



Spontaneous sputum discriminates inflammatory phenotypes in patients with asthma

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ABSTRACT

Background: Eosinophils in induced sputum are not only a useful biomarker for diagnosing asthma but are also associated with severe asthma. However, little is known about the association between eosinophils in spontaneous sputum and asthma severity.

Objective: To investigate whether spontaneous sputum eosinophils are related to severe asthma in adult patients with asthma.

Methods: We conducted a retrospective cross-sectional study on 86 people with asthma whose spontaneous sputa were successfully collected. Patients were classified into 4 phenotypes according to the eosinophil and neutrophil levels in spontaneous sputum. We determined the association between inflammatory phenotypes and severe asthma. Moreover, we also compared asthma severity among the phenotypes classified according to blood eosinophils and spontaneous sputum eosinophils.

Results: Asthma phenotypes were as follows: paucigranulocytic, 30.2%; neutrophilic, 18.6%; eosinophilic, 32.6%; and mixed, 18.6%. People with eosinophilic asthma had the highest blood eosinophils, total immunoglobulin E (IgE), and fractional exhaled nitric oxide among the 4 phenotypes. Significant differences were observed in asthma severity between the phenotypes ($P = .019$). In particular, 57.2% and 56.2% of patients had severe eosinophilic asthma and mixed asthma, respectively. The logistic regression analysis revealed that spontaneous sputum eosinophilia represented the strongest association with severe asthma among the inflammatory variables. Finally, more patients with severe asthma were included in the phenotype with spontaneous sputum eosinophils greater than 3% and blood eosinophils less than or equal to $300/\mu\text{L}$ and in the phenotype with spontaneous sputum eosinophils greater than 3% and blood eosinophils greater than $300/\mu\text{L}$.

Conclusion: Spontaneous sputum can provide helpful information on airway inflammatory phenotyping in patients with asthma.

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Introduction

Bronchial asthma is characterized by chronic airway inflammation, bronchial hyperresponsiveness, and reversible airflow limitation.^{1,2} The most efficient treatment for asthma is an inhaled corticosteroid (ICS) which improves asthma control in most patients and dramatically reduces asthma death. Severe asthma is defined as asthma requiring maximum approved dosage of ICS plus

an additional controller therapy to achieve and maintain disease control, or asthma that is uncontrolled with the same therapy, or asthma that requires oral corticosteroid therapy more than half of the time.³ Severe asthma is estimated to comprise approximately 5% to 10% of the total asthma population.^{3–5} Currently, treatment for patients with severe asthma is variable.⁶ In particular, the progress of biologics, including anti-IgE monoclonal antibodies (mAbs), anti-interleukin 5 (IL-5) mAb, anti-IL-5 receptor α mAb, and anti-IL-4 receptor α mAb, has been remarkable. To effectively use these biologics, simple phenotyping for severe asthma in clinical practice is essential.

Airway inflammation can be histologically assessed in patients with asthma using bronchial biopsies, bronchial-alveolar lavage, or induced sputum. Induced sputum is a less invasive assessment than

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bronchial biopsies and bronchial-alveolar lavage, and analysis of induced sputum cellularity is a reliable method for identifying airway inflammatory phenotypes in an individual with asthma. The presence of sputum eosinophils can discriminate people with asthma from those without asthma⁷ and is a good predictor of ICS response.^{8,9} Sputum eosinophils (SpE) are increased in most patients with severe asthma compared with those with mild to moderate asthma.^{10,11} In addition, increased gene expression of the T-helper 2 (T_H2) cytokines, such as IL-4 and IL-5, occurs in the sputum of people with asthma.^{12,13} Fractional exhaled nitric oxide (FeNO) correlates with eosinophilic inflammation in peripheral airways¹⁴ and is the most convenient marker to measure airway inflammation in clinical practice. Although anti-IL-5 mAb and anti-IL-5 receptor α mAb do not appreciably affect FeNO, subcutaneous IL-4 mAb decreased FeNO approximately 30% to 40%,¹⁵ suggesting airway inflammation is regulated by a variety of factors.

Inducing sputum using hypertonic saline has been performed for half a century and can be performed safely using inhaling bronchodilator before hypertonic saline exposure. Previous reports revealed that hyperosmolarity did not affect the cell counts, cellularity, and levels of the inflammatory mediators and chemokines measured in induced sputum.¹⁶ Sputum is a frequently observed feature in patients with asthma and chronic obstructive pulmonary disease; therefore, spontaneous sputum can be collected with little burden compared with induced sputum. Pizzichini et al¹⁷ have reported that induced sputum has a higher proportion of viable cells than spontaneous sputum; however, no difference in total and differential cell counts were observed between induced sputum and spontaneous sputum in patients with asthma and those with chronic obstructive pulmonary disease.¹⁸

The inflammatory phenotype of asthma is generally classified by eosinophilic, neutrophilic, mixed, and paucigranulocytic airway inflammation. The aim of this study was to investigate the characteristics of phenotypes classified by spontaneous sputum inflammatory cells.

