

Original Article



Relationship between airway inflammation and airflow limitation in elderly asthmatics

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ABSTRACT

Background: The prevalence of asthma in elderly population has been increasing. Previous studies have demonstrated clinical characteristics of elderly asthmatics (EA). However, little is known regarding the influence of immunological change on the physiological status of EA.

Objective: We investigated the relationship between inflammatory mediators and the pulmonary function (PF) of EA.

Methods: Eligible adult asthmatics recruited from the Allergy Center of Saitama Medical University Hospital were classified into a non-EA group (<40 years old, n = 15) and an EA group (≥60 years old, n = 43). Sputum induction and PF tests were performed. Concentrations of an eosinophil-derived neurotoxin (EDN) and neutrophil elastase (NE) in sputum supernatants were measured by enzyme-linked immunosorbent assay and a fluorometric assay using a commercial assay kit, respectively. Cell counts and EDN and NE concentrations in sputum were compared between the 2 groups. The association among those parameters and PF were analyzed in each group.

Results: The EA group had a significantly higher severe asthmatics proportion ($p = 0.01$), a lower current smokers proportion ($p = 0.002$), lower sensitization rate to aeroallergens ($p = 0.012$), several PFs deterioration ($p < 0.0001$) and lower total IgE levels ($p = 0.001$) than the non-EA group. Sputum neutrophil counts and NE concentrations were significantly higher in the EA group than those in the non-EA group (median neutrophil: 4.11 vs. 2.74×10^5 /mL, $p = 0.03$; NE: 2.0 vs. 1.6 $\mu\text{g}/\text{mL}$, $p < 0.05$, respectively), whereas sputum eosinophil counts and EDN concentrations were not. Sputum EDN concentrations were significantly positively correlated with sputum neutrophil counts ($r = 0.39$, $p = 0.031$) and NE concentrations ($r = 0.57$, $p < 0.0001$) only in the EA group. Eosinophil-related parameters were negatively correlated with several PFs in the 2 groups. Neutrophil-related parameters were negatively correlated with PFs only in the non-EA group.

Conclusion: This study determines that in EA, persistent active eosinophilic airway inflammation is accompanied by advanced neutrophilic inflammation, which may contribute to deteriorated PF. This distinct airway inflammation may increase the severity of asthma in EA.

Keywords: Aging; Asthma; Eosinophil-derived neurotoxin; Neutrophil elastase; Pulmonary function

Conflict of Interest

The authors have no financial conflicts of interest.

Author Contributions

Conceptualization: Tomoyuki Soma. Data curation: Tomoyuki Soma, Yoshitaka Uchida, Takehito Kobayashi, Kazuyuki Nakagome. Formal analysis: Tomoyuki Soma. Funding acquisition: Tomoyuki Soma. Methodology: Tomoyuki Soma, Makoto Nagata. Project administration: Tomoyuki Soma, Yoshitaka Uchida, Takehito Kobayashi, Kazuyuki Nakagome. Visualization: Tomoyuki Soma, Yuki Hoshino, Kazuki Katayama. Writing - original draft: Tomoyuki Soma. Writing - review & editing: Kazuyuki Nakagome, Makoto Nagata.

INTRODUCTION

Asthma has been recognized as a common disease in older adults since we now live in an aging society. Epidemiologic studies have demonstrated that the prevalence of asthma in elderly individuals is estimated to range from 4.5% to 12.7% [1]. Many factors affect clinical practices toward elderly asthmatic patients. Compared with younger individuals, elderly individuals are less perceptive of asthmatic symptoms because physical or mental changes related to aging occur or comorbidity is more frequent, which likely makes it difficult to diagnose asthma in older patients. As a result, elderly asthmatics (EA) receive either insufficient or inappropriate treatment. A weakening of the chest wall, respiratory muscle strength, and lung structure related to aging modify lung function, which results in fixed airflow limitation in asthma or the additional onset of chronic obstructive pulmonary disease [2]. The complexities of those physical changes in elderly asthmatic patients contribute to mortality, hospitalization, medical costs. In 2013, 90% of patients who died of asthma were older than 65 years in Japan [3].

Aging is considered a major modifier in the immune system. A reduced number and diminished function of peripheral memory CD4⁺T cells and diminished cytotoxicity of Natural Killer cells and Natural Killer T cells would contribute to an increased susceptibility to microbes in older individuals [4]. One *ex vivo* study demonstrates that peripheral blood mononuclear cells stimulated by anti-CD3 and anti-CD28 expressed lower FoxP3 mRNA in EA compared with healthy elderly individuals [5]. Those immunosenescence-related changes are involved in low-grade chronic inflammation, namely, “inflammaging,” showing systemically increased levels of IL-1 β , IL-6, and tumor necrosis factor (TNF)- α . Also, increased levels of IL-6 are observed in bronchoalveolar lavage fluid (BALF) in healthy elderly individuals [6]. Likewise, elderly asthmatic patients have been reported to have higher levels of IL-6 and IL-8 in sputum [7]. Increased sputum IL-6 levels are associated with uncontrolled status in elderly asthmatic patients and the hospitalization of those patients [7]. Similarly, increased numbers of neutrophils in sputum and BALFs and increased levels of IL-8, neutrophil elastase (NE), and matrix metalloprotease (MMP) in BALFs are observed [8]. Nevertheless, no associations of increased eosinophils or interleukin (IL)-5 levels with indices of asthma control are observed unless the number of eosinophils and IL-5 levels was also increased in sputum of elderly asthmatic patients [7].

