EVALUATION OF SERUM EOSINOPHIL-DERIVED NEUROTOXIN IN CHILDREN AS A BIOMARKER FOR ASTHMA DIAGNOSIS AND CONTROL

By

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ABSTRACT

Background: *Asthma symptoms include wheezing, coughing, dyspnea, and chest tightness, as well as variable degrees of airway blockage and hyper-responsiveness. Many biomarkers have been widely studied in asthmatic children for asthma diagnosis and monitoring. Clinical decisions related to asthma control status have traditionally been based on subjective symptoms, rescue medication use, and lung function changes. Biomarkers for reflecting asthma control status remain controversial. Although a standard definition of eosinophilic asthma has not been developed yet, peripheral blood eosinophil counts of ≥150 cells/μL, ≥300 cells/μL, or ≥400 cells/μL have been used in trials on adult populations to describe eosinophilic asthma and can readily be identified in a primary care setting. Specific biomarkers of eosinophilic asthma in children have been widely used, including blood eosinophil count, sputum eosinophil count (%), fractional excretion of nitric oxide (FeNO), and serum periostin level. One of the emerging biomarkers is Eosinophil-derived neurotoxin (EDN), which is a degranulated eosinophil protein. Bronchoconstriction and hyper-responsiveness are more closely associated with the presence of eosinophil-derived neurotoxic and the eosinophil-cationic protein (ECP) in the inflamed airways.*

Aim of the work: *To assess Serum EDN levels as a potential biomarker for the detection of asthma control status in children as well as to distinguish asthmatic from those healthy non allergic children.*

Subjects& methods: *this is a cross sectional study carried out on 62 asthmatic children who are on regular follow-up at Pediatric Allergy Outpatient Clinics, Al-Azhar university Hospitals (Al-Hussein & Sayed-Galal Hospitals). The study was conducted between August 2021 and March 2022. Serum EDN concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit, with results expressed in nanograms per milliliter (ng/ mL).*

Results: *There was a highly significant value of serum EDN in discrimination between asthmatic and healthy non allergic children with a sensitivity of 91.94 % and specificity of 96.67% at cutoff point is 55 ng/ml. there was a prognostic performance value of eosinophil derived neurotoxin in discrimination between uncontrolled and controlled asthmatic children with a sensitivity of 75.0% and specificity of 56.67% when the cutoff point is 75 ng/ml.*

Conclusion: *Serum EDN may be used as helpful biomarker beside clinical assessment to distinguish between asthmatic children from healthy non allergic children .also can be used as marker for discrimination of control status in asthmatic children and monitoring controller drugs.*

Keywords: *EDN, major basic protein, asthma Biomarker, children.*

INTRODUCTION

Asthma is the most prevalent chronic illness affecting children and adolescents worldwide, and it significantly increases morbidity and mortality rates in this age group **(Jones et al., 2022)**. An important component of allergy illness is the eosinophil. As a result, for the diagnosis, treatment, and monitoring of asthma, direct assessment of eosinophilic inflammation is required **(Kim et al., 2017)**. The prevailing thinking on eosinophilic inflammation monitoring is that eosinophil counts give a limited understanding of the activity of these cells, whereas the secretory activity of eosinophils (their tendency to release mediators) gives a more accurate and complete picture of the situation **(Kim et al., 2017)**. The increased production of various cytokines from T-helper 2 cells, such as IL-3, IL-4, and IL-5, which are key elements in allergic asthma leads to increased blood eosinophil count Airway remodeling, is a frequent hallmark of asthma, even in young children, although not in the very early stages **(Fehrenbach et al., 2017)**. Asthma therapy aims to achieve clinical control and minimize the patient's future risks. In order to achieve this aim in children with asthma, continuous monitoring is required **(Szefler et al., 2020)**. Interleukins IL-4, IL-5, and IL-13 are three types of type 2 cytokines that have been linked to asthma so far. Eosinophilic inflammation is best treated with corticosteroids (CS), however biologics that target type 2 inflammation are helpful in sever asthma with a high total eosinophilic count (TEC) **(Porsbjerg et al., 2020)**. Conventional measures such as serum periostin levels, TEC, sputum eosinophil counts (percent), and fractional excretion of nitric oxide (FeNO) concentration have all been used