Methods

Subjects and Study Design

This study was conducted in the pulmonary and allergy units of the Showa University Hospital, Tokyo, Japan, between September 2016 and August 2018. A total of 160 patients, regularly followed at Showa University Hospital for at least 2 years and aged 20 to 80 years, were recruited in this study. The diagnosis of asthma was based on symptoms of cough, breathlessness, or dyspnea together with the demonstration of airflow variability. The latter was based on a reversible airflow limitation that represented an increase of at least 12% and 200 mL in forced expiratory volume in 1 s (FEV1) after the inhalation of salbutamol or the clinical treatment targeting asthma. The treatment steps were referenced from the Global Initiative for Asthma.¹⁹ Patients with poor adherence to treatment (<80%) were excluded. Adherence to treatment was assessed by the amount of drugs prescribed per year and the amount of drugs remaining. Diagnoses of perennial allergic rhinitis and seasonal cedar pollinosis were based on clinical history and a positive result for serum allergen-specific IgE. Medical history was collected, and all subjects underwent a physical examination by an experienced respiratory physician, measurement of body mass index, a lung function test, FeNO, and a blood test for peripheral eosinophils, total IgE, and specific IgE against the following 6 aeroallergens: cocksfoot, ragweed, *Cryptomeria japonica*, *Dermatophagoides pteronyssinus*, cat dander, and dog dander. Sensitization was defined as a specific IgE level greater than 0.10 IU/mL. Blood tests were performed one time in the morning at 8 AM to 10 AM.

This study complied with the Good Clinical Practice guidelines and the Declaration of Helsinki and was approved by the Institutional Review Board at Showa University School of Medicine on

April 11, 2019 (No. 2842). All patients provided written informed consent before their participation.

Sputum Processing

A total of 160 patients were instructed to bring to clinic a spontaneous sputum obtained in their home in a sterile, screw-cap cup. The sputa were collected after rinsing their mouths with water to prevent saliva contamination. Sputum was not collected during a respiratory infection or asthma exacerbation. Sputum samples were processed within 4 hours of collection as previously described.²⁰ Using a microscope, adequate plugs of sputum were selected to reduce contamination with saliva. Then, 1% of dithiothreitol (Fujifilm Wako, Osaka, Japan), freshly prepared in a dilution of 1 in 10 with distilled water, was added to the sputum sample. After incubation at 37°C for 15 minutes, phosphate-buffered saline was added to the samples in a volume equal to the sputum plus dithiothreitol. The samples were centrifuged at 600g and 4°C for 10 minutes. Cells were resuspended in phosphate-buffered saline or bovine serum albumin (1%) and were placed in a Shandon 3 cytocentrifuge (Cytospin 3; Shandon, Pittsburgh, Pennsylvania). Cytospins were prepared at 500 rpm for 10 minutes. The cytospin slides were air-dried and stained with DiffQuik (Dade Behring Inc, Newark, Delaware) to obtain differential cell counts made by counting 300 cells per slide. Samples with squamous cell contamination greater than 30% were not accepted as previously described.²¹

Measurement and Assessments

Spirometry was performed using a CHESTAC-8900 (Chest MI, Inc, Tokyo, Japan). Measurements were obtained in accordance with the guidelines for pulmonary function tests issued by the Japanese Respiratory Society.²² The forced expiration maneuver was conducted with the subject in a standing position. The highest value from 3 technically satisfactory attempts was recorded. FEV1 and FVC values were expressed as measured values and percentages of predicted values. Percent predicted FVC and percent predicted FEV1 were calculated with prediction equations of FVC and FEV1 provided by the Japanese Respiratory Society.²² Equations used are as follows:

Male: predicted FVC (L) = $0.045 \times \text{height (cm)} - 0.023 \times \text{age (years)} - 2.258$, predicted FEV1 (L) = $0.036 \times \text{height (cm)} - 0.028 \times \text{age} - 1.178$.

Female: predicted FVC (L) = $0.032 \times \text{height (cm)} - 0.018 \times \text{age (years)} - 1.178$, predicted FEV1 (L) = $0.022 \times \text{height (cm)} - 0.022 \times \text{age} - 0.005$.

FeNO was measured by a portable device (NIOX VERO; Circassia AB, Oxford, United Kingdom), according to the recommendations of the American Thoracic Society - European Respiratory Society.²³ The sensor on the device was changed periodically, in line with the manufacturer's guidance. Measurements of FeNO were performed before spirometry.

Severe asthma was defined according to the American Thoracic Society - European Respiratory Society Task Force as asthma requiring treatment with high ICS dose together with add-on medication or oral corticosteroids to prevent it from becoming uncontrolled or which remains uncontrolled despite these therapies.³ Uncontrolled asthma was defined as one of the following at the 12-month follow-up visit: (1) poor symptom control (asthma control test score of <20); (2) frequent exacerbations (≥ 2 bursts of oral corticosteroids in the previous year); (3) serious exacerbations (≥ 1 hospitalization in the previous year because of asthma); and (4) airflow limitation (FEV1 of <80% predicted).³

Inflammatory Phenotypes Classified by Spontaneous Sputum Cell Composition

Patients were classified into the following 4 phenotypes using SpE threshold of greater than or equal to 3% and a sputum

