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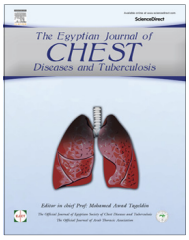

ORIGINAL ARTICLE

Nasal lavage fluid nuclear factor kappa B and cytology in asthmatic children and their correlation with severity and control

Eman M. Fouda [a], **Terez B. Kamel** [a,*], **Manal M. Abd Al-Aziz** [b], **Amro N. Atyia** [a]

[a] *Department of Pediatrics, Faculty of Medicine, Ain Shams University, Cairo, Egypt*
[b] *Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt*

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KEYWORDS

Nasal lavage;
NFκB;
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Abstract *Background:* Asthma is the most common chronic inflammatory disease in childhood. The relevance of NFκB which is known to be an inflammatory marker in upper airway epithelium and its relation to lower airway inflammation has not been fully studied in childhood asthma.

Aim of study: The study aimed at evaluating the diagnostic value of nasal lavage nuclear factor kappa B and cells as a less-invasive bench-side maneuver and inflammatory biomarkers in asthmatic children and correlating with asthma severity.

Methods: This case-control study recruited 60 asthmatic children from Pediatric Chest Clinic, Children's Hospital; Ain Shams University. Thirty healthy non-asthmatic children-age and sex-matched were included as a control group. Nasal lavage cytology, nasal lavage NFκB and forced expiratory volume in 1 s (FEV1) % of predicted for age and sex were estimated.

Results: Nasal lavage NFκB levels were significantly higher in asthmatics than in controls with a mean of 0.129 ± 0.113 μg/μg nuclear proteins and 0.0176 ± 0.013 μg/μg nuclear proteins, respectively. Nasal lavage NFκB and eosinophil levels were significantly higher with increasing asthma severity and with worsening levels of asthma control. Nasal lavage NFκB showed a sensitivity of 87% and a specificity of 87% in predicting asthma severity.

Conclusions: Despite that spirometry and clinical classification are the gold standards for grading of asthma, Nasal lavage NFκB and cells can be considered as a new less-invasive non-subjective inflammatory marker for assessment of different grades of asthma severity and control.

* Corresponding author. Cell: +20 1224035744, tel.: +20 (2) 24843479.
E-mail addresses: foudaeman@gmail.com (E.M. Fouda), terez2003@hotmail.com (T.B. Kamel), manal_aaziz@yahoo.com (M.M. Abd Al-Aziz), drmero2007@hotmail.com (A.N. Atyia).

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Introduction

Bronchial asthma, a clinical complication of persistent inflammation of airways and subsequent airway hyperresponsiveness, is a leading cause of morbidity and mortality [1]. Despite that airway inflammation is a useful marker of

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disease activity, there is less information concerning airway inflammation in young children, in whom access to the lower airway is a major obstacle and in whom the asthma phenotype is thought to be more heterogeneous [2].

The upper airway, especially nasal epithelium, shares several properties with the lower airways. Moreover, it is easily accessible and nasal lavage (NAL) is well tolerated even by infants as young as 4 wk old. In comparison with serum biomarkers, measurements of NAL inflammatory markers can be assumed to reflect airway pathology more directly. Therefore, the determination of inflammatory markers in the nasal epithelium fluid would be attractive for both epidemiological and clinical purposes [3].

Nuclear factor kappa B (NF-kappa B) is a transcription factor that is critical for production of many inflammatory cytokines. It is activated in airway epithelium of both human asthmatics and mice after allergic stimulation [4]. It has been considered a master regulator of both innate and adaptive immune responses and it might play a cardinal role in allergic airway diseases [5]. Therefore, understanding various facets of regulation of NFκB and its targets may offer the potential to advance our knowledge of immune processes in asthma [6]. Moreover; no studies to date have identified its level in nasal lavage in correlation with asthma severity in children.

Aim of the work

This study aimed at evaluating diagnostic value of nasal lavage (NAL) nuclear factor kappa B and cytology, as a less-invasive bench-side maneuver, as inflammatory biomarkers in asthmatic children and to correlate it with asthma severity assessed clinically and by forced expiratory second 1 (FEV1) % of predicted for age and sex [7]; together with NAL cellularity as an indicator of airway inflammation.

