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Original Article

Urine Neutrophil Gelatinase-associated Lipocalin as a Prognostic Biomarker in the First Episode of Idiopathic Nephrotic Syndrome in Children

Geethanjali Pradeepchandran[1], Susy Joseph[1], Susan Uthup[2], Geetha Saradakutty[1]

[1]Department of Pediatrics, Medical College, Thiruvananthapuram, India
[2]Department of Pediatric Nephrology, Medical College, Thiruvananthapuram, India

ABSTRACT

Aim: Idiopathic nephrotic syndrome (NS) is the most common glomerular disorder of childhood. Its prognosis is correlated with treatment responsiveness and not renal histopathology. Most of the children who suffer from NS experience multiple relapses and there is a risk of long-term drug dependence with possible side effects. Hence, there is always a need for markers to assess its long-term outcome even in steroid responders. Urine neutrophil gelatinase-associated lipocalin (uNGAL) is an early risk marker of acute kidney injury and also a marker of progression of chronic kidney disease. Our aim was to determine if urine NGAL could predict steroid responsiveness at the onset of NS, which would help in the planning and monitoring of the treatment in idiopathic NS. The aims of this study were to determine the levels of uNGAL in children who were having their first episode of NS and to study its relation with steroid resistance at 3 months.

Materials and Methods: A prospective observational study was conducted in children diagnosed with their first episode of idiopathic NS in a tertiary care teaching hospital from January, 2019 to July, 2020. Urinary NGAL measurements were conducted before starting steroids.

Results: Seventy-nine children satisfying the inclusion criteria were included in this study. Their mean age was 7.18 (±2.86) years. The male to female ratio was 1.25:1. All 63 children who had urine NGAL less than 10 ng/mL responded to the standard dose of steroids at 8 weeks and attained remission. Out of the 16 children with NGAL over 10 ng/mL, 56.3% (n=9) responded to steroids within 8 weeks (intermediate or late steroid responders) and 43.8% (n=7) were steroid resistant NS (SRNS). Urine NGAL below 10 ng/mL was associated with steroid responsiveness in the first episode of NS at 3 months ($p<0.001$).

Conclusion: Urine NGAL below 10 ng/mL is an early predictive biomarker of steroid responsiveness in the first episode of idiopathic NS.

Keywords: Neutrophil gelatinase-associated lipocalin (NGAL), idiopathic nephrotic syndrome, steroid responsiveness, biomarker, prognosis

Introduction

Nephrotic syndrome (NS) is the most common glomerular disease in children. The prevalence of NS in children is 12-16 per 100,000 individuals and the underlying cause is idiopathic in 95% of cases (1). Oral glucocorticoids form the mainstay of treatment. Invasive renal biopsy remains the standard for diagnosis of NS in adults. Unlike in adults, steroid responsiveness is a better predictor than histopathological diagnosis in the long-term prognosis of idiopathic NS in children (2). Although most children undergoing their first episode of NS respond to standard steroid therapy, many have multiple relapses, a few have drug dependence with drug side effects and 5-10% are steroid resistant. Steroid-resistant NS is associated with a 50% risk of end-stage kidney failure and poor quality of life in childhood. An early non-invasive marker to predict

Address for Correspondence
Geetha Saradakutty, Department of Pediatrics, Government Medical College, Thiruvananthapuram, India
Phone: +91 04712528437 E-mail: geethapmohan@gmail.com ORCID: orcid.org/0000-0001-8905-9292
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steroid responsiveness would be helpful in better planning treatment, thereby resulting in fewer side effects of steroids.

