

# Evaluation of Urinary Neutrophil Gelatinase-associated Lipocalin as a Biomarker in Pediatric Vesicoureteral Reflux Assessment

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**Introduction.** Defining a non-invasive marker to predict the risk of kidney damage is very helpful to manage vesicoureteral reflux (VUR) in children. This study aimed to investigate the predictive value of urinary neutrophil gelatinase-associated lipocalin (uNGAL) as a non-invasive biomarker of reflux nephropathy in pediatric with primary VUR.

**Methods.** This case-control study was performed on 63 children with primary VUR and 72 age- and sex-matched healthy children as controls. Urinary NGAL and creatinine levels were measured and compared between patients and control groups as well as among the subgroups of patients in terms of disease severity.

**Results.** Among patients, 9 cases were diagnosed as mild, 35 as moderate, and 19 with a severe form of VUR. We observed higher but insignificant levels of uNGAL in patients versus healthy controls ( $P > .05$ ), in cases with severe form compared to mild and moderate forms of VUR ( $P > .05$ ) as well as in those patients with bilateral versus unilateral involvement ( $P > .05$ ). The mean ratios of uNGAL/uCr were significantly higher in patients group than controls ( $P < .05$ ) as well as in patients with severe form compared to moderate and mild forms of VUR ( $P < .05$  and  $P < .05$ , respectively). Also, ROC analysis revealed the sensitivity of 61% and specificity of 53% for uNGAL/uCr ratio for prediction of VUR.

**Conclusion.** Our findings indicate a potential predictive value of uNGAL/uCr ratio as a non-invasive biomarker for the management of VUR although, its clinical application has been challenging and needs to be confirmed by further investigations.

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## INTRODUCTION

Vesicoureteral reflux (VUR) is the most common congenital risk factor for urinary tract infection (UTI) in children.<sup>1-4</sup> The incidence of primary VUR is 1.0% in the normal population and 30 to 50% in pediatric with recurrent UTI.<sup>5,6</sup>

Renal scarring may develop in children with reflux-related urinary tract infection and it leads to reflux nephropathy. Scars in these patients

could also be caused by developmental failures during the fetal period and by postnatal urinary tract infections, which the latter is acquired and preventable. Early diagnosis along with appropriate imaging and treatment have been emphasized as effective attempts to reduce the incidence and severity of renal scarring. Generally, radiological findings and laboratory tests are used for diagnosis and monitoring of kidney injury<sup>3,7,8</sup> whereas,

diagnosis of reflux nephropathy often requires imaging studies. Ultrasonography as a non-invasive method but with non-specific results can be considered beside the  $^{99m}\text{Tc}$ -dimercaptosuccinic acid (DMSA) renal scintigraphy as a standard but invasive and costly procedure.<sup>9,10</sup>

In this context, defining and implementation of a non-invasive and precise method to predict the risk of kidney parenchyma damage could be very helpful in the clinical settings. Preclinical studies in the rat models have shown an up-regulation of neutrophil gelatinase-associated lipocalin (NGAL) gene expression within two weeks post pyelonephritis that begins to decrease by 4 to 6 weeks but never returns to the baseline level.<sup>11</sup>