Table 1
Demographic Characteristics of the Patients

	Absence (n = 34)	Insufficient (n = 40)	Eligible (n = 86)	P value
Age: y	55.5 ± 15.0	50.8 ± 13.5	64.4 ± 13.3	<.001 ^a
Male: n (%)	21 (61.8)	15 (37.5)	34 (39.5%)	.056
BMI	23.5 ± 3.4	22.7 ± 3.1	23.4 ± 3.1	.549
Age of asthma onset: y	31.8 ± 22.7	27.8 ± 20.7	39.1 ± 20.4	.016 ^a
Smoking	2/7/25 (5.9/20.6/73.5)	6/5/29 (15.0/12.5/72.5)	7/26/53 (8.1/30.2/61.7)	.459
Current/Prior/Never: n (%)				
Allergic rhinitis: n (%)	22 (35.3)	19 (47.5)	51 (59.3)	.052
Treatment step 1/2/3/4/5: n (%)	3/7/22/1/1 (8.8/20.6/64.7/2.9/2.9)	1/5/21/9/4 (2.5/12.5/52.5/22.5/10.0)	0/9/25/29/23 (0/10.5/29.1/33.7/26.7)	<.001 ^b
ACT	21.7 ± 3.23	21.8 ± 2.64	20.8 ± 3.4	.279
Peripheral blood eosinophils: /μL (%)	277.6 ± 208.2 (4.74 ± 3.59)	300.7 ± 198.9 (5.25 ± 3.40)	462.7 ± 381.4 (7.06 ± 5.5)	.043 ^a
				.099
Total IgE: IU/mL	922.4 ± 2158.8 (2.34 ± 0.77)	1775.1 ± 4012.6 (2.59 ± 0.83)	692.6 ± 1027.4 (2.46 ± 0.61)	.494
				.451
IgE-Der p positive: n (%)	20 (58.8)	30 (75.0)	47 (54.7)	.023 ^b
FVC: % predicted	104.8 ± 16.3	102.4 ± 14.1	102.0 ± 16.6	.599
FEV1: % predicted	83.2 ± 20.3	84.2 ± 17.1	82.9 ± 20.2	.903
FEV1/FVC: %	66.3 ± 15.1	67.9 ± 11.1	65.5 ± 12.0	.604
FeNO: ppb	40.9 ± 33.9	53.3 ± 45.9	55.1 ± 41.9	.163
ICS: μg/d	347.8 ± 233.9	436.3 ± 260.9	586.6 ± 298.0	<.001 ^a
Severe asthma: n (%)	4 (11.8)	14 (35.0)	35 (40.7)	.021 ^b

Abbreviations: ACT, asthma control test; BMI, body mass index; Der p, *Dermatophagoides pteronyssinus* allergen; FeNO, fraction of exhaled nitric oxide; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; ICS, inhaled corticosteroid; ppb, ppb, parts per billion.

NOTE: Values are mean ± SDs. The treatment steps were referenced from Global Initiative for Asthma.¹⁸ Percentages predicted were calculated based on the normal provided by the Japanese Respiratory Society.²¹ Sensitization to Der p was defined as a specific IgE level greater than 0.10 IU/mL. ICS doses were converted to FP according to GINA.¹⁸ Absence group, patients who did not bring sputum samples; insufficient group, patients whose sputum samples were insufficient to measure differential cell counts; eligible group, patients whose sputum samples could be measured.

^aP values given for the analysis of variance indicated statistically significant differences among study groups.

^bP values given for the Pearson χ^2 test indicated statistically significant differences among study groups.

neutrophil threshold of greater than or equal to 60%, according to the previous studies^{24–26}: paucigranulocytic phenotype (<3% SpE and <60% sputum neutrophils); neutrophilic phenotype (polymorphonuclear leukocytes [PMN phenotype]) (<3% SpE and ≥60% sputum neutrophils); eosinophilic phenotype (Eos phenotype) (≥3% SpE and <60% sputum neutrophils); and mixed phenotype (≥3% SpE and ≥60% sputum neutrophils). Clinical variables were compared among the 4 phenotypes. Patients were also classified into the following 4 phenotypes using SpE and peripheral blood eosinophils, according to previous studies^{27,28}: noneosinophilic phenotype was defined as less than 3% SpE and less than 300/μL blood eosinophils; blood eosinophilic (b-eosinophilic) phenotype as less than 3% SpE and greater than or equal to 300/μL blood eosinophils; sputum eosinophilic (s-eosinophilic) phenotype as greater than or equal to 3% SpE and less than 300/μL blood

eosinophils; and mixed phenotype as greater than or equal to 3% SpE and greater than or equal to 300/μL blood eosinophils. Clinical variables were compared among the 4 phenotypes.

Statistical Analyses

The results are expressed as mean ± SD for continuous variables. Values that were not normally distributed, such as total IgE, were log-transformed to obtain normal distribution. All analyses were performed using JMP system version 14 (SAS Institute, Inc, Cary, North Carolina). The differences in the continuous variables were analyzed using Kruskal–Wallis test, and the differences in the categorical variables were analyzed using Pearson χ^2 test. To examine the association between severe asthma and inflammatory variables, logistic regression analyses were performed using severe

