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SERUM NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN (NGAL) AND URINARY NGAL EXCRETION AS A BIOMARKER OF RENAL INJURY IN CHILDREN WITH BETA THALASSEMIA MAJOR

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ABSTRACT **Background:** Frequent blood transfusions among patients with beta thalassemia major leads to iron overload state and leads to damage of various organs including kidney. Very few studies have explored on Serum and urinary NGAL as a biomarkers of renal injury in thalassemia major children. Therefore, this study is planned to investigate the renal injury in beta thalassemic children by measuring serum and urinary NGAL levels and correlating it with cystatin c and creatinine clearance.

Methods: The study was a cross-sectional conducted among 25 patients with β thalassemia major, aged 1-18 years, having undergone regular blood transfusion and chelation therapy. Levels of plasma and urinary NGAL were measured and compared to the standard values of the normal range. Linear regression analysis was done.

Results: Mean(SD) serum NGAL value in 1- 5 years of age was 1.6(0.26) , in 5-10 years was 2.15(0.23), in 10- 15 years was 2.6 (0.11) and > 15 years it was 18.11(33.76). (p value <0.005).Mean (SD) urine NGAL value in 1- 5 years of age was 0.66 (0.11) , in 5-10 years was 1.13(0.13), in 10- 15 years was 1.38 (0.18) and > 15 years it was 1.94(0.25). (p value <0.005).The mean values of plasma N-GAL, and Urinary N-GAL were significantly higher in our patients as compared to that of standard population values($p<0.05$).

Conclusions: Serum and urine NGAL values are found to be much higher in those with longer duration of transfusion and chelation. Positive correlation was found between urine NGAL levels and cystatin C. Serum and urine NGAL values are fair markers of renal injury in thalassemia major patients on multiple transfusions.

KEYWORDS : NGAL, Cystatin, Creatinine Clearance, Thalassemia Major

INTRODUCTION

Beta thalassemia major(β TM) is an inherited hemoglobinopathy that causes reduced or absent globin synthesis, resulting in an imbalanced accumulation of globin chains and ineffective erythropoiesis with hemolysis. This leads to a state of severe anemia requiring repeated blood transfusions.[1] It is estimated that about 100,000 children with transfusion dependent thalassemia are born worldwide annually. Out of these 8,000 to 10,000 born in India alone.[2,4]

This therapy results in iron overload leading to iron deposition in various organs such as, liver, kidney, and endocrine glands. The mechanism of renal injury has been thought to be multifactorial. It could be due to direct toxicity of free iron molecules causing cytopathic effects of cells within the kidney, chronic anemia, free radicals released as a result of activation of the oxidative stress pathways or indirect drug toxicity of chelators. [3]

Detection of the progressive renal damage using conventional parameters, such as serum creatinine levels or creatinine clearance is often misleading ,as it presents as raised values later. Due to this reason, the identification of markers that indicate early renal injury is highly desirable. Neutrophil gelatinase-associated lipocalin (NGAL), also known as oncogene 24p3(presents on long arm of chromosome 24) or Lipocalin-2 (LCN2), is a protein that in humans is encoded by the LCN2 gene. It is expressed in neutrophils and in low levels in the kidney, prostate, and epithelia of the respiratory and alimentary tracts. NGAL is involved in innate immunity by sequestrating iron containing siderophores that in turn limits bacterial growth. Lipocalin-2 also functions as a growth factor.[4]

Plasma NGAL is freely filtered by the glomerulus, and largely reabsorbed in the proximal tubules allowing only low levels of NGAL to be detectable in the urine of normal individuals. Excretion of NGAL should occur only when there is a concomitant proximal renal tubular injury that precludes NGAL reabsorption and/or synthesis.[5] Induction of NGAL after renal injury precedes the elevation of classical markers for kidney damage like serum creatinine,urinary N-acetyl glucosaminidase, and b2 microglobulin.[6]

Therefore, this study was planned to investigate the renal injury in beta thalassemic children by measuring serum and urinary NGAL levels and correlating it with cystatin c and creatinine clearance.

MATERIAL AND METHODS

Study Design And Setting:

The study was a cross sectional study conducted at the Department of Pediatrics, Post Graduate Institute of Medical Education and Research, Dr. RML Hospital, New Delhi.

