

Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis

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Background: Clinical cluster analysis from the Severe Asthma Research Program (SARP) identified 5 asthma subphenotypes that represent the severity spectrum of early-onset allergic asthma, late-onset severe asthma, and severe asthma with chronic obstructive pulmonary disease characteristics. Analysis of induced sputum from a subset of SARP subjects showed 4 sputum inflammatory cellular patterns. Subjects with concurrent increases in eosinophil ($\geq 2\%$) and neutrophil ($\geq 40\%$) percentages had characteristics of very severe asthma. **Objective:** To better understand interactions between inflammation and clinical subphenotypes, we integrated inflammatory cellular measures and clinical variables in a new cluster analysis.

Methods: Participants in SARP who underwent sputum induction at 3 clinical sites were included in this analysis

($n = 423$). Fifteen variables, including clinical characteristics and blood and sputum inflammatory cell assessments, were selected using factor analysis for unsupervised cluster analysis. **Results:** Four phenotypic clusters were identified. Cluster A ($n = 132$) and B ($n = 127$) subjects had mild-to-moderate early-onset allergic asthma with paucigranulocytic or eosinophilic sputum inflammatory cell patterns. In contrast, these inflammatory patterns were present in only 7% of cluster C ($n = 117$) and D ($n = 47$) subjects who had moderate-to-severe asthma with frequent health care use despite treatment with high doses of inhaled or oral corticosteroids and, in cluster D, reduced lung function. The majority of these subjects ($>83\%$) had sputum neutrophilia either alone or with concurrent sputum eosinophilia. Baseline lung function and sputum neutrophil percentages were the most important variables determining cluster assignment.

Conclusion: This multivariate approach identified 4 asthma subphenotypes representing the severity spectrum from mild-to-moderate allergic asthma with minimal or eosinophil-predominant sputum inflammation to moderate-to-severe asthma with neutrophil-predominant or mixed granulocytic inflammation. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Severe Asthma Research Program, severe asthma, sputum, eosinophils, neutrophils, phenotype, cluster analysis

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Understanding asthma heterogeneity and phenotypic subpopulations is important in defining the spectrum of asthma severity. Guidelines have used approaches that rely on descriptive characteristics to classify asthma severity and patient disease risk primarily by using current therapeutic requirements and symptom assessment.¹⁻⁴ Cluster analysis, an unsupervised statistical approach in which individual subjects are grouped based on multiple similarities in clinical or biologic measures without *a priori* hypotheses, has been used to characterize asthma severity.⁵⁻¹² Although these analyses have described new clinical and pathobiologic asthma phenotypes, none have included multivariate analyses of both clinical and multiple biologic measures in a single-cluster analysis on a large number of subjects who have been characterized comprehensively by using standardized protocols.

A previous cluster analysis performed on a large cohort of subjects from the National Heart, Lung, and Blood Institute's Severe Asthma Research Program (SARP) included 34 clinical variables derived from comprehensive clinical phenotyping.⁷ This analysis defined clinically relevant asthmatic subpopulations that include an increasingly severe spectrum of early-onset allergic asthma, a late-onset severe asthma subpopulation, and a subgroup of subjects with severe asthma with characteristics

Abbreviations used

ATS: American Thoracic Society
 BMI: Body mass index
 FVC: Forced vital capacity
 HCU: Health care utilization
 ICS: Inhaled corticosteroid
 OCS: Oral corticosteroid
 SARP: Severe Asthma Research Program

of chronic obstructive pulmonary disease despite being non-smokers. However, at the time of the clinical cluster analysis, the subset of SARP subjects who had assessments of inflammatory cellular measures was limited, and thus these variables were not included in the analysis. Consequentially, the clinical asthma phenotypes described could not be associated with any pathobiologic mechanism. Blood and sputum inflammatory cell counts are now available on a large subset of SARP subjects, allowing inclusion of these inflammatory assessments with clinical variables in a new cluster analysis. We hypothesized that integration of pathobiologic measures and subjects' characteristics in a single multivariate analysis would lead to the identification of groups of subjects who share not only clinical phenotypic traits but also similar pathobiologic mechanisms that might determine these phenotypes.

To accomplish this goal, we analyzed the subjects in SARP with complete clinical characterizations, including blood and induced sputum cellular assessments. These subjects represent a broad spectrum of asthma severity but are enriched for severe asthma, as defined by the American Thoracic Society (ATS) workshop on refractory asthma.³ Application of factor analysis and clustering approaches to this group of SARP subjects identified 4 asthma subphenotypes with different clinical characteristics and inflammatory cell patterns.

METHODS

SARP

After establishing standard operating procedures, including a review by an independent data safety monitoring board and approval by the institutional review boards of all sites, study participants underwent comprehensive phenotypic characterization, as previously described.⁴ Briefly, 2 groups of nonsmoking asthmatic subjects (<5 pack years of tobacco use) were recruited. Those with severe asthma met the ATS definition of severe asthma.⁵ A second group of subjects with mild-to-moderate asthma who did not meet these severity criteria were recruited as a comparator group. Thus the phenotyped SARP subjects included a spectrum of mild-to-severe asthma. After informed consent, clinical staff administered questionnaires that assessed demographic information, asthma symptoms and medication use, medical history, and health care utilization (HCU). Physiologic testing of lung function included "baseline" prebronchodilator spirometry with defined withholding of appropriate medications before testing, maximal bronchodilation to assess postbronchodilator lung function (using 6-8 puffs of short-acting β -agonists), and bronchial hyperresponsiveness to methacholine in subjects with an FEV₁ of greater than 50% of predicted value before inhalational challenge. Atopy was assessed by means of skin prick testing and measurement of serum total IgE levels and blood eosinophil percentages. Exhaled nitric oxide levels were measured by using ATS-approved online devices at a constant flow rate. Sputum was induced in SARP by using the methodology used by other National Heart, Lung, and Blood Institute-sponsored asthma networks, as previously described.¹³⁻¹⁵ In brief, an ultrasonic nebulizer with 3% saline was used to induce sputum. Samples were processed by using the whole sputum method. All slides were overread by an independent investigator at Wake Forest (>500 cells per slide); slides with

