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Association of Serum Neutrophil Gelatinase-Associated Lipocalin with Childhood Asthma in Benha University Hospitals

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Abstract

Background: In various nations, asthma affects 1–18% of the population. It is a common, chronic respiratory condition. Fluctuating wheezing, shortness of breath, chest tightness, and/or coughing symptoms, as well as variable expiratory airflow limitation, are the hallmarks of bronchial asthma. The aim of the study was to evaluate the association of serum NGAL with childhood bronchial asthma for diagnosis, prognosis and assessment of severity. Methods: This prospective, cross-sectional study was conducted on 40 asthmatic cases. Cases were subdivided into four asthma groups (intermittent, mild persistent, moderate persistent and severe persistent) and 20 healthy children representing the control group. Cases were subjected to history taking, Routine Laboratory investigations, special laboratory investigations as serum NGAL level. NGAL concentrations were determined with Human Neutrophil – Gelatinase - Associated Lipocalin (NGAL) ELISA Kit. Results: NGAL showed significant positive correlations with eosinophil ($r = 0.676$, $P < 0.001$), CRP ($r = 0.729$, $P < 0.001$), and asthma severity ($r = 0.712$, $P < 0.001$), while it showed significant negative correlations with FEV 1 ($r = -0.723$, $P < 0.001$) and FEV/FVC ($r = -0.767$, $P < 0.001$). No significant correlations were detected with other parameters. ROC analysis was done for NGAL to distinguish severe cases. It revealed a significant AUC of 0.945 with a 95% CI ranging from 0.841 -1 ($P < 0.001$). The best cut-off point was > 190, at which sensitivity and specificity were 87.5% and 100%, respectively. Conclusions: Serum NGAL level seems to be significantly elevated in asthmatic children and can be related to asthma severity.

Keywords: Neutrophil Gelatinase-Associated Lipocalin, Childhood, Asthma.

1. Introduction

In various nations, asthma affects 1–18% of the population. It is a common, chronic respiratory condition. Fluctuating wheezing, shortness of breath, chest tightness, and/or coughing symptoms, as well as variable expiratory airflow limitation, are the hallmarks of bronchial asthma. Airflow restrictions and symptoms both typically change over time and in terms of severity. Exercise, allergy or irritant exposure, and other factors are frequently responsible for these variances, changes in the weather or respiratory virus illnesses.

The presence of symptoms and airflow restriction may occasionally last for several weeks or months at a time. They may also go away on their own or in response to medication. On the other hand, patients may endure sporadic asthma attacks (exacerbations), which can be dangerous and place a heavy strain on both the patient and the community. Asthma is typically accompanied by persistent airway inflammation and airway hyperresponsiveness to direct or indirect stimuli. Even when symptoms are absent or lung function is normal, these characteristics typically continue; however, with therapy, they may return to normal [1].

Asthma is the most common chronic disease among children. According to the World Health Organization, approximately 300 million people currently have asthma and approximately 250,000 patients die each year. Aside from its increasing prevalence, the severity of asthma also seems to be increasing in pediatric and adolescent patients, which is based on the observed increase in rates of consultations and visits into clinics, hospitals, and emergency departments [1].

The 25 kDa glycoprotein known as neutrophil gelatinase-associated lipocalin (NGAL) was first discovered as a matrix protein of certain granules of human neutrophils. In addition to neutrophils, NGAL is also secreted by adipose tissue, macrophages, respiratory and intestinal epithelial cells, vascular endothelial cells, and renal tubuli cells [2].

There are a number of theories put out there to explain Lipocalin2's functional role in inflammation. One such theory holds that granulocytes accumulate at the sites of acute or chronic inflammation (such as bronchial asthma), release their granules, which contain NGAL, and so cause local tissue injury. This suggests that serum NGAL levels are correlated with the severity of bronchial asthma. There is an up regulation in NGAL seen in the lungs of patients with bronchial inflammation both in the epithelial cells and in the type-II alveolar pneumocytes[3].

Increased MMP-9 and NGAL levels were found in broncho-alveolar lavage (BAL) fluid samples from individuals with asthma, pulmonary emphysema, and chronic obstructive pulmonary disease (COPD), likely as a result of structural changes in the airways, according to several investigations. However, it is still unclear how NGAL functions as a potential indicator of the severity of the childhood asthmatic condition [4].

