



CYSTATIN C AN EARLY MARKER OF GLOMERULAR DYSFUNCTION IN THALASSEMIA MAJOR.

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ABSTRACT... Objectives: The objective of the present study is to investigate the role of cystatin C as an early marker of glomerular dysfunction in thalassemia major. **Study Design:** Cross sectional comparative study. **Setting:** Department of Biochemistry Post Graduate Medical institute and the Thalassemia Centre in Sir Ganga Ram Hospital Lahore. **Period:** July 2017 and July 2018. **Material & Methods:** This study examined 90 male children all between the ages of 5-11, using non probability sampling techniques. The children were grouped as 21 healthy male children as control group I and 69 diagnosed male thalassemia major children further subdivided on the basis of serum ferritin level as group II, serum ferritin level <2500ng/ml, group III, serum ferritin level 2500-5000ng/ml and group IV serum ferritin level >5000ng/ml respectively. Individuals with hereditary renal diseases, on steroid therapy, or other co-morbid renal diseases were excluded from the study. Complete blood analysis, serum ferritin, creatinine and cystatin C were measured by Micro lab 300 and solid phase enzyme linked immune sorbent assay (ELISA) respectively. The results were compared by using SPSS version 20. **Results:** Group I: n=21 healthy children with ferritin between normal range 105.33 ± 30.03, serum creatinine 0.41 ± 0.05, serum cystatin C 0.57 ± 0.14. eGFR-creatinine 133.38±7.63, eGFR-cystatin C 122.9±17.63. Group II: n=20 (β-TM with ferritin <2500ng/ml). Mean ferritin was 1997.5±300.68 ng/ml (P<0.001), creatinine was 0.43± 0.05, serum cystatin C 0.66 ± 0.05 (P<0.05). eGFR-creatinine 121.45±4.89 P<0.05, eGFR-cystatin C 105.15±6.49 P<0.001. Group III: n= 25 (β-TM with ferritin 2500-5000 ng/ml). Mean ferritin level was 3850.0± 718.18 ng /ml (P<0.001), creatinine was 0.5±.07, cystatin C 0.96±0.13 (P<0.001). eGFR-creatinine 103.29±8.26(P<0.001), eGFR-cystatin C 75.75±10.67 (P<0.001). Group IV: n=24 (β-TM with ferritin >5000 ng/ml). Mean ferritin level was 6311.67±1060.61 ng/ml P value (P<0.001), creatinine was 0.57 ± 0.07, cystatin C 1.11 ± 0.09 (P<0.001). eGFR-creatinine 94.42±8.69 (P<0.001), eGFR cystatin C 64.67±4.23(P<0.001). **Conclusion:** A highly significant positive relation was found between serum ferritin-cystatin C as compared to creatinine among the study groups II, III and IV and significant inverse relationship between cystatin C and eGFR that concluded cystatin C as an early marker of glomerular dysfunction than creatinine in thalassaemia major children.

Key words: Cystatin C, EGFR, Transfusion Dependent Thalassemia Major Children Ferritin Creatinine.

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INTRODUCTION

Thalassemia's the heterogeneous inherited disorders of hemoglobin synthesis presenting with severe anemia early in life. The carrier population of thalassemia is up-to 150 million in more than 60 countries.¹ The estimated carrier rate of thalassemia major (β-TM) in Pakistan is about 5-7%.² An imbalance in the globin chain synthesis and an excess of unmatched alpha (α) globin chain underlies the pathophysiology of

anemia in β-TM children. Long standing anemia, iron overload cause epithelial and endothelial injury with progressive glomerular injury.³

β-TM children with severe anemia require long-term blood transfusion and chelation therapy to maintain Hb concentrations at around 9-10.5g/dl.⁴

Chronic anemia, hypoxia, toxicity of iron chelators

and iron overload due to repeated blood transfusion play an imperative role in developing complications in thalassaemic population. To avoid the deleterious effects of iron overload, iron chelation therapy is started at around 1000 ng/ml of ferritin level, which is a standard practice in β -TM children and it usually occurs after 10th to 12th blood transfusions. Total body iron stores are routinely evaluated by serum ferritin estimation that has a therapeutic and prognostic role in the treatment of β -TM children estimation and is routinely done at interval of least 3 months.⁵

However, in transfusion- dependent thalassaemic children serum ferritin level below 2500 ng/ml is reported to have a better prognosis.⁶