Patients and methods

Study population and design

During the period from April, 2012 to October, 2014, this case–control study was carried out at the Pediatric Chest Clinic, Children's Hospital, Ain Shams University; Cairo, Egypt. It included 60 asthmatic children and 30-age and sex-matched- healthy children as a control group. Patients were selected according to the global initiative for asthma "GINA" [7]. An informed consent was obtained from parents of both patients and controls. The Pediatric Department Board ethically approved the study. None of the patients had a respiratory tract infection or exacerbation of asthma during enrollment. None of the asthmatics gave history for allergic rhinitis or previous nasal steroid intake (to assess local nasal epithelium as a representative for the lower airway inflammation). Neither asthmatics nor healthy children were exposed to second-hand tobacco smoking which may increase airway inflammation and NFκB level.

Asthmatic patients were further subdivided into allergic and non-allergic; based on atopic history and/or previous positive skin prick test [8]. Levels of asthma control were systematically assessed using GINA guidelines [7] during past three months: controlled versus partially/uncontrolled asthma. Severe exacerbations, according to ATS/ERS definition [9] and the number of days with symptoms (GINA guidelines) were specifically recorded.

Both patients and controls were examined, and then underwent spirometry and venous blood samples were collected to determine complete blood count for eosinophilia and nasal lavage fluid sampling for cytology and measurement of NFκB level.

Spirometry

A dynamic spirometry (Jaeger, Germany) was done for measurement of FEV1% of predicted; according to standards of the European Respirator Society and the American Thoracic Academy [10]. We used the highest values of FEV1 of three forced expiratory maneuvers.

Nasal lavage sampling

We clearly explained the maneuver for the child and parents as described [3]. Older children ($\geqslant$7 y) sat on a chair, with their head bent backward; they were instructed to hold their breath and to make a hiccup sound thus to close soft palate and prevent fluid being swallowed. We instilled 5 ml of pre-warmed isotonic using a needleless syringe. Then the child was asked to hold his/her breath for 10 s and then expel the fluid into a specimen cup. For younger children, lavages were performed in a supine position. Pre-warmed (2 ml) isotonic saline was instilled into each nostril and immediately aspirated into a specimen trap by inserting a flexible suction catheter. After washing each nostril, 1 ml of saline was aspirated through the catheter to rinse secretions into the trap. A proteinase inhibitor "phosphoramidon" was added to the specimen cup before collection of the expelled fluid [11–13].

NAL cytology

For analysis of cellular components, samples were processed quickly. A mucolytic agent; dithioerythritol (Sputalysin) was added to the NAL sample. After centrifugation, total cell count was obtained by a manual hemocytometer and differential cell counts by preparation of cytocentrifuge slides. Specimens were stained using the May-Grünwald-Giemsa method. Eosinophil, macrophage, lymphocyte and neutrophil counts are then expressed as cell count $\times 10^4$/ml [12–14]. Aliquots of the supernatant were stored at 70 °C for later analysis of NFκB.

Nasal lavage fluid NFκB measurement

Nuclear protein was isolated from the thawed cell pellet in a three step process according to the manufacturer's instructions (Nuclear Extract Kit; Cayman chemical company, Ann Arbor, MI, USA). The protein concentration was determined by Coomassie protein assay. NFκB in nuclear protein extract was determined by enzyme linked immunosorbent assay (Cayman chemical company). Assay specificity was confirmed by using the provided competitive oligonucleotides. The provided HeLa cell extract was used to produce a standard curve. Results were expressed as concentration of NFκB μg/μg nuclear protein.

Statistics

Statistical analysis was performed using (SPSS, Inc, Chicago, Illinois, USA) version 15. Data were presented as mean and standard deviation. Categorical variables were expressed as count and percentage. Unpaired *t* test and Mann–Whitney *U*-test were used to analyze differences between two groups. Comparison of three groups was performed using analysis of variance and Fisher's protected least significant difference test or chi-squared test. Correlations between data were analyzed using Pearson correlation test. Receiver operating characteristic "ROC" curve was performed to define best cut-off, sensitivity and specificity. Analyses with resultant $P \leqslant 0.05$ were determined significant.

Results

Characteristics of asthmatic and healthy control groups are shown in Table 1. Twenty asthmatic children had history of atopy, however after considering a previously positive skin prick test, the allergic asthmatics represented (42/60; 70%). Most studied children belonged to an urban residence near to our center (Cairo, Egypt), so residence cannot be attributed as a predisposing factor for asthma in our study. FEV1% of predicted for age and sex was lower with statistical significance among asthmatics when compared to the control group and among asthmatics with increasing asthma severity ($P < 0.05$). Blood eosinophil percent was significantly higher among asthmatics when compared to healthy control

Table 1 Characteristics of asthmatic patients and healthy controls.