Recent years have seen a tremendous increase in the number of studies that have investigated NGAL as a biomarker for both diagnosis and prognosis. Its secreted nature and the availability of commercially available robust immunoassays have contributed to NGAL emerging as a potential biomarker in a wide array of benign and malignant human diseases [5].

The aim of the study was to evaluate the association of serum NGAL with childhood bronchial asthma for diagnosis, prognosis and assessment of severity.

2. Patients and methods

This prospective, cross-sectional study was conducted on 40 asthmatic cases in Pediatrics pulmonology and Microbiology Departments, Benha University Hospital in the period from October 2021 to September 2022.

The study was done after being approved by the institutional ethical committee and informed consent was obtained from all participants included.

Cases were subdivided into four Asthma groups (intermittent, mild persistent, moderate persistent and severe persistent) and 20 healthy children representing the control group.

Inclusion criteria were a) the Asthma groups: both sexes and age below 18 years old children who were diagnosed clinically and classified according to guidelines for the diagnosis and treatment of asthma (NHLBI 2007). **b)** Control group: A control group of 20 age and sex matched healthy children were recruited from the outpatient clinic presenting with minimal medical or surgical disorders, with no history of allergic disease or wheezing.

Exclusion criteria were children with chronic diseases (e.g., malnutrition, anatomic malformation of the respiratory system, chronic lung disease, chronic heart disease, chronic kidney disease, gastro-esophageal reflux disease, cystic fibrosis, polycythemia vera) and malignancy.

Methods

Establish asthma diagnosis: adapted from 2007 NHLBI (National Heart, Lung and Blood Institute) Guidelines for the Diagnosis and Treatment of Asthma Expert Panel Report 3.

Determine that symptoms of recurrent airway obstruction are present based on history and clinical examination. History of cough, recurrent wheezing, recurrent difficulty breathing, recurrent chest tightness. Symptoms occur or worsen at night or with exercise, viral infection, exposure to allergens and irritants, changes in weather, hard laughing or crying, stress or other factors. In all patients ≥5 years of age, use spirometry to determine that airway obstruction is at least reversible. Consider other causes of obstruction.

Classification of Asthma Severity: adapted from Guidelines for the diagnosis and Treatment of Asthma Expert panel Report 3; 2007 (NHLBI).

The following entry variables were uniformly recorded: Demographic variables: Age, Sex. Full Clinical examination: Full history, examination and evaluation with pulmonary function tests (FEV1, FVC and FEV/FVC). Routine Laboratory investigations: CBC, CRP, Liver Function Test, Renal Function Tests. Special laboratory investigations: Serum NGAL level. Serum NGALconcentrations were determined with Human Neutrophil Gelatinase- Associated Lipocalin (NGAL) ELISA Kit.

Technique: Samples were taken under complete aseptic conditions, 3ml of Peripheral venous blood samples were collected by venipuncture from all patients and healthy controls. 3 ml blood in serum separate tube, then the serum was separated by centrifugation for 10 minutes at room temperature. The samples were stored at -20° C.

Principle: This ELISA kit uses Sandwich–ELISA as the method. The micro-Elisa strip plate provided in this kit has been pre-coated with an antibody specific to NGAL. Standards or samples are added to the appropriate micro-Elisa strip plate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)-conjugated antibody specific for NGAL is added to each micro-Elisa strip plate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain NGAL and HRP conjugated NGAL antibody will appear blue in color and then tum yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of NGAL. NGAL concentration is then calculated by comparing the OD of the samples to the standard curve.

Calculation of Results: Known concentrations of Human NGAL Standard and its corresponding reading OD is plotted on the log scale (x-axis) and the log scale (y-axis).

respectively. The concentration of human NGAL in sample is determined by plotting the samples OD on the y axis. The original concentration is calculated by multiplying the dilution factor.

Precision: Intra-assay precision (precision within an assay): 3 samples with low, middle and high-level Human NGAL were tested 20 times on one plate respectively, with a detection range: 8 pg/ ml - 400 pg /ml. Inter-assay precision (precision between assays): 3 samples with low, middle and high-level Human NGAL were tested on 3 different plates, 8 replicates in each plate.