Urine neutrophil gelatinase-associated lipocalin (uNGAL) is upregulated in cases of renal injury and acts as a highly sensitive, early biomarker for acute kidney injury (3). Also, higher urine and plasma NGAL levels are associated with disease severity and progression in chronic kidney disease (CKD). Since children with steroid resistant NS (SRNS) have a greater risk for progressive CKD, the urinary NGAL levels may be higher than in those with steroid sensitive NS (SSNS) (4). Acute kidney injury induces rapid upregulation of NGAL mRNA within the thick ascending limb of Henle's loop and the collecting ducts. Following this, the accumulation of NGAL in the distal nephron causes an increase in urine NGAL levels (5). Zhang et al. (6) concluded that NGAL is a better indicator than plasma creatinine and has a satisfactory early predictive value for acute kidney injury. NGAL increases rapidly in both serum and urine after kidney tissue damage (up to 1,000-fold).

The present study was conducted to determine if there was a correlation between urine NGAL and steroid responsiveness in children who were undergoing with first episode of idiopathic NS. Being able to predict steroid unresponsiveness based on uNGAL would help to plan early alternate treatment strategies.

Materials and Methods

This study was conducted to determine the levels of urine NGAL in the first episode of idiopathic NS and its relation with steroid responsiveness. All consecutive cases of first-episode idiopathic NS in patients aged 1 to 12 years admitted between January, 2019 and July, 2020 to a tertiary teaching institution were enrolled in this study. Out of the 85 cases, 79 children satisfying the criteria of NS as per ISPN Guidelines were enrolled (7). Children with congenital NS, secondary NS, concomitant urinary tract infections, acute kidney injury, CKD, or children on nephrotoxic medications were excluded from this study. The patients were evaluated for clinical and biochemical parameters including the presence of haematuria, hypertension, the severity of edema, levels of urine NGAL, urine protein creatinine ratio, serum albumin, and cholesterol levels. Urine samples were collected from patients in the early morning before the initiation of steroid therapy.

Figure 1 shows the flow chart used in this study. Urine NGAL was measured by a commercially available ELISA kit (Elabscience®, Houston, USA) which specifically detects human urine NGAL. This ELISA kit applies to the *in vitro* quantitative determination of human NGAL concentrations in serum, plasma, and other biological fluids. The specifications of this kit include Sensitivity: 0.10 ng/mL, Detection range: 0.16-10 ng/mL, Specificity: No significant cross-reactivity or interference between human NGAL and analogues was observed, and Repeatability: Coefficient of variation is <10%. In our study, the cut-off value of uNGAL was taken as 10 ng/mL based on previous studies (8,9).

Urine samples, collected in sterile containers, were centrifuged for 20 min and the supernatant was collected into clean tubes, aliquoted, and frozen at -20 °C until the time of urine NGAL assay. Samples were thawed and mixed thoroughly just before the assay to avoid erroneous results of repeated freeze/thaw cycles.

All children were followed up to assess their outcomes at 3 months. The children were assigned into subgroups based on their initial response to steroids. Kidney Disease Improving Global Outcomes (KDIGO) guidelines were followed for definitions (10).

Initial responder; Attainment of complete remission within the initial 4 weeks of corticosteroid therapy.

Initial non-responder/steroid resistance; Failure to achieve complete remission after 8 weeks of corticosteroid therapy.

Additionally, ISKDC data suggest that an absence of response to steroid therapy at 8 weeks indicates non-response (11), but a lack of response at the completion of 6 weeks often prompts many nephrologists to pursue renal biopsy.

According to ISPN guidelines, renal biopsy was carried out on all children who failed to attain remission by 4 weeks.

We classified the children into three groups based on their steroid response as:

Early responder; If the steroid response was obtained within the first two weeks.

Intermediate responder; When the steroid response was noted between 2-4 weeks.

Late responder; When the steroid response was obtained between 4 and 8 weeks.

Steroid resistant; When there was no steroid response even after 8 weeks.

Patients were treated as per the Indian Society of Paediatric Nephrology recommendations (7,12).

Institutional ethical clearance was obtained from Human Ethics Committee of Medical College, Thiruvananthapuram (approval no: 02/38/2019/MCT, dated on 16.01.2019), and

written informed consent was obtained prior to this study. Confidentiality was ensured and maintained throughout this study.

