Heterogeneity of Paucigranulocytic Asthma: A Prospective Cohort Study with Hierarchical Cluster Analysis

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What is already known about this topic? Asthma is a heterogeneous disease that can be divided into 4 different inflammatory phenotypes, of which paucigranulocytic asthma (PGA) remains largely unexplored.

What does this article add to our knowledge? We identified 3 important clinical subtypes of PGA ("mild PGA," "PGA with psychological dysfunction and rhinoconjunctivitis and other allergic diseases," and "smoking-associated PGA"). Of them, "smoking-associated PGA" has high risk of severe exacerbation.

How does this study impact current management guidelines? Our study indicates that heterogeneity definitely exists in PGA, the subtypes of which are characterized by distinct features and are of relevance in clinical practice.

BACKGROUND: Asthma, a heterogeneous disease, can be divided into 4 inflammatory phenotypes using induced sputum cell counts—eosinophilic asthma (EA), neutrophilic asthma (NA), mixed granulocytic asthma, and paucigranulocytic asthma (PGA). Although research has focused on EA and NA, there is little known about PGA.

OBJECTIVE: To study the heterogeneity of PGA and identify possible PGA clusters to guide clinical treatment. METHODS: Patients with PGA were grouped by hierarchical cluster analysis and enrolled into a prospective cohort study to validate the clusters, relative to future risk of asthma exacerbations in a real-world setting. Clusters were validated by tree analysis in a separate population. Finally, we explored PGA stability. RESULTS: Cluster analysis of 145 patients with PGA identified 3 clusters: cluster 1 (n [110, 75.9%) was "mild PGA," cluster 2 (n [20, 13.8%) was "PGA with psychological dysfunction and rhinoconjunctivitis and other allergic diseases," and cluster 3 (n [15, 10.3%) was "smoking-associated PGA." Cluster 3 had significantly increased risk of severe exacerbation (relative risk [RR] [6.43, P[.01), emergency visit (RR [8.61, P[.03), and hospitalization (RR [12.94, P< .01). Results of the cluster analysis were successfully validated in an independent PGA population classified using decision tree analysis. Although PGA can transform into or develop from other phenotypes, 70% were stable over time. CONCLUSIONS: Among 3 identified PGA clusters, cluster 3 had a higher risk of severe exacerbation. PGA heterogeneity indicates the requirement of novel targeted interventions. (J Allergy Clin Immunol Pract 2021;9:2344-55)

Key words: Asthma; Paucigranulocytic asthma; Phenotypes; Cluster analysis; Heterogeneity

Asthma is a heterogeneous disease with various clinical phenotypes.1,2 Sputum induction, as a noninvasive technique, has been used to study the features and phenotypes of airway inflammation in asthma. According to the inflammatory cell counts in induced sputum, 4 different inflammatory phenotypes of asthma have been well recognized: eosinophilic asthma (EA), neutrophilic asthma (NA), mixed granulocytic asthma (MGA), and paucigranulocytic asthma (PGA).3 The identification of these phenotypes has been successfully used in the optimization of asthma treatment.4 In recent years, the eosinophilic

and neutrophilic phenotypes of asthma have been well characterized. Sputum eosinophilia is associated with allergic symptoms and a good response to inhaled corticosteroids (ICS)4; although neutrophils in sputum are usually related to infection (viral, bacterial) or environmental irritant exposure (such as cigarette smoking),5 they are less responsive to ICS6 and favorably respond to long-term macrolide therapy.7 However, there remains an unmet clinical need in the understanding and treatment of noneosinophilic forms of asthma,8 and specifically, PGA remains largely unexplored.

The prevalence of PGA in asthma is 31% to 51.7%, which makes it one of the most common phenotypes of asthma.3,9-11 However, its characteristics and pathobiology are not well delineated.12 Some studies suggest that PGA may represent a cross-sectional view related to a stage of disease activity or that represents the effect of successful (corticosteroid) treatment and, as such, is not a specific phenotype, but the result of successful therapeutic intervention.9,12 Other studies suggest that it may be driven by processes other than eosinophils and neutrophils, such as macrophages or mast cells.13,14 These studies indicate that PGA may itself be heterogeneous and comprise several subtypes. Recognizing the heterogeneity of PGA is essential to guide management and determine priorities for clinical care, especially regarding potentially modifiable elements or “treatable traits” that impact asthma-related outcomes.15,16 However, few studies have systematically explored the heterogeneity of PGA.

In this study, we hypothesized that there would be several clusters or subtypes of PGA based on multidimensional assessments. First, we identified these subtypes using cluster analysis in a cross-sectional study. Second, we determined the prognostic importance of these clusters in a 12-month prospective cohort study that was conducted in a real-world setting to assess whether the identified clusters would predict future risk of exacerbation and to identify clusters that were more prone to experience exacerbations in the future. Third, we validated the results of the cluster analysis in another separate population. Fourth, the stability of PGA was investigated before and after 1-month fixed antiasthma treatment.