Statistical Methods:

Data management and statistical analysis were done using SPSS version 28 (IBM, Armonk, New York, United States). Quantitative data were assessed for normality using the Shapiro-Wilk test. According to normality, quantitative data were summarized as means and standard deviations or medians and ranges. Categorical data were summarized as numbers and percentages. Quantitative data were compared between the studied groups using independent t-test or Mann-Whitney U test. Categorical data were compared using the Chi-square test. ROC analysis was done for NGAL to distinguish those with severe asthma. AUC with 95% CI and diagnostic indices were calculated. Correlations were done using Spearman's correlation. NGAL was compared according to gender using the

Mann-Whitney U test and severity grades using the Kruskal Wallis test. Multivariate logistic regression analysis was done to predict severe asthma. Odds ratios with 95% confidence intervals were calculated. All statistical tests were two-sided. P values less than 0.05 were considered significant.

3. Results

No significant differences were observed between the studied groups regarding age (P = 0.116) and gender (P = 0.582).

Table (1) Demographic characteristics in the studied groups

	Cases (n= 40)	Controls (n = 20)	P-value
Age (years)	9 ±3	10 ±3	0.116
Sex			
Males	23 (57.5)	10 (50)	0.582
Females	17 (42.5)	10 (50)	

Data were presented as mean ±SD or number (percentage)

Regarding asthma severity: About one-third of the patients had mild intermittent asthma (37.5%), while about one-quarter had mild persistent asthma (27.5%). Only 15% and 20% had moderate and severe asthma, respectively. **Table 2**

Table (2) Asthma severity in the studied patients

	n (%)
Mild intermittent	15 (37.5)
Mild persistent	11 (27.5)
Moderate	6 (15)
Severe	8 (20)

Regarding laboratory findings: the cases group revealed significantly higher NGAL (median=74vs50, P<0.001), eosinophil (median = 398 vs. 285, P < 0.001) and CRP (median = 6 vs. 4, P = 0.026). No significant differences were observed regarding TLC (P = 0.104), creatinine (P = 0.219), AST (P = 0.616), ALT (P=0.627), bilirubin (P = 1.0), BUN (P = 0.251). **Table (3)**

Table (3) Laboratory findings in the studied groups

	Cases (n= 40)	Controls (n = 20)	P-value
TLC	6500 (3087 - 14000)	8300 (4500 - 12000)	0.104
Eosinophil	398 (188 - 1001)	285 (90 - 411)	<0.001*
FEV1	81 ±33	101.2±7.3	<0.001*
FEV/FVC	78 ±25	100.1±7.3	<0.001*
CRP	6 (2 - 25)	4 (0 - 8)	0.026*
ALT	29 ±8	29 ±7	0.627
Creatinine	0.92 ±0.24	1 ±0.22	0.219
AST	24 (5 - 51)	23 (4 - 42)	0.616
Bilirubin	1 ±0.1	1 ±0.1	1.0
BUN	14 (6 - 22)	12 (4 - 22)	0.251
NGAL	74 (42 - 255)	50 (45 – 60)	<0.001*

* Significant; Data were presented as mean ±SD or median (range)

NGAL showed significant positive correlations with eosinophil (r = 0.676, P < 0.001), CRP (r = 0.729, P < 0.001), and asthma severity (r = 0.712, P < 0.001), while it showed significant negative correlations with FEV 1 (r = -0.723, P < 0.001) and FEV/FVC (r = -0.767, P < 0.001). No significant correlations were detected with other parameters. **Table 4**

Table (4) Correlation between NGAL and other parameters in the studied patients

	NGAL	
	r	P
Age (years)	-0.01	0.949
TLC	-0.119	0.465
Eosinophil	.676	<.001*
FEV1	-.723	<.001*
FEV/FVC	-.767	<.001*
CRP	.729	<.001*

ALT	0.28	0.08
Creatinine	0.09	0.582
AST	-0.213	0.186
Bilirubin	-0.079	0.627
Asthma severity	.712	<.001*
BUN	0.265	0.099