Statistical Analysis

Statistical analyses were performed using SPSS26. All quantitative variables are expressed as mean and standard deviation and qualitative variables as proportions. Groups were compared using non-parametric Fisher's exact test and p-values <0.05 were considered significant.

Results

Seventy-nine children were included in this study. The male to female ratio was 1.25:1. The mean age was 7.18 (±2.86) years. Out of these, 30.37% (n=24) were less than 5 years old, 56.96% (n=45) were between 5 and 10 years old, and 12.65% (n=10) above 10 years of age.

Urine NGAL was measured before starting steroids. All sixty-three children out of the seventy-nine (79.7%) who had urine NGAL ≤10 ng/mL responded to standard doses of steroids by 8 weeks. This shows that 100% of children with uNGAL ≤10 ng/mL had attained remission by 8 weeks. Out of the 16 children with NGAL >10 ng/mL, 56.3% (n=9) responded to steroids and attained remission within 8 weeks [either as intermediate (n=2) or late responders (n=7)] and 43.8% (n=7) did not show any response to steroids within the 8 weeks (SRNS). Fisher's exact test was performed and this showed that there was a statistically significant relation between NGAL value ≤10 ng/mL and increased steroid response and also between values >10 mg/dL and steroid unresponsiveness with a p-value <0.001. Table I shows the comparison of SSNS & SRNS.

91.13% (n=72) patients achieved remission within 8 weeks of steroid therapy (SSNS). Among these, 82.3% (n=65) attained remission within 4 weeks. In those who attained remission, 49.4% (n=39) attained remission within 2 weeks (early responders), 32.9% (n=26) attained remission within 2 to 4 weeks (intermediate responders) and 8.9% (n=7) attained remission after 4 weeks but within 8 weeks (late responders). However, 8.9% (n=7) of the children did not attain remission within 8 weeks (SRNS). Table II shows the patient characteristics for the low NGAL and high NGAL groups.

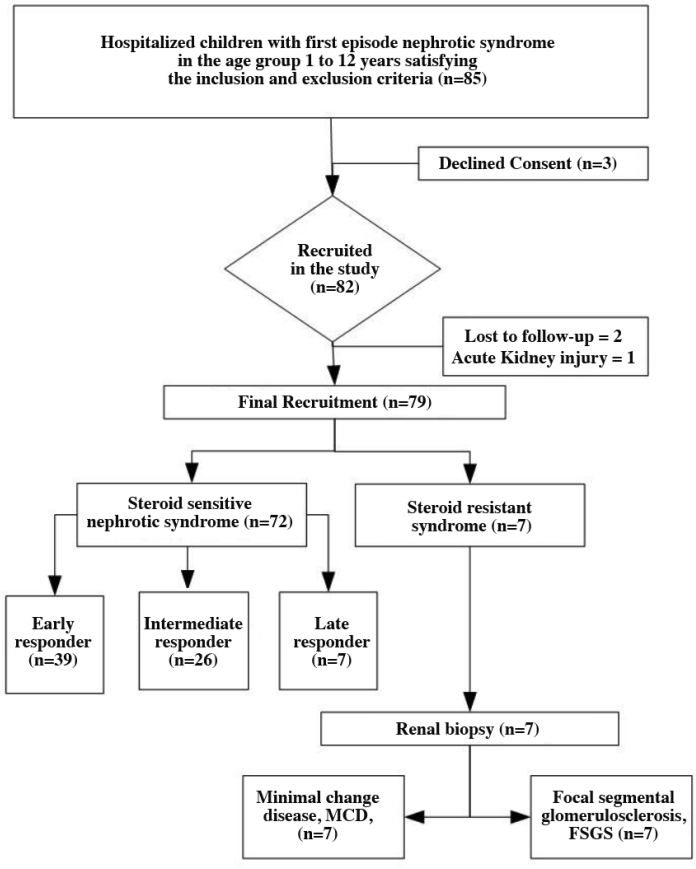

Figure 1. Flow of patients in the study

Table I. Comparison of steroid sensitive nephrotic syndrome & steroid resistant nephrotic syndrome

