

Lung function decline and variable airway inflammatory pattern: Longitudinal analysis of severe asthma

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Background: Eosinophilic airway inflammation measured by using induced sputum is an important treatment stratification tool in patients with severe asthma. In addition, sputum eosinophilia has been shown to be associated with severe exacerbations and airflow limitation.

Objectives: We sought to identify whether eosinophilic inflammation in sputum is associated with FEV₁ decrease in patients with severe asthma and whether we could identify subgroups of decrease behavior based on the variation of eosinophilic airway inflammation over time.

Methods: Ninety-seven patients with severe asthma from the Glenfield Asthma Cohort were followed up with scheduled 3-month visits; the median duration of follow-up and number of visits was 6 years (interquartile range, 5.6-7.6 years) and 2.7 visits per year. Induced sputum was analyzed for eosinophilic inflammation at scheduled visits. Linear mixed-effects models were used to identify variables associated with lung function and overall decrease. In addition, using individual patients' mean and SD sputum eosinophil percentages over time, a 2-step cluster analysis was performed to identify patient clusters with different rates of decrease.

Results: FEV₁ decrease was -25.7 mL/y in the overall population. Postbronchodilator FEV₁ was also dependent on

exacerbations, age of onset, height, age, sex, and log₁₀ sputum eosinophil percentages ($P < .001$). Three decrease patient clusters were identified: (1) noneosinophilic with low variation (mean decrease, -14.0 mL/y), (2) eosinophilic with high variation (mean decrease, -40.9 mL/y), and (3) hypereosinophilic with low variation (mean decrease in lung function, -19.2 mL/y).

Conclusion: The amplitude of sputum eosinophilia was associated with postbronchodilator FEV₁ in asthmatic patients. In contrast, high variability rather than the amplitude at baseline or over time of sputum eosinophils was associated with accelerated FEV₁ decrease. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key Words: Severe asthma, airway inflammation, lung function decline, eosinophilia, induced sputum

Asthma is a common disease that affects approximately 300 million persons worldwide and has a prevalence of between 7% and 15% in European nations.¹ A proportion of patients with asthma ($\leq 10\%$) have severe persistent disease and are at a particularly high risk of exacerbations, hospitalization, and death and often have severely impaired quality of life.² Although this group represents a relatively small proportion of the asthmatic population, they consume two thirds of the health care costs attributed to asthma.³

Eosinophilic airway inflammation is an important biomarker in patients with severe asthma. Sputum eosinophilia is associated with a favorable response to both inhaled and oral corticosteroids in asthmatic patients.⁴ A number of previous reports have identified that strategies aimed at optimizing eosinophil counts in sputum with corticosteroids significantly reduce the frequency of asthma exacerbations.^{5,6} More recently, targeting eosinophilic airway inflammation in sputum selectively with an mAb to IL-5 (mepolizumab) has been shown to significantly reduce the frequency of asthma exacerbations and might allow down-titration of oral corticosteroids in patients with refractory asthma.⁷⁻⁹ In addition, we have identified phenotypes of severe asthma enriched for eosinophilic airway inflammation that might respond to therapies directed toward T_H2 immune pathways. Therefore induced sputum eosinophils are important stratification and risk biomarkers in patients with severe asthma.¹⁰

A number of previous studies have identified that severe asthma is associated with an accelerated rate of lung function, lung function decline with exacerbations,¹¹ disease duration, cigarette smoking,¹² sex, inhaled corticosteroid therapy,¹³ and CD8⁺ T-cell infiltration¹⁴ of the airway wall being associated with lung function decline in adult patients. Immunopathologic studies in patients with severe asthma have demonstrated that the presence of sputum or tissue eosinophilia is associated with remodeling of the airway wall and airflow limitation.^{15,16} In

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Abbreviations used

BDP: Beclomethasone dipropionate
 BIC: Bayesian information criterion
 LME: Linear mixed effects

addition, 2 reports have identified sputum eosinophilia cross-sectionally as being associated with impaired lung function.^{17,18}

However, the presence of eosinophils in sputum might be highly variable and reflect fluctuation in immune activation, natural biological variation, and/or the rate of passage from the tissue compartment to the luminal airway compartment. This is important because airway inflammation measured based on induced sputum¹⁹ and exhaled nitric oxide²⁰ in asthmatic patients is known to undergo temporal oscillations, which might influence important clinical outcomes, such as exacerbations. Therefore it is possible that therapeutic strategies targeting T_H2 inflammation in asthmatic patients could benefit from optimizing both the amplitude and variability of eosinophilic airway inflammation over time. It is currently unknown whether either the amplitude or variation over time in patients with sputum eosinophilia is associated with lung function decline in patients with severe asthma. Furthermore, the rate of lung function decline in phenotypic subgroups of the population with severe asthma stratified by airway inflammation has not been clearly examined.

We hypothesized that (1) lung function in patients with severe asthma is associated with eosinophilic airway inflammation when accounting for common cofactors, such as exacerbations, that are known to influence lung function decline trajectories and (2) phenotyping patients based on both the amplitude and variation in sputum eosinophil counts over time would identify populations associated with a differential rate of FEV₁ decrease. We tested these hypotheses in a population with severe asthma derived from patients attending the Glenfield Hospital Difficult Asthma Clinic.

We selected postbronchodilator FEV₁ percent predicted as the *a priori* lung function trait of interest to evaluate airway structural changes rather than lung function effects that might have been confounded by airway smooth muscle tone, as would be expected with prebronchodilator spirometry.

METHODS

Patients

We identified patients from our Leicester Difficult Asthma Database cohort at Glenfield Hospital who met the following screening criteria: (1) a physician's diagnosis of asthma with objective evidence (≥ 1 peak flow variation of $\geq 20\%$ over a 2-week period, bronchodilator reversibility of $\geq 12\%$ /200 mL, or airway hyperresponsiveness [methacholine PC₂₀ ≤ 8 mg/mL]); (2) a minimum of 5 years of follow-up with scheduled 3-month visits assessing airway inflammation based on induced sputum and postbronchodilator spirometric results; (3) a minimum of 3 or more visits with sputum cell counts and postbronchodilator FEV₁ measurements over the follow-up period; and (4) complete baseline demographic data (Fig 1).

All patients had a less than 10 pack-year smoking history.

