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NOD₂ expression, DNA damage and oxido-inflammatory status in Egyptian patients with atopic bronchial asthma: Exploring their nexus to disease severity

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Abstract:

Background: Allergic asthma is a chronically relapsing inflammatory airway disease with a complex pathophysiology. **Aim:** This study was undertaken to investigate the potential contribution of NOD₂ signaling, proinflammatory cytokines, chitotriosidase (CHIT1) activity, oxidative stress and DNA damage to atopic asthma pathogenesis, as well as to explore their possible role as surrogate noninvasive biomarkers for monitoring asthma severity. **Methods:** Sixty patients with atopic bronchial asthma who were divided according to asthma severity into 40 mild-moderate, 20 severe atopic asthmatics, in addition to thirty age-matched healthy controls were enrolled in this study. NOD₂ expression in PBMCs was assessed by quantitative real-time RT-PCR. DNA damage indices were assessed by alkaline comet assay. Serum IgE, IL-17, IL-8 and 3-Nitrotyrosine levels were estimated by ELISA. Serum CHIT1 and GST activities, as well as MDA levels, were measured. **Results:** NOD₂ mRNA relative expression levels were significantly decreased in atopic asthmatic cases relative to controls with lower values among severe atopic asthmatics. On the other hand, IL-17 and IL-8 serum levels, CHIT1 activity, DNA damage indices and oxidative stress markers were significantly increased in atopic asthmatic cases relative to controls with higher values among severe atopic asthmatics. The change in these parameters correlated significantly with the degree of decline in lung function. **In conclusion,** the interplay between NOD₂ signaling, proinflammatory cytokines, CHIT1 activity, heightened oxidative stress and DNA damage orchestrates allergic airway inflammation and thus contributing to the pathogenesis of atopic asthma. These parameters qualified for measurement as part of new noninvasive biomarker panels for monitoring asthma severity.

Keywords: Atopic bronchial asthma; NOD₂ expression; DNA damage; proinflammatory cytokines; CHIT1 enzyme; 3-Nitrotyrosine.

1. Introduction:

Allergic asthma is a complex and heterogeneous disease characterized by spontaneous airflow limitation, lung tissue remodeling, mucus hypersecretion, bronchial hyper-responsiveness and increased plasma IgE concentration [1]. The identification of the molecular mechanisms engaged in allergic airway inflammation and remodeling that lead to chronic limitation of airflow is critical for a better understanding of the pathogenesis of asthma and for the development of novel therapeutics aimed at limiting the onset and progression of the disease[2].

Evidence is accumulating to suggest that the innate, non-antigen-dependent immunity crucially participates in the pathogenesis of allergic asthma [3]. Nod (nucleotide-binding oligomerization domain) 2 is an intracellular pattern-recognition molecule of the NLR (Nod-like receptor) family that detects conserved microbial components called pathogen-associated molecular patterns (PAMPs) in mammalian species [4]. It has been implicated as ancient cellular sentinels in mediating protective immune responses against a repertoire of intracellular pathogens[5]. Dysregulation of NOD₂ signaling is involved in the pathogenesis of a variety of allergic and inflammatory disorders; however, its precise role in atopic bronchial asthma is far from being fully characterized [6].

Cytokines play an integral role in the coordination and persistence of the chronic airway inflammation and remodeling in asthma by promoting immune cell recruitment and activation [7]. T helper 17 (Th17), a distinct subset of CD4⁺ T cells with IL-17 as their major cytokine, contribute to the pathogenesis of inflammatory and autoimmune diseases [8]. IL-8, a C-X-C chemokine, has major actions as a neutrophil chemoattractant and activator [9]; however, the mechanisms by which these cytokines are associated with severe asthma have not yet been verified.

Chitotriosidase enzyme (CHIT1), the major active chitinase in human plasma, is mainly expressed by different lineages of activated blood and tissue macrophages, in which its expression is regulated by multiple intrinsic and extrinsic signals[10]. CHIT1 has been

associated with the pathological conditions that are characterized by tissue inflammation and remodeling, including atherosclerosis and asthma[11]. The absence of its substrate chitin in the human body and the exclusive production by immunologically active cells confirmed its vital role in the innate immunity against chitin-containing organisms such as fungi and protozoa [12]. However, its immunomodulatory effects are not limited to its chitinolytic activity against the engulfed chitin-containing organisms, or even to innate immunity but its function extends far beyond them as it has been involved in the activation of macrophages, as well as other immune cells such as T helper cells and eosinophils [13].

Oxidative stress is a characteristic of chronic inflammatory diseases. Reactive oxygen species (ROS) are highly reactive molecules; they can easily oxidize cellular components such as lipid, protein, and DNA[14]. In bronchial asthma, oxidative stress-mediated damage may aggravate airway inflammation by inducing diverse proinflammatory mediators, enhancing bronchial hyperresponsiveness, stimulating bronchospasm, as well as increasing vascular permeability, epithelial shedding and mucin secretion [15]. Tyrosine nitration occurs when a reactive derivative of ONOO oxidizes tyrosine to form tyrosyl radicals, which then react with nitrogen dioxide to form 3-nitrotyrosine (3-NT)[16]. This modified protein is an easily quantifiable biomarker for in vivo biological nitration and NO-dependent damage [17]. Host antioxidant systems are generally activated in response to the oxidant attack, but individuals have different capacities of antioxidant defense[18].

