Original Article

DOI: 10.7860/JCDR/2024/66093.19318

Biochemistry Section

Correlation of Serum Neutrophil Gelatinase-associated Lipocalin with Clinical Severity Score in Patients with Acute Coronary Syndrome: A Cross-sectional Study

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ABSTRACT

Introduction: Acute Coronary Syndrome (ACS) is a term that encompasses various clinical presentations such as ST-Segment Elevation Myocardial Infarction (STEMI), Non STEMI (NSTEMI), or unstable angina. Neutrophil Gelatinase-associated Lipocalin (NGAL), also known as lipocalin-2, belongs to the lipocalin category of extracellular proteins. Recent research indicates that NGAL levels are increased in different cardiac situations, regardless of the presence of acute renal injury.

Aim: To compare the levels of serum NGAL and Global Registry of Acute Coronary Events (GRACE) scores between cases with ACS and Angio-negative cases, and to study the correlation of serum NGAL with the GRACE score (clinical severity score) in ACS patients.

Materials and Methods: A cross-sectional study was conducted with 128 patients at the Department of Biochemistry, MKCG Medical College and Hospital, Berhampur, Odisha, India, presenting to the Cardiac Emergency Department from November 2020 to August 2021. Coronary Angiograms (CAG) were done to confirm the presence of Coronary Artery Disease (CAD), and patients were grouped accordingly as Group-1 with ACS patients and Group-2 with angiogram-negative cases. Apart from the routine work-up, including serum urea and creatinine, serum electrolytes, fasting blood sugar, lipid profile, and management, all patients underwent determination of serum NGAL levels and GRACE score at admission. The data were analysis using student's t-test and Pearson's correlation test.

Results: Among the 128 participants (64 cases in Group-1 and 64 cases in Group-2), Group-1 comprised 37 cases of STEMI, 26 cases of NSTEMI, and one case of unstable angina. Serum NGAL levels were significantly elevated in patients with CAG-proven ACS (140.89±56.47 ng/mL) without any renal dysfunction, sepsis, or overt infection compared to patients without CAD on CAG (52.01±18.39 ng/mL) ($p<0.0001$). The serum NGAL level exhibited a positive correlation of 0.359 ($p=0.004$) with the severity of ACS, as measured by the GRACE score.

Conclusion: The present study suggests that serum NGAL in patients with ACS may serve as a potential novel biomarker for risk stratification and predicting the severity of the disease.

Keywords: Coronary angiograms, Enzyme-linked immunosorbent assay, Kidney injury, Unstable angina

INTRODUCTION

The ACS is a major clinical outcome of coronary atherosclerosis. The clinical spectrum includes different presentations, ranging from STEMI to presentations seen in NSTEMI or unstable angina [1]. Cardiac damage caused by a sudden decrease in blood supply to the heart poses a significant risk to both the patient's well-being and lifespan. The absence of a curative remedy for this prevalent and perilous ailment, together with the varied results of treatment, has emphasised the significance of risk evaluation. The multinational registry known as the Global Registry of Acute Coronary Events (GRACE), which encompasses a vast number of observations, has been utilised to develop regression models for predicting the prognosis of patients with ACS. Nonetheless, there was a requirement for a thorough risk model founded on preliminary presentations to anticipate the overall risk of mortality in myocardial infarction within the initial six-month period. Given the significance of triage and care in the early stages of ACS, a risk tool has been developed based on the features of patients at initial presentation within the first hours or days [2]. NGAL (lipocalin-2) is a small extracellular protein with a variety of functions and is a representative of the lipocalin family [3]. This protein is an acute-phase protein that weighs 25 kDa and consists of a glycosylated monomer of simple protein chains [4]. Neutrophil gelatinase is an enzyme belonging to the Matrix Metalloproteinase (MMP) group, namely collagenase IV, and is covalently attached to it. This enzyme is found in neutrophils [5]. NGAL up-regulation has been demonstrated in diverse clinical situations such as infections, inflammation, intoxication, ischaemia, Acute Kidney Injury (AKI), and some neoplastic transformations [6-10]. The levels of urinary NGAL also differ according to age, gender, and hepatic function, and they are directly correlated with changes in the inflammatory markers [11].

The NGAL may contribute to the vascular remodelling and instability of atherosclerotic plaques in atherosclerotic arteries. The occurrence of NGAL in the vascular medium has been documented, existing both in its unbound state and in a compound with MMP-9. The interaction between NGAL and MMP-9 leads to the formation of a complex that inhibits the degradation of MMP-9 and enhances its proteolytic activity. This activity is implicated in the development of an unstable atherosclerotic plaque. The complex is found within atherosclerotic plaques, and its concentration has been observed to be higher in plaques with intramural haematoma and central necrosis [12-15].