NGAL also called human neutrophil lipocalin or uterocalin is a member of lipocalins family which is secreted from activated neutrophils and mediates several physiologically (e.g. apoptosis and organogenesis) and pathologically processes in different conditions.<sup>11,12</sup> This secretory protein is expressed physiologically at very low levels in trachea, stomach, lungs, kidneys and colon tissues in healthy subjects. NGAL is secreted into the urine by the thick ascending limb of the loop of Henle and collecting ducts of the kidney and its expression is induced in the injured tubular or epithelial cells of the kidney.<sup>11</sup> For this reason, urinary NGAL (uNGAL) has been investigated as a non-invasive biomarker for evaluation of either acute or chronic kidney injuries and even UTI.<sup>12</sup> The results of a pilot study have demonstrated that the children with ureteropelvic junction obstruction (UPJO) had increased urinary kidney injury molecule-1 (KIM-1) and NGAL levels.<sup>13</sup> Moreover, a recent study by Nickavar *et al.* on a group of Iranian children with or without primary VUR demonstrated that urinary NGAL/Cr ratio can be considered as a sensitive, specific, and more accurate biomarker to diagnose primary VUR in pediatric.<sup>14</sup> Alternatively, a prospective controlled observational study showed that the NGAL/Cr and KIM-1/Cr ratios were effective urinary diagnostic markers for febrile UTI<sup>15</sup> and a meta-analysis demonstrated that NGAL has excellent potential for diagnosis as well as monitoring and prognosis of acute kidney injury.<sup>16-18</sup>

The present study was designed to further investigate this correlation and to determine the predictive value of uNGAL as a non-invasive

marker for reflux nephropathy in pediatric with primary VUR.

## MATERIALS AND METHODS

This cross sectional study with a comparison control group was conducted on children with primary VUR who were diagnosed based on bottom-up approach protocol (all patients with recurrent febrile UTI underwent nuclear voiding cystourethrography) in the Department of Pediatric Nephrology at a University hospital between March 2017 and December 2017. The study was approved by our institutional research ethics committee (No. IR.UMSHA.REC.1396.422) and all parents provided a signed and approved written informed consents before enrollment of their children in this study. During the study, 85 patients aged two months to 12 years with VUR who were referred to the pediatric nephrology clinic were evaluated. All cases had VUR based on nuclear voiding cystourethrography (RNC). Among all cases, 22 cases were excluded since they did not refer for follow up and finally 63 cases were recruited for evaluations. Also, 72 age- and sex-matched healthy children who were referred to the university health care centers for annual health care monitoring were included as control group. None of the cases in both groups had urinary tract infection concurrently or in the past six months, based on their medical history as well as urine culture examinations.

In order to rule out obstructive diseases and secondary vesicoureteral reflux, patients underwent ultrasonography and those cases who had abnormal ultrasonography underwent Voiding Cystourethrography (VCUG) and Intravenous Pyelogram (IVP). Children with normal ultrasonography or abnormal ultrasonography but without obstruction according to VCUG and IVP results were entered to our study. Also, patients with known congenital or acquired immunodeficiency, metabolic disorder and recent surgical manipulations of the kidneys, ureters and bladder were excluded from the study.

Urinary reflux was classified as mild (grade1), moderate (grade2 and 3), and severe (grade4 and 5) based on the return of radiotracer into the ureter during nuclear voiding cystourethrography.<sup>19</sup> All patients underwent DMSA scan to detect scar formation at least six months after the last episode of UTI.

### Samples Collection and Laboratory Measurements

The first morning voided urine samples were obtained from all study subjects for analysis of urine creatinine (Cr) and NGAL levels. Urine samples with pyuria were omitted from the analysis. The samples were collected in sterile polypropylene containers. One-milliliter aliquots were centrifuged at 4000 ×g for 10 minutes and the supernatant fractions were stored at -80 °C until analysis of biomarkers. Also, urine samples were tested for the presence of blood or leukocytes by using microscopic analysis and samples containing blood or leukocytes were excluded. Measurement of urine creatinine levels (mg/dL) in the fresh urine samples was performed by CREA Creatinine Jaffe method compensated (Roche Diagnostics, Mannheim, Germany). Urinary NGAL levels (ng/mL) were determined using quantitative Enzyme-Linked Immunosorbent Assay (ELISA) kits (Human NGAL Lipocalin-2/NGAL ELISA kit; Bio Vendor Diagnostic, Brno, Czech Republic) according to the manufactures' instructions. Additionally, the ratio of uNGAL/uCr (ng/mg) was calculated for all samples. Urinary levels of biomarkers were expressed as the ratio of uNGAL to urinary creatinine.

