

Association of Neutrophil Gelatinase Associated Lipocalin and Cystatin-C with Kidney Function in Children with Nephrotic Syndrome

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ABSTRACT

Background: Nephrotic syndrome (NS) is a major clinical concern in human health, especially in children. Despite of the etiology, the prediction of remission in different treatment regimens based on suitable biomarkers is under development. The goal of this evaluation was the demonstration of correlation between serum level of Neutrophil gelatinase associated lipocalin (NGAL) and cystatin-C with kidney function in patients with NS.

Methods: During the period between September 2008 and December 2011, 52 patients admitted to St. Al Zahra University Hospital were selected for evaluation. The measured parameters consisted of NGAL, cystatin-C, creatinine, albumin, blood urea nitrogen, urine protein, glomerular filtration rate. Demographic data were collected and considered in comparisons. Comparison between variables and their correlations were examined.

Results: Means of serum NGAL and cystatin-C were significantly higher in case than the control group, $P < 0.05$. The mean of serum NGAL in patients without remission and who achieved remission were 23.09 (standard deviation [SD] ± 10.11) and 36.26 (SD ± 20.10) ng/ml respectively; $P < 0.05$. Serum NGAL levels had a correlation with the following factors: Systolic blood pressure, diastolic blood pressure (DBP), cystatin-C, remission. Linear regression analysis showed a significant correlation between cystatin-C and systolic and DBP.

Conclusions: Based on the results, serum NGAL can be used as a prognostic marker for remission. In addition, NGAL and cystatin-C are biomarkers of kidney injury in NS.

Keywords: Children, cystatin-C, glomerular filtration rate, Nephrotic syndrome, Neutrophil gelatinase associated lipocalin

INTRODUCTION

Nephrotic syndrome (NS) is characterized by massive proteinuria, hypoalbuminemia, edema and hyperlipidemia with 2-3/100,000 annual incidences. Steroid has a main role in treatment, but patients who do not have appropriate response to steroid, have a greater chance to attain end stage renal

disease (ESRD).^[1,2] Resistant NS has been widely accepted as an increasing etiology of ESRD.^[3,4] Therefore, detecting early stages of chronic kidney diseases (CKD) has greatest importance in treating patients at risk.^[5-7] The available standard biochemical markers are not sensitive enough to determine high-risk patients.^[8] However, a variety of factors including response to steroid, hypertension, extent of glomerular sclerosis and interstitial fibrosis have been accepted as prognostic markers of getting ESRD.^[9,10] Recently, new biomarkers have been introduced to define patients at risk.^[11-13] Among these biomarkers, Neutrophil gelatinase associated lipocalin (NGAL) and cystatin-C have greatest significance.^[14-20] There is few data regarding the role of NGAL and cystatin-C in children with NS.^[21] In this study, we evaluated serum NGAL and cystatin-C in both Steroid Sensitive Nephrotic syndrome (SSNS) and steroid resistant Nephrotic syndrome (SRNS) children. In addition, we evaluated any possible correlation between these two biomarkers and response to steroid.

METHODS

In this cross-sectional study, 104 participants aged 1-18 years were enrolled i.e., 52 cases and an equal number of control. Since normal value for these two parameters in normal children is varied widely, a control group was enrolled to compare the results. Cases were selected from children with idiopathic NS who had been hospitalized from September 2008 to December 2011 in the only referral Pediatric Nephrology Department, affiliated to Isfahan University of Medical Sciences, Isfahan, Iran. Histopathologic findings mostly consisted of minimal change disease and or focal segmental glomerular sclerosis (FSGS). The histopathologic diagnosis of FSGS was based on the following criteria: (i) A lesion affecting some of the glomeruli in the renal biopsy while others remain unaffected and (ii) the affected glomeruli having a portion that has undergone capillary collapse with obliteration of capillary lumina with or without adhesions.