Patients with angiographically confirmed CAD had considerably elevated levels of NGAL serum compared to individuals with normal arteries [16,17]. Patients suffering from acute myocardial infarction also experience elevated expression of NGAL. It is regarded as an active mediator of postischaemic inflammation and the remodelling reaction. The level of NGAL is elevated in the necrotic zone of the infarction and the surrounding healthy tissue [18,19]. To date, many

studies have been conducted regarding NGAL and the severity of ACS [20-23]. However, only two studies have been done correlating the GRACE score and serum NGAL by Nymo SH et al., and Soylu K et al., but they only included NSTEMI patients in their studies [20,21].

Hence, the present study was conducted to compare the levels of serum NGAL and GRACE scores between two groups: Group-1 (ACS patients) and Group-2 (Angio-negative cases), and to study the correlation of serum NGAL with the GRACE score (clinical severity score) in ACS patients.

MATERIALS AND METHODS

The study was conducted as a cross-sectional investigation at the Department of Biochemistry, in partnership with the Department of Cardiology at MKCG Medical College and Hospital in Berhampur, Odisha, India. The study period spanned from November 2020 to August 2021. The study received approval from the Institutional Ethical Council, identified as (IEC no 904). A written informed consent form in both English and the local language was signed by all the participants.

Inclusion criteria: Patients aged 18 years and older with any form of ACS were included in the study, irrespective of their glycaemic status, and age- and sex-matched angio-negative cases were taken as controls. In all the subjects, CAG was done to confirm the presence of CAD.

Exclusion criteria: Patients with hepatic disease, renal disease, overt infection or sepsis, and critically-ill individuals were excluded from the study.

Sample size: A total of 64 ACS cases (Group-1) were taken from the Department of Cardiology after angiography confirmation, and age- and sex-matched 64 angio-negative cases were taken as Group-2. Amid the challenges due to the Coronavirus Disease-2019 (COVID-19) pandemic during the study period, a total of 128 participants (Group-1 comprising 64 ACS patients and Group-2 comprising 64 angiogram-negative participants) were enrolled. The researcher visited the inpatient Department of Cardiology and employed a convenient purposeful random sampling method. The impacts of the pandemic were acknowledged and managed to ensure participant safety and data quality.

Study Procedure

A brief history was collected as per the data collection sheet. Anthropometric values like height, weight, and Body Mass Index (BMI) were recorded for each participant in the study. Echocardiography was conducted utilising a Philips CX50 ultrasound apparatus (manufactured by Philips, Netherlands). The Left Ventricular Ejection Fraction (LVEF) was determined to be 121 using Simpson's biplane approach [22]. Angiographic information was recorded in individuals from both groups for diagnostic purposes.

Clinical risk assessment: The ACS clinical risk assessment was conducted by the Cardiology Department utilising the GRACE risk score. The GRACE score is a predictive logistical model that utilises eight prognostic variables: age, systolic blood pressure, heart rate, plasma creatinine, Killip class, ST-segment depression, elevation in myocardial markers, and cardiac arrest on admission [2]. The GRACE score was calculated using the GRACE score calculator (https://www.mdcalc.com/calc/1099/grace-acs-risk-mortality-calculator). In terms of in-hospital mortality, a GRACE score of less than 109 was classified as low-risk, a score between 109 and 140 was considered intermediate risk, and a score greater than 140 was deemed high-risk, based on a previous study [23].

Biochemical parameters measurement: On the day of admission, 3 mL in a serum vial was used for the estimation of the lipid profile, Renal Function Tests (RFT) electrolytes, and NGAL. A 2 mL blood sample was collected in a fluoride vial for the estimation of fasting blood glucose. The quantitative assay for NGAL was done using an Enzyme-linked Immunosorbent Assay (ELISA) kit in the Erba Transasia ELISA plate reader. All biochemical parameters were measured by maintaining Quality Control. The reference range for the above parameters was taken from [Table/Fig-1] [24-34].