* Significant; r: Correlation coefficient

There is statistically significant difference between the studied groups in NGAL except between; (Control group and Mild intermittent group), (Control group and Mild persistent group) and (Mild intermittent group and Mild persistent group) which show no significant difference in-between. **Table 5**

Table (5) Multiple comparisons table showing the significance difference within the studied groups in NGAL

Compared Groups		
(A)	**(B)**	**p**
Control	**Mild intermittent**	**0.3**
	Mild persistent	**0.5**
	Moderate persistent	**0.002***
	Severe persistent	**0.001****
Mild intermittent	**Mild persistent**	**0.1**
	Moderate persistent	**0.002***
	Severe persistent	**0.001****
Mild persistent	**Moderate persistent**	**0.001****
	Severe persistent	**0.001****
Moderate persistent	**Severe persistent**	**0.001****

NGAL showed an overall significant difference between different asthma grades (P < 0.001). Post hoc analysis revealed significantly lower NGAL in mild cases (median = 60) than in moderate and severe cases (median = 162 and 233, respectively). No significant difference was observed in NGAL according to gender (P = 0.607). **Figure 1**

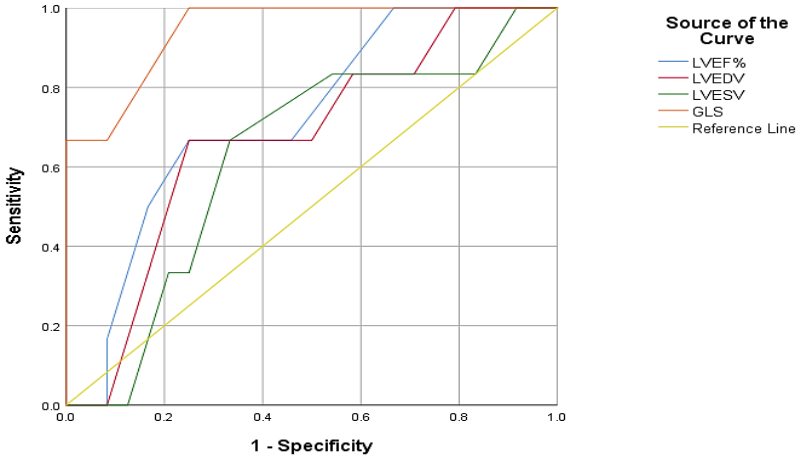

Fig. (1) NGAL according to severity grades

ROC analysis was done for NGAL to distinguish severe cases. It revealed a significant AUC of 0.945 with a 95% CI ranging from 0.841 -1 (P < 0.001). The best cut-off point was > 190, at which sensitivity and specificity were 87.5% and 100%, respectively. **Figure 2**

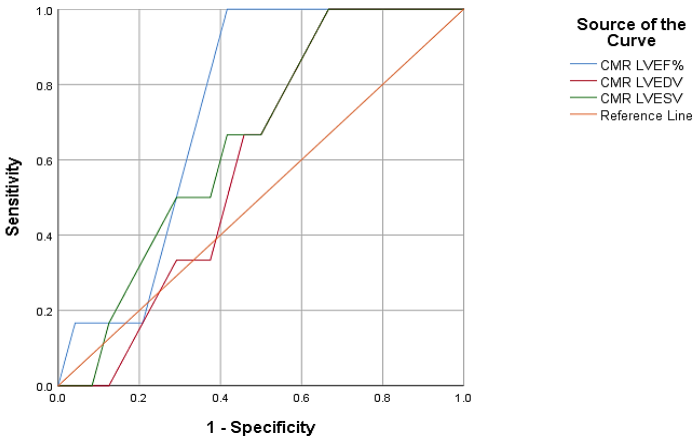

Fig. (2) ROC analysis of NGAL to predict severe asthma

Multivariate logistic regression analysis was done for the prediction of severe cases. It revealed that NGAL was a significant predictor for severe asthma (OR = 1.037, 95% CI =1.012 – 1.064, P = 0.004), controlling for age and gender **Table 6.**

Table (6) Multivariate logistic regression analysis to predict severe asthma

	OR (95% CI)	P-value
Age (years)	0.867 (0.558 - 1.348)	0.526
Sex	0.935 (0.083 - 10.554)	0.956
NGAL	1.037 (1.012 - 1.064)	0.004*

4. Discussion

Asthma is the most common chronic disease among children. According to the World Health Organization, approximately 300 million people currently have asthma and approximately 250,000 patients die each year. Aside from its increasing prevalence, the severity of asthma also seems to be increasing in pediatric and adolescent patients, which is based on the observed increase in rates of consultations and visits into clinics, hospitals, and emergency departments [6].