Less is known about the effects of thalassemia on kidneys, however, anemia, chronic hypoxia, oxidative stress, iron excess and harmful effects of iron chelators can influence the normal functioning of the kidneys.⁷

Hyperdynamic circulation due to chronic anemia may increase renal plasma flow and glomerular filtration rate (GFR), however, long-standing anemia and hypoxia can also cause epithelial and endothelial injury with transudation of macromolecules into the mesangium thus contributing to progressive glomerular injury and decrease in GFR.³

GFR is measured by the plasma clearance of a substance in a unit of time by the kidney. Renal GFR an excellent guide to measure kidney function. Clearance of inulin and other exogenous techniques such as iohexol,⁵¹

Cr-EDTA, ^{99m}Tc-labeled diethylene triamine pentaacetic acid (DTPA) and ²⁵¹I labeled iothalamate are considered as the Gold standard exogenous techniques for GFR estimation.⁸

Therefore, in clinical settings use of endogenous marker for GFR estimation is routinely done.⁹ Estimation of GFR with serum and plasma creatinine is a widely used endogenous marker in routine clinical practice.¹⁰

Scr concentration also remains unchanged with a mild to moderate decrease in GFR. Subjects with moderate impairment of renal function present with unchanged Scr levels.¹¹ Change in muscle mass, age, gender and race also influence creatinine level.¹²

Clinically cystatin-C (cys-C) cysteine proteinase inhibitor due its small size, continuous production by all cells in the body, free filtration at the glomeruli and metabolism in the proximal tubule, not affected by age, sex, gender and change in muscle mass is thought to be close to an ideal and sensitive endogenous indicator to estimate GFR as compared to creatinine in high risk patients.¹³ Cys-C has an advantage of being an early and accurate indicator of glomerular dysfunction than creatinine in the pediatric population.¹⁴ Early detection of glomerular dysfunction with cys-C can decrease the rate of renal failure and mortality.¹⁵ It was reported in a study conducted in 2014 that serum cys-C can detect stage II chronic kidney disease earlier than creatinine.¹⁶ In children with normal and reduced GFR, cys-C is more precise than creatinine to estimate GFR.⁹ Cys-C has a positive correlation with Serum creatinine and negative relationship with GFR in thalassaemic population.¹⁷

Estimation of GFR in relation to serum ferritin levels using new endogenous marker cys-C may also help to delay the occurrence of the renal failure.⁶

The study was performed to ascertain whether there was glomerular damage due to anemia and iron overload in thalassemia major population and to investigate the potential utility of cystatin-C as an early marker of glomerular dysfunction in thalassemia major children.

MATERIALS AND METHODS

This Cross sectional Comparative study was held between July 2017 and July 2018 after approval by the Ethical Review committee, Advanced Studies and Research Board of University of Health Sciences Lahore (attached as annexure I) at Post Graduate Medical Institute Lahore with Thalassemia Center in Sir Ganga Ram Hospital

Lahore.

An informed verbal and written consent from the care takers of the patients was taken and their demographic data was recorded on a proforma. The inclusion criteria for the study was male children all between the ages of 5-11 of age. Children with the history hereditary renal diseases, recurrent urinary tract infections, valvular heart disease, liver diseases, and diabetes mellitus on steroids therapy and nephrotoxic drugs were excluded from the study. The study population was divided as group I control with 21 healthy male children and 69 thalassaemic children on regular blood transfusion with history of at least 10-12 blood transfusion/year, further subdivided as group II, III and IV on the basis of varying ferritin level.

Group 2: n = 20 β -TM male children with ferritin < 2500ng/ml.

Group 3: n = 25 β -TM male children with ferritin 2500-5000ng/ml.

Group 4: n = 24 β -TM male children with ferritin >5000ng/ml.

The sample size was calculated, with 80% power of study and 5% level of significance

Purposive blood sample was taken and 5 ml of blood was collected from the median cubital vein by aseptic technique then poured in two vacutainers, 2 ml in a vacutainer with EDTA for complete blood analysis and the test were carried out at the clinical pathological laboratory of Sir Ganga Ram Hospital Lahore. Remaining 3ml blood was taken in vacutainer without anticoagulant the test samples were allowed to clot at room temperature for 30 minutes centrifuged for 5 minutes at 4000 rpm at PGMI Biochemical laboratory Lahore.

