Research Article

Sputum Interleukin-32 in Childhood Asthma: Correlation with IL-1β

Sabrine Louhaichi[1], Tarak Cherif[1], Besma Hamdi[1], Kamel Hamzaoui[1], Agnes Hamzaoui[1]

1. Medicine Faculty of Tunis, Department of Basic Sciences, University of Tunis El Manar, Tunis, Tunisia

Background: Asthma is an airway disorder where inflammatory cytokines are partly responsible for exacerbating the disease. Interleukin (IL)-32, generally referred to as natural killer cell (NK4) transcript 4, is described as an immunoregulator involved in the stimulation of anti-/pro-inflammatory cytokines. The abnormal presence of IL-32 has been observed during inflammatory diseases, particularly in asthma. This study aims to characterize IL-32 in the inflammatory process in patients with severe asthma.

Methods: IL-32 and IL-1β levels in the supernatant of induced sputum obtained from 59 asthma patients (mild: 20 patients; moderate: 20 patients; severe: 19 patients) were measured using ELISA. Sputum IL-32 mRNA expression was measured by RT- PCR.

Results: Sputum IL-32 was significantly elevated at the protein (*p= 0.00019*) and mRNA expression (*p < 0.0001*) in asthmatics compared to non-asthmatic controls. IL-32 was elevated in severe asthmatic patients (*p < 0.0001*) compared to mild-moderate asthma. Severe asthmatics allergic to house dust mites expressed higher sputum IL-32 levels than severe asthmatics without allergy (*p = 0.0001*). A significant association was found between sputum IL-32 and IL-32 mRNA (r =0.531; *p = 0.0159*) in severe asthma. In the same way, sputum IL-1β and IL-32 were significantly correlated (r = 0.476; *p = 0.0337*).

Conclusion: The level of IL-32 in induced sputum may be associated with asthma severity.

Corresponding author: Kamel Hamzaoui, kamel.hamzaoui@gmail.com

1. Introduction

Childhood asthma is a chronic inflammatory disease of the airways [1], where innate and adaptive immune cells interacting with resident epithelial cells cause bronchial hyper-reactivity [2]. Childhood asthma manifests as recurrent attacks of wheezing, breathlessness, chest tightness, and coughing, particularly at night and in the early morning. Asthma clinical manifestations can vary from mild to severe, and the phenotypical presentation is very heterogeneous. This diversity in clinic presentation reflects the complexity of the different basic mechanisms that lead to asthma development. Childhood asthma prevalence worldwide is rising dramatically making it regarded as a major healthcare problem in children [3]. Immune response in the asthmatic respiratory tract is mainly driven by CD4⁺ T helper (Th) cells, represented by Th1, Th2, and Th17 cells, especially Th2 cells. The balance between cytokines, as well as transcription factors associated with Th, is compromised in asthma. The heterogeneity and plasticity of Th cell subsets represent part of the immune cells involved in the pathogenesis of asthma. The question to be answered is whether so-called

pathogenic Th subpopulations and other immune cells exhibit functional redundancy. Furthermore, how complex signals in vivo regulate the generation of pathogenic Th cytokines (IL–17, IL–1β, IL–26) [4][5] or non–pathogenic Th subsets warrants increasing investigation [6]. Restoring the balance between the responses of Th2/Th1 as well as Treg cells, and their respective transcription factors T-bet/STAT6 and Foxp3 considerably improves asthma. Other T cell populations and cytokines also play a role as alarmin in (IL–33, TSLP) [7][8] or as anti–inflammatory cytokines including IL–37 [9], IL35 [10] and regulatory T (Treg) cells [11].

Interleukin (IL–)32, known as the Natural Killer (NK) cell transcript 4 (NK4), is a cytokine produced by macrophages, T lymphocytes, NK cells, monocytes, mast cells, keratinocytes, and epithelial cells [12]. It was first depicted as a pro–inflammatory cytokine [13]. IL–32 by the presence of its different isoforms shows pro-/anti–inflammatory properties and regulatory properties [13]. The role of IL–32 has been, so far, investigated in several inflammatory and infectious diseases [14][15][16][17] including asthma [18]. IL–32 stimulates macrophages to produce pro–inflammatory factors (TNF–α, IL–1β, and IL–6) via the p38–MAPK and NF–κB pathways [6][19]. Human IL–32 can promote the production of IL–1β, TNF–α. The production of IL–1β, IL–6, IL–8, and TNF–α was down–regulated by silencing IL–32 expression in monocytes [19]. IL–32 has increasingly been suggested as a key player in the pathophysiology of asthma. In asthmatics, the airway presence of IL–32 was negatively correlated with the forced expiratory volume in 1 s (FEV1) and positively correlated with the annual exacerbation rate [20]. In that study, the authors concluded that the increased IL–32 level in the induced sputum samples correlated with an increased risk of asthma exacerbation.

IL–32 synergizes with the NOD1– and NOD2–specific mucopeptides of peptidoglycans for the release of IL–1β and IL–6 [21]. IL–1β is a potent inflammatory cytokine implicated in asthma. We recently reported its overexpression in asthma [5]. Extending this, we found increased sputum IL–1β in asthmatics. IL–1β was recently found associated with IL–32 production during the inflammatory process [1]. The clinical implications of IL–32 are not fully revealed to date and remain controversial in terms of asthma severity in childhood. This prompted us to measure IL–32 levels in induced sputum supernatants from asthmatic patients and to analyze the relationship of IL–32 with asthma severity.

We measured IL–32 and IL–1β at the protein and mRNA levels in severe asthma.