In the present study no significant difference was found between male and female in the healthy controls and four asthmatic groups regarding serum NGAL level. Our findings were consistent with a previous study of analytical characterization and clinical testing of serum lipocalin-2 by Stejskal et al. [7] which showed that there was no difference between male and female serum NGAL level. In contrast, a previous study by Cullen et al. [8] showed a significant gender-related differences for NGAL where female levels were significantly higher. The difference in serum NGAL level between genders may be explained as recent evidence suggests that the use of a single cut-off value may be inappropriate under some circumstances and leads to confounding results.

In the study of acute phase reactants in allergic airway disease by Buyukozturk et al. [9] & the study of Nacaroglu HT, et al. [10] The mean CRP in asthma groups were not significantly different when compared to the control group. The relationship between CRP levels and asthma is a complicated interaction affected by multiple parameters which may explain the discrepancy between different studies.

In the present work, we compared the mean absolute eosinophilic count (AEC) between healthy controls and asthmatic children's groups, and it was found to be significantly higher in asthmatic children. We also found that there was high significant elevation in AEC in severe persistent group than in other asthmatic groups.

In accordance with our study Chaudhary et al. [11] in a study aimed to compare total serum immunoglobulin E and absolute eosinophil count levels among asthmatic and non-asthmatic children, conducted in the Department of Pediatrics and TB and Chest of the Medical College of North India from October 2015 to November 2016, where children from 3 years to 18 years (30 with bronchial asthma and 30 healthy children) were included in his study randomly. The mean AEC was significantly higher in cases as compared to healthy children. In bronchial asthma group, mean AEC increased with the increasing severity of asthma. This finding was in accordance with the finding of many previous studies done by El-Zohery et al. in [12] and Devi et al. in [13].

However, in comparison to our study regarding eosinophils count but upon analysis of bronchial biopsy specimens between different asthmatic children's groups and non-asthmatic children demonstrated in four previous

reports: Cokugras H et al. [14]; Payne DN et al. [15]; Jenkins HA et al. [16]; Payne DN et al. 2004

reported that there were no significant differences in eosinophils numbers between any of the groups.

A possible explanation for the discrepancy between different studies regarding eosinophil level in bronchial asthma is that children in some studies may have been undergoing treatment with high-dose anti-inflammatory agents, which may have substantially reduced eosinophil numbers [17].

Similar to our study regarding eosinophils count; Yi-Giien Tsai et al. [18] demonstrated that there is significantly higher serum IgE and Eosinophils count in severe persistent asthmatic children compared to milder asthmatic and healthy children. Also, Liu W et al. [19] reported higher blood eosinophils and IgE levels in severe asthmatic patients. Akelma et al. [4] also reported that total serum eosinophil numbers and IgE levels were significantly higher in the asthma group than in the control group.

High AECs and serum IgE levels were constantly associated with bronchial asthma as compared to non-asthmatics.

Our study revealed that there was highly significant elevation in serum NGAL level in asthmatic patients in comparison to control group. In accordance with our study Karakoc GB et al. [20] demonstrated that MMP-9 and NGAL levels in BAL in children with asthma were significantly higher than those in healthy controls. Other several studies performed on patients with asthma, pulmonary emphysema, chronic obstructive pulmonary disease (COPD) revealed increased MMP-9 and NGAL levels in broncho-alveolar lavage (BAL) fluid samples [21-23].

In a previous study by Cockayne et al. [24] to investigate Systemic Biomarkers of Neutrophilic Inflammation in COPD Patients. Serum NGAL was found to be higher in COPD patients and related to disease severity. In contrast to our study regarding serum NGAL; Akelma et al. [4] reported that the difference in serum NGAL levels between the asthma groups was insignificant demonstrating that similar NGAL levels between the subgroups (mild intermittent, mild persistent and moderate persistent) were found but severe persistent asthma group was not included in the study.