Parameter	SSNS (n=72) n (%)	SRNS (n=7) n (%)	p-value
Gender Male Female M:F ratio	 40 (55.6) 32 (44.4) 1.32:1	 4 (57.1) 3 (42.8) 1.33:1	 0.62
Family history (renal)	5 (6.9)	1 (14.2)	0.65
Edema Anasarca Pl effusion	 4 (5.5) 9 (12.5)	 0 1 (14.2)	 0.68 0.62
Hypertension	21 (29.1)	5 (71.4)	0.03*
Hematuria	18 (25)	5 (71.4)	0.02*
Allergy	11 (15.2)	1 (14.2)	0.71
Urine NGAL ≤10 ng/mL >10 ng/mL	 63 (87.5) 9 (12.5)	 0 7 (100)	 0.000*
Serum cholesterol <500 mg/dL ≥500 mg/dL	 60 (83.3) 12 (16.7)	 1 (14.2) 6 (85.8)	 0.008*
Serum albumin <2 g/L ≥2 g/L	 64 (88.8) 8 (11.2)	 5 (71.4) 2 (28.6)	 0.35
Biopsy MCD$^{\varsigma}$ FSGS	 --	 7 (50) 7 (50)	 --

*Statistically significant association between the study variable and SRNS
$^{\varsigma}$In 14 steroid resistant patients (Biopsy was done - 50% MCD and 50% FSGS)
NGAL: Neutrophil gelatinase-associated lipocalin, SSNS: Steroid sensitive nephrotic syndrome, SRNS: Steroid resistant nephrotic syndrome, FSGS: Focal segmental glomerulosclerosis, MCD: Minimal change disease

A renal biopsy was performed in those children (17.8%) who did not attain remission within 4 weeks as per institutional protocol. In the 14 children who underwent renal biopsy, minimal change disease was found in 50% (n=7) and focal segmental glomerulosclerosis (FSGS) was found in 50% (n=7). Of the late responders (n=7), 85.71% (n=6) were minimal change disease and 14.2% (n=1) was FSGS. Of the steroid resistant cases (n=7), 85.71% (n=6) had FSGS and 14.2% (n=1) had minimal change disease.

Discussion

NS is the most common renal disorder encountered in children and its course cannot be predicted for steroid resistance until at least after the 6th week of a steroid course. This usually paves the way for complications of steroid therapy without any clinical improvement for the patient. Renal biopsy usually arrives at a histopathological diagnosis, but it is an invasive procedure in children.

Table II. Patient characteristics between low NGAL and high NGAL groups

Parameters	NGAL≤10 ng/mL (n=63) n (%)	NGAL>10 ng/mL (n=16) n (%)	Signature
Steroid responsiveness <2 weeks 2-4 weeks 4-8 weeks >8 weeks	 39 (61.9) 24 (38.1) 0 0	 0 2 (12.5) 7 (43.8) 7 (43.8)	 <0.001*
Anasarca	4 (6.3)	0	0.39
Pleural effusion	8 (12.6)	2 (12.5)	0.67
Allergy	10 (15.8)	2 (12.5)	0.54
Hematuria	13 (20.6)	10 (62.5)	0.002*
Hypertension	13 (20.6)	13 (81.2)	<0.001*
Preterm	10 (15.8)	0	0.08
Biopsy done	0	14 (100)	<0.001*
Serum cholesterol <500 mg/dL ≥500 mg/dL	 31 (49.2) 32 (50.8)	 5 (31.2) 11 (68.8)	 0.02
Normal urea	46 (73.01)	8 (50)	0.07
Normal creatinine	34 (53.9)	9 (56.2)	0.77

*Statistically significant
NGAL: Neutrophil gelatinase-associated lipocalin

Moreover, in children, steroid responsiveness is a better prognostic marker for the disease course than renal biopsy. Therefore, if we had a non-invasive test for determining steroid responsiveness in the initial phase, we could personalize our treatment interventions in a child friendly manner. uNGAL is not only an early marker of acute kidney injury, but also a marker for progression of CKD and there is evidence in the literature about the use of this as a prognostic marker for steroid responsiveness. There are studies highlighting the role of uNGAL in SSNS, SRNS and normal children but no studies measuring uNGAL in the first episode before starting steroids. Therefore, this study was undertaken to determine whether urine NGAL could predict steroid responsiveness in the first episode of NS, which would help in the planning and monitoring of the treatment of idiopathic NS.

