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## Serum Neutrophil Gelatinase-associated Lipocalin as a Predictor of Acute Kidney Injury in Critically-ill Neonates

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**Abstract:** Early detection of evolving Acute Kidney Injury (AKI) in critically ill neonates can lead to better preventive and therapeutic interventions. Neutrophil Gelatinase-associated Lipocalin (NGAL) is a promising biomarker of AKI, which was also shown to increase in inflammation. The objective of this study was to assess the utility of serum NGAL (sNGAL) as an early marker of evolving AKI in critically-ill neonates with and without sepsis. sNGAL levels were estimated in 60 critically-ill neonates at the time of admission to Neonatal Intensive Care Unit (NICU), in comparison to 20 healthy matched control. Patients were categorized as sepsis (n = 35) and no-sepsis (n = 25) subgroups on basis of clinical and laboratory criteria. They were subsequently discriminated according to creatinine and urine output criteria of the Acute Kidney Injury Network (AKIN), into AKI (n = 34) and no-AKI (n = 26) subgroups. sNGAL levels were significantly higher in the patient group as compared to control ( $132.7 \pm 67.8$  vs.  $55 \pm 10.3$  ng mL<sup>-1</sup>, p = 0.0001). Elevated levels were comparable between sepsis and no-sepsis groups ( $130.1 \pm 69.4$  vs.  $136.5 \pm 66.6$  ng mL<sup>-1</sup>, p = 0.7) and they positively correlated with 48-hour post-admission serum creatinine (p = 0.0001). Patients of AKI group had significantly higher sNGAL than those of no-AKI group ( $176.2 \pm 55.9$  vs.  $75.9 \pm 28.3$  ng mL<sup>-1</sup>, p = 0.0001). A cut-off value for sNGAL of 117.5 ng mL<sup>-1</sup>, was predictive of AKI with a sensitivity of 82% and a specificity of 88.5%. It could be speculated that measurement of serum NGAL can serve as a clinically useful marker for early prediction of evolving AKI in critically-ill neonates with and without sepsis.

**Key words:** Neutrophil gelatinase-associated lipocalin, critically-ill neonate, acute kidney injury

### INTRODUCTION

Acute Kidney Injury (AKI) in neonates is a complex disorder that lacks a uniform clinical presentation (Workeneh, 2011). It is seen more commonly in critically-ill neonates because they are liable to have multi-organ failure and commonly exposed to nephrotoxic drugs (Andreoli, 2004).

The need for early diagnosis together with the many shortfalls of using changes in serum creatinine (sCr) urged the search for new biomarkers for earlier detection of AKI (Askenazi *et al.*, 2009).

Neutrophil Gelatinase-associated Lipocalin (NGAL) is a promising evolving early biomarker of AKI (Mishra *et al.*, 2003). It was originally isolated from specific neutrophil granules and was later located in the bone marrow cells, lungs, proximal renal tubules and colonic epithelium (Miklaszewska *et al.*, 2006). It is generally expressed in very low concentrations but increases in the presence of epithelial injury or inflammation (Schmidt-Ott *et al.*, 2006; Zappitelli *et al.*, 2007). It can be even considered as a marker of leukocyte

activity or an acute phase protein (Sveger *et al.*, 2003). It also acts as a potent bacteriostatic agent and represents a novel and important iron-depleting antimicrobial defense strategy (Goetz *et al.*, 2002). Its expression was found to increase specifically in sepsis (Parravicini, 2010), in cancer breast, colon, pancreas, ovaries (Kalousek *et al.*, 2006) and brain (Smith *et al.*, 2008) and in renal tubular dysfunction (Venkataraman and Kellum, 2007). It was also suggested to be a marker for obstructive nephropathy (Wasilewska *et al.*, 2011). NGAL also has a physiological role in kidney development and as a growth factor (Soni *et al.*, 2010).

The utility of urinary NGAL was validated in adults and children as a sensitive and specific earlier biomarker of AKI than conventional renal function tests. Studies on NGAL in critically-ill neonates are limited and one of the most recent studies claimed that the prevalence of sepsis in those neonates may limit its value as a specific marker of AKI (Parravicini, 2010).

This study was designed to evaluate the utility of serum NGAL measurement for early detection of AKI in critically-ill neonates with and without sepsis.