We found that there was high significant elevation in serum NGAL level in severe persistent asthmatic group than in healthy controls and other asthmatic groups.

In agreement with our results Karakoc et al. [20] demonstrated that broncho-alveolar lavage (BAL) MMP-9 and NGAL levels in children with persistent asthma were significantly higher than those in children with intermittent asthma and healthy controls.

In our study no significant correlation was found between serum NGAL and age or sex of the studied subjects. On the other hand, serum NGAL level showed statistically high significant negative correlation with FEV1/FVC and FEV1 especially in severe persistent asthmatic group.

Our observation agrees with Nacaroglu et al. [10] study in which there was a statistically significant negative correlation between FEV1/FVC and serum NGAL level.

Also, in consistence with our findings, broncho alveolar lavage NGAL levels were inversely correlated with both FEV1 and peak expiratory flow (PEF) values in children with asthma Karakoc et al. [20]. Similarly, Eagan TM et al. 2010 in the study of NGAL as a biomarker in COPD found that pulmonary function tests showed negative correlation with NGAL Plasma level. In contrast with our results Sarra Bchir et al. [25] in a study of Concomitant elevations of MMP-9, NGAL, proMMP-9/NGAL and neutrophil elastase in serum of patients with COPD, found that FEV/FVC and FEV1 was not correlated with serum NGAL level. Also, that was reported in three previous studies carried out by Bolton CE et al. [26]; Kwiatkowska S et al.[27]; Ropcke S et al.[28] in which serum MMP-9 and NGAL levels in COPD were not significantly correlated with the spirometry variables (FEV/FVC, FEV1).

Comparing the results of different studies, it is interesting that some identified correlation of systemic biomarkers such as NGAL with pulmonary function parameters, while others did not. This could be due to a number of reasons. Systemic biomarkers association with lung function parameters could be affected by the presence or absence of other factors such as coexisting systemic inflammation and extra pulmonary comorbidities such as in metabolic syndrome and whether that the studied subjects were well-matched and did not display any significant differences with respect to demographic covariates or not , also drug intake as theophylline and/or oral systemic steroids has a possible confounding effect [24].

Regarding NGAL level correlation with CRP level and absolute eosinophilic count our results showed a statistically significant positive correlation in asthma groups. On the other hand, NGAL did not show correlation with the other studied parameters in our study (age, sex and liver & kidney function tests) in asthmatic groups.

In agreement with our results in children with asthma Akelma et al. [4], measured serum CRP levels to exclude possible co-morbid infections, stated that a weak positive correlation was noted between NGAL and CRP levels. Similarly in a study purposed to demonstrate the importance of NGAL for COPD-AE as a current inflammation marker and reveal its link to other acute inflammation indicators such as CRP level. On the other hand, Eagen TM et al. 2010, found no significant correlation between CRP level and NGAL levels.

Several hypotheses have been suggested to explain the functional role of Lipocalin2 in inflammation. One hypothesis is that acute (predominantly) or chronic inflammation (e.g., bronchial asthma) leads to the accumulation of granulocytes at the sites of inflammation. These granulocytes undergo apoptosis,

release their granules (containing NGAL) and there by mediate local tissue injury which suggests serum NGAL correlation to bronchial asthma severity [29].

Cowland JB et al. [30] reported that in normal human lung tissue, NGAL is present constituently within tracheal goblet cells and type II pneumocytes, there is an up regulation in NGAL seen in the lungs of patients with bronchial inflammation both in the epithelial cells and in the type-II alveolar pneumocytes, also Mori K et al. [31] reported that NGAL expression is increased in case of bowel and respiratory inflammation, in particular in the setting of bacterial infections.

5. Conclusion

Based on our results, serum NGAL level seemed to be higher in asthmatic children and could be related to asthma severity. We suggest that NGAL may be used as a potential marker of bronchial asthma in children and can be helpful in determining the severity of the disease. However, these results need to be confirmed by further studies conducted on larger groups to identify the role of NGAL in childhood asthma and to determine its potential use as a clinical marker.

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