Among the 79 participants, 91.45% (n=72) achieved remission with steroid therapy by 8 weeks and the remaining 7 (8.9%) were steroid resistant (SRNS). In our study, 82% (n=65) of the children attained remission within the first 4 weeks of steroid therapy. This is similar to the International Study of Kidney Disease in Children (ISKDC) study and Indian study where 80% of children achieved clinical remission

within 4 weeks of corticosteroid therapy (11,12). In a study conducted by Mortazavi and Khiavi (13), 75.2% of patients responded to standard steroid therapy within 4 weeks.

In the sixteen children with an NGAL value >10 ng/mL, remission was attained in 56.3% (n=9) [intermediate (n=2) and late responders (n=7)] and 43.8% (n=7) did not attain remission. There is a significant relationship between NGAL >10 ng/mL and steroid unresponsiveness (p<0.001). In two recent studies, Bennett et al. (14,15) demonstrated the capacity of urine NGAL to predict the degree of response to steroid therapy in children with idiopathic NS, allowing health care professionals to discriminate between steroid-sensitive and steroid resistant children. There was a significant positive relationship between increasing uNGAL levels and the severity of disease, as measured by eGFR (14). In a study by Bennett et al. (15), urine NGAL is markedly increased in those patients with SRNS versus SSNS patients (in relapse or in remission of proteinuria), and versus healthy controls (p<0.001) and uNGAL also showed a high discriminatory power (AUC 0.91, p<0.0001) between SRNS and SSNS patients. In a study by Nickavar et al. (5), in 52 children with idiopathic NS (n=27 were steroid resistant; and n=25 were steroid responsive) aged from 1 to 16 years, urine NGAL was significantly higher in the steroid resistant patients in comparison to the steroid sensitive patients and they considered uNGAL to be a marker of steroid resistance in children with idiopathic NS.

Additionally, several previous studies have demonstrated that urine NGAL concentrations are not affected by age or gender in the paediatric population, lending support to the conclusion that the elevated urine NGAL levels seen in the SRNS children were not influenced by these factors (16-18).

In a study by Cangemi et al. (8), the calculated limits of blank (LOB) and detection (LOD) values were 0.5 ng/mL and 0.95 ng/mL, respectively. The distribution of uNGAL values approximated a log-normal distribution (median 5.2 ng/mL, interquartile range 2.5-12.8 ng/mL) (17). Another study by Bennett et al. (9) revealed a median of 6.6 ng/mL with IQR 2.8 to 17 ng/mL (8).

Nishida et al. (19) measured serum and urinary NGAL levels in children with renal diseases such as renal dysfunction (estimated glomerular filtration rate <90 mL/min 1.73 m^2), proliferative glomerulonephritis, steroid-resistant NS, steroid-sensitive NS, and tubular dysfunction.

They found that both serum and urinary NGAL levels showed significant inverse correlations with an estimated glomerular filtration rate in the analysis with all subjects, and also in the analysis with the renal dysfunction group. Additionally, in those patients with tubular dysfunction, the increase of the urinary NGAL level was remarkable compared with the other disease groups (19). This is in line with our hypothesis as SRNS is associated with a greater risk of progression and increased tubular damage, resulting in the excretion of low molecular weight proteins such as NGAL in urine. The elevated levels of urinary NGAL represent a "real-time" indicator of active inflammation and tubular injury with ongoing proteinuria (4,20,21).

