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

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Online Data Supplement

Material and 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). Details of the Material and Methods are described in our previous reports (E1).

1. Participants

Patients with severe asthma were enrolled at Hokkaido University Hospital and 29 affiliated hospitals and clinics between February 2010 and September 2012. The study inclusion and exclusion criteria are shown in our previous report (E1). Several clinical parameters were evaluated in all subjects during their two-day stay at Hokkaido University Hospital (E1). All subjects were confirmed that they had been stable conditions without respiratory infection nor severe asthma exacerbation at least 4 weeks before examinations.

2. Definition of asthma and severe asthma

The diagnosis of asthma was made by respiratory physicians according to GINA criteria (E2) on the basis of recurrent episodes of respiratory symptoms associated with demonstrable reversible airflow limitation. Variable airflow limitation was confirmed at some time during the history with excessive bronchodilator reversibility or/and significant increase in FEV₁ after controller treatment (increase in FEV₁ >12% and >200mL). Severe asthma was defined based on the ATS criteria of refractory asthma in 2000 (E3), with slight modifications as follows;

Major Characteristics

In order to achieve asthma control:

1. Treatment with continuous or near continuous (>50% of year) oral corticosteroids
2. Requirement for treatment with high-dose inhaled corticosteroids:

The inhaled corticosteroid doses were modified due to differences in their availability in Japan as follows:

Flutide® ≥ 800 µg, Pulmicort® ≥ 1200 µg, QVAR® ≥ 600 µg, Alvesco® ≥ 600 µg, Asmanex® ≥ 600 µg, Adair® ≥ 1000 µg, Symbicort® ≥ 960 µg

Minor Characteristics

1. Requirement for daily treatment with a controller medication in addition to inhaled corticosteroids, e.g., long-acting β -agonist, theophylline, or leukotriene antagonist
2. Asthma symptoms requiring short-acting β -agonist use on a daily or near daily basis
3. Persistent airway obstruction ($FEV_1 < 80\%$ predicted; diurnal PEF variability $> 20\%$)
4. One or more urgent care visits for asthma per year
5. Three or more oral steroid “bursts” per year
6. Prompt deterioration with $< 25\%$ reduction in oral or inhaled corticosteroid dose
7. Near fatal asthma event in the past

Definition of severe asthma requires one or both major criteria and two minor criteria.

When patients were well-controlled under the current medications (not fulfilled any of minor characteristics 2, 4, and 5 at the entry), these subjects were confirmed that they experienced episodic deterioration of symptoms, urgent care visits, and rescue use of short-acting bronchodilators when current medication was reduced within one year.

3. Pulmonary function tests

Because of the severity of asthma in all subjects, no respiratory medicines were prohibited, except for the use of short-acting bronchodilators for at least 12 h before all measurements. Spirometry (including bronchodilator response: BDR), diffusing capacity for carbon monoxide of the lung (DLCO), and lung volumes were performed as described previously (E1).

4. Measurements of biomarkers

Total serum IgE levels (IU/mL) and specific IgE responses to common inhaled allergens, including *Dermatophagoides farinae*, grass pollens, animal dander, and molds, were determined. Atopy was defined on the basis of positive multiple antigen simultaneous test

(MAST) scores (>1.01 lumicount) to at least one common inhaled allergen (E3, E4). FeNO concentrations were measured with the NIOX MINO[®] (Aerocrine, Stockholm, Sweden) using a single-breath online method, according to the American Thoracic Society guidelines (E6).

Sputum was induced by inhalation of 4.5% saline for 15 minutes with an ultrasonic nebulizer after inhalation of oxytropium and salbutamol for pulmonary function testing. Subjects were instructed to rinse their mouths with water to avoid contamination with saliva. Induced sputum was placed in a 50-mL polystyrene tube, and a 2-fold volume of 0.1% dithiothreitol (DTT)/phosphate-buffered saline (PBS) was added. Tubes were then shaken in a water bath for 30 min at 37°C, and then a 2-fold volume of PBS was added. Tubes were shaken again in a water bath for 10 min at 37°C. After centrifuging for 10 min, the supernatant was collected, and a smear was prepared and stained using Diff-Quik (International Reagent Corp., Green Cross, Osaka, Japan). Two respiratory physicians (SK and NT) and a medical laboratory technician independently blinded to the clinical information of the subjects counted at least 250 nonsquamous cells. Disagreement between the respiratory physicians was resolved in conference. Samples with a percentage of epithelial cells $\geq 80\%$ were excluded from analyses.