METHODS

Design overview

This study consisted of 4 parts (Figure 1). Part I was a crosssectional study to consecutively collect the demographic and clinical features of patients with PGA to identify PGA clusters using cluster analysis. Part II was a prospective noninterventional cohort study with a 12-month follow-up period, which was designed to validate these clusters in relation to future risk of asthma exacerbations in a realworld setting. Part III was a cross-sectional study to validate the results of identified clusters by tree analysis in another separate population. Part IV was a post hoc analysis to assess the stability of the inflammatory phenotypes and explore the transition of PGA based on prospectively collected data. Parts I, II, and IV were based on the Australasian Severe Asthma Network (ASAN),17 which is still ongoing. The study was approved by the institutional review board at West China Hospital, Sichuan University (Chengdu, China) (No. 2014-30). All participants provided written informed consent.

Subjects

In parts I, II, and IV, subjects aged 18 years with asthma were consecutively recruited from the asthma clinic of the West China Hospital, Sichuan University from March 2014 to November 2017. In part III, we recruited an independent population aged 18 years with asthma at West China Hospital, Sichuan University from March 2017 to September 2018, to

validate the identified clusters in part I. The detailed inclusion and exclusion criteria of the subjects are described in the Online Repository text at www.jaci-inpractice.org.
Patients with PGA identified by induced sputum analysis were recruited in part I from March 2014 to May 2016. In part II, all the included patients from part I were asked to enter a prospective cohort study. These patients were followed at 1, 3, 6, 9, and 12 months within the 12-month period. In part III, the patients with PGA were from another separate population, and the detailed information is described in the Online Repository text at www.jaciinpractice.org. In part IV, based on the ASAN design, patients who had visits 1 and 2 after a fixed 1-month antiasthma treatment with successful sputum induction were enrolled in the study from June 2016 to November 2017.

Definition of inflammatory phenotypes

Based on the cutoff percentage of eosinophils (3%) and neutrophils (61%) in induced sputum determined in our previous studies,17,18 all included subjects were classified into 4 different inflammatory phenotypes. Patients with a sputum proportion of <61% neutrophils and <3% eosinophils were classified as PGA, with 61% neutrophils and <3% eosinophils classified as NA, with <61% neutrophils and 3% eosinophils classified as EA, and with 61% neutrophils and 3% eosinophils classified as MGA.

Data collection and assessments

All included patients underwent multidimensional asthma assessments19 including demographics, asthma history, current treatment, history of previous exacerbations, comorbidities, asthma control (Asthma Control Questionnaire [ACQ]20), quality of life (Asthma Quality of Life Questionnaire [AQLQ]21), spirometry, psychological dysfunction, atopy, and systemic inflammation, which was described in detail in our recently published study.17 Psychological dysfunction was defined as having either a score of at least 8 on the anxiety subscale or depression subscale by the 14-item Hospital Anxiety and Depression Scale (HADS).22,23 Atopy was confirmed by at least 1 positive skin prick test to common aeroallergens including house dust mites (Dermatophagoides pteronyssinus, Dermatophagoides farinae), mold (Alternaria tenuis, Aspergillus species), dog hair, cat hair, pollen (ragweed, birch, London plane), and cockroach.24 Detailed information is described in the Online Repository text at www.jaci-inpractice.org.

Sputum induction and processing

All enrolled patients underwent sputum induction based on standard methods as described in our previous studies.14,18 Detailed information is described in the Online Repository text at www.jaciinpractice.org.

Inflammatory biomarkers in sputum supernatant The sputum supernatant was aspirated and stored at 80C. Levels of tumor necrosis factor-alpha (TNF-a), interleukin (IL)-1b, IL-4, IL-5, IL-6, IL-8, IL-13, IL-17A, and interferon (IFN)-g in sputum supernatants were measured using a Luminex-based MILLIPLEX MAP Human Cytokine/Chemokine Magnetic bead Panel Kit (EMD Millipore Corporation, Billerica, Mass). The minimum detectable concentrations of cytokines in sputum supernatants are described in the Online Repository text at www.jaci-inpractice.org.

Management of subjects with asthma in parts II and IV of the study

All patients enrolled in parts II and IV received antiasthma treatment in a real-world setting that was determined by the standard practice of their treating physicians. Detailed patient management is described in the Online Repository text at www.jaciinpractice.org.

Cluster analysis

Detailed information about cluster analyses is described in the Online Repository text at www.jaci-inpractice.org.

Validation by a silhouette plot and random forest

A cluster plot was used to visualize the results of the cluster analysis. A silhouette plot was used to validate the results of the cluster analysis. Random forest was used to identify the key predictors, and the key predictors were used to rerun the cluster analysis. A consistency check was used to compare the 2 results by calculating Cohen's kappa statistic.

Discrimination of clusters by sputum inflammatory biomarkers

We explored discrimination of clusters using sputum inflammatory biomarkers, which were significantly different among the 3 clusters. Receiver operating characteristic (ROC) analysis was performed to determine the best cutoff value for discriminating these clusters. The area under the curve (AUC), sensitivity, and specificity of the models were calculated.