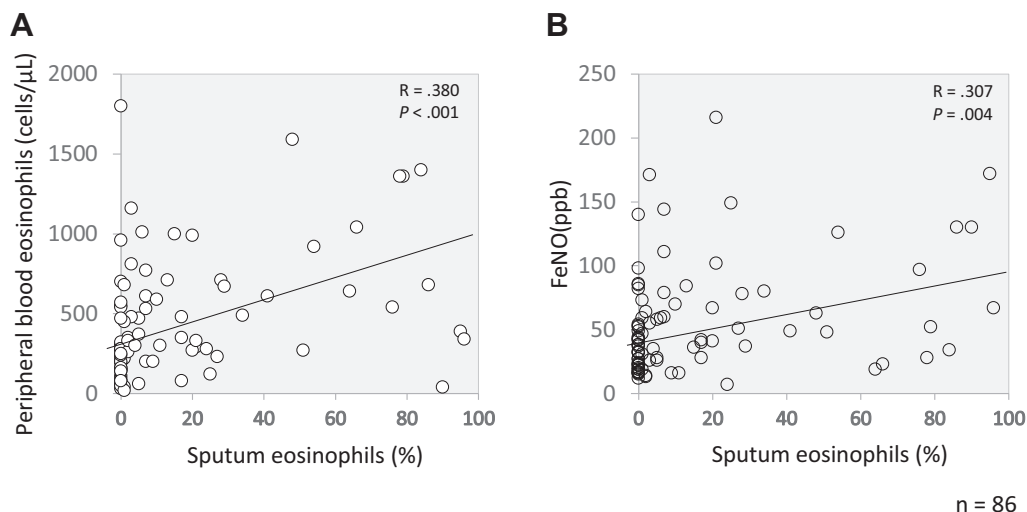


Figure 1. Correlation between spontaneous sputum eosinophils and peripheral blood eosinophils (A) and spontaneous sputum eosinophils and FeNO (B). FeNO, fraction of exhaled nitric oxide.

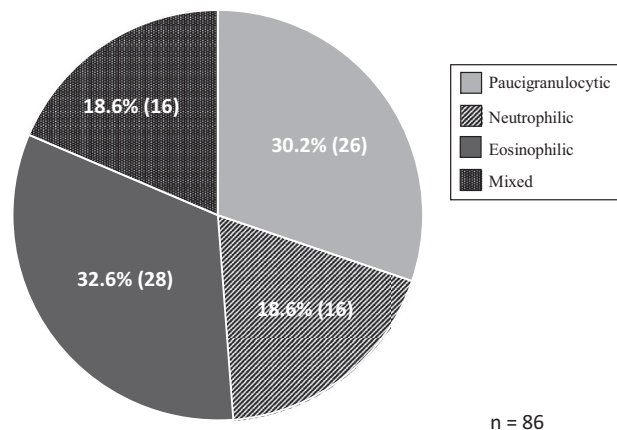


Figure 2. Distribution of the patients classified by differential cell counts in spontaneous sputum. Patients were classified into 4 phenotypes using a sputum eosinophil threshold of $\geq 3\%$ and a sputum neutrophil threshold of $\geq 60\%$. The numbers in the pie chart are frequency (%) of the phenotypes. Total number of patients were 86.

asthma as the outcome variable. These models were adjusted for age and sex. A value of P less than .05 was considered significant for all statistical assessments.

Results

Subject Characteristics and Study Course

In this study, sputa were collected from 126 of 160 patients who were requested to bring spontaneous sputum. Of the 126 collected sputa, 40 were excluded owing to 10 insufficient sputum contents, 6 insufficient sputum cell counts, or 24 squamous cell contamination greater than 30%. In total, 86 patients were analyzed. The demographic characteristics of the patients are listed in Table 1. The patients in the absence group and insufficient group were likely younger, less blood eosinophilic, less positive to IgE–Der p, and less severe. Eligible patients with a mean age 64.4 ± 13.3 years were predominantly women (60.5%). Regarding T_H2 biomarkers, peripheral blood eosinophils were $462.7 \pm 381.4/\mu\text{L}$ ($7.06 \pm 5.5\%$), FeNO was 55.1 ± 41.9 ppb, and the geometric total IgE level was 692.6 ± 1027.4 IU/L. All patients were being treated with an ICS, and the treatment steps of the Global Initiative for Asthma¹⁸ were at step 2 for 9 (10.5%), step 3 for 25 (29.1%), step 4 for 29 (33.7%), and step 5 for 23 (26.7%) patients. There were 11 patients using omalizumab for a year or longer.

Spontaneous Sputum Eosinophils in People With Asthma

Of the 86 patients, 44 (51.2%) had 3% or more eosinophils in their spontaneous sputum. The SpE revealed a weak but significant correlation with peripheral blood eosinophils ($r = 0.380$, $P < .001$, Fig 1A) and FeNO ($r = 0.307$, $P = .004$, Fig 1B). Conversely, no correlation was found between peripheral blood eosinophils and FeNO ($r = 0.130$, $P = .232$, data not indicated).

Inflammatory Phenotype With Spontaneous Sputum

Of the 86 people with asthma, 35 (40.7%) had severe asthma. Significant association was observed between spontaneous SpE and severe asthma ($P = .002$, data not indicated). Next, the patients were classified into the following 4 groups by the composition of granulocytes: paucigranulocytic, PMN, Eos, and mixed phenotypes. In this study, people with paucigranulocytic asthma were 26 (30.2%), PMN 16 (18.6%), Eos 28 (32.6%), and mixed 16 (18.6%) (Fig 2). The demographic characteristics of the 4 inflammatory phenotypes are listed in Table 2. There were significant differences in sex among the 4 phenotypes, whereby more women were included in the mixed asthmatics ($P = .019$). There were also significant differences in peripheral blood eosinophils, logarithmic total IgE, FeNO, and the treatment levels between the groups ($P < .001$, $P = .012$, $P = .007$, and $P = .025$, respectively). Both Eos and mixed phenotypes tended to have high peripheral blood eosinophil and FeNO compared with the other two phenotypes, paucigranulocytic and PMN phenotypes. In particular, the Eos phenotype had the highest blood eosinophil, total IgE, and FeNO among the 4 phenotypes. Current smokers were 4 (15.4%) among the paucigranulocytic asthmatics, 1 (6.3%) among the PMN asthmatics, and 2 (12.5%) among the mixed asthmatics (data not indicated). Among the 11 patients using omalizumab, 3 patients had more than 3% of SpE, including 1 Eos and 2 mixed phenotypes (eTable 1).