DNA is a particular target for oxidation which generates DNA adducts, strand breaks and other lesions [19]. DNA damage might contribute to increased mutation rates, apoptosis and associated tissue regeneration and cell proliferation [20]. Recent studies have revealed the association between asthma and increased oxidative DNA damage [21] however, information is still lacking regarding the nature of this link.

In view of these considerations, this study was performed to investigate the potential contribution of NOD2 signaling, some proinflammatory cytokines, CHIT1 activity, heightened oxidative stress and DNA damage to the pathogenesis of atopic bronchial

asthma as well as to explore their possible role as surrogate noninvasive biomarkers for monitoring asthma severity.

2. Subjects and methods:

2.1. Study population:

Sixty patients with atopic bronchial asthma were recruited from the Internal Medicine and Chest Departments in Tanta University Hospital during the period between January and October 2017. Diagnosis of asthma was based on the criteria of the Global Initiative on Asthma (GINA) guidelines[22]. The severity of asthma in these patients was assessed based on daytime symptoms, nocturnal symptoms and lung functions.

Thirty age-matched healthy community-based controls with no history of asthma or any allergic disease, currently nonsmokers and not receiving any drug at their participation in this study were also included. Patients were divided according to asthma severity into 40 mild-moderate and 20 severe atopic asthmatics. The atopic status of patients and control subjects was ascertained by positive skin prick allergen tests (wheal diameter ≥ 3 mm greater than saline control) to one or more of common aeroallergen extracts (house dust mite, house dust, cotton dust, mixed pollens, cat fur, dog fur, feathers) and/or total serum IgE levels ≥ 100 IU/mL [23].

All subjects had no change of asthma medications 4 weeks prior to recruitment to the study. All subjects were nonsmokers and free from upper respiratory tract infection for at least 4 weeks preceding the study. The study was approved by the scientific and ethics committees of the Tanta University Hospital, Tanta University, Tanta, Egypt, was in accordance with the principles of the Declaration of Helsinki II and informed consent was obtained from each subject before the start of this study.

2.2. Methods:

- All enrolled subjects were submitted to history taking, thorough clinical examination, and then subjected to the followings:-

2.2.1. Pulmonary Function Tests:

Spirometric study was done for all cases using Morgan's transfer apparatus with Della computer (Buckingham, England) according to the American Thoracic Society standards

[24]. Forced expiratory volume in 1 sec (FEV₁), forced vital capacity (FVC), and the FEV₁/FVC ratio were measured before and 15 min after bronchodilation induced by inhalation of salbutamol (400 µg). The results were recorded as the percentage of the predicted values. Subsequently, asthmatic patients were categorized into severity groups by using level of FEV₁ % predicted. The patients were labeled as having mild-moderate (FEV₁ % predicted > 60 %), or severe (FEV₁ % predicted < 60%) airflow obstruction [22].

2.2.2. Blood sampling:

After 12 hours of overnight fasting, 7 ml of venous blood samples were collected from all patients and controls. A portion of blood was collected into a dry sterile centrifuge tube, allowed to clot at room temperature, centrifuged at 3500g for 10 min, and the serum was kept frozen at -80 °C after collection until analysis. Another portion of blood collected in heparinized tubes was stored at -80 °C until preparation of peripheral blood mononuclear cells.

2.2.3. Biochemical assay:

1. **Serum C-reactive protein (CRP) levels** were quantified by using the latex slide semi-quantitative test (#CAT: 514 004, Egyptian Company for Biotechnology (S.A.E), Cairo, Egypt) according to the manufacturer's instructions.
2. **Erythrocyte sedimentation rate (ESR)** was determined using Westergren tube [25].
2. **Eosinophil counts** were determined according to Burrows et al [26].
3. **Assay of serum total IgE:** Total IgE level was measured by ELISA using AccuBind IgE Quantitative Kits, (Lake Forest, California, USA).
4. **Preparation of Peripheral blood mononuclear cells (PBMCs):** PBMCs were prepared by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Briefly, heparinized blood was carefully layered on Ficoll, and PBMCs were harvested from the white interphase after centrifugation for 30 minutes at 400 g, at room temperature and washed with phosphate buffered saline (PBS). The PBMCs samples were stored at -80° C till the samples were further processed for RNA isolation and Comet assay.

5. Quantitative measurement of nucleotide-binding oligomerization domain-containing protein 2 (NOD₂) gene expression by quantitative real-time reverse transcription PCR (RT-PCR) :

- i **RNA extraction:** Total RNA was extracted from PBMCs using Gene JET RNA Purification Kit (Thermo Scientific, # K0731, USA) according to the manufacturer protocol. Total RNA concentration and purity were determined by measuring OD260 and OD260/280 ratio, respectively, on a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA), RNA was then stored at -80°C.
- ii **cDNA synthesis :** Total RNA samples were reverse-transcribed using the RevertAid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) to produce cDNA as previously described[27]. The cDNA was then stored at -20°C until used for PCR.

iii **Real-time quantitative PCR:**

This cDNA was used as a template to determine the relative expression of the DUOX2 gene using StepOnePlus real-time PCR system (Applied Biosystem, USA). Primer sequences specific for NOD₂ (NCBI GenBank Nucleotide accession # NM_001293557) were designed according to **Liu et al.,[28]**; as follows: (forward) 5'- GCCTTCCTTCTACAGCACGT-3', and (reverse) 5'- TGGCAGGGCTCTTCTGCAAG -3'. Primers for β -actin (NCBI GenBank Nucleotide accession # NM_001101.4), which was used as a reference to calculate fold change in target gene expression, were: (forward) 5'- CTCTTCCAGCCTTCCTTCCT-3' and (reverse) 5'- AGCACTGTGTTGGCGTACAG-3'.

