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## **Distinct Phenotypes of Smokers with Fixed Airflow Limitation Identified by Cluster Analysis of Severe Asthma**

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**Key words:** severe asthma, smoking, cluster analysis, phenotypes, eosinophils

**Author Contributions:** NT, SK, study concept and design, statistical analysis, acquisition of data, interpretation of data, and drafting the manuscript; HM, study concept and design, interpretation of data, and finalizing of the manuscript; KS, NS, SF, KT, MO, YM,

acquisition of data; MS, KN, HK, interpretation of data; YN, evaluation of sinusitis score; YI, statistical analysis; SW, interpretation of data, and finalizing of the manuscript; MN, study concept and design, interpretation of data, and finalizing of the manuscript.

## Abstract

**Background:** Smoking may have multifactorial effects on asthma phenotypes, particularly in severe asthma. Cluster analysis has been applied to explore novel phenotypes, which are not based on any *priori* hypotheses.

**Objective:** To explore novel severe asthma phenotypes by cluster analysis when including smoking asthmatics.

**Methods:** We recruited a total of 127 subjects with severe asthma, including 59 current or ex-smokers, from our university hospital and its 29 affiliated hospitals/pulmonary clinics. Clinical variables obtained during a two-day hospital stay were used for cluster analysis. After clustering using clinical variables, the sputum levels of 14 molecules were measured to biologically characterize the clinical clusters.

**Results:** Five clinical clusters, including 2 characterized by low FEV1/FVC, were identified. When characteristics of smoking subjects in these two clusters were compared, there were marked differences between the two groups; one had high levels of circulating eosinophils, high immunoglobulin E levels, and a high sinus score, while the other was characterized by low levels of the same parameters. Sputum analysis revealed intriguing differences of cytokine/chemokines pattern in these two groups. The other three clusters were similar to those previously reported: young onset/atopic, nonsmoker/less eosinophilic, and female/obese. Key clinical variables were confirmed to be stable and consistent three years later.

**Conclusion:** This study reveals two distinct phenotypes with potentially different biological

pathways contributing to fixed airflow limitation in cigarette smokers with severe asthma.

## **Introduction**

Smoking may have multifactorial effects on asthma phenotypes, particularly on severe asthma, although it is generally considered to have deleterious effects on asthma (1). For this clarification, investigators have classified the disease into two or three groups, such as smokers vs. nonsmokers or current smokers vs. past smokers vs. nonsmokers (2-5), and compared the asthma phenotypes between two (or three) groups. However, reports are highly inconsistent, especially with regard to the effects of smoking on airway inflammation in asthma; some studies have demonstrated that smoking attenuates eosinophilic inflammation, whereas others describe eosinophilic inflammation induced by smoking (4-10).

In contrast, clustering of characteristics using a “data-dependent classification approach” has recently been applied to explore and characterize novel asthma phenotypes, which are not based on any *priori* hypotheses (11-17). In some studies, variables used for analysis were intentionally selected by researchers (13-15), whereas in others, they were selected statistically in an attempt to reduce redundancy of variables and to enhance the stability of the analysis (16-18). However, either way, the results would be highly affected by the variables selected. Thus, cluster analysis should be considered to generate hypotheses for searching for novel phenotypes of the disease. Given the inconsistent data on the association of smoking

with airway inflammation in asthma (4-10), it would be intriguing to apply cluster analysis to subjects with severe asthma, focusing particularly on smoking subjects.

In many asthma studies, smokers, particularly heavy smokers, are excluded based on the hypothesis that smoking may affect the characteristics of asthma (11,12,16,17). In the current study, patients with severe asthma, intentionally including smokers, were recruited. Data were obtained from all subjects during their two-day stay in Hokkaido University hospital to ensure data quality. The focus was on subjects with severe asthma alone, because it was anticipated that such an approach would more likely elucidate and characterize novel phenotypes, if any, in severe asthma, rather than including a wide variety of mild to moderate asthmatic patients, most of whom are well-controlled. Specifically, we hypothesized that the effects of smoking on inflammation in asthma would vary and would not affect all subjects in the same way. It was anticipated that cluster analysis would clarify this complexity. Further, one objective was to determine whether severe asthma clusters in Japan would be similar to those identified in previous studies from Western countries. Finally, after classifying severe asthma by clinical clustering analysis, sputum levels of cytokines and/or chemokines were measured in an attempt to characterize the identified phenotypes biologically.

## **Methods**

This study was approved by the ethics committees of all hospitals, and all subjects provided

their written, informed consent. This study was registered in the UMIN Clinical Trials Registry (UMIN-CTR) system ([https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr\\_view.cgi?recptno=R000003917](https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000003917)). Details of the Material and Methods are described in our previous reports (19) and the online supplement.