Validation of prespecified subtypes of PGA

To assess the ability of subtypes of PGA identified by cluster analysis to predict future outcomes, all included subjects with PGA were followed up in a prospective 12-month cohort study. The primary outcome was moderate-to-severe exacerbation. A moderate asthma exacerbation in this study was defined by an event that, when recognized, should result in a temporary change in treatment to prevent the exacerbation from becoming severe, such as an unscheduled visit. Severe asthma exacerbation was defined by events including emergency visit, hospitalization, or intensive care unit (ICU) admission, using systemic corticosteroids for at least 3 days.25 The secondary outcomes included unscheduled visit, emergency visit, hospitalization, and ICU admission. Detailed definitions are described in the Online Repository text at www.jaci-inpractice.org.

Decision tree analysis and validation

First, decision tree analysis in subjects with PGA from part I was performed using all 10 variables to predict the cluster assignment for each subject, and misclassification rates were calculated. Second, to further validate the accuracy of the clustering results, we used the variables of the decision tree analysis to group the subjects from another separate population enrolled in part III. The sociodemographic and clinical characteristics of clusters from parts I and III were compared.

Inflammatory phenotype and PGA stability

To explore the stability of the inflammatory phenotype, in part IV, we first analyzed the variability of inflammatory phenotype. Second, the transition of PGA was also analyzed. Meanwhile, we recorded ACQ, AQLQ, Asthma Control Test (ACT), and preeforced expiratory volume in 1 second (FEV1) at visits 1 and 2, and calculated DACQ, DACT, DAQLQ, and DFEV1.

Statistical analyses

Detailed information about statistical analyses is described in the Online Repository text at www.jaci-inpractice.org.

RESULTS Subject characteristics

A total of 458 patients with documented evidence of diagnosed asthma (Figure E1, available in this article's Online

FIGURE 1. A, Flowchart of participants with asthma in this study. B, Flowchart of variable selection in cluster analysis. ASAN,
Australasian Severe Asthma Network; EA, eosinophilic asthma; MGA, mixed granulocytic asthma; NA, neutrophilic asthma; PGA,
paucigranulocytic asthma.

Repository at www.jaci-inpractice.org) were consecutively recruited, and 145 eligible subjects with PGA were included in part I (Figure 1). The demographic and clinical characteristics of the included subjects are listed in Table E1 (available in this article's Online Repository at www.jaci-inpractice.org). Ninety patients (62.1%) were females, with a mean age of 46.21 13.45 years and a mean body mass index of 23.86 3.40 kg/m2. Of these, 26.2% (n ¼ 38) were smokers, 60.0% (n ¼ 87) had atopy, 62.1% (n ¼ 90) had rhinoconjunctivitis and other allergic diseases, and 14.5% (n ¼ 21) had psychological dysfunction. The mean age of asthma onset was 34.10 17.92 years and the median duration of asthma was 7.00 years (interquartile range [IQR], 3.00-20.50). In our study population, the median FEV1% predicted was 78.00 (IQR, 62.00-92.00), and the median ACQ score and AQLQ score were 0.50 (IQR, 0.00-1.17) and 6.13 (IQR, 5.43-6.56), respectively.

Cluster analysis

Based on the cubic clustering criterion (CCC), pseudo F-statistic, and pseudo T2-statistic (Table E4, available in this article's Online Repository at www.jaci-inpractice.org), 3 clusters were identified. The demographic and clinical characteristics of each cluster are shown in Table I. Differential inflammatory cells in sputum and blood of the 3 clusters are shown in Table II. Cluster 1 was characterized by nonsmoking females with mild airway obstruction. Cluster 2 was characterized by psychological dysfunction and rhinoconjunctivitis and other allergic diseases. Cluster 3 was characterized by late-onset, smoking males with fixed airflow limitation.

Cluster 1: mild PGA. Cluster 1 (n ¼ 110) was the largest cluster comprising 75.9% of the population and were predominantly females (n ¼ 79, 71.8%) with nonsmoking status. This cluster had nearly normal lung function, indicating mild airway obstruction. The subjects with PGA in cluster 1 presented better lung function and less airway obstruction than those in clusters 2 and 3 (FEV1% predicted: 80.50 [69.00, 94.25] vs 75.00 [58.25, 94.00] vs 62.00 [45.00, 71.00]%, respectively, P¼ .002; FEV1/ forced vital capacity [FVC]: 0.73 [0.62, 0.79] vs 0.69 [0.63, 0.78] vs 0.55 [0.43, 0.64]%, respectively, P< .001). Furthermore, patients in cluster 1 had the highest AQLQ score than clusters 2 and 3 (6.18 [5.62, 6.60] vs 5.52 [4.51, 6.29] vs 5.80 [5.47, 6.69], P¼ .024).

Cluster 2: PGA with psychological dysfunction and rhinoconjunctivitis and other allergic diseases. Cluster 2 consisted of 20 (13.8%) subjects, of whom 75.0% had atopy. The patients with this subtype of PGA had the youngest age of asthma onset (28.75 14.82 years) in comparison with clusters 1 (32.51 17.65 years) and 3 (52.87 11.98 years) (P < .001). The greatest proportion of patients presented with psychological dysfunction (100.0% vs 0.9% vs 0.0%, P < .001) and rhinoconjunctivitis and other allergic diseases (80.0% vs 64.5% vs 20.0%, P¼ .001) in this cluster compared with the other 2 clusters. In addition, the sputum IL-6 level (in pg/mL) in cluster 2 was significantly elevated compared with that in cluster 1 (61.48 [25.04, 98.94] vs 21.37 [10.69, 49.61], P ¼ .011) (Table E5, available in this article's Online Repository at www.jaci-inpractice.org, and Figure 2).