Severity Classified by Inflammatory Phenotype With Spontaneous Sputum

Figure 3 reveals the distribution of asthma severity by the 4 inflammatory phenotypes. Significant differences were observed among the phenotypes ($P = .019$). In particular, 57.2% and 56.2% of patients had severe asthma in Eos and mixed phenotypes, respectively. However, the asthma control test score did not differ among the phenotypes (20.3 ± 2.6 in paucigranulocytic, 20.7 ± 4.0 in PMN, 21.6 ± 2.7 in Eos, and 20.8 ± 3.4 in mixed asthmatics). There were no significant differences among the phenotypes in pulmonary function (Table 3).

Table 2
Demographic Characteristics of Inflammatory Phenotypes Classified by Differential Cell Counts in Spontaneous Sputum

	Paucigranulocytic (n = 26)	PMN (n = 16)	Eos (n = 28)	Mixed (n = 16)	P value
Age: y	62.3 $\pm$ 13.3	62.6 $\pm$ 14.7	65.4 $\pm$ 12.5	68.1 $\pm$ 13.3	.520
Male : n (%)	14 (53.9)	7 (43.8)	12 (42.9)	1 (6.3)	.019 ^b
BMI	23.5 $\pm$ 3.0	22.6 $\pm$ 2.8	24.0 $\pm$ 3.2	22.9 $\pm$ 3.3	.468
Allergic rhinitis: n (%)	12 (46.2)	8 (50.0)	21 (75.0)	10 (62.5)	.147
Atopic: n (%)	22 (84.6)	11 (68.7)	24 (85.7)	9 (56.2)	.089
Peripheral blood eosinophils: / μL (%)	314.9 $\pm$ 368.8 (5.2 $\pm$ 5.5)	273.3 $\pm$ 205.5 (4.2 $\pm$ 3.0)	669.3 $\pm$ 408.7 (10.3 $\pm$ 5.9)	456.3 $\pm$ 318.9 (7.2 $\pm$ 4.0)	<.001 ^a
Total IgE: IU/mL (log IgE)	640.3 $\pm$ 822.3 (2.44 $\pm$ 0.60)	573.9 $\pm$ 957.2 (2.24 $\pm$ 0.77)	1024.6 $\pm$ 1381.2 (2.75 $\pm$ 0.46)	314.4 $\pm$ 356.2 (2.23 $\pm$ 0.52)	.146
FeNO: ppb	43.2 $\pm$ 29.8	35.8 $\pm$ 22.8	74.3 $\pm$ 44.7	60.7 $\pm$ 55.0	.007 ^a
ICS: $\mu\text{g/day}$	526.0 $\pm$ 278.3	531.3 $\pm$ 355.7	635.7 $\pm$ 259.9	654.7 $\pm$ 327.1	.363
Treatment step 2/3/4/5: n (%)	5/10/8/3 (19.2/38.5/30.8/11.5)	3/7/2/4 (18.8/43.8/12.5/25.0)	0/4/14/10 (0/14.3/50.0/35.7)	1/3/6/6 (6.3/18.8/37.5/37.5)	.025 ^b

Abbreviations: BMI, body mass index; Eos, eosinophilic; FeNO, fraction of exhaled nitric oxide; PMN, polymorphonuclear; ppb, parts per billion.

NOTE: Values are mean $\pm$ SDs. ICS doses were converted to FP according to GINA.¹⁸ Treatment step were defined by GINA.¹⁸ Paucigranulocytic, PMN, Eos, and mixed phenotype were defined as less than 3% sputum eosinophils and less than 60% sputum neutrophils, less than 3% sputum eosinophils and greater than or equal to 60% sputum neutrophils, greater than or equal to 3% sputum eosinophils and less than 60% sputum neutrophils, and greater than or equal to 3% sputum eosinophils and greater than or equal to 60% sputum neutrophils, respectively.

^a P values given for the analysis of variance indicated statistically significant differences among study groups.

^b P values given for the Pearson χ^2 test indicated statistically significant differences among study groups.