### **Subject enrollment**

Patients with severe asthma diagnosed by respiratory physicians were enrolled at Hokkaido University Hospital and 29 affiliated hospitals and pulmonary specialist clinics between February 2010 and September 2012. The study inclusion and exclusion criteria are shown in our previous reports (19). Smokers and subjects with co-occurrence of COPD and emphysema were not excluded from this study when they were diagnosed as having asthma (19). Severe asthma was defined based on the ATS criteria of refractory asthma in 2000 (20), with slight modifications (see on-line supplement and our previous report, ref 19).

### **Clinical evaluations at Hokkaido University Hospital**

A total of 127 subjects with severe asthma underwent procedural evaluations during their two-day stay at the Hokkaido University Hospital (19). Chest and sinus CT scans were performed with patients in the supine position by a multidetector-row spiral CT scanner with a 64-detector array (Aquilion64; Toshiba Medical Systems, Otowara, Japan). The sinus CT findings were evaluated using the Lund-Mackay score (LMS) (21,22). The questionnaires

included questions about: age at onset of asthma, smoking status, asthma quality of life (the asthma quality of life questionnaire; AQLQ), gastroesophageal reflux disease (GERD), daytime somnolence, depression, and rhinitis symptoms. FeNO concentrations were measured with the NIOX MINO<sup>®</sup> (Aerocrine, Stockholm, Sweden) according to the American Thoracic Society guidelines (23). Sputum was induced by the inhalation of 4.5% saline with an ultrasonic nebulizer after inhalation of oxytropium and salbutamol for pulmonary function testing. Levels of 14 cytokines/chemokines in sputum supernatant were determined using Luminex multi-analyte technology (24,25) or specific ELISA. Three-years after the entry, 101 of 127 subjects were completed the re-evaluations of clinical data including spirometry, blood biomarkers (eosinophil counts and total IgE), FeNO and sputum induction.

### **Statistical analysis**

Statistical analyses were performed using the statistical packages SYSTAT for Windows (Version 11, Systat Software, Inc., Chicago, IL) and JMP Pro10<sup>®</sup> (SAS Institute Inc., Cary, NC). For cluster analysis, Ward's minimum-variance hierarchical clustering method was performed using an agglomerative (bottom-up) approach. Thirteen clinical variables (①)-(⑬) were selected for the analysis as follows: (①) peripheral eosinophil count (cells/ $\mu$ L) (②) fraction of exhaled nitric oxide level (FeNO)(ppb) (③) predicted values of forced expiratory volume in one second (%FEV1)(maximum value) (④) FEV1/forced vital capacity (FVC)

(with the value corresponding to the maximum FEV1) (⑤) predicted values of transfer coefficient of the lung for carbon monoxide (%Kco), which is diffusing capacity for carbon monoxide of the lung (DLco) corrected by alveolar volume (VA) (⑥) smoking history (never or ex, current) (⑦) pack-years (⑧) body mass index (BMI)(kg/m<sup>2</sup>) (⑨) total serum IgE (IU/mL) (⑩) atopic status (allergen-specific IgE) (⑪) sex (⑫) age (⑬) Age at onset of asthma. To compare differences between clusters, analysis of variance (ANOVA), Kruskal-Wallis, and chi-square tests followed by *post hoc* analyses were used for parametric continuous, nonparametric continuous, and categorical variables, respectively. In an attempt to create a comprehensible overview of all the results combined, radar charts were made for each cluster (Figure 2).

## Results

Detail characteristics of all subjects are described in the online supplement.

### Cluster Analysis

Figure 1 shows the dendrogram generated by hierarchical clustering analysis and Table E2 shows the important variables which determine each bifurcation by stepwise discriminant analysis. In the first bifurcation ( I in Figure 1), subjects were classified into two groups based on the %FEV1 value (preserved %FEV1 group and low %FEV1 group). Low %FEV1 group was further classified into two groups (cluster 3 and cluster 4) (Ⅲ) according to the

FEV1/FVC ratio and BMI; The next bi-classification of the low FEV1/FVC group (IV) was determined by the degree of peripheral eosinophil count and pack-years; cluster 3 was characterized by intense Th2-related indices, whereas cluster 4 was characterized by low Th2-related asthma indices with extremely high pack-years. A schematic diagram of the characteristics of all 5 clusters is shown in Figure 1. The detailed characteristics of each cluster are described below.