Study Population:

The study population included children of age from 1year to 18 years with thalassemia major. Children who had urinary tract infection, respiratory tract infection, recent or regular consumption of nephrotoxic drugs except chelating agents and children with sepsis were excluded from the study. Given the lower prevalence of thalassemia major in India and case load in our hospital, we were able to recruit a total of 25 study participants.

Study Duration:

The study was conducted during November 2014 To March 2016.

Data Collection Procedure:

After obtaining written informed consent from parents or guardians for the enrolment in the study. At the first clinical visit all children were evaluated clinically and age , sex ,blood pressure, height and weight were recorded. General physical examination and systemic examination were done. An account of diabetes, hypertension, any febrile illness and history of transfusion and chelation therapy were taken. Investigations done were complete blood count with Peripheral smear and reticulocyte count, blood urea, creatinine, Serum proteins(albumin, globulin and total), random blood sugar and urine analysis includes : Urine Routine /microscopy, Urine culture/ sensitivity.

Sample Collection :

For serum NGAL and cystatin C the blood sample (3ml) were collected by venipuncture into a vacutainer tube (Plain) at hospital .After collection it was allowed to clot for atleast ½ hr and centrifuged at 3000rpm for 10 minutes. Separated serum then stored in different tubes at -70^{0}C and was analyzed by ELISA. For urinary NGAL, spot mid stream morning urine samples were collected at the hospital. After collection, it was immediately centrifuged at 3000 rpm for 5-10 minutes . After centrifugation supernatant was collected and stored at -70^{0}C and was analyzed by ELISA.

ELISA Test For Individual Proteins:

Principle:

In this method, an enzyme, which reacts with a colourless substrate to

produce a coloured product, is covalently linked to a specific antibody that recognizes a target antigen. If the antigen is present, the antibody-enzyme complex will bind to it, and the enzyme component of the antibody-enzyme complex will catalyze the reaction generating the coloured product. Thus, the presence of the coloured product indicates the presence of the antigen. The intensity of the reaction determines the antigen or analyte concentration and is measured as optical density by spectrophotometer. The optical density (OD) of the sample is compared to a standard curve, which is typically a serial dilution of a known-concentration solution of the target molecule.

Sandwich ELISA

Microwell is coated with a capture antibody and when sample is added, any antigen present binds to capture antibody. Then primary antibody, secondary antibody and substrate are added subsequently with wash in-between using buffer solution. A surface is prepared to which a known quantity of capture antibody is bound. The name Sandwich is given because antigen is sandwiched between two antibodies. Quantitative estimation of the markers was done using ELISA technique. Kits were used for the respective markers for cystatin C and neutrophil gelatinase-associated lipocalin (USCN Life Science Inc, Boston). The kits contained pre-coated antibody-plates, detection antibodies, buffers, diluents, standards, and substrates. The measurement was done on microplate reader (Infinite®, Tecan Group Ltd., Switzerland) machine.

Measurement Of Neutrophil Gelatinase Associated Lipocalin :

This assay employed a quantitative sandwich enzyme immunoassay technique. Microplates precoated with an antibody specific for NGAL were used. Sample or standard containing NGAL were sandwiched by biotinylated antibody specific for NGAL. Then, Avidin conjugated with enzyme Horseradish peroxidise was added and 3,3',5,5'-Tetramethylbenzidine(TMB) substrate was added . The minimum detectable level of NGAL was 0.156-10 ng/ml. The intra-assay and inter-assay coefficients of variation were <10% and <12% respectively.

40µl of sample diluent plus 10µl of testing sample
⇩
Incubation (30 min) at 37°C
⇩
Washed thrice with 300µl of wash buffer
⇩
50 µl of Avidin Horseradish-Peroxidase Conjugate was added to each well.
⇩
Incubation 30 minutes at 37°C followed by repeat wash 5 times
⇩
Addition 50µL of TMB substrate & incubation (30minutes)
⇩
Addition 50 µl of Stop Solution. The colour changed from blue to yellow.
⇩
The absorbance was read on a microplate reader at 450 nm immediately.

Measurement Of Cystatin C

This assay employed a quantitative sandwich enzyme immunoassay technique. Microplates precoated with an antibody specific for human cystatin C were used. Sample or standard containing cystatin C were sandwiched by biotinylated antibody specific for cystatin C. Then, Avidin conjugated with enzyme Horseradish peroxidise was added and 3,3',5,5'-Tetramethylbenzidine(TMB) substrate was added. The minimum detectable level of NGAL was 0.156-10 ng/ml. The intra-assay and inter-assay coefficients of variation were <9% and <11% respectively.