## MATERIALS AND METHODS

This cross-sectional study was conducted between November, 2010 and August, 2011. It comprised 60 full-term neonates; completed 37-41 weeks of gestation, who were clinically evaluated as critically-ill and were subsequently admitted to the Neonatal Intensive Care Unit (NICU). A group of 20 healthy neonates matched for gestational age were included as a control group.

Critically ill neonates had been suffering a variety of critical conditions including congenital heart diseases, surgical problems (mostly abdominal), hypoxic ischemic encephalopathy, respiratory distress (meconium aspiration, infections) and one patient had an inborn error of metabolism (maple syrup urine disease). Small for gestational age (SGA) neonates and those with congenital malformations of the kidney, as detected by abdominal ultrasound, were not included.

Initial venous blood samples were withdrawn at the time of NICU admission for assessment of sNGAL levels and initial serum creatinine (sCr) concentrations. Meanwhile, comparable blood samples were collected from neonates of the control group at matched postnatal ages. Sepsis work-up included complete blood counts, serum C-reactive protein and blood cultures. Measurement of sCr was repeated for critically ill neonates, at least once, within 48 hours from the initial sample.

Critically-ill neonates were categorized as sepsis ( $n = 35$ ) and no-sepsis ( $n = 25$ ) subgroups on basis of clinical and laboratory criteria. They were subsequently discriminated, within 48 h of admission, according to the criteria proposed by the Acute Kidney Injury Network in 2007 (Mehta *et al.*, 2007), into AKI ( $n = 34$ ; 9 in stage I, 15 in stage II, 10 in stage III) and no-AKI ( $n = 26$ ) subgroups. Creatinine criteria were met in all AKI patients, while urine output criteria were met in only 12 of them as:

- Stage I AKI was diagnosed when sCr increased  $> 0.3$  mg dL<sup>-1</sup> or = 150-200% from initial sCr (and urine output  $< 0.5$  mL kg<sup>-1</sup> h<sup>-1</sup> for  $> 6$  h)
- Stage II AKI was diagnosed when sCr increased  $> 200$ -300% from initial sCr (and urine output  $< 0.5$  mL kg<sup>-1</sup> h<sup>-1</sup> for  $> 12$  h).
- Stage III AKI was diagnosed when sCr increased  $> 300\%$  from initial sCr (and urine output  $< 0.3$  mL kg<sup>-1</sup> h<sup>-1</sup> for  $> 24$  h)

**Laboratory methods:** Venous blood samples were collected under complete aseptic precautions and each was divided among an EDTA tube for complete blood count, a plain test tube for serum separation and the

bottle assigned for blood culture. Samples in the plain test tube were left to clot for 30 min, then centrifuged at 1000 xg for 15 min and sera were separated for the assay of creatinine and stored at -20°C till analyzed for sNGAL or lipocalin 2; as advised by the manufacturer. Hemolysed samples were discarded.

- **CBC:** It was done on Coulter Counter T660 (Beckman Instruments Inc., Scientific Instruments Division; Fullerton; CA 92634; 3100; USA)
- **CRP:** RapiTex CRP is the latex reagent for detection of CRP. The test is based on immunochemical reaction between CRP and antibodies to CRP bound to latex particles. Elevated CRP concentration more than 6 mg dL<sup>-1</sup> leads to visible agglutination of the latex particles
- **Serum creatinine:** It is measured on Synchron CX9 (Beckman Instruments Inc, Scientific Instruments Division; Fullerton; CA 92634; 3100; USA)

**sNGAL Assay:** The assay was carried out by an ELISA technique using reagents provided by Quantikine (R and D International; Inc, 614 McKinly Place; Minneapolis; MN55413, USA). In this technique, a monoclonal antibody specific for NGAL has been precoated on a microplate. Standards and samples were pipetted into the wells and any Lipocalin-2 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme linked monoclonal antibody specific for Lipocalin - 2 was added to the wells. Following a wash step to remove any unbound antibody- enzyme reagent, a substrate solution was added to the wells and color developed proportionately to the amount of Lipocalin-2 bound in the initial step. The intensity of color was measured at 450 nm wave length.