A 25- μ L PCR mix was prepared by adding 12.5 μ L of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 2 μ L of cDNA template, 1 μ L forward primer, 1 μ L reverse primer, and 8.5 μ L of nuclease free water. The thermal cycling conditions were as follows: Initial denaturation at 95°C for 10 minutes was followed by 40 cycles with denaturation

at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. At the end of the last cycle, the temperature was increased from 60 to 95 °C for melting curve analysis. A control reaction without a DNA template was performed in parallel to detect genomic DNA contamination. The determination of the relative levels of gene expression was performed using the comparative cycle threshold($\Delta\Delta Ct$) method and normalized to the reference gene β -actin [29].

6. Comet assay: Alkaline comet assay was performed on PBMCs from asthmatic patients or their matched controls using a commercial kit (Trevigen's Comet Assay kit, 4250-050-k, Gaithersburg, MD, USA) according to the manufacturer's instructions. DNA was stained with propidium iodide. Stained slides were examined using a fluorescent microscope with excitation filter 420-490 nm (Olympus BX51, Tokyo, Japan). Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool1,UK) linked to a CCD camera was used to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration, the percentage of migrated DNA, and finally, the tail moment. Generally, 50 to 100 randomly selected cells are analyzed per sample.

7. Estimation of serum Chitotriosidase (CHIT1) enzyme activity:

Serum chitotriosidase enzyme activity was assayed by a spectrofluorometric method according to **Hollak et al.[30]**. This assay is based on the enzymatic hydrolysis of the synthetic substrate 4-methylumbelliferyl- β -D-NN,N'- triacetylchitotriose by chitotriosidase resulting in the release of the fluorescent molecule 4-methylumbelliferone (4MU), which can be measured fluorimetrically at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The emitted fluorescence is proportional to the amount of molecules hydrolyzed.

8. Proinflammatory cytokines: Serum IL-8 and IL-17 levels were assayed using commercially available ELISA kits (Cat # ab46032, Abcam, Cambridge, MA, USA) and (Cat # KAC1591, Biosource Europe, S.A., Nivelles, Belgium) respectively; following the manufacturer's protocols.

9. Redox status parameters:

- a) **3-nitrotyrosine (3-NT) levels:** 3-nitrotyrosine is a surrogate index of peroxynitrite mediated protein oxidation/nitration. Serum 3-NT levels were determined using a commercially available ELISA kit (#CAT: NWK-NTR01-02, Northwest, Life Science Specialties, Vancouver, Canada) for human nitrotyrosine, following the manufacturer's instructions.
- b) **Malondialdehyde (MDA) Levels:** Lipid peroxidation product MDA was determined by a spectrophotometric method based on measurement of the color occurring at 532 nm during the reaction of MDA with thiobarbituric acid (TBA) [31].
- c) **Glutathione-S-transferase assay (GST) activity:** GST enzyme activity was determined using Biodiagnostic assay kit (Biodiagnostic, Giza, Egypt), which measures total GST activity by measuring the conjugation of 1- chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation was accompanied with an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample [32].

2.2.4. Statistical analysis: Results are expressed as mean $\pm$ standard deviation (SD). The intergroup variation was measured by one-way analysis of variance (ANOVA) followed by the Tukey's test. The statistical evaluation of the data was performed using Graph Pad Prism 4.03 (GraphPad Software, San Diego, California, USA). P values less than 0.05 were considered statistically significant.

3. Results:

3.1. Demographic and clinical characteristics of the studied groups:

The demographic characteristics and the clinical data of the studied population are reported in Table 1. There was no significant difference in age or sex distribution between asthmatic patients and controls. Disease duration (years) showed also a non-significant difference between the two patient groups ($p > 0.05$). Pulmonary function parameters (FEV_1 (% predicted), FVC(% predicted) and FEV_1 / FVC ratio) were significantly different in the

three groups, along with being lower in severe atopic asthmatics than in mild–moderate atopic asthmatics or in control subjects ($P < 0.0001$). Likewise, erythrocyte sedimentation rate (mm/hr), C-reactive protein (mg/L), were significantly different in the three groups; however, their levels showed a significant elevation in severe atopic asthmatics than in mild–moderate atopic asthmatics or in control subjects ($P < 0.0001$). Notably, total serum IgE concentrations, peripheral blood Neutrophil %, and Eosinophils % were significantly increased in all atopic asthmatic patients compared to control subjects ($p < 0.0001$); however, no significant difference was found among the different grades of asthma severity.

3. 2. Nucleotide-binding oligomerization domain-containing protein 2 (NOD 2) mRNA relative expression:

Our data revealed that NOD₂ mRNA expression (assessed by qRT-PCR) in peripheral blood mononuclear cells of the atopic asthmatic patients (both mild-moderate and severe groups) were significantly downregulated compared to the healthy control subjects ($p < 0.0001$). Moreover, the severe atopic asthmatic patients exhibited a significant decrease in NOD2 expression as compared to the mild-moderate atopic asthmatic patients ($p < 0.0001$). These data are illustrated in Figure 1.