Control group was selected from age- and sex-matched children who had been referred to pediatric clinics for routine health examination. The demographic, clinical and laboratory data were recorded for all participants. These data for

case group comprised of height, weight, age at the onset of disease, 24 h urinary protein excretion, serum albumin, blood urea nitrogen (BUN), serum creatinine, serum NGAL, serum cystatin-C, glomerular filtration rate (GFR) (based on both updated Schwartz equation and new cystatin-C based Schwartz formula)^[22-25] systolic and diastolic blood pressure (DBP) and medications record. Data was updated regularly every 3-6 months, during the follow-up visit for each patient. Data sheet for the control group consisted of demographic data, BUN, serum albumin, serum creatinine, serum NGAL, serum cystatin-C, GFR and systolic and DBP.

Serum cyclosporine-A level was measured by ELISA method (Abnova-USA) in patients to follow its toxic concentration.

Serum creatinine and BUN were measured by enzymatic methods on an auto-analyzer instrument (Hitachi 917, Japan). Serum NGAL was measured by ELISA method (Bioproto Kit). Serum cystatin C was measured by particle-enhanced immunoturbidimetric method (Dako, Glostrup, Denmark).^[26] A cystatin C level of lower than 1.38 mg/l is suggested as normal in the general population.^[27,28]

To calculate GFR for each subject, we applied the following equations:

The updated 2009 Schwartz equation:^[24]

$$\text{GFR (ml/min/1.73 m}^2\text{)} = 0.413 \times \text{Height (cm)/Serum Creatinine (mg/dl)}$$

The new 2009 Schwartz equation:^[22,23]

$$\begin{aligned} \text{GFR (ml/min/1.73 m}^2\text{)} &= 39.1 \\ &\times (\text{Height (m)/Creatinine (mg/dl)})^{0.516} \\ &\times (1.8/\text{Cystatin C (mg/L)})^{0.294} \\ &\times (30/\text{BUN(mg/dl)})^{0.169} \\ &\times (1.099)^{\text{if male}} \times (\text{Height (m)/1.4})^{0.188} \end{aligned}$$

Definitions

CKD was defined as GFR lower than 90 ml/min/1.73 m²; Hypertension was defined as blood pressure higher than 95th percentile for age and height according to data from the task force report on high blood pressure in children and adolescents.^[29] Response to treatment was classified as complete remission, partial remission and non-remission: Complete remission was defined

as negative or trace proteinuria adjusted by body surface area $<350 \text{ mg/m}^2/\text{day}$. Partial remission was defined as a reduction in proteinuria, but still remaining in the supranormal range. Non-responder (SRNS) was considered as the inability to induce remission and/or partial remission within 4 weeks of daily steroid therapy following by three pulses of methylprednisolone. SSNS was defined as remission after 4-6 weeks of full dose of steroid therapy.^[1,29]

Treatment protocols

All patients underwent similar initial treatment strategy. They were initially treated with oral prednisolon ($60 \text{ mg/m}^2/\text{day}/4$ to 6 weeks), with or without three consecutive doses of methyl prednisolone pulses ($10\text{-}30 \text{ mg/kg/dose}$). The medication was then tapered in an alternate-day regimen over 6-9 months.

Patients were categorized into following subgroups: (1) subgroup A: those who were SSNS and remained steroid sensitive until the end of the study, (2) subgroup B: those who were SSNS and gradually turned to SRNS, (3) subgroup C: those who were SRNS and remained steroid resistant until the end of the study.

Patients who responded partially to prednisolon or demonstrated steroid side-effects received cyclophosphamide ($2\text{-}3 \text{ mg/kg/day}$ for 2 to 3 months). For those patients who did not respond to mentioned medications, cyclosporine-A ($3\text{-}5 \text{ mg/kg/day}$) was commenced after performing kidney biopsy. In cases that did not respond to cyclosporine A in a 6 month course, mycophenolate mofetil ($500\text{-}1000 \text{ mg/m}^2/\text{day}$) was replaced. Angiotensin-converting enzyme inhibitor (ACEI) was added as an adjuvant therapy to control hypertension or proteinuria.

Statistical analysis

Statistical analyses were performed using the software (SPSS: An IBM Company, version 16.0, IBM Corporation, Armonk, New York, USA). The categorized data were reported as frequencies and percentages. Continuous data were reported as mean and standard deviation.