greater than 80% squamous cells were deemed inadequate for analysis. Subjects with an FEV₁ of less than 45% of predicted value after albuterol administration did not undergo sputum induction, but a spontaneous sputum sample was collected when possible. Only subjects with complete blood and sputum cell counts were used for this analysis.

Cluster analyses

Orthogonal varimax factor analysis was performed to select variables for cluster analysis. This analysis identified 15 variables that included baseline and maximal FEV₁ percent predicted values, eosinophil and neutrophil percentages in blood and sputum, sex, race, age, age of onset, asthma duration, body mass index (BMI), and the 3 previously described composite variables (ie, total number of asthma controllers, dose of corticosteroids, and HCU in the past year⁷). The Ward minimum-variance hierarchical clustering method was performed by using an agglomerative (bottom-up) approach and Ward linkage (see the [Methods](#) section in this article's Online Repository at www.jacionline.org for additional statistical methods). At each generation of clusters, samples were merged into larger clusters to minimize the within-cluster sum of squares or maximize the between-cluster sum of squares. Stepwise discriminant analysis was used to identify the most influential variables in cluster assignment. ANOVA, Kruskal-Wallis, and χ^2 tests were used for parametric continuous, nonparametric continuous, and categorical variables, respectively, to compare differences between clusters.

RESULTS

Subject selection

Four hundred twenty-three subjects in SARP underwent sputum induction and had complete data for analysis (244 subjects from Wake Forest University, 103 subjects from the University of Wisconsin, and 76 subjects from the University of Pittsburgh). A comparison of the demographics and baseline clinical characteristics of this subset of subjects with those of the larger SARP cohort used in the previous cluster analysis is shown in [Table E1](#) in this article's Online Repository at www.jacionline.org.⁷ There are no significant differences in age, sex, race, age of asthma onset, or disease duration between the subjects with nonsevere and those with severe asthma in these 2 cohorts. Both the nonsevere and severe asthma groups in the current analysis had a slightly higher mean BMI when compared with the larger cohort ($P < .02$). Subjects with severe asthma in the sputum subset had higher baseline FEV₁ percent predicted values than subjects with severe asthma in the larger cohort (mean, 68% vs 62%; $P = .007$) because of exclusion of subjects with low lung function from sputum induction. The severe asthma group in the sputum cohort reported less oral corticosteroid (OCS) use (32% vs 43% subjects, $P < .0001$) and fewer hospitalizations for asthma exacerbations in the past year (18% vs 31%, $P = .007$). This group was more atopic than the severe asthma group in the larger cohort, with 80% of subjects with severe asthma having 1 of more positive skin prick test responses (80% vs 67%, $P = .009$).

Selection of variables

Factor analysis identified 7 factors that accounted for 70% of variance in the analysis (see [Table E2](#) in this article's Online Repository at www.jacionline.org). The most influential factors (factors 1-3) were characterized by highly correlated lung function parameters and measures of asthma control (medication use and HCU). Inflammatory cell measures correlated in blood (factor 4)

TABLE I. Important variables in cluster analysis by stepwise discriminant analysis

Step	Variable	Partial R^2	F value	P value
1	Baseline FEV ₁ (% predicted)*	0.59	201.22	<.0001
2	Sputum neutrophils (%)	0.52	151.58	<.0001
3	Current age (y)	0.16	27.01	<.0001
4	Maximal FEV ₁ (% predicted)†	0.07	9.75	<.0001
5	Asthma duration (y)	0.03	4.36	.005
6	Sputum eosinophils (%)	0.04	5.82	.0007
7	BMI	0.03	4.58	.004
8	No. of asthma controllers‡	0.02	3.17	.02

*Prebronchodilator values with a greater than 6-hour withhold of bronchodilators.

†Postbronchodilator values after 6 to 8 puffs of albuterol.

‡Controllers include leukotriene modifiers, ICSs, long-acting β -agonists, theophyllines, OCSs, and omalizumab.

or sputum (factor 7); however, they did not correlate with each other.

Cluster analysis

A dendrogram was generated, and 4 groups of subjects were identified (see Fig E1 in this article's Online Repository at www.jacionline.org). Nearly 90% of the subjects were equally distributed between clusters A, B, and C, whereas the remaining subjects (11%) were assigned to cluster D. Subjects classified as having severe asthma based on the ATS workshop on refractory asthma were found in all 4 of the inflammatory clusters.³ By using discriminant analysis, the 2 most influential variables for cluster assignment were baseline FEV₁ percent predicted values ($F = 201$, $P < .0001$) and sputum neutrophil percentages ($F = 151$, $P < .0001$, Table I).