3. 3. DNA damage :

DNA damage in peripheral blood mononuclear cells was determined by alkaline single cell electrophoresis (comet assay). Two critical factors including the percentage of DNA in the tail and tail moment (% DNA in tail $\times$ tail length) were assessed (Fig. 2). Based on these criteria, DNA damage was increased in cells from atopic asthmatic patients (both mild-moderate and severe groups) compared to healthy control subjects ($p < 0.0001$) (Fig. 3). Moreover, the severe atopic asthmatic patients suffered from greater DNA damage as depicted by the significant increase in the percentage of DNA in the tail and tail moment compared to the mild-moderate atopic asthmatic patients ($p < 0.0001$).

3.4. Markers of the oxido-inflammatory status:

Our results demonstrated significant elevations of the serum levels of the oxidative stress biomarkers MDA (as a marker for lipid peroxidation) and 3-NT (as a marker for protein nitration) concomitantly with significant increase in the GST activity in atopic asthmatic

patients (both mild-moderate and severe groups) compared to the healthy control subjects ($p < 0.0001$), thus providing an evidence for an prooxidant/ antioxidant imbalance. Meanwhile, this altered redox status was more evident in the severe atopic asthmatic patients compared to the mild-moderate atopic asthmatic patients ($p < 0.0001$). Furthermore, our data revealed that the serum concentrations of the proinflammatory cytokines (IL-17, IL-8) as well as serum CHIT1 enzyme activity were significantly elevated in atopic asthmatic patients (both mild-moderate and severe groups) compared to the healthy control subjects ($p < 0.0001$), with being prominently higher in the severe atopic asthmatic patients compared to the mild-moderate atopic asthmatic patients ($p < 0.0001$). These data are summarized in table (2).

3.5. Correlations between the studied parameters in atopic asthmatic patients:

The correlations between the different parameters in atopic asthmatic patients were performed using Pearson's correlation ($n = 60$), where a significant negative correlation was revealed among FEV_1 (% predicted), as a monitor for the degree of airway obstruction, and all studied parameters except for NOD_2 expression which exhibited a significant positive correlation with FEV_1 (% predicted). Other positive correlations were also observed between CHIT1 activity and proinflammatory cytokines (IL-8, IL-17) as well as between oxidative stress markers (MDA, 3-NT, GST) and DNA damage indices. Additionally, linear regression analysis was performed analysis using FEV_1 (% predicted) as a dependent variable, and other studied parameters as independent variables, where relative NOD_2 expression, DNA damage indices, IL-8, IL-17 and 3-NT behaved as independent predictors that affect FEV_1 (% predicted) in atopic asthmatic patients. These data are summarized in Tables 3 and 4.

4. Discussion:

Allergic asthma is a chronically relapsing inflammatory airway disease with a complex pathophysiology. Bacterial sensor NOD_2 can trigger a potent antigen-specific immune response with a Th2-type polarization cytokine profile; thus acting as a link between innate and adaptive immunity [5]. Despite its irrefutable importance as a key mediator of immune

responses to bacteria and inflammation, its precise role and clinical significance in atopic asthma are yet to be elucidated.

To the best of our knowledge, our study is the first to investigate NOD2 expression in human peripheral blood and to explore its nexus to asthma severity along with DNA damage indices and some oxido-inflammatory markers in Egyptian patients with atopic bronchial asthma. Our data revealed that NOD₂ mRNA expression levels were significantly downregulated in PBMCs from the atopic asthmatic patients compared to the control subjects, with lower NOD₂ expression levels in the severe compared to the mild-moderate atopic asthmatics; underscoring a role for NOD₂ impaired expression in modulating the development and amplification of the disease state in allergic asthma.

In harmony with this finding; Wong et al. [33] reported a significantly defective expression of NOD2 in allergic asthmatics and hence suggested that this downregulation might be a predisposition factor for the development of allergic asthma. Furthermore, the previous reports on a similar lowered expression pattern for NOD₂ in other allergic diseases such as atopic dermatitis bolstering our finding. Likewise, prevailing independent studies have displayed that loss-of-function variants of NOD₂ gene polymorphisms are significantly associated with a heightened risk of an increase in allergen-specific serum IgE levels and a variety of atopy-related traits, such as atopic dermatitis [34].

Moreover, our data unveiled that NOD₂ expression in PBMCs correlated significantly with reduced lung functions; supporting the notion that NOD₂ acts as a brake to dampen immune responses, and the impaired recognition of microbial components via NOD₂ may result in an insufficient downregulation of excessive immune responses thus contributing to the pathogenesis of inflammation and disease severity in allergic asthma. It might also have an association with the potential changes in the microbiome both in the gut and lungs that may impact changes in FEV1 and disease severity.

In this context, given that NOD₂ activation drives innate inflammatory responses that are dependent on the activation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways [35]; it is tempting to speculate that lowered NOD2 expression in asthmatic patients might lead to defective activation of NF- κ B. Conceivably, NF- κ B

inhibition, in turn, will result in deficient Th1 responses and impaired autophagy [36], thereby hindering the clearance of bacterial pathogens that reach the alveolar cell cytoplasm and culminating into exacerbations of allergic asthma.