**Statistical methods:** Standard program for social sciences, release 13.0 (SPSS Inc, USA) was used for data entry and analysis. Comparison of different variables in various groups was done using Mann Whitney test for the non-parametric variables. Chi-square ( $\chi^2$ ) test was used to compare frequency of qualitative variables among various groups. Spearman's correlation test was used for correlating the non-parametric variables. For all tests, a probability  $p < 0.05$  was considered significant and  $p < 0.001$  was considered highly significant.

## RESULTS

The clinical and laboratory characteristics of patients and controls are shown in Table 1. Serum NGAL levels (sNGAL) were significantly higher in patients ( $132.7 \pm 67.8$ )

Table 1: Clinical and laboratory characteristics of patients and controls

Variables	Patients				Controls			
	Median	Min.-max.	IQR	Mean±SD	Median	Min.-max.	IQR	Mean±SD
GA (weeks)	40	37-42	3.0	39.1±2.4	39	37-42	4.0	39.2±2.7
BW (kg)	3.4	3.2-4.3	1.0	2.97±0.87	3400	3.2-4.2	1.1	2.9±0.7
Sex								
*Male	34 (57%)	--	--	--	12 (60%)	--	--	--
*Female	26 (43%)	--	--	--	8 (40%)	--	--	--
Delivery mode								
*Vaginal	35 (58%)	--	--	--	11 (55%)	--	--	--
*Cesarean	25 (42%)	--	--	--	9 (45%)	--	--	--
sCr (mg dL <sup>-1</sup> )	0.3	0.1-0.6	0.2	0.3±0.12	0.2	0.1-0.3	0.1	0.2±0.06
sCr (mg dL <sup>-1</sup> )**	0.55	0.1-2.1	0.5	0.71±0.46	0.2	0.1-0.4	0.1	0.17±0.07

\*Data presented as number (percentage), sCr: Serum creatinine, \*\*Subsequent sCr, IQR: Interquartile range

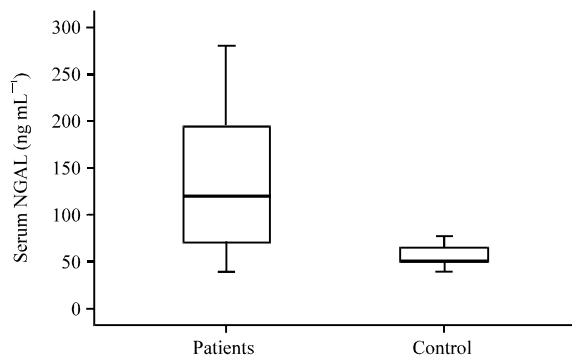


Fig. 1: Comparison of sNGAL levels in patients and controls revealed significantly higher values in the former. In this box plot, there were no extreme values (>3 IQR), no outliers (1.5-3 IQR), the whiskers represent the maximum and minimum and the box represents the 25th and 75th percentiles (in between is the IQR) and the median

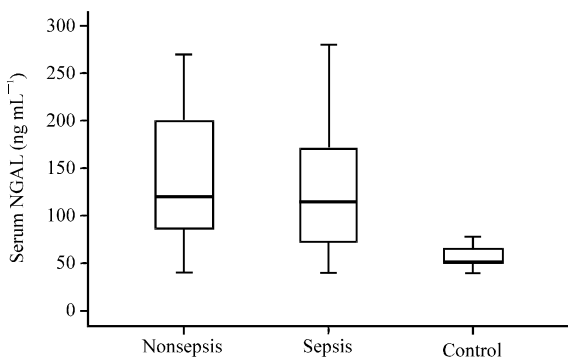


Fig. 2: Comparison of sNGAL levels in sepsis and non-sepsis patients revealed no significant difference

than control (55±10.3) ( $Z = -5.25, p = 0.0001$ ) (Fig. 1), however, these elevated levels were comparable between sepsis (130±69.4) and no-sepsis (136.5±66.6) subgroups ( $Z = -0.36, p = 0.7$ ) (Fig. 2).