Cluster 3: smoking-associated PGA. Cluster 3 comprised the smallest proportion (n ¼ 15, 10.3%) of subjects with PGA. They were older in age (in years) (58.07 10.61 vs 45.46 13.40 vs 41.45 10.91, P¼ .001) and had a later age of asthma onset (52.87 11.98 vs 32.51 17.65 vs 28.75

14.82, P < .001). All of them were smokers (100.0%) with increased smoking pack-years compared with the other 2 clusters. Moreover, patients with this subtype of PGA had an increased IL-6 level (in pg/mL) in comparison with those in cluster 1

TABLE I. Demographic and clinical characteristics of the included patients with PGA grouped by cluster analysis in part I

Variables	Cluster 1	Cluster 2	Cluster 3	F/c2/H	P value
n	110	20	15		
7.952					
.001					
Age (y)	45.46 13.40	41.45 10.91	58.07 10.61#,z		
Sex, males/females	31/79	9/11	15/0†††,**	29.411	
<.001					
BMI (kg/m2)	23.71 3.44	24.09 3.34	24.62 3.26	0.525	.593
Smoking status, n (%)				50.665	
<.001					
Current smoker	5 (4.5)	5 (25.0)†	7 (46.7)†††,***		
Ex-smoker	11 (10.0)	2 (10.0)	8 (53.3)		
Never	94 (85.5)	13 (65.0)	0 (0.0)		
Smoking pack-yearsx	0.00 (0.00, 0.00)	0.00 (0.00, 9.23)			
35.00 (25.00, 46.00)†††,***	67.950	<.001			
Age of asthma onset (y)	32.51 17.65	28.75 14.82	52.87 11.98#,z	10.856	
<.001					
Asthma duration (y)	7.00 (3.00, 22.00)	10.00 (3.00, 22.50)			
4.00 (1.00, 9.00)	5.280	.071			
ICS dose (BDP equivalent; mg/d)	314.55 479.07	260.00 361.87	533.33		
686.26 1.829	.401				
Treatment step based on GINA					
1.661	.460				
Steps 1/2/3	94 (85.5)	17 (85.0)	11 (73.3)		
14.294					
.001					
Steps 4/5	16 (14.5)	3 (15.0)	4 (26.7)		
Moderate-to-severe exacerbation in past year, n (%)	56 (50.9)				
71 (64.5)	12 (60.0)				
16 (80.0)	6 (40.0)				
3 (20.0)††,***					
Comorbidities, n (%)					
Rhinoconjunctivitis and other allergic diseases					
Psychological dysfunction	1 (0.9)	20 (100)†††	0 (0)***		
100.892	<.001				
Nasal polyps	8 (7.3) 3 (15.0)	0 (0)	2.363	.248	
Gastroesophageal reflux disease	11 (10.0)	4 (20.0)	0		
(0)	3.313	.147			
Osteoporosis	4 (3.6) 2 (10.0)	1 (6.7)	2.340	.278	
Obesity	12 (10.9)	3 (15.0)	1 (6.7)	0.646	
.812					
Diabetes	3 (2.7) 1 (5.0)	1 (6.7)	1.782	.349	

Chronic obstructive pulmonary diseasek			11 (10.0)		3 (15.0)			
	3 (20.0)		1.990		.371			
Spirometry								
Prebronchodilator FEV1 (% predicted)			80.50 (69.00, 94.25)			75.00		
(58.25, 94.00)		62.00 (45.00, 71.00)†††			12.628		.002	
Prebronchodilator FVC (% predicted)	93.51	15.51	92.65	17.87	92.60	15.42	0.041	
	.960							
FEV1/FVC		0.73 (0.62, 0.79)			0.69 (0.63, 0.78)			0.55 (0.43,
0.64)†††,**		18.259		<.001				
Change in FEV1 (%)		8.06 (3.14, 15.68)		10.90 (0.81, 22.26)		13.33 (7.60, 17.77)		
	1.352	.509						
ACQ score		0.42 (0.00, 1.00)			0.92 (0.00, 1.79)			0.83 (0.00,
1.33)	2.710		.258					
AQLQ score	6.18 (5.62, 6.60)		5.52 (4.51, 6.29)†		5.80 (5.47, 6.69)		7.500	
	.024							
IgE (IU/mL)		92.17 (41.45, 271.43)			115.50 (46.89, 298.84)			
	47.40 (36.20, 90.30)		4.436		.109			
Atopy, n (%)	66 (60.0)		15 (75.0)		6 (40.0)		4.375	.112

ACQ, Asthma Control Questionnaire; AQLQ, Asthma Quality of Life Questionnaire; BDP, beclomethasone dipropionate; BMI, body mass index; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; GINA, Global Initiative for Asthma; ICS, inhaled corticosteroid; PGA, paucigranulocytic asthma.