Dysregulation of cytokines and T cell functions have been linked to the immune-pathogenesis of allergic asthma[7]. Our data revealed that IL-17 and IL-8 serum levels were significantly elevated in severe atopic asthmatics compared to mild-moderate atopic asthmatic or control subjects, signing a possible connection to the decline in lung functions. Recently, Th17 cells have been found to contribute to the development of system inflammation in allergic asthma[8]. In accordance with our results, previous reports proved that plasma IL-17 concentrations were significantly higher in asthmatic patients without or with steroid treatment compared to control group[33, 37]. This elevation could be partially attributed to the increased frequency and activation of circulating Th17 cells, which are the major source of IL-17 in vivo [38]. Additionally, the increased expression of IL-17 has been described in bronchial submucosa from asthmatic patients, with its expression levels correlating with asthma severity, especially in patients with neutrophilic corticosteroid-resistant disease [39]. Well in line, animal models of asthma proposed that Th17 cells promote neutrophilic inflammation, and, in concert with Th2 cells, are essential for the development of airway hyper-responsiveness in asthma [40]. Moreover, it has been reported that polymorphism in IL-17F that results in a loss-of-function is inversely related to asthma risk [41].

IL-8 (CXCL8) is a chemoattractant cytokine involved in the chemoattraction and activation of neutrophils through the CXCR2 receptor[9]. Consistent with our data, Jiang et al. [42] showed that elevated serum levels of IL-8 can be involved in the development and progression of bronchial asthma. Similarly, Hosoki et al. [43] reported that neutrophils and IL-8 were the only inflammatory components in bronchoalveolar lavage (BAL) fluids that distinguish controlled asthma from uncontrolled asthma, and both correlate inversely with FEV₁; lending support to our finding.

Inasmuch as IL-17 is essential for stimulating airway structural cells to secrete neutrophil chemo-attractants such as IL-8 [44]; it is conceivable that circulating IL-17 and IL-8 levels

showed a strong positive association with each other along with being significantly correlated with the degree of decline in lung functions in our cohort of atopic asthmatic patients. This correlation was recently observed at both the mRNA and protein levels [39], signifying a cause-effect relationship.

CHIT1 enzyme is associated with innate immunity against chitin coated pathogens and was identified as one of the major protein products of activated macrophages [13]. Our data displayed that serum CHIT1 activity was significantly increased in severe atopic asthmatics compared to mild-moderate atopic asthmatic or control subjects, along with its correlation with reduced lung function, strengthens its association with disease severity. One hypothesis to explain this augmented CHIT1 activity in allergic asthma may be based on the fact that chitin, either phagocytized by the macrophage in the cell wall of bacteria or protozoa, or directly introduced to macrophages through an unidentified receptor [26] can promote the production of TNF- α and IFN- γ which can both activate CHIT1 expression and activity inside macrophages [45]. Alternatively, bacterial lipopolysaccharide and peptidoglycan product muramyl dipeptide [46] can also stimulate CHIT1 transcription mainly through the NF- κ B signaling pathway [47].

Consistent with our data, Gavala et al. [48] depicted that CHIT1 activity is enhanced in allergic airway inflammation; corroborating the notion that CHIT1 might contribute to the up-regulation of Th2-mediated inflammation and airway remodeling as important components of asthma pathophysiology. Well in line, increased levels of CHIT1 mRNA and protein were detectable in nasal mucosa from allergic donors compared with normal donors [49]. Moreover, individuals homozygous for a relatively common 24 bp duplication in exon 10 of *CHIT1* (rs3831317) which results in a complete lack of chitotriosidase activity are less likely to suffer from atopic conditions [50]. Noteworthy, our data depicted that serum levels of CHIT1 activity strongly correlated with those of proinflammatory cytokines (IL-17 and IL-8) in atopic asthmatic patients, lending credence the hypothesis of a mutual regulation cascade in the production of these pro-inflammatory mediators.

Reactive oxygen and nitrogen species produced by inflammatory cells in the lung play a prominent role in the pathogenesis as well as amplification of inflammation in asthmatic

airways[51]. In line with this premise, our data provided an evidence for altered redox status as judged by significant elevations of the serum levels of the oxidative stress biomarkers MDA (as a marker for lipid peroxidation) and 3-NT (as a marker for protein nitration) concomitantly with significant increase in the GST activity in atopic asthmatic patients compared to control subjects. Meanwhile, this oxidative milieu was more evident in the severe compared to the mild-moderate atopic asthmatic patients.

Noteworthy, our data lay emphasis on the enhanced lipid peroxidation and its correlation with reduced lung functions in atopic asthmatics. Consistent with our data, a recent study demonstrated that serum concentration of MDA was the most predictive biomarker which has a significant association with disease severity in asthmatic children [52]. Regarding protein nitration, our data corroborate earlier reports on the marked increase of 3-NT in the BAL fluid of patients with severe asthma [53] as well as in exhaled breath condensate of allergic asthmatic children [54]. Intriguingly, it has been shown that oxidative stress could aggravate inflammation and consequently increases asthma severity via decreasing histone deacetylase 2 activity secondary to its nitration [55], our findings lend credence to such scenarios.