Pearson correlation test was used to control the association between demographic parameters. Student *t*-test was performed to comparing the means between biochemical factors and GFR.

Linear regression test was applied to examine the correlations between quantitative variables. *P* value of <0.05 was considered as significant.

Ethics

This study was approved by the Research and Ethics Committee of Isfahan University of Medical Sciences. Written informed consent from all parents and oral assent from children were obtained. This study was performed in accordance with the ethical standards of the Helsinki declaration.

RESULTS

Overall, 104 participants (52 cases and 52 controls) were enrolled. Male to female ratio in case group was 2/1. This ratio was similar in the control group.

The means of age were not significantly different in both groups. The body mass index (BMI) was not different between groups, $P = 0.42$. Systolic blood pressure (SBP) was significantly higher in case than the control group; $100.76 \pm 13.59 \text{ mmHg}$ versus $95.57 \pm 8.49 \text{ mmHg}$, respectively, $P = 0.021$. Means of DBP were $66.25 \pm 12.73 \text{ mmHg}$ in the case and $57.78 \pm 7.16 \text{ mmHg}$ in the control group, $P < 0.0001$. Demographic parameters including age, height, weight, SBP, DBP and BMI are pointed out in Table 1.

The mean for serum albumin, serum NGAL, serum cystatin-C, BUN, creatinine and GFR estimated by new Schwartz formula were significantly different in case from the control group [Table 2].

Patients' medications were categorized to the following regimens including prednisolone

Table 1: Clinical and demographic features of the participants

Demographic findings	Case (52) mean±SD	Control (52) mean±SD	P value
Age (year)	10.4±3.73	9.23±2.9	0.2
Height (cm)	134.14±18.6	126±15.07	0.03*
Weight (kg)	37.14±17.09	33.11±10.45	0.15
SBP (mm Hg)	100.76±13.59	95.57±12.73	0.02*
DBP (mm Hg)	66.25±12.73	57.78±7.16	0.0001*
BMI (kg/m ²)	19.46±4.72	20.09±3.10	0.42

* $P < 0.05$ with 95% confidence interval. SBP=Systolic blood pressure, DBP=Diastolic blood pressure, BMI=Body mass index, SD=Standard deviation

(51.3%), prednisolone/cyclosporine-A (47.5%), prednisolone/cyclosporine-A/cellcept (9.6%) and one of the above regimen + ritoximab (7.8%).

Mean serum level of cyclosporine was 25.65 ± 22.54 and no one found in toxic level (>450 ng/ml).^[30,31]

There was no difference in either NGAL or cystatin-C among three case-subgroups; $P > 0.05$ [Table 3].

The means of serum NGAL in patients without remission and who achieved remission were 23.09 ± 10.11 and 36.26 ± 20.10 ng/ml respectively, $P = 0.004$.

Nonetheless, there was no significant difference between serum NGAL in patients with or without fibrosis; 27.39 ± 15.7 ng/ml and 22.80 ± 8.35 ng/ml respectively; $P = 0.17$.

Table 2: Laboratory findings of the participants

Biochemical markers	Case (52) mean±SD	Control (52) mean±SD	P value
Cr (mg/dl)	0.54±0.24	0.44±0.99	0.0001*
BUN (mg/dl)	21.44±7.39	13.63±2.66	0.0001*
Cystatin (mg/ml)	0.96±0.4	0.53±0.1	0.0001*
Alb (g/dl)	4.34±0.52	4.83±0.24	0.0001*
NGAL (ng/ml)	25.62±13.45	12.86±3.77	0.0001*
GFR ^a (ml/min/1.73 m ²)	186.91±87.31	188.05±61.53	0.0001
GFR ^b (ml/min/1.73 m ²)	90.54±27.11	115.98±18.17	0.0001*

Cr=Creatinine, BUN=Blood urea nitrogen, SD=Standard deviation, NGAL=Neutrophil gelatinase associated lipocalin. ^aCreatinine-based glomerular filtration rate, ^bCystatin-based glomerular filtration rate. * $P < 0.05$ with 95% confidence interval