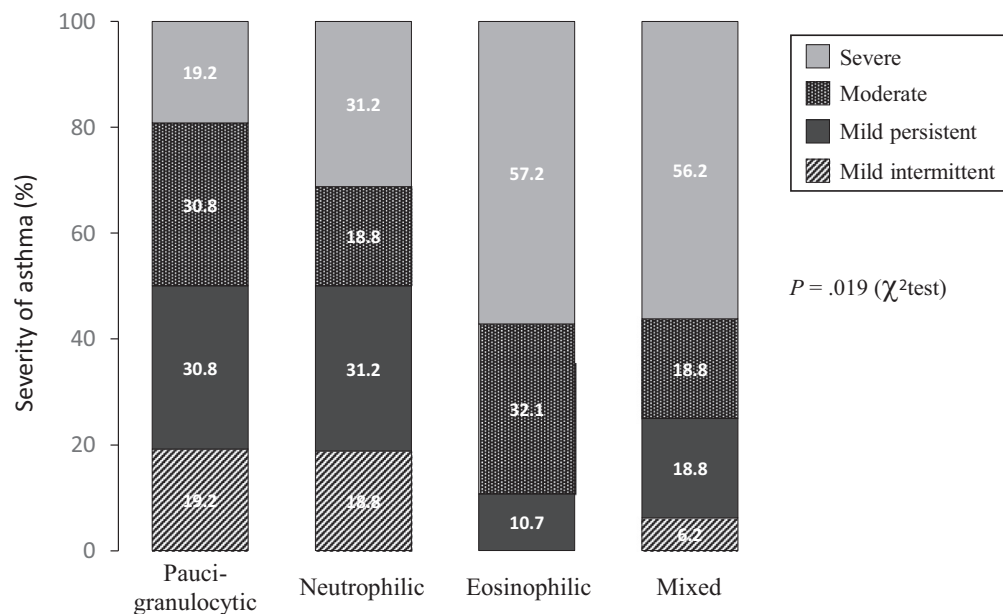


Figure 3. Distribution of the patients according to severity of asthma. Severe asthma was defined based on the International European Respiratory Society/American Thoracic Society guidelines on definition, evaluation, and treatment of severe asthma. A χ^2 test revealed a significant difference between the phenotypes ($P = .019$).

Association Between Asthma Severity and Inflammatory Indices

Table 4 indicates the association of inflammatory indices with severe asthma. The logistic regression analysis revealed that the area under the curve (AUC) index was the highest for spontaneous SpE (AUC = 0.713, $P = .001$). For spontaneous SpE, a value of 2.0% discriminated those with severe asthma from those without severe asthma with a sensitivity of 77.1% and specificity of 60.8%. Peripheral blood eosinophils also revealed a significant association with severe asthma (AUC = 0.618, $P = 0.046$), with a cut-off point of 270 calculated by the Youden's index that maximizes the AUC value of sensitivity plus specificity minus 1. Conversely, IgE, FeNO, and sputum neutrophils were not associated with severe asthma.

Inflammatory Phenotyping With a Combination of Sputum Eosinophils and Blood Eosinophils

Finally, the participants were classified into 4 phenotypes by a combination of SpE and blood eosinophils. Demographic details are listed in Table 5. Significant differences were observed in the prevalence of allergic rhinitis ($P = .037$) and severe asthma ($P = .021$) and the levels of FeNO ($P = .004$) among the four phenotypes. Patients were more likely classified as severe asthma if categorized as s-eosinophilic or mixed phenotype. In addition, the mixed phenotype had a trend of low %FEV1 compared with the other 3 phenotypes, despite no significance. The patients with a mixed phenotype had a trend of higher total serum IgE compared with the other 3 phenotypes.

Table 3
Pulmonary Function Tests in Four Inflammatory Phenotypes Classified by Differential Cell Counts in Spontaneous Sputum

	Paucigranulocytic (n = 26)	PMN (n = 16)	Eos (n = 28)	Mixed (n = 16)	P value
%FVC	104.9 ± 14.4	105.1 ± 17.6	95.9 ± 15.6	104.9 ± 18.9	.129
%FEV1	85.1 ± 3.9	85.9 ± 4.9	75.5 ± 3.7	89.5 ± 4.9	.103
FEV1/FVC: %	104.9 ± 18.9	67.1 ± 16.3	63.8 ± 9.9	67.1 ± 10.7	.765

Abbreviations: Eos, eosinophilic; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; PMN, polymorphonuclear.

NOTE: The highest value from 3 technically satisfactory attempts was recorded. Values are mean ± SDs and %FVC and %FEV1 were calculated based on the normal provided by the Japanese Respiratory Society.²¹

Discussion

The novel finding of this study is that patients with asthma could be classified by the cellular composition of granulocytes in spontaneous sputum, and eosinophils in spontaneous sputum are positively associated with severe asthma.

In this study, data from 86 of 160 patients were eligible and analyzed. The patients excluded from the analyses were younger, had lower peripheral blood eosinophils, and were less severe than the eligible patients. Therefore, prevalence of severe asthma was 40.7%, which was considered extremely high, among the patients finally analyzed in this study. This is consistent with the daily practice that patients with severe asthma tend to have more symptoms, including sputum. Meanwhile, the proportion of SpE in the patients of the absence group and insufficient group is unknown; in other words, inflammatory phenotyping with spontaneous sputum may be difficult in patients with mild asthma.

In previous studies, thresholds for sputum eosinophilia and neutrophilia were 3% and 40% to 76%, respectively.^{24–26} Accordingly, we defined greater than or equal to 3% sputum eosinophils as sputum eosinophilia and greater than or equal to 60% sputum neutrophils as sputum neutrophilia, respectively. We first reported that spontaneous sputum can discriminate inflammatory phenotypes in patients with asthma using similar thresholds to those of previous studies^{24–26}; the proportion of inflammatory phenotypes was 30.2% with the paucigranulocytic phenotype, 18.6% with the PMN phenotype, 32.6% with the Eos phenotype, and 18.6% with the mixed phenotype. Exposure to hypertonic saline to obtain induced sputum is safe; however, it takes time and a degree of burden. Therefore, demonstrating agreement between spontaneous sputum, which can be easily collected, and induced sputum may increase further usefulness of measuring spontaneous sputum.