Blood and sputum supernatants samples were immediately frozen after collection and stored at -80°C until assay. Initially, the concentrations of 21 molecules in randomly selected 30 samples from 127 subjects were determined using Luminex multi-analyte technology

(Luminex Corporation, Austin, TX, USA) (E7,E8) and specific ELISA (R&D Systems, Minneapolis, MN, USA). The assay sensitivities of each molecule are shown in our previous report (E1). Analytes with more than 50% undetectable values were excluded from further analysis (E1), and 14 molecules were subsequently measured in all subjects. All samples were measured in duplicate. Undetectable values were assigned an arbitrary value of half the sensitivity limit. Scaled-out values were assigned an arbitrary value of two-fold the upper limit.

5. CT imaging

On admission (day 1) around 3:30 PM, patients inhaled 400 µg of oxitropium, followed by inhalation of 400 µg of salbutamol 30 min after starting the inhalation of oxitropium (E1). A CT scan was performed 30 min after starting the inhalation of salbutamol. Chest, sinus, and abdominal 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).

5-1. Chest CT imaging

i. Procedure for the CT scan

Chest high-resolution CT scans were performed in the supine position with patients holding their breath at full inspiration. All subjects were instructed to take a full inspiration and hold

by a technician who confirmed compliance visually. Some subjects had received training in breath holding before the examination. No patient received intravenous contrast medium.

ii. Technical parameters of CT scanners: Technical parameters were as follows: 0.5 mm collimation; 120 kV; 75-350 mA; 0.5 s gantry rotation; and 1.0 mm thickness.

iii. Computerized assessment of low attenuation volume (LAV): CT raw data sets were reconstructed into isometric voxel data using both soft-tissue and bone algorithms. The length of one voxel side was 0.625 mm. Reconstructed data were transferred to the workstation and subsequently reconstructed further into three-dimensional chest images (Virtual place Fujin rajin 310; AZE Ltd., Tokyo, Japan). In brief, the whole lungs containing airways (A) were extracted from the 3D image of the thorax, deleting the heart and major vessels in the lungs. The bronchial skeleton (B) was subsequently extracted from the whole lungs, leaving the lungs consisting of parenchyma without major vessels or proximal bronchial trees. Total lung volume was defined as (A) – (B). The severity of emphysema was assessed as low attenuation volume (LAV), based on the threshold value of –950 HU. LAV% was defined as lung low attenuation volume divided by total lung volume assessed by CT, consistent with previous reports (E9, E10).

5-2. Sinus CT imaging

All subjects underwent sinus CT examination in a supine position using a 64-slice CT scanner (Aquilion64; Toshiba Medical Systems, Otawara, Japan). The same otolaryngologist evaluated and

scored the CT scans according to the Lund-Mackay score (10). A score of 0, 1, or 2 was assigned to each sinus region (maxillary, anterior/posterior ethmoidal, sphenoidal, and frontal), and a score of 0 or 2 was assigned for each osteomeatal complex to give a possible scoring of 24. A score of 0 was assigned for a completely aerated sinus, 1 for a partially opacified sinus, and 2 for a completely opacified sinus. For the osteomeatal complex, 0 was assigned if it was unobstructed, whereas 2 was assigned if an obstruction was detected. (E1).

6. Questionnaires

CRCs administered comprehensive questionnaires directly to all subjects on day 1 of admission.

i. Age at onset of asthma

The age at onset of asthma was self-reported. To ensure that this was as accurate as possible, patients were asked to describe episodes of dyspnea, wheezing, or coughing they had experienced during childhood and puberty. In cases of uncertainty, the time of the earliest respiratory symptoms was designated as the age at onset of asthma symptoms and used to calculate the duration of asthma.

ii. Smoking status

The smoking status of all patients was also assessed by CRCs. Patients were divided into three groups: current continuous smokers (Current), former smokers who had stopped smoking for a minimum of 1 month before the initial visit (Ex), and lifetime non-smokers

(Never).

iii. Asthma quality of life questionnaire (AQLQ)

Asthma-related quality of life (QOL) was assessed using the Japanese version of the AQLQ developed by Juniper (E11,E12) in all participants. The AQLQ consists of 32 items separated into four sections: symptoms, activity limitations, emotional function, and exposure to environmental stimuli. The overall score of the AQLQ was calculated as the mean of the sum of all items. Permission to use the Japanese version of the AQLQ was obtained from Dr. Juniper.