Characteristic data		Asthmatics ($n = 60$)	Controls ($n = 30$)
Age in years, Mean ± SD		8.7 ± 3.74	8.15 ± 3.19
Sex (male/female)		36/24	19/11
Positive history for atopy**		10 (33.3%)	0.00
Residence	Urban	46 (76.7%)	27 (90%)
	Rural	14 (23.3%)	3 (10%)
BMI kg/m^2, Mean ± SD		17.80 ± 1.52	17.03 ± 0.94
FEV1% pred., Mean ± SD**		80.96 ± 12.72	97.90 ± 1.74
Blood eosinophils %, Mean ± SD**		5.11 ± 3.81	0.70 ± 0.75
NAL NFκB (μg/μg nuclear proteins), Mean ± SD**		0.129 ± 0.113	0.0176 ± 0.013
NAL Cell count (cells × 10^4/ml)	Neutrophils, Mean ± SD**	6.8 ± 11.48	4.133 ± 4.32
	Eosinophils, Mean ± SD**	7.86 ± 2.116	0 ± 0.40
	Squamous epithelium, Mean ± SD	16.0 ± 14. 81	15.0 ± 21.48

ICS, inhaled corticosteroid; FEV1, forced expiratory volume in 1 s; % pred., percentage of the predicted value; BMI, body mass index; NAL, Nasal lavage; NFκB, nuclear factor kappa b.
** $P < 0.05$ (significant).

Table 2 Nasal lavage of asthmatic children in relation to severity; atopy and control.

		Asthmatics ($n = 60$)		
		Mild intermittent ($n = 22$)	Mild persistent ($n = 12$)	Moderate to severe ($n = 26$)
NAL cell count (cells × 10^4/ml)	Neutrophils, Mean ± SD**	4.8 ± 1.48	5.5 ± 1.92	5.8 ± 1.74
	Eosinophils, Mean ± SD**	6.84 ± 3.01	7.01 ± 5.08	7.86 ± 4.63
NAL NFκB, Mean ± SD** (μg/μg nuclear proteins)		0.056 ± 0.036	0.087 ± 0.051	0.184 ± 0.091
		Uncontrolled ($n = 14$)	Partial-control ($n = 28$)	Controlled ($n = 18$)
NAL cell count (cells × 10^4/ml)	Neutrophils, Mean ± SD**	6.1 ± 8.74	5.3 ± 2.91	4.4 ± 1.48
	Eosinophils, Mean ± SD**	7.82 ± 3.46	6.99 ± 5.18	4.94 ± 1.01
NAL NFκB, Mean ± SD** (μg/μg nuclear proteins)		0.059 ± 0.030	0.078 ± 0.047	0.26 ± 0.117
			Allergic ($n = 42$)	Non-allergic ($n = 18$)
NAL cell count (cells × 10^4/ml)	Neutrophils, Mean ± SD		5.21 ± 1.87	4.97 ± 1.66
	Eosinophils, Mean ± SD**		7.66 ± 1.24	3.789 ± 2.17
NAL NFκB, Mean ± SD** (μg /μg nuclear proteins)			0.145 ± 0.105	0.126 ± 0.123

** $P < 0.05$ (significant).

($P < 0.05$), and also among asthmatics with increasing asthma severity ($P < 0.05$). The nasal lavage fluid eosinophils were higher with statistical significance among asthmatics when compared to healthy controls (Table 1) ($P < 0.05$), and among asthmatics with increasing asthma severity (Table 2) ($P < 0.05$). In addition, nasal lavage fluid eosinophil count was higher with statistical significance among allergic asthmatics when compared to non-allergic asthmatics ($P < 0.05$) (Table 2).

Nasal lavage fluid NFκB levels were significantly higher in asthmatics than in controls with a mean of 0.129 ± 0.113 μg/μg nuclear proteins and 0.0176 ± 0.013 μg/μg nuclear proteins, respectively (Table1).

NAL NFκB levels were significantly higher in moderate to severe followed by mild persistent then mild intermittent asthmatics with a mean of 0.184 ± 0.091 μg/μg proteins and 0.087 ± 0.051 μg/μg proteins and 0.056 ± 0.036 μg/μg proteins, respectively (Table 2) (Fig. 1) .