to assess eosinophilic inflammation in asthma. However, investigations of TEC predicting sputum eosinophil levels in eosinophilic asthma have showed different results. Sputum eosinophil count and FeNO concentration are generally difficult to obtain and vary by CS therapy **(Lee et al., 2019)**. The most significant role in asthma pathogenesis is played by the eosinophil granule proteins [Eosinophil-derived neurotoxin (EDN), Eosinophil cationic protein (ECP), major basic protein (MBP), and eosinophil peroxidase (EPO)] **(Gleich & Adolphson, 1999)**. But eosinophils are the only cells that primarily produce EDN and ECP. Therefore, any variation in EDN or ECP level would be a good indication of eosinophilic inflammation **(Kim et al., 2017)**. Some studies have shown a stronger relationship between eosinophil granule proteins, such as eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), and eosinophil airway inflammation, which causes bronchoconstriction and hyper responsiveness **(Granger et al., 2022)**. Biomarkers like EDN, which measures eosinophil activity by counting and grading granules released by the eosinophils, are rather uncommon. Eosinophilic airway inflammation develops more rapidly when EDN levels rise, and this might signal that asthma medication is failing (Rodriguez Del Rio et al., 2022). The purpose of this study was to assess the role of serum EDN levels beside clinical assessment to distinguish asthmatic from healthy non allergic children as well as to evaluate its use as a potential biomarker for detecting control status in asthmatic children.

Sample size:

Assuming that mean + SD of Eosinophil Derived Neurotoxin in bronchial asthma group and in control group was 76.23 ± 14.37 versus 34.77 ± 10.69 respectively. So, sample size was calculated by Open Epi program to be 84 cases (28 in each group): confidence level 95 % & power of test 80%.

Ethical consideration:

Ethical Approval and Consent to participate:

The Faculty of medicine –Al-Azhar University Ethics Research Committee approved the study, which was conducted following the Declaration of Helsinki. Written informed consent was obtained from the pregnant women who enrolled in this study .Also, the study was approved by

the pediatric department Ethics Committee.

Participants or their guardians were given informed consent that includes the aim and steps of the study.

The patient had the right to withdraw from the study at any time.

Availability of data and materials:

The data that support the findings of this study are available on request from the corresponding author.

Competing interests:

The authors declare that they have no competing interests.

Funding:

The authors received no financial support for the research, authorship, and/or publication.

SUBJECTS AND METHODS

This study carried out on 92 school children ,62 asthmatic children aged asthmatic children (7-16 y) who were on regular follow up at Pediatric Allergy Outpatient Clinics, Al-Azhar University Hospitals (Al-Hussein & Sayed-Galal Hospitals) and 30 apparently healthy child age matched with no history of asthma, atopy or other allergic diseases were enrolled as control group.

It was conducted between August 2021 and March 2022. All children who participating in the study were diagnosed as having asthma according to the Global Initiative for Asthma guidelines **(GINA., 2009)**.

Inclusion criteria: Eosinophilic Asthmatic with blood eosinophil count more than 150/µl was used to determine the type of asthma according to the guidelines of the International European Respiratory Society/American Thoracic Society **(Chung, 2016)**.

Children aged 7-16y with confirmation of the asthma diagnosis by dynamic spirometry pre & post- bronchodilators.

Exclusion criteria:

1. Children who receive any medications regularly for asthma other than bronchodilators in last 3 months.
2. Children with history of low birth weight or NICU admission, or who needed prolonged oxygen therapy.
3. Children with respiratory tract infections.
4. Children with acute or chronic systemic disorders, and who refusal to do spirometry technique, or suffer from respiratory conditions other

than asthma were excluded from the study.