Inflammatory cell patterns in the 4 clusters

Although there were differences in blood eosinophil percentages ($P = .03$) and blood neutrophil percentages ($P = .055$) between the clusters (see Fig E2 in this article's Online Repository at www.jacionline.org), differences in sputum eosinophil and neutrophil percentages were more significant ($P = .001$ and $P < .0001$, respectively; Fig 1), and the latter had greater influence on cluster assignment (Table I). To better define patterns of granulocytes in sputum, subjects were classified into 4 inflammatory subgroups based on whether the eosinophil and neutrophil percentages were greater than or less than the median for these measures based on our prior publications (median eosinophil percentage, 2%; median neutrophil percentage, 40%).^{13,15} The most frequent inflammatory pattern in subjects from clusters A and B was a low or paucigranulocytic sputum cell pattern (<2% eosinophils and <40% neutrophils, Fig 2). An eosinophil-predominant pattern ($\geq 2\%$ eosinophils and <40% neutrophils) was most frequent in cluster B (41% of subjects). In contrast, the paucigranulocytic and eosinophil-predominant patterns collectively are present in only 7% of subjects in clusters C and D. The majority of subjects in these clusters have increased sputum neutrophil percentages (97% and 83% of subjects in clusters C and D, respectively), with some subjects showing concurrent increases in sputum eosinophil percentages. Taken together, there is a marked difference in cellular patterns between clusters A and B compared with clusters C and D, in which a neutrophil-predominant pattern (63% and 38%, respectively) or a mixed granulocytic pattern

(34% and 45%, respectively) was present in the majority of subjects.

Clinical characteristics of the 4 clusters

Cluster A. The youngest subjects with the lowest BMI are in cluster A, which consists of a greater proportion of white women than the other clusters (Table II). Two thirds of these subjects have early-onset asthma before the age of 12 years, and nearly 90% are allergic to common aeroallergens, as determined by using skin prick testing. All subjects in cluster A had normal lung function (baseline FEV₁ percent predicted: mean, 97%; range, 91% to 105%; postbronchodilator FEV₁ percent predicted: mean, 107%; range, 99% to 113% [25% to 75% interquartile range]). Half of these subjects were treated with inhaled corticosteroids (ICSs), and a few ($n = 26$) required higher doses of ICS or OCSs (Table III). HCU was less frequent in this cluster, although 13% of subjects reported treatment with OCSs or evaluation in the emergency department for an asthma exacerbation in the past year. One third of the subjects reported recurrent upper and lower respiratory tract infections, but a similar frequency was observed in the other subject clusters (Table III).

Cluster B. Subjects in cluster B are slightly older, almost half are obese (BMI, >30 kg/m²), and one third are African American. Similar to cluster A, most subjects had early-onset allergic asthma, but disease duration is longer because subjects are currently older. Cluster B subjects had mildly decreased baseline lung function (baseline FEV₁ percent predicted: mean, 73%; range, 64% to 82%) but reverse to normal or near-normal lung function after maximal bronchodilation (postbronchodilator FEV₁ percent predicted: mean, 86%; range, 78% to 95%). The use of corticosteroids was more frequent in this group, and one third required treatment with high doses of ICSs (26%), OCSs (6%), or both. HCU in the past year is similar to that seen in cluster A, but 41% of cluster B subjects report a prior history of a hospitalization for asthma "ever," 15% of these in the intensive care unit.

Cluster C. Cluster C subjects are older and have the highest BMI (half with BMI, >30 kg/m²), and 38% are classified as having severe asthma (Table II). Half of the subjects reported the onset of asthma after the age of 12 years, although most are atopic based on skin prick test responses. Lung function was similar to that seen in cluster B (baseline FEV₁ percent predicted: mean, 76%; range, 66% to 84%; postbronchodilator FEV₁ percent predicted: mean, 88%; range, 79% to 97%). In contrast to cluster B, however, 41% of subjects in cluster C were being treated with high doses of ICSs, and 16% were being treated with concurrent OCSs ($P = .02$ compared with cluster B). Despite this therapeutic requirement for high-dose corticosteroid treatment, HCU in the past year was not different between clusters B and C, although historical HCU ("ever") was higher for cluster C ($P = .04$). Although subjects in this cluster reported a similar frequency of sinopulmonary infections, medical comorbidities, such as gastroesophageal reflux disease (37%), hypertension (28%), and diabetes (9%), are more frequent in the older subjects in this cluster compared with clusters A and B (Table III).

Cluster D. Subjects in the smallest cluster are clinically different from those in the previous clusters. They are the oldest subjects with a higher proportion of men, the majority of the subjects are white, and most are obese, with a BMI of greater than 30 kg/m² (57%, Table II). Two thirds of the subjects in cluster D

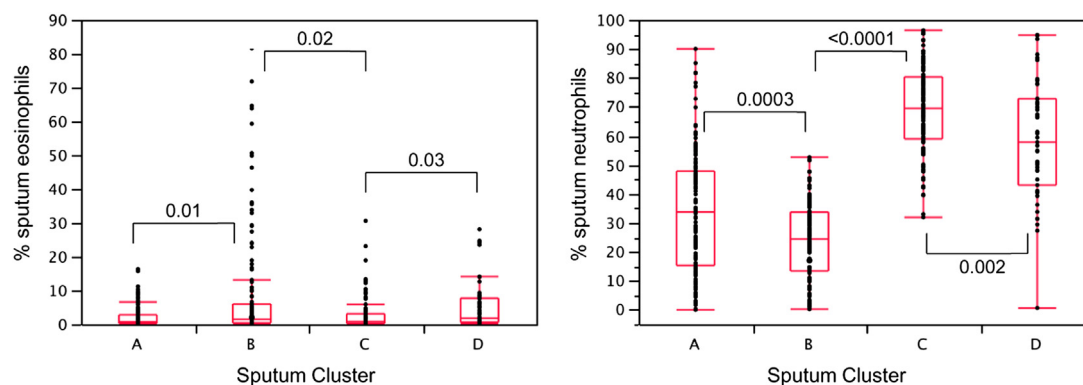


FIG 1. Sputum inflammatory cells. Shown are box plots of eosinophil and neutrophil percentages from sputum specimens. There was a wide range of values for both cell types in all sputum clusters. There are statistically significant differences among the 4 groups ($P = .001$ for eosinophils and $P < .0001$ for neutrophils), with differentiation between clusters in contrast to blood inflammatory cells (see Fig E2). P values shown on the graph are paired comparisons between 2 clusters of similar disease severity.