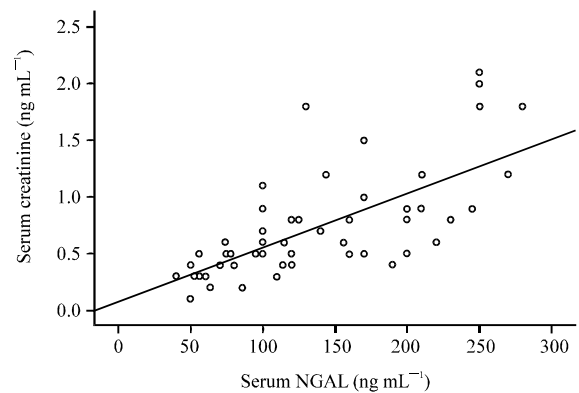


Fig. 3: sNGAL levels correlated significantly with subsequent sCr levels measured within 48 h after admission

More importantly, sNGAL levels-measured at admission-correlated significantly ( $r = 0.78, p = 0.0001$ ) with the subsequent sCr levels, measured within the next 48 h (Fig. 3).

Comparing AKI and no-AKI subgroups revealed significantly increased sNGAL levels in AKI (176.2±55.9) than no-AKI patients (64.1±18.2) ( $Z = -5.98, p = 0.0001$ ) (Fig. 4).

Neonates with stage II AKI had their sNGAL levels significantly higher (159.5±51) than neonates with stage I (98.3±31.2) ( $Z = -2.87, p = 0.003$ ) and non-significantly lower than neonates with stage III (197.3±56) ( $Z = -1.9, p = 0.056$ ) (Fig. 5).

Important to emphasize is that stage I patients had significantly higher sNGAL levels (98.3±31.2) than no-AKI patients (64.1±18.2) ( $p = 0.013$ ).

The cut-off value of sNGAL for differentiating critically-ill AKI and no-AKI neonates was calculated as (117.5 ng mL<sup>-1</sup>) with an area under the receiver-operating characteristic curve (AUC) of 0.95, sensitivity of 82% and specificity of 88.5% (Fig. 6).

Within the AKI patients, twenty (58.8%) had sepsis and fourteen (41.2%) had no sepsis ( $\chi^2 = 0.008, p = 0.9$ ).

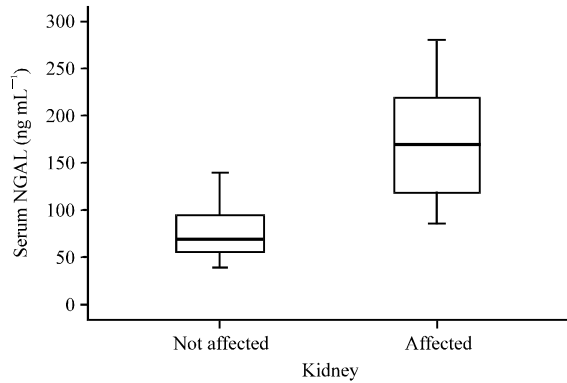


Fig. 4: Comparison of sNGAL levels in AKI and no-AKI patients revealed significantly higher levels in the former

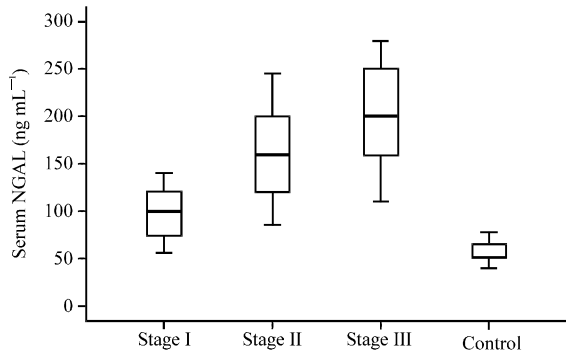


Fig. 5: Comparison of sNGAL levels in subsequent stages of AKI revealed increasing levels with increasing severity as indicated by stages

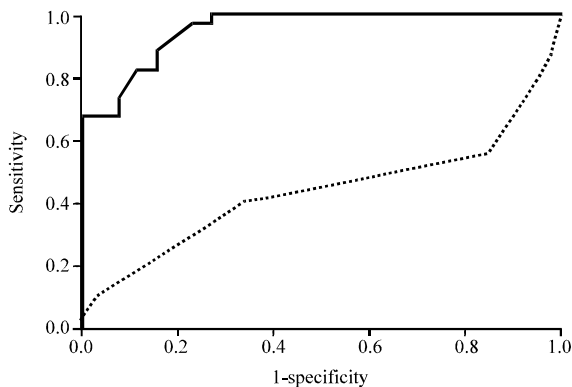


Fig. 6: ROC curve for sNGAL to detect AKI at a cut-off value of 117.5 ng mL<sup>-1</sup> showed AUC of 0.95

sNGAL levels in the former (175.5±57.2) were comparable to those in the latter (173±56.1) ( $Z = -0.02, p = 0.9$ ).