### Statistical Analysis

To compare the quantitative variables across two groups, independent samples t-test was used. To assume to perform t-test, we used the logarithmic scale of the quantitative data. Also, analysis of variance (ANOVA) with post hoc Tukey test was used to compare more than two groups. We used receiver operating curve (ROC) and area under curve (AUC) analysis as a measure of the ability of uNGAL and/or uNGAL/uCr ratio to

discriminate healthy controls from the patients group. We also implemented an artificial neural network (ANN) model<sup>20-22</sup> for a better comparison of age/sex-adjusted uNGAL and/or uNGAL/Cr ratio between the study groups. Given a set of independent variables (such as age, sex and uNGAL/Cr), ANN can determine nonlinear relation between independent and output variables. All computation was performed using SPSS version 22.0 and the  $P < .05$  was considered significant.

### RESULTS

Totally, 135 cases including 63 patients (13 males and 50 females) and 72 healthy children (25 males and 47 females) were enrolled in this study. The age range of the control group was 6 to 144 months ( $49.6 \pm 41.7$ ) and for the patients group was 2 to 132 months ( $59.1 \pm 34.7$ ) ( $P > .06$ ). Considering the bilateral or unilateral forms of VUR, 12 patients had unilateral and 51 had bilateral VUR. Moreover, based on the severity of the disease, 9 cases were diagnosed as mild, 35 as moderate, and 19 with a severe form of VUR. All 63 patients underwent DMSA scan examination at least 6 months after the last febrile UTI, of which, 31 (8 males and 23 females) had a renal scar and the remaining 32 cases had normal DMSA scan. Out of 8 male patients with a renal scar, four had severe bilateral VUR, two cases had severe unilateral VUR, and other two cases showed moderate unilateral VUR. Among 23 female patients with a renal scar, 4 severe bilateral, 15 moderate bilateral, and two moderate unilateral VUR were diagnosed and two cases were surgically treated.

Comparison of the mean and median levels of uNGAL and uNGAL/uCr ratio are shown in Table.

Comparison of the Urinary NGAL levels and uNGAL/uCr Ratios Between the Study Groups

Groups	n	uNGAL			uNGAL/uCr		
		Mean (SD)	Median	P	Mean (SD)	Median	P
Controls	72	3.21 (4.41)	1.26	> .05	0.07 (0.13)	0.02	< .05
Patients	63	7.10 (15.78)	2.00		0.20 (0.41)	0.04	
Severity							
Mild	9	3.59 (6.64)	1.49	> .05	0.05 (0.08)	0.03	< .05
Moderate	35	4.86 (7.18)	2.00		0.11 (0.17)	0.05	
Sever	19	12.89 (26.7)	2.67		0.43 (0.66)	0.05	
Lateral							
Unilateral	12	4.73 (8.52)	2.08	> .05	0.07 (0.11)	0.04	> .05
Bilateral	51	7.66 (17.07)	2.00		0.23 (0.45)	0.05	

Note: All comparisons were performed by independent sample t-test as well as ANOVA with LSD as a post hoc test based on the logarithmic scales of the data.

Abbreviations: uNGAL, urinary neutrophil gelatinase-associated lipocalin; uCr, urinary creatinine.

We observed higher but insignificant levels of uNGAL in the patients group versus healthy controls ( $P > .05$ ), in the cases with severe form compared to mild and moderate forms of VUR ( $P > .05$ ), as well as, in those patients with bilateral versus unilateral involvement ( $P > .05$ , Table). Comparison of the mean ratios of uNGAL/uCr between study groups revealed the significant differences between patients and controls ( $P < .05$ ) and between the patients subgroups according to the severity of disease ( $P < .05$ ) which the later was more evident between severe and mild forms of VUR ( $P < .05$ , Table). There were no statistical differences for the mean levels of uNGAL ( $P > .05$ ) and uNGAL/uCr ratios ( $P > .05$ ) between the patients with ( $n = 31$ ) and without ( $n = 32$ ) a renal scar.

uNGAL/uCr ratio for prediction of VUR were calculated by ROC analysis. Discrimination of the patients group from healthy controls by considering the quantitative data in ROC analysis revealed that the definition of a cut-off point value of 0.03 for uNGAL/uCr ratio showed 61.0% sensitivity and 53.0% specificity to predict VUR (Figure). A similar analysis by considering a cut-off point value of 1.33 for uNGAL levels showed 64% sensitivity and 54% specificity for this biomarker (Figure).