**Cluster 1 (*n* = 18, 14.2%): early-onset, atopic, mild eosinophilic**

This group had the youngest mean age at onset (22.6 years), and 83.3 percent of subjects were atopic. 72.2% of subjects were smokers (11.1% current, 61.1% ex), and the median pack-years was 5.2. The mean peripheral eosinophils count was 307.1 cells/ $\mu$ L, and FeNO was 60.0 ppb. Sputum eosinophils (median 9.4%) and total serum IgE levels (mean 448.5 IU/mL) were elevated. The mean %FEV1 (mean 104.3%, max value) and FEV1/FVC (mean 75.2%) were normal and higher compared with the 4 other clusters. The median sinus score (LMS) was 6.

**Cluster2 (*n* = 41, 32.2%): late-onset, low Th2-related indices (low blood/sputum eosinophil, low FeNO, low IgE and low sinus score)**

The mean age at onset was 41.6 years. 51.2% of subjects were smokers (4.9% current, 46.3% ex), and the median pack-years was 0.1. Peripheral eosinophil count (mean 82.3 cells/ $\mu$ L),

FeNO (mean 19.2 ppb), and total serum IgE levels (mean 42.9 IU/mL) were low. Sputum eosinophil counts were also low (median 0.8%), whereas the neutrophil count was high (median 63.8%). The LMS score (median 0) and the prevalence of nasal polyps (9.8%) were low.

**Cluster 3 and 4: late-onset, fixed airflow limitation (low FEV<sub>1</sub>/FVC)**

**Cluster 3 (*n* = 40, 31.5%): intense Th2-related indices (high blood/sputum eosinophil, high FeNO, high IgE and high sinus score), 72.5% of subjects were smokers**

The mean age at onset was 36.4 years. 72.5% of subjects were smokers (10% current, 62.5% ex). The median pack-years in all subjects was 11.0 and in smoking subjects was 21.0. Peripheral and sputum eosinophils (mean 329.8 cells/ $\mu$ L, median 24.0%, respectively), FeNO (mean 29.1 ppb), and total serum IgE (mean 284.8 IU/mL) levels were high. The mean FEV<sub>1</sub>/FVC was low (mean 55.6%). The sinus score (LMS) (median 7) was also high.

**Cluster 4 (*n* = 8, 6.3%): low Th2-related indices (low blood/sputum eosinophil, low FeNO, low IgE and low sinus score), all smokers**

The mean age at onset was 44.6 years. All subjects were smokers (50% current, 50% ex), and the median pack-years was the highest (72.0). Similar to cluster 3, the mean FEV<sub>1</sub>/FVC was low (mean 55.8%). The mean low attenuation volume (LAV) value (mean 3.89%) was the

highest, and %KCO (mean 84.8%) was the lowest, although within normal limit, among the 5 clusters. In contrast with cluster 3, peripheral and sputum eosinophils (mean 69.0 cells/ $\mu$ L, median 0.8%, respectively), FeNO (mean 21.5 ppb), and total serum IgE (mean 51.0 IU/mL) levels were low. LMS score (median 1) and the prevalence of nasal polyps (0%) were very low. Thus, these subjects appeared to have mild to moderate COPD together with the clinical phenotype of severe asthma.

**Cluster 5 ( $n = 20$ , 15.7%): female predominance, high BMI and intense Th2-related indices (high blood/sputum eosinophil, high FeNO, high IgE and high sinus score)**

The mean age at onset was 46.4 years. This cluster was female-dominant (85%) with a high mean BMI (31.0 kg/m<sup>2</sup>), and 80.0% of subjects were never smokers (5.0% current, 15.0% ex). The mean blood and sputum eosinophils (mean 526.4 cells/ $\mu$ L, median 24.8%, respectively) and FeNO (mean 62.7 ppb) were high. The mean %FRC (mean 83.0%), and %VC (mean 89.9%), were the lowest among the 5 clusters. The sinus score (LMS: median 11) was also high.

**Comparisons of characteristics between two smoking groups with low FEV<sub>1</sub>/FVC**

**[smoking subjects in Cluster 3 (N=29) vs. Cluster 4 (N=8)]**

Table 4 shows the comparisons of representative characteristics between two smoking groups characterized by low FEV<sub>1</sub>/FVC. Despite similarities of FEV<sub>1</sub>/FVC and symptoms score

(AQLQ), features related with Th2-related indices, such as blood/sputum eosinophils, FeNO, total IgE and sinus score showed in a sharp contrast between two groups.

### **Biomarker measurements in serum and sputum supernatant**

To address biological differences among the 5 clinically defined clusters, biomarkers in sputum were compared among the 5 clusters. IL-5, IL-6, EGF (epidermal growth factor), MCP-1 (monocyte chemoattractant protein-1) and OPN (osteopontin) showed significant differences ( $P < 0.05$ ) among the 5 groups (Table E6). IL-5 levels were high in clusters 3 and 5, whereas OPN and IL-6 levels were high in cluster 4. In addition, radar charts provided the distinct pattern of biomarkers in the sputum visually for each cluster (Figure 2).