2. Materials and methods

2.1. Ethical approval of the research

The present study was approved by the Institutional Review Board of our hospital (A. Mami hospital of respiratory diseases) in compliance with the ethics committee of the Medicine Faculty of Tunis. Informed consent was obtained from all participating subjects. This study was approved by the Ethics Review Board at the Medicine University of Tunis and the ethics board of the Abderrahmane Mami hospital (MES19SP/02/2022) and was conducted in accordance with the Helsinki declaration.

2.2. Patients

Patients were investigated by the Department of Pediatrics Respiratory Disease and the laboratory research (Chronic Pulmonary Pathologies: From Genome to Management, Abderrahman Mami Hospital (Ariana, Tunisia). The diagnosis and severity of asthma were established according to the current Global Strategy for Asthma Management and Prevention (GINA) report [22]. Patients with severe (n = 19), moderate (n = 20) and mild (n = 20) asthma were scored according to GINA 2014 were included. The major diagnostic criteria for asthma were as follows: a medical history of episodic breathlessness, wheezing, cough, and chest tightness; spirometric features of airway obstruction with positive bronchial reversibility test and/or a positive result of methacholine challenge test. The control group consisted of volunteers with no history of obstructive lung diseases, with normal spirometry test results. Exclusion criteria for all study participants were symptoms of respiratory tract infection or asthma exacerbation within 6 weeks preceding the study onset. Lung function was assessed by spirometry with bronchial reversibility testing. The measurements were performed in accordance with the recommendations of the European Respiratory Society (ERS) and the American Thoracic Society (ATS) [23][24]. A positive bronchodilator response was defined as an increase of >200 mL and ≥12% of the predicted value in either FEV1 or FVC. The methacholine challenge test was performed consistently with the ATS guidelines, a minimum of one day before sputum induction. Atopy was defined as a positive skin prick test (3 mm in diameter in the presence of positive histamine and negative diluent controls) to at least one of 15 extracts of

common local aeroallergens [21]. Total IgE concentration was evaluated in serum using ELISA Biomerieux mini Vidas (France) according to the manufacturer's instruction (measurement range 0.5–1000 kIU/L).

2.3. Sputum processing

Sputum induction was preceded by inhalation of 400 μg of salbutamol and subsequent spirometry. Induction was performed with sterile hypertonic saline (NaCl) at increasing concentrations (3%, 4%, and 5% solutions) via an ultrasonic nebulizer (ULTRA–NEBTM2000, DeVilbiss, Port Washington, NY, USA) in accordance with the ERS guidelines [24][25]. Spirometry was repeated after each inhalation. The induction was stopped after a decrease in FEV1 by at least 20% from the baseline (post-bronchodilator) value. Sputum plugs were isolated from saliva and processed with a 0.1% solution of dithiothreitol (DTT, Sigma Aldrich, St. Louis, MO, USA). Total cell count was assessed manually using a haemocytometer, and cell viability was determined by the trypan blue exclusion method. After centrifugation, the obtained supernatants were stored at –80 ∘C for IL–32 measurements. The criteria for appropriate IS quality were as follows: <50% epithelial cells, total cell count >0.5 cells × 10^6/g sputum and >300 non-epithelial cells on one slide [24][25].

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of IL–32 were quantified with a quantitative sandwich enzyme immunoassay using an ELISA DuoSet kit (R&D, CA) as recently reported [18]. Briefly, the plates were coated with goat anti–human IL–32 antibody as the capture antibody for 16 h at room temperature. Subsequently, 100 μl of standard sputum was added, and the procedure was performed according to the manufacturer's instructions. Reference concentrations of IL–32 were used to prepare assay calibration. The absorption was determined with an ELISA reader (Biotek ELX800, USA) at 450 nm. The concentrations were interpolated from standard curves expressed in pg/ml. Inter– and intra–assay coefficients of variation were below 10%. To avoid any bias, all samples were analyzed blindly without knowledge of the clinical status. All samples were run in duplicate with the appropriate standards on Nunc Maxi Sorb 96–well microplates (Sigma-Aldrich, Germany). The detection range was 5 ng/ml − 100 ng/ml. Values below this level were scored as 0 ng/mL for statistical analysis.

Sputum IL–1β levels were determined by ELISA following the manufacturer's instructions and as we reported recently [9]. The concentration of IL–1β was measured by human Duoset ELISA kits (R&D Systems) according to the manufacturer's instructions. The sensitivity of the assay was high (pg/ml) and the limit of detection (LOD) was as follows: IL–1β (0.02 pg/ml). Standards provided by the manufacturer were used to generate a standard curve for each sample and data were analyzed using Bioplex Manager software.

2.5. Isolation of Sputum Fluid Mononuclear Cells

Induced mononuclear cells were isolated from asthmatic patients and healthy donors by density centrifugation using a lymphocyte separation medium (PAA Laboratories). Sputum mononuclear cells were washed twice in RPMI1640 and were stimulated with 1 μg/mL anti–CD3 plus anti–CD28 mAbs or 10 μg/mL recombinant soluble CD40L (R&D Systems), respectively, in order to analyze IL–32 mRNA expression by reverse transcription (RT)–quantitative PCR (RT–qPCR) [5].