40µl of sample diluent plus 10µl of testing sample
⇩
Incubation (30 min) at 37°C
⇩
Washed thrice with 300µl of wash buffer
⇩
50 µl of Avidin Horseradish-Peroxidase Conjugate was added to each well.
⇩
Incubation 30 minutes at 37°C followed by repeat wash 5 times
⇩
Addition 50µL of TMB substrate & incubation (30minutes)
⇩
Addition 50 µl of Stop Solution. The colour changed from blue to yellow.
⇩
The absorbance was read on a microplate reader at 450 nm immediately.

Measurement Of Urinary Creatinine And Creatinine Clearance

Urinary creatinine was measured using Jaffe's method on calorimetric method and measurement interpreted by automated (COBAS INTEGRA® 400 plus, Roche Diagnostics Ltd., Switzerland) analyser. Creatinine clearance was calculated by modified Schwartz formula.

Statistical Analysis:

Data was entered and analysed with Statistical Package for Social Sciences (SPSS IBM) version 21.0 software. Normality of data was tested by Kolmogorov-Smirnov test. If the normality was rejected then non parametric test was used. Quantitative variables were compared using Unpaired t-test/Mann-Whitney Test (when the data sets were not normally distributed) between the two groups and anova/kruskal wallis test(for non parametric data) between more than two groups. Pearson correlation coefficient was used to find out correlation between two quantitative variables. Univariate and multivariate linear regression was used to assess the association of Urine NGAL, Serum NGAL and Serum Cystatin C with age, sex, height and weight. A p value of <0.05 was considered statistically significant.

RESULTS

The age of the study population ranged from a minimum of 1 year to a maximum of 18 years with a mean(SD) of 8.08 (± 5.45).Majority of children (32%) were in age group of 1- 5 years, while 28%were in age group of 5-10 years and 20% were in age group of 10-18 years. Among the 25 children 40% were female sand 60% were males. All the participants presented with pallor.(100%). Family history of thalassemia was found in 18(72%)(Table 1).

Table 1 Baseline Characteristics Of The Study Participants. (N=25)

Variables	Mean ± SD	Min-Max	Inter quartile Range
Age (years)	8.24 ± 5.45	1-18	3.750 - 12.500
Weight (kg)	24.76 ± 11.98	8-45	13.750 - 38
Height (cms)	113.92 ± 26.79	68-155	93 - 135.500
Haemoglobin(g/ dl)	7.27 ± 1.4	5-10	6 - 8.275
TLC	9188.4 ± 1388.04	6890-12440	8300 - 9872.500
Neutrophil	56.04 ± 5.84	42-67	53.500 - 60
Lymphocytes	40.64 ± 6.31	30-55	36.750 - 44.500
Platelets(lacs)	4.46 ± 0.88	3-6	3.800 - 5.075
Urea (mg/ dl)	32.84 ± 9.18	12-46	27.250 – 40
Creatinine (mg/ dl)	0.35 ± 0.14	0.2-0.7	0.275 - 0.400
Total bilirubin (mg/ dl)	1.06 ± 0.33	0.7-2	0.800 - 1.125
SGOT(U /L)	34.88 ± 10.55	18-50	28.500 - 45
SGPT (U/ L)	42.36 ± 10.88	20-62	33.500 - 49.250
ALP(U/ L)	110.28 ± 23.25	65-180	95.500 - 120
Total protein (mg/ dl)	5.86 ± 0.38	5.1-7	5.600 - 6.025
Albumin (mg/ dl)	3.86 ± 0.41	3.1-4.5	3.550 - 4.200
Globulin (mg/dl)	2.24 ± 0.25	2-3	2 - 2.400
serum NGAL(ng/dl)	5.26 ± 15.27	1.03-78.5	1.775 - 2.666
Urine NGAL(ng/ml)	1.19 ± 0.49	0.53-2.33	0.697 - 1.526
Serum Cystatin C (ng/ml)	2.99 ± 2.22	0.6-12.62	1.990 - 3.480
Creatinine clearance (ml/min)	143.24 ± 37.44	90.3-217	105.750 - 173.250
Duration in years of chelators received	7.09 ± 5.12	0.5-16	2 – 13
Duration in years of Transfusion received	7.19 ± 5.34	0.33-17	2.750 - 10.500