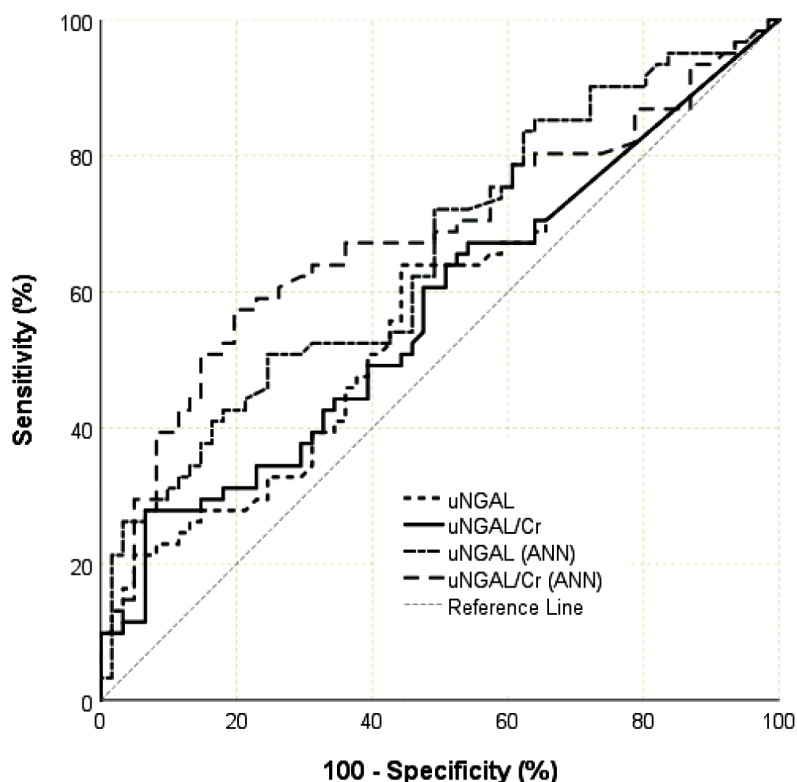
We also used the artificial neural network (ANN) to compare the results and we found that using a cut-off point value of 0.50 for uNGAL and 0.49 for uNGAL/uCr ratio, the sensitivities and specificities were 51.0% and 76.0% for uNGAL and 58.0% and 81.0% for uNGAL/uCr ratio; respectively (Figure).

### Receiver Operating Curve (ROC) Analysis

The sensitivity and specificity of uNGAL and

### DISCUSSION

Vesicoureteral reflux (VUR) is the most common



Test Result Variable(s)	Area (SD)	P	Cut point	Sensitivity (%)	Specificity (%)
uNGAL	0.572 (.052)	0.169	1.335	64	54
uNGAL/Cr	0.572 (.052)	0.168	0.031	61	53
uNGAL (ANN)	0.654 (.049)	0.003	0.509	51	76
uNGAL/Cr (ANN)	0.680 (.049)	0.001	0.498	58	81

Discrimination of the patients group from healthy controls by considering the quantitative data in ROC analysis revealed that the definition of a cut-off point value of 0.03 for uNGAL/uCr ratio showed 61.0% sensitivity and 53.0% specificity to predict VUR.

