sample study; although patients were asymptomatic. Since renal dysfunction may not be detected by traditional tests, the use of early markers is recommended.

Based on these results we recommend careful monitoring of kidney dysfunction in transfusion-dependent BTM patients as the BTM patients' life expectancy has increased which put the patients at a higher risk of end-stage kidney disease. Routine renal function tests are not sensitive enough to detect early renal damage, therefore it is preferable to use better biomarkers such as cystatin C and NAG to monitor the stage of kidney damage in BTM patients and prevent the progression of kidney disease, hopefully, doctors would detect kidney damage early enough to reverse

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