Mishra et al. (22) showed that uNGAL had significant positive correlations with the duration of illness (r=0.342, p=0.006), the urine protein creatinine ratio (r=0.594, p<0.001), and a negative correlation with serum albumin (r=0.470, p<0.001) and their conclusion was that the uNGAL/creatinine level correlated with the activity of the disease and it can distinguish not only SRNS from SSNS, but also FSGS and minimal change disease histopathological sub-types of SRNS in children. In our study, all children had cholesterol values above 200 mg/dL. Among the 18 children who had serum cholesterol ≥500 mg/dL, 31.2% (n=6) did not achieve remission, whereas 68.8% (n=11) attained remission. There was a statistically significant association between very high serum cholesterol ≥500 mg/dL and steroid unresponsiveness (p=0.008). This is similar to the observations made by Krishanamurthy et al. (23) that serum cholesterol in SRNS cases shows statistically significant elevation compared to other types.

In the study group, haematuria was present in 27% (microscopic) and 73% of the children did not have haematuria. This is similar to a study carried out by the international kidney disease foundation which showed that microscopic haematuria can present in 20% of cases, while macroscopic haematuria is rare in idiopathic NS (11). There is a statistically significant relation between haematuria and steroid unresponsiveness with a p-value of 0.002. A similar observation was made in a study conducted by Mortazavi and Khiavi (13) where patients with SRNS had a higher frequency of haematuria (p=0.001) and higher mean age (p=0.017) compared with the SSNS group.

Among our study group, 33% of the children were found to have hypertension at admission, whereas 67% of children had normal blood pressure. In a study conducted by Tapia and Bashir (24), moderate arterial hypertension was present in 25% of cases. In our study, of the 26 children who had hypertension, 21/72 (29%) were in the steroid sensitive group and 5/7 (71%) were in the steroid resistant group. A statistically significant association between the presence of

hypertension and steroid unresponsiveness was observed with a p-value of 0.03. A similar study conducted by Manasa et al. (25) also showed a significant association between hypertension and steroid unresponsiveness with a p-value of 0.0001.

Study Limitations

Our study was from a single centre, with a short-term follow-up of 3 months. Additionally, we did not follow-up these children to find out whether those with initial steroid response developed steroid resistance in subsequent relapses. This study was performed with an ELISA kit with a detection range of 0.1 to 10 ng/mL only. Values above 10 ng/mL could not be measured as a quantitative figure in our study, which was a major limitation.

Conclusion

A single measurement of urine NGAL in the first episode of NS before starting treatment helps to predict steroid responsiveness. Higher urine NGAL levels (>10 ng/mL) are seen in late steroid responders and steroid-resistant NS. Larger cohort studies with a longer duration of follow-up are required to objectively assess the role of urinary NGAL levels in the first episode in predicting the course of idiopathic NS.

We could use this study model to determine whether higher NGAL levels predict steroid responsiveness in the initial phase but could not utilize it as a marker for difficult NS including frequently relapsing NS or steroid dependent NS, since we only followed up the children for 3 months. The quantitative estimation, though costlier, may provide better understanding. But, screening by a semi-quantitative (cut-off value of uNGAL being 10 ng/mL) test as used in this study may be useful in resource-limited centres. Although the gold standard is renal biopsy, it is an invasive procedure. Therefore, the utility of markers such as urine NGAL coupled with predictive clinical variables such as hypertension and haematuria may provide the physician with valuable information. A more child-friendly personalized evaluation and subsequent treatment strategies can be planned based on screening tools such as urinary uNGAL.

Ethics

Ethics Committee Approval: Institutional ethical clearance was obtained from Human Ethics Committee of Medical College, Thiruvananthapuram (approval no: 02/38/2019/MCT, dated on 16.01.2019).

Informed Consent: Written informed consent was obtained prior to this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.J., S.U., G.S., Concept: G.P., S.J., Design: G.P., S.U., G.S., Data Collection or Processing: G.P., S.U., Analysis or Interpretation: G.P., S.J., S.U., G.S., Writing: G.P., S.J., G.S.

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