Our Difficult Asthma Database population consisted of 908 patients with asthma (Table I). From this population, 97 patients had 5 or more years of follow-up and at least 3 visits with sputum and postbronchodilator lung function measurements. To benchmark our population to a reference population with refractory asthma, we extracted data from patients who met the American Thoracic Society workshop consensus criteria²¹ for refractory asthma (minus the lung function decline population) as a comparator group

(Table I). Our lung function decline population was matched for body mass index, sex, and all lung function metrics to the American Thoracic Society refractory asthma group; this is important with respect to the potential for selection bias of lung function traits. In contrast, our lung function decline population was typical of the population that would be recruited into phase 2/phase 3 studies of T_H2-targeted strategies (eg, mepolizumab^{7,8} and lebrikizumab²²) and patients described with refractory asthma in a recent United Kingdom registry population.²³ Specifically, the population was a mixture of patients with early- and later-onset eosinophilic asthma, primarily never smokers, receiving high-dose inhaled corticosteroids and in some cases systemic steroids and add-on therapy. Therefore it would appear that our lung function decline population differed from the refractory asthma database cohort with respect to these parameters, which is not surprising in view of our sputum management protocol, which aims to optimize eosinophilic airway inflammation and therefore might selectively retain such patients.

Approval from the Leicestershire, Northamptonshire, and Rutland research ethics committee was obtained for data analysis of the clinical database for patients attending the Glenfield Hospital Difficult Asthma Clinic.

Asthma management protocol

Patients were managed with a protocol directed at normalizing eosinophilic airway inflammation measured in induced sputum, which was adopted by our clinical service after the report of Green et al.⁵ Scheduled clinical assessments with sputum and lung function occurred at 3-month intervals over the lung function decline follow-up period. In the sputum management group decisions about anti-inflammatory treatment were made in accordance with an algorithm based on maintenance of a sputum eosinophil count of less than 3% with a minimum of anti-inflammatory therapy. The threshold of 3% was chosen because it identifies patients with corticosteroid-responsive asthma.⁴ If the sputum eosinophil count was less than 1%, we reduced anti-inflammatory treatment irrespective of asthma control. If the sputum eosinophil count was 1% to 3%, no changes were made to anti-inflammatory treatment, and if it was greater than 3%, we increased anti-inflammatory treatment. Modification of individual bronchodilator therapy was performed according to traditional assessments of symptoms, peak expiratory flow, and use of β -agonists.

Baseline investigations

Patients were phenotyped¹⁰ by undergoing extensive evaluation at baseline, including a full medical history, height, weight, allergy, smoking status, and lung function testing with bronchial challenge using methacholine, where appropriate, and sputum inflammation.²⁴

Allergy testing

Atopic status was assessed by using both skin prick tests to the common airborne allergens cat, dog, house dust mite, and *Aspergillus fumigatus* (ALK-Abelló, Hørsholm, Denmark) and total and specific IgE testing. Sensitization was defined as having positive skin test results with a wheal of 3 mm or greater or a positive IgE level of 0.35 kU/L or greater by using the UniCAP250 system (Pharmacia, Milton Keynes, United Kingdom) in patients taking regular antihistamines.

Pulmonary function testing

For each clinic visit, patients underwent standard spirometry (Vitalograph, Maids Moreton, United Kingdom), according to American Thoracic Society/European Respiratory Society guidelines,²⁵ with reversibility after 15 minutes by using 200 μ g of inhaled salbutamol administered through a spacer.

Sputum induction

Sputum induction was performed with previously described methods.²⁴ Before testing, patients inhaled 200 μ g of salbutamol. Patients were asked to blow the nose and rinse the mouth with water. Sputum induction was undertaken with normal saline administered through an ultrasonic nebulizer, followed by successive concentrations of saline from 3% up to 5% to aid expectoration. The expectorate was processed within 2 hours. Sputum free

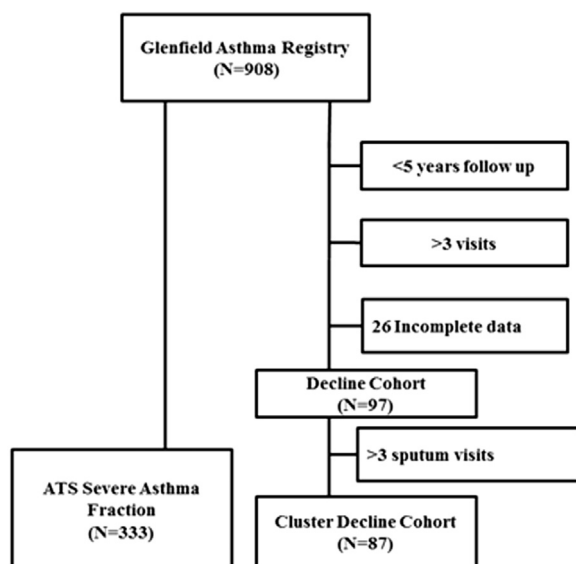


FIG 1. The lung function decline cohort was selected from our Glenfield asthma registry by using the following filters: (1) severe asthma, (2) 5 or more years of longitudinal follow-up, and (3) 3 or more serial visits with paired induced sputum and postbronchodilator spirometry. ATS, American Thoracic Society.

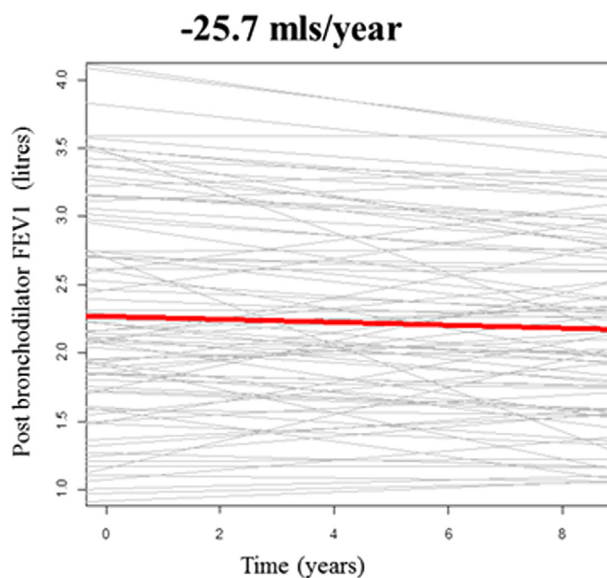


FIG 2. Postbronchodilator FEV₁ (y-axis) versus time (x-axis) in the final lung function decline model (model 30, see Table E1). The solid black line represents the overall lung function decline trajectory (decline value, -25.7 mL/y). The gray lines indicate patient-specific trajectories and were significantly influenced by $\log_{10}$ sputum eosinophils, which were modeled as both fixed and random effects.

from saliva was weighed and added to a 4× volume of 0.1% dithiothreitol (Sigma, Poole, Dorset, United Kingdom). The sputum was dispersed by means of gentle aspiration into a Pasteur pipette, vortexed for 15 seconds, and then rocked on a bench spiromix for 15 minutes. After the addition of an equal volume of Dulbecco PBS (Sigma), sputum was filtered through a 48- μ m nylon gauze and centrifuged at 2000 rpm for 10 minutes. An aliquot was removed, and total cell count, squamous cell contamination, and viability were assessed by using a Neubauer hemocytometer with the trypan blue exclusion method. Cell cytospin preparations were stained with Romanowski stain. A differential cell count was obtained, counting more than 400 nonsquamous cells.