iv. Gastroesophageal reflux disease (GERD)

The severity of GERD was assessed using the frequency scale for the symptoms of GERD (FSSG) (E13). The FSSG consisted of 12 questions that were scored to indicate the frequency of symptoms as follows: never = 0, occasionally = 1, sometimes = 2, often = 3, and always = 4. Patients with scores ≥ 8 were considered to have GERD.

v. Daytime somnolence

Daytime somnolence was assessed using the Epworth Sleepiness Scale (ESS) as a self-administered questionnaire (E14,E15). Patients with scores ≥ 11 were considered to be at risk of sleep apnea syndrome (SAS).

vi. Depression

The severity of depression was assessed using the Self-Rating Depression Scale (SDS) (E16,E17). The SDS consisted of 20 questions that were scored to indicate the frequency of

symptoms as follows: never = 0, occasionally = 1, sometimes = 2, often = 3, and always = 4.

Patients with scores ≥ 50 were considered to have depression.

vii. Rhinitis symptoms

The presence of rhinitis symptoms was assessed using questionnaires according to the Practical Guideline for the Management of Allergic Rhinitis in Japan 2009 (E18).

7. Statistical analysis

Statistical analyses were performed using the statistical packages SYSTAT for Windows (Version 11, Systat Software, Inc., Chicago, IL) and JMP Pro10[®] (SAS Institute Inc., Cary, NC). For cluster analysis, Ward's minimum-variance hierarchical clustering method was performed using an agglomerative (bottom-up) approach (see the dendrogram in Figure 1). For each generation of clusters, samples were merged into larger clusters to minimize the within-cluster sum of squares or to maximize the between-cluster sum of squares. Thirteen clinical variables [(1)-(13)] were selected for the analysis as follows:

(A) Eosinophilic inflammation – (1) peripheral eosinophil count (cells/ μ L), (2) exhaled nitric oxide level (FeNO)(ppb)

(B) Pulmonary function – (3) %FEV1 (maximum value), (4) FEV1/FVC (with the value corresponding to the maximum FEV1), (5) %KCO

(C) Smoking status – (6) smoking history (never or ex, current), (7) pack-years

(D) (8) body mass index (BMI)

(E) IgE – (9) total serum IgE (IU/mL), (10) atopic status (allergen-specific IgE)

(F) Others – (11) sex, (12) age, and (13) Age at onset of asthma

All measurements were standardized using z scores for continuous variables and as 0 or 1 for categorical variables. Stepwise discriminant analysis was used to identify the most influential variables in cluster assignment.

As described above, spirometry was performed four times (twice each day, before and after the inhalation of bronchodilators, see ref. E1). The maximum FEV1 obtained from the four procedures was used. FEV1/FVC also used the value that corresponded to the maximum FEV1. To compare differences among clusters, analysis of variance (ANOVA), the Kruskal-Wallis test, and the chi-square test 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). The center circle line (0) indicates the average values for all subjects, and then the score indicates the SD. So, for example, a score of 0.4 indicates mean+0.4SD values.

8. Clinical evaluations at three-year follow-up

Three-years after the entry, 101 of 127 subjects were completed the re-evaluations of clinical data at outpatient clinic in Hokkaido University, including spirometry, blood biomarkers

(eosinophil counts and total IgE), FeNO and sputum induction.

Results

Subject demographics

Tables 1 and E1 show the demographics of all patients. The demographics were similar between subjects recruited from Hokkaido University and from the other cities. At the time of enrolment, the mean age was 58.0 ± 13.1 years (range, 26–83 years). The age at asthma onset encompassed a wide range (range, 0–76 years; mean $\pm$ SD, 38.2 ± 17.7 years.), and the mean duration of asthma was 19.8 ± 14.9 years (range, 1–61 years). Most patients were female ($n = 76$, 59.8%), and 46 (36.2%) were non-atopic. Thirteen (10.2%) subjects were current smokers, and 62 (48.8%) were ex-smokers. Forty-five (35.4%) used daily oral corticosteroids (OCS). Seventy-three (57.5%) subjects had an FEV1/FVC $<70\%$ (maximum value, as defined in Material and Methods).

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Figure legends

Fig. E1

Comparison of baseline characteristics among five clusters.

