

PLASMA NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN AS AN EARLY BIOMARKER OF ACUTE KIDNEY INJURY IN SNAKE BITER. Thamarai¹, K. Sivakumar²**HOW TO CITE THIS ARTICLE:**

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ABSTRACT: INTRODUCTION: Acute kidney injury due to snake bite represents a frequent and devastating problem. Currently, Acute Kidney Injury is diagnosed by biochemical monitoring of increase in serum creatinine. Increase in serum creatinine represents a late indication of a functional change in glomerular function rate. Studies have shown that Neutrophil Gelatinase Associated Lipocalin has been found to be very useful for the detection of acute kidney injury within few hours of nephrotoxic insult. Limited information, however, is available regarding the study of plasma Neutrophil Gelatinase-Associated Lipocalin in snake bite. **AIM:** The purpose of the study was to estimate the diagnostic accuracy of plasma Neutrophil Gelatinase-Associated Lipocalin as an early biomarker of Acute Kidney Injury in patients with snake bite and to correlate with serum creatinine. If early detection of Acute Kidney Injury occurs, it can be followed by effective treatment modalities to abort the development or limit the severity of AKI. Therefore this study was designed to explore the importance of pNGAL in cases of snake bite induced AKI. **MATERIALS AND METHODS:** A prospective observational study was designed to study the patients admitted for the treatment of snakebite within 6 hours in a tertiary care hospital. Patients admitted for snake bite were followed by estimation of pNGAL on day 1 and serum creatinine from the period of admission for up to 5 days. A total of 130 snake bite patients were enrolled and 100 were included in the final study. Snake bite patients were classified into two groups based on the occurrence and absence of AKI. Plasma NGAL and serum creatinine was estimated by solid phase Enzyme Linked Immunosorbent Assay method and Jaffe's method respectively. Data were entered into the excel sheet and analyzed statistically using statistical package for the social sciences (SPSS) version 17. **RESULTS:** Among 100 snake bite patients 64 individuals had elevated pNGAL levels with mean of 356.7 were found to have developed AKI according to Risk-Injury-Failure-Loss of function-End stage renal disease (RIFLE) criteria. 36 individuals had pNGAL level with mean of 83 were found to be non AKI patients with 'p' value of 0.001. Using Pearson's correlation, pNGAL level was not significantly correlated with the serum creatinine at the time of admission with 'r'-0.19, but correlated with serum creatinine only after 72 hours ('r'0.82). With an area under the curve of 0.914 and a standard error of 0.027, pNGAL shows an asymptotic significance of 0.000 proving it as a better predictor over creatinine, in diagnosing AKI. **CONCLUSION:** Plasma NGAL were elevated in snake bite induced AKI than non AKI individuals even before the elevation of serum creatinine, indicating the marker of renal injury. However further studies are suggested with large number of samples to confirm or refute the present observation. **KEYWORDS:** Neutrophil Gelatinase Associated Lipocalin, Acute Kidney Injury, Nephrotoxic insult, Pearson's correlation.

INTRODUCTION: Acute kidney injury is seen in the majority of the cases of snake bite especially viper¹. The gravity, spectrum and the outcome are variable. India is home to some of the most

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poisonous snakes in the world. Most of the Indians are victims of Russell's viper or echiscarinatus bites¹. Viper bites are more common than other poisonous snakebites in human beings.²

SNAKE BITE AND AKI: Viper bite is the commonest and it is seen in 91.6% cases among the AKI patients. Acute kidney injury complicates the course in 5% to 30% of victims of severe viper poisoning.³ The factors that contribute AKI due to snake bite are Direct cytotoxicity, Bleeding, Hypotension, Circulatory collapse, Intravascular hemolysis, Disseminated Intravascular Coagulation and Micro Angiopathic Hemolytic Anemia (MAHA).³ Urinary beta-N acetylglucosaminidase excretion in patients bitten with Russell's viper, indicating a direct toxic effect of venom on the kidney.⁴ The sub lethal dose of *Russell's viper* or *E.carinatus* venom causes hemorrhages in the kidneys and mild acute tubular necrosis in 20% of the glomeruli within 24 hrs of envenomation.⁵ However, lethal dose of venom causes acute tubular necrosis and fibrin thrombi in 50-75% of glomeruli.⁵ Russell's viper venom causes changes in renal plasma flow, glomerular filtration rate and filtration fraction. It also causes reduced tubular reabsorption of sodium and increased fractional excretion of sodium.⁴ Myoglobinuria, sepsis and hypersensitivity to venomous or anti-venom protein also contribute towards renal failure. Hypotension and circulatory collapse set in motion a chain of hemodynamic disturbances, which culminate in ischemic AKI.⁴ Intravascular hemolysis plays a role in the pathogenesis of snake bite induced AKI.⁴ The renal failure following snake bite should be considered as an example of the hemolytic uremic syndrome.⁶ Viper venom contains many proteins that interact with members of the coagulation cascade and the fibrinolytic pathway. *Russell's viper* venom (RVV) contains a factor V activates serine proteinase, which has been separated from a factor X-activating protein, also present in this venom. Disseminated intravascular coagulation (DIC) is a consistent feature in patients bitten by *Russell's viper*, *E.carinatus*, *boomslang* and *pit vipers*.⁴