Sputum samples met the following quality control criteria: (1) a plug weight of 50 mg or greater, (2) A minimum of 400 cells, (3) viability of 50% or greater, and (4) squamous contamination of 20% or less.

All of the lung function and sputum measurements were performed in the “stable” state (≥ 4 weeks after an exacerbation) and sequentially on the same day or within 1 week of the patient’s clinical assessment.

Asthma control

Asthma control was captured at each study visit by using the 7-item Juniper Asthma Control Questionnaire.²⁶

Statistical analysis

Statistical analysis was performed with R (R Development Core Team [2012]) and SPSS version 16 (SPSS, Chicago, Ill) software. Parametric and nonparametric data are presented as means (SEMs) and medians (interquartile ranges), unless stated. For comparison of multiple groups characterized by using parametric and nonparametric variables, 1-way ANOVA or the Kruskal-Wallis test was used, and the χ^2 test was used for proportions. For log-transformed variables, 0 values were converted to 0.25 to preserve the values efficiently.

We used linear mixed-effects (LME) modeling to identify the relationship between sputum eosinophilia and decrease in postbronchodilator FEV₁. The LME model is similar to a multiple regression model, but unlike the multiple regression model, it allows for multiple entries across time for patients. Variables can either be static over time, which we call fixed effects in the model (eg, height), or variables can change over time, which we call random effects (eg, FEV₁).

The LME model is ideal for irregularly spaced data with static and time-varying covariates. In addition, individual patients are allowed to have their own specific decrease trajectories²⁷ represented as random effects. For further details, see the Methods section and Fig E1 in this article’s Online Repository at www.jacionline.org.

The lung function decline model was built iteratively (30 discrete lung function decline models) by using the Akaike information criteria, a method of quantifying the fit of the model. Akaike information criteria were used to identify the model that best described the decrease in postbronchodilator FEV₁ within the population (see Table E1 in this article’s Online Repository at www.jacionline.org). The final lung function decline model included the following fixed effects: (1) time (in years); (2) height (in centimeters); (3) age of onset of asthma symptoms (in years); (4) exacerbations, which were defined as an increase in symptoms for 2 or more consecutive days requiring pulsed prednisolone (0.5 mg/kg/d) or admission to the hospital for emergency care; (5) mean $\log_{10}$ sputum eosinophil percentage over time; and (6) sex. In addition, the random effects of time (in years) and $\log_{10}$ sputum eosinophil percentage were modeled to allow us to model patient-specific lung function decline. We did not adjust for treatment effects because they did not significantly influence lung function in our models (for models 11 and 14–28, see Table E1).

The final expression for the lung function decline model is outlined in Equation 1 in this article’s Online Repository at www.jacionline.org.

To determine individual means and SDs for the $\log_{10}$ sputum eosinophil percentage over time for each patient, we determined a minimum number of observations needed. Because 2 observations are not sufficient to evaluate an SD and the percentage error of the SD reduces asymptotically with the number of observations, 4 sputum eosinophil measurements over time were used to evaluate the mean and SD because this allowed a good trade-off between accuracy of statistics while preserving the majority of the patients in the lung function decline cohort ($n = 87$). A 2-step cluster analysis statistical algorithm in SPSS software²⁸ was then used to cluster patients based on their mean (representing the amplitude of eosinophilic airway inflammation) and SD $\log_{10}$ sputum eosinophil counts (representing the variation of eosinophilic airway inflammation) over time to identify whether we could generate clusters of differential lung function decline within our population with severe asthma. Fifteen models were evaluated by using between 1 and 15 clusters. The cluster algorithm works by finding the optimal number of clusters that fit the data structure rather than relying on *a priori* assumptions of cluster number/structure.