In this study, the Eos phenotype and mixed phenotype had more patients with severe asthma than those without sputum eosinophilia. This result is consistent with previous studies,^{24–26} indicating not only induced SpE but also spontaneous SpE can be a reliable biomarker for severe asthma. Although the prevalence of severe asthma was similar between the Eos phenotype and the mixed phenotype, pulmonary function, such as %FEV1, was lower in the Eos phenotype compared with the mixed phenotype. Therefore,

Table 4
Logistic Regression Analyses for Severe Asthma in Patients With Asthma

	AUC	Cut-off point	Sensitivity	Specificity	Accuracy	Youden index	P value
Sputum eosinophils	0.713	2.0%	77.1%	60.8%	32.3%	0.379	.001 ^a
Blood eosinophils	0.618	270/ μ L	77.1%	47.1%	35.3%	0.242	.046 ^a
IgE (log)	0.558	2.1	80.0%	37.2%	36.9%	0.173	.251
FeNO	0.508	63 ppb	37.1%	74.6%	40.7%	0.117	.964
Sputum neutrophils	0.519	73/ μ L	31.4%	78.4%	43.8%	0.098	.596

Abbreviations: AUC, area under the curve; FeNO, fraction of exhaled nitric oxide; ppb, parts per billion.

NOTE: AUCs for detecting severe asthma were calculated using receiver operating characteristics curve. Cut-off point was calculated by the Youden index that maximizes the AUC value of sensitivity plus specificity minus 1.

^aP values given for the analysis of variance indicated statistically significant differences between severe asthma and the variable.

the Eos phenotype may include people with more severe asthma than the mixed phenotype. The Eos phenotype appeared to have more patients with atopy, higher peripheral blood eosinophils, higher IgE, and higher FeNO than the mixed phenotype. Accordingly, it is assumed that the Eos phenotype includes more people with T_H2-type severe asthma than the mixed phenotype. Previous reports have reported that airway neutrophilic inflammation was found in severe asthma;²⁹ however, another report did not confirm this result.³⁰ In this study, spontaneous sputum neutrophilia was not associated with severe asthma. Smoking may not be associated with PMN inflammation because most current smokers were classified in the paucigranulolytic phenotype. In this study, the number of patients receiving omalizumab were only 11; however, the patients were distributed to all phenotypes with the largest number in PMN phenotype. Because these 11 patients had been using omalizumab for at least 1 year, they were considered to be responders to omalizumab in varying degrees. These observations are consistent with the real world data reporting a clinical response with omalizumab in diverse patient types.^{31,32}

We must take into consideration the differences between spontaneous sputum and induced sputum. First, spontaneous sputum is more likely to include nasal secretions and saliva contamination than induced sputum, and we cannot deny that this contamination might have affected the results of this study. However, in accordance with the previous findings with induced sputum,^{24–26} the Eos phenotype classified by spontaneous sputum included more patients with severe asthma. With respect to saliva, contamination can occur in any phenotype, and Simpson et al³³ found that the addition of saliva to induced sputum did not change the differential cell count in vitro. Therefore, it is unlikely that nasal discharge and saliva contamination had a major effect on the results of this study. Second, there was a greater time interval, although less than 4 hours, between the collection and analysis of

spontaneous sputum compared with induced sputum. Unfortunately, there have been no reports regarding the time course of changes in the sputum cell fraction. Thus, we cannot predict that this difference in interval time could cause differences in cellular composition between spontaneous sputum and induced sputum. Third, induced sputum was exposed to hypertonic saline during the period between collection to measurement. Previous reports found that induced sputum exposed to hypertonic saline has a higher proportion of viable cells, but no difference was observed in cellular composition compared with spontaneous sputum.^{17,18} These considerations could support the evidence that spontaneous SpE and induced SpE are associated with severe asthma.

The spontaneous SpE revealed a weak correlation with peripheral blood eosinophils and FeNO, and no correlation was found between peripheral blood eosinophils and FeNO. Our results are in agreement with Gao et al³⁴ who utilized induced sputum. In this study, there were more severe patients in the s-eosinophilic phenotype than those in the b-eosinophilic phenotype. Comparing the two phenotypes, FeNO was higher in patients with s-eosinophilic phenotype than those with b-eosinophilic phenotype. It has been found that FeNO levels and blood eosinophil values provide independent information in patients with asthma.³⁵ Biologics targeting eosinophils, such as anti-IL-5 Ab and anti-IL-5 receptor Ab, do not decrease FeNO,^{36,37} whereas biologics targeting IL-4/13 decrease FeNO,¹⁵ suggesting that FeNO is regulated by IL-4/IL-13 but not IL-5. Taken together, IL-4/IL-13-mediated mechanisms may be contained in s-eosinophilic asthma compared with in b-eosinophilic asthma, and this may render s-eosinophilic asthma more severe.