When calculating area under ROC curve (Fig. 2), the best cut-off value for nasal lavage fluid NFκB was 0.034 μg/μg nuclear proteins to predict asthma with a sensitivity of 87% and specificity 87%.

There were positive significant correlations between NAL NFκB and blood eosinophil percent, NAL eosinophils, and NAL neutrophils among asthmatics (Table 3). Moreover, it showed a significant negative correlation with FEV 1% among them while, there were no significant correlations between NAL NFκB and age, duration of illness, body mass index (BMI), dose and duration of inhaled steroid therapy.

Discussion

Asthma is a series of complex, overlapping individual diseases or phenotypes, each defined by an interaction between genetic and environmental factors. NFκB exerts a key role in perpetuating inflammatory reactions in asthma, and levels of NFκB expression increase with severity [15]. There is an association between concentration of inflammatory markers and cells in samples from lower airways and clinical severity of asthma. Hence, the upper airway, specifically the nasal epithelium, shares many properties with the lower airway. Moreover, nasal lavage (NAL) is easily accessible and well tolerated [3]. The current study was designed to assess the role of NAL NFκB as an inflammatory biomarker in grading of asthma severity and control.

In the present study, nasal lavage fluid NFκB levels were significantly higher in all asthmatics compared to healthy controls, and among asthmatics with increasing asthma severity. In addition, it correlated negatively with indices of forced expiratory volume second 1(FEV1).

Although all patients were compliant on inhaled steroid therapy, not all of them show full control. NAL NFκB levels were higher in uncontrolled/partially controlled asthmatics than those fully controlled. Our study reported 70% of asthmatics being allergic; they also had higher levels of NAL NFκB when compared to non-allergic children.

These results are in relative concordance with many lines of evidence that indicate enhanced NFκB pathway activation in asthmatic tissues. Peripheral blood mononuclear cells (PBMCs) of adult uncontrolled, severe and moderate asthmatics have higher levels of NFκB p65 protein expression than normal individuals [16]. Also in children, NFκB p65 protein abundance is also higher in moderate asthmatic peripheral blood mononuclear cells when compared to normal individuals [17]. Furthermore, when compared to non-asthmatic individuals, both nuclear extracts from sputum cells, bronchial

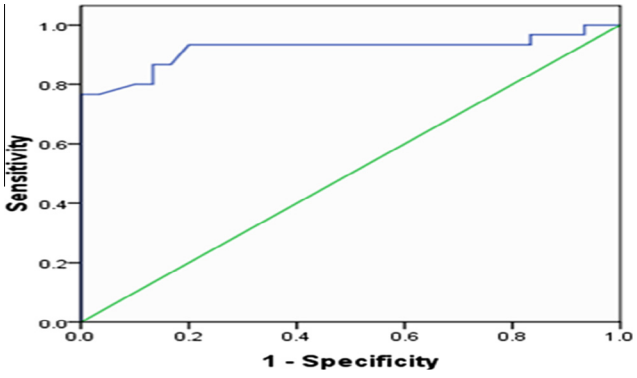

Figure 2 Receiver operating characteristic (ROC) curve for NAL NFκB to predict bronchial asthma.

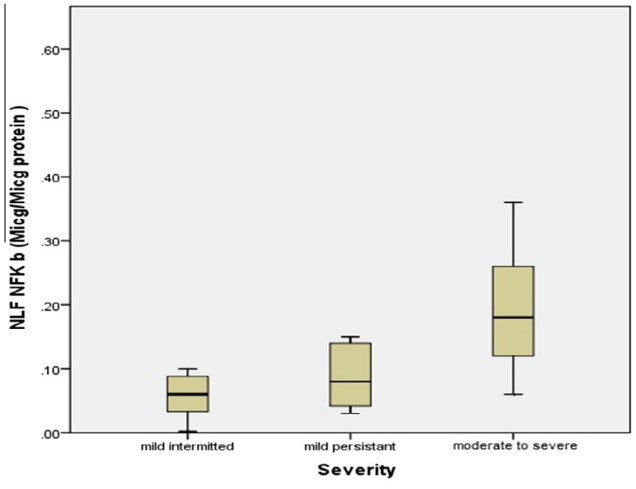

Figure 1 Nasal lavage NFκB in different grades of asthma.

Table 3 Correlations between NLF NFκB and Other Variables of Asthmatic Group.