Data are represented in mean standard deviation, median (quartile 1, quartile 3), or frequency (%). Other allergic diseases include eczema and hay fever. *P < .017, **P < .005, ***P < .001 vs cluster 2, with the Bonferroni correction. †P < .017, ††P < .005, †††P < .001 vs cluster 1, with the Bonferroni correction.

#P < .05 vs cluster 1, zP < .05 vs cluster 2; least significant difference was used as post hoc tests. xSmoking pack-years ¼ pack per day smoking years. kChronic obstructive pulmonary disease was defined as a postbronchodilator FEV1/FVC less than 0.70 persistently.

(62.21 [5.20, 110.93] vs 21.37 [10.69, 49.61]), but this was not statistically significant (P¼ .308).

Validation by a silhouette plot and random forest

Cluster plot (Figure E2, available in this article's Online Repository at www.jaci-inpractice.org) and silhouette plot (Figure E3, available in this article's Online Repository at www. jaci-inpractice.org) indicated a reasonable structure of our cluster analysis. Smoking pack-years and psychological dysfunction were key predictors of the random forest (Figure E4, available in this article's Online Repository at www.jaci-inpractice.org). There was an almost perfect agreement (kappa ¼ 0.916) with a consistent rate of 96.6% between the results of cluster analysis by 10 variables and 2 key predictors (Table E6, available in this article's Online Repository at www.jaci-inpractice.org).

Discrimination of clusters by inflammatory biomarkers

Significant differences in the levels of IL-6 and IL-17A biomarkers in sputum were found among the 3 clusters. We further explored the discrimination of clusters by IL-6 and IL-17A levels in induced sputum. When applying IL-6 to distinguish clusters 2 and 3 from cluster 1, the cutoff value was 57.36 pg/mL for IL-6 and the AUC of the ROC was 0.661 (95% confidence interval [CI], 0.530-0.792, P¼ .015), with a sensitivity of 56.0% and a specificity of 81.7% (Figure E5, A, available in this article's

TABLE II. Differential inflammatory cells in sputum and blood in subjects with PGA grouped by cluster analysis in part I

Variables	Cluster 1	Cluster 2	Cluster 3	F/c2/H	P value
n	110	20	15		
Sputum					
Total cells (106/mL)	2.33 (1.46, 3.46)	3.16 (1.60, 4.95)	2.88 (2.16, 5.94)	3.986	.136
Neutrophils (%)	22.00 (7.69, 40.75)	17.50 (8.63, 24.63)	36.75 (24.50, 53.50)†,**	8.916	.012
Neutrophils count (104/mL)	49.31 (12.53, 110.82)	40.59 (20.76, 120.32)	126.68 (35.05, 311.85)†	6.152	.046
Eosinophils (%)	0.00 (0.00, 0.50)	0.00 (0.00, 0.50)	0.00 (0.00, 0.25)	1.666	.435
Eosinophils count (104/mL)	0.00 (0.00, 0.93)	0.00 (0.00, 1.09)	0.00 (0.00, 1.49)	0.311	.856
Lymphocytes (%)	1.00 (0.25, 2.00)	0.63 (0.50, 1.81)	0.75 (0.25, 2.00)	0.735	.692
Lymphocytes count (104/mL)	2.24 (0.57, 5.27)	2.08 (0.70, 4.85)	2.05 (0.72, 11.18)	0.407	.816
Macrophages (%)	75.38 (57.25, 90.06)	81.50 (74.50, 90.44)	62.25 (44.75, 75.00)†,**	8.529	.014
Macrophages count (104/mL)	153.63 (102.27, 269.03)	214.01 (134.15, 434.76)	200.77 (118.08, 265.82)	4.399	.111
Blood					
Leukocytes (109/L)	5.56 (4.95, 6.62)	6.26 (4.69, 7.15)	6.26 (5.60, 7.42)	3.264	.196
Eosinophils (%)	2.85 (1.64, 5.00)	3.58 (1.47, 5.10)	2.43 (0.74, 3.26)	3.512	.173
Eosinophils (109/L)	0.17 (0.09, 0.31)	0.20 (0.09, 0.33)	0.14 (0.06, 0.21)	2.383	.304
Neutrophils (%)	60.82 (54.44, 66.76)	58.68 (53.24, 63.11)	61.24 (58.95, 65.41)	1.794	.408
Neutrophils (109/L)	3.35 (2.86, 4.21)	3.68 (2.66, 4.17)	3.83 (2.93, 4.55)	1.506	.471
Monocytes (%)	5.58 (4.59, 6.95)	5.82 (4.93, 6.91)	7.00 (6.15, 9.43)††	11.828	.003
Monocytes (109/L)	0.31 (0.25, 0.41)	0.34 (0.28, 0.44)	0.48 (0.37, 0.68)††	13.895	.001
Lymphocytes (%)	29.19 (24.07, 33.66)	31.41 (28.16, 34.96)	28.67 (25.73, 30.85)	2.313	.315
Lymphocytes (109/L)	1.67 (1.32, 1.96)	1.92 (1.45, 2.17)	1.85 (1.40, 2.69)	2.770	.250
Basophils (%)	0.47 (0.33, 0.70)	0.47 (0.40, 0.67)	0.42 (0.31, 0.70)	0.117	.943
Basophils (109/L)	0.03 (0.02, 0.04)	0.03 (0.02, 0.04)	0.03 (0.02, 0.05)	0.677	.713