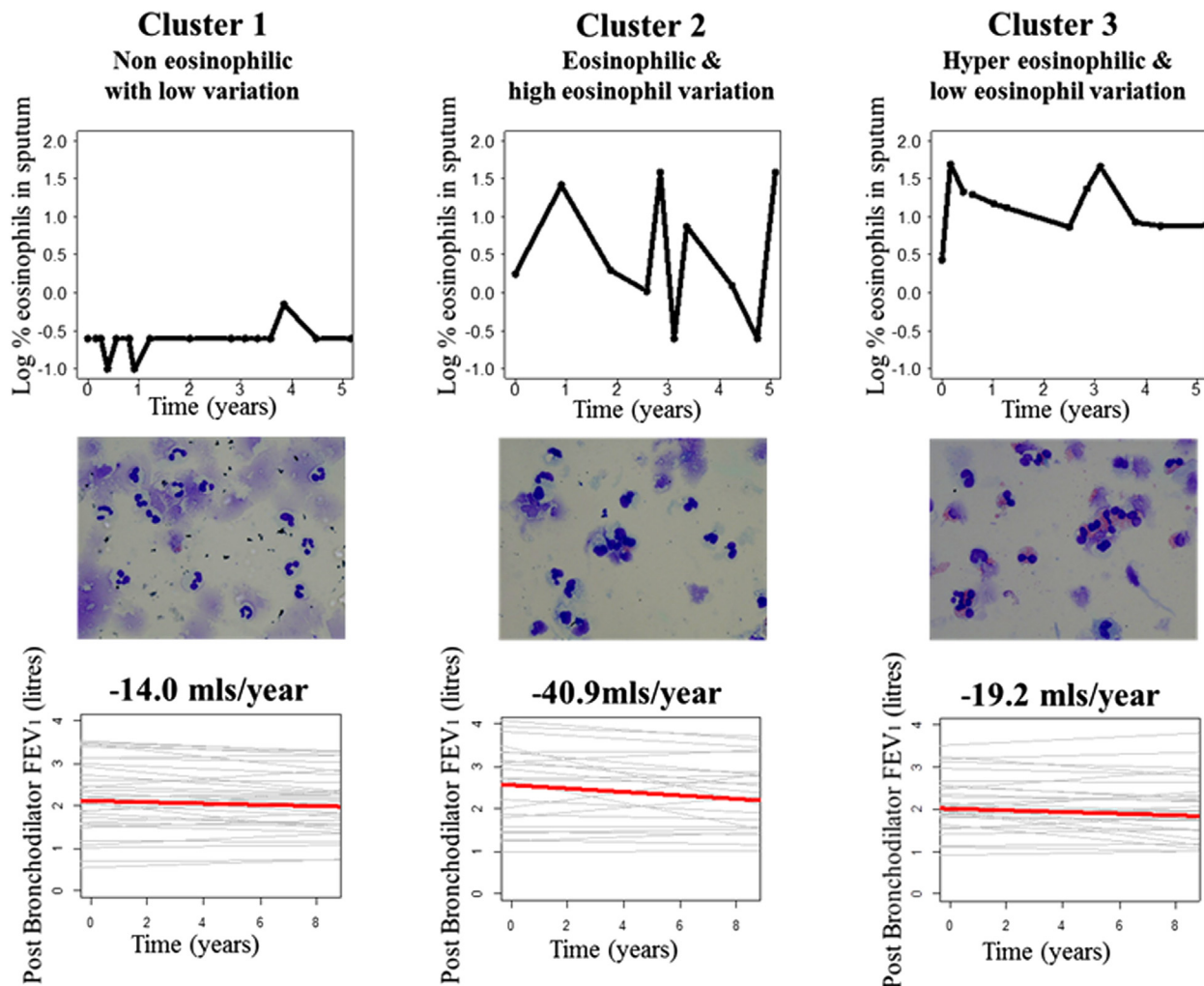


FIG 3. Results of clustering decrease in postbronchodilator FEV₁ according to the mean log₁₀ percentage sputum eosinophil counts and variation in eosinophilic inflammation (SD of the mean log₁₀ percentage sputum eosinophil count). Three clusters were identified. Cluster differences are described through typical individual patient plots of log₁₀ sputum eosinophil percentage and time (*top*), sputum smears (*middle*), and FEV₁ decrease over time (*bottom*).

The best-fitting model and thus the number of clusters was determined by first calculating the Bayesian information criterion (BIC). This is another parameter that evaluated model fit, which uses both the fit of the model and the number of parameters used to determine the best-fitting model. The change in BIC was calculated by using the BIC values for m clusters with $m-1$ clusters. The ratio (R) of change in BIC for each model step to change of BIC for the first cluster model was calculated and used to determine the best-fitting number of clusters. The least number of clusters that satisfy the condition of a ratio less than or equal to 0.04 is defined as the best-fitting number of clusters; in most cases this is the cluster model with the smallest BIC. On this basis, a 3-cluster model was identified as best fitting the data structure. The cluster membership variable derived for the 87 patients was then used to determine FEV₁ decrease trajectories for the clusters.

RESULTS

Eosinophilic airway inflammation and postbronchodilator FEV₁ in patients with severe asthma

We observed a significant decrease in postbronchodilator FEV₁ of 25.7 mL/y in our final lung function decline model ($P = .0059$;

model 30, see [Table E1](#); [Fig 2](#)). Eosinophilic airway inflammation significantly influenced postbronchodilator FEV₁ in our final lung function model, with a mean postbronchodilator FEV₁ reduction of 81.4 mL per log₁₀ unit increase in sputum eosinophil percentages ($P < .0001$). In addition, age of onset, exacerbations, sex, and height all significantly influenced postbronchodilator FEV₁, as previously reported. Our lung function models did not identify an association with *A fumigatus* sensitization (as a fixed effect) and lung function decline (see [Table E1](#)).

Variation of sputum eosinophil counts over time and lung function decline in patients with severe asthma

As demonstrated in [Tables II and III](#), we identified that both (1) the mean log₁₀ sputum eosinophil percentage and (2) its variation over time (SD) were significantly associated with postbronchodilator FEV₁ in our asthmatic population. To identify whether these 2 parameters were associated with lung function decline, we used a mixture modeling approach known as 2-step cluster analysis.

TABLE I. Baseline clinical demographics

	Lung function decline cohort portion of DAC cohort (n = 97)	Severe refractory portion of DAC cohort (n = 333)	P value
Female sex (%)	60	65	.433
Age (y [IQR])*	57.5 (46.7-65.1)	50.7 (37.5-60.6)	<.001
BMI (kg/m ² [IQR])*	30.9 (30.6-35.1)	29.2 (24.7-32.1)	.070
Smoking status (%)			
Current smoker	1.1	13.6	<.01
Exsmoker	13.7	30.0	
Never smoker	85.3	56.3	
Pack-year history	0.00 (0.00-0.00)	8 (0.625-19.250)	<.01
Exacerbations in previous calendar year	5.00 (3.00-8.00)	4 (2-6)	.123
Atopy to common aeroallergens (%)†	63.4	58.2	.55
Total IgE (kU/L [IQR])*	168 (54-483)	122 (28-389)	.086
Blood eosinophil count (× 10 ⁹ [IQR])*	0.69 (0.49-1.22)	0.64 (0.4-1.05)	.092
Age of asthma onset (y [IQR])*	24.00 (5.00-38.00)	18.00 (2.00-38.00)	.144
Baseline Juniper ACQ score (IQR)*	2.92 (1.71-3.71)	2.71 (1.71-3.57)	.618
BDP equivalent dose (μg [IQR])*	2000 (1000-2000)	2000 (2000-3000)	<.01
Oral steroid dose (mg [IQR])*	0.0 (0.0-0.0)	0.0 (0.0-7.6)	<.01
Post-BD FEV ₁ (%)‡	75.35 (69.87-80.83)	81.80 (0.7895-0.8465)	.443
Post-FEV ₁ /FVC	70.24 (66.45-74.02)	71.40 (69.86-72.94)	.581
Log ₁₀ sputum eosinophils§	5.45 (3.62-8.20)	2.57 (1.29-3.85)	<.001
Sputum neutrophils (%)	55.55 (49.28-61.82)	60.08 (56.47-63.69)	.038
No./% receiving omalizumab (Xolair)	0/0.0	3/0.90	.8171
No./% receiving methotrexate	2/2.1	7/2.1	1.000
No./% receiving LTRAs	19/20	90/27	.210
No./% receiving theophylline	15/16	80/24.1	.779

ACQ, 7-Point Juniper asthma control questionnaire; BD, bronchodilator; BDP, beclomethasone dipropionate equivalents; BMI, body mass index; DAC, Difficult Asthma Clinic; FVC, forced vital capacity; IQR, interquartile range; LTRA, leukotriene receptor antagonist.

*Median (IQR).

†Common aeroallergens, including house dust mite, cat, dog, timothy grass.

‡Lung function at baseline visit.

§Geometric mean (95% CI).

TABLE II. Fixed effects for final mixed-effects model on FEV₁ decrease

Fixed effects	Value	SE	P value
Intercept	-5.19	1.82	.0044
Age	-0.0258	0.00560	<.0001
Time	-0.0257	0.00931	.0059
Height	0.0509	0.00968	<.0001
Age of symptom onset	0.0121	0.00370	.0015
Exacerbations	-0.0261	0.00774	.0008
Log ₁₀ % sputum eosinophils	-0.0814	0.0194	<.0001
Sex (male)	0.397	0.206	.0568

Two-step cluster analysis based on the mean log₁₀ sputum eosinophil percentage and SD of the log₁₀ sputum eosinophil percentage identified 3 distinct clinical clusters that best fitted the data structure. The cluster model included cluster ID, time, and the interaction of cluster ID with time (cluster ID*time) as fixed effects (see Fig E2, Table E2, and Equation 2 in this article's Online Repository at www.jacionline.org).