Parameters	Method of estimation	Normal reference range
Serum urea [24]	Urease method	15-40 mg/dL
Fasting Plasma Glucose (FPG) [25]	GOD-PAP	70-110 mg/dL
Serum creatinine [26,27]	Enzymatic method	0.6-1.2 mg/dL in men; 0.5-1.1 mg/dL in women
Serum total cholesterol [28]	CHOD-PAP	<200 mg/dL
Serum triglycerides [29]	GPO-TOPS	<150 mg/dL
Serum HDL [30]	Selective inhibition method	35-80 mg/dL
Serum LDL [31,32]	Calculated by Friedwald's formula	<100 mg/dL
Serum sodium [33]	Ion sensing electrode	136-146 mEq/L
Serum potassium [33]	Ion sensing electrode	3.5-51 mEq/L
Serum NGAL [34]	ELISA	47-55 ng/mL

[Table/Fig-1]: Normal range of different parameters [24-34].
GOD-PAP: Glucose oxidase-peroxidase coupled method; CHOD-PAP: Cholesterol oxidase PAP; GPO-TOPS: Gycerol-3-phosphate-peroxidase-to-triglyceride

ELISA method for the estimation of NGAL: The estimation of serum NGAL was done by the commercial kit available from BioVendor Human Lipocalin-2/NGAL ELISA Kit. The standards, quality controls, and samples were incubated for one hour in the microplate wells of an ELISA kit that had been pre-coated with polyclonal anti-human lipocalin-2 antibody. Following the process of washing, a biotin-150 labelled polyclonal anti-human lipocalin-2 antibody was introduced and allowed to incubate with the captured lipocalin-2 for a duration of one hour. Subsequently, the mixture was subjected to another round of washing. Next, the Streptavidin-HRP 152 conjugate was introduced and incubated for 30 minutes and washed for the last time. The remaining conjugate reacted with the substrate solution (TMB), followed by the addition of the stop solution, and the absorbance was measured [34].

STATISTICAL ANALYSIS

The statistical analysis involved representing the data as the mean value plus or minus the Standard Deviation (SD). The statistical analysis was conducted using International Business Machines (IBMs) Statistical Package for the Social Sciences (SPSS) version 22.0 software. Mean data of cases and controls were compared using a student's t-test, and p-values were calculated. The correlation was computed using Pearson's correlation coefficient, and the association between variables was visualised through a scatterplot. A p-value of <0.05, which is below the threshold for significance, was deemed significant.

RESULTS

Among the 128 participants (64 cases in Group-1 and 64 cases in Group-2), out of Group-1, 37 cases were STEMI, 26 cases were NSTEMI, and one case was of unstable angina. There was no statistically significant difference in mean age, height, weight, and BMI between the two groups. The mean heart rate and systolic blood pressure were significantly greater in Group-1 compared to Group-2 ($p<0.05$). The mean LVEF in Group-1 was significantly lower than that in Group-2 ($p<0.05$) [Table/Fig-2].

The mean levels of fasting plasma glucose, creatinine, total cholesterol, and LDL were higher and statistically significant in Group-1 compared to Group-2 with a p-value <0.05. The mean estimated Glomerular Filtration Rate (eGFR) was higher and statistically significant in Group-2 compared to Group-1. The mean value of HDL cholesterol was

Parameters	Group-1 (n=64) Mean±SD			Group-2 (n=64) Mean±SD			p-value
Age (years)	55.38±9.98			52.56±8.36			0.0870
Gender (M/F) n (%)	M	41	64%	M	37	58%	0.469
	F	23	36%	F	27	42%	
Height (cm)	164.45±7.67			163.5±7.129			0.4693
Weight (kg)	69.17±8.70			67.66±7.23			0.2876
BMI (kg/m^2)	25.57±2.67			25.83±2.20			0.5488
Pulse rate (Beats/min)	86.60±12.97			81.03±8.02			**0.0041***
SBP (mm of Hg)	129.6±21.14			121.11±7.03			**0.0028***
DBP (mm of Hg)	80.3±10.33			77.13±7.92			0.0535
LVEF (%)	54.47±9.78			65.34±2.39			**0.0001****

[Table/Fig-2]: Anthropometric and clinical parameters of Group-1 and Group-2.
del=significant at p<0.05 SBP: Systolic blood pressure; DBP: Diastolic blood pressure

lower in Group-1 compared to Group-2, and it was also statistically significant (p<0.05). The mean serum NGAL values in Group-1 were higher than in Group-2, which was statistically significant (p<0.05). The mean GRACE score in Group-1 (127.19±38.67) was significantly higher compared to Group-2 (74.10±15.72) with a p-value <0.0001 [Table/Fig-3].