A. Age at onset of asthma B. FEV₁/FVC C. Peripheral Eosinophils (cells/ μ L)

D. FeNO (ppb) E. Total serum IgE (IU/mL) F. Sputum Eosinophils (%)

G. Sputum Neutrophils (%) H. LMS (Lund-Mackay score)

Fig. E2

Application of parameters obtained at three-year follow-up to each cluster

A. FEV₁/FVC B. Peripheral Eosinophils (cells/ μ L) C. Total serum IgE (IU/mL)

D. FeNO (ppb) E. Sputum Eosinophils (%)

Table E1. Comparisons of patients' demographics between Hokkaido University and other sites

N	All 127	Hokkaido Univ. 35	Other sites§ 92	P-value
Age, years	58.0±13.1	59.3±11.8	57.5±13.5	0.46
Male, N (%)	51 (40.2%)	13 (37.1%)	38 (41.3%)	0.67
Age at onset of asthma, years	38.2±17.7	41.7±16.2	36.9±18.1	0.14
Asthma duration, years	19.8±14.9	17.6±13.5	20.6±15.3	0.29
Smoking status, N (%)				0.74
Never	52 (40.9%)	13 (37.1%)	39 (42.4%)	
Ex	62 (48.8%)	19 (54.3%)	43 (46.7%)	
Current	13 (10.2%)	3 (8.6%)	10 (10.9%)	
Pack-years	5.0 (0-22.7)	4.5 (0-25.5)	5.2 (0-19.1)	0.54
Body mass index, kg/m ²	25.6±5.4	25.8±5.7	25.6±5.4	0.85
ICS dose, µg	1649±574	1617±394	1661±631	0.64
OCS use, N (%)	45 (35.4%)	12 (34.3%)	33 (35.9%)	0.87
Atopy, N (%)	81 (63.8%)	22 (62.9%)	59 (64.1%)	0.89
FEV ₁ %predicted (prebronchodilator)	82.8±19.4	83.3±18.3	82.6±19.6	0.85
FEV ₁ %predicted (max*)	91.4±19.1	91.6±17.8	91.4±19.6	0.95
FEV ₁ /FVC, %†	67.1±13.0	67.2±12.1	67.0±13.3	0.95
FEV ₁ /FVC<70%, N (%)	73 (57.5%)	19 (54.3%)	54 (58.7%)	0.65
KCO %predicted	108.2±24.3	104.8±22.2	109.4±25.1	0.32
Blood eosinophils, cells/µL	203.4 (0.50)	235.9 (0.47)	192.3 (0.51)	0.36
Total serum IgE, IU/mL	141.6 (0.73)	104.9 (0.72)	158.6 (0.74)	0.22
Eosinophils in sputum (%)‡	9.6 (1.2-31.2)	14.8 (1.3-35.4)	8.5 (1.2-30.6)	0.74
FeNO, ppb	31.2 (0.36)	25.8 (0.37)	33.6 (0.36)	0.12
AQLQ	5.4 (4.8-6.2)	5.3 (4.8-6.1)	5.4 (4.8-6.2)	0.90

Mean ± SD Geometric mean (log₁₀ SD) Median (interquartile range)

* Maximum value of FEV₁ among 4 procedures (see Material and Methods)

† FEV₁/FVC used the value corresponding to the maximum FEV₁. (see Material and Methods)

‡ N=115

§ 29 affiliated hospitals/pulmonary clinics

|| P-values were obtained by the chi-square test, the unpaired *t*-test, or the Kruskal-Wallis test, as appropriate. (Hokkaido Univ. vs. other sites)

Table E2. Discriminant models for each cluster splitting

Variable	Model 1		Model 2		Model 3		Model 4	
	F value	P value	F value	P value	F value	P value	F value	P value
Sex	13.2	0.0004						
Age			19.2	<0.0001				
Age at onset of asthma					4.77	0.033		
Atopy	6.45	0.012					6.22	0.016
Smoking status							10.8	0.0021
Pack-years			9.87	0.0030	5.3	0.024	46.9	<0.0001
BMI	3.53	0.062	2.32	0.13	15.7	0.0002	3.63	0.064
%FEV ₁	27.1	<0.0001			5.32	0.025		
FEV ₁ /FVC	10.2	0.0018	4.14	0.047	7.42	0.0084	14.6	0.00046
%KCO					4.56	0.039		
Peripheral eosinophil	25.4	<0.0001	6.95	0.011			27.0	<0.0001
Total IgE	2.89	0.091	19.1	<0.0001			9.35	0.040
FeNO			6.49	0.014	21.6	<0.0001	4.79	0.035