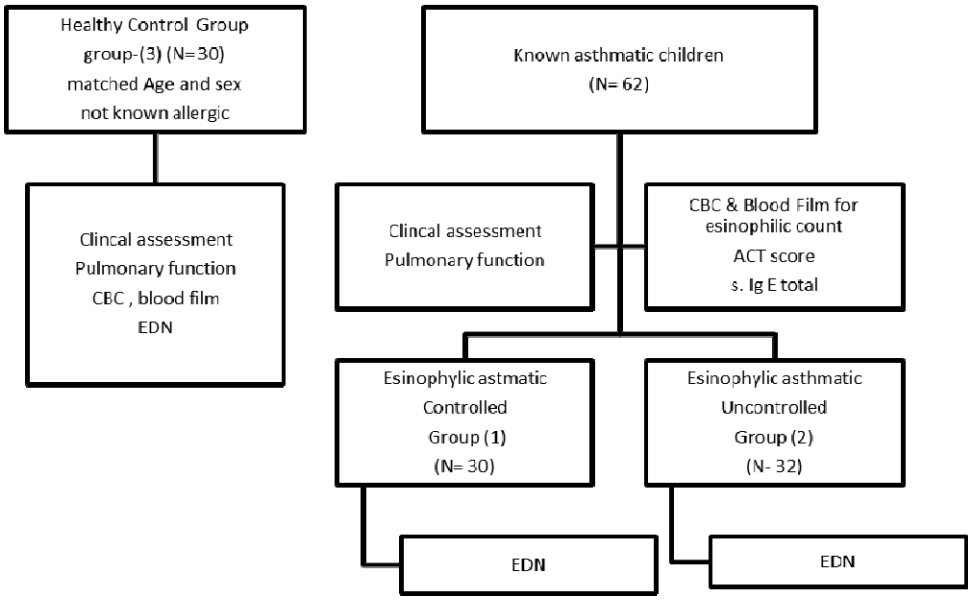
5. Children who had upper airway blockage, also excluded from the trial.

Methodology: all children subjected to:

1. Detailed history taking.
2. Thorough Physical examination, anthropometric assessment,
3. Oxygen saturation (SO_2) measurement by pulse oximetry, and,
4. Laboratory assessment including:
   a. Complete blood count (CBC) with differential,
   b. Serum EDN assay were done for all participants., and serum IgE .(Venous blood samples (6 ml) were withdrawn from all subjects of the study under complete aseptic conditions from the antecubital vein. The first portion of the sample (2 ml) was collected in EDTA tubes for CBC analysis and eosinophil counting. The second portion (4 ml) was collected in sterile red (plain) tubes, centrifuged, and the serum was collected and refrigerated at -80°C for the evaluation of EDN).
5. Pulmonary function were done for all children by a handled computerized spirometer *(Spirostik equipment and blue cherry software from Gerathem Respiratory, 2016)*.

Asthmatic children were divided into 2 subgroups according to Childhood Asthma Control Test (C-ACT) for children 4-11 years old, and Asthma Control Test (ACT) for children 12 years or older. Both questionnaires are consistent with The National Asthma Education and Prevention Program (NAEPP) and GINA guidelines. A cut-off point of 19 for C-ACT and ACT indicates uncontrolled asthma *(Koolen, 2011)*.

Study Design:



Statistical analysis of the data:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp).Comparisons between groups for categorical variables were assessed using Chi-square test was applied to compare between two groups. Alternatively, Student t-test was used to compare two groups for normally distributed quantitative variables. Fisher Exact correction test was applied when more than 20% of the cells have expected count less than. Mann Whitney test, ANOVA and Post Hoc test (Tukey) were used as needed.

RESULTS

Our result showed 62 asthmatic children who were divided into two groups group (1): 30 asthmatic controlled children. Group (2) 32 uncontrolled asthmatic children and 30 apparently healthy children as control group.