2.5.1. Real-time polymerase reaction (PCR) analysis

Total RNA was isolated from unfractionated sputum cells using an RNeasy Mini kit (Sigma-Aldrich) as we reported [18][26]. RNA purity was determined by the OD 260/OD 280 ratio. Real-time semi-quantitative RT–PCR was performed in a single 50μl reaction volume containing 25 μl of One-step RT-PCR SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.25 μl of 40 × Multi Scribe reverse transcriptase (Applied Biosystems), and the following sense and antisense primers at 10 nM: IL–32: 5'-TGAGGAGCAGCACCCAGAGC-3'; 5'-CCGTAGGA CTGGAAAGAGGA-3'; IL–1β: 5'-CATCTGGAGGCGGTGGAGGA-3'; 5'-GGGACAGACCTGAGGGTGGT-3' and β-actin: 5-GGACTTCGAGCAAGAGATG and 3-AGCACT GTGTTGGCGTACA. The terminal cycling conditions were 50 ∘C for 2 min and 95 ∘C for 10 min, followed by 40 cycles of complication at 95 ∘C for 15 s and 60 ∘C for 1 min for denaturing and annealing-extension, respectively. Expression of the message level was measured with an ABI PRISM 7500 Sequence Detection System (Applied BioSystems) and normalized to β-actin mRNA.

2.5.2. Western blotting analysis

After adjusting the protein concentration, 10 μg of each protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 4-15% gradient gels, followed by analysis using a Trans-Blot Turbo Transfer Pack (Bio-Rad) and Trans-blot Turbo Blotting System (Bio-Rad). For immunoblotting, the primary antibody reaction was performed by stirring with Can Get Signal Solution1 (Toyobo) at room temperature for 1 h. The IL-32 and the IL-1b antibodies were rabbit polyclonal anti-IL-32 antibodies (dilution, 1:1000; Protein tech). The secondary antibody reaction was performed using a goat anti-rabbit IgG, horseradish peroxidase-linked antibody (dilution, 1:10000; Bio-Rad). Anti-β actin antibody (dilution, 1:3000; Sigma) was used as a loading control followed by the reaction with the corresponding horseradish peroxidase-linked secondary antibody (dilution, 1:10000; Bio-Rad). Detection and visualization were performed with an Image Quant LAS500 (GE Healthcare) system using ECL Prime Western Blotting detection reagent (GE Healthcare) as the chemiluminescence detection reagent.

2.6. Analysis

Data were expressed as means ± SD. Nonparametric statistical comparison between groups was performed by using the Mann-Whitney U test. Correlations between IL-32 levels and sputum cell percentages and other parameters were determined using Spearman's rank correlation coefficient analysis. The data are presented as the median and 25% and 75% quartiles for skewed variables and as the mean ± SEM for variables with a normal distribution. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software, release 20.0 (IBM Corp., Armonk, NY, USA). A p-value < 0.05 was considered to indicate statistical significance.

Results

2.7. Subjects' characteristics

The studied subjects are summarized in Table 1. The 59 asthma patients are distributed as follows; 20 patients have mild asthma; 20 patients have moderate asthma and 19 patients have severe asthma. Patients with severe asthma had significantly increased total IgE ($p < 0.001$) compared to the other two groups. (Table 1) ($p = 0.002$). Among the 20 patients with severe asthma, 9 patients were allergic to dust mites

	Mild Asthma	Moderate Asthma	Severe Asthma	Controls	*p–value*
Subjects n = 59	20	20	19	20	
Mean age (year)	7.72 ± 2.36	9.52 ± 3.47	12.7 ± 3.24	7.86 ± 2.72	*<0. 028*
Gender (M/F)	33.8%	33.8%	32.2%	32.4%	N.S.
IgE (IU/mL)	593.8 ± 118.2	737.8 ± 157.5	1322.5 ± 127.0*	162.7 ± 33.2	*<0.001*
FEV1 % pred	77.5 ± 4.72	72.6 ± 4.28	86.72 .62	58.30 .32	*<0.001*
FVC % pred	83.3 ± 6.2	72.6 ± 4.28	58.7 .62	82.3 ± 5.2	*<0.001*
BMI (Kg/m^2)	24.56 ± 7.2	32.2 ± 4.6	34.95 ± 5.72	22.5 ± 2.5	*=0.0004*
Sputum Characteristics					
Total cells x 10^6 per mL	1.82 (1.07 -2.37)	1.95 (1.77 – 3.63)	2.88 (1.89 – 4.59) *	1.30 (1.22 – 2.46)	*<0. 001*
Cell viability%	77 (66 – 82)	74 (55 – 86)	71 (55 – 82)	69 (59 – 82)	*N.S.*
Neutrophils%	19.2 (18.5 – 20. 5)	32.4 (29.3 – 40.5)	51.7(30 –53.4) *	35.6 (13.3 – 41.8)	*=0.0001*
Eosinophils%	0.7 (0.4 – 1.1)	0.5 (0.3 – 1.8)	0.7 (0.2 – 1.2)	0.3 (0.0 – 0.4)	*N.S.*
Macrophages%	38 (25 – 49.5)	32 (29 – 44)	65.2 (63 –70,5) *	48.8 (32 – 65.5)	*<0. 001*
Lymphocytes%	0.6 (0.3 – 0.9)	0.5 (0.2 – 1.0)	4.8 (2.2 – 5.6) *	3.5 (0.5 –4.2)	*<0. 001*
Squamous%	3.9 (1.5 – 5.8)	2.7 (1.0 –4.8)	2.7 (2.0 – 6.5)	3.7 (2.0 – 5. 9)	*N.S.*

Table 1. Patient characteristics and pulmonary function in asthmatic patients with different severity.

Data are presented as median (interquartile range) or mean ± SD, unless otherwise stated. FEV1: forced expiratory volume in 1s; FVC: forced vital capacity. N.S. Not significant. [*]: Values in severe asthma were significantly different from healthy controls (HC).