Concomitantly, the present study shows evidence of an elevated GST activity in atopic asthmatic cases relative to controls with higher values among severe atopic asthmatics. This elevation might be a signal of a compensatory mechanism for the excessive ROS generation [56]. However, this finding raises the question about the interpretation of this elevation and whether it is a pathogenic or protective factor.

Oxidative DNA damage has been linked to a number of pathological conditions including asthma [21]. Our data unveiled that DNA damage indices, assessed by alkaline single cell electrophoresis, were increased in PBMCs from atopic asthmatics patients compared to control subjects. Moreover, the severe atopic asthmatic patients suffered from greater DNA damage as depicted by the significant increase in the percentage of DNA in the tail and tail moment compared to the mild-moderate atopic asthmatic patients. There may be biological plausibility for these findings since ROS generation by inflammatory macrophages leads to DNA damage in the surrounding epithelial cells [20] and enhanced proliferative signals

mediated by cytokines released by inflammatory cells increase the number of cells at risk for mutations [57]. Likewise, increased DNA damage indices were found in children with asthma as compared to control group[58, 59], claiming that there is a link between the increased levels of oxidative stress and DNA damage seen in those patients. High levels of DNA strand breaks are produced either during the direct interaction of ROS with DNA or during the repair process of damaged DNA as an intermediate step[60]. Nevertheless, our observed correlation between DNA damage indices and reduced lung functions allows us to portray DNA damage response as an underlying driver of asthma pathophysiology via mediating allergic airway inflammation and exacerbating disease severity.

In conclusion, the insight gained through this investigation provides critical understanding for the interplay between NOD2 signaling, proinflammatory cytokines, CHIT1 activity, heightened oxidative stress and DNA damage in orchestrating allergic airway inflammation and remodeling and thus profoundly contributing to the pathogenesis of atopic asthma. These parameters qualified for measurement as part of new noninvasive biomarker panels for monitoring asthma severity; such role is supported by the demonstration of their significant correlation with the degree of decline in lung function. Lastly, the present data may have clinical relevance in order to support future therapeutic strategies aimed at treating severe asthma through preventing structural airway changes that are potentially linked to allergen exposure.

5. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure legends:

Fig. 1. Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) mRNA relative expression levels. Values are expressed as mean $\pm$ SD. P was considered significant at <0.05 . a Significance vs. control group, b Significance vs. mild-moderate atopic asthmatic patients using One way ANOVA followed by Tukey's post hoc test for multiple comparison.

Fig (2) Photomicrographs representation of DNA damage detected by alkaline single cell electrophoresis (comet assay) of peripheral blood mononuclear cells from (A) control subjects; (B) mild-moderate atopic asthmatics and (C) severe atopic asthmatic patients. The double headed arrows matches the comet tail.

Fig (3) Comparison of (A) Tail DNA percentages (B) Tail moment percentages among control subjects, mild-moderate atopic asthmatics and severe atopic asthmatic patients. Values are expressed as mean $\pm$ SD. P was considered significant at <0.05 . a Significance vs. control group, b Significance vs. mild-moderate atopic asthmatic patients using One way ANOVA followed by Tukey's post hoc test for multiple comparison. The tail moment was calculated by multiplying the percent of DNA in the tail by the length of the tail.

Table (1): Demographic and clinical characteristics of the studied groups:

Parameters/ Groups	Control group (n =30)	Atopic Asthmatics (n= 60)		One way ANOVA	
		Mild-moderate (n= 40)	Severe (n= 20)	F /t value	p-value
Age	48.3 ± 7.6	49.1 ± 8.4	51.6 ± 9.3	0.979	> 0.05
Sex (M:F)	8:22	10:30	7:13	0.692	> 0.05
Disease duration (years)	N/A	17.2 ± 9.3	18.9 ± 11.5 ^b	t=0.616	> 0.05
Eosinophils %	1.37 ± 0.42	2.99 ± 1.12 ^a	3.86 ± 2.34 ^a	22.86	<0.0001*
Neutrophil %	48.49 ± 5.78	69.31 ± 6.75 ^a	69.78 ± 7.50 ^a	100.4	<0.0001*
CRP (mg/L)	0.46±0.14	1.91 ± 0.74 ^a	4.12 ± 0.21 ^{a,b}	307.2	<0.0001*
ESR ^{1st} hr (mm/hr)	10.00 ± 3.85	25.31 ± 9.72 ^a	33.89 ± 13.47 ^{a,b}	43.63	<0.0001*
Total serum IgE (IU/ml)	39.8±25.9	399.5±54.6 ^a	435.2±185.4 ^a	153.2	<0.0001*
FEV ₁ (% predicted)	98.91 ± 12.51	82.25 ± 9.48 ^a	46.84 ± 11.17 ^{a,b}	137.5	<0.0001*
FVC (% predicted)	89.47 ± 10.14	72.63 ± 8.21 ^a	44.53 ± 7.60 ^{a,b}	157.3	<0.0001*
FEV ₁ / FVC (%)	90.54 ± 4.13	85.86 ± 10.22 ^a	63.15 ± 2.75 ^{a,b}	91.19	<0.0001*

Values are expressed as mean ± SD. CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity. P was considered significant at <0.05. a Significance vs. control group, b Significance vs. mild-moderate atopic asthmatic patients using One way ANOVA followed by Tukey's post hoc test for multiple comparison.