PGA, Paucigranulocytic asthma.
Data are represented in mean standard deviation, median (quartile 1, quartile 3), or frequency (%).
*P < .017, **P < .005, ***P < .001 vs cluster 2, with the Bonferroni correction.
†P < .017, ††P < .005, †††P < .001 vs cluster 1, with the Bonferroni correction.
Online Repository at www.jaci-inpractice.org). When applying IL-17A to distinguish clusters 1 and 3 from cluster 2, the cutoff value was 2.85 pg/mL for IL-17A and the AUC of the ROC was
0.665 (95% CI, 0.529-0.801, P ¼ .047), with a sensitivity of 41.9% and a specificity of 92.9% (Figure E5, B, available in this article's Online Repository at www.jaci-inpractice.org).
Validation of the prespecified clusters and logistic regression analyses
To validate the prespecified clusters, a prospective 1-year cohort study was conducted to follow these subjects from part I, and a total of 133 patients who completed the 1-year follow-up in a real-world setting were analyzed (Figure 1). Within the 12 months of follow-up, it was found that, compared with cluster 1, patients in cluster 3 had a greater proportion of severe exacerbation (25.0% vs 3.9%, P¼ .024) and hospitalization (25.0% vs 1.9%, P ¼ .008) and experienced more severe exacerbations (0.50 1.00 vs 0.10 0.71, P ¼ .010), emergency visits (0.25 0.62 vs 0.08 0.70, P¼ .015), and hospitalizations (0.50 1.00 vs 0.02 0.14, P¼ .001) (Table E7, available in this article's Online Repository at www.jaci-inpractice.org).
We further established logistic regression models to analyze the future risk of asthma exacerbation, unscheduled visit, emergency visit, and hospitalization across the prespecified subtypes by cluster analysis (Figure 3). These analyses indicated that when cluster 1 was taken as the reference, cluster 3 had a significantly increased risk of severe exacerbation (relative risk [RR] ¼ 6.43, 95% CI ¼ [1.24, 33.33], P ¼ .01), emergency visit (RR ¼ 8.61, 95% CI ¼ [1.09, 67.89], P ¼ .03), and hospitalization (RR ¼ 12.94, 95% CI ¼ [1.91, 87.80], P< .01).
Validation of identified clusters using decision tree analysis in a separate population
To predict the accuracy of cluster assignment in subjects with PGA, we developed a tree diagram using 2 variables (Figure 4). The 2 variables identified were psychological dysfunction and smoking pack-years, which was consistent with the results of the random forest. The misclassification rate of the decision tree for each cluster was calculated, and 97.2% of subjects were correctly assigned to each cluster using the decision tree.
In part III, 115 patients with PGA from another separate population were recruited to validate the identified clusters using a 2-variableebased tree diagram. As a result, it was found that the subtypes grouped by decision tree analysis were almost similar to clusters identified by cluster analysis (Tables E8 and E9, available in this article's Online Repository at www.jaciinpractice.org).
Inflammatory phenotype and PGA stability
A total of 175 patients of asthma with successful sputum induction between 2 visits from ASAN data were included in part IV (Table E10, available in this article's Online Repository at www.jaci-inpractice.org). First, the variability of inflammatory phenotypes of asthma was analyzed (Figure 5). At baseline, there were 92 subjects with PGA, 53 with EA, and 30 with NA. After 1 month of fixed antiasthma treatment, it was found that there were 45.1% patients (n ¼ 79) whose inflammatory phenotypes

FIGURE 2. Sputum (A) IFN-g, (B) IL-1b, (C) IL-4, (D) IL-5, (E) IL-6, (F) IL-8, (G) IL-13, (H) IL-17A, and (I) TNF-a levels in patients with PGA grouped by cluster analysis in part I. The horizontal

line in the box indicates the median, the box indicates the interquartile range, the vertical lines indicate the 5th to 95th percentiles, and the black dots indicate samples that are outside the 5th to 95th percentile range.
IFN, Interferon; IL, interleukin; TNF, tumor necrosis factor.
shifted (c2 ¼ 26.72, P< .001). In addition, changes in ACQ, ACT, AQLQ, and FEV1 in patients grouped by different shifts of inflammatory phenotypes groups are shown in Table E11 (available in this article's Online Repository at www.jaciinpractice.org).
Second, patients with PGA in part IV were grouped by 2-variableebased tree diagram to observe the stability of PGA after 1-month fixed asthma treatment (Table III). As a result, we found that nearly 70.0% of patients with PGA remained unchanged in the inflammatory phenotype. There was no difference in the variability of phenotypes among the 3 clusters (P¼ .162). Intriguingly, it was found that the PGA subjects with unchanged phenotype had better improvement of DACT scores (3.5 [1.0,
6.0] vs 0.5 [1.0, 4.0]D,ACT (59.4% vs 35.7%,P¼ .025) and a greater proportion ofP¼ .037) fithanxed
3 scores increase in those with a changeable phenotype after 1 month of