Table (1): Comparison between the studied groups according to clinical and laboratory data

	Controlled asthma (n = 30)	**Uncontrolled asthma (n = 32)**	**Healthy Control group (n = 30)**	**P**
Age (years)				
Mean ± SD.	10.20 ± 2.89	10.69 ± 2.89	10.37 ± 2.82	0.711
Gender				
Male	19 (63.3%)	15 (46.9%)	16 (53.3%)	0.426
Female	11 (36.7%)	17 (53.1%)	14 (46.7%)	
BMI (kg/m^2)				
Mean ± SD.	22.83 ± 3.27	22.56 ± 3.88	23.37 ± 2.37	0.616
WBC (1000/mL)				
Mean ± SD.	7.07 ± 1.66	7.47 ± 1.62	7.07 ± 1.66	0.538
Neutrophil count (1000/mL)				
Mean ± SD.	3.47 ± 0.46	3.59 ± 0.54	3.32 ± 0.28	0.096
Eosinophil count (1000/mL)				
Mean ± SD.	289.0 ± 66.38	291.81 ± 55.94	45.97 ± 12.33	<0.001*
Sig. bet .Grps.	p_1=0.974,p_2<0.001*,p_3<0.001*			
Esinophil derived neurotoxin (ng/mL)				
Mean ± SD.	70.23 ± 14.37	80.88 ± 11.16	34.77 ± 10.69	<0.001*
Sig. bet .Grps.	p_1=0.026*,p_2<0.001*,p_3<0.001*			
Total IgE (IU/ml)				
Mean ± SD.	234.0 ± 60.14	209.8 ± 71.59	30.87 ± 10.69	<0.001*
Sig. bet .Grps.	p_1=0.514,p_2<0.001*,p_3<0.001*			

p: p value for comparing between the three studied groups, p1: p value for comparing between Group 1& Group 2
p2: p value for comparing between Group 1 & Group 3, p3: p value for comparing between Group 2& Group 3

Table (1) shows the basic clinical and laboratory data of the studied asthmatic children and controls. Our results showed statistically significant difference among the three groups regarding eosinophilic count, Eosinophil derived neurotoxin and total serum IgE which were higher among uncontrolled asthmatics children than controlled asthmatic and healthy control group .no statistically significant difference in absolute neutrophilic count among groups.

Table (2): Comparison between controlled and non-controlled asthmatic children

	Controlled asthma (n = 30)	Uncontrolled asthma (n = 32)	P
Duration of asthma (years)			
Mean ± SD.	5.80 ± 1.69	6.25 ± 1.70	0.301
Allergic Rhinitis	9 (30.0%)	8 (25.0%)	0.659
Atopy	4 (13.3%)	2 (6.3%)	^{FE}p=0.418
Severity of asthma			
Mild	20 (66.7%)	25 (78.1%)	0.312
Moderate	10 (33.3%)	7 (21.9%)	
ACT score			
Mean ± SD.	23.50 ± 1.57	12.59 ± 2.75	<0.001*
ICS	13 (43.3%)	10 (31.3%)	0.325
SABA (oral or inhaled)	19 (63.3%)	18 (56.3%)	0.570
Nasal steroid	6 (20.0%)	3 (9.4%)	^{FE}p=0.294

Table (2) shows other significant data about multi-allergic comorbidity and medication .there was statically significant difference in ACT score between uncontrolled asthmatic and controlled asthmatics.