AKI AND SERUM CREATININE: The diagnosis of AKI following viper bite hinges on functional biomarkers such as serial measurements of serum creatinine. The rise in serum creatinine is delayed by a few days, because the serum creatinine concentration does not increase until half of the kidney function is lost.⁷ Serum creatinine is influenced by several non-renal factors such as age, gender, muscle mass, muscle metabolism, medications, hydration status, nutrition status and tubular secretion.⁸ Acute kidney conditions can exist with no increase in serum creatinine owing to the concept of renal reserve until the 50% of kidney function must be lost.⁸ Increase in serum creatinine represents a late indication of a functional change in glomerular function rate (GFR) that lags behind important structural changes that occur in the kidney during the early-damage stage of AKI.⁹

AKI and NGAL: Human NGAL (synonyms: lipocalin 2, siderocalin, neutrophil lipocalin) is 25kilo Daltons¹⁰ (kDa) secretory glycoprotein belongs to lipocalin superfamily¹¹ expressed in kidney cells and undergo an early dramatic up regulation in response to nephrotoxic injury. NGAL has been originally isolated from the secretory granules of activated human neutrophils.¹² Human NGAL consists of a single disulfide bridged polypeptide chain of 178 amino acid residues with a calculated molecular mass of 22kDa and its glycosylation increases its apparent molecular mass to 25kDa.¹¹ They are characterized by their ability to bind small, hydrophobic molecules in a structurally conserved pocket formed by 8 antiparallel beta pleated sheets; it binds to specific cell surface receptors to form macromolecular complexes. The major ligands for NGAL are small iron-binding

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molecules¹³ called siderophores. NGAL is responsible for growth and differentiation of renal tubular epithelial cells and exerts bacteriostatic effects in the distal urogenital tract by interfering with bacterial siderophores-mediated iron acquisition.^{13,14} This siderophores iron complex limits proximal tubular damage and reduces apoptosis.¹⁴ NGAL is a protease resistant polypeptide, released from the distal tubules, secreted with the urine or back leaking to the plasma, freely filtered, reabsorbed in the proximal tubules through endocytic megalin receptors or finally secreted with the urine. The promoter region of the NGAL gene contains binding sites for a number of transcription factors, including nuclear factor- κ B-light-chain-enhancer of activated B cells (NF- κ B).¹⁵ NF- κ B is known to be rapidly activated in kidney tubule cells after acute injury and plays a central role in controlling cell survival and proliferation.⁹ These findings provide a potential molecular mechanism for the documented role of NGAL in enhancing the epithelial phenotype, both during kidney development and following AKI.¹⁶ It is now well known that AKI results in a dramatically increased NGAL mRNA expression in distant organs especially the liver and lungs, and the over expressed NGAL protein released into the circulation may constitute a distinct systemic pool.⁹ Additional contributions to the systemic pool in AKI may derive from the fact that NGAL is a known acute phase reactant and released from neutrophil, macrophages, and other immune cells.¹⁷ Any decrease in glomerular filtration rate resulting from AKI would be expected to decrease the clearance of NGAL, with further accumulation in the systemic pool.¹⁶ These factors are responsible for the elevated levels of NGAL in the plasma during AKI. Plasma NGAL has emerged as a biomarker that predicts the development of AKI in patients 1-3 days earlier than serum creatinine.⁹ NGAL is easily measured, unaffected by other biological variables and capable of both early detection and risk stratification.