The clustering algorithm identified 3 distinct clusters that differed in the nature of eosinophilic airway inflammation (Fig 3) and the rate of postbronchodilator FEV₁ decrease, as outlined below and summarized in Table IV. The addition of baseline postbronchodilator FEV₁ as an additional input variable in the cluster model did not alter the cluster model significantly and was associated with poor cluster separation. In addition, baseline

TABLE III. Random effects for final lung function decline model

Random effects	SD	P value
Intercept	0.6124	<.0001
Time	0.0622	<.0001
Log ₁₀ % sputum eosinophils	0.116	<.0001
Residual	0.207	NA

NA, Not applicable.

postbronchodilator FEV₁ did not correlate with either the mean or SD of log₁₀ sputum eosinophil percentage over time.

Cluster 1: Noneosinophilic with low variation. This cluster consisted of a group of predominantly female subjects with an increased body mass index (mean body mass index, 32.3 kg/m² [SD, 7.3 kg/m²]) and neutrophilic inflammation (mean sputum neutrophil percentage at baseline, 67.91% [SD, 24.66%]; geometric mean sputum eosinophil percentage at baseline, 1.26% [95% CI, 0% to 4.8%]). This group of patients had a rate of decrease of -14.0 mL/y.

Cluster 2: Eosinophilic with high eosinophilic variation. This cluster consisted of subjects with low-grade but significant sputum eosinophilia (geometric mean sputum eosinophil percentage, 4.28% [95% CI, 1.07% to 7.49%]) at baseline with a high variance of their log₁₀ normalized sputum eosinophil percentage over time (SD, 0.84). This cluster accounted for 26% of the population, had well-preserved lung function (mean postbronchodilator FEV₁ at baseline, 79% [SD, 22.8%]), were

TABLE IV. Clinical outcome and demographics for the 3 eosinophilic clusters

Variable	Cluster 2: Low-grade eosinophilic and high variation			P value
	Cluster 1: Noneosinophilic with low variation	Cluster 2: Low-grade eosinophilic and high variation	Cluster 3: Hypereosinophilic and low variation	
No. of patients (%)	34 (39)	23 (26)	30 (34)	NA
No. of visits, median (IQR)	10.5 (7-13)	11 (9-12.5)	12 (7.25-14)	.773
Age (y), mean (SD)	52.63 (12.17)	54.28 (13.83)	54.27 (13.98)	.853
Height (cm), mean (SD)	162.38 (9.82)	169.04 (10.75)	165.70 (10.06)	.056
BMI (kg/m ²), mean (SD)	32.30 (7.73)	30.51 (7.78)	28.33 (5.46)	.088
Baseline post-BD FEV ₁ /FVC	0.72 (0.20)	0.72 (0.13)	0.70 (0.13)	.797
Baseline post-BD FEV ₁ (% predicted)	76.40 (28.51)	79.00 (22.77)	69.48 (21.12)	.342
Baseline FEV ₁ bronchodilator response, mean (SD)	7.39 (15.17)	9.82 (17.74)	10.42 (17.54)	.018
Age of symptom onset (y), mean (SD)	23.62 (18.27)	24.39 (19.40)	24.30 (20.47)	.986
Sex (female), no. (%)	24 (71)	11 (48)	17 (57)	.204
<i>Aspergillus</i> sensitization, no. (%)	9 (26)	7 (30)	10 (33)	.704
Atopy, no. (%)	25 (74)	16 (70)	17 (57)	.323
Pack-years smoked, mean (SD)	0.03 (0.177)	0.91 (1.974)	1.12 (2.638)	.061
Juniper asthma control score, total at baseline	2.8 (1.2)	2.4 (1.3)	3.3 (0.6)	.251
BDP equivalent, mean (SD) at baseline	1578 (894)	2145 (1445)	2375 (1256)	.091
BDP equivalent, mean (SD) over follow-up period	1988 (995)	2060 (1094)	1836 (932)	.011
Oral dose, mean (SD) over follow-up period	8.27 (10.93)	7.36 (10.28)	7.85 (9.16)	.149
Sputum neutrophils (SD) at baseline (%)	67.91 (24.66)	54.70 (26.71)	49.66 (27.00)	.046
Sputum eosinophils (GEM [95% CI]) at baseline (%)	1.26 (0-4.8)	4.28 (1.07-7.49)	10.24 (6.93-13.55)	<.001
Log ₁₀ (SD) sputum eosinophils over duration of follow-up (%)	0.10 (0.60)	0.63 (0.84)	1.01 (0.54)	<.001
Log ₁₀ (SD) sputum neutrophils over duration of follow-up (%)	1.78 (0.12)	1.65 (0.17)	1.69 (0.13)	.002
Exacerbations over follow-up period, mean (SD)	2.24 (1.44)	2.55 (1.70)	1.93 (1.64)	.397
FEV ₁ decrease (mL/y [SEM])	-14.0 (0.008)*	-40.9 (0.012)	-19.2 (0.011)*	.030

One-way ANOVA and Kruskal-Wallis and χ^2 tests were used, where appropriate.

BD, Bronchodilator; BDP, beclomethasone dipropionate equivalents; BMI, body mass index; FVC, forced vital capacity; GEM, Geometric mean; NA, not applicable.

* $P < .05$ for the comparison against cluster 2.

receiving the highest daily dose of beclomethasone dipropionate equivalent of inhaled corticosteroids over the lung function decline period (mean inhaled corticosteroid beclomethasone dipropionate equivalent/24 h at baseline, 2060 μ g [SD, 1094 μ g]), and had the highest rate of lung function decline of -40.9 mL/y.

Cluster 3: Hypereosinophilic and low variation. This group had a very high amplitude of eosinophilic inflammation (geometric mean sputum eosinophil percentage, 10.24% [95% CI, 6.93% to 13.55%]) at baseline, as well as over the follow-up lung function decline period and low temporal variability in sputum eosinophil counts over time. Despite this cluster having the poorest lung function at baseline (mean postbronchodilator FEV₁ percentage at baseline, 69.5% [SD, 21.1%]), this group had a lung function decline over time of -19.2 mL/y similar to cluster 1.

There were significant differences in the rate of decrease between individual clusters (Table IV).