Table (3): Comparison between the three studied groups according to pulmonary function parameters pre and post bronchodilators (B2 agonist)

	Controlled asthma (n = 30)	Uncontrolled asthma (n = 32)	Healthy group (n = 30)	p
FVC (% of predicted)				
Pre-BD				
Mean ± SD.	81.37 ± 5.68	83.13 ± 4.98	93.63 ± 2.88	<0.001*
Sig. bet .Grps.	p_1=0.367,p_2<0.001*,p_3<0.001*			
Post-BD				
Mean ± SD.	90.20 ± 8.54	92.94 ± 6.35	95.03 ± 4.11	0.053
Z(p_0)	4.037* (<0.001*)	4.622* (<0.001*)	1.514 (0.130)	
FEV1				
Pre-BD				
Mean ± SD.	73.57 ± 3.73	71.03 ± 3.54	92.03 ± 8.21	<0.001*
Sig. bet .Grps.	p_1=0.123,p_2<0.001*,p_3<0.001*			
Post-BD				
Mean ± SD.	95.97 ± 8.17	96.50 ± 8.30	96.73 ± 7.70	0.917
Z(p_0)	4.784* (<0.001*)	4.941* (<0.001*)	1.735 (0.083)	
FEV1 / FVC ratio				
Pre-BD				
Mean ± SD.	78.27 ± 4.48	80.56 ± 4.78	96.07 ± 5.38	<0.001*
Sig. bet .Grps.	p_1=0.219,p_2<0.001*,p_3<0.001*			
Post-BD				
Mean ± SD.	92.10 ± 9.70	94.88 ± 6.52	96.67 ± 5.40	0.124
Z(p_0)	4.370* (<0.001*)	4.956* (<0.001*)	0.478 (0.632)	

p0: p value for comparing between Pre-BD and Post-BD .p1: p value for comparing between Group 1 and Group 2. p2: p value for comparing between Group 1 and Group 3. p3: p value for comparing between Group 2 and Group 3

Table (3) shows that different pulmonary function parameters which were significantly lower in asthmatic children than healthy control with significant improvement after bronchodilator inhalation.

Table (4): Correlation between eosinophil derived neurotoxin and different parameters in patients with asthma (n = 62)

		EDN (ng/mL)	
		r_s	P
Age (years)		0.262	0.040*
BMI (kg/m²)		0.121	0.348
WBC (1000/mL)		-0.163	0.205
Neutrophil count (1000/mL) (ns		0.132	0.308
Eosinophil count (1000/mL) (si		0.252	0.048*
Total IgE (IU/ml)		-0.001	0.996
FVC (% of predicted)	**Pre-BD**	0.137	0.287
	Post-BD	0.132	0.305
FEV1	**Pre-BD**	-0.343	0.006*
	Post-BD	0.065	0.614
FEV1 / FVC ratio	**Pre-BD**	0.104	0.422
	Post-BD	0.157	0.223

Table (4): shows the correlation between eosinophil derived neurotoxin and different parameters in asthmatic children (n = 62) is positively correlated with total IgE, and eosinophilic count and negatively with neutrophilic count and all spirometer parameters.

Table (5): Univariate and multivariate Logistic regression analysis for the parameters affecting uncontrolled vs controlled asthmatic children

	Univariate		#Multivariate	
	P	**OR (LL – UL 95%C.I)**	**P**	**OR (LL – UL 95%C.I)**
Eosinophil count (1000/mL)	**0.854**	1.001(0.993 – 1.009)		
Eosinophil derived neurotoxin (ng/mL)	**0.004***	1.068(1.021 – 1.118)	0.026*	1.054(1.006 – 1.104)
Total IgE (IU/ml)	**0.159**	0.994(0.987 – 1.002)		
Duration of asthma (years)	**0.296**	1.174(0.869 – 1.586)		
FEV1 (Pre-BD)	**0.011***	0.830(0.720 – 0.958)	0.099	0.879(0.755 – 1.025)
FEV1 / FVC ratio (Pre-BD)	**0.060**	1.115(0.995 – 1.249)		

#: All variables with $p<0.05$ was included in the multivariate

Table (5) shows univariate and multivariate Logistic regression analysis for the parameters affecting asthma control showed that significant value of EDN as multivarient factor in assessment of control status.