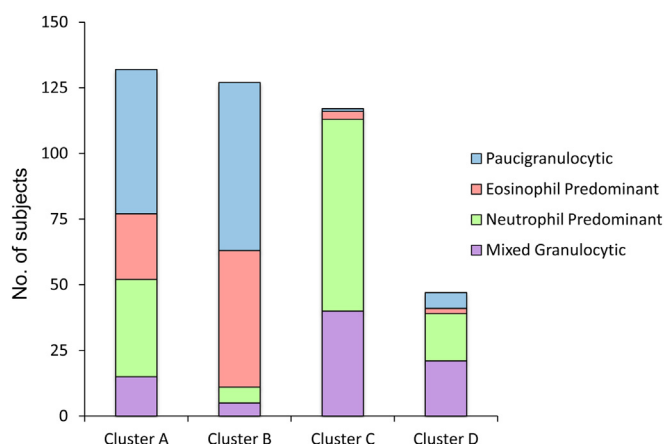


FIG 2. Sputum granulocyte patterns in the 4 clusters. Subjects were assigned to one of 4 possible patterns based on sputum eosinophil percentages ($<2\%$ or $\geq 2\%$) and sputum neutrophil percentages ($<40\%$ or $\geq 40\%$). All 4 granulocyte patterns are present in every cluster, but in general, as asthma severity increases (clusters C and D), sputum neutrophil percentages increase in nearly all subjects and sputum eosinophil percentages persist in a third to half of these subjects, despite increasing doses of ICSs, OCSs, or both.

have severe asthma. Onset of asthma after the age of 12 years (later-onset asthma) is more common. Despite later onset of disease, measures of atopy (IgE levels and skin prick test results) were increased in most subjects. Lung function is markedly reduced, and although there is reversibility to short-acting β -agonists, there is persistent airflow obstruction after acute maximal bronchodilation (baseline FEV₁ percent predicted: mean, 47%; range, 36% to 57%; postbronchodilator FEV₁ percent predicted: mean, 61%; range, 50% to 71%). This group of subjects reported complex medication regimens, 66% required treatment with high doses of ICS, 26% required daily OCSs, and 70% were treated with at least 1 additional asthma controller medication (Table III). Urgent HCU was frequent, with 17% of subjects hospitalized for an asthma exacerbation in the past year and more than two thirds reporting a history of pneumonia in the past. Comorbidities were frequent in cluster D subjects (44% with gastroesophageal reflux disease, 37% with hypertension, 9% with diabetes, and 11% with osteoporosis).

DISCUSSION

The original SARP clinical cluster analysis identified 5 asthma phenotypes that differed in phenotypic characteristics, medication use, HCU, lung function, and responsiveness to bronchodilators.⁷ The current analysis integrates measures of cellular inflammation, clinical characteristics, and physiologic variables in a new cluster analysis on 423 subjects who had both blood and induced sputum cell differentials performed at 3 SARP clinical centers. Using a similar clustering approach, we identified 4 novel asthma subpopulations differentiated by inflammatory cell phenotypic patterns and clinical characteristics.

These 4 clusters represent the range of asthma severity, from mild allergic asthma with normal lung function, limited medication requirements, rare HCU, and minimal airway inflammation in cluster A to progressively more severe asthma. Cluster D was characterized by decreased lung function, high medication requirements and frequent HCU, medical comorbidities, and complex airway inflammation. Clusters A and D represent the ends of this spectrum; they are expectedly very different from each another. In contrast, clusters B and C represent the midrange of clinical asthma severity, with similar levels of lung function and HCU but a more frequent requirement for treatment with higher ICS or OCS regimens in cluster C subjects to achieve similar levels of asthma control. Although subjects in clusters B and C might appear clinically similar, there is a marked shift in airway cellular inflammation from minimal or eosinophil-predominant airway inflammation to neutrophil-predominant or mixed granulocytic patterns in the latter cluster. Clusters B and C illustrate the importance of integrating clinical disease characteristics and inflammatory cell measures into a single multivariate analysis not only to identify novel subphenotypes but also to understand underlying pathophysiologic mechanisms. It is important that in this multivariate approach no single biomarker or cell type delineated individual cluster groups. Measurements of exhaled nitric oxide and the frequency of atopy (assessed by means of skin prick testing) did not differ among the 4 clusters.