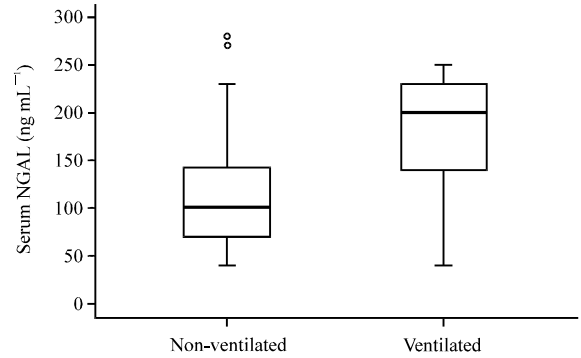


Fig. 7: Comparison of sNGAL levels in ventilated and non-ventilated patients revealed significantly higher levels in the former

Seventeen patients were ventilated; 7 had sepsis and 10 had no sepsis ( $\chi^2 = 2.8, p = 0.09$ ). Upon comparison of ventilated (17) and non-ventilated (43) babies regarding sNGAL level, it was significantly higher in the former (174.5±69) than the latter (116.2±60.4) group ( $Z = -2.8, p = 0.006$ ) (Fig. 7).

## DISCUSSION

Immediately after a kidney injury, blood urea or creatinine levels may be normal (Devarajan, 2010) and the only sign may be decreased urine production. At the same time, the degree of oliguria doesn't necessarily correlate with the severity of injury and interventions inside NICUs including diuretics, dopamine and mannitol can cause disproportionately higher urine output (Venkataraman and Kellum, 2007).

Moreover, AKI can occur without affection of urine output and classifying AKI as oliguric or nonoliguric has a prognostic value rather than a diagnostic indication (Workeneh, 2011).

Meanwhile, the desirable biomarker needed for detection of AKI should be non-invasive, sensitive, specific, increases proportionate to degree of damage, can predict clinical outcomes and efficacy of therapy and its results are available while damage is limitable (Devarajan, 2010).

For the last two decades, NGAL has been studied extensively in urine-and to a lesser extent in plasma-because it dramatically rises in urine after renal injury; being concentrated in the distal tubules (Uttenenthal, 2005). In this study, sNGAL levels were assessed in 60 critically-ill neonates. We applied the creatinine and urine output criteria of AKIN classification for detection and staging of AKI because the absolute and percentage change in serum creatinine-that these criteria include-can

accommodate variations related to age and reduce the need for a baseline creatinine level (Mehta *et al.*, 2007). Urine output criteria were fulfilled in about 35% of our AKI patients. Many studies in neonates report over 50% of AKI cases to be non-oliguric, which highlights the insensitivity of oliguria to predict AKI in this population (Aggarwal *et al.*, 2005; Gupta *et al.*, 2005). One recent research work (Xiao-yu *et al.*, 2011) applied the same criteria for retrospective staging of AKI in asphyxiated neonates and suggested the system as a reliable and sensitive indicator for AKI in neonates.

The sNGAL levels were generally higher in critically-ill neonates compared to healthy controls; a finding that could be related to any of the causes of elevated sNGAL as sepsis or renal impairment. However, neonates with sepsis didn't have higher levels of sNGAL as expected in infections (Xu *et al.*, 1995) and in septic shock; as shown in critically-ill children by Wheeler *et al.* (2008). No-sepsis patients still had significantly higher sNGAL levels than healthy neonates, suggesting that factors independent of sepsis in these patients led to the increased sNGAL levels. Having significantly higher subsequent sCr levels in no-sepsis patients compared to healthy neonates ( $p < 0.001$ ) suggests kidney impairment as the possible cause of elevated sNGAL levels in the former group.