FIGURE 3. Relationship of the 3 identified clusters with (A) moderate-to-severe exacerbation, (B) severe exacerbation, (C) unscheduled visit, (D) emergency visit, and (E) hospitalization within a 12-month follow-up with cluster 1 as the reference. As there was no emergency visit in cluster 2, the RR of cluster 2 was zero in Figure 3, D. CI, Confidence interval; RR, relative ratio.
treatment (Table E12, available in this article's Online Repository at www.jaci-inpractice.org).
DISCUSSION
To the best of our knowledge, this is the first study to identify subtypes of PGA with respect to demographic, clinical, functional, and inflammatory biomarkers using cluster analysis, and to prospectively evaluate the significance of the prespecified clusters in a real-worldsetting.Wealsovalidatedourclusteringresultsinanother separate population and explored the stability of inflammatory phenotypes over time. Three subtypes of PGA were identified as "mild PGA," "PGA with psychological dysfunction and rhinoconjunctivitis and other allergic diseases," and "smoking-associated PGA." Of these 3 subtypes, cluster 3 had high risk of severe exacerbation, emergency visit, and hospitalization within a 12-month follow-up period, and cluster 1, mild PGA, was the most prevalent. Decision tree analysis identified that cluster assignment could be predicted in subjects using only 2 variables, with an accuracy of 97.2%. In addition, the variability of inflammatory phenotypes implied that PGA could result from or convert to other phenotypes during antiasthma treatment. Our study indicates that heterogeneity definitely exists in PGA, the subtypes of which are characterized by distinct features and are of relevance in clinical practice.
It has been previously reported that PGA most likely represents a "benign" phenotype of asthma, related to a good response to antiasthma treatment. A study by Ntontsi et al9 found that PGA had better lung function based on pre-FEV1 (% predicted) (80.5 [69.7-95.0]; P ¼ .009) and FEV1/FVC (%) (0.71 [0.67-0.76]; P¼ 0.041), which was consistent with the results of our study, such as pre-FEV1 (% predicted) (78.00 [62.00-92.00]) and FEV1/ FVC (%) (0.70 [0.60-0.77]). However, the study also found that
14.8% of patients with PGA had poor asthma control despite optimal treatments, which indicates the heterogeneity within PGA. Moreover, Demarche et al26 showed that PGA may

display a low-grade airway and systemic inflammation, and PGA might be heterogeneous because of almost two-thirds of the patients receiving ICS, which might have attenuated airway inflammation initially present before the start of the treatment. In addition, Tliba and Panettieri12 suggested that PGA might represent a crosssectional view related to disease activity rather than a stable phenotype, and further studies are needed to examine the stability of PGA. All of these studies indicated that the heterogeneity of PGA needs to be studied further.

This study confirms the heterogeneity of PGA. The results clearly identified 3 subtypes of PGA using cluster analysis and show that these can be accurately predicted using 2 variables in a decision tree analysis. Cluster 1 in subjects with PGA represents a “benign” subtype with well-controlled asthma, whereas clusters 2 and 3 had more severe airway obstruction and an increased future risk of asthma outcomes, which contained 24.1% of PGA. Our study further extends the recognition of PGA and distinguishes the “severe” from “benign” subtypes of PGA using a combination of decision tree analysis and molecular signature (ie, IL-6 and IL-17A), which would be of great relevance to clinical practice.

Of the 3 clusters identified, cluster 1 (75.9% of the overall population with PGA) would be widely recognized as well-treated asthmatic patients. Not surprisingly, patients in this cluster had mild airway obstruction. This indicates that cluster 1 can manifest as mild asthma with a lower future risk of exacerbation.

All of the subjects in PGA cluster 2 had neuropsychological asthma.18,27,28 Thesepatientshadpsychologicaldysfunction,which indicated increased local and systemic inflammation that would be associated with severe asthma and had poor response to antiasthma treatment.27-29 It has been shown that IL-1b, IL-6, TNF-a, and IFN-g levels correlate with the severity of psychological

FIGURE 4. Decision tree analysis and misclassification rate for each cluster. Decision tree analysis using 2 variables including psychological dysfunction and smoking pack-years. Blue, cluster 1, mild PGA; green, cluster 2, PGAwith psychological dysfunction and rhinoconjunctivitis and other allergic diseases; red, cluster 3, smoking-associated PGA. The size of the circle represents the sample size. PGA, Paucigranulocytic asthma.

dysfunction, especially depression.18 Furthermore, this is most likely explained by IL-1b and TNF-a as 2 key proinflammatory cytokines that mediate the correlation of psychological dysfunction with impaired bronchodilator response and neutrophilic airway inflammation. Although our study did not confirm the elevation of these psychological dysfunction-associated inflammatory cytokines in cluster 2 (Tables E13-E16, available in this article’s Online Repository at www.jaci-inpractice.org), we found that sputum IL-8 and TNF-a were positively associated with anxiety (HADS-A scores), in all participants whether adjusted or not (Tables E15 and E16, available in this article’s Online Repository at www.jaciinpractice.org), which could be explained by the small size of the sample. Sputum IL-6 in cluster 2 subjects was higher than that in cluster 1. Several studies have suggested that IL-6 may participate in thepathogenesisofasthmaandpsychologicaldysfunction.30-32 This might explain the higher levels of IL-6 in cluster 2 to some extent.