The serum was removed, aliquoted and stored at -20° C at the Mid-City Pathological laboratory Hospital Lahore until assayed. The separated serum was used for the assays of cystatin-C by Human cystatin C ELISA kit (Glory science Co. Ltd) for research only, quantitative serum ferritin estimation by Human Ferritin Enzyme Immune

Assay Test Kit Bio Check, INC. Catalog Number, BC-1025 and serum creatinine by creatinine liquid color Jaffe-Reaction, semi- automatic analyzer 5010.

Statistical Analysis

Statistical Analysis of the data was carried out using SPSS 20.0 for windows. Data were shown as the mean and standard deviation (SD). Independent student t-test was used for the comparison of two quantitative variables pair wise.

One way ANOVA was used for the comparison between the variables among the study groups. Findings were elaborated using, tables and graphs. P values < 0.05 was considered statistically significant.

RESULTS

A total of 90 subjects were included in the study consisting of 21 controls in group I and 69 thalassaemia major on regular blood transfusion in group II, III and IV. The mean serum ferritin levels were 105.33 ± 30.03 in the control and 1997.5 ± 300.68 ng/ml, 3850.0 ± 718.18 ng/ml and 6311.67 ± 1060.61 ng/ml among the thalassaemic groups respectively (see Table-I,II).

eGFR calculations were carried out using Schwartz pediatric equation using creatinine and cystatin C among the control and group II,III and IV with varying Ferritin level.

The results concluded significant decrease in eGFR with $P < 0.001$ among the thalassaemic groups II,III and IV with high ferritin levels as compared to creatinine which showed decrease eGFR in group IV as compared to group II and III but eGFR value remained within normal limits thus concluding cystatin C as an accurate and early marker of glomerular dysfunction. (Table-III)

There was significant difference in values of serum ferritin among the control and study groups on regular blood transfusion with or without iron chelation therapy. The frequency and number of blood transfusion per month was high in group III and IV as compared to group II, Table-IV & V.

The mean serum creatinine level was $0.41 \pm$

0.05mg/ml and in the control and 0.43 ± 0.05 mg/ml, 0.5 ± 0.07 mg/ml 0.57 ± 0.07 mg/ml in the study groups II, III and IV respectively (Table-I&II).

Serum cystatin C 0.57 ± 0.14 mg/l in the control group, and 0.66 ± 0.05 ($P < 0.05$), 0.96 ± 0.13 mg/l ($P < 0.001$), 1.11 ± 0.09 mg/l ($P < 0.001$) in the group II, III and IV children respectively (Table-I&II).

Results: Serum ferritin was significantly high with ($P < 0.001$) in group II than the control group. Value of serum cystatin- C was higher ($P < 0.05$) in group II than the control group.

Results: Level of serum ferritin was significantly high P value ($P < 0.001$) in group IV than in group III. Group IV had high mean value P value ($P < 0.001$) of serum cystatin- C and serum creatinine than group III.

Variables	Group I (control) (21)	Group II.(20)
Serum ferritin ng/ml	105.33+ 30.33	1997.5+300.68
Serum creatinine mg/dl	0.41+ 0.05	0.43+0.05
Serum Cystatin C	0.57+0.14	0.66 + 0.05

Table-I. Biochemical parameters Serum ferritin, creatinine and cystatin C group I (Control) and group II.

Variables	Group III (25)	Group IV(24)
Serum ferritin ng/ml	3850 \pm 718.18	6311.67 \pm 1060.61
Serum creatinine mg/dl	0.5 \pm 0.07	0.57 \pm 0.07
Serum cystatin C	0.96 \pm 0.13	1.11 \pm 0.09

Table-II. Biochemical parameters serum ferritin, creatinine and cystatin C group III and group IV.

Variables	Control groupI	Group II	Group III	Group IV
eGFR creatinine 1.73 ml/min	133.38 \pm 7.63	121.45 \pm 4.89 $P < 0.05$	103.29 \pm 8.26 ($P < 0.001$),	94.42 \pm 8.69 ($P < 0.001$),
eGFR cystatin C 1.73 ml/min	122.9 \pm 17.63	105.15 \pm 6.49 $P < 0.001$	eGFR-cystatin C 75.75 \pm 10.67 ($P < 0.001$).	GFR cystatin C 64.67 \pm 4.23($P < 0.001$)

Table III. Variation in eGFR with creatinine and cystatin C.

Group II n= (20).	Number of Blood transfusion/ month	Frequency of transfusion/ month	Percentage of blood transfusion
	1	4	20%
	2	12	60%
	3	3	15%
	4	1	5%
Group III n= 25			
	1	4	16%
	2	12	48%
	3	8	32%
	4	1	4%
Group IV n= 24			
	1	5	20%
	2	7	29%
	3	6	25%
	4	6	25%

Table-IV. Frequency and percentage of blood transfusion per month in group II, III and IV.