2.8. Expression of sputum fluid IL–32 strongly associates with severe asthmatics

Sputum IL–32 was detected in all asthmatic groups and healthy controls. IL–32 sputum levels were significantly higher in asthmatic patients (9.77 ± 8.99 pg/ml; *p = 0.0019*) compared with healthy non–asthmatics controls (3.26 ± 0.72 pg/ml) (Figure 1A). The expression of IL–32 in severe asthma (21.79 ± 2.56 pg/ml) is higher than the values observed in patients with mild asthma (3.14 ± 0.82 pg/ml; *p < 0.0001*) and moderate asthma (3.38 ± 0.80 pg/ml; *p < 0.0001*). No significant differences were observed between mild and moderate asthma compared to healthy controls (*p = 0.624; p = 0.632* respectively).

In the same way, IL–32 mRNA in childhood asthma was increased compared to non–asthmatic control (Figure 1B). IL–32 mRNA in severe asthma was more expressed than in patients with mild and moderate asthma (*p < 0.0001*). Moderate and mild asthmatics expressed a similar level of IL–32 at the protein and mRNA levels (*p > 0.05*). IL–32 protein level was significantly correlated with IL–32 mRNA expression (r = 0,531; *p = 0.0159*) (Figure 1C).

Patients with severe asthma, allergic to dust mites expressed more IL–32 (24.25 ± 2.35 pg/ml) compared to non–allergic patients (19.77 ± 1.46 pg/ml, p = 0.0001) (Figure 1D).

Using Western blotting analysis results confirmed the abundant IL–32 and IL–1β protein in severe asthmatic patients compared to healthy controls. IL–32 and IL–1β proteins (Fig. 2D, E) were highly expressed in severe asthmatic samples compared to healthy controls (Fig. 2E).
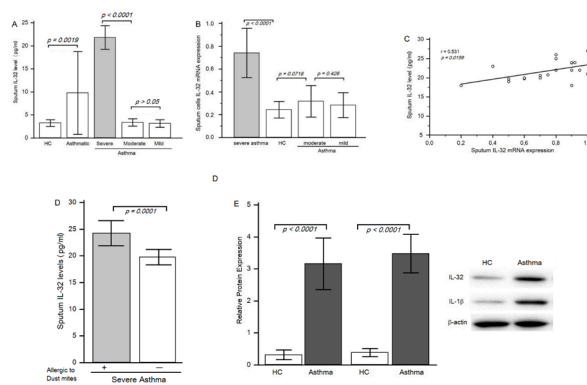
Figure 1. IL-32 levels in childhood asthma patients. (A): Sputum IL-32 in asthmatic patients and non-asthmatic controls (*p = 0.0009*). (B): IL-32 mRNA expression was increased in severe asthmatic patients compared to healthy participants. (C): Sputum IL-32 protein correlated significantly with IL-32 mRNA expression in severe asthmatic (r = 0.531; p = 0.0159). (**D**): Increased IL-32 level in severe asthma positive for dust mite allergy compared to non-allergic severe asthmatics (*p = 0.0001*). (**E**): Western blot analysis of IL-32 and IL-1β. High IL-32 and IL-1β expression was observed in severe asthmatic patients. The statistical analysis was performed using the Mann-Whitney U test. *p* values are indicated and the correlation was analyzed by Spearman's rank correlation test.
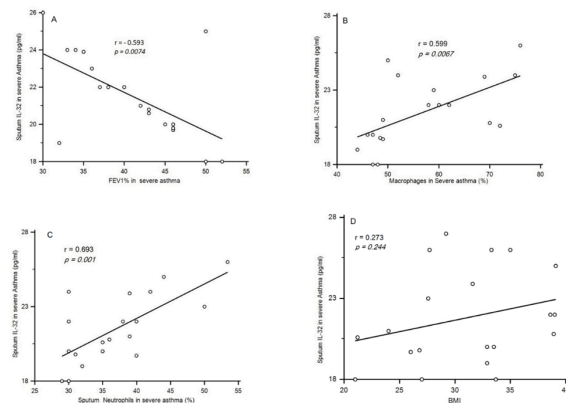

Figure 2. Sputum expression of IL-32 in severe asthma, correlations with FEV1%, sputum polynuclear neutrophils (PNN), sputum macrophages and Body Mass Index (BMI Kg/m^2). (A) Significant correlation between sputum IL-32 and FEV1% in severe asthma (r = −0.593; *p =0.0074*). (B) Positive correlation between sputum IL-32 and macrophages (%) (r =0.599; *p= 0.0067*). (C) A significant correlation was reported between sputum IL-32 and PNN % (r = 0.693; *p = 0.001*). (D): Correlation between sputum IL-32 and BMI (Kg/m^2). Statistical significance was analyzed using Spearman's rank correlation test for the studied correlations.

2.9. Correlations of sputum IL-32 and FEV1%, macrophages (%), PNN (%) in severe asthma

Sputum IL-32 protein levels were inversely correlated with FEV1 (% pred.) (r = − 0.593; *p = 0.0074*) (Figure 2A). Associations were found between IL-32 protein and sputum macrophages (r = 0.599; *p = 0.0067*) and neutrophils (r = 0.693; *p = 0.001*) (Figure 2B, 2C). However, no correlation was observed between IL-32 and BMI (Kg/m^2) (r = 0.273; *p = 0.244*).