Table 2 shows the baseline characteristics of the study participants

Table 2 Age Wise Distribution Of Cystatin C And NGAL Among Study Participants. (n=25)

	1 – 5 years	6- 10 years	10-15 years	15 years	P value
Serum Cystatin C (Ng/ml)					<.0005
Mean ± SD	1.7 ± 0.55	2.39 ± 0.3	3.07 ± 0.48	5.81 ± 3.85	
Median	1.89	2.4	2.9	4.03	
Inter quartile Range	1.473 - 1.970	2.230 - 2.425	2.733 - 3.504	3.686 - 6.979	

serum NGAL (ng/ml)					<.0005
Mean ± SD	1.6 ± 0.26	2.15 ± 0.23	2.6 ± 0.11	18.11 ± 33.76	
Median	1.64	2.13	2.56	3.04	
Inter quartile Range	1.542 - 1.795	2.085 - 2.321	2.523 - 2.666	2.991 - 21.946	
Urine NGAL(Ng/ml)					<.0005
Mean ± SD	0.66 ± 0.11	1.13 ± 0.13	1.38 ± 0.18	1.94 ± 0.25	
Median	0.66	1.12	1.38	1.9	
Inter quartile Range	0.585 - 0.695	1.008 - 1.260	1.244 - 1.526	1.782 - 2.089	

Mann Whitney test applied, p value of <0.05 is significant.

It was observed that with the increasing age , the values of NGAL and cystatin C also increased .For serum Cystatin C mean value in 1- 5 years of age was 1.7(SD=0.55) , in 5-10 years was 2.39(SD=0.3), in 10- 15 years was 3.07 (SD=0.48) and > 15 years it was 5.81(SD=3.85). This was statistically significant.(p value <0.005).For serum NGAL mean value in 1- 5 years of age was 1.6 (SD=0.26) , in 5-10 years was 2.15(SD=0.23), in 10- 15 years was 2.6 (SD=0.11) and > 15 years it was 18.11(SD=33.76). It was observed that that it was statistically significant (p value <0.005). For urine NGAL mean value in 1- 5 years of age was 0.66 (SD=0.11) , in 5-10 years was 1.13(SD=0.13), in 10- 15 years was 1.38 (SD=0.18) and > 15 years it was 1.94(SD=0.25). This was statistically significant (p value <0.005).(Table 3). Analysis of sex with the measures found no significant association.There was increase in serum cystatin C, serum NGAL and urine NGAL levels with increase in duration of transfusion as well as with increase in duration of chelation, and this correlation was statistically significant as shown in Table 3.

Table 3 Correlation Of Cystatin C, Serum And Urinary NGAL With Duration Of Chelators And Transfusion Received. (N=25)

		log Serum Cystatin C	log serum NGAL	Urine NGAL
Duration in years of chelators received	**Correlation Coefficient***	0.851	0.606	0.967
	P value	<0.0001	0.0028	<0.0001
Duration in years of Transfusion received	**Correlation Coefficient**	0.847	0.654	0.977
	P value	<0.0001	0.0004	<0.0001

**Pearson correlation coefficient was used*

Multivariate regression analysis after adjusting the confounding factors, showed no affect of independent variables on serum NGAL levels, urine NGAL and cystatin C (p>0.05).

Table 4 Multivariate Regression For Log Serum NGAL.

Variables	**Unstandardized Coefficients**		**P value**	**95.0% Confidence Interval for B**	
	B	**Std. Error**		**Lower Bound**	**Upper Bound**
Age (years)	.246	.347	.488	-.489	.981
height (cms)	-.006	.018	.747	-.044	.032
log weight(kg)	-1.876	1.445	.213	-4.939	1.187
Duration in years of chelators received	-0.069	.122	.577	-0.328	0.189
Duration in years of Transfusion received	-0.043	.254	.868	-0.582	0.496

Table 5 Correlation Of Serum NGAL With Creatinine Clearance

Parameters	**Creatinine clearance**		**Cystatin C**	
	Correlation coefficient	**p value**	**Correlation coefficient**	**p value**
Serum NGAL	-0.254	.2214	0.808	<0.0001
Urinary NGAL	-0.277	0.1797	0.866	<0.0001

**Pearson correlation coefficient was used.*

Serum and urine NGAL was well correlated with cystatin C.(p value <0.05)

DISCUSSION

NGAL has not been studied extensively in beta thalassemia major children. There are very few studies about NGAL in transfusion –dependent beta thalassemia major patients. The results from a few studies have shown that NGAL, m RNA, and protein levels are increased in transfusion dependent thalassemia major as a result of iron overload ,while other studies suggested that elevated NGAL levels in these patients are mainly due to renal injury.[7]

In our study levels of serum and urinary NGAL were not high as compare to the reference values given in few studies. The multivariate analysis had shown no significant correlation with age, sex ,height, weight , duration of chelation and transfusion.