Parameters	Group-1 (n=64) Mean±SD	Group-2 (n=64) Mean±SD	p-value
FPG (mg/dL)	124±53.20	90.53±8.38	**0.0001****
Urea (mg/dL)	28.1±11.40	24.48±5.67	0.024
Creatinine (mg/dL)	0.79±0.22	0.70±0.11	**0.008***
eGFR (mL/min/m^2)	96.61±19.18	104.3±16.54	**0.007***
Total cholesterol (mg/dL)	189.17±54.47	165.42±40.22	**0.006***
Triglyceride (mg/dL)	164.03±89.37	142.34±46.069	0.087
HDL (mg/dL)	42.89±10.60	50.13±10.61	**0.0001****
LDL (mg/dL)	114.37±45.75	93.48±33.12	**0.004***
Serum sodium (mEq/L)	138.63±3.65	138.39±3.96	0.722
Serum potassium (mEq/L)	4.314±0.45	4.00±0.59	**0.001***
Serum NGAL (ng/mL)	140.89±56.47	52.01±18.39	**p<0.0001****
GRACE score	127.19±38.67	74.10±15.72	**p<0.0001****

[Table/Fig-3]: Mean biochemical data in Group-1 and Group-2.
*Significant at p<0.05

In Group-1, 25 (39.06%) of subjects had low-risk, 21 (32.8%) had medium-risk, and 18 (28.1%) had high-risk. In Group-2, all the study subjects had low-risk [Table/Fig-4].

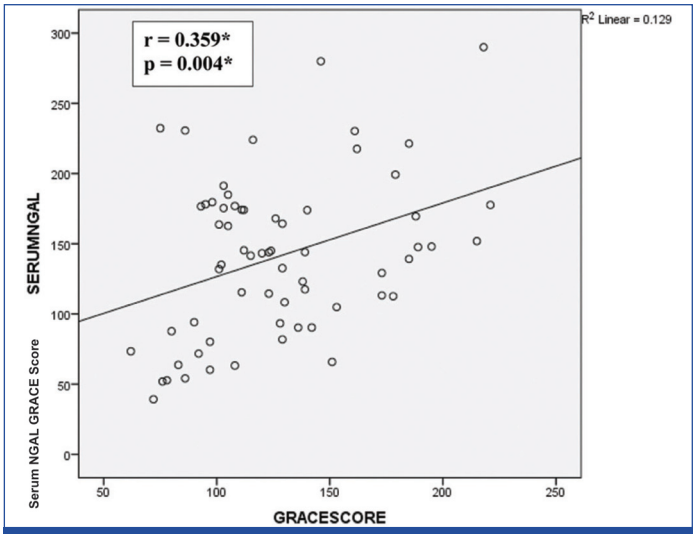
In present study, a significant positive correlation of serum NGAL with the clinical severity indicator GRACE score was found [Table/Fig-5,6].

Grace score	Group-1 n (%)	Group-2 n (%)
Low-risk (<109)	25 (39.06%)	64 (100%)
Medium-risk (109-140)	21 (32.8%)	0
High-risk (> 140)	18 (28.1%)	0

[Table/Fig-4]: Distribution of GRACE score according to low, medium and high-risk in Group-1 and Group-2.

Variables	Serum NGAL levels in Group-1	
	r-value	p-value
GRACE score	0.359*	0.004*

[Table/Fig-5]: Correlation of serum NGAL with clinical risk score.



[Table/Fig-6]: Scatterplot showing correlation between serum NGAL and GRACE score.
Shows serum NGAL has a positive linear correlation with GRACE SCORE

DISCUSSION

The study found that serum NGAL levels were elevated in all patients in the ACS cohort (Group-1) compared to Group-2. This finding is similar to the study conducted by Soylu K et al., (2015), which included 47 non-ST elevation ACS patients and 45 subjects with normal coronary arteries, showing significantly higher serum NGAL levels compared to the control group [20]. In a study conducted by Zografos T et al., it was found that the median serum NGAL levels in patients with angiographically diagnosed CAD were considerably greater compared to those with normal coronary arteries [17]. However, a study published by Sivalingam Z et al., measured plasma NGAL in 876 high-risk patients with stable CAD. They found a low value in ACS patients and concluded that the sample should be collected in stable CAD patients [35]. Sahinarslan A et al., showed that levels of leukocytes and serum NGAL are higher in patients with ACS compared to stable CAD patients [36].