2.10. Expression of sputum fluid IL-1β strongly associates with severe asthmatics

IL-32 can induce interleukin 1β (IL-1β) and other pro-inflammatory cytokines as reported by Netea et al. [21]. At the protein level IL-1β was more expressed in severe asthmatic patients (78.15 ± 11.13 pg/ml; *p < 0.0001*) than in moderate and mild asthmatics (16.0 ± 4.18pg/ml; 14.25 ± 2.5 pg/ml respectively) (Figure 3A). No difference was found between mild and moderate asthma (*p > 0.05*). IL-1β was significantly correlated to IL-32 (r = 0.476; *p = 0.0337*) (Figure 3B).
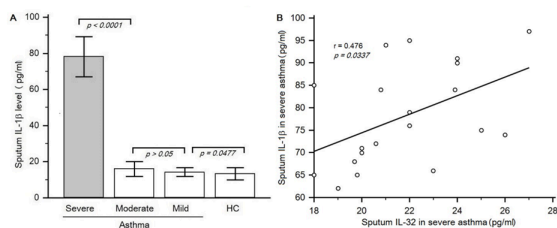
Figure 3. IL–1β levels in asthmatic patients. (A) Sputum IL–1β in asthmatic patients and non–asthmatic controls. The statistical analysis was performed using the Mann–Whitney U test. *p* values are indicated. (B) Sputum IL–1β protein was correlated with IL–32 expression in severe asthma (r = 0.476; *p = 0.033*). Statistical significance was analyzed using Spearman's rank correlation test.

3. Discussion

Our data reported that childhood patients with severe asthma have increased expression of IL–32 and increased release of IL–1β protein compared to mild–moderate asthmatic patients and non–asthmatic controls. To our knowledge, this work is likely to be the first study involving IL–32 in induced sputum in severe asthma in children. IL–32 mRNA was increased in severe asthmatics. In addition, IL–32 protein levels were significantly correlated with neutrophils and macrophages. In contrast, no correlation was found with IgE levels. Additionally, IL–32 levels in the airways were inversely correlated with FEV1%. These results indicate a relationship between IL–32 levels in the airways and disease severity.

We designed this work to investigate levels of the newly described pro–inflammatory cytokine IL–32 in airway compartments. Our data are consistent with those of other authors who have found increased IL–32 levels in asthma [27][28]. Meyer *et al.* found that serum IL–32 levels were elevated in asthmatic patients [29]. Other research groups have shown that IL–32 may play a role in allergic diseases. Patients with allergic rhinitis differed significantly from healthy control individuals in having increased expression of IL–32 in serum [30]. IL–32 has been expressed in atopic dermatitis lesional skin, whereas it has not been detected in skin biopsy specimens from controls [31]. The role of IL–32 in inflammation can be explained by the participation of the immune mechanisms of Th1 cells in an endotype according to the classification thus influencing angiogenesis [29][32]. Our study did not allow us to differentiate the categories of endotypes, because the number of asthmatics in our series was relatively small. We cannot confirm or refute the role of IL–32 in mild to moderate asthma. But what seems plausible is that IL–32 certainly plays a more important role in severe asthma and the Receiver operating characteristic (ROR) curve further demonstrated that IL–32 could be a potent inflammatory cytokine implicated in asthma as reported in chronic inflammatory diseases [33][34].

IL–32 is a key modulator in the pathogenesis of various clinical conditions and is mostly induced by IL–8 [15][35]. Recombinant IL–32 induces the proinflammatory cytokines TNF–α, IL–8, and macrophage inflammatory protein 2 in monocytes, macrophages, and PBMCs [13]. IL–8 has been discovered to be an inflammatory cytokine that is a potent activating and chemotactic factor of neutrophils, suggesting an important reciprocal relationship between neutrophils and IL–8 in the pathogenesis of a variety of neutrophil–infiltrating chronic inflammatory diseases [36]. Heinhuis et al. reported that. The overexpression of IL–32 results in enhanced expression of IL–8 [37]. IL–32 modulates important inflammatory pathways (including TNF–α, IL–6 and IL–1β), contributing to the pathogenesis of inflammatory diseases [15]. Mature IL–1β activates the IL–1 receptor (IL–1R) to induce the production of pro–inflammatory cytokines including IL–8, which is typically elevated in neutrophilic asthma [38]. IL–8 is widely known as a potent neutrophil chemoattractant, but recently it also has been used as an activator of neutrophils. Such stimulation favors the degradation of cell membranes and nuclei and leads to the formation of extracellular web–like structures from released nucleic acids and histones [39]. IL–1β is a potent inflammatory cytokine implicated in several chronic inflammatory diseases, including asthma and chronic obstructive pulmonary disease [33]. The number of neutrophils in the airways of asthmatic individuals depends on IL–8 and tumor necrosis factor (TNF–α) concentrations, both being chemotactic cytokines released from macrophages, epithelial cells and neutrophils [40][41]. The role of neutrophils in the development of asthma is multi–dimensional. Their presence and overactivation are associated with increased asthma severity. Studies regarding neutrophils were performed in either induced sputum or bronchoalveolar fluid, and peripheral blood. Mediators normally released from neutrophils as IL–8, playing important role in innate immunity, contribute to the development of asthma. The role of IL–32 in inflammation is pleiotropic since it is involved in not

only promoting pro-inflammatory cytokines but also stimulating anti-inflammatory cytokines [42][43][44][45].