DISCUSSION

For the first time, we have shown that the variability rather than amplitude of sputum eosinophils over time is significantly associated with the rate of postbronchodilator FEV₁ decrease in patients with severe asthma. These observations might have an important effect on future therapies that use eosinophilic biomarkers to modulate asthma risk. Our findings extend previous cross-sectional observations in asthmatic patients, showing that sputum eosinophil counts are increased in patients with accelerated FEV₁ decrease.^{17,18}

We have demonstrated an overall rate of decrease of 25.7 mL/y in our population with severe asthma. Previous reports in asthma

have demonstrated rates of decrease of 31.5 mL/y in asthmatic patients with frequent exacerbations versus 14.6 mL/y in those with infrequent exacerbations,¹¹ 26.6 mL/y in an population with occupational asthma who were exposed to low-molecular-weight sensitizers,²⁹ 28.4 to 39.7 mL/y in adult asthmatic patients who had never smoked in the Busselton cohort,¹² and 16.1 to 21.5 mL/y in asthmatic patients receiving inhaled corticosteroids.¹³ We speculated this heterogeneity in lung function decline parameters might be related to the heterogeneity of eosinophilic airway inflammation in our population.

In keeping with these aforementioned lung function studies in asthmatic patients, we identified that baseline sputum eosinophil counts, exacerbations, age of onset, and sex significantly influence postbronchodilator FEV₁ in populations with severe asthma.

Specifically, we identified that for every log₁₀ unit increase in the percentage of sputum eosinophils, there was an 81.4-mL decrease in postbronchodilator FEV₁, which is approximately 3-fold greater than the annual rate of FEV₁ decrease observed in our overall population.

However, we were not powered to assess the interaction of these individual effects with time to identify their role in influencing lung function decline rather than postbronchodilator FEV₁ *per se*. Further research is needed in this area because previous studies that have evaluated interaction effects with time (sex, treatment, and exacerbations) in asthmatic patients have had small sample sizes^{11,13} and have not specifically evaluated patients with severe asthma, whereas other have not evaluated lung function decline with modeling approaches in the context of eosinophilic airway inflammation.^{17,18} Finally, we did not identify an association between *A fumigatus* sensitization and lung function decline, suggesting that our previously reported observation of fixed

airflow obstruction in patients with *Aspergillus* species sensitization³⁰ are unlikely to be indicative of a causal relationship and might be driven by the association of sensitization to filamentous fungi and eosinophilic airway inflammation.

In light of our observation that individual variations in sputum eosinophil counts over time demonstrated a highly significant association with postbronchodilator FEV₁, we evaluated the role of eosinophilic variation over time and FEV₁ decrease by using 2-step clustering algorithms. We identified a low-grade eosinophilic cluster with high eosinophil variation over time and a decrease of -40.9 mL/y. In contrast, we identified a hypereosinophilic cluster with low eosinophilic variation (-19.2 mL/y) and a noneosinophilic neutrophilic cluster that exhibited a similar rate of decrease (-14.0 mL/y). The 3 clusters did not differ in their baseline inhaled/oral corticosteroid use, baseline level of asthma control, or airflow limitation. These results clearly identify phenotypes based on airway inflammation by using sputum that have a differential rate of lung function decline within the overall population with severe asthma and have implications for future studies examining asthma risk and also potentially for T_H2-directed clinical trials in asthmatic patients that aim to normalize the amplitude of eosinophilic inflammation over time rather than its variation. Our findings are consistent with the observations made by McGrath et al,¹⁹ who identified different patterns of airway inflammation in sputum over a 1-year period in asthmatic patients, notably high-grade sputum eosinophilia, low-grade sputum eosinophilia, and noneosinophilic disease. However, the latter report did not evaluate the role of eosinophilic variation over time in asthmatic patients.

One potential mechanism for the difference in lung function decline and observed eosinophilic variance between the 3 clusters could be related to adherence to inhaled/oral corticosteroids. In particular, the low-grade eosinophilic group with high eosinophilic variation might be intermittently adherent to therapy. Further studies to establish adherence behavior within asthma clusters are required to address this hypothesis.

An alternative explanation for the differential rate of lung function decline across the 3 clusters is that the nature and/or sensitivity to common environmental exposures, such as allergens, particulate matter, or pathogens, might have differed among the 3 clusters. However, the clusters did not differ significantly in their degree of atopic sensitization, and the patients were all from the same geographic location within Leicestershire. Therefore it would be unlikely that differences in exposure could account for the differences in eosinophilic variation observed, although a previous report has reported marked differences in airway inflammation within a short geographic radius in highly urbanized areas because of differences in diesel exposure.³¹

A third possibility is that the underlying biological mechanism of airway inflammation might differ among the 3 reported clusters. Oscillations of eosinophilic inflammation in cluster 2 might have been related to susceptibility to environmental exposures, variable susceptibility of airway structural cells to corticosteroids, or oscillations in key effector cytokines and chemokines, such as IL-5, GM-CSF, and eotaxin, resulting in cyclical eosinopoiesis/tissue recruitment. Previous reports have identified cyclical oscillations of blood eosinophil counts, with peak levels occurring at midnight in healthy volunteers; it remains unclear whether periodicity is observed at longer time scales.³² In addition, a previous report in a 16-month-old patient with cyclical neutropenia revealed evidence of cyclical fluctuations in blood eosinophil counts

associated with cyclical oscillations in bone marrow lymphoid and myeloid lineages.³³ This would suggest that period failure of myeloid differentiation factors or impaired responsiveness of myeloid precursors might underpin the oscillatory behavior of eosinophils. Clearly, these assertions are purely speculative and warrant further research in asthmatic patients.

We selected postbronchodilator FEV₁ as the dependent variable in our linear mixed models because it is more likely to reflect structural changes within the airways rather than increased smooth muscle tone *per se*. The association between eosinophils in sputum and postbronchodilator FEV₁ decrease is biologically plausible. Eosinophils within the airway wall are associated with features of remodeling, particularly thickening of the basement membrane.¹⁵ Patients with severe eosinophilic asthma have narrowing of the airway lumen of proximal conducting airways (secondary to thickening of the airway wall) that correlates with the degree of airway hyperresponsiveness and is absent in patients with nonasthmatic eosinophilic bronchitis.³⁴ Targeting eosinophils in sputum with anti-IL-5 increased luminal patency when compared with placebo.⁷ Therefore eosinophilic disease might modulate lung function decline through a number of discrete mechanisms, including airway wall remodeling, altered airway compliance, and mucus hypersecretion.

Our study has a number of methodological limitations. First, a number of patients did not attend their regularly scheduled 3-month clinical visits over the 5-year lung function decline period. To account for this, we chose linear mixed modeling, which is suitable for irregularly spaced data with static and time-varying covariates and missing data. Linear mixed models are a recognized statistical technique to account for data that have this structure without resorting to imputation of missing data that could compromise data integrity and would be difficult to perform in a study of this nature.