Increased sputum eosinophil percentages have been described in subjects with asthma of all severity and are associated with decreased asthma control and exacerbations in some asthmatic subjects.¹⁶ In general, treatment with ICSs decreases sputum eosinophilia and improves asthma control in most subjects.^{14,17-19}

TABLE II. Demographics and clinical characteristics of subjects in clusters

No. of subjects	Cluster A 132	Cluster B 127	Cluster C 117	Cluster D 47	P value
Age at enrollment (y)	27 (10)	35 (11)	42 (14)	50 (10)	<.0001
Sex (% female)	73	61	62	43	.003
Race (% white)	70	58	62	79	.03
BMI (kg/m ²)	28 (6)	31 (9)	32 (10)	31 (6)	.0001
BMI >30 kg/m ² (%)	28	46	50	57	<.0001
Age of asthma onset (y)	12 (13)	12 (12)	17 (16)	22 (18)	.001
Onset ≥12 y (%)	37	41	52	60	.01
Asthma duration (y)	15 (8)	23 (13)	24 (14)	28 (18)	<.0001
Subjects with severe asthma (%)	15	24	38	66	<.0001
Baseline lung function*					
FEV ₁ (% predicted)	97 (10)	73 (13)	76 (13)	47 (15)	<.0001
FVC (% predicted)	104 (11)	87 (12)	87 (12)	65 (14)	<.0001
FEV ₁ /FVC ratio	0.80 (0.1)	0.70 (0.1)	0.71 (0.1)	0.59 (0.1)	<.0001
Postbronchodilator lung function†					
FEV ₁ (% predicted)	107 (10)	86 (11)	88 (12)	61 (14)	<.0001
FVC (% predicted)	108 (12)	96 (13)	96 (11)	79 (15)	<.0001
Change in percent predicted FEV ₁	12 (13)	20 (20)	19 (23)	31 (28)	<.0001
PC ₂₀ methacholine (mg/mL)‡§	1.66 (0.7)	0.89 (0.8)	1.07 (0.7)	0.93 (0.5)	.02
Atopy status					
Total serum IgE (IU/mL)‡	120 (0.6)	178 (0.6)	135 (0.6)	118 (0.7)	.09
No. of positive SPT responses	4.0 (2.9)	4.8 (3.2)	4.2 (3.1)	4.0 (3.5)	.14
≥1 Positive SPT response (%)	85	90	83	76	.13
Exhaled nitric oxide (ppb)‡	30 (0.4)	34 (0.4)	26 (0.4)	32 (0.4)	.22

Numeric data are expressed as mean (SDs).

SPT, Skin prick test.

*Prebronchodilator values with a greater than 6-hour withhold of bronchodilators.

†Postbronchodilator values after 6 to 8 puffs of albuterol.

‡Geometric mean (log₁₀ SD).

§Only subjects with FEV₁ of 55% of predicted value or greater before testing underwent methacholine challenge (n = 129, 112, 104, and 11, respectively).

||P value from ANOVA or χ^2 analysis among 4 clusters.

Subjects with severe asthma and persistent sputum eosinophilia despite chronic use of high doses of ICSs or OCSs might represent a specific eosinophilic subpopulation.^{13,15,18,20} The increasing frequency of an eosinophilic inflammatory phenotype in cluster B, a group with fair asthma control (normal lung function and low HCU requiring treatment with ICSs [64% subjects]) supports the importance of eosinophils as one pathobiologic biomarker in these subjects with mild-to-moderate asthma. In contrast, the increasing frequency of a mixed granulocytic pattern of airway cellular inflammation despite higher corticosteroid doses in cluster D subjects suggests complex biologic mechanisms that affect asthma severity. These findings imply that a single biomarker alone is unlikely to be sufficient to accurately subclassify these groups with more severe asthma.

There are numerous reports of “noneosinophilic” asthma based on induced sputum, but it is difficult to ascertain from these studies whether these subjects had primarily paucigranulocytic or predominantly neutrophilic airway inflammation. The finding of sputum neutrophilia in asthmatic patients has led to a number of clinical trials with antibiotics (macrolide), antifungals (itraconazole), or specific antineutrophilic compounds, such as CXCR2 or anti-TNF immunomodulators, targeting innate immunity pathways in addition to traditional allergic or eosinophilic pathobiologic mechanisms.²¹⁻²⁶ In the current study the neutrophil-predominant and mixed granulocytic patterns were the most frequent patterns observed in the severe asthma clusters. It is interesting that there was a relatively high frequency of atopy in these clusters, suggesting that the presence of atopy does

not exclude a neutrophilic phenotype in subjects with severe asthma.

Cluster C and D subjects were treated with the highest doses of ICSs or OCSs, and it has been suggested that sputum neutrophils might represent a specific biologic “side effect” of exposure to high doses of corticosteroids.¹⁷ Our group has previously published a factor analysis in which corticosteroid use was not associated with either sputum eosinophil or neutrophil percentages, suggesting minimal effects of continuous corticosteroid exposure on sputum granulocytes.^{13,15} Analysis of sputum and blood neutrophil percentages in subjects with severe asthma in the current study stratified by the intensity of corticosteroid exposure also did not show statistical differences in sputum neutrophilia between subjects receiving high-dose ICSs or chronic OCSs.