### **Stability of clinical variables after three-year follow-up**

Of the 127 subjects, 101 subjects were successfully re-evaluated using several clinical parameters, including peripheral eosinophil count, FeNO, total serum IgE level, and pulmonary function tests. Table E7 and E8 shows the clinical characteristics for 5 clusters using the data obtained at three-year follow-up. When parameters obtained at three-year follow-up were applied and plotted to each cluster, similar results were confirmed, as shown in Figure E2.

## **Discussion**

In this study, we applied a clinical clustering approach to 127 adult patients with severe asthma, a large percent of whom had a current or past smoking history. We identified five distinct phenotypes, two of which clearly showed low FEV<sub>1</sub>/FVC (Cluster 3 and 4). 72.5% of subjects (29/40) in Cluster 3 and all of 8 subjects in Cluster 4 were smokers. When two smoking groups with low FEV<sub>1</sub>/FVC were compared, they appeared to differ by type of peripheral and airway inflammation. The amount and pattern of cytokines and/or chemokines in sputum in the 5 clusters suggested distinct airway inflammation patterns behind the 5 clusters. Considering the high smoking rate in Japan and worldwide (26), inclusion of smokers in the severe asthma study more precisely reflects the “real world” of severe asthma, where smoking rates are reported to reflect those in the general population

Asthma was defined on the basis of the patients’ episodic respiratory symptoms together with demonstrable reversible airflow obstruction at some time during their history. Low mean BDR values of patients (Table 2) at the entry were likely attributable to the response measured while they were on full medication. Airway remodeling may also explain the low mean BDR values (27). Importantly, subjects with characteristics of co-existing COPD were not excluded in this study, if they also had features of asthma.

In many previous studies, smoking was associated with a less eosinophilic inflammatory pattern (3-5). Such a conclusion was largely drawn based on the comparison between

smokers and nonsmokers. In this study, the subjects classified into Cluster 4 were all smokers, and their median pack-years were the highest among the 5 clusters. Subjects in this cluster were characterized by a fixed low FEV<sub>1</sub>/FVC ratio (average 55.8%) and low levels of eosinophils, IgE, FeNO, and sinus score, results which differ markedly from typical eosinophilic asthma. In addition, relatively low %KCO and high LAV in Cluster 4 subjects evidently support a co-existence of emphysema. On the other hand, some previous studies describe smoking asthmatics characterized by eosinophilic inflammation (6-8) and these observations are consistent with the characteristics of the smoking subjects classified into Cluster 3 (N=29) in the present study. Similar to Cluster 4, there was a fixed airflow limitation; the mean FEV<sub>1</sub>/FVC was as low as 55.6%. However, this group differed markedly from Cluster 4 based on the presence of blood and sputum eosinophils. High levels of FeNO and serum IgE were another feature, which are likely linked to a Type 2 T-helper (Th2) cell-high inflammatory response. Like typical eosinophilic asthma, the sinus score was high, and the prevalence of nasal polyps was also high in the subjects of this cluster. In short, although this group seems to be heavily affected by smoking, like Cluster 4, these 2 groups differ in the nature of airway inflammation. Meanwhile, non-smoking subjects in Cluster 3 (N=11) represent typical characteristics of severe asthma with fixed airflow limitation associated with eosinophilic inflammation.

Moreover, comprehensive measurements of cytokine and/or chemokine levels in sputum also

showed differing profiles between the two clusters (Table E6, Figure 2): Cluster 3 subjects had high CCL11 and IL-5 levels, whereas Cluster 4 subjects had high IL-6 and OPN levels. Indeed, recent studies have also shown increased levels of IL-6 and OPN in sputum from asthma subjects who smoke (24,28,29), and this was correlated with the number of neutrophils. These results are consistent with those for Cluster 4 from the present current study, but not necessarily so for all of the smoking subjects. These results may explain the aforementioned inconsistent results of the previous reports on the effect of smoking on asthma phenotypes and suggest that two distinct biological pathways exist for development of fixed airflow limitation in severe asthma.