IL–1β is a potent inflammatory cytokine. Extending this, we found a significant correlation between IL–32 and IL–1β. Simpson et al. reported that sputum IL–1β was significantly elevated in severe asthma compared with other asthma (mild-moderate), suggesting a key role for IL–1β in asthma severity. IL–1β concentrations were independently associated with the neutrophil chemoattractant IL–8 [34]. Our results support recent findings, which show increased IL–1β in asthma, especially in severe asthma [33] and provide evidence of the involvement of IL–32. The activation of the innate immune cells such as macrophages, dendritic cells and PNN can activate caspase-1 through inflammasome assembly, which subsequently leads to mature IL–1β through the cleavage of pro-IL–1β and finally results in cascade inflammatory response. IL–32 stimulated the production of pro-inflammatory cytokines TNF–α, IL–1β, IL–8 and IL–6 by activating nuclear factor-kappa B (NF–kB) and mitogen-activated protein kinase (MAPK) p38.2 [21]. We reported increased IL–32 levels in severe asthma positive for dust mites compared to non-allergic patients. Recent data reported that IL–32 expression may be elevated in allergic diseases during Th2-type eosinophilic inflammation [31].

Pulmonary macrophages control inflammatory manifestations through the release of chemokines and cytokines that attract inflammatory cells and the release of proteases [45]. Macrophages are the main source of IL–32 [45][46]. We reported a significant correlation between IL–32 production and the percentage of macrophage in severe asthma. In asthma, alveolar macrophages are inappropriately activated and are involved in the development and progression of the disease. Airway macrophages can be activated by allergens via low-affinity IgE receptors to release inflammatory mediators amplifying responses [47][48]. We did not find a significant correlation between IL–32 expression in severe asthmatics and BMI. Segura et al. reported that BMI and asthma severity showed a moderate correlation (r = 0.528), according to Colton's criteria [49]. They also observed that there was a non-significant correlation between BMI and asthma severity with IL–4, and finally there was no correlation with IL–1β [49]. However, more recent results indicated that BMI was significantly correlated with IL–1β but not with other measured indicators of inflammation including neutrophilia, eosinophilia, IL–6, and IL–8 [50]. Our result should include more severe asthmatic

patients for the possible correlation between BMI and the expression of IL–32 and IL–1β. Grandi et al reported a trial of weight loss in patients with a BMI of 35 to 40 kg/m2 and moderate to severe asthma to assess the effect of dynamic hyperinflation [51].

There are some limitations to this study. First, the dosage of IL–32 at the time of exacerbation and remission of the disease associated with the treatment is missing in our work to see the degree of evolution of this cytokine in relation to the evolution of asthma severity in childhood. This suggests that the measurements taken at any given time may be insufficient to reliably capture the extent of potential inflammatory activity. A Mendelian randomization study may provide further information to explain the evolution of the pathology according to the inflammatory process. Second, this is a retrospective study with relatively few samples, highlighting the need for future large-scale prospective analyzes designed to validate and expand on these results.

4. Conclusions

In conclusion, this study shows that significant increases in IL–32 protein and mRNA expression can be observed in the induced sputum of severe asthmatic subjects compared to mild and moderate asthmatic patients. A significant correlation was observed between sputum protein IL–32 levels and IL–1β in severe asthmatics. IL–32 was also correlated with macrophages and neutrophils. These results provide information on key mechanisms of airway injury in severe childhood asthma. Our study suggests that induced sputum, a relatively non-invasive sampling method, can be an interesting tool to study bronchial inflammatory and remodeling events.

Conflict of interest

All authors declare no conflicts of interest in this paper.

Author contributions

The study was designed by AH, KH and SL. Sample and patient-related data collection were performed by SL, TCh, and BH. Laboratory work and data collection were performed by KH, SL, Tch and BH. Data analysis was

performed by SL, and TCh. Data interpretation and writing of the manuscript were done by AH, KH and SL. All authors read and approved the final manuscript.

Abbreviations

IL: Interleukin; ELISA: enzyme-linked immunosorbent assay; (RT) PCR: Reverse transcription polymerase chain reaction; ROC: receiver operating curve; NK4: natural killer cell transcript 4; DCs: Dendritic cells; FEV: Forced expiratory volume; NF-κB: Nuclear factor-kappa B; NOD: Nucleotide-binding oligomerization domain; Th: Helper T cell; TNF: Tumor necrosis factor.

References

1. [a, b]Frei R, Heye K, Roduit C. (2022) Environmental influences on childhood allergies and asthma The Farm effect. Pediatr Allergy Immunol. 33(6):e13807. doi: 10.1111/pai.13807.

2. [△]Lambrecht BN, Hammad H. (2015) The immunology of asthma. Nat Immunol 16:45–56.

3. [△]Yangzong Y, Shi Z, Nafstad P, et al. (2012) The prevalence of childhood asthma in China: a systematic review. BMC Public Health 12:860.

4. [△]Hu Y, Chen Z, Zeng J, et al. (2020) Th17/Treg imbalance is associated with reduced indoleamine 2,3 dioxygenase activity in childhood allergic asthma. Allergy Asthma Clin Immunol. 16:61. doi:10.1186/s13223-020-00457-7.

5. [a, b, c]Louhaichi S, Mlika M, Hamdi B, et al. (2020) Sputum IL-26 Is Overexpressed in Severe Asthma and Induces Proinflammatory Cytokine Production and Th17 Cell Generation: A Case-Control Study of Women. J Asthma Allergy. 3;13:95-107. doi: 10.2147/JAA.S229522.

6. [a, b]Luo W, Hu J, Xu W, Dong J. Distinct spatial and temporal roles for Th1, Th2, and Th17 cells in asthma. Front Immunol. 2022; 13: 97406

7. [△]Kim MH, Kwon JW, Hahn et al. (2022) Circulating IL-32 and IL-33 levels in patients with asthma and COPD: a retrospective cross-sectional study. J Thorac Dis.14(6):2437-2439. doi: 10.21037/jtd-21-524.

