Cytokine 140 (2021) 155421

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Sputum IL-25, IL-33 and TSLP, IL-23 and IL-36 in airway obstructive diseases. Reduced levels of IL-36 in eosinophilic phenotype

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ARTICLE INFO

Keywords:
Airway obstructive diseases
Alarmins
Sputum
IL-23
IL-36

ABSTRACT

Introduction: Alarmins ((IL-25, IL-33 and thymic stromal lymphopoietin (TSLP)) are known to promote Th2 inflammation and could be associated with eosinophilic airway infiltration. They may also play a role in airway remodeling in chronic airway obstructive diseases such as asthma and chronic obstructive pulmonary disease (COPD). IL-23 and IL-36 were shown to mediate the neutrophilic airway inflammation as seen in chronic airway obstructive diseases. **Objectives:** The purpose of this project was to determine the expression and the production of these cytokines from induced sputum (IS) in patients with chronic airway obstructive diseases including asthmatics and COPD. The relationship of the mediators with sputum inflammatory cellular profile and the severity of airway obstruction was assessed.

Methods: The alarmins (IL-25, IL-33 and TSLP) as well as IL-23 and IL-36 concentrations were measured in IS from 24 asthmatics and 20 COPD patients compared to 25 healthy volunteers. The cytokines were assessed by ELISA in the IS supernatant and by RT-qPCR in the IS cells.

Results: At protein level, no difference was observed between controls and patients suffering from airway obstructive diseases regarding the different mediators. IL-36 protein level was negatively correlated with sputum eosinophil and appeared significantly decreased in patients with an eosinophilic airway inflammation compared to those with a neutrophilic profile and controls. At gene level, only IL-36, IL-23 and TSLP were measurable but none differed between controls and patients with airway obstructive diseases. IL-36 and IL-23 were significantly increased in patients with an neutrophilic inflammatory profile compared to those with an eosinophilic inflammation and were correlated with sputum neutrophil proportions. None of the mediators were linked to airway obstruction.

Conclusions: The main finding of our study is that patients with eosinophilic airway inflammation exhibited a reduced IL-36 level which could make them more susceptible to airway infections as IL-36 is implicated in antimicrobial defense. This study showed also an implication of IL-36 and IL-23 in airway neutrophilic inflammation in chronic airway obstructive diseases.

1. Introduction

As asthma and COPD combine multiple phenotypes and overlap between them, it has been recently suggested to consider these pathologies together as “chronic airway obstructive diseases” to select appropriate therapies. Moreover, it was proposed to consider patients suffering from airway diseases according to “treatable traits” such as eosinophilic airway inflammation rather than using the conventional diagnostic labels [1–3].

Alarmins ((namely thymic stromal lymphopoietin (TSLP), Interleukin (IL)-33 and IL-25)) are cytokines released by the bronchial epithelial cells, among others, in case of danger signal that can be endogenous or exogenous. Indeed, damage-associated molecular patterns (DAMPs) which include IL-33 itself [4], induce a pro-inflammatory

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https://doi.org/10.1016/j.cyto.2021.155421
Received 7 September 2020; Received in revised form 15 December 2020; Accepted 15 December 2020
Available online 22 January 2021

and immune response in the same way than exogeneous pathogen-associated molecular patterns (PAMPs) [5]. The released alarmins can in turn initiate a Th2 response and local eosinophil recruitment in asthma context [6]. Targeting those mediators was recently thought to be of interest as they are up-stream of the Th2 cascade [7,8] and promising clinical studies are ongoing [9]. Inhibition of the cytokine triad could also represent a treatment opportunity in the future [10].

TSLP is an IL-2 family member shown to be linked to atopic diseases [11] but also to non-atopic pathologies such as chronic obstructive pulmonary disease (COPD) [12]. Tezepelumab, an anti-TSLP antibody, has recently shown beneficial effects in a clinical trial targeting severe asthma [13]. IL-33 is an IL-1 family cytokine, and once secreted and fixed to its ST2 receptor (an IL-1 receptor like-1 also known as IL1RL1 and which possesses transmembrane and soluble isoforms), mediates the group 2 innate lymphoid cells (ILC2) activation and inflammatory cytokines production. Increase in lung IL-33 and ST2 expressions have been observed both in asthma and COPD [14]. Moreover, single nucleotide polymorphism (SNPs) in TSLP and IL-33 genes were identified to be linked to asthma susceptibility [15]. IL-25, also known as IL-17E, although being part of IL-17 cytokine family, has a pleiotropic nature and possess distinct functions [16]. It is implicated in Th2-dependent immunity and is involved in the development of "Th2-high allergic asthma", a subtype of asthma showing good inhaled corticosteroids (ICS) response [17]. In the same manner, sputum high IL-25 gene expression level appeared closely related to high IL-5 expression pattern [18]. IL-25 was observed to be increased in the blood of COPD patients and its production was promoted by TSLP [19]. Finally, the triad TSLP, IL-33 and IL-25 were also reported to be involved in the airway remodeling, a classical asthma feature [20].

IL-36, an IL-1 superfamily member, is also produced by the lung epithelium in response to stress such as viral or bacterial infections or house dust mite stimulation [21]. It is known to be linked to a Th-1 and Th-17 response and may contribute to the neutrophilic infiltration seen in asthma and COPD [22].

Finally, IL-23, an IL-12 family member, regulates chronic inflammatory processes. Anti-IL-23 (Risankizumab) are used for chronic skin diseases such as psoriasis [23]. IL-23 drives the Th-17 inflammation and a mouse asthma model revealed that an increased airway IL-23 expression induced a local eosinophil and neutrophil recruitment and Th2 cytokines production [24]. It could then be considered as an interesting potential target for therapies in severe asthma [25] as it has been related to the severity of airway obstruction [26]. IL-23 appeared also increased in bronchial biopsies of COPD patients compared to healthy controls [27].

Induced sputum (IS) is a non-invasive method to collect cells from airways that is useful both as a research tool and in the management of asthmatic patients in clinical practice [28]. In addition, induced sputum has been pivotal developing the concept of airway inflammatory phenotype [29].

As the literature regarding the sputum levels of these mediators is limited in chronic airway diseases, this study aimed to measure the alarmins (TSLP, IL-33 and IL-25) as well as IL-23 and IL-36 protein and gene expression levels in sputum of patients suffering from chronic airway obstructive diseases and in a group of healthy controls. In addition, the relationship between these proteins production and specific traits such as sputum inflammatory cellular profile and the severity of airway obstruction was assessed in order to detect particular phenotypes/endotypes.

2. Material and methods

2.1. Subjects

Patient characteristics are given in Table 1. Twenty-four asthmatic

Table 1
Demographic and functional characteristics.

	Controls (n = 25)	Airway obstructive diseases (n = 44)	P value	COPD (n = 20)	Asthmatic (n = 24)	P value
Sex (M/F)	11/14	17/27	0.80	9/11	8/16	0.67
Age (years)	55 ± 14	54 ± 16	0.81	64 ± 8$^{\$\$\$}$	46 ± 17	0.0002
Smoking status(NS/ExS/S)	19/4/2	17/15/12	0.02	0/12/8	17/3/4	<0.0001
Pack-years	5 ± 9	20 ± 20	<0.001	35 ± 17 ***$^{\$\$\$}$	9 ± 14	0.0024
BMI (kg/m^2)	25 ± 3	25 ± 4	0.68	24 ± 3	26 ± 4	0.28
Atopy (yes/no)	5/20	22/22	0.02	6/14	16/8	0.0023
FeNO (ppb)	20 (16–29)	27 (10–46)	0.30	29 (10–56)	26 (10–44)	0.55
FEV1 (% predicted)	111 ± 15	64 ± 26	<0.0001	48 ± 18***$^{\$\$\$}$	77 ± 25***	<0.0001
FEV1 post BD (% predicted)	117 ± 16	69 ± 27	<0.0001	52 ± 20***$^{\$\$\$}$	88 ± 23***	<0.0001
FVC (% predicted)	116 ± 19	82 ± 22	<0.0001	72 ± 20***$^{\$}$	90 ± 21***	<0.0001
FVC post BD (% predicted)	116 ± 19	86 ± 20	<0.0001	77 ± 20***$^{\$\$\$}$	102 ± 14	<0.0001
FEV1/FVC (%)	79 ± 5	64 ± 14	<0.0001	55 ± 10***$^{\$\$\$}$	71 ± 12**	<0.0001
FEV1/FVC post BD (%)	84 ± 5	65 ± 17	<0.0001	54 ± 10***$^{\$\$\$}$	80 ± 11	<0.0001
RV (%predicted)		147 ± 52		187 ± 37	125 ± 38	0.0038
TLC (% predicted)		101 ± 23		115 ± 16	105 ± 13	0.17
DLCO (% predicted)		57 ± 21		44 ± 13	74 ± 14	0.0004
KCO (% predicted)		76 ± 28		55 ± 15	84 ± 11	0.0007
ICS (yes/no)	0/25	29/15		11/9	18/6	<0.0001
OCS (yes/no)	0/25	5/39		3/17	2/22	0.15
LABA (yes/no)	0/25	35/9		14/6	21/3	<0.0001
LAMA (yes/no)	0/25	12/32		11/9$^{\$\$\$}$	1/23	<0.0001
SABA (yes/no)	0/25	12/32		2/18$^{\$}$	10/14	0.0004
SAMA (yes/no)	0/25	20/24		11/9	9/15	0.0001
LTRA (yes/no)	0/25	7/37		1/19	6/18	0.01
Severity status				GOLD 1: 1 GOLD 2: 10 GOLD 3: 7 GOLD 4: 2	6 mild 7 moderate 11 severe- refractory	

Results are expressed as mean ± SD or median (25–75%). NS: non-smoker; ExS: ex-smoker; S: smoker; BMI: body mass index; FeNO: exhaled nitric oxide. FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; DLCO: diffusing capacity of the lung for carbon monoxide; KCO: gas transfer coefficient; ICS: inhaled corticosteroids; OCS: oral corticosteroids; LABA: long acting beta 2 agonist, LAMA: long acting muscarinic antagonist. SABA: short acting beta agonist; SAMA: short acting muscarinic antagonist; LTRA: leukotriene receptor antagonist. *P < 0.05, **P < 0.01, ***P < 0.001 vs healthy subjects. $^{\$}$P < 0.05, $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001 vs asthmatic subjects.

and 20 COPD patients were recruited through the outpatient clinic and pulmonary rehabilitation centre (CHU, Sart-Tilman, Liege). Characteristics of patients classified using classical diagnosis can be found in Table 1. Asthma was diagnosed following the GINA guidelines (http://ginasthma.org/). Mild and moderate asthma were defined as patients without maintenance treatment or with a dose of ICS lower than 1000 μg beclomethasone per day, associated with a $FEV_1 \geq 80\%$ predicted. Severe and refractory asthma were defined according to ATS criteria [30]. Diagnosis of COPD was made according to GOLD criteria (http://goldcopd.org/).

Twenty-five healthy volunteers were enrolled by advertisement among the hospital and staff. This study was approved by the ethics Committee of CHU Liège and all subjects gave written informed consent for participation.

2.2. Lung function tests

Lung function assessment was performed for all patients as previously described and according to ATS/ERS standard criteria [31–33].

2.3. Sputum induction and processing

The sputum was induced and processed as previously described [34,35]. Cell viability was determined by trypan blue exclusion and the differential cell count was performed by counting 500 non-squamous cells on cytospins stained with May-Grünwald-Giemsa (Table 2).

2.4. Immunoassays

The concentrations of alarmins (IL-25, IL-33 and TSLP) and IL-23 contained in the sputum supernatant were assessed by ELISA multiplex using Fluorokine® Multianalyte Profiling (MAP) Kits and a Duoset kit was used for IL-36 (R and D systems, Minneapolis, USA) according to the manufacturer's instructions. Detection limits were 50, 0.2, 0.55, 75 and 33 pg/ml for IL-25, IL-33, TSLP, IL-23 and IL-36 respectively. Spiking experiments of cytokines in sputum supernatants showed that recovery was between 80% and 120% for all the analytes. Results are displayed in Table 3.

Table 2
Sputum cell counts.

	Controls (n = 25)	Airway obstructive diseases (n = 44)	P value
Sputum weight (g)	3.7 (2.9–5.2)	2.6 (1.5–5.1)	0.08
Squamous cells (%)	16 (8–26)	3 (1–11)	<0.0001
Viability (%)	80 (75–89)	76 (64–85)	0.20
10^6cells/g	0.9 (0.5–1.9)	3.3 (1.6–7.0)	<0.0001
Macrophages %	17 (13–44)	10 (4–26)	<0.05
Macrophages 10^3/g	188 (124–713)	395 (173–2711)	0.25
Neutrophils %	76 (46–82)	67 (40–92)	0.80
Neutrophils 10^3/g	682 (281–1236)	1781 (865–5781)	<0.01
Eosinophils %	0.0 (0.0–1.0)	3.4 (0.0–13.6)	<0.05
Eosinophils 10^3/g	0.0 (0.0–16.9)	64.5 (0.0–516.0)	<0.01
Epithelial cells %	2.2 (0.9–4.4)	1.2 (0.0–5.5)	0.28
Epithelial cells 10^3/g	25.0 (6.7–82.5)	69.5 (0.5–120.3)	0.71
Lymphocytes %	1.8 (0.6–2.9)	1.4 (0.2–2.0)	0.19
Lymphocytes 10^3/g	18.0 (7.0–32.2)	36.0 (6.7–122.5)	0.16
Inflammatory phenotypes			
Eosinophilic (≥3%)		17	
Neutrophilic (≥76%)		13	
Mixed granulocytic		5	
Paucigranulocytic		9	

Results are expressed as median (25–75%).

Table 3
Alarmins (IL-25, IL-33, TSLP), IL-23 and IL-36 protein and gene expression levels.

	Controls	Airway obstructive diseases	P value
Detectable IL-33 (%)	6/20 (30)	15/44 (34)	>0.99
IL-33 (pg/ml)	0.0 (0.0–0.2)	0.0 (0.0–0.2)	0.98
Detectable IL-25 (%)	6/20 (30)	9/44 (20)	0.53
IL-25 (pg/ml)	0.0 (0.0–163.2)	0.0 (0.0–0.0)	0.58
Detectable TSLP (%)	10/20 (50)	18/44 (41)	0.59
TSLP (pg/ml)	0.3 (0.0–0.5)	0.0 (0.0–0.5)	0.48
Detectable IL-23 (%)	9/20 (45)	18/44 (41)	0.79
IL-23 (pg/ml)	0.0 (0.0–88.7)	0.0 (0.0–116)	0.34
Detectable IL-36 (%)	12/13 (92)	26/32 (81)	0.65
IL-36 (pg/ml)	172.4 (74.5–424.7)	310.6 (160.8–468.3)	0.34
Detectable IL-33 gene (%)	0/8 (0)	0/29 (0)	>0.99
Detectable TSLP gene (%)	5/8 (62)	23/29 (79)	0.37
TSLP (Fold change)	1.0 ± 2.6	1.5 ± 2.6	0.38
Detectable IL-23 gene (%)	8/8 (100)	27/29 (93)	>0.99
IL-23 (Fold change)	1.1 ± 4.0	1.2 ± 3.3	0.89
Detectable IL-36 gene (%)	7/7 (100)	26/29 (90)	>0.99
IL-36 (Fold change)	1.0 ± 4.9	1.4 ± 3.8	0.92

Detectable proportions were compared using Fisher tests. Mediators levels are expressed as median (25–75%) and were compared with Mann-Whitney tests. Data for gene expression are expressed as geometric mean ± SD and were compared with Mann-Whitney tests.

2.5. RNA extraction and RT-qPCR methods

Mediator gene expression levels were assessed in a subgroup of patients composed of 14 asthmatics, 15 COPD patients and 8 healthy controls.

These steps were performed exactly as recently described [36]. Sequences of primers and probes are listed in the online supplementary material. They were all obtained from IDT (Integrated DNA Technologies, Skokie, IL, USA). PCR efficiencies were calculated using qbase + qPCR analysis software (Biogazelle, Zwijnaarde, Belgium) and were all between 1.9 and 2.1 except for IL-25 which was impossible to obtain. The same program was used to obtain relative quantitation in gene expression using the $2^{-\Delta\Delta Ct}$ method. HPRT1 and GNB2L1 were used as reference genes as also previously reported [36]. Finally, the expression of IL-5 was used as control as this gene was known to be linked with eosinophilic airway inflammation in asthma [37,38]. IL-8 was assessed as marker of neutrophilic inflammation as its expression was correlated with neutrophilic phenotype [39]. Results can be found in Table 3.

2.6. Statistical analysis

Patient demographic and functional characteristics were expressed as mean ± SD. Comparisons between patient groups were performed using unpaired t tests. Chi-square test was applied for categorical variables. Sputum cell counts and cytokines levels were expressed as median (25–75%). Mann-Whitney tests were used to compare 2 groups and Kruskal Wallis tests were applied when more than 2 groups were compared. Correlations were calculated with Spearman's rank correlation analysis. All statistical analyses were performed with Graphpad Prism 7.0 (Graphpad Software San Diego, CA, USA). Differences were considered statistically significant when a two-sided p-value was < 0.05.

3. Theory

Little is known about sputum alarmins (IL-25, IL-33 and TSLP) as well as IL-23 and IL-36 in the context of chronic obstructive diseases such as asthma and COPD. The objective of this study was to determine

the expression and the production of the alarmins (IL-25, IL-33 and TSLP), and IL-23 and IL-36 in induced sputum of patients with chronic airway obstructive diseases including asthmatics and COPD and to compare them to healthy subjects. The relationship of the mediators with sputum inflammatory cellular profile and the severity of airway obstruction was also assessed.

4. Results

4.1. Demographic and functional patient characteristics

Patients were well matched according to sex, age and BMI but not for tobacco habits. Patients suffering from asthma and COPD exhibited pulmonary function parameters significantly impaired compared to healthy controls (Table 1).

4.2. Sputum cell counts

Patients had significantly a higher sputum total cell number and a higher number of neutrophils compared to healthy subjects. Moreover, the percentage and number of eosinophils were significantly higher. In counterpart, the proportion of squamous cells and macrophages appeared significantly lower (Table 2).

4.3. Mediators

At protein level, among all the analyzed mediators, the levels of IL-25 were poorly detectable in the majority of the samples. Between a third and a half of patients gave measurable results for TSLP, IL-23 and IL-33 but the levels of these mediators did not appear different between groups. IL-36 was detectable in the majority of the samples but did not differ between groups neither (Table 3).

At gene level, only IL-36, IL-23 and TSLP gave measurable expressions and none of them were different between groups (Table 3).

4.4. Relationship with demographic characteristics

None of the mediators differed between men and women at protein or gene level. None correlated with age, BMI or pack-year values. No differences was noted between the different tobacco status groups or the atopic status.

4.5. Relationship with airway obstruction

There was no relationship between any of the mediator at the protein or gene expression levels and the lung function parameters including pre and post bronchodilation FEV1 and FVC but also TLC, RV, DLCO and KCO values.

In addition, patients presenting fixed airway obstruction (defined as post bronchodilation FEV1 % predicted $< 80\%$ and FEV1/FVC $< 70\%$, n $= 19/34$ subjects with available post bronchodilation values) did not differ from those patients without (n $= 15/34$) for alarmins and cytokines at protein and gene level (data not shown).

4.6. Relationship with airway inflammation

In all subjects combined (n $= 69$), IL-36 protein level was negatively correlated with sputum eosinophil percentage and number ($r = -0.44$ and $r = -0.43$, $p < 0.01$ respectively, Fig. 1A). The correlation obtained between IL-36 level and sputum neutrophil count was not significant ($r = 0.25$, $p = 0.11$).

When the patients were classified according to inflammatory phenotypes, IL-36 appeared significantly decreased in patients with a eosinophilic inflammation compared to those with a neutrophilic airway inflammation and controls (Fig. 1B).

There was no correlation between alarmins or IL-23 protein levels and sputum cellular inflammatory profile.

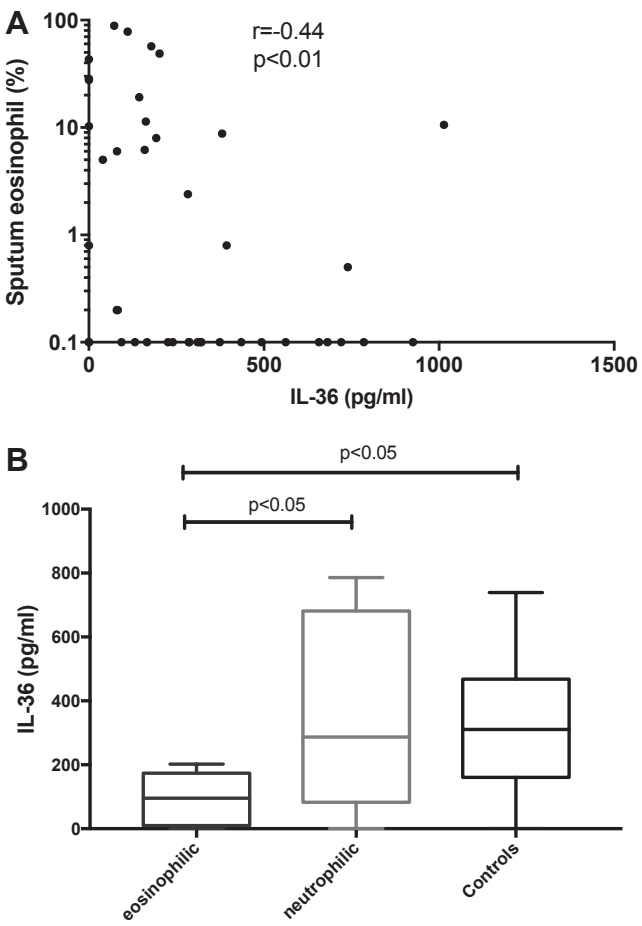

Fig. 1. (A) Correlation between IL-36 protein level and sputum eosinophil percentage. (B) Sputum IL-36 concentrations according to airway inflammatory phenotype. Data were displayed as Box and Whiskers (Min-Max) and were compared with Kruskal Wallis ($p < 0.05$) then Dunn's multiple comparisons tests gave a p value < 0.05 as indicated. Eosinophilic phenotype: sputum eosinophil count $\geq 3\%$, neutrophilic: sputum neutrophil count $\geq 76\%$. Those who combined both were not considered in this analysis.

Regarding the genes, the IL-5 sputum expression used as control gene of airway eosinophilia was notably increased in patients presenting an eosinophilic inflammation compared to other phenotypes (Fig. 2A). Similarly, IL-8 expression, a control gene for airway neutrophilia, was significantly increased in patients with neutrophilic inflammation compared to others (Fig. 2B).

In all subjects combined IL-36 gene expression was correlated with the sputum neutrophil percentage ($r = 0.58p < 0.001$, Fig. 3A) but not with eosinophil percentage ($r = -0.21$ and $p = 0.24$). Similarly to what is seen at protein level, IL-36 gene expression was significantly increased in patients with a neutrophilic airway inflammation compared to those with an eosinophilic inflammation (Fig. 3B). In the same manner, IL-23 expression exhibited a significant correlation with the sputum neutrophil percentage in all subjects ($r = 0.57$, $p < 0.001$, Fig. 4A) and patients with a neutrophilic inflammation had higher expression level compared to those with eosinophilic airway inflammation (Fig. 4B). There was no correlation between any sputum cell type and TSLP gene expression in the whole cohort. However, in obstructive airway diseases, TSLP expression appeared negatively correlated with sputum eosinophil absolute value ($r = -0.51$, $p < 0.05$, Fig. 5A) and a trend was observed for a correlation with eosinophil percentage ($r = -0.39$, $p = 0.07$, Fig. 5B).

None of the mediators appeared correlated with the exhaled nitric oxide (FeNO) values.

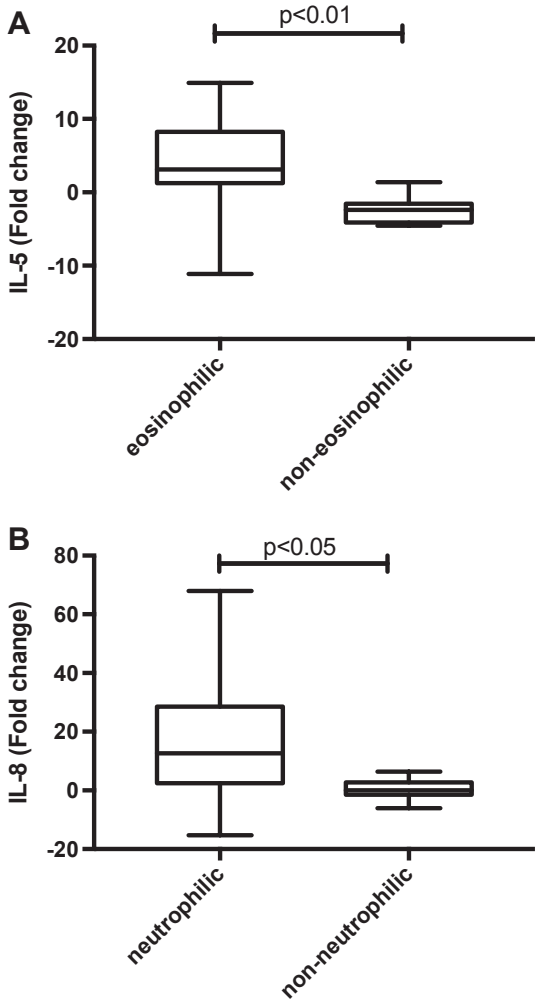

Fig. 2. (A) Sputum IL-5 gene expression according to airway inflammatory phenotype (B) Sputum IL-8 gene expression according to airway inflammatory phenotype. Data were displayed as Box and Whiskers (Min-Max) and were compared with Mann-Whitney tests. Eosinophilic phenotype: sputum eosinophil count $\geq$ 3%, neutrophilic: sputum neutrophil count $\geq$ 76%.

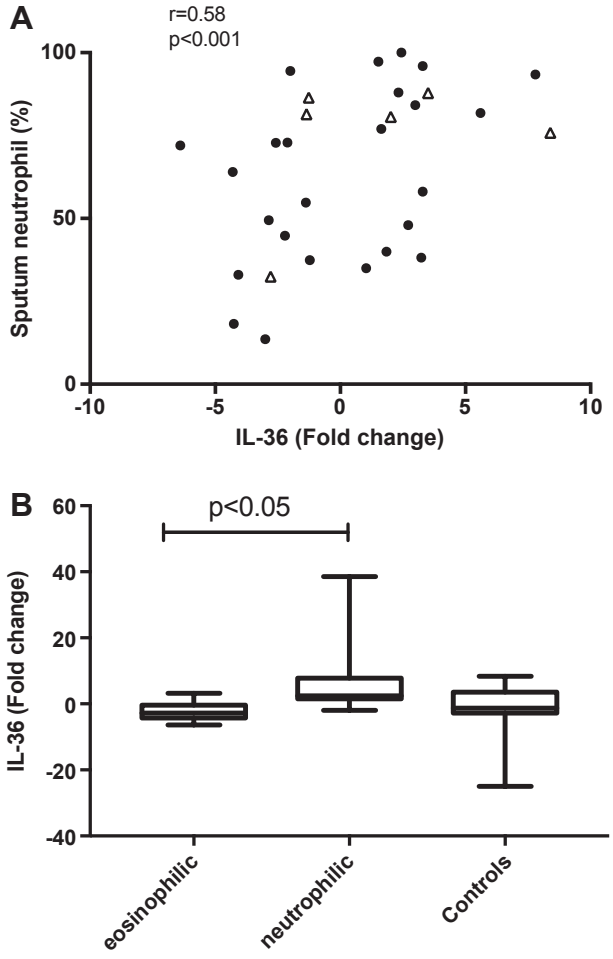

Fig. 3. (A) Correlation between IL-36 gene expression level and sputum neutrophil percentage. (B) Sputum IL-36 gene expression according to airway inflammatory phenotype. (A) • represent subjects with chronic airway diseases, Δ represent healthy controls. (B) Data were displayed as Box and Whiskers (Min-Max) and were compared with Kruskal Wallis ($p < 0.05$) then Dunn's multiple comparisons tests gave a p value < 0.05 as indicated. Eosinophilic phenotype: sputum eosinophil count $\geq$ 3%, neutrophilic: sputum neutrophil count $\geq$ 76%. Those who combined both were not considered in this analysis.

4.7. Relationship with treatments

Among patients, no differences were observed between those treated with beta-2 agonists and those without. IL-36 gene expression in patients treated with ICS showed a reduced expression compared to those not receiving ICS (0.7 ± 3.3 vs 2.1 ± 3.6, $p = 0.05$). Likewise, IL-23 gene expression in patients with ICS showed a lower expression than steroid naïve patients (0.8 ± 2.8 vs 2.0 ± 3.4, $p < 0.05$).

5. Discussion

Overall our study showed that the sputum protein and gene levels of alarmins (IL-25, IL-33 and TSLP), IL-23 and IL-36 did not differ between patients suffering from chronic airway obstructive diseases and healthy controls. The main finding from our study is that there is an inverse relationship between the level of IL-36 and the magnitude of the airway eosinophilic inflammation while it positively correlated with airway neutrophilic inflammation. To the best of our knowledge, our study is the first to report on the levels of sputum IL-36 in patients suffering from chronic airway obstructive diseases.

IL-36 is well known to be activated in psoriasis where it induces neutrophil and Th17 cells recruitment and activation [40]. In asthma and COPD, literature appeared limited. Murine asthma models showed airway neutrophil but not eosinophil recruitment induced by IL-36 as well as an increase of chemokines linked to neutrophil chemotaxis [22]. In vitro studies highlighted the IL-36 production by human airway epithelial cells [41] and only one study described an elevated IL-36 gene expression in rhinovirus infected human bronchial epithelial cells in a context of asthma [42]. Interestingly, in our study, patients displaying an eosinophilic airway inflammation have lower IL-36 protein levels that healthy subjects which could make them more prone to bacterial and viral infections as IL-36 is crucial for lung innate immunity [43]. Supporting this hypothesis, the eosinophilic phenotype in airway obstructive diseases were shown to be more susceptible to exacerbations [44–46]. Our current finding is in the same line as what has been reported by Da Silva et al. who showed that gene of antiviral defense were decreased in eosinophilic asthmatics [47]. Finally, ICS treatment is known to reduce IL-1-like cytokines [48] and this may explain the decrease of IL-36 level in our patients treated with ICS compared to those without.

IL-23, for its part, induces IL-17 release which in turn stimulates neutrophil chemoattractant agents [49]. As expected we found a significant correlation between IL-23 gene levels and sputum neutrophil proportions. As for IL-36, we found a significant decrease of IL-23 expression level induced by corticosteroids. Some authors showed a

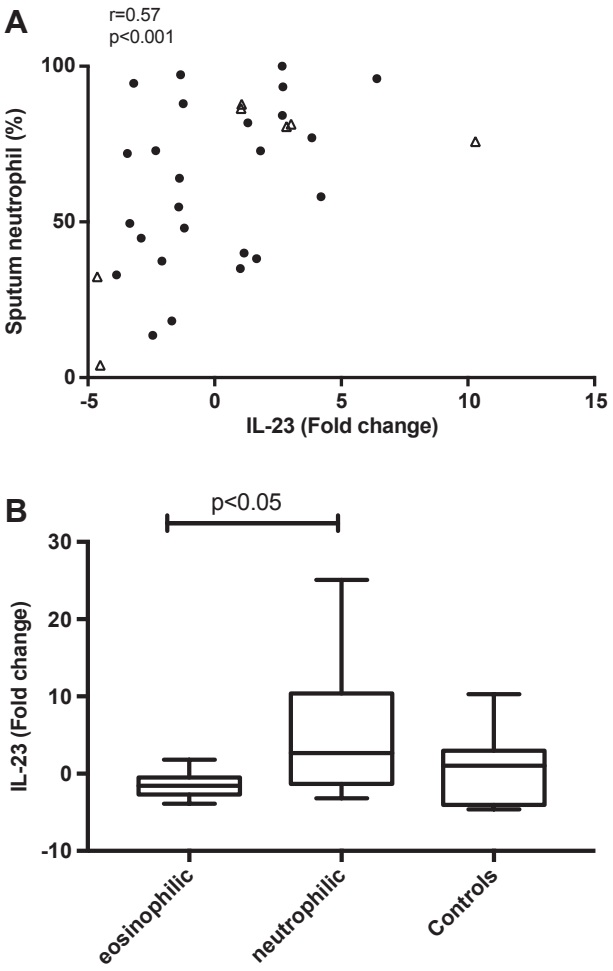

Fig. 4. (A) Correlation between IL-23 gene expression level and sputum neutrophil percentage. (B) Sputum IL-23 gene expression according to airway inflammatory phenotype. (A) • represent subjects with chronic airway diseases, Δ represent healthy controls. (B) Data were displayed as Box and Whiskers (Min-Max). Mann-Whitney test was used to compare eosinophilic and neutrophilic phenotype. Eosinophilic phenotype: sputum eosinophil count ≥ 3%, neutrophilic: sputum neutrophil count ≥ 76%. Those who combined both were not considered in this analysis.

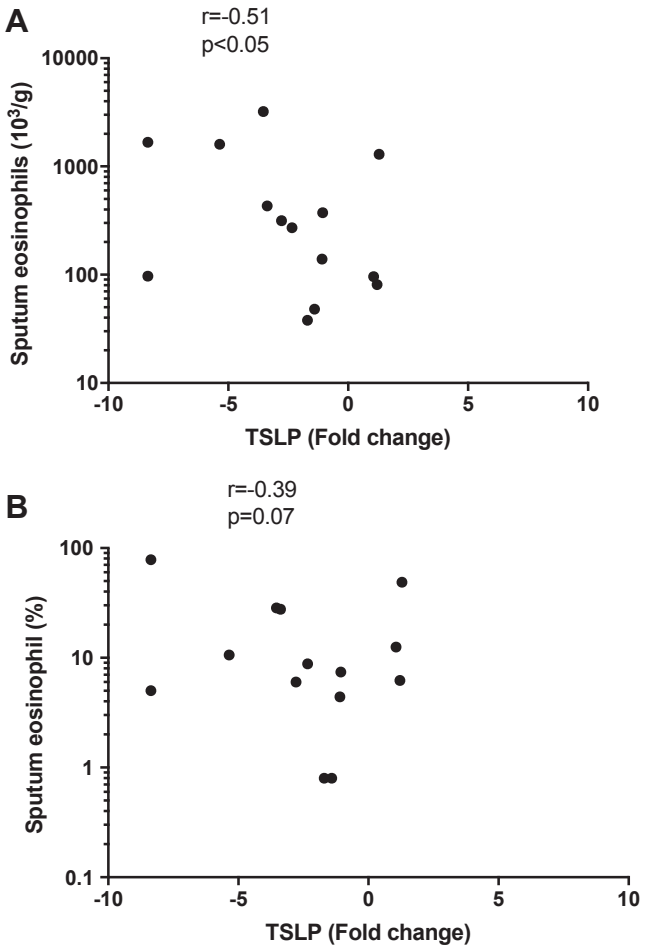

Fig. 5. (A) Correlation between TSLP gene expression level and sputum eosinophil count. (B) Correlation between TSLP gene expression level and sputum eosinophil percentage.

decreased IL-23 expression in human blood macrophages induced by glucocorticoids, which was mediated by p38 MAPK inhibition [50]. However, the underlying mechanisms that repress airway IL-23 in our study needs more investigation.

Another original aspect of our work is the search for the presence of alarmins in the airways of patients suffering from chronic airway obstructive diseases as these mediators have been recently put forward as key epithelial derived mediators in regulating eosinophilic airway inflammation [51]. Unfortunately, TSLP, IL-33 and IL-25 were poorly detectable not only at protein level but also at gene level and did not show difference between groups.

Surprisingly, a negative correlation was noted between TSLP expression and sputum eosinophils in our study. However, it has been recently observed that high sputum TSLP mRNA level in viral induced asthma exacerbation was not associated with sputum eosinophils [52]. Moreover, bronchoalveolar lavage TSLP expression in asthmatics has been also recently shown to be rather linked to lung neutrophil infiltration [53]. In addition TSLP was shown to be involved in neutrophilic, Th2 low airway inflammation and link to IL-17 release [54].

IL-33, although not detectable in the complete set of patients, was not different from healthy controls. IL-33 has been reported to be quickly oxidized after its secretion which could have alter the detection [55]. Finally IL-25 was also hard to detect in sputum in our study.

The explanations for the low detection levels of alarmins in our study are multiple. Firstly, as alarmins are epithelial-derived cytokines, and the proportion of epithelial cells is rather low in sputum, it could be that the amount produced is insufficient for detection. Secondly, the time window of investigation may not be optimal for detection as alarmins were shown to be essentially released by the epithelium during an exacerbation period [55] whereas our patients were stable at the time of sputum sampling. Thirdly, the treatment effect may have play a role as a large proportion of the patients are treated with ICS and long acting beta 2 agonist (LABA). However, although glucocorticoids have been shown to decrease IL-25 expression [56,57], alarmins are mainly considered as poorly sensitive to corticosteroids and linked to cortico-resistance [9,56,58,59]. Beta 2 receptor agonists for their parts, were observed to increase the release of alarmins [60,61] but no effect was noticed in our study maybe due to the low patient number in the no LABA group. Indeed, the last limitation of this study was the limited sample size which may have reduced the power of the sub-analyses to detect significant differences between controls and patients. These findings are then needed to be confirmed in larger cohorts.

In conclusion, the main finding of our study is the demonstration of reduced IL-36 level in the patients with eosinophilic airway inflammation. We believe that it could make the patients more susceptible to airway infections as IL-36 has a role in antimicrobial defense and partly explain why eosinophilic airway inflammation may make patients with obstructive airway disease prone to exacerbate.

CRediT authorship contribution statement

C. Moermans: Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision. **K. Damas:** Investigation. **J. Guiot:** Resources. **M.S. Njock:** Methodology. **J.L. Corhay:** Resources. **M. Henket:** Resources, Data curation. **F. Schleich:** Resources. **R. Louis:** Conceptualization, Resources, Writing - review & editing, Visualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This project was financially supported by the European Union (Interreg 5-a Euregio Meuse Rhine).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2021.155421.

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