NGAL appears to be an early potential 'real time' biomarker for AKI and its expression is proportional to the severity of renal injury. Our main objective was to estimate the diagnostic accuracy of plasma Neutrophil Gelatinase-Associated Lipocalin as an early biomarker of AKI in patients with snake bite and to correlate with serum creatinine. Early detection of renal injury is of key importance to impact morbidity and mortality and the introduction of potential therapeutic agents in the course of renal disease. However NGAL performance in a snake bite injury care setting has not been well described. We performed this study to estimate the diagnostic accuracy of pNGAL for early detection of AKI.

MATERIALS AND METHODS:

Study Population: This was the prospective observational study conducted in the tertiary care hospital. A detailed history was elicited for Co-morbid diseases, concomitant drug intake and species of snake, time of the bite, treatment before hospitalization and the history of reduced urine output. Patients admitted within 6 hours of snake bite were included in the study. Patients excluded were snake bite patients with elevated serum creatinine on admission, known cases of hypertension, diabetes, chronic kidney disease, systemic and urinary tract infections. We enrolled 130 snake bite patients admitted and we excluded 8 patients with diabetes, 5 patients with hypertension, 1 patient with end stage renal disease on chronic Renal Replacement Therapy, 4 patients with elevated serum creatinine levels at the time of admission and 12 patients with previous hospitalization for treatment of snakebite, leaving 100 patients for analysis. The patients were classified into two groups according to the occurrence and absence of AKI. For comparative purposes, pNGAL was measured in the plasma of apparently healthy 20 (12 males and 8 females) controls who had come for master health checkup.

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Consent and Ethical clearance: All participants signed an informed, written consent to participate in the clinical examinations and biochemical investigations before entering the study. Ethical clearance was obtained.

Collection of Specimens: Under strict aseptic precautions, 4ml of venous blood was collected from the snake bite patients at the time of admission and serially for up to 5 days for the estimation of pNGAL and serum creatinine respectively. One part of the blood was transferred to plain tube and serum was separated for the estimation of serum creatinine. The other part of the sample was collected in Ethylene diamine tetra acetic acid (EDTA) coated polypropylene tubes for the estimation of plasma NGAL.

Processing of Samples: Laboratory assistants were blinded to sample sources and clinical information until the end of the study. Calibration of instruments and reagents had been done before entering the study. Serum creatinine was measured by modified jaffe's kinetic method (ident I, CPC Diagnostics, Biosystems) in the fully automated analyzer (BS 380). Plasma NGAL was measured by Solid phase enzyme linked immunosorbent assay method (NGAL Rapid ELISA KIT 037, Antibody shop ELISA kit, BioPorto Diagnostics DK-2820 Gentofte, Denmark). For estimation in ELISA, samples were diluted according to the expected NGAL concentrations, 1/100 for plasma. The absorbances were read at 450nm in an ELISA reader. Calibration curve was constructed by plotting the mean of duplicate absorbance values for each NGAL Calibrator on the y-axis against the corresponding pNGAL concentrations in ng/ml on the x-axis. The pNGAL concentration of each diluted sample was found by locating the point on the curve. The concentration of pNGAL in diluted specimens was calculated by multiplying the result by the sample dilution factor. All reagents were stored at 2-8°C. The reference value of NGAL varies from 33 -106 ng/ml.

Statistical Analysis: Continuous variables were expressed as mean \pm standard deviation and compared between the two groups using t-test, and among three or more groups using analysis of variance, wherever appropriate. Diagnostic characteristics of pNGAL were evaluated with receiver-operating characteristic (ROC) curves. For all analyses, 'p' values <0.05 were considered as significant. pNGAL was correlated with serum creatinine using Pearson's correlation. Data were entered into the excel sheet and analyzed statistically using statistical package for the social sciences (SPSS) version 17.

RESULTS: AKI, defined as an increase in creatinine of at least 50% from baseline or a reduction in urine output to <0.5 ml/kg/h for >6 h. AKI was defined using the creatinine and urine output criteria of the RIFLE (Risk-Injury-Failure-loss of function-End stage renal disease) classification. Patients were monitored daily using the RIFLE criteria.