8. [△]Hamzaoui A, Berraies A, Kaabachi W, et al. (2013) Induced sputum levels of IL-33 and soluble ST2 in young asthmatic children. J Asthma. 50(8):803-9. doi: 10.3109/02770903.2013.816317.

9. [a, b]Charrad R, Berraïes A, Hamdi B, et al (2016) Anti-inflammatory activity of IL-37 in asthmatic children: Correlation with inflammatory cytokines TNF-α, IL-β, IL-6 and IL-17A. Immunobiology. 221(2):182-7. doi: 10.1016/j.imbio.2015.09.009.

10. [△]Mansour AI, Abd Almonaem ER, Behairy OG, et al. (2017) Predictive value of IL-35 and IL-17 in diagnosis of childhood asthma. Scand J Clin Lab Invest.77(5):373-378. doi: 10.1080/00365513.2017.1328739.

11. [△]Hamzaoui A, Maalmi H, Berraïes A, et al. (2011) Transcriptional characteristics of CD4 T cells in young asthmatic children: RORC and FOXP3 axis. J Inflamm Res. 4:139-46. doi: 10.2147/JIR.S25314.

12. [△]Dinarello, CA S.H. Kim SH. (2006) IL-32, a novel cytokine with a possible role in disease, Ann. Rheum. Dis. 65 (Suppl 3) iii61eiii64.

13. [a, b, c]Kim S, Han S, Azam T, et al. (2005) Interleukin-32 a Cytokine and Inducer of TNFα. Immunity. 22:131–42. doi: 10.1016/S1074-7613(04)00380-2.

14. [△]Ribeiro-Dias F, Saar Gomes R, de Lima Silva LL, et al. (2017) Interleukin 32: A Novel Player in the Control of Infectious Diseases. J Leukoc Biol 101:39–52. doi: 10.1189/jlb.4RU0416-175RR.

15. [a, b, c]Joosten LAB, Netea MG, Kim S-H, et al. (2006) IL-32, a Proinflammatory Cytokine in Rheumatoid Arthritis. Proc Natl Acad Sci 103:3298–303. doi: 10.1073/pnas.0511233103.

16. [△]Shioya M, Nishida A, Yagi Y, et al. (2007) Epithelial Overexpression of Interleukin-32α in Inflammatory Bowel Disease: IL-32α and IBD. Clin Exp Immunol 149:480−6. doi: 10.1111/j.1365-2249.2007.03439.x.

17. [△]Montoya D, Inkeles MS, Liu PT, et al. (2014) IL-32 is a Molecular Marker of a Host Defense Network in Human Tuberculosis. Sci Transl Med. 6(250):250ra114. doi: 10.1126/scitranslmed.3009546.

18. [a, b, c]Hamzaoui K, Borhani-Haghighi A, Dhifallah IB, Hamzaoui A. (2022) Elevated levels of IL-32 in cerebrospinal fluid of neuro-Behcet disease: Correlation with NLRP3 inflammasome. J Neuroimmunol. 365:577820. doi: 10.1016/j.jneuroim.2022.577820.

19. [a, b]Nold-Petry CA, Nold MF, Zepp JA, Kim SH, Voelkel NF, Dinarello CA. (2009) IL-32- dependent effects of IL-1 beta on endothelial cell functions. Proc Natl Acad Sci U S A. 106:3883−8

20. [△]Kwon JW, Chang HS, Heo JS, Bae DJ, Lee JU, Jung CA, et al. (2017) Characteristics of asthmatics with detectable IL-32gamma in induced sputum. Respir Med. 2017; 129:85−90

21. [a, b, c, d]Netea MG, Azam T, Ferwerda G, et al. (2005) IL-32 synergizes with nucleotide oligomerization domain (NOD) 1 and NOD2 ligands for IL-1beta and IL-6 production through a caspase 1-dependent mechanism. Proc Natl Acad Sci U S A. 102(45):16309-16314

22. [△]GINA (2022 GINA Report, Global Strategy for Asthma Management and Prevention. Available from: http://www.ginaasthma.org).

23. ᐱPellegrino R, Viegi G, Brusasco V, et al. (2005) Interpretative strategies for lung function tests. Eur Respir J. 26(5):948-68. doi: 10.1183/09031936.05.00035205.

24. ᵃ, ᵇ, ᶜMiller MR, Hankinson J, Brusasco V, et al. (2005) ATS/ERS Task Force. Standardisation of spirometry. Eur Respir J. 26(2):319-38. doi: 10.1183/09031936. 05.00034805.

25. ᵃ, ᵇCrapo RO, Casaburi R, Coates AL, et al. (2000) Guidelines for methacholine and exercise challenge testing -1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. Am J Respir Crit Care Med. 161(1):309-29. doi: 10.1164/ajrccm.161.1.ats11-99.

26. ᐱSalhi M, Tizaoui K, Louhaichi S, et al. (2020) IL-26 gene variants and protein expression in Tunisian asthmatic patients. Cytokine. 134:155206. doi: 10.1016/j.cyto.2020.155206.

27. ᐱXin T, Chen M, Duan L, et al. (2018) Interleukin-32: its role in asthma and potential as a therapeutic agent. Respir Res. 19(1):124. doi: 10.1186/s12931-018-0832-x.

28. ᐱBoreika R, Sitkauskiene B. (2021) Interleukin-32 in Pathogenesis of Atopic Diseases: Proinflammatory or Anti-Inflammatory Role? J Interferon Cytokine Res. 41(7): 235-243. doi: 10.1089/jir.2020.0230.

29. ᵃ, ᵇMeyer N, Christoph J, Makrinioti H, et al. (2012) Inhibition of angiogenesis by IL-32: possible role in asthma. J Allergy Clin Immunol. 129(4):964-73.e7. doi: 10.1016/j.jaci.2011.12.1002.