Second, we had a small sample size and as such were not powered to study the role of multiple interactions of fixed effects on lung function decline trajectories, although no clear guidance exists for sample size calculations in linear mixed modeling studies.

Third, we did not record adherence at clinic visits, making it difficult to assess whether the variance of eosinophilic inflammation in cluster 2 was a real biological effect or related to intermittent treatment use. In support of the former hypothesis, cluster 1 was similar demographically to cluster 2 and did not exhibit the typical profile of early-onset atopic asthma associated with suboptimal treatment adherence. However, future studies that evaluate variability in inflammation over time will need to record adherence to therapy (either through evaluation of prescription pick-up records or exhaled breath biomarkers)³⁵ and potentially account for adherence as a fixed effect in the modeling to fully interpret the data.

Fourth, our postbronchodilator FEV₁ measurements at baseline and serially were not recorded after a period of intensive oral anti-inflammatory therapy to identify maximal postbronchodilator FEV₁. As such, the FEV₁ measurements might not have truly been reflective of lung structural changes underpinning airflow obstruction in asthmatic patients. However, our aim was to evaluate the relationship between that natural variation in eosinophilic disease over time, and as such, normalizing eosinophils with prednisolone therapy at scheduled lung function visits would have confounded our measurements. Therefore our approach was a real-world observational approach.

Fifth, we did not capture sputum eosinophils with sufficient temporal granularity to determine periodicity phenomena.

Sixth, our patients were assessed during the stable state, and the level of asthma control was matched in patients within the 2 eosinophilic clusters. Therefore it is unlikely (but not inconceivable) that patients with high eosinophilic variation had more severe disease and subsequently had further assessments. However, the median number of observations did not differ across eosinophilic clusters over the follow-up period, further supporting the validity of our observations.

Seventh, the absolute numeric value of the percentage of sputum eosinophils might be highly misleading with respect to lung damage in asthmatic patients. Specifically, the absolute value does not reflect (1) the state of activation of eosinophils or (2) the rate of resolution of eosinophilic inflammation; high values in sputum might simply reflect low tissue retention. Indeed, we have recently demonstrated that obese asthmatic patients have high levels of tissue eosinophils despite low absolute sputum percentages of eosinophils.³⁶

In conclusion, for the first time, we have demonstrated that variability rather than amplitude over time of eosinophilic inflammation in sputum is associated with accelerated lung function decline in patients with severe asthma and that within this population exist discrete eosinophilic clusters that have a differential rate of decline. Further work to validate our observations in large asthmatic populations is warranted. In addition, further research is required to examine the mechanisms driving eosinophilic variation in sputum and lung function decline in our reported eosinophilic clusters.

Key messages

- The variability of eosinophilic inflammation rather than the amplitude over time is associated with accelerated lung function decline in patients with severe asthma.
- Further research is required to determine whether targeting variable eosinophilic airway inflammation in patients with severe asthma will improve patient outcomes.

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METHODS

Linear mixed modeling approaches: Background

Longitudinal observational data are associated with a number of potential limitations, including (1) irregularly spaced data (not all patients attend their regularly scheduled follow-up visits [in this case 3-month intervals]); (2) not all relevant data are captured at each visit (in this case sputum eosinophils and postbronchodilator lung function); (3) the presence of static (eg, age of onset and sex) and time-varying covariates (eg, treatment, FEV₁, and sputum eosinophil counts); and (4) different levels of data at the population and patient levels. LME models are an extension of linear regression models, which are useful in evaluating irregularly spaced and timed data, as is often the case in “real-world longitudinal observational” studies. LME models are characterized by both fixed effects, which are constant over time for individual patients, and random effects, which change over time. The concept is illustrated for FEV₁ decrease in Fig E1.

Table E1 summarizes the iterative approaches to model FEV₁ decrease in our population. This approach maximized the information content of the lung function decline model by iteratively fitting fixed effects that might influence lung function decline and random effects that we wanted to study. The final lung function decline model (model 30 in Table E1) is outlined below.

Lung function decline model 30: Numeric algebraic expression of the final postbronchodilator FEV₁ lung function decline model

In the model below, b_{0i} , b_{1i} (time), and b_{2i} (sputum eosinophil percentage) are random effects, and sputum eosinophil percentage, time, height, sex, exacerbations, and age of onset are fixed effects. The expression below therefore conforms to the general expression for an LME model, as outlined in Fig E1, B.

$$\begin{aligned} \text{Post bronchodilator FEV1}_i \approx & (-5.19 + b_{0i}) + (-0.0258 + b_{1i}) \cdot \text{time} \\ & + (-0.0814 + b_{2i}) \cdot \log_{10} \text{Eosinophil\%} + 0.0509 \cdot \text{height} \\ & + 0.397 \cdot \text{sex} (\text{male} = 1, \text{female} = 0) - 0.0261 \cdot \text{exacerbations} \\ & + 0.0121 \cdot \text{age of onset} + \varepsilon_i \end{aligned}$$

$$b_{0i} \sim N(0, 0.6124)$$

$$b_{1i} \sim N(0, 0.0622)$$

$$b_{2i} \sim N(0, 0.116)$$

$$\varepsilon_i \sim N(0, 0.207)$$

where $N(\mu, \sigma)$ is the normal distribution with mean μ and SD σ .

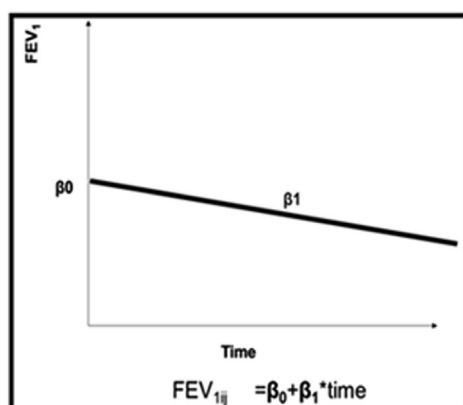
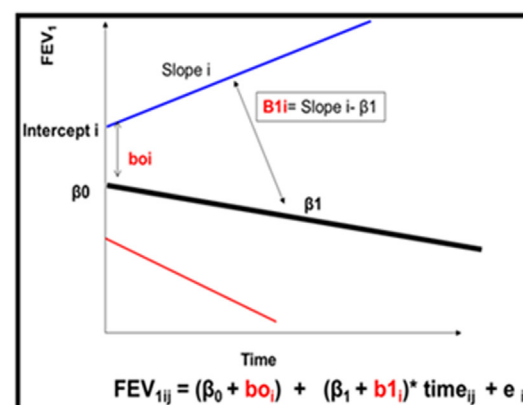
A Standard linear regression**B Linear mixed effects model**

FIG E1. Linear mixed models for FEV₁ decrease. **A**, Standard expression for a simple linear regression. The FEV₁ for a particular patient (*i*) at the time point (*j*) is equal to the intercept of the population (β_0) plus the gradient of the population (β_1) times time. **B**, The linear mixed model extends the simple regression, such that individual patients might have their own lung function decline trajectories. In this figure there are 2 patients (blue and red) who have distinct lung function decline trajectories. In this case the lung function decline for the patient in blue is accounted for by the intercept of the blue patient (b_{0i}), the difference in the slope of the blue patient from the population slope term (b_{1i}), and an error term (*e*). The population terms (β_0 and β_1) are known as fixed effects, and the individual slope and intercept terms (b_{1i} and b_{0i}) are known as random effects. Random effects are usually assigned to time-varying variables of interest.