Besides the two groups that were characterized by smoking and low FEV<sub>1</sub>/FVC, we recognize that among the other three clusters, two are similar to those identified in several studies conducted in Western countries. Cluster 1 alone was characterized by predominantly atopic and younger onset-age compared with that of the 4 other clusters. Cluster 5, which is characterized by a high body mass index (BMI) and female predominance, is similar to that identified in Western people (11,12). A high BMI-associated increase in abdominal fat may decrease lung volumes such as %FRC in these patients; thus, obesity directly affects respiratory mechanics, and this may partly explain the severity of asthma in patients in this cluster. Interestingly and unexpectedly, the subjects in Cluster 5 showed mainly eosinophilic inflammation, which is in contrast with some previous studies that reported predominantly

non-eosinophilic inflammation in the cluster with high BMI and female predominance (11,12,30,31). The reason for this discrepancy is unclear, but differences in genetic background, the inclusion of smokers, and the relatively-lower mean BMI in the present population may have influenced these results. Considering other reports, which demonstrated a significant association of obesity with eosinophilic airway inflammation and high IL-5 levels in sputum (32,33), the results in the present study seem to support such an association. Cluster 2 is characterized by less eosinophilic inflammation (predominantly neutrophilic), despite fewer pack-years. The inflammatory nature of this cluster is similar to that of Cluster 4, but the cytokine/chemokine profiles in sputum appear to differ on the radar charts (Figure 2), suggesting biological differences in the pathogenesis of these two clusters. Although the efficacy of several biologics targeted against Th2-high related molecules, such as IgE, IL-5, or IL-4/13, has been recently demonstrated (34), the subjects in these two clusters may be relatively resistant to these therapies. Future studies should also be focused on these less-eosinophilic phenotypes, and the cytokines/chemokines profiles shown by radar charts in this study may help identify the candidate targets for subjects in these two clusters.

This study has several significant limitations. First, the sample size is small, despite recruitment of subjects from 29 affiliated hospitals/pulmonary clinics. However, a unified protocol in the 2-day stay at Hokkaido University hospital ensured a high quality of data acquisition. Second, we used the old definition of severe asthma that was developed by

American Thoracic Society (ATS) workshop in 2000 (20) because the new definition by European Respiratory Society (ERS) / ATS guidelines (35) had not been officially announced when this study was designed. However, we confirmed that subjects experienced episodic deterioration of symptoms or an increase in urgent care visits or in rescue use of short-acting bronchodilators when their current medication was reduced within one year (on-line Material and Methods). Thus, all subjects were certainly in conditions where high doses of ICS or OCS were required for asthma control. Third, even if we intentionally attempted to recruit smoking asthmatic subjects regardless of co-existing COPD, it is likely that physicians did not include heavy smokers who had predominantly COPD characteristics such as emphysema, leading to omission of heavy smokers in this study. It should be noted that %KCO values are reported to be normal to high in asthma (36). Lastly, because of the cross-sectional nature of this study, we were unable to discuss the trajectory of change in pulmonary functions, particularly for subjects in clusters 3 and 4; how these patients developed fixed airflow limitation. To prospectively evaluate the declines in the FEV<sub>1</sub>, our study is ongoing to reach up to six years of follow-up. Although we completed a three-year follow-up for all subjects, we believe that three years are insufficient to determine individual changes in FEV<sub>1</sub> because of the variable nature of pulmonary function in severe asthmatic patients. Accordingly, longitudinal changes in FEV<sub>1</sub> for subjects in each cluster can be clarified in future analyses.

The cluster analysis employed here may have some inherent limitations, including the

somewhat intentional variable selection, with potential biases for smoking-related and pulmonary function parameters. The final selection of five clusters was meaningful and attractive, but not appropriately confirmed of its stability. We only confirmed the reproducibility of some key parameters over three years in this study. However, it is well known that any cluster analyses most often contain some clinical or scientific biases. Rather, these studies are best used for hypothesis generation.

In conclusion, the cluster analysis in this study, which focused only on patients with severe asthma, provided two distinct phenotypes with potentially different biological pathways contributing to fixed airflow limitation in cigarette smokers with severe asthma. These results represent the first cluster analysis of smoking in relation to severe asthma, and this should facilitate further studies.

### **HiCARAT (Hokkaido-based Investigative Cohort Analysis for Refractory Asthma)**

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## Figure legends

### Fig. 1

Dendrogram and schematic diagram of five clusters. Density of red color indicate the mean value of peripheral eosinophil count in each cluster.

In the first bifurcation ( I), subjects were classified into two groups based on the %FEV<sub>1</sub> value (preserved %FEV<sub>1</sub> group and low %FEV<sub>1</sub> group). Low %FEV<sub>1</sub> group was further classified into two groups (cluster 3 and cluster 4) (III) according to the FEV<sub>1</sub>/FVC ratio and BMI; The next bi-classification of the low FEV<sub>1</sub>/FVC group (IV) was determined by the degree of peripheral eosinophil count and pack-years; cluster 3 was characterized by intense Th2-related indices, whereas cluster 4 was characterized by low Th2-related asthma indices with extremely high pack-years. Table E2 shows the important variables which determine each bifurcation by stepwise discriminant analysis.