It has also been suggested that the presence of sputum neutrophils in asthmatic subjects is caused by concurrent chronic sinopulmonary infections, especially in patients with severe disease with poor baseline lung function. In the current study subjects were required to be free from respiratory tract infectious symptoms for at least 4 weeks before sputum induction and thus did not have an acute sinopulmonary infection. In addition, there was no difference in subject-reported history of a diagnosis of sinusitis or bronchitis among the clusters, implying that there was not an increased frequency of airway infections in the more severe clusters (C and D). More cluster D subjects did report a prior history of pneumonia, however, which is consistent with our previous publication showing a relationship between decreased pulmonary function and serious lung infections in the past.⁴

TABLE III. Medication use, HCU, and comorbidities

No. of subjects	Cluster A 132	Cluster B 127	Cluster C 117	Cluster D 47	P value‡
Corticosteroid use (%)					<.0001§
None	45	36	37	11	
Low- to moderate-dose ICS	39	37	22	23	
High-dose ICS*	16	26	41	66	
OCS or systemic corticosteroids*	4	6	16	26	
≥2 Controllers† (%)	40	43	47	70	.008§
HCU, past year (%)					.007§
Emergency department visit	20	18	24	19	
≥3 OCS bursts	13	13	18	28	
Hospitalized for asthma	5	4	9	17	
Hospitalized in ICU	2	4	3	2	
HCU, ever (%)					
Emergency department visit	64	65	79	68	.07
Hospitalized for asthma	28	41	55	64	<.0001
Hospitalized in ICU	6	15	22	34	<.0001
History of sinopulmonary infections (%)					
Pneumonia	38	45	44	68	.007
Bronchitis	35	31	36	37	.78
Sinusitis	41	47	42	53	.44
Associated allergic diseases (%)					
Aspirin sensitivity	10	9	11	10	.98
Nasal polyps	5	11	12	21	.02
Coexisting medical problems (%)					
Gastroesophageal reflux	19	28	37	44	.003
Hypertension	8	13	28	37	<.0001
Osteoporosis	2	3	6	11	.04
Diabetes	5	3	9	9	.27

All data are presented as percentages of subjects.

ICU, Intensive care unit.

*The high-dose ICS dose is equivalent to 1000 µg or greater fluticasone propionate daily; chronic OCS represents 20 mg/d or greater or other systemic steroids in the past 3 months.

†Controllers include leukotriene modifiers, ICSs, long-acting β-agonists, theophyllines, OCSs, and omalizumab.

‡P value from χ^2 analysis among 4 clusters.

§P value from χ^2 analysis of ranked ordinal composite variables among 4 clusters.

A potential limitation to this analysis is the cross-sectional design of SARP with the collection of 1 induced sputum sample as part of the comprehensive clinical characterization. Some recent studies have shown a degree of variability in sputum inflammatory cell counts when collected at different times.^{14,27-29} It is possible that subjects could change inflammatory cell patterns, and this temporal variability could confound interpretation of inflammatory cell pattern heterogeneity. Although repeated measures of any clinical variable are invaluable, sputum induction with accurate assessment of inflammatory cells can be difficult to obtain in most health care settings, even at tertiary referral health care systems. Thus it is important to investigate the phenotypic implications of sputum inflammatory cell assessments performed during comprehensive phenotypic characterization on asthma severity and determine the operational aspects and cost-effectiveness of sputum induction in clinical settings.

In contrast, spirometry with assessment of the bronchodilator response to short-acting β-agonists can be performed easily in most clinical settings. Interestingly, lung function measures remain the most important variables for cluster assignment in both the current sputum and previous SARP clinical cluster analyses. The current analysis confirms that baseline FEV₁ percent predicted and bronchodilator reversibility are pivotal clinical variables that aid in the identification of clinical subphenotypes of asthma.

The following approach could be considered to attempt to use the results of this cluster analysis in a clinical setting. The current analysis confirms the importance of spirometry in the “real-world” clinic and advocates for the performance of prebronchodilator and postbronchodilator assessments with appropriate withholding of bronchodilator medications before testing to adequately characterize airflow limitation in these patients. In the current study the cluster D subjects with the lowest lung function and the most complex medication regimens were characterized by a mixed granulocytic (45%) or neutrophilic (38%) inflammatory cell pattern. These findings suggest that clinic patients with decreased baseline FEV₁ percent predicted values despite treatment with high-dose ICSs or OCSs and a second controller might be considered for sputum induction to guide further therapeutic options in the management of their severe disease.

In conclusion, a multivariate approach using both clinical phenotypes and inflammatory cellular information together identified 4 sputum clusters (A to D). These phenotypes represent the severity spectrum from mild-to-moderate allergic asthma with minimal or eosinophil-predominant sputum inflammation to moderate-to-severe asthma with complex neutrophil-predominant or mixed granulocytic inflammation. Sputum neutrophil percentages were the second most influential variable in this multivariate analysis, suggesting that this cell likely has an

important role in the pathobiology of patients with severe asthma, either alone or in conjunction with airways eosinophils. These findings imply that increased sputum neutrophil percentages might be a biomarker of different pathobiologic mechanisms in patients with more severe asthma, a finding that requires further investigation. As new biologic therapies are developed for the management of severe asthma, both neutrophilic and eosinophilic components of airway inflammation should be evaluated and potentially targeted by using a personalized medicine paradigm.

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Clinical implications: In this analysis severe asthma was associated with sputum neutrophilia with or without eosinophilia, suggesting that new biologic therapies for these patients might need to target both inflammatory cell types.