Stepwise discriminant analysis (P < 0.15)

Model 1: Cluster 1,2 vs. 3,4,5 (I in Figure1)

Model 2: Cluster 1 vs. 2 (II in Figure1)

Model 3: Cluster 3,4 vs. 5 (III in Figure1)

Model 4: Cluster 3 vs. 4 (IV in Figure1)

Bold: P < 0.05

Table E3. Health care utilization and comorbidities

N	Total 127	Cluster 1 18	Cluster 2 41	Cluster 3 40	Cluster 4 8	Cluster 5 20	P-value†
Health care utilization, N (%)							
Hospitalization after 20 years of age	72 (56.7%)	11 (61.1%)	17 (41.5%)	28 (70.0%)	5 (62.5%)	11 (55.0%)	0.087
Hospitalization in previous year	19 (15.0%)	2 (11.1%)	6 (14.6%)	6 (15.0%)	1 (12.5%)	4 (20.0%)	0.96
History of intubation	7 (5.5%)	2 (11.1%)	1 (2.4%)	4 (10.0%)	0 (0%)	0 (0%)	0.29
Daily SABA use	33 (26.0%)	6 (33.3%)	9 (22.0%)	9 (22.5%)	5 (62.5%)	4 (20.0%)	0.13
ER visit $\geq$ once/year	90 (70.9%)	16 (88.9%)	27 (65.9%)	28 (70.0%)	5 (62.5%)	14 (70.0%)	0.46
CS burst $\geq$ 3 times/year	45 (35.4%)	6 (33.3%)	15 (36.6%)	14 (35.0%)	0 (0%)	10 (50.0%)	0.18
Near fatal asthma	32 (25.2%)	7 (38.9%)	7 (17.1%)	13 (32.5%)	2 (25.0%)	3 (15.0%)	0.24
Comorbidities, N (%)							
Allergic conjunctivitis	29 (22.8%)	5 (27.8%)	9 (22.0%)	6 (15.0%)	2 (25.0%)	7 (35.0%)	0.50
Atopic dermatitis	16 (12.6%)	4 (22.2%)	4 (9.8%)	5 (12.5%)	1 (12.5%)	2 (10.0%)	0.74
Food allergy	22 (17.3%)	4 (22.2%)	9 (22.0%)	6 (15.0%)	0 (0%)	3 (15.0%)	0.59
Oral allergy syndrome	18 (14.2%)	2 (11.1%)	9 (22.0%)	3 (7.5%)	0 (0%)	4 (20.0%)	0.24
Drug allergy	30 (23.6%)	4 (22.2%)	9 (22.0%)	8 (20.0%)	4 (50.0%)	5 (25.0%)	0.48
GERD (FSCG $\geq$ 8)	66 (52.0%)	10 (55.6%)	23 (56.1%)	22 (55.0%)	1 (12.5%)	10 (50.0%)	0.26
Depression (SDS $\geq$ 50)	24 (18.9%)	4 (22.2%)	11 (26.8%)	5 (12.5%)	1 (12.5%)	3 (15.0%)	0.51
Sleepiness (ESS $\geq$ 11)	23 (18.1%)	6 (33.3%)	6 (14.6%)	9 (22.5%)	1 (12.5%)	1 (5.0%)	0.18

SABA: Short-acting β_2 agonist, ER: Emergency room, CS: Corticosteroid,

COPD: Chronic obstructive pulmonary disease, GERD: Gastroesophageal reflux disease