30. ᐱJeong HJ, Shin SY, Oh HA, et al. (2011) IL-32 up-regulation is associated with inflammatory cytokine production in allergic rhinitis. J Pathol. 224(4):553-63. doi: 10.1002/path.2899.

31. ᵃ, ᵇMeyer N, Zimmermann M, Bürgler S, et al. (2010) IL-32 is expressed by human primary keratinocytes and modulates keratinocyte apoptosis in atopic dermatitis. J Allergy Clin Immunol. 2010 Apr;125(4):858-865.e10. doi: 10.1016/j.jaci.2010.01.016.

32. ᐱLötvall J, Akdis CA, Bacharier LB, et al. (2011) Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol. 127(2):355-60. doi: 10.1016/j.jaci.2010.11.037.

33. ᵃ, ᵇ, ᶜBaines KJ, Simpson JL, Wood LG, et al. (2011) Transcriptional phenotypes of asthma defined by gene expression profiling of induced sputum samples. J Allergy Clin Immunol 127: 153–160.

34. ᵃ, ᵇSimpson JL, Phipps S, Baines KJ, et al. (2014) Elevated expression of the NLRP3 inflammasome in neutrophilic asthma. Eur Respir J. 43(4):1067-76. doi: 10.1183/09031936.00105013.

35. ᐱKhawar MB, Abbasi MH, Sheik N, et al. (2016) IL-32: A Novel Pluripotent Inflammatory Interleukin, toward s Gastric Inflammation, Gastric Cancer, and Chronic Rhino Sinusitis, Mediators of Inflammation. 1–8.

36. ᐱTomassen P, Vandeplas G, Van Zele, et al. (2016) Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. J Allergy Clin Immunol 137:1449e56.e4.

37. ᐱHeinhuis TS, Plantinga G, Semango B, et al. (2016) Alternatively spliced isoforms of IL-32 differentially influence cell death pathways in cancer cell lines, Carcinogenesis 37 (2)197–205.

38. ᐱGibson PG, Simpson JL, Saltos N, et al. (2001) Heterogeneity of airway inflammation in persistent asthma. Chest 119: 1329–1336.

39. ᐱSchorn, C, Janko C, Latzko M, et al. (2012) Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. Front. Immunol. 3, 277.

40. ᐱDrost EM, Mac Nee W, et al (2002) Potential role of IL-8, platelet-activating factor and TNF-alpha in the sequestration of neutrophils in the lung: effects on neutrophildeformability, adhesion receptor expression, and chemotaxis. Eur. J. Immunol. 32 (2), 393–403.

41. ᐱLavinskiene S, Bajoriuniene I, Malakauskas K, et al. (2014) Sputum neutrophil count after bronchial allergen challenge is related to peripheral blood neutrophil chemotaxis in asthma patients. Inflamm. Res. 63 (11), 951–959.

42. ᐱHeinhuis B, Koenders MI, van Riel PL, et al. (2011) Tumour necrosis factor alpha-driven IL-32 expression in rheumatoid arthritis synovial tissue amplifies an inflammatory cascade. Ann Rheum Dis. 70:660–7.

43. ᐱHeinhuis B, Koenders MI, van de Loo FA, Netea MG, (2011) Inflammation-dependent secretion and splicing of IL-32{gamma} in rheumatoid arthritis. Proc Natl Acad Sci U S A.108:4962-7.

44. ᐱKang JW, Choi SC, Cho MC, Kim HJ, Kim JH, Lim JS, et al. (2009) A proinflammatory cytokine interleukin-32 beta promotes the production of an anti-inflammatory cytokine interleukin-10. Immunology. 128:e532–40

45. ᵃ, ᵇ, ᶜTillie-Leblond I, Pugin J, Marquette CH, et al. (1999) Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus. Am J Respir Crit Care Med. 159(2): 487-94. doi: 10.1164/ajrccm.159.2.9805115.

46. ᐱBabusyte A, Stravinskaite K, Jeroch J, et al. (2007) Patterns of airway inflammation and MMP-12 expression in smokers and ex-smokers with COPD. Respir Res. 8 (1):81. doi: 10.1186/1465-9921-8-81.

47. ᐱBarnes PJ. Mechanisms in COPD: Differences from asthma. Chest (2000); 117(2 Suppl):10 S–14 S. doi: 10.1378/chest.117.2_suppl.10s.

48. ^Netea MG, Azam T, Lewis EC, et al. (2006) Mycobact erium tuberculosis induces interleukin-32 production t hrough a caspase- 1/IL-18/interferon-gamma-depend ent mechanism. PLoS Med. 3(8):e277. doi: 10.1371/jour nal.pmed.0030277.

49. ^a, ^b Segura MN, Murillo GE, Rojas-Dotor S, Rico G, Mart ínez HL, Sandoval SC. Marcadores inflamatorios relaci onados con asma e índice de masa corporal [Inflamm atory markers associated with asthma and body mass index]. Rev Alerg Mex. 2007;54(6):196-200.

50. ^Todoric K, Zhou H, Zhang H, Mills K, Peden DB, Hern andez ML. Body mass index correlates with pollutant-induced interleukin-1β in sputum and blood. Ann Aller gy Asthma Immunol. 2015;114(3):251-253.

51. ^Grandi Silva A, Duarte Freitas P, Ferreira PG, et al. Eff ects of weight loss on dynamic hyperinflation in obese women asthmatics. J Appl Physiol. 2019;126(2): 413-42 1.

Declarations