Table 3: Serum biomarkers levels in case subgroups

Biomarker	Subgroup A	Subgroup B	Subgroup C	Significance
Cystatin-C (mg/ml)	0.98±0.54	0.96±0.31	0.92±0.21	*Not significant
NGAL (ng/ml)	24.47±12.05	27.86±13.14	26.98±18.78	*Not significant

* $P < 0.05$ with 95% confidence interval was significant. NGAL=Neutrophil gelatinase associated lipocalin

Table 4: Correlation among biomarkers and biochemical parameters in case group

Biomarker	Cystatin-C	NGAL	Albumin	Urine protein	Cyclosporin level	GFR Cr-based	GFR cystatin-based	SBP	DBP	BMI
Cystatin-C (mg/ml)	-	0.418*	-0.023	-	0.07	-0.359*	-0.673*	0.418*	0.291*	0.476*
NGAL (ng/ml)	0.418*	-	-0.212	0.488*	0.23	0.024	-0.189	0.341*	0.410*	-

*Asterisk means that two variables have good correlation with $P < 0.05$. NGAL=Neutrophil gelatinase associated lipocalin, GFR=Glomerular filtration rate, SBP=Systolic blood pressure, DBP=Diastolic blood pressure, BMI=Body mass index

Considering serum cystatin-C, no significant difference was shown between patients with and without fibrosis, 0.96 ± 0.41 mg/ml and 0.96 ± 0.38 mg/ml respectively; $P = 0.99$. In addition, there was not any significant difference between patients who achieved or did not achieve remission, 0.92 ± 0.31 mg/ml versus 1.1 ± 0.65 mg/ml respectively; $P = 0.32$.

Bivariate correlation coefficient test showed that SBP had significant positive correlations with BMI and cyclosporine levels; $r = 0.75$, $P = 0.0001$ and $r = 0.33$, $P = 0.014$ respectively. The same positive correlation was true for DBP.

Applying bivariate correlation showed that serum cystatin-C level had significant positive correlation with the following factors; serum NGAL: $r = 0.42$, $P = 0.002$; SBP: $r = 0.425$, $P = 0.002$; DBP: $r = 0.29$, $P = 0.03$ [Table 4]. However, significant positive correlation was found between serum NGAL and the followings; SBP: $r = 0.34$, $P = 0.013$; DBP: $r = 0.43$, $P = 0.001$; remission: $r = 0.39$, $P = 0.002$ and cystatin-C as the above [Table 4]. Nevertheless, serum NGAL level had a negative correlation with GFR based cystatin-C: $r = -0.358$, $P = 0.0001$.

Regression analysis (linear method) demonstrated that serum NGAL had significant correlation with DBP and serum cystatin-C levels; $P = 0.05$ and $P = 0.009$ respectively.

Applying regression analysis showed that serum cystatin-C levels had significant correlation with the following factors; SBP: $P = 0.008$ and NGAL: $P = 0.009$.

DISCUSSION

NS as one of the most frequent glomerular disease in children may result in ESRD.^[2] SRNS

patients are at higher risk to develop ESRD. To determine the patients at risk, a variety of predisposing factors have been introduced, such as genetic mutations, response to steroid, severity of interstitial fibrosis and glomerular sclerosis.^[1,8] Since diagnosis of steroid-resistant patients help physicians to choose the best modalities, finding new predictor biomarkers is mandatory.^[32] In this study, we evaluated two novel biomarkers (NGAL, cystatin-C) in SSNS and SRNS.

Cystatin-C, a low molecular weight protein of cysteine protease family is produced by all nucleated cells and catabolized by proximal tubules.^[17] Using a meta-analysis, Dharnidharka *et al.*, showed that cystatin-C had superiority to creatinin in estimating GFR.^[28] Cystatin-C has been widely accepted as a predictor biomarker in kidney and cardiovascular diseases.^[5,33] In addition, serum cystatin-C has been introduced as a more sensitive biomarker than serum creatinin in predicting renal dysfunction in primary NS.^[27,28] Indeed, this marker may reflect tubular injury in NS. It is demonstrated that urine cystatin-C levels was higher in NS with relapses.^[28,34] Although, we did not assess the relapses, there was no significant difference between serum cystatin-C in patients with and without remission.