SDS: Self-rating depression scale, ESS: Epworth sleepiness scale

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

Table E4. Medication use

N	Total 127	Cluster 1 18	Cluster 2 41	Cluster 3 40	Cluster 4 8	Cluster 5 20	‡P-value
*ICS dose, µg	1649±574	1561±482	1763±507	1603±677	1678±594	1573±566	0.62
OCS use, N (%)	45 (35.4%)	9 (50.0%)	9 (22.0%)	16 (40.0%)	5 (62.5%)	6 (30.0%)	0.086
†OCS dose, mg	0 (0-5.0)	1.5 (0-5)	0 (0-0)	0 (0-5.0)	3.91 (0-5.0)	0 (0-4.8)	0.31
LABA, N (%)	99 (78.0%)	14 (77.8%)	34 (82.9%)	25 (62.5%)	7 (87.5%)	19 (95.0%)	0.041
Theophylline, N (%)	85 (66.9%)	13 (72.2%)	25 (61.0%)	30 (75.0%)	8 (100%)	9 (45.0%)	0.035
LTRA, N (%)	106 (83.5%)	15 (83.3%)	41 (100%)	27 (67.5%)	6 (75.0%)	17 (85.0%)	0.031
Tiotropium, N (%)	21 (16.5%)	3 (16.7%)	4 (9.8%)	6 (15.0%)	6 (75.0%)	2 (10.0%)	0.0002
H1 blocker, N (%)	56 (44.1%)	10 (55.6%)	23 (56.1%)	9 (22.5%)	4 (50.0%)	10 (50.0%)	0.023
Nasal steroid, N (%)	23 (18.1%)	4 (22.2%)	8 (19.5%)	8 (20.0%)	0 (0%)	3 (15.0%)	0.67
Macrolide, N (%)	8 (6.3%)	2 (11.1%)	4 (9.8%)	0 (0%)	1 (12.5%)	1 (5.0%)	0.31
Omalizumab, N (%)	5 (3.9%)	0 (0%)	4 (9.8%)	1 (2.5%)	0 (0%)	0 (0%)	0.21

Median (interquartile range)

ICS: inhaled corticosteroid, OCS: oral corticosteroid, LABA: long-acting β₂ agonist, LTRA: leukotriene receptor antagonist

*Equivalent to budesonide dose

†Equivalent to prednisolone dose

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

Table E5. Comorbidities

	Total	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	P-value†
N	127	18	41	40	8	20	
Comorbidities							
*Sinus score (LMS)	3 (0-10)	6 (2-12)	0 (0-2)	6.5 (2-12)	1 (0-3)	10.5 (0.3-14)	<0.0001
*Nasal polyps, N (%)	33 (26.0%)	10 (55.6%)	4 (9.8%)	14 (35.0%)	0 (0%)	5 (25.0%)	0.001
Aspirin sensitivity, N (%)	19 (15.0%)	6 (33.3%)	4 (9.8%)	5 (12.5%)	1 (12.5%)	3 (15.0%)	0.21
Allergic rhinitis, N (%)	99 (78.0%)	16 (88.9%)	32 (78.0%)	30 (75.0%)	7 (87.5%)	14 (70.0%)	0.62

Median (interquartile range)

LMS: Lund-Mackay score

* detected by CT

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

Table E6. Cytokine/chemokine levels in sputum supernatant

	Total	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	P-value*
N	115	16	34	39	7	19	
EGF	86.6 (0.58)	100.2 (0.54)	65.3 (0.58)	129.4 (0.45)	164.8 (0.53)	43.9 (0.74)	0.017
CCL11	3.72 (0.70)	2.51 (0.64)	4.64 (0.69)	4.43 (0.69)	2.40 (0.84)	2.88 (0.75)	0.49
IL-1 β	16.4 (0.61)	21.2 (0.52)	14.1 (0.64)	15.4 (0.70)	10.9 (0.59)	23.2 (0.41)	0.60
IL-5	2.25 (0.63)	1.63 (0.57)	1.53 (0.54)	3.27 (0.60)	1.35 (0.44)	3.28 (0.87)	0.025
IL-6	30.0 (0.50)	20.7 (0.51)	27.5 (0.56)	34.5 (0.39)	92.5 (0.58)	23.8 (0.46)	0.037
IL-8	1916.7 (0.45)	1375.8 (0.40)	1687.6 (0.48)	2244.3 (0.46)	3248.4 (0.56)	1895 (0.34)	0.30
IL-12p70	1.89 (0.40)	1.36 (0.40)	2.34 (0.34)	1.63 (0.36)	1.87 (0.47)	2.32 (0.37)	0.14
IL-17A	0.58 (0.34)	0.45 (0.25)	0.66 (0.38)	0.56 (0.36)	0.62 (0.27)	0.60 (0.36)	0.66
IP-10	41.2 (1.35)	38.8 (1.34)	24.7 (1.19)	81.1 (1.30)	190.3 (1.86)	15.3 (1.48)	0.24
MCP-1	37.2 (0.73)	28.8 (0.76)	20.9 (0.65)	69.7 (0.68)	72.3 (0.86)	28.0 (0.78)	0.011
CCL5	3.52 (0.33)	2.81 (0.14)	3.17 (0.24)	4.23 (0.42)	4.94 (0.50)	3.13 (0.24)	0.46
VEGF	13.8 (0.82)	14.5 (0.81)	12.1 (0.78)	20.5 (0.71)	39.9 (0.50)	4.97 (1.08)	0.46
TNF- α	4.01 (0.69)	2.97 (0.72)	3.32 (0.73)	5.77 (0.77)	5.09 (0.85)	3.16 (0.55)	0.46
OPN	0.32 (0.51)	0.22 (0.48)	0.29 (0.45)	0.33 (0.43)	1.54 (0.92)	0.27 (0.43)	0.035