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METHODS

Factor analysis: Variables used in cluster analysis

Factor analysis was used to identify variables that were highly correlated to allow reduction in the number of variables. Table E2 shows the 7 independent factors identified by using orthogonal varimax rotation of the 20 initially selected variables (shown in the left column).^{E1} Four composite variables from the original clinical cluster analysis were used in this factor analysis. These composite variables were derived from questionnaire data that were transformed to capture multiple questions in a ranked ordinal scale as a method of variable reduction.^{E2} Factor 1 shows correlation between multiple lung function measures (FEV₁ and forced vital capacity [FVC], both baseline and after bronchodilators), and as such, FVC variables were eliminated from further analysis. Similarly, factor 3 shows correlation between baseline FEV₁ and baseline airflow obstruction (FEV₁/FVC ratio) and bronchodilator responsiveness (percentage change in FEV₁). Given the close association between baseline FEV₁ and the ability to respond more robustly to bronchodilators when FEV₁ is lowest, only FEV₁ was selected for the cluster analysis. Three factors (factors 2, 5, and 6) show correlations among variables that were important in the initial SARP clinical cluster analysis and thus were not eliminated. Factors 4 and 7 show correlation in blood and sputum inflammatory cell counts, respectively. The inclusion of inflammatory cell counts in this new cluster analysis was the primary purpose of the analysis, and thus despite their correlation, they were not eliminated. In summary, after elimination of redundant lung

function measures, the remaining 15 variables (marked with “†” in the left column of Table E2) were included in cluster analysis.

Cluster analysis

Ward minimum-variance hierarchical clustering method was performed by using an agglomerative (bottom-up) approach and Ward linkage (see dendrogram below).^{E3} At each generation of clusters, samples were merged into larger clusters to minimize the within-cluster sum of squares or maximize between-cluster sum of squares. Table I shows the results from stepwise discriminant analysis of the 15 variables to determine the 8 most influential variables for cluster assignment. Similar to the original SARP cluster analysis, the baseline FEV₁ percent predicted value is identified as an important variable.

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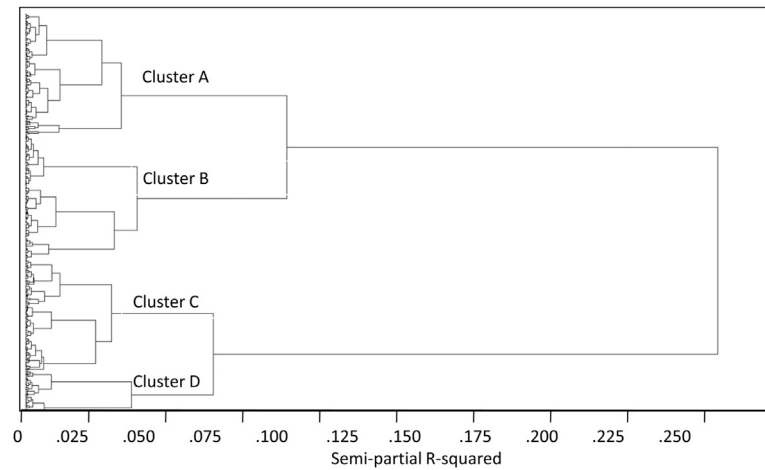


FIG E1. Dendrogram. Four hundred twenty-three subjects were clustered into a single final group. With successive clustering, 4 groups were identified.^{E3}

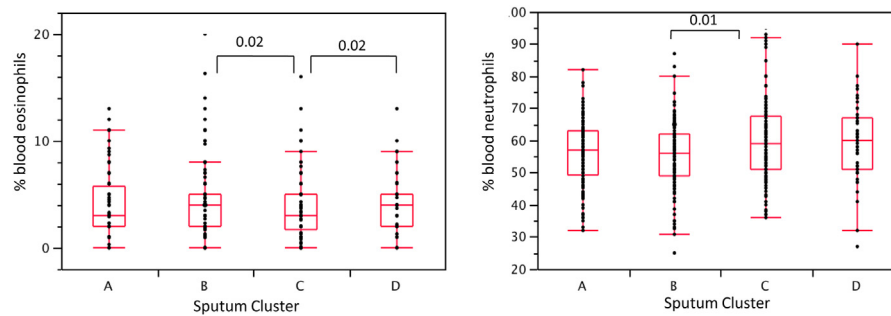


FIG E2. Blood inflammatory cells. Shown are box plots of eosinophil and neutrophil percentages from blood specimens. There was a wide range of values for both cell types in all clusters. Although there are differences among the 4 groups ($P = .055$ for eosinophils and $P = .03$ for neutrophils), there is less differentiation between clusters compared with that seen with sputum inflammatory cells (see Fig 1). P values shown on the graph are paired comparisons between 2 clusters of similar disease severity.

TABLE E1. Clinical characteristics of subjects in sputum cluster analysis compared with original clinical cluster analysis