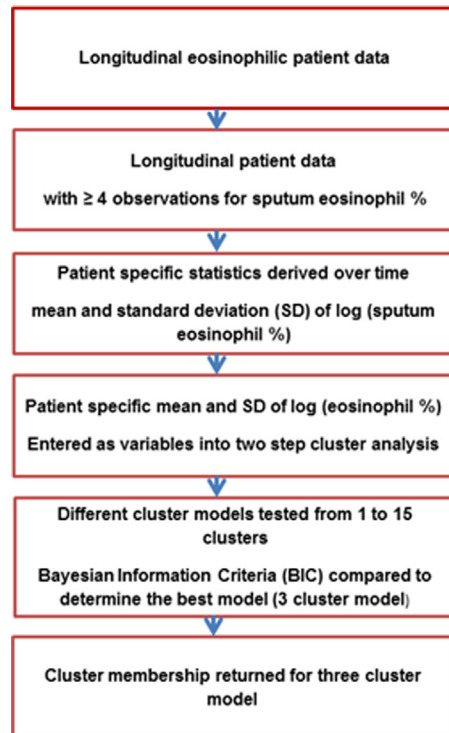


FIG E2. Step-by-step algorithms for 2-way cluster analysis.

TABLE E1. Iterations for mixed effects with postbronchodilator FEV₁

Model	Fixed effects	Random effects	AIC	BIC	Log Lik	Value of decline	P value
1	1	1	471	485	−233	NA	NA
2	1 + time	1	457	476	−225	−0.03043	<.001
3	1	1 + time	393	416	−192	NA	<.001
4	1 + time	1 + time	394	422	−191	−0.03160	.0023
5	1 + time + BDP	1 + time	417	449	−201	−0.03147	.0026
6	1 + time + BDP + BDP*time	1 + time	439	476	−212	−0.04677	.0064
7	1 + time + logeos%	1 + time	367	400	−176	−0.02978	.0027
8	1 + time + logeos% + BDP	1 + time	390	427	−187	−0.02990	.0028
9	1 + time + logeos% + time*logeos%	1 + time	377	414	−181	−0.02853	.0084
10	1 + time + neut%	1 + time	403	436	−195	−0.03177	.0019
11	1 + time + neut% + BDP	1 + time	426	463	−205	−0.03169	.0021
12	1 + time + neut% + time*neut%	1 + time	419	456	−201	−0.01813	.2727
13	1 + time + neut% + logeos	1 + time	383	420	−183	−0.02965	.0029
14	1 + time + neut% + logeos + BDP	1 + time	405	447	−194	−0.02977	.0030
15	1 + time + logeos + BDP + age of onset	1 + time	416	463	−198	−0.02977	.0030
16	1 + time + logeos + BDP + age of onset + height	1 + time	403	453	−190	−0.02995	.0027
17	1 + time + logeos + BDP + age of onset + height + sex	1 + time	392	448	−184	−0.0299	.0027
18	1 + time + logeos + BDP + age of onset + height + sex + atopy	1 + time	396	455	−185	−0.0300	.0026
19	1 + time + logeos + BDP + age of onset + height + sex + aspergillus	1 + time	395	455	−185	−0.02964	.0029
20	1 + time + logeos + BDP + age of onset + height + sex + exacerbations	1 + time	392	452	−183	−0.02733	.0057
21	1 + time + logeos + BDP + age of onset + height + sex + exacerbations + atopy	1 + time	395	460	−183	−0.02748	.0054
22	1 + time + logeos + BDP + age of onset + height + sex + exacerbations + atopy	1 + time + logspeos	383	462	−175	−0.02519	.0074
23	1 + time + logeos + BDP + age of onset + height + sex + exacerbations + atopy	1 + time + BDP	395	473	−180	−0.02818	.0043
24	1 + time + logeos + BDP + age of onset + height + sex + exacerbations + atopy	1 + time + logeos + BDP	385	482	−172	−0.02607	.0049
25	1 + time + neut% + BDP + age of onset + height + sex + exacerbations + atopy	1 + time + neut%	394	473	−180	−0.026923	.0085
26	1 + time + logeos + neut% + BDP + age of onset + height + sex + exacerbations + atopy	1 + time	395	460	183	−0.02748	.0054
27	1 + time + logeos + neut% + BDP + age of onset + height + sex + exacerbations + atopy	1 + time + logspeos	383	462	−175	−0.02519	.0074
28	1 + time + logeos + neut% + BDP + age of onset + height + sex + exacerbations + atopy	1 + time + logeos + neut%	NA	NA	NA	NA	NA
29	1 + time + logeos + age of onset + height + sex + exacerbations + atopy	1 + time + logspeos	360	434	−164	−0.02500	.0075
30	1 + time + logeos + age of onset + height + sex + exacerbation + age	1 + time + logspeos	334	403	−152	−0.0257	.0059

Final iterative model reporting the association of sputum eosinophils with post bronchodilator FEV₁% predicted is indicated with boldface text.

AIC, Akaike information criteria; BDP, beclomethasone dipropionate; Log Lik, Log Likelihood method; NA, not applicable.

TABLE E2. Fixed effects for final mixed-effects model on FEV₁ decrease for eosinophilic clusters

Fixed effects	Value	SE	P value
Intercept	2.5777	0.1648	<.0001
Time	−0.04086	0.009439	<.0001
(Cluster 2)	−0.4213	0.2192	.0580
(Cluster 3)	−0.3549	0.2134	.1001
Time*(Cluster 2)	0.02168	0.01224	.0768
Time*(Cluster 3)	0.02684	0.01222	.0282

Equation E2. Numeric algebraic expression of the eosinophilic cluster lung function decline model

For cluster 1:

*Post bronchodilator FEV*_{1*i*} $\approx 2.58 + (-0.014).time$

For cluster 2:

*Post bronchodilator FEV*_{1*i*} $\approx 2.16 + (-0.0409).time$

For cluster 3:

*Post bronchodilator FEV*_{1*i*} $\approx 2.22 + (-0.0192).time$