A high value of NGAL in ACS patients is mainly due to inflammation and neutrophil activation. Inflammation is a crucial factor in the advancement of atherosclerosis and in the process of destabilising atherosclerotic plaque, which ultimately results in ACS. Macrophages and neutrophils infiltrate and contribute to the conversion of stable coronary artery plaques into unstable lesions characterised by a thin fibrous crown. NGAL has been demonstrated to create a complex with MMP-9, safeguarding the latter from deterioration and maintaining MMP-9's ensymatic function. MMP-9 and other members of the MMP family have been linked to vascular remodelling, the development of atherosclerosis, and the rupture of plaques. This suggests that NGAL may play a role in the development of CAD [37]. NGAL protein is produced by activated neutrophils and vascular wall cells. The increased levels of NGAL in ACS patients may be attributed to the presence of coronary thrombosis, more severe coronary atherosclerosis, and a greater extent of myocardial ischaemia. Neutrophil activation is observed in people with unstable angina and acute myocardial infarction, but not in individuals with stable angina. This is due to impaired neutrophil response, including a reduced capacity to generate reactive oxygen species in response to strong stimuli in patients with stable angina [38].

In the present study, the mean GRACE score of Group-1 was found to be significantly higher compared to the mean GRACE score of Group-2. As per the eight prognostic variables of the GRACE Scoring system, such as age, systolic blood pressure, heart rate, plasma creatinine, Killip class, ST-segment depression, elevation in myocardial necrosis marker, and cardiac arrest on admission, the mean±SD of heart rate, systolic blood pressure, and serum creatinine was higher in Group-1 than in Group-2, and the difference was statistically significant. Elneihoum AM et al., demonstrated a significant correlation between NGAL levels and cardiovascular risk factors such as age, hypertension, and smoking. The variables, including age, blood pressure, and creatinine, that are utilised in the computation of the GRACE risk score, also serve as triggers for NGAL expression [39]. The intricate interplay described here is a component of cardiorenal syndrome, a complex illness involving both the heart and the kidneys. In this syndrome, dysfunction in one

organ can lead to dysfunction in the other organ, either acutely or chronically [40].

A study conducted by Rahmani R et al., in 330 ACS patients indicated that the GRACE score had a significant predictive value in determining the severity and extent of coronary artery stenosis [41]. As studied by Hu C et al., a GRACE score >91 was associated with a greater risk of cardiovascular events [42]. In the study conducted by Georgios S et al., a total of 539 individuals with confirmed ACS were included. The study suggested that the average GRACE score was indicative of a greater level of complexity in the coronary anatomy [43]. The findings of the present study indicate that the utilisation of the GRACE score can provide insights into the expected severity of CAD prior to undergoing coronary angiography. During a clinical assessment of patients with ACS, particularly NSTEMI, in the emergency department, a higher GRACE score may suggest the presence of severe CAD. This information can alert clinicians to consider a more aggressive therapeutic approach or an early percutaneous coronary intervention.

A strong positive connection was observed between the levels of serum NGAL and the GRACE risk score in the ACS cohort (Group-1) in this investigation. In a study conducted by Soylu K et al., it was found that serum NGAL levels were positively correlated with the GRACE risk score in NSTEMI ACS cases [20]. Nymo SH et al., published a study where they grouped ACS patients into four groups as per the serum NGAL levels, and the findings suggested that the GRACE score increasing with an increase in serum NGAL levels [21].

The conventional biomarkers presently accessible are troponins and Creatine Kinase-myoglobin Binding (CK-MB), which have significantly contributed to diagnosis. Although there have been advancements in laboratory assays for cardiac-specific biomarkers, the early diagnosis of coronary ischaemia in individuals with ACS is still a significant concern. Therefore, novel biomarkers such as NGAL are becoming increasingly fascinating in the field of diagnosis and risk stratification.

Limitation(s)

In the present study, the sample size was smaller due to the COVID-19 pandemic, and convenient purposeful sampling was adopted during the study. Other inflammatory markers may be evaluated to find out the correlation between NGAL and the inflammatory markers.

CONCLUSION(S)

The mean serum NGAL level and GRACE Score were found to be elevated in all patients of the ACS cohort (Group-1) compared to Group-2. There was a significant positive correlation of serum NGAL with the GRACE Score in the ACS cohort. NGAL is highly modulated in ACS, and increased serum NGAL levels at admission were strongly associated with clinical risk scores. Therefore, serum NGAL may be a useful biomarker for early diagnosis, and along with the GRACE Score, it may further be used as a marker for risk stratification in ACS patients.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jun 20, 2023
- Manual Googling: Jan 29, 2024
- iThenticate Software: Feb 01, 2024 (11%)

ETYMOLOGY: Author Origin

EMENDATIONS: 9

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jun 17, 2023**
Date of Peer Review: **Aug 28, 2023**
Date of Acceptance: **Feb 02, 2024**
Date of Publishing: **Apr 01, 2024**