About 57% (34) of the critically ill neonates in this study developed AKI; according to the AKIN classification (Mehta *et al.*, 2007). These results are close to the incidence of AKI reported in critically-ill neonates by Guerin *et al.* (2000). In their study, the authors mentioned that up to 60% of patients may have already sustained AKI on admission to NICU. Furthermore, AKI may develop after admission; being a frequent complication in this population and it results in hospital mortality of 45-60%.

The sNGAL increased significantly in neonates with evolving AKI before sCr elevation and it correlated significantly with the subsequent and not with initial sCr. Similar results were reported in some studies such as those done to diagnose AKI following cardiac surgery (Mishra *et al.*, 2005; Krawczeski *et al.*, 2011). Most of the studies in neonates, however, reported the utility of urine rather than serum or plasma NGAL as an early diagnostic marker of AKI (Lavery *et al.*, 2008; Huynh *et al.*, 2009; Gabbard *et al.*, 2010; Mussap *et al.*, 2010). On the other hand, some authors reported moderate efficacy of NGAL as a predictor of AKI in critically-ill adults (Siew *et al.*, 2009).

Among the studies that measured NGAL in plasma was the one done by Cruz *et al.* (2010). They measured NGAL in a large population of critically-ill adults and ascertained its value as an early marker for the development of AKI and for prediction of the need for

renal replacement therapy. Tuladhar *et al.* (2009) and Koyner *et al.* (2008) also measured sNGAL after adult cardiothoracic surgery and (Niemann *et al.*, 2009) measured it in adults during liver transplantation. In addition, sNGAL proved its predictive value in contrast-induced AKI in the study of Hirsch *et al.* (2007).

Present study ascertains the predictive value of sNGAL in severity staging of AKI in neonates. The levels were increasing with more severe renal impairment. This is similar to the studies done by Krawczeski *et al.* (2011) in children and by Haase-Fielitz *et al.* (2009) in adults following cardiac surgery. Haase *et al.* (2009) also found that the discriminatory ability of NGAL for AKI increased with increasing severity according to AKIN criteria as shown by increasing AUC in subsequent stages.

The cut-off value for sNGAL to diagnose AKI was ( $117.5 \text{ ng mL}^{-1}$ ) with specificity of 89%, sensitivity of 82% and AUC of 0.95. Comparable levels; ( $100 \text{ ng mL}^{-1}$ ) 2 h after the insult, were suggested by Krawczeski *et al.* (2011). This small difference could be related to inclusion of children in their study while neonates are probably more liable to AKI. Other studies showed different cut-off values as that of Dent *et al.* (2007); sNGAL to predict AKI was  $150 \text{ ng mL}^{-1}$  with AUC of 0.96. The difference of this value from ours may be related to the difference in study population, number of patients enrolled or prevalence of other factors modifying serum levels of NGAL such as sepsis. In another study, Makris *et al.* (2009) found that both plasma NGAL and serum creatinine baseline measurements could predict AKI but AUC for NGAL was significantly larger than that for creatinine ( $p = 0.024$ ).

About 57% of neonates with sepsis (20 out of 35) developed AKI. This was comparable to the incidence of AKI in septic patients; 30-50% reported by Bagshaw *et al.* (2008) in adults and 50% by Bagshaw *et al.* (2010a) in critically-ill patients but different from the incidence reported in neonatal sepsis (26%) by Mathur *et al.* (2006). The researchers in the latter study studied 200 septic patients and they defined Acute Renal Failure (ARF) according to blood urea nitrogen and urine output; this could explain the difference from our results.

Upon comparison of sNGAL in septic ( $175.5 \pm 57.2$ ) and non-septic ( $177 \pm 56$ ) AKI patients, non-significant difference was found ( $p > 0.05$ ). On the other hand, Bagshaw *et al.* (2010b) measured plasma and urine NGAL levels in septic and non-septic AKI patients and found higher levels in the former than latter group.

The current study also revealed highly significant elevation of sNGAL levels in ventilated versus non-ventilated neonates; probably because the former were more critically ill and this was similar to the study done by Zapitelli *et al.* (2007).

We conclude that serum NGAL is useful for early detection of evolving AKI and for grading of its severity even in the presence of sepsis in critically-ill neonates.

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