Mehryar Habibi et al[7] studied the upregulation of NGAL in 25 adult and 9 paediatrics patients where they found that there was no upregulation of NGAL in paediatrics patients which indicates that the inducing factor, iron overload, was not sufficient, probably due to fewer blood transfusion therapies, to induce NGAL expression compared to adults.

Another study done by Behairy OG et al[8] reported that serum cystatin-C and β-2 microglobulin had positive correlation with urea, creatinine, serum ferritin, albumin/creatinine ratio. In addition, duration of chelation therapy and frequency of blood transfusion/year had negatively correlation with creatinine clearance, hemoglobin, and eGFR. In previous studies, similar findings were reported.[9,10]We found similar results in our study too.

Many reasons other than renal injury can be contemplated for elevated NGAL levels, including anemia/hypoxia,iron homeostatic disturbances. In paediatrics patients inducing factors ,iron overload are not sufficient to cause raised level because of fewer transfusion therapy, less duration of use of chelators. So the age, number of transfusion and duration of chelation plays a major role to raise the NGAL levels.

The study has few limitations such as smaller sample size and conducted in a single tertiary hospital, A multicentric prospective studies, measuring urinary and serum levels of these markers are needed for better understanding of the pathological importance of proteins and their usefulness in predicting early diagnosis of renal injury.

CONCLUSION

Serum and urine NGAL values are found to be much higher in those with longer duration of transfusion and chelation. No influence of other confounding factors on serum NGAL levels, urine NGAL and cystatin C. Positive correlation was found between urine NGAL levels and cystatin C.

REFERENCES

1. Bagga A, Mantan M. Nephrotic syndrome in children. Indian J Med Res.2005;122: 13-28
2. McKinney PA, Feltbower RG, Brocklebank JT, Fitzpatrick MM. Time trends and ethnic patterns of childhood nephrotic syndrome in Yorkshire, UK. Pediatr Nephrol. 2001;16:1040.
3. Eddy AA, Symons J.M. Nephrotic syndrome in childhood. Lancet 2003; 362:629-39.
4. Bagga A, Srivastava RN. Nephrotic syndrome. Pediatric Nephrology 4th edition, New Delhi, Jaypee 2006: 161-200.
5. Robert P.Woroniecki, Tatyana N. Orlova, Natasha Mendelev,*et al.* Urinary proteome of steroid sensitive and steroid resistant idiopathic nephrotic syndrome of childhood. Am J Nephrol 2006; 26: 258-67.
6. Hodson E, Knight J, Willis N, Craig J. Corticosteroid therapy for nephrotic syndrome in children. Cochrane database syst rev; CD001533: 2005.
7. Roudkenar MH, Halabian R, Oodi A, Roushandeh AM, Yaghmai P, Najar MR, Amirizadeh N, Shokrgozar MA. Upregulation of neutrophil gelatinase-associated lipocalin, NGAL/Lcn2, in beta-thalassemia patients. Arch Med Res. 2008 May;39(4):402-7.
8. Behairy OG, Almonaem ERA, Abed NT, Abdel OM, Zakaria RM, AbdEllaty RI. Role of serum cystatin-C and beta-2 microglobulin as early markers of renal dysfunction in children with beta thalassemia major. Int J Neph Renovas Dis. 2017:10 261–268
9. Fouad IZ, ElNahid MS, Youssed MF, Amroussey YM.Urinary neutrophil gelatinase-associated lipocalin as a marker of kidney injury in Egyptian patients with thalassemia. Egypt J Intern Med 2019; 31: 343-52
10. Ozek G, Carti O, Serdaroglu E, Cuhadar S, Yaman Y, Oymak Y. Renal Dysfunction in Pediatric Thalassemia Major Patients and Evaluation of Urine NGAL Levels in Thalassemia. *Blood* 2014; 124 (21): 4049