Table (2): Comparison between biochemical parameters in the different grades of asthma severity:

Parameters/ Groups	Control group (n =30)	Atopic Asthmatics (n= 60)		One way ANOVA	
		Mild-moderate (n= 40)	Severe (n= 20)	F value	p-value
Serum CHIT1 enzyme activity (nmol/ml/h)	29.68 ± 9.22	54.39±7.46 ^a	86.11 ± 8.25 ^{a,b}	281.1	<0.0001*
IL-8 levels (pg/ml)	0.15±0.01	11.86±0.12 ^a	85.2±9.45 ^{a,b}	2508	<0.0001*
IL-17 levels (pg/ml)	25.11±9.32	38.43±12.25 ^a	54.9±11.57 ^{a,b}	42.62	<0.0001*
MDA Levels (nmol/L)	58.84± 28.31	185.44±76.52 ^a	275.32± 98.15 ^{a,b}	59.84	<0.0001*
3-Nitrotyrosine (ng/ml)	14.9±5.8	95.3±16.7 ^a	139.5±27.4 ^{a,b}	344.9	<0.0001*
GST activity (U/l)	4.51±0.21	5.69±0.43 ^a	8.92±0.87 ^{a,b}	458.3	<0.0001*

Values are expressed as mean ± SD. CHIT1: chitotriosidase; MDA: malondialdehyde; GST: glutathione S transferase, *P was considered significant at <0.05. a significance vs. control group, b significance vs. mild-moderate atopic asthmatic patients using One way ANOVA followed by Tukey's post hoc test for multiple comparison.

Table (3): Correlations (Pearson's) between the studied parameters in atopic asthmatic patients.

<i>Variables</i>	NOD2	CHIT1	IL-8	IL-17	Tail DNA %	Tail moment %	MDA	3-NT	GST
CHIT1									
<i>r</i>	-0.325								
<i>P</i>	> 0.05								
IL-8									
<i>r</i>	-0.583	+ 0.740							
<i>P</i>	<0.001*	<0.001*							
IL-17									
<i>r</i>	-0.693	+0.763	+ 0.706						
<i>P</i>	<0.001*	<0.001*	<0.001*						
Tail DNA %									
<i>r</i>	-0.576	+0.488	+0.467	+0.492					
<i>P</i>	<0.01*	<0.01*	<0.001*	<0.001*					
Tail moment %									
<i>r</i>	-0.524	+0.532	+0.499	+0.518	+0.8848				
<i>P</i>	<0.01*	<0.01*	<0.001*	<0.001*	<0.001*				
MDA									
<i>r</i>	-0.562	+0.421	+0.723	+0.614	0.656+	+0.725			
<i>P</i>	<0.01*	<0.01*	<0.001*	<0.01*	<0.001*	<0.001*			
3-NT									
<i>r</i>	-0.611	+0.409	+0.754	+0.619	+0.705	+0.732	+0.813		
<i>P</i>	<0.01*	<0.01*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*		
GST									
<i>r</i>	+0.334	-0.396	-0.508	-0.504	-0.529	-0.478	-0.632	-0.647	
<i>P</i>	>0.05	<0.05*	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*	
FEV1 (% predicted)									
<i>r</i>	+ 0.754	-0.688	-0.710	-0.658	-0.584	-0.569	-0.642	-0.598	0.388
<i>P</i>	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.05*
Total serum IgE									
<i>r</i>	-0.734	+0.687	+0.542	+0.511	+0.245	+0.318	+0.327	0.296	0.233
<i>P</i>	<0.001*	<0.01*	<0.001*	<0.001*	>0.05	>0.05	>0.05	>0.05	>0.05

r : Pearson's coefficient of correlation. **P* was considered significant at <0.05. NOD2: Nucleotide-binding oligomerization domain-containing protein 2; CHIT1: chitotriosidase; MDA: malondialdehyde; 3-NT: 3-nitrotyrosine; GST: glutathione S Transferase; FEV1: forced expiratory volume in one second.

Table (4): Multiple linear regression analyses using FEV1 (% predicted) as a dependent variable in atopic asthmatic patients

<i>Variables</i>	Unstandardized coefficients	standardized coefficients		t value	<i>P value</i>
	Beta	S.E.	Beta		
NOD2	- 12.89	4.85	-0.43	2.67	<0.001*
CHIT1	0.05	0.01	0.26	1.54	>0.05
IL-8	9.45	3.74	0.81	1.59	<0.001*
IL-17	4.9	3.22	0.195	0.95	<0.001*
Tail DNA %	3.26	0.32	0.27	1.43	<0.001*
Tail moment %	2.18	0.47	0.14	0.56	<0.001*
MDA	0.11	0.19	0.1	0.65	>0.05
3-NT	2. 15	0.42	0.47	4.91	<0.001*

S.E. standard error; *P was considered significant at <0.05. NOD2: Nucleotide-binding oligomerization domain-containing protein 2; CHIT1: chitotriosidase; MDA: malondialdehyde; 3-NT: 3-nitrotyrosine.