NGAL in AKI and non-AKI: Out of 100 snake bite individuals, 73 % were males and 27 % were females. Mean age group of snake bite individuals in males and females were 37 and 34 respectively. Mean body weight of snake bite individuals in males and females were 60 and 55 respectively as given in Table 1. pNGAL was estimated in age, sex and body weight matched apparently healthy controls for comparative purposes. In the snake bite patients, plasma NGAL investigation was carried

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out at the time of admission. Serum creatinine was measured from the period of admission for up to 5 days. Patients with elevated pNGAL values developed AKI and those with comparatively lower pNGAL values (< 83 ng/ml) had not developed AKI during the study period ($P < 0.001$ at each time point). The mean of pNGAL in snake bite induced AKI was 356.7 ng/ml which was higher than the non AKI individuals of snake bite (83 ng/ml) and apparently healthy adults (32 ng/ml). Diagnosis of AKI was made based on the RIFLE criteria. A summary of pNGAL values among healthy controls, AKI and non AKI individuals as shown in Table 2. pNGAL was a good diagnostic marker for AKI development within the 24 hours from the time of admission, with an area under the ROC (AuC-ROC) of 0.91 (95% CI 0.86–0.96). Using a threshold value of pNGAL 83 ng/ml, the sensitivity was 88 % (CI: 0.78-0.94) and specificity was 86% (CI: 0.72-0.94).

NGAL and severity of illness and AKI: Peak pNGAL concentrations increased with severity of AKI. Among the pNGAL values, peak level was 825 ng/ml. There was a similar relationship between mean pNGAL levels and RIFLE-max class (Non-AKI 83 ng/ml; Risk 106.58 ng/ml; Injury 298.47 ng/ml; Failure 517 ng/ml; $P < 0.001$) as given in Table 3. Patients with pNGAL of mean 83 at the time of admission did not show any rise in serum creatinine level up to > 72hrs. Those with pNGAL values greater than the mean of 517 ng/ml, >298 ng/ml, and >106 ng/ml developed AKI within 24 - 48 hrs; 48-72 hours and more than 72 hours respectively. Using Pearson's correlation, pNGAL level was not significantly correlated with the serum creatinine at the time of admission with $r = -0.19$, but correlated with serum creatinine only after 72 hours ($r = 0.82$) as shown in table 4.

DISCUSSION: This study revealed the importance of using plasma NGAL as a marker of acute kidney injury in snake bite. Snake bite induced AKI is an important cause of mortality in rural areas. We observed that pNGAL levels were significantly higher in snake bite patients compared to healthy adults and are significantly higher in AKI patients compared to non-AKI patients of snake bite with p value of 0.001. Those patients with elevated pNGAL level on initial hours of admission with mean of 356.7 is found to have developed AKI. pNGAL is found to rise in AKI patients earlier than serum creatinine and there is a positive correlation between the rise in NGAL with the development of AKI in severity and time period. Our study evaluates pNGAL is a better predictor than serum creatinine in snake bite induced AKI, because NGAL rises even before renal cell necrosis¹⁸ which occurs after the nephrotoxic insult. Parikh et al. demonstrated that NGAL, an early biomarker of AKI after cardio-pulmonary bypass surgery, is markedly induced within 2–6 hour after the initiation of the operation in patients destined for AKI. Makris K et al¹⁹ have proved that pNGAL as a reliable predictor of early AKI in multi-trauma patients on the 1st day of injury. Any decrease in glomerular filtration rate resulting from AKI would be expected to decrease the renal clearance of NGAL, with subsequent accumulation in the systemic circulation.^{9,20} The relative contribution of these mechanisms to the rise in pNGAL after AKI remains to be determined. As shown in Table 3, with rise in pNGAL level, there is a proportional increase in the deterioration of renal function with elevated creatinine level which shows NGAL as a positive definite marker of acute kidney injury. These results show that pNGAL is not only a predictor of kidney injury but also it reflects the degree of the severity. pNGAL with mean of 517 ng/ml is found to have elevated serum creatinine between the duration of 24-48 hrs and these individuals fell into the category of “failure” (RIFLE).²¹ Those with mean of 298.47 in the duration of 48-72 hrs fell into the category of injury and those with mean of 106ng/ml developed AKI after 72 hours comes under the category of “risk” (RIFLE).²¹

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In the Pearson's correlation analysis, as shown in Table 5, there is a no correlation noticed between the rise in pNGAL and serum creatinine on the day of admission but significant correlation was obtained between pNGAL and serum creatinine after 72 hours with `r` value of 0.82. Creatinine becomes abnormal when more than 50% of GFR is lost,²² and it takes up to 2-3 days to rise. The traditional laboratory test to diagnose AKI such as estimation of serum creatinine will be detected in serum only after 50% of renal cell death has occurred. Serum creatinine was elevated only after 48 hours which was proved in many studies. Devarajan et al²³ have proved that after cardiopulmonary bypass, NGAL was increased significantly in plasma and in urine 2–6 h after surgery in those patients who subsequently developed AKI; the rise in creatinine was not evident until 48–72 h later.