Table (6): Prognostic performance for Eosinophil derived neurotoxin (ng/mL)

	Discrimination of asthmatics from healthy children	Discrimination of uncontrolled from controlled asthmatics
AUC	0.997	0.740
P	<0.001*	0.001*
95% C.I	0.990 – 1.0	0.612 – 0.867
Cut off	>55	>75
Sensitivity	91.94	75.0
Specificity	96.67	56.67
PPV	98.3	64.9
NPV	85.3	68.0

Table (6) shows Prognostic performance for Eosinophil derived neurotoxin (ng/mL) to discriminate asthma from healthy group with cutoff value of 55ng/ml and also discrimination uncontrolled asthma from controlled asthma with cutoff value of 75ng/ml.

ROC curve for Eosinophil derived neurotoxin (ng/mL) to discriminate asthma from healthy group. **Figure (2):** ROC curve for Eosinophil derived neurotoxin (ng/mL) to discriminate uncontrolled asthma from controlled asthma.

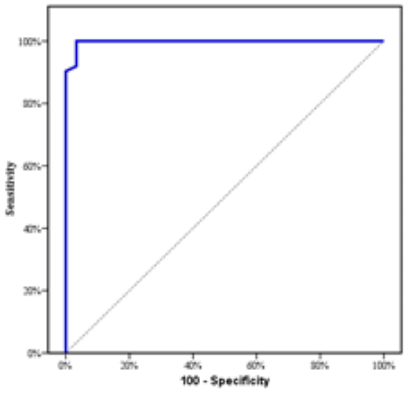


Figure (1)

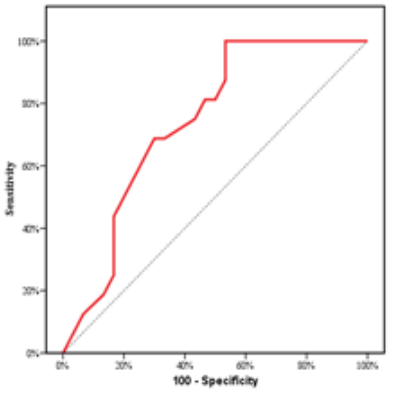


Figure (2)

DISCUSSION

Accurate Asthma diagnosis and establishment of asthma symptoms control is the goal of asthma management. Type 2 bronchial inflammation (eosinophilic type) is pivotal in allergic children. Many studies were conducted to validate useful and available biomarkers in clinical practice for asthma diagnosis and control grading. There are many therapeutic options are available and all doctors are in need for a biomarker to evaluate the effect of asthma treatment and patient response. EDN level has been proposed as a valuable marker to assess eosinophilic inflammation **(Kim et al., 2017)**. This study aimed to assess the serum eosinophil derived neurotoxin (EDN) level as a helping biomarker to distinguish asthmatic from non-allergic healthy children also to assess it value to determine the control status in children who are already diagnosed.

As regard demographic and laboratory data **(Table 1)** of the studied groups we found that there is no statically difference among studied groups regarding sex, age ,absolute neutrophilic count HbA1c and BMI our data agreed with **(Al-Adawy et al., 2018**; **Szefler et al., 2020)**.

Our study showed that all asthmatic children with controlled asthma symptoms (ACT score less than 19) had significantly lower level of serum EDN, total IgE and eosinophilic count (p-value <0.001 for each) compared with uncontrolled asthmatic children. **(Table 1)** These results agreed with **(Nagao et al., 2018)** who reported that total serum EDN (p-value <0.001), IgE (p-value <0.001), and eosinophilic count (p value <0.001) is higher in both well controlled and non-controlled asthmatics than the healthy controls. And explained by elevated EDN level has been linked to eosinophilia and Th2-mediated inflammation and type 2 airway inflammation is the common type in childhood asthma.

As regard duration of asthma, allergic-comorbidities and the airway inflation and medication used by the asthmatic children **(Table 2)** there was no statistically significant difference between groups as serum EDN level may be affected by some medication as systemic corticosteroid and leukotriene antagonist which was reported by **(Howarth et al., 2020)** and **(Jones et al., 2022)**.