Group II n=20		Group III		Group IV	
Type of Iron chelation	Percentage of iron chelation therapy	Type of Iron chelation	Percentage of iron chelation therapy	Type of Iron chelation	Percentage of iron chelation therapy
Oral	20%	Oral	5	Oral	4%
Cap hydraea	20%	Cap hydraea	2	Cap hydraea	8%
Irregular	35%	Irregular	8	Irregular	16%
No chelation	20%	No chelation	5	No chelation	25%
Intravenous chelation	5%	Intravenous chelation	5	Intravenous chelation	8%

Table-V. Frequency and percentage of Iron chelation therapy in group II, III and IV.

DISCUSSION

Blood transfusion is important for the normal growth and development in thalassemia major patients. Repeated blood transfusions can inevitably lead to iron overload which could cause organ damage thus increasing the mortality. During the last decade, a large number of studies and available literature revealed an interest in cys-C a new endogenous marker of glomerular dysfunction. Previously some studies and recent study by Hamad and El Meglegly in 2010 also stated that the early detection of renal dysfunction with these specific early markers may interrupt the progression of renal damage in thalassaemic population.¹⁸

In past β -TM children did not live long to develop conditions linked to kidney dysfunction. However, the advent of iron chelation therapy increased the life expectancy of β -TM children and had allowed previously unnoticed renal complications to emerge.¹⁹

Qari and his co-workers in 2013 reported that in thalassemia major children, complications of iron overload resulting in excess of free iron with the production of injurious hydroxyl radicals thus increasing the complexity of the disease. A study by reported progressive renal failure to be one the main complications in β TM children.^{20,21}

Mishra and Tiwari in 2013 documented about 5 gm of hepatic iron deposition within 1 year of regular blood transfusions in a child who gets 25 units of blood transfusion per year without any iron chelation therapy. Markedly high serum ferritin level of 1997.5 ± 300.68 , 3850.0 ± 718.18 ng/ml and 6311.67 ± 1060.6 ng/ml was found

among the study groups II, III and IV, the results were similar to recent work by Riaz et al., in 2011, which reported mean ferritin levels of 4236.5 ng/ml in transfusion dependent thalassemia major children.^{22,23}

The use of iron chelation also affect functioning of glomeruli as DFO (Deferoxamine) given intravenously can also cause renal toxicity when given in high dose. However, DFO has decreased the rate of mortality by decreasing the cardiac and other complications.²⁴ The same year in 2015 Ricchi and Marsella also stated the use of oral iron chelator DFP where iron chelation with DFO is not appropriate for treating iron overload.²⁵

Financial constraints observed in this study, with monthly low family income around 14000-16000 rupees/ month also contributed to the non-compliance of the treatment/chelation therapy among the thalassaemic study groups. The results observed in the study were similar to work published by Arif and his co-workers in 2008, which documented an alarming impact of socioeconomic conditions and distressing psychological burden on the families of thalassaemic children.²⁶ According to a study by Cunningham in 2004, lifelong transfusion and chelation treatment is a challenging task especially in developing countries where the prevalence of disease and related to health problems is high.²⁷

It was observed in the present study that blood transfusions were repeated irregularly (3 or 3-4 times/month) with an interval of less than 1-2 weeks in children with ferritin level between 2000 ng/ml to >5000ng/ml. It was also found that almost all the patients were under transfused

with hemoglobin level below 10gm/dl. Increase in frequency of blood transfusion and iron mediated toxicity greater than 2500 ng/ml observed in the present study was similar to high mean serum ferritin level in thalassemia major children patients with and without chelation therapy, reported by Shah and his colleagues in 2010.²⁸

A recent work by Filler and his co-team in 2012 stated, even though creatinine is commonly used in clinical practice to estimate GFR, yet it does not share the properties of an ideal marker of GFR estimation like inulin. Hence due to the certain limitation with the use of serum creatinine as a marker for GFR estimation, use of small molecular weight protein cys- C, produced constantly by all nucleated cells in the body is in clinical trials as an early and potent marker to assess glomerular damage.²⁹