Variables	*R*	*P*	Sig*
Age (years)	0.162	0.216	NS
Duration of illness (years)	0.191	0.313	NS
BMI (kg/m^2)	0.051	0.699	NS
Blood eosinophils%	0.874	0.00	HS
FEV 1% of predicted	−0.838	0.00	HS
NAL eosinophils (cell × 10^4/ml)	0.635	0.00	HS
NLF neutrophils (cell × 10^4/ml)	0.541	0.00	HS
Dose of inhaled steroid (mcg/day)	0.236	0.209	NS
Duration of inhaled steroid (months)	0.013	0.947	NS

BMI, body mass index; *S, significant; HS, highly significant; NS, non significant.

biopsies [18], and cultured bronchial epithelial cells from stable, untreated asthmatics have greater levels of NFκB p65 [19].

Furthermore, being higher among allergic asthmatics comes in agreement with Cristen et al. [20], who demonstrate that selective activation NF-kappa B in airway epithelium is sufficient to induce airway hyper-responsiveness and smooth muscle thickening, which are both critical features of allergic airway disease.

These results are also in concordance with Abdulamir et al. [15] who found that NFκB exerts a key role in perpetuating inflammatory reactions in asthma, and levels of NFκB expression increase with severity.

In the present work, NAL cytology revealed higher counts of eosinophils (denoting eosinophilic airway inflammation), then neutrophils, and lymphocytes in asthmatic patients when compared to the control group, as the contribution of eosinophils, neutrophils and lymphocytes has been well established in the patho-physiology of asthma [21,22].

These results are in concordance with Noah et al. [13,23] who reported high levels of NAL eosinophils in asthmatic children when compared to non-asthmatic children. Also, they come in relative agreement with Carroll et al. [24] who reported increased levels of lymphocytes and eosinophils in airway biopsy specimens in asthmatic patients. Similarly, both Jung et al. [21] and Spahn et al. [25] reported that, sputum eosinophilia predicts asthma relapse in asthmatic children.

However in another study, Fahy [26] reported that neutrophils accumulate in the airway in more severe forms of chronic severe asthma, and neutrophil numbers are associated with chronic airway narrowing. In addition, neutrophils are prominent during acute severe asthma exacerbations, where it is possible that they have roles in both the initiation and resolution of attacks.

Current results also disagree with Hart et al. [18] who reported that sputum neutrophil numbers were similar in both asthmatic and non asthmatic group.

Also NAL eosinophils were significantly higher in allergic asthmatic patients when compared to the non-allergic asthmatic group, which is in concordance with Venge [27] who reported that eosinophils are found at increased numbers in asthma and so in allergic as compared with non-allergic asthma. NAL eosinophils were also higher in relation to increased asthma severity and worsening levels of control.

These findings are in relative agreement with Bousquet et al. [28] who reported an increase in the numbers of eosinophils in bronchoalveolar lavage fluid in asthmatics as compared with the controls, and eosinophil counts in bronchoalveolar lavage fluid significantly correlated with the clinical asthma score and increase with severity.

Moreover, our study calculated the best cut-off value for nasal lavage NFκB of 3.40 μg/μg nuclear proteins to predict asthma severity with a sensitivity of 87% and specificity 87%. Thus NAL NFκB can be an objective biomarker for monitoring airway inflammation. However, further studies are required in order to better elucidate its clinical significance and association to childhood asthma with co-morbid nasal allergy, in addition to its responsiveness to treatment.

Furthermore, as the technology and therapeutic agents become increasingly available, therefore, the potential of targeting the NFκB pathway in asthma is a challenge for further clinical pharmacology studies.

Conclusion

Despite that spirometry and GINA guidelines are gold standards for grading of asthma, they cannot assess the degree of airway inflammation. Nasal lavage is a safe bench-side maneuver that can give an idea about airway inflammation by assessing its cellularity in different grades of asthma severity. Nasal lavage fluid cytology can be done as a prognostic test before stepping up or down treatment. NAL NFκB level can predict asthmatics from non-asthmatics. It can be considered as a new relatively non-invasive marker for assessment of different grades of asthma severity and control. It can be used for indirect detection and monitoring of airway inflammation, disease severity, and response to steroid treatment in asthmatic children.

Authors' agreement

I (Terez Boshra Kamel), The Corresponding Author of this article (the Contribution") has the right to grant on behalf of all authors and does grant on behalf of all authors, a license to "The Egyptian Journal of Chest diseases and Tuberculosis" to permit this Contribution (if accepted) to be published.

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Conflict of interest

There was no conflict of interest.

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