### Fig. 2

Radar charts representing the cytokines/chemokines levels in sputum supernatant.

Table 1. Characteristics of subjects

	Total	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	P-value*
N	127	18	41	40	8	20	
Male, N (%)	51 (40.2%)	8 (44.4%)	8 (19.5%)	25 (62.5%)	7 (87.5%)	3 (15.0%)	<0.0001
Age, years	58.0±13.1	42.6±10.1	55.8±13.3	62.9±8.7	70.1±4.5	61.6±11.8	<0.0001
Age at onset of asthma, years	38.2±17.7	22.6±13.8	41.6±17.6	36.4±17.0	44.6±18.6	46.4±13.2	0.0001
Smoking status, N (%)							
Current	13 (10.2%)	2 (11.1%)	2 (4.9%)	4 (10.0%)	4 (50.0%)	1 (5.0%)	<0.0001
Ex	62 (48.8%)	11 (61.1%)	19 (46.3%)	25 (62.5%)	4 (50.0%)	3 (15.0%)	
Never	52 (40.9%)	5 (27.8%)	20 (48.8%)	11 (27.5%)	0 (0%)	16 (80.0%)	
Pack-years	5.0 (0-22.7)	5.2 (0-19.9)	0.1 (0-11.6)	11.0 (0-34.9)	72.0 (57.1-96.0)	0 (0-0)	<0.0001
Body mass index, kg/m <sup>2</sup>	25.6±5.5	25.5±4.5	25.6±4.1	23.4±3.7	23.7±4.8	31.0±8.0	<0.0001
Atopy, N (%)	81 (63.8%)	15 (83.3%)	22 (53.7%)	26 (65.0%)	5 (62.5%)	16 (80.0%)	0.30
AQLQ	5.4 (4.8-6.2)	6.0 (5.4-6.3)	5.3 (4.7-6.0)	5.4 (4.5-6.2)	5.5 (4.8-6.4)	5.3 (4.6-5.8)	0.38

Mean ± SD Median (interquartile range)

\*P-values were obtained by the chi-square test, ANOVA, or the Kruskal-Wallis test, as appropriate.

Table 2. Pulmonary function test results and low attenuation volume (LAV)

N	Total	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	P-value§
127		18	41	40	8	20	
VC %predicted (prebronchodilator)	104.2±15.2	112.0±14.6	105.0±13.2	105.4±14.6	113.0±17.0	89.9±10.8	<0.0001
FEV <sub>1</sub> %predicted (prebronchodilator)	82.8±19.4	94.1±16.4	96.6±15.3	69.7±15.7	76.2±16.7	72.7±12.2	<0.0001
FEV <sub>1</sub> %predicted (max*)	91.4±19.1	104.3±14.1	105.9±14.1	77.9±14.7	81.0±15.3	81.4±11.9	<0.0001
FEV <sub>1</sub> /FVC, %†	67.1±13.0	75.2±10.2	76.7±8.9	55.6±12.5	55.8±12.5	67.6±9.8	<0.0001
Bronchodilator response: BDR							
ΔFEV <sub>1</sub> , mL (400 µg salbutamol)	88.7±133.9	175.0±238.2	74.1±113.1	93.5±100.4	23.8±44.4	55.3±93.9	0.023
Reversibility, % (400 µg salbutamol)	4.7±6.7	6.4±7.5	4.0±6.6	5.8±7.1	1.6±2.7	3.6±6.2	0.49
KCO %predicted	108.2±24.3	96.7±12.5	111.6±17.1	99.0±19.6	84.8±27.4	139.2±23.0	<0.0001
Low attenuation volume: LAV%‡	2.02 (0.72)	0.26 (0.68)	0.22 (0.51)	1.30 (0.63)	3.89 (0.68)	0.26 (0.60)	<0.0001
TLC %predicted	111.8±14.5	122.7±15.8	110.2±13.3	112.7±15.4	113.0±6.6	103.3±10.1	<0.0001
FRC %predicted	98.4±22.0	113.5±22.1	91.3±19.0	104.6±21.5	108.0±21.4	83.0±13.8	<0.0001
RV %predicted	114.6±22.1	123.9±23.2	106.4±20.2	118.8±23.1	118.8±21.8	112.8±18.5	0.026
RV/TLC, %	36.6±7.2	30.7±5.0	34.8±7.5	38.2±5.5	39.3±6.8	41.6±6.7	<0.0001

Mean ± SD Geometric mean (log<sub>10</sub> SD)

\*Maximum value of FEV<sub>1</sub> among 4 procedures (see on-line Material and Methods)

†FEV<sub>1</sub>/FVC was applied the value corresponding to the maximum FEV<sub>1</sub>.(see on-line Material and Methods)

‡measured by computed tomography

§P-values were obtained by ANOVA.