This study found that patients in cluster 3 were characterized by airflow limitation and an increased risk of asthma exacerbation, which indicates that these patients had more severe asthma. We found that 26.6% of patients in cluster 3 had Global Initiative for Asthmaedefined severe asthma. There were some issues that would accountfortheclinicalfeaturesofcluster3inPGA.First,therewasa greater proportion (100.0%)

of smokers in cluster 3. Several studies33-38 have reported that current smokers have worse asthma control and more frequent exacerbations than never smokers with asthma. As suggested by Westerhof et al,38 smokers had an altered airway immune response leading to an increased susceptibility to infections, which may result in exacerbations.39 In addition, smoking can alter the microbiome and lead to bacterial colonization in the airway, resulting in neutrophilic airway inflammation.40 Furthermore, smokers have lower sensitivity to corticosteroids.41,42 Alloftheabovefeaturescouldcontributetoworseasthma control and a higher exacerbation rate. Second, the subjects in cluster 3 were older, which indicates that older patients have an increased risk of developing complications from asthma. Older people have alterations in both innate and adaptive immune responses termed "immunosenescence,"43 which showed an increase in sputum neutrophils and neutrophil mediators. Furthermore, older individuals have a chronic, low-grade, systemic inflammation with increased inflammatory mediators including IL-6, IL-1b, and TNF-a, termed as "inflammaging."44 Owing to increased levels of sputum neutrophils and inflammatory mediators, "immunosenescence" and "inflammaging" might lead to less response to corticosteroid treatment, resulting in worse control in older asthmatic patients. Third, most of the subjects in this cluster were nonatopic, with decreased eosinophils in blood, which may indicate less response to corticosteroids. Fourth, there were elevated local inflammatory markers such as IL-1b, IL-6, and IL-17A in cluster 3 compared with cluster 1, although it was not statistically significant. This would most likely be explained by the small sample size in this cluster, which could result in less power to detect this potential difference across clusters. As this was a real-world study, we did not exclude patients with chronic obstructive pulmonary disease (COPD). Patients in cluster 3 had some features resembling COPD, such as being older, smokers, and having fixed airflow limitation. However, there was no difference in the distribution of asthma-COPD overlap among these 3 clusters (clusters 1, 2, and 3 were 10.0%, 15.0%, and 20.0% in part I, respectively [P¼ .371], and were 10.5%, 7.7%, and 18.8% in part III, respectively [P¼ .604]). The differences across the 3 clusters could be largely attributed to heterogeneity of asthma rather than COPD.

Part IV illustrated a clear picture of development of PGA and variability of inflammatory phenotypes. Importantly, we found that approximately 70% of subjects with PGA in part IV were stable over time, which does not support the hypothesis that all subjects with PGA represent a cross-sectional view related to disease activity or represent a treatment success. Rather, this indicates that most patients with PGA could constitute an independent phenotype. Furthermore, it was also found that PGA could shift to one of the other3inflammatoryphenotypes,andthattheseotherinflammatory phenotypes could also convert to PGA. Other published studies45-47 also support our findings, which might partly explain the heterogeneityofPGA.TheanalysesofourprospectivecohortstudywithPGA on 1-month fixed treatments demonstrated a better improvement of ACT in subjects with unchanged phenotypes than those with

FIGURE 5. The distribution of inflammatory phenotypes identified by induced sputum observed at visits 1 and 2, which indicated the transition of PGA. EA, Eosinophilic asthma; MGA, mixed granulocytic asthma; NA, neutrophilic asthma; PGA, paucigranulocytic asthma.

TABLE III. Conversion of inflammatory phenotypes for PGA grouped by 2-variableebased tree diagram after 1-month fixed treatment in part IV

Variables	Cluster 1	Cluster 2	Cluster 3	c2	P value

n 67 16 9
8.177 .162
Conversion of inflammatory phenotypes
PGA 47 (70.10) 12 (75.00) 5 (55.56)
EA 3 (4.50) 1 (6.30) 0 (0)
NA 17 (25.40) 2 (12.50) 3 (33.33)
MGA 0 (0) 1 (6.30) 1 (11.11)
EA, Eosinophilic asthma; MGA, mixed granulocytic asthma; NA, neutrophilic asthma; PGA, paucigranulocytic asthma.
Data are represented in frequency (%).

changed phenotypes. One possible reason is that the change from PGA to granulocytic asthma might induce more aggressive inflammation. Moreover, it was foundthat most patients with a conversion of phenotype after a 1-month fixed treatment (n ¼ 22, 78.6%) were converted from PGA to NA. This was