A work published by Inker et al., 2012 also recognized the diagnostic inaccuracy of creatinine for GFR estimation due to variation in creatinine level with the change in muscle mass, age and gender and stated cys- C as a substitute and better marker than creatinine for eGFR.³⁰ Earlier work by Tanaka and his co-workers in 2007 also reported that serum cys-C remains unaffected with a change in age, gender and muscle mass and is a better and superior marker than creatinine to predict GFR.³¹ Shilpak and his co-team in 2013 also discovered the significance of cys-C as a predictor of cardiovascular problems in diabetics and in metabolic syndromes.³²

Results of the present study revealed 0.43 to 0.57 mg/dl of serum creatinine level among the study groups, the level of serum creatinine was normal even with 50% reduction in GFR. Whereas, an increase in cys-C from 0.66 to 1.2 mg/l was observed in relation to high ferritin levels. A decrease in GFR was observed among the study groups with or without chelation therapy. The increase in serum cys-C observed in this study also indicated an indirect correlation of cys-C with GFR and a positive correlation of cys-C with serum ferritin level. The results of this study were similar to work published by Westhuizen in 2006 which also reported cystatin C as a useful biomarker

of renal dysfunction. These authors established that serum cys-C was a sensitive marker for renal impairment defined as GFR <80 ml/min/1.73 m.²

The present study signifies the usefulness of cys-C in identifying reasonably impaired renal function in transfusion dependent β -TM children due to iron overload. Similar results were reported by Peiris and his co-workers in 2008 which documented the diagnostic cut-off value of 1.25mg/l of cystatin C for the recognition of patients with moderately impaired renal function with a single random blood sample.¹¹ Murty and his colleagues signifies the accuracy of cystatin c in estimating a decrease in GFR in creatinine blind range (between 40 and 70 ml/min/1.73 m²).

Earlier a work by Safaie and co-workers in 2008 demonstrated a positive correlation between serum ferritin and predictors of tubular toxicity. Whereas, another study by Koliakos and his co-fellows in 2003 reported glomerular rather than tubular damage with a high level of serum ferritin in β -TM children.³³ Recent work in 2017 by Behairy and his co-workers concluded that glomerular dysfunction in beta thalassemia is not an uncommon complication and cys-C may be a better marker that could predict earlier slight changes in glomerular filtration rate, as compared to creatinine.

The results of this study were similar to work done by Ali and Mahmoud in 2014, who found that serum cys-C, is superior for GFR estimation when compared to other estimates in thalassemia major children. An inverse relationship of cys-C and eGFR was found in the present study among the study groups with serum ferritin > 2000 ng/ml. Moreover, a significant decrease in eGFR from 105-64 ml/min/1.72m² calculated with cy-C was also observed among the study groups with ferritin level from > 2500 ng/ml to >5000ng/ml and eGFR calculated with creatinine had values from 121-94 ml/min/1.72 m² respectively.¹⁶

CONCLUSION

Iron overload due to irregular iron chelation, low socioeconomic status, lack of education and insufficient health care facilities among the

thalassemia major patients were the main factors which contributed to ineffective treatment.

In terms of pre-transfusion low Hb (7-9gm/dl) observed in our study was due to ineffective erythropoiesis. The frequency of blood transfusion increased with the increase in age from one transfusion/month to 3-4 per month.

The present data confirmed that glomerular dysfunctions exist in beta thalassemia major children. These abnormalities were mainly sub-clinical.

Highly significantly positive correlation of cys- C with serum ferritin level as compared to serum creatinine was found in thalassemia major children on regular blood transfusions.

The study concluded a rise in serum cystatin C level greater than normal when GFR was 71ml/min/m² on the other hand serum creatinine level increased within normal limits at GFR was 88ml/min/m². This study thus concluded the significance of serum cystatin C in the estimation of mild reduction in GFR which was overlooked with creatinine.

The comparison between eGFR calculated with serum cystatin-C and creatinine in thalassemia major children concluded a significant inverse correlation of cys-C as compared to creatinine with eGFR in group II III and IV, the results and data of the present study thus concluded cystatin C as an early marker of glomerular dysfunction than creatinine in transfusion dependent thalassemia major children.

Early identification of renal dysfunction with cys-C for the high-risk thalassaemic population on regular blood transfusion may help to take specific measure to avoid renal complications. Therefore, the routine use of new marker cys-C for early detection of glomerular dysfunction is recommended for all thalassemia major patients on regular blood transfusions, to diagnose and manage the subclinical renal involvement and its related complications, present study data might be a help for further research regarding

glomerular complications due to iron overload in thalassemia major population.

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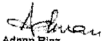



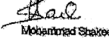
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