Table 3. Serum biomarkers and sputum cell differentiation

	Total	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	P-value*
Blood / Serum, N	127	18	41	40	8	20	
Eosinophils, cells/ $\mu$ L	203.4 (0.50)	307.1 (0.23)	82.3 (0.48)	329.8 (0.34)	69.0 (0.29)	526.4 (0.37)	<0.0001
Neutrophils, cells/ $\mu$ L	4554.4 (0.17)	4169.8 (0.14)	4715.9 (0.19)	4415.8 (0.17)	5596.2 (0.21)	4497.1 (0.15)	0.47
Total serum IgE, IU/mL	141.6 (0.73)	448.5 (0.54)	42.9 (0.71)	284.8 (0.60)	51.0 (0.63)	214.9 (0.47)	<0.0001
FeNO, ppb	31.2 (0.36)	60.0 (0.39)	19.2 (0.32)	29.1 (0.28)	21.5 (0.18)	62.7 (0.26)	<0.0001
Sputum analysis, N	115	16	34	39	7	19	
Eosinophils, %	9.6 (1.2-31.2)	9.4 (4.1-17.8)	0.8 (0-9.4)	24.0 (2.7-46.4)	0.8 (0.5-3.4)	24.8 (6.8-41.6)	<0.0001
$\geq$ 3% Eosinophils, N (%)	72 (62.6%)	13 (81.3%)	11 (32.4%)	29 (74.4%)	2 (28.6%)	17 (89.5%)	<0.0001
Neutrophils, %	53.2 (34.5-71.1)	54.7 (39.2-72.3)	63.8 (36.9-77.8)	47.6 (32.4-65.3)	62.4 (50.9-73.8)	40.0 (26.4-55.6)	0.054
$\geq$ 61% Neutrophils, N (%)	43 (37.4%)	6 (37.5%)	19 (55.9%)	11 (28.2%)	4 (57.1%)	3 (15.8%)	0.087

Mean  $\pm$  SD Geometric mean (log<sub>10</sub> SD) Median (interquartile range)

\*P-values were obtained by chi-square test, ANOVA, or Kruskal-Wallis test, as appropriate.

Table 4. Characteristics of subjects in Cluster 3 and 4

	Cluster 3		Cluster 4	P-value*
	Never smokers	Smokers	Smokers	
N	11	29	8	
Male, N (%)	2 (18.2%)	23 (79.3%)	7 (87.5%)	0.99
Age, years	64.5±6.6	62.3±9.4	70.1±4.5	0.029
Smoking status, N (%)				0.49
Current	0 (0%)	4 (13.8%)	4 (50.0%)	
Ex	0 (0%)	25 (86.2%)	4 (50.0%)	
Never	11 (100%)	0 (0%)	0 (0%)	
Pack-years	0	21.0 (10.8-40.5)	72.0 (57.1-96.0)	<0.0001
AQLQ	5.8 (5.3-6.2)	5.1 (4.4-6.3)	5.5 (4.8-6.4)	0.66
Pulmonary functions and LAV				
FEV <sub>1</sub> % predicted (max†)	84.0±13.1	75.7±14.9	81.0±15.3	0.63
FEV <sub>1</sub> /FVC, %‡	56.6±5.0	55.2±8.0	55.8±12.5	0.86
KCO % predicted	109.0±12.1	95.2±20.7	84.8±27.4	0.25
LAV%§	1.00 (0.58)	1.43 (0.66)	3.89 (0.68)	0.20
Inflammation				
Blood Eosinophils, cells/μL	393.3 (0.23)	308.5 (0.38)	69.0 (0.29)	<0.0001
Total serum IgE, IU/mL/μL	212.9 (0.57)	318.2 (0.51)	51.0 (0.63)	0.006
FeNO, ppb	28.0 (0.13)	29.5 (0.32)	21.5 (0.18)	0.21
Sputum Eosinophils, %	29.6 (14.8-54.8)	22.3 (1.7-39.6)	0.8 (0.5-3.4)	0.012
Sinus score (LMS)	9 (7-13)	6 (2-11)	1 (0-3)	0.021
Sputum cytokines				
CCL11	N=11 5.66 (0.66)	N=28 4.03 (0.70)	N=7 1.87 (0.84)	0.073
IL-5	2.66 (0.48)	3.54 (0.64)	1.34 (0.38)	0.11
IL-6	26.7 (0.36)	42.7 (0.37)	77.7 (0.58)	0.064
OPN	0.24 (0.29)	0.38 (0.47)	1.15 (0.92)	0.017

\*Cluster 3 (Smokers) vs. Cluster 4

\*P-values were obtained by Fisher's exact test, ANOVA, or Kruskal-Wallis test, as appropriate.