Geometric mean (log₁₀ SD)

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

Table E7. Characteristics of subjects at three-year follow-up

	Total	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	P-value‡
N	101	14	35	35	6	11	
Male, N (%)	42 (41.6%)	6 (42.9%)	7 (20.0%)	22 (62.9%)	6 (100%)	1 (9.1%)	<0.0001
Body mass index, kg/m ²	25.6±4.9	27.0±5.2	26.1±4.3	23.6±3.7	23.3±4.7	30.0±6.9	0.0001
Pulmonary functions							
FEV ₁ %predicted (prebronchodilator)	81.2±21.3	88.9±14.3	95.1±17.2	67.8±19.0	68.3±18.3	77.1±18.9	<0.0001
FEV ₁ %predicted (max*)	86.3±20.3	95.0±11.6	100.4±14.4	72.3±18.8	74.0±16.3	81.9±18.5	<0.0001
FEV ₁ /FVC, %†	65.3±13.9	72.6±8.0	76.1±7.8	52.7±10.2	52.2±11.2	68.7±9.5	<0.0001
Blood / Serum, N							
Eosinophils, cells/μL	233.1 (0.49)	359.6 (0.29)	156.3 (0.58)	295.6 (0.40)	116.2 (0.49)	303.7 (0.43)	0.023
Total serum IgE, IU/mL	133.3 (0.70)	384.5 (0.74)	54.4 (0.63)	214.2 (0.66)	85.6 (0.62)	168.6 (0.38)	<0.0001
FeNO, ppb	28.8 (0.32)	52.1 (0.38)	23.1 (0.27)	28.2 (0.28)	19.4 (0.38)	35.7 (0.30)	0.003

Mean ± SD Geometric mean (log₁₀ SD) Median (interquartile range)* Maximum value of FEV₁ among 2 procedures (before and after the inhalation of bronchodilators)†FEV₁/FVC was applied the value corresponding to the maximum FEV₁.

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

Table E8. Characteristics of subjects in Cluster 3 and 4 at three-year follow-up

	Cluster 3		Cluster 4	P-value*
	Never smokers	Smokers	Smokers	
N	8	27	6	
Male, N (%)	1 (12.5%)	21 (77.8%)	6 (100%)	0.99
Pulmonary functions				
FEV ₁ %predicted (max†)	78.5±17.9	70.5±19.0	74.0±16.3	0.68
FEV ₁ /FVC, %‡	53.3±9.5	52.5±10.6	52.2±11.2	0.94
KCO %predicted	108.8±13.8	88.1±19.1	82.2±31.2	0.55
Inflammation				
Blood Eosinophils, cells/μL	444.6 (0.20)	261.9 (0.43)	116.2 (0.49)	0.087
Total serum IgE, IU/mL	200.8 (0.76)	218.3 (0.68)	85.6 (0.62)	0.17
FeNO, ppb	35.5 (0.15)	26.4 (0.30)	19.4 (0.38)	0.35
Sputum Eosinophils, %§	16.7 (13.2-27.1)	12.1 (2.4-21.4)	1.4 (0.7-5.4)	0.028

*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 (log₁₀ SD) Median (interquartile range)†Maximum value of FEV₁ among 2 procedures (before and after the inhalation of bronchodilators)‡FEV₁/FVC was applied the value corresponding to the maximum FEV₁.

§ N=7(Cluster3, Never smokers), N=24(Cluster 3, Smokers), N=5 (Cluster4)

Figure E1

A.

B.

C.

D.

E.

F.

G.

H.

I.

Figure E2