Kim et al., reported that leukotriene receptor antagonists showed reduction in EDN levels from elevated to normal values

(Kim et al., 2018) and no any child included in our study received leukotriene receptor antagonists in last 3 months.

Jin an et al., 2020 reported that eosinophil derived neurotoxin can be considered as biomarker for control state and treatment efficacy. Likewise, in adult asthmatic patients, EDN level might be a valuable biomarker to determine the administration and monitoring the efficacy of biologics treatment.

Our study also revealed significant positive correlation between serum EDN and Age (P-value=0.040), eosinophil counts (p-value=0.048) **(Table 4)** and negative correlation with FEV1 pre-BD (p-value=0.006), and no correlation with absolute neutrophil counts (p-value=0.308) among all asthmatic children regardless control status (table 4) this data agreed with **(Amer et al., 2020)** and **(Kim et al., 2017)** who reported that serum EDN level was higher in asthmatic when compared to healthy controls. This agreed also with **(Nagao et al., 2018)** who studied Eosinophil-Derived Neurotoxin (EDN) as biomarkers for diagnosis of bronchial asthma in children. They reported that EDN was significantly increased in asthmatic children when compared to controls.

(Kim, 2013) study also reported a significant increase in serum EDN levels in symptomatic versus asymptomatic asthmatic patients.

An et al., 2020 showed a significant negative correlation between serum EDN levels and pulmonary function (FEV1 and FEV1/FVC) with asthma in adult. Our findings of the correlation between FEV1 pre-BD and serum EDN levels were similar to those of previous studies however, the difference was not statistically significant this can be explained by small number of patients in those studies and larger studies are needed to confirm the correlation between EDN levels and lung function.

Regression analysis in our study **(Table 5)** univariate and multivariate showed that EDN can be used as promising biomarker for diagnosis of control status in asthmatic children this data agreed with Jin an et al., who reported that serum EDN level was an independent biomarker for indicating uncontrolled asthma status in regression analysis **(An et al., 2020)**.

According to AUC in the ROC analysis **(Table 6)** between asthmatic and healthy children our

result showed that EDN level with cutoff value of 55 ng/ml can distinguish asthmatic form non asthmatic children. Our result differ from data reported **kim et al., 2017** who reported the cutoff value for asthma diagnosis was 44.2 ng/mL, sensitivity was 81.3%, specificity was 87.1%, positive predictive value was 90.7%, and negative predictive value was 75.0%. **(Kim et al., 2017)**. This can be explained by difference in inclusion and exclusion criteria and some children in our study had allergic multi-morbidity (asthma and allergic rhinitis).

As regard EDN value for diagnosis of control status in asthmatic children ROC analysis and AUC =0.740 (table 6) showed cutoff value of 75 ng/ml can distinguish controlled asthmatic children from uncontrolled asthmatics .our result agreed with Jin an et al., who carried his study in adult and reported that ROC analysis, serum EDN level showed a significantly better performance for predicting uncontrolled asthma status (area under the curve, (0.726) and EDN can be used not only for monitoring the treatment, but also for diagnosis of asthma.

CONCLUSION

Serum EDN level is a helpful biomarker for diagnosis of uncontrolled asthma status with cutoff value 75ng/ml and practically is better than subjective data obtained by scoring systems. serum EDN level is as powerful diagnostic tool for confirming diagnosis of asthma aided by clinical assessment and pulmonary function with cutoff value 55ng/ml.

Limitations of the study:

Cross-sectional design, the lack of direct bronchial inflammatory biomarkers, and the limited number of patients. Limitation of calculation of cumulative dose of inhaled corticosteroids or nasal steroids used by the both asthmatic groups of children and its correlation with EDN level.

In addition, we did not track changes in serum EDN levels over time and their relation to asthma treatment.

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