This study has several limitations. First, although we reported that spontaneous SpE is associated with asthma severity, there is no direct comparison between the inflammatory phenotypes classified by spontaneous sputum and those by induced sputum in a

Table 5
Demographic Characteristics of Inflammatory Phenotypes Classified by Blood Eosinophilia and Spontaneous Sputum Eosinophilia

	Noneosinophilic (n = 28)	b-eosinophilic (n = 14)	s-eosinophilic (n = 11)	Mixed (n = 33)	P value
Age : y	62.6 $\pm$ 12.8	62.1 $\pm$ 15.7	66.2 $\pm$ 14.0	66.4 $\pm$ 12.5	.605
Male : n (%)	13 (46.4)	8 (57.1)	2 (18.2)	11 (33.3)	.172
Allergic rhinitis: n (%)	13 (46.4)	7 (50.0)	5 (45.5)	26 (78.8)	.037 ^b
Peripheral blood eosinophils: / μ L (%)	150.8 $\pm$ 83.6	595.8 $\pm$ 393.5	185.5 $\pm$ 94.2	727.3 $\pm$ 353.0	<.001 ^a
IgE: IU/mL (log IgE)	614.9 $\pm$ 905.7 (2.36 $\pm$ 0.68)	615.2 $\pm$ 810.7 (2.36 $\pm$ 0.65)	364.5 $\pm$ 404.6 (2.29 $\pm$ 0.56)	900.8 $\pm$ 1305.3 (2.65 $\pm$ 0.51)	.444
FeNO: ppb	33.1 $\pm$ 19.5	54.9 $\pm$ 34.8	70.9 $\pm$ 51.8	68.8 $\pm$ 48.2	.004 ^a
%FEV1:	84.4 $\pm$ 20.4	87.4 $\pm$ 19.3	90.6 $\pm$ 16.3	77.3 $\pm$ 20.7	.172
ICS: μ g/d	533.0 $\pm$ 287.7	517.9 $\pm$ 350.5	586.8 $\pm$ 292.4	671.2 $\pm$ 277.8	.221
Severe asthma: n (%)	7 (25.0%)	3 (21.4%)	6 (54.6%)	19 (57.6%)	.021 ^b

Abbreviations: FeNO, fraction of exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; ICS, inhaled corticosteroid; ppb, parts per billion.

NOTE: Values are mean $\pm$ SDs and %FEV1 were calculated based on the normal provided by the Japanese Respiratory Society.²¹ ICS doses were converted to FP according to GINA.¹⁸ Noneosinophilic, b-eosinophilic, s-eosinophilic, and mixed phenotypes were defined as less than 3% sputum eosinophils and less than 300/ μ L blood eosinophils, less than 3% sputum eosinophils and greater than or equal to 300/ μ L blood eosinophils, greater than or equal to 3% sputum eosinophils and less than 300/ μ L blood eosinophils, and greater than or equal to 3% sputum eosinophils and greater than or equal to 300/ μ L blood eosinophils, respectively.

^aP values given for the analysis of variance.

^bP values given for the Pearson χ^2 test indicated statistically significant differences among study groups.

single population. Investigating whether inflammatory phenotypes classified by spontaneous sputum and those by induced sputum reveal similar distribution may further support the usefulness of the spontaneous sputum fraction. Second, the proportion of females was significantly higher in the mixed phenotype than the other phenotypes. This result is inconsistent with previous reports.^{22–24} We currently do not have an explanation of this disparity. Additional studies with increased number of patients are needed. Third, in this study, the patients were relatively older and limited to a single racial group. To evaluate our findings, further studies are needed to investigate whether similar results can be obtained in younger patients and other racial groups.

Asthma is diagnosed mainly based on respiratory symptoms, including wheezing, cough, dyspnea, and chest discomfort. It is sometimes difficult to diagnose asthma, or it may be misdiagnosed, particularly in severe asthma.^{38,39} Measurement of eosinophils in sputum may help not only in the assessment of inflammatory phenotype of asthma but also in the confirmation of an asthma diagnosis. Spontaneous sputum requires less burden to obtain than induced sputum in patients with severe asthma.

In conclusion, severe asthma was associated with spontaneous SpE. Although the differences of characteristics between those who had induced SpE and those who had spontaneous SpE have not been fully studied, we believe that our classification using spontaneous sputum may be clinically useful for inflammatory phenotyping of severe asthma.

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Supplementary Data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jana.2020.06.017>.

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Supplementary Data

eTable 1

Demographic Characteristics of the Patients Using Omalizumab

	Omalizumab Group (n = 11)
Age: y	64.7 ± 11.9
Male: n (%)	4 (36.4)
BMI	23.0 ± 3.3
Allergic rhinitis: n (%)	7 (63.6)
ACT	18.9 ± 3.3
Peripheral blood eosinophils: / μ L (%)	464.5 ± 330.9 (7.5 ± 5.6)
Total IgE (log IgE): IU/mL	552.9 ± 562.8 (2.54 ± 0.46)
%FEV1	75.3 ± 20.8
FEV1/FVC: %	58.5 ± 12.5
FeNO: ppb	43.2 ± 22.5
Sputum phenotype (Pau/Neu/Eos/Mix): n	3/5/1/2
Phenotype with sputum and blood eosinophils (non-Eos/b-Eos/s-Eos/Mixed): n	2/6/1/2

Abbreviations: ACT, asthma control test; BMI, body mass index; FeNO, fraction of exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; ppb, parts per billion; Pau, paucigranulocytic; Neu, neutrophilic; Eos, eosinophilic.

NOTE: Values are mean ± SDs and %FEV1 was calculated based on the normal provided by the Japanese Respiratory Society.²¹