In our study, with an area under the ROC (AuC-ROC) of 0.91 (95% CI 0.86–0.96), shows an asymptotic significance of 0.000 proving it as a better predictor over creatinine, in diagnosing AKI. This study correlates with the reported AUC in the 0.93-1 range²³ given in previous studies done by *Mishra et al* and *Benett et al*. *Dent C et al*²⁴ found that both urine and plasma NGAL were excellent independent predictors of AKI, with an area under the curve (AUC) of >0.9 for the 2–6-h urine and plasma NGAL measurements. *Dinna N. Cruz et al.*,²⁵ also found that pNGAL was a good diagnostic marker of AKI development within the next 48 h (area under ROC 0.78, 95% CI 0.65–0.90) and peak pNGAL concentrations increased with worsening of AKI severity (R=0.554, `p`0.001).

Ours is the first study to prospectively evaluate plasma NGAL in patients admitted for snake bite, making the results more readily applicable. Finally, we proved that pNGAL correlates with severity of AKI, yet remains an independent predictor for AKI. Various epidemiological and clinical studies have shown a strong association between rise in NGAL and early detection of AKI than serum creatinine which is delayed by several days. Serum creatinine does not accurately depict kidney function until a steady state has been reached, which requires more than 3 days. Hence it does not allow for early detection of acute renal injury. The change in serum creatinine, however, does not discriminate the time and type of renal insult or the site and extent of glomerular or tubular injury. In addition, in cases of more extensive tubular injury, there is a lag in time between the injury and an increase in serum creatinine. A sensitive bio marker like NGAL is needed to detect early kidney injury.

CONCLUSION: First, our results clearly indicate that plasma NGAL is a highly sensitive predictor of acute kidney injury in viper snake bite patients. pNGAL is a substantial early biomarker of AKI which allows diagnosing AKI 48 hours prior to the diagnosis based on RIFLE criteria. Second, pNGAL is elevated earlier than traditional biomarker, serum creatinine. Therefore pNGAL is useful to detect acute tubular injury in a timely manner, so that early intervention can be initiated.

SCOPE FOR THE FUTURE: It will be important in future studies to validate the pNGAL concentration measurements in clinical samples from large cohorts. In future, if standardized commercial tools would be easily available at a reasonable cost with reduced turnaround time, it is possible to diagnose AKI as early as possible with this novel biomarker.

LIMITATIONS: This study enrolled patients from a single center and small numbers of subjects; data from a multicenter study would be more useful. The test to demonstrate AKI (tissue biopsy) is highly not feasible in this study. We only assessed pNGAL once at a time and therefore we could not able to

follow up the serial pNGAL measurements and the differentiation from prerenal states remains a confounding factor that is open to further research. The lack of specific cut-off values for NGAL and differences in clinical assay characteristics are additional limitations to the widespread use of NGAL in laboratory practice at the present time.

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Demographic characters are shown in Table 1:

Demographic characters	All	Non-AKI	AKI	p` value
Number of individuals	100	36	64	0.001*
Male sex (%)	73(73%)	27(37%)	46(63%)	0.01*
Female sex (%)	27(27%)	11(40.78%)	16(59.25%)	0.001*
Males age (years)	37.90±7.76	35.46±7.86	39.33±7.62	0.47
Females age (years)	34.72±6.89	36.09±7.28	33.35±6.19	0.16
Males body weight (kg)	60.7 ± 10.15	59.4 ± 9.48	61.5 ± 10.4	0.15
Females body weight (kg)	55.4 ± 9.15	54.4 ± 8.54	56.5 ± 9.57	0.09

Table: 1 Demographic characters of snake bite patients

*-significant

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Biomarker	Healthy controls	Non-AKI	AKI	p` value
pNGAL	32 ± 5.7	83.02 ± 12.7	356.7 ± 180	<0.001*