Mean ± SD Geometric mean (log<sub>10</sub> SD) Median (interquartile range)

†Maximum value of FEV<sub>1</sub> among 4 procedures (see on-line Material and Methods)

‡FEV<sub>1</sub>/FVC was applied the value corresponding to the maximum FEV<sub>1</sub>. (see on-line Material and Methods)

§measured by computed tomography

Figure 1

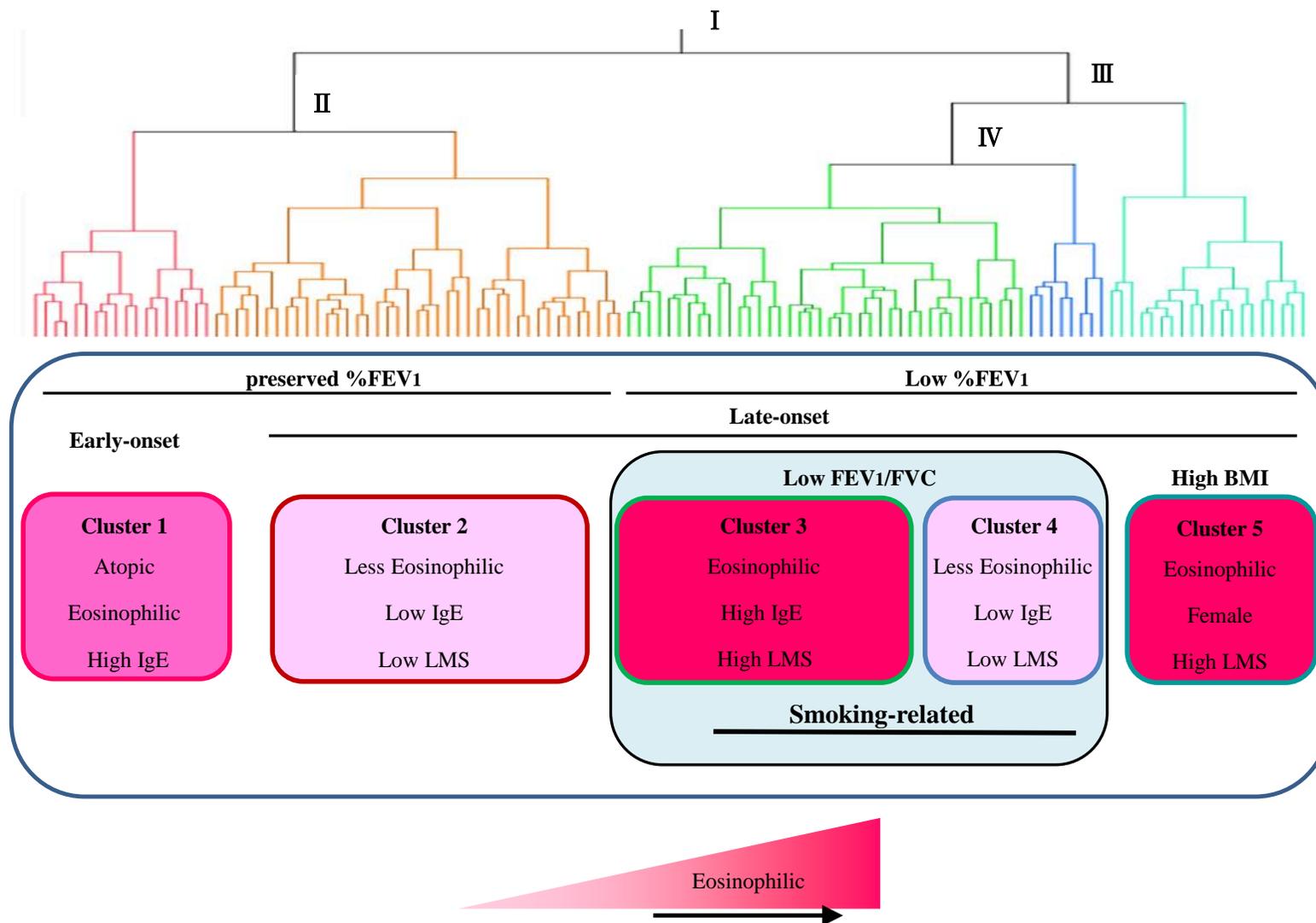


Figure 2