congenital risk factor for UTI that leads to the kidney scar, so-called reflux nephropathy, in 50% of pediatric patients. Pathological consequences of reflux nephropathy include tubular destruction, loss of cortical tissue and interstitial fibrosis. These complications are often asymptomatic and should be exactly pursued.<sup>23-28</sup> Management of UTI patients with regard to screening for VUR is still under debate. There is disagreement about the screening of the VUR following urinary tract infection. This controversy has been mainly focused on the recommendations that RNC should not be routinely performed in children with the first-time febrile UTIs between 2 and 24 months.<sup>29,30</sup> NGAL as a secretory protein belonged to the lipocalins family is produced by ischemic renal tubules and damaged epithelium of the nephrons and is one of the newest markers of renal injury. Unlike Cr levels and urine output that measure the renal function, NGAL is specifically produced by damaged nephrons and released into the blood and urine as a pathological biomarker which can be measured easily.<sup>31</sup>

In the present study, we evaluated the predictive values of uNGAL level and uNGAL/uCr ratio in VUR patients. We observed the high levels of uNGAL and uNGAL/uCr ratios in the patients group compared to control subjects but the only significant difference was found for uNGAL/uCr ratio. More importantly, we found a highly significant ratio of uNGAL/uCr in patients with severe VUR compared to mild and moderate VUR. Moreover, ROC analysis to determine the sensitivity and specificity of both uNGAL levels and uNGAL/uCr ratios for prediction of VUR revealed 64.0% sensitivity and 54.0% specificity for uNGAL and 61.0% sensitivity plus 53.0% specificity for uNGAL/uCr ratio (Table). Similar ROC analysis based on the ANN model revealed lower sensitivities and higher specificities for both variables to predict VUR. These findings may support the recent study on a group of Iranian pediatric with VUR that showed the potential of urinary NGAL/Cr ratio as a sensitive, specific, and more accurate biomarker for diagnosis of primary VUR in pediatric.<sup>14</sup>

Likewise, our results are in line with Parmaksiz *G et al.* study that showed uNGAL as a more sensitive biomarker than other factors for prediction of renal scarring in VUR.<sup>32</sup> Alternately, Forster *et al.* demonstrated that both uNGAL and plasma NGAL

can be used as novel sensitive markers for early prediction of UTI.<sup>33</sup> There is mounting evidence that, measurement of both plasma NGAL and urine NGAL levels in various disorders such as UTI, VUR, renal scarring, and obstructive uropathy has great importance for the management of these kidney disorders.<sup>34-38</sup> These two types of NGAL have different implications in the clinical setting so that, pNGAL acts as a marker of systemic inflammation; while uNGAL is indicative for renal epithelium injury.<sup>38</sup>

With this in mind, we found that the higher uNGAL/uCr ratios in the patients versus controls and those patients with a severe form of VUR could be indicative for the potential predictive value of this biomarker in conjunction with urine creatinine measurement. Although, its sensitivity and specificity appeared not to be so enough for this purpose. For this reason, further studies by recruiting larger samples are needed to confirm these results.

Of note, we did not observe significant differences for uNGAL levels in terms of the severity of disease and even between patients and healthy subjects. Consistently, Nickolas *et al.* depicted that uNGAL does not increase in the mild cases of chronic kidney disease.<sup>39</sup> Due to the lack of enough data regarding clinical relevance and predictive value of uNGAL in VUR patients, our results in comparison with similar studies should be interpreted cautiously and, definitely further well-designed studies by recruiting larger samples are needed to determine the diagnostic, predictive and even prognostic values of uNGAL for management of VUR.

## CONCLUSION

In conclusion, our findings indicate a potential predictive value of uNGAL/uCr ratio as a non-invasive biomarker in VUR. More importantly, regardless of the presence or absence of renal scar, the severity of VUR was significantly associated with uNGAL/uCr ratio. Although this urinary biomarker has been explored and almost validated for management of UTI, its value in the point of clinical application for assessment of renal damage in VUR still needs to be confirmed by further investigations.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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