Table 2: pNGAL values among healthy controls, snake bite induced AKI and non AKI individuals at the time of admission

*-significant

Parameters	Non AKI	Risk	Injury	Failure	p`
pNGAL (admission)	83	106.58	298.47	517	0.001**
Creatinine (admission)	0.95	0.959	1.012	1.014	0.001**
Creatinine (< 24 hrs)	0.98	0.981	1.013	1.017	0.001**
Creatinine (24 - 48hrs)	1	0.975	1.08	1.75*	0.001**
Creatinine (48-72hrs)	1.1	0.994	1.69*	2.46	0.001**
Creatinine (>72 hrs)	1	1.56*	2.34	3.84	0.001**

Table 3: pNGAL and Serum creatinine concentrations in the snake bite patients

pNGAL-ng/ml; Serum Creatinine-mg/dl.

*-AKI development based on RIFLE Criteria.

** - significant.

Parameters	Duration from admission	Pearson`s correlation
pNGALVs Serum creatinine	< 24 hrs	-0.19
pNGALVs Serum creatinine	>72 hrs	0.82*

Table 4: Correlation of pNGAL with serum creatinine

*Highly significant correlation

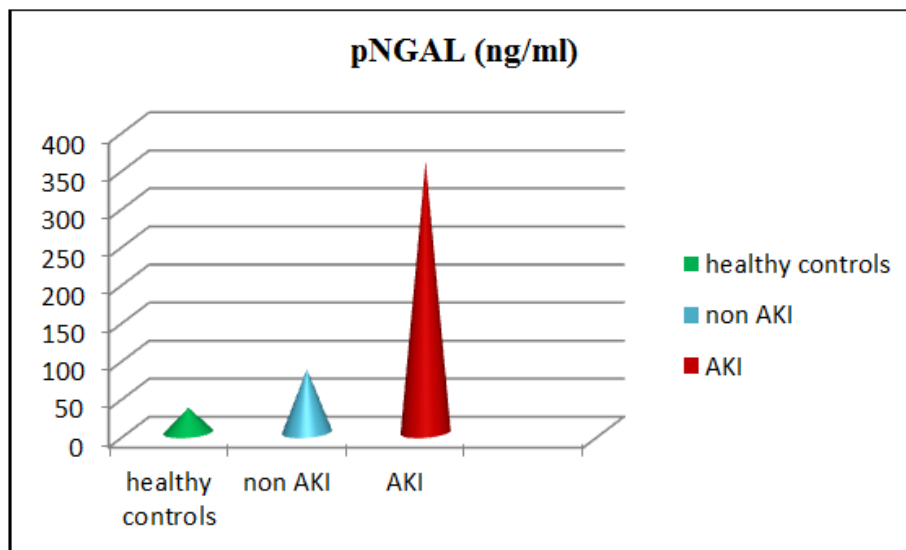


Fig. 1: pNGAL values among 3 groups

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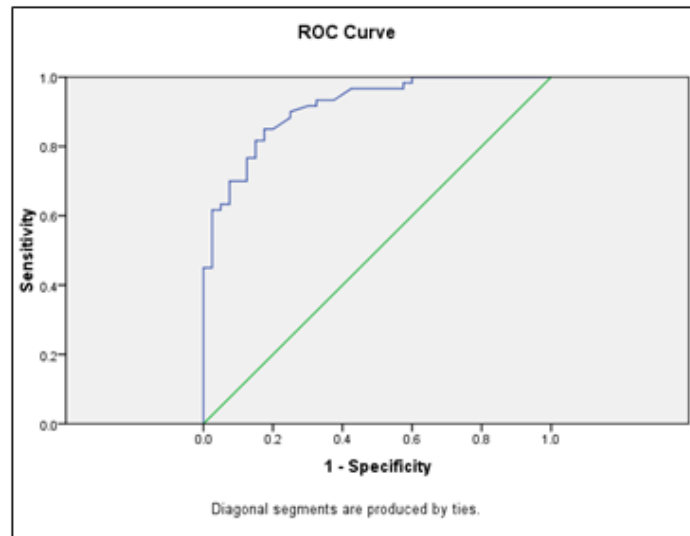


Fig. 2: AUC-ROC curve of pNGAL

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