No. of subjects	Sputum cluster analysis			Original cluster analysis			Cohort comparison, P value†	
	Not severe	Severe	P value*	Not severe	Severe	P value*	Not severe vs not severe	Severe vs severe
	297	126		422	304			
Age at enrollment (y)	34 (13)	41 (14)	<.0001	34 (12)	41 (15)	<.0001		
Sex (% female)	66%	56%	.08	69%	63%	.08		
Race (% white, African American, other)	65/26/9	66/32/2	.04	65/28/7	62/29/9	.052		
BMI (kg/m ²)	30 (8)	33 (9)	.0004	29 (8)	30 (8)	.0001	.02	.01
BMI >30 kg/m ² (%)	37	56	.0002	31	47	<.0001	.09	.07
Age of asthma onset (y)	13 (13)	17 (16)	.17	14 (13)	16 (16)	.081		
Onset ≥12 y (%)	42	52	.09	43	49	.11		
Asthma duration (y)	21 (13)	24 (14)	.06	20 (13)	25 (14)	<.0001		
Baseline lung function‡								
FEV ₁ (% predicted)	83 (16)	68 (23)	<.0001	83 (18)	62 (21)	<.0001		.007
FVC (% predicted)	92 (14)	83 (20)	<.0001	93 (16)	77 (20)	<.0001		.006
FEV ₁ /FVC ratio	0.74 (0.1)	0.66 (0.1)	<.0001	0.75 (0.1)	0.65 (0.1)	<.0001		
Maximal lung function§								
FEV ₁ (% predicted)	95 (15)	81 (21)	<.0001	95 (15)	77 (21)	<.0001		
FVC (% predicted)	99 (14)	94 (17)	<.0001	100 (14)	90 (18)	<.0001		.05
Change in percent predicted FEV ₁	15 (15)	26 (29)	.0006	14 (14)	21 (21)	<.0001		
PC ₂₀ methacholine (mg/mL)**	1.21 (0.7)	1.09 (0.9)	.93	1.26 (0.7)	0.8 (0.8)	.02		
Atopy status								
Total serum IgE (IU/mL)**¶	122 (0.8)	129 (0.8)	.33	113 (0.7)	120 (0.8)	.66		
No. of positive skin prick test responses	4.3 (3.0)	4.2 (3.5)	.45	3.9 (2.9)	2.9 (3.0)	<.0001	.04	.0002
≥1 Positive skin prick test response (%)	87	80	.10	84	67	<.0001		.009
Exhaled nitric oxide (ppb)**#	29 (0.4)	32 (0.4)	.68	29 (0.4)	27 (0.4)	.40		
Corticosteroid use (%)			<.0001			<.0001		.01
None	52	0		43	0			
High-dose ICS††	2	99		4	100			
OCS or systemic corticosteroids††	1	32		2	48			
Controller medications (%)††			.003			<.0001	.003	<.0001
None	45	7		37	0			
≥2 Controllers	33	79		47	95			
HCU, past year (%)			<.0001			<.0001		.007
Emergency department visit	8	12		10	9			
≥3 OCS bursts	6	24		8	26			
Hospitalized for asthma	3	18		2	31			

Severe asthma, as defined by the ATS workshop on refractory asthma.^{E4} Numeric data are expressed as means (SDs), except when data are expressed as **geometric mean (log₁₀ SD).

P values are from ANOVA or χ^2 analysis:

*Comparison of not severe versus severe within each cohort.

†Comparison of not severe versus not severe and severe versus severe between the 2 cohorts.

‡Prebronchodilator values with a greater than 6-hour withhold of bronchodilators.

§Postbronchodilator values after 6 to 8 puffs of albuterol.

||Only subjects with FEV₁ of 50% or greater of predicted value before testing underwent methacholine challenge.

• Sputum cluster cohort: not severe, n = 272; severe, n = 82.

• Original cluster cohort: not severe, n = 357; severe, n = 129.

¶Not all subjects had serum IgE measurements.

• Sputum cluster cohort: not severe, n = 295; severe, n = 119.

• Original cluster cohort: not severe, n = 361; severe, n = 211.

#Not all subjects had exhaled nitric oxide measurements.

• Sputum cluster cohort: not severe, n = 242; severe, n = 117.

• Original cluster cohort: not severe, n = 364; severe, n = 238.

††Medication key: The high-dose ICS dose is equivalent to 1000 μ g or greater of fluticasone propionate daily; chronic OCS represents 20 mg/d or greater of other systemic steroids in the past 3 months. Controllers include leukotriene modifiers, ICSs, long-acting β -agonists, theophyllines, OCSs, and omalizumab.

TABLE E2. Factor analysis: Orthogonal varimax rotation of 20 variables

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7
Current age [†]	−46	22	0	−10	−1	68*	2
Sex [†]	1	5	27	−39	7	8	66*
Race [†]	0	2	−3	3	0	−65*	4
BMI [†]	−48	28	22	−20	1	−22	10
Age of onset [†]	−23	16	2	−6	−82*	36	3
Asthma duration [†]	−22	5	−2	−4	87*	32	−2
Baseline lung function							
FEV ₁ (% predicted) [†]	71*	−15	61*	−9	−6	−7	12
FVC (% predicted)	90*	−9	21	−2	2	−14	4
FEV ₁ /FVC ratio	20	−20	80*	−13	−12	−11	21
Maximal lung function							
FEV ₁ (% predicted) [†]	81*	−14	41	−2	−10	−9	16
FVC (% predicted)	91*	−1	−6	3	3	−12	5
Change in percent predicted FEV ₁	−8	6	−78*	−7	−6	−13	11
Composite variables							
Corticosteroid use [†]	−13	85*	−16	1	−6	12	−9
HCU, past year [†]	−12	66*	−6	−11	−7	−11	9
HCU, ICU ever	−6	39	−31	−7	28	−19	−11
No. of controllers [†]	−5	82*	−3	2	1	21	0
Blood eosinophils (%) [†]	6	5	−14	82*	0	6	15
Blood neutrophils (%) [†]	3	19	−15	−76*	−3	22	11
Sputum eosinophils (%) [†]	2	15	−38	35	−15	14	57*
Sputum neutrophils (%) [†]	−19	16	−5	−22	6	30	−56*
Variance explained by individual factor	3.45	2.32	2.28	1.67	1.59	1.51	1.25

Varimax rotation is a transformation of the coordinates used in factor analysis that maximizes the sum of the variances of the squared loadings on factors. Orthogonal rotation makes all factors uncorrelated with one another.^{E1} Through varimax orthogonal rotation, all factors are independent of each other, and the first factor explains more variance of correlations among variables than the following factors.

ICU, Intensive care unit.

*Values shown are $\times 100$; those greater than 0.5 are marked with asterisks.

[†]Final variables used in the cluster analysis.