Abbreviations list

3-NT: 3-nitrotyrosine

CHIT1: Chitotriosidase enzyme

FEV1: Forced expiratory volume in 1 sec

FVC: forced vital capacity

GST: Glutathione-S-transferase

MDA: Malondialdehyde

NLR: Nod-like receptor

Nod2: nucleotide-binding oligomerization domain 2

PAMPs: pathogen-associated molecular patterns

ROS: Reactive oxygen species

Highlights

- Decreased NOD₂ expression levels along with increased IL-17, IL-8 levels, CHIT 1 activity, DNA damage indices and oxidative stress markers were found in atopic asthmatic cases relative to controls.
- The change in these parameters correlated significantly with the degree of decline in lung function.
- The interplay between NOD₂ signaling, proinflammatory cytokines, CHIT1 activity, heightened oxidative stress and DNA damage orchestrates allergic airway inflammation and thus contributing to the pathogenesis of atopic asthma.
- These parameters qualified for measurement as part of new noninvasive biomarker panels for monitoring asthma severity.

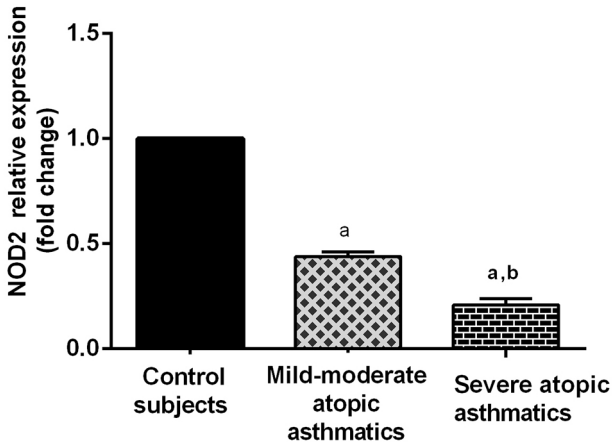


Figure 1

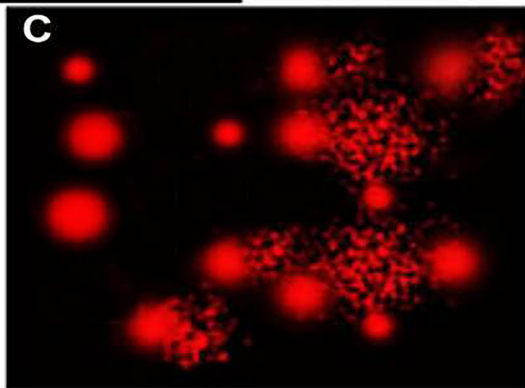
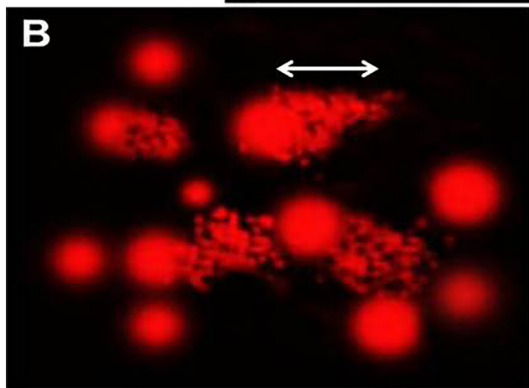
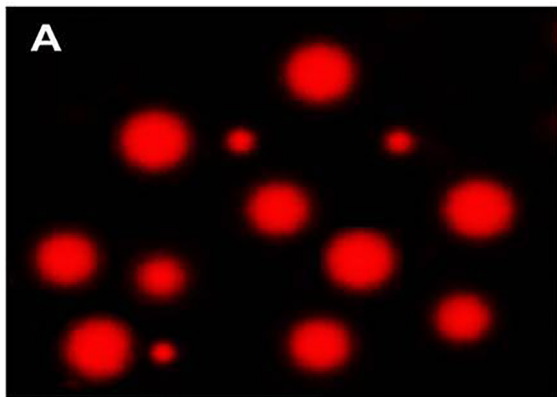
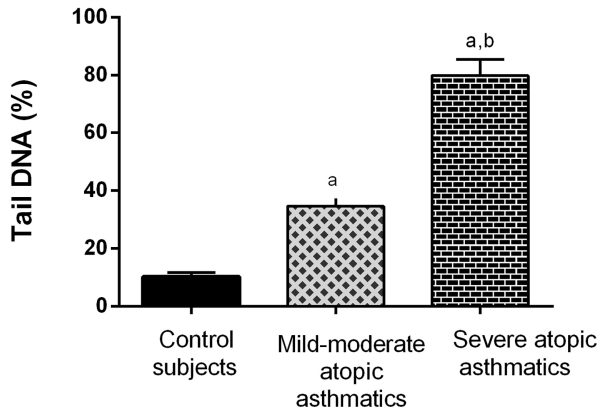


Figure 2

A



B

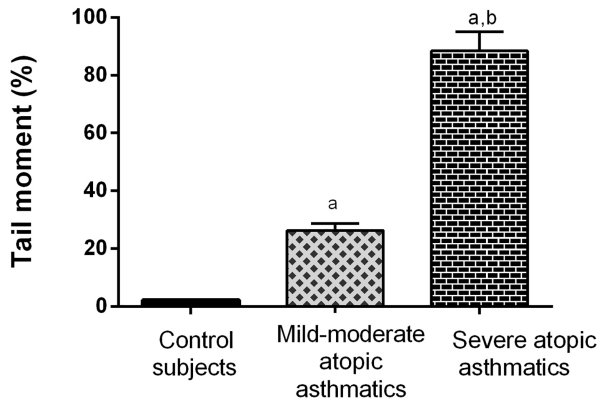


Figure 3