While GFR based serum cystatin-C was significantly lower in case comparing with the control group, it was not significantly different among sub-groups of case group. In addition, there was no significant difference in GFRs between those who achieved remission and those who did not.

We found that GFR based on either serum creatinin and or cystatin-C is higher in control than in case group. In addition, cystatin-C based GFR was lower than creatinin based GFR in case group. It asserted the previous claim that cystatin-C was a marker of early renal dysfunction.^[27,28,35] However, we did not demonstrate any difference between cystatin-C levels in patients with or without fibrosis. Furthermore, cystatin-C could not differentiate between patients with and without remission.

Although, proteinuria might effect on urine cystatin-C levels, hypoalbuminemia had no effect on serum cystatin-C.^[36,37] In our study, applying regression analysis did not show any correlation between serum albumin and serum cystatin-C levels. Furthermore, in accordance with the

previous studies, we did not find any correlation between corticosteroid consumption and serum cystatin-C levels.^[38]

NGAL has been introduced as a new risk marker of acute kidney injury and a predictive marker of CKDs.^[32] Increased urine NGAL levels in FSGS patients comparing with the control group have been shown.^[26,39,40] In addition, in diseases related to proximal and distal tubules and glomerular filtration barrier, NGAL levels may be useful for observing the response to treatment.^[41,42]

In a recent study on NS, Bennett *et al.*, showed that that median NGAL was significantly higher in SRNS than both SSNS and healthy controls.^[21] Consistent with Bennett *et al.*'s study, the higher amount of serum NGAL was detected in SRNS comparing with both SSNS and control group [Table 3].

Urine NGAL has been demonstrated as an early marker of tubulointerstitial impairment in CKDs.^[43] Animal studies revealed that serum NGAL had a higher level in rats with kidney fibrosis. However, prescribing ACEI dramatically decreased serum NGAL levels.^[44]

In this study, comparing serum NGAL levels between patients with and without fibrosis showed that serum NGAL was not significantly different in both groups. Although, regression analysis demonstrated a significant correlation between serum NGAL and remission ($P = 0.037$), no correlation was found between serum NGAL levels and severity of fibrosis. Nonetheless, consuming ACEIs by most of our patients may interfere with interpreting the results.

Increased serum level of NGAL has been described in chronic renovascular hypertension perhaps a result of kidney injury or ischemia.^[45] Blumczynski *et al.*, showed that in children with primary hypertension and kidney injury had higher levels of serum NGAL.^[46]

In concert with the above studies, we reported that serum NGAL levels had a positive correlation with systolic and diastolic hypertension.

Whilst toxic levels of cyclosporine, A may increase serum NGAL, no patient in our study had toxic cyclosporine level.^[31] Therefore, we did not find any correlation between toxic levels of cyclosporine-A and serum NGAL levels.

Our study revealed that serum NGAL had only a significant correlation with remission ($P = 0.004$).

Therefore, higher serum NGAL levels may be a risk factor for diagnosis patients who did not achieved remission.

Urine NGAL levels had significant correlation with urine protein and inverse correlation with GFR.^[32] Nishida *et al.*, showed significant inverse correlations between both serum and urinary NGAL levels with GFR.^[47] In consistent with these studies, we demonstrated that serum NGAL level had a positive correlation with serum cystatin-C and negative correlation with GFR based cystatin-C. Furthermore, we found that serum NGAL had no correlation with serum albumin.

It is suggested to evaluate a large number of patients categorized based on various underlying NS.

CONCLUSION

Serum NGAL is a novel biomarker that can predict the remission in NS patients. In addition, serum cystatin-C and GFR based cystatin-C might be used as markers of early prediction of kidney injury in NS. Considering the obtained data, it seems the major application of this study may be lead to shortening course of disease and hospitalization period as well as a reduction of cost of treatment and patient family stress.

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