Those evidences suggest that chronic inflammation due to aging affects the clinical progression of chronic lung disease, and that non-type-2 immunological cascades predominantly occur in aged patients. However, how neutrophils and eosinophils are involved in the pathophysiology of EA has yet to be determined since previous studies show associations not of those activated leukocytes but of inflammatory cytokines with a clinical aspect in elderly patients with asthma. In contrast to chronic obstructive pulmonary disease, representative chronic lung diseases, of which aging is a risk factor, feature airway inflammation in EA, which has not been sufficiently analyzed; thus, the appropriate treatment has not been determined. Therefore, we conducted this observational study to examine the characteristics of airway inflammation, especially granulocytes, in elderly patients with asthma. To address this, we measured levels of granule proteins and leukocyte counts in sputum in patients with asthma, evaluated their association with pulmonary function, and compared them between elderly patients with asthma (≥ 60 years old) and younger patients with asthma (< 40 years old).

MATERIALS AND METHODS

Study subjects

Adult patients with asthma were recruited from the Allergy Center of Saitama Medical University Hospital from 2014 to 2016, all of whom have been participating in our ongoing clinical asthma research since 2014 (approval numbers: 08-043 and 13-083). Asthma was diagnosed according to the definition stated in the Global Initiative for Asthma guidelines: those who have asthmatic symptoms, experiences of exacerbation and wheezing, and have bronchial reversibility that responded to bronchodilator and/or bronchial hyperresponsiveness using the methacholine challenge [9]. Candidates matching the following criteria were excluded: other pulmonary diseases; asthma exacerbation or respiratory infections during the 4 weeks prior to the day of outpatient consult; pregnancy; or severe comorbid diseases, such as cancer, renal failure, and heart failure.

Study design

This study was a retrospective cohort to determine the characteristics of elderly patients with asthma aged ≥ 60 years (the EA group) and compare them with those of younger patients with asthma (< 40 years, the non-EA group). The study was approved by The Institutional Review Board of the Saitama Medical University Hospital (approval number: 16-046). Written informed consent was obtained from all individuals.

Eligible patients underwent pulmonary function tests using an AS307 spirometer (Minato Medical Science, Osaka, Japan) [10] and induction sputum, and the fraction of exhaled nitric oxide (FeNO) was measured using a chemiluminescence analyzer (SiEVER 280i NIPPON MEGACARE Co, Ltd., Tokyo, Japan) with a resolution of one part per billion according to the recommendations of the American Thoracic Society (ATS) [11]. They answered an asthma control test (ACT) on their visit days.

We assessed pulmonary function, cell distribution in sputum, eosinophil-derived neurotoxins (EDNs) and neutrophil elastase (NE) using sputum serum and the ACT.

Sputum induction and processing

Sputum induction was performed by the subjects according to the previously described method [12]. After salbutamol was inhaled with a metered-dose inhaler, sterile hypertonic saline (4.5%) was briefly inhaled at room temperature using an ultrasonic nebulizer (MU-32, Azwell, Osaka, Japan). Subsequently, sputum was induced for 5 minutes up to 4 times.

Sputum was immediately processed. After equal weights of 0.1% dithiothreitol in sterile Hanks' balanced salt solution (HBSS; 1 mL) were added to sputum, the sample was gently vortex-mixed and repeatedly aspirated at an ambient temperature until it became homogeneous. After the sample was diluted to 5 mL with HBSS and centrifugated at 400 g for 10 minutes to prepare cytospin slides, cells on the slides were stained with May-Grünwald-Giemsa. At least 500 inflammatory cells were counted using 2 independent investigators. The sample with $< 50\%$ squamous epithelial cells was considered adequate for analysis [13, 14].

Measurement of sputum inflammatory mediator levels

The sputum supernatant EDN was quantified using enzyme-linked immunosorbent assay kits (Medical and Biological Laboratory Co., Ltd., Nagoya, Japan), whereas the sputum supernatant NE was measured using an NE activity assay kit according to the manufacturer's

instructions (Cayman Chemicals, Ann Arbor, MI, USA). Measurements were performed in duplicate. The lower limit of detection for the EDN assay was 0.62 ng/mL.

Statistical analysis

A number of eosinophils and neutrophils and concentrations of EDN and NE in sputum were log-transformed for statistical analysis. The normality of the values was determined using the Kolmogorov-Smirnov test. The Student *t* test was used for parametric continuous variables, and the Mann-Whitney *U* test was used for nonparametric variables. Associations between the 2 groups were determined by Spearman correlation test. Categorical variables were examined by either the χ^2 test or Fisher exact test, as appropriate. Values were described as mean \pm standard deviation or as median with a 25th–75th percentile range, if not normally distributed.

The data were analyzed using IBM SPSS Statistics ver. 20.0 (IBM Co., Armonk, NY, USA).

RESULTS

Characteristics of patients

A total of 87 patients with asthma enrolled in the study. Of them, 35 were classified into the non-EA group, and 52 were classified into the EA group. Of all the sputum specimens, 15 (42.9%) in the non-EA group and 43 (82.7%) in the EA group were appropriate for the analysis.

The patient characteristics are presented in **Table 1**. The prevalence of current smokers was significantly lower and that of previous smokers was significantly higher in the EA group than in the non-EA group. The prevalence of asthmatics having sensitized environmental allergens was lower in the EA group than in the non-EA group. Subjects in the EA group received significantly higher doses of ICS than those in the non-EA group. Compared with non-EAs, more EAs had severe asthma based on the European Respiratory Society (ERS)/ATS severe asthma guidelines [15], although the ACT scores did not differ between the 2 groups. There were no differences between the 2 groups in terms of sex, body mass index, or duration of asthma.

EAs had significantly lower pulmonary function indices, including forced expiratory volume in 1 second (FEV₁), forced vital capacity (FVC), and FEV₁/FVC, than non-EAs. Levels of total serum IgE were significantly lower and percentages of neutrophil in the blood were significantly higher in the EA group than in the non-EA group. There were no differences in following general and airway inflammatory indices between the 2 groups regarding FeNO, percentages of eosinophil in the blood, or percentages of leukocyte.

Differences of inflammatory cells and leukocyte granule proteins in sputum between EA and Non-EA groups

The total number of cells in sputum was significantly higher in the EA group than in the non-EA group (**Table 1**). Log numbers of neutrophils (but not eosinophils) in sputum were significantly higher in the EA group than in the non-EA group (**Fig. 1, Table 2**).

Log concentrations of NE in sputum were significantly increased in the EA group than those in the non-EA group unlike log concentrations of EDN in sputum (**Fig. 1, Table 2**).

Table 1. Demographics of subjects

Variable	Non-EA (n = 15)	EA (n = 43)	p value
Age (yr)	35 ± 3	70 ± 5	NA
Sex, female:male	5 (33.3):10 (66.6)	13 (30.2):30 (69.8)	NS
Duration of disease (yr)	4.5 (1.3–8.0)	5.5 (2.7–20.0)	NS
Smoking history, never:previous:current smokers	9 (60.0):2 (13.3):4 (26.7)	18 (41.9):24 (55.8):1 (2.3)	0.002
Body mass index (kg/m ²)	24.0 (21.5–33.3)	24.0 (23.0–27.0)	NS
Sensitization to environmental allergens	15 (100)	29 (67)	0.012
ICS (FP µg/day)	360 (160–500)	600 (320–1000)	0.026
OCS	0 (0)	2 (4.7)	NA
Severe asthma	2 (13.3)	22 (51.2)	0.01
ACT (point)	22 (21–24)	24 (20–25)	NS
FeNO (ppb)	25.0 (20.0–51.0)	24.0 (15.0–35.0)	NS
IgE (IU/L)	698 (457–2,562)	153 (38–485)	0.001
Blood neutrophils (%)	54.0 ± 8.9	60.7 ± 9.2	0.034
Blood eosinophils (%)	6.9 ± 4.8	6.3 ± 6.0	NS
FEV ₁ (L)	2.98 ± 0.77	1.73 ± 0.50	<0.0001
%FEV ₁ (% of predicted)	90.0 ± 18.0	79.5 ± 26.8	NS
FVC (L)	3.82 ± 0.97	2.70 ± 0.63	<0.0001
%FVC (%)	102.5 ± 19.4	92.2 ± 19.2	NS
FEV ₁ /FVC (%)	79.5 ± 7.4	65.4 ± 11.6	<0.0001
Sputum total cells (×10 ⁵ /mL)	4.5 (2.4–8.9)	7.5 (5.0–11.3)	0.03
Sputum eosinophils (%)	4.0 (1.2–18.1)	4.0 (0.7–13.8)	NS
Sputum neutrophils (%)	46.8 (32.0–60.8)	59.6 (28.4–68.8)	NS
Sputum macrophages (%)	13.7 (11.1–36.0)	3.2 (3.3–32.0)	NS
Sputum lymphocytes (%)	1.9 (1.2–2.8)	2.1 (0.9–5.0)	NS

Values are presented as mean ± standard deviation, number (%), or median (interquartile range).

ICS = inhalation corticosteroid (fluticasone), where 2 µg beclomethasone = 2 µg budesonide = 1 µg fluticasone
 p values were calculated using the Student *t* test for variables with a parametric distribution, the Mann-Whitney *U* test for nonparametric data, and the χ^2 test or Fisher exact test for comparison of proportions, as appropriate.
 EA, elderly asthmatics; BMI, body mass index; OCS, oral corticosteroid; ACT, asthma control test; FeNO, fraction of exhaled nitric oxide; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; NA, not applicable; NS, not significant.

Association among sputum granulocytes and leukocyte granule proteins

In both groups, increased numbers of log sputum eosinophils and neutrophils were significantly correlated with increased levels of log sputum EDN and NE, respectively (**Fig. 2A and B**). Increased numbers of log sputum neutrophils and levels of NE were significantly correlated with increased levels of log sputum EDN in the EA group (**Figs. 2D and 3D**) but not in the non-EA group (**Figs. 2D and 3B**). No significant correlations were observed between increased numbers of log sputum eosinophils and neutrophils or between increased levels of log sputum NE and the number of log sputum neutrophils in the 2 groups (**Figs. 2C, 3A, and 3C**).

Association between sputum granulocytes and clinical markers

In the EA group, higher numbers of log sputum eosinophils were correlated with lower %FEV₁ and %FVC, whereas numbers of log sputum neutrophils were not correlated with any pulmonary function parameters (**Table 3**). In the EA group, higher log sputum eosinophil numbers were correlated with lower ACT points, whereas log sputum neutrophil numbers were not (**Table 3**). In the non-EA group, higher log sputum eosinophil numbers were correlated with lower %FEV₁ and FEV₁/FVC, whereas higher numbers of log sputum eosinophils and neutrophils were correlated with lower FEV₁ and FVC (**Table 3**). In the non-EA group, higher log sputum neutrophil numbers were correlated with lower ACT points, whereas log sputum eosinophil numbers were not (**Table 3**).

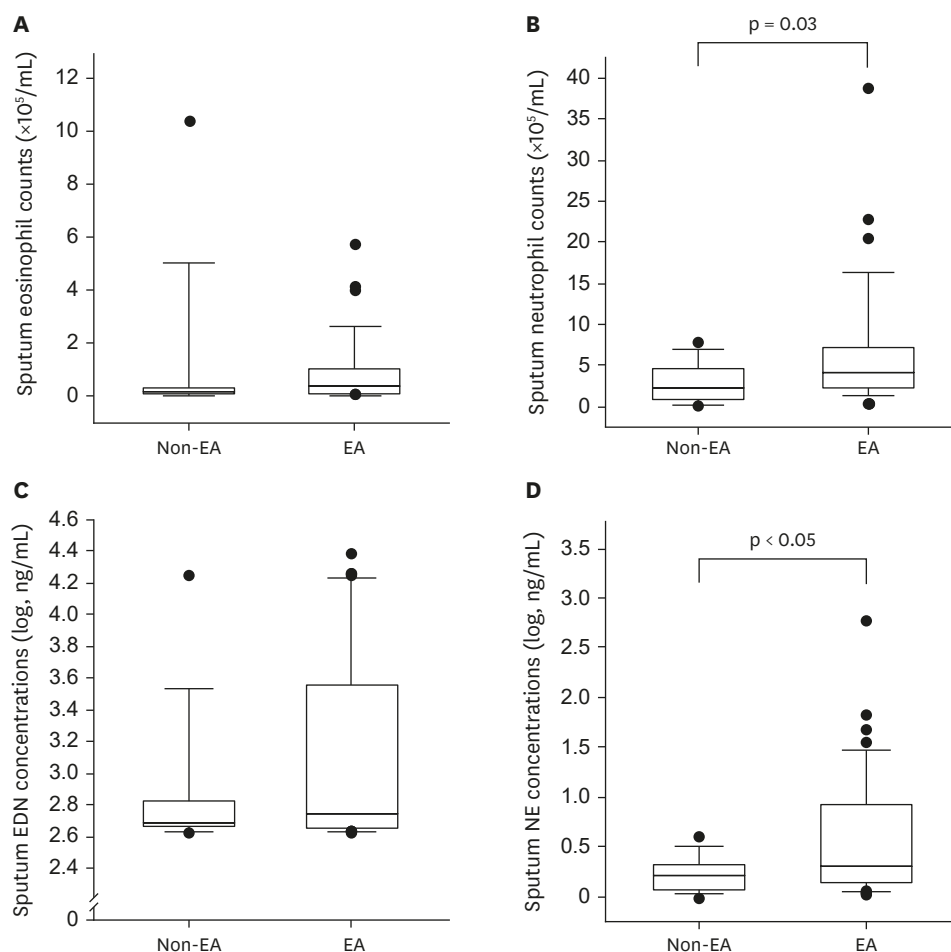


Fig. 1. Differences in numbers of granulocytes and concentrations of granule proteins in sputum between non-elderly and elderly asthmatics. (A) Number of eosinophils, (B) number of neutrophils, (C) log concentrations of EDN, and (D) log concentrations of NE in sputum in the non-EA and EA groups. The number of eosinophils and neutrophils and concentrations of EDN and NE in sputum were statistically analyzed after being log-transformed. The statistical significance of the differences was determined by a Student *t* test. Values of sputum EDN and NE are expressed as log-transformed. EA, elderly asthmatics; EDN, eosinophil-derived neurotoxin; NE, neutrophil elastase.

Table 2. Comparison of sputum granulocyte numbers and granule protein concentrations between non-EA and EA

Variable	Non-EA (n = 15)	EA (n = 43)	<i>p</i> value
Eosinophils (×10 ⁵ /mL)			NS
Median (IQR)	0.15 (0.06–0.33)	0.31 (0.02–1.17)	
Log ± SD	4.1 ± 1.2	4.1 ± 1.4	
Neutrophils (×10 ⁵ /mL)			0.03
Median (IQR)	2.74 (1.0–5.19)	4.11 (2.16–7.15)	
Log ± SD	5.3 ± 0.5	5.7 ± 0.6	
EDN (ng/mL)			NS
Median (IQR)	488.6 (460.7–712.3)	554.8 (446.4–3,866.8)	
Log ± SD	2.9 ± 0.4	3.1 ± 0.6	
NE (ng/mL)			0.01
Median (IQR)	1.6 (1.1–2.2)	2.0 (1.3–8.7)	
Log ± SD	0.2 ± 0.2	0.7 ± 0.6	

p values were calculated using the Student *t* test for variables with a parametric distribution and the Mann-Whitney *U* test for nonparametric data.

EA, elderly asthmatics; IQR, interquartile range; SD, standard deviation; EDN, eosinophil-derived neurotoxin; NE, neutrophil elastase; NS, not significant.

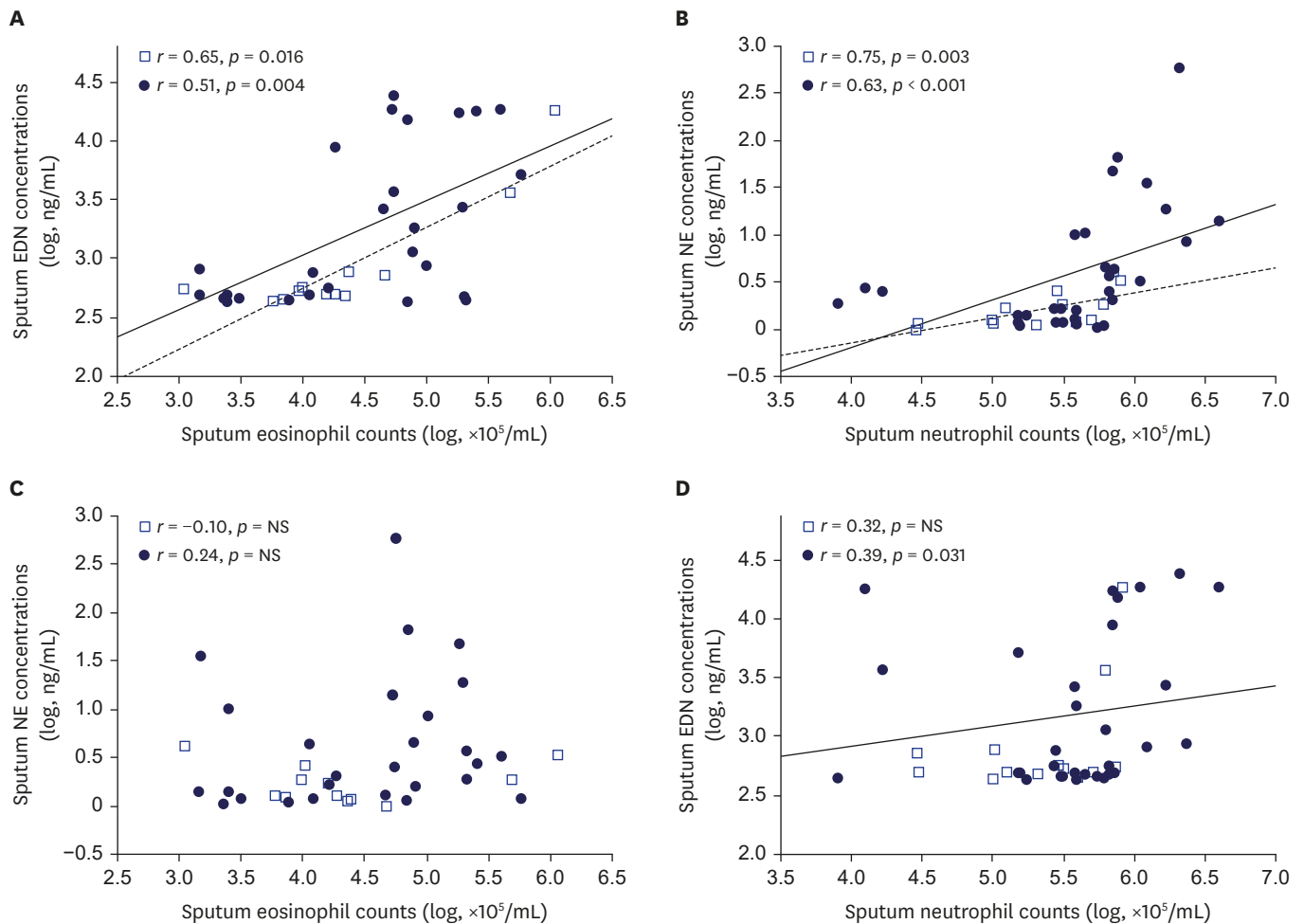


Fig. 2. Correlations of numbers of sputum granulocytes with concentrations of sputum granule proteins in non-elderly and elderly asthmatics. Correlations of (A) log sputum eosinophil numbers and log sputum EDN concentrations, (B) log sputum neutrophil numbers and log sputum NE concentrations, (C) log sputum eosinophil numbers and log sputum NE concentrations, and (D) log sputum neutrophil numbers and log sputum EDN concentrations in non-elderly and elderly asthmatics. Statistical significance of the correlations was determined by Spearman correlation test. Open squares and the dotted line indicate non-elderly asthmatics. Solid circles and the solid line indicate elderly asthmatics. All values are expressed as log-transformed. EDN, eosinophil-derived neurotoxin; NE, neutrophil elastase.

Association between sputum leukocyte granule proteins and clinical markers

In the EA group, increased levels of log sputum EDN were correlated with decreased %FEV1 and FEV1/FVC, whereas log sputum NE levels were not correlated with any pulmonary function parameters (Table 4). In the EA group, both log sputum EDN and NE levels were not correlated with ACT points. In the non-EA group, increased log sputum EDN levels were correlated with decreased FEV1/FVC, whereas increased log sputum NE levels were correlated with decreased FEV1 and FVC (Table 4). In the non-EA group, increased log sputum NE levels were correlated with lower ACT points, whereas log sputum EDN levels were not.

DISCUSSION

Our findings demonstrated distinct characteristics of airway inflammation in elderly asthmatic patients. Compared with younger asthmatic patients, the number of sputum neutrophils and NE levels were increased in elderly asthmatic patients, but the number of

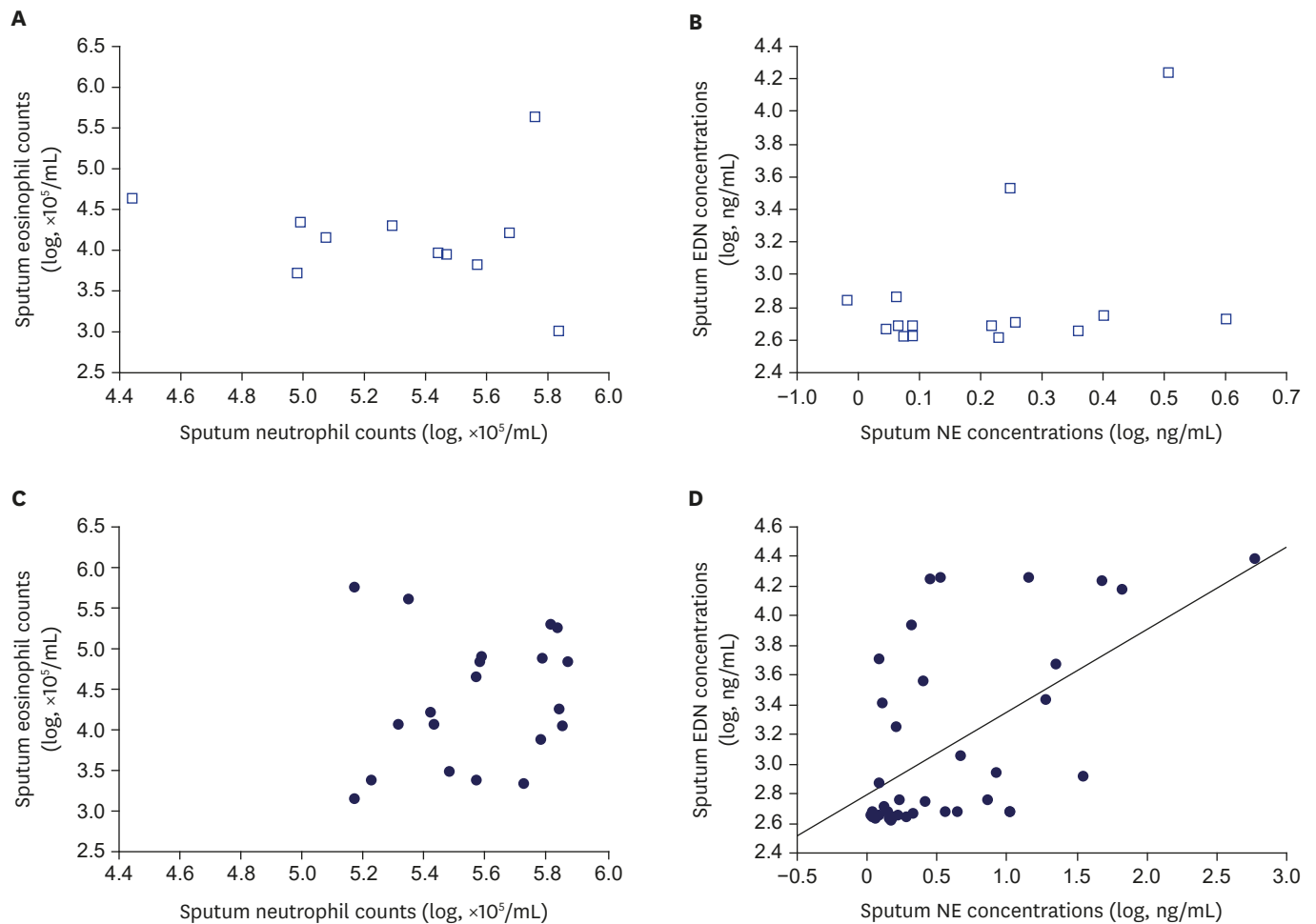


Fig. 3. Correlations between numbers of sputum neutrophils and eosinophils and between concentrations of sputum NE and EDN in non-elderly and elderly asthmatics. Correlations of (A) log sputum neutrophil and eosinophil numbers and (B) log sputum NE and EDN concentrations in non-elderly asthmatics. (C) Log sputum neutrophil and eosinophil numbers and (D) log sputum NE and EDN concentrations in elderly asthmatics. Statistical significance of the correlations was determined by Spearman correlation test. All values are expressed as log-transformed. EDN, eosinophil-derived neurotoxin; NE, neutrophil elastase.

Table 3. Correlations of sputum granulocyte numbers with pulmonary functions in non-EA and EA

Variable	Non-EA				EA			
	Log sputum eosinophils		Log sputum neutrophils		Log sputum eosinophils		Log sputum neutrophils	
	r	p value	r	p value	r	p value	r	p value
FEV ₁	-0.37	NS	-0.83	< 0.001	-0.23	NS	-0.24	NS
%FEV ₁	-0.65	0.016	-0.23	NS	-0.33	0.025	-0.03	NS
FVC	-0.22	NS	-0.89	< 0.001	-0.24	NS	-0.25	NS
%FVC	-0.47	NS	-0.12	NS	-0.30	0.041	-0.07	NS
FEV ₁ /FVC	-0.59	0.036	-0.12	NS	-0.21	NS	0.04	NS
ACT	-0.30	NS	-0.86	< 0.001	-0.39	0.040	0.01	NS

Numbers of eosinophil and neutrophils in sputum were log-transformed for statistical analysis. Statistical significance of the correlations was determined by Spearman correlation test.

EA, elderly asthmatics; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; ACT, asthma control test; NS, not significant.

sputum eosinophils and EDN levels were not. Additionally, only in elderly asthmatic patients were increased sputum NE levels associated with increased sputum EDN levels, although increased sputum neutrophil counts were not associated with increased sputum eosinophil counts. Our findings suggest that activated neutrophils possess some potent effective relations with activated eosinophils in airway neutrophilia in elderly asthmatic patients. In

Table 4. Correlations between sputum NE and EDN concentrations and pulmonary functions in non-EA and EA

Variable	Log non-EA				Log EA			
	Log EDN		Log NE		Log EDN		Log NE	
	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value
FEV ₁	-0.27	NS	-0.54	0.040	-0.21	NS	-0.02	NS
%FEV ₁	-0.43	NS	0.25	NS	-0.35	0.030	0.02	NS
FVC	-0.03	NS	-0.54	0.040	-0.13	NS	-0.12	NS
%FVC	-0.35	NS	0.24	NS	-0.35	0.029	-0.02	NS
FEV ₁ /FVC	-0.62	0.014	0.20	NS	-0.21	NS	0.15	NS
ACT	-0.31	NS	-0.57	0.028	0.07	NS	-0.09	NS

Concentrations of NE and EDN in sputum were log-transformed for statistical analysis. Statistical significance of the correlations was determined by Spearman's correlation test.

EA, elderly asthmatics; EDN, eosinophil-derived neurotoxin; NE, neutrophil elastase; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; ACT, asthma control test; NS, not significant.

fact, our findings are clinically relevant because increased sputum EDN levels were associated with decreased %FEV₁ in elderly asthmatic patients. Neutrophil-predominant inflammation in airways might contribute to the effect of eosinophils on airflow deterioration in elderly asthmatic patients.

In this study, EA demonstrated airway neutrophilia. Our findings coincide with those of several studies, showing increased neutrophil counts and IL-8 in sputum and bronchoalveolar lavage (BAL), increased NE and MMP levels in BAL, increased IL-17F in sputum, and an association between increased neutrophil counts in sputum and aging [7, 8, 16]. Therefore, the predominance of neutrophils seems to be reliable in EA.

In this study, EA showed elevated airway NE levels. Airway neutrophilia in EA is possibly influenced by multiple triggers, including proinflammatory cytokines and microbiota. An aged, house dust mite (HDM)-sensitized mouse intratracheally exposed to HDMs results in increased IL-6 and IL-1 β , namely, proinflammatory as well as Th2 cytokines, in BAL [17]. Proinflammatory cytokines, such as IL-6, TNF- α , and IL-1 β , which are representative markers of "inflammaging," are systemically increased in elderly persons. Along with aging, inflammaging exhibited low-grade systemic inflammation without infection, which is attributed to damage associated with molecular patterns delivered from damaged cells and tissue, harmful products produced by microbial constituents, cell senescence, the activation of the coagulation system, and age-related changes to the immune system [6]. Although proinflammatory cytokines were not measured in our study, inflammaging potentially progressed the activation of neutrophils in the airways of EA. Likewise, there is a possibility of potent activation of neutrophils due to senescence. One *in vivo* study demonstrated that aged neutrophils circulating in blood upregulated CXCR4 and lost L-selectin expression and a higher expression of toll-like receptor (TLR) 4, adhesion molecules, and intercellular interactions compared with neutrophils delivered from wild mice stimulated by TNF- α . This suggests that circulating neutrophils are constantly exposed to priming signals and have been activated along with aging in circulation [18]. Additionally, that study demonstrated that aging of neutrophil was driven by microbiota through TLRs and Myd88 signaling. Considering the higher frequency of bacteria colonization in airways of elderly individuals, EA might have more aged neutrophils primed by bacteria, leading to an increased number of activated neutrophils.

This study demonstrated that both higher sputum neutrophil counts and higher sputum NE levels were associated with higher sputum EDN levels in EA unless sputum neutrophil counts were not associated with sputum eosinophil counts in those patients. Those associations

between 2 inflammatory mediators delivered from neutrophils and eosinophils suggest that both activated granulocytes collaborate on airway inflammation, resulting in declined pulmonary function in EA. Our findings support that an elderly, ovalbumin (OVA)-sensitized mouse challenged by OVA showed increased eosinophils, IL-5, and interferon (IFN)- γ in BAL [19] and that an elderly, HDM-sensitized mouse exposed to HDM showed Th1-, Th2-, and Th17-type immune responses, including increased eosinophils and neutrophils and elevated levels of IFN- γ , IL-5, IL-13, and IL-17A [17]. Our previous study demonstrated that neutrophils from human peripheral blood stimulated with IL-8 or lipopolysaccharide promoted the transmembrane migration of eosinophils [20, 21]. To the contrary, human peripheral blood eosinophils migrated by chemoattractants did not induce the transmembrane migration of neutrophils [22]. Considering these findings, in the airways of EA, infiltrated neutrophils are energized and not only contribute to bronchial inflammation but also might be engaged in the accumulation of eosinophils, leading to complex neutrophil and eosinophil airway inflammation.

Our study demonstrated that increased sputum EDN levels but not sputum NE levels were associated with decreased %FEV₁ and %FVC in EA, which was not observed in the younger asthmatic patients. Our findings coincided with those of several studies, showing an association between increased urinary EDN and decreased FEV₁ in children [23, 24] or decreased FEV₁/FVC in adults [25]. In particular, the significant correlation between increased urinary EDN and decreased FEV₁/FVC was observed in asthmatics ≥ 54 years old [25]. Additionally, 2 studies demonstrated that type-2 inflammation is amplified in EA [26, 27]. Elderly patients with severe asthma, as defined by ERS/ATS guidelines, exhibited increased blood eosinophils [26] and type-2 gene mean, which is the averaged gene expression of IL4, IL5, and IL13 in sputum cells [27]. Similar to such evidence, our study demonstrated that sputum EDN concentrations were inversely correlated with more pulmonary function parameters in EA than in non-EA. Association between increases of sputum eosinophil counts and higher ACT points in EA, but not in non-EA, in this study, also supports the evidence. Consequently, energetic eosinophils rather than neutrophils in airways might contribute to decline of pulmonary function in EA.

In contrast to EA, in non-EA, 2 declined pulmonary function parameters were associated with increased sputum NE levels. These results suggest that effect of active neutrophils on pulmonary function is more conspicuous than that of eosinophils in younger asthmatics. No association of sputum neutrophil counts or NE levels with sputum eosinophils or EDN support those results in our study. Additionally, higher current smoker rates in non-EA might influenced our results because smoking promotes neutrophilic airway inflammation. Otherwise, treatment might be already enough to reduce eosinophilic airway inflammation. Taken together, our findings suggest that neutrophils and eosinophils independently promote airway inflammation in non-EA.

Unique airway inflammation in EA in our study presented features similar to those of severe asthma. High rates of patients with severe asthma exhibit predominant neutrophilia in airways [28]. Genotype cluster analysis demonstrated that several detected gene expressions related to neutrophilic inflammation; specifically, IL-1 β , IL-1 receptor, IL-17, and CXCR2 were detected in classified into neutrophil-predominant inflammation asthmatics with low %FEV₁ [29-31]. In our study, we observed that severe asthma was more prevalent in EA than in younger asthmatics (**Table 1**) and the enhanced accumulation of neutrophils and neutrophil activation in airways of EA (**Fig. 1**). Therefore, the unique immune response in severe asthmatics might be involved in advanced neutrophil inflammation in EA.

This study is limited in that it is a small retrospective study in a single hospital. There were fewer younger asthmatics than EA, which might be a potential selection bias. The duration of disease, smoking history and severity of asthma might be affected while IgE and pulmonary function were similar to the previous studies [26, 32]. The differences of distribution in smoking history between the 2 groups might be marginal because the EA who had the low prevalence of current smokers showed increases of neutrophil-related molecules and comparable levels of eosinophil-related ones, compared with the non-EA. The higher prevalence of severe asthmatics might be involved in our results. However, what immunological cascades accelerate neutrophilic inflammation in elderly severe asthmatics remains unclear. Therefore, further established studies might be needed.

In conclusion, this study addresses that in the elderly asthmatic patients, activated neutrophils are increased and associated with activated eosinophils in airways. Moreover, an elevated inflammatory mediator delivered from eosinophils is associated with airflow limitation. This distinct feature in airways demonstrates that treatment is necessary for both neutrophilic and eosinophilic inflammation in elderly asthmatic patients. Furthermore, functional neutrophils might promote the activation of an eosinophilic immune response, leading to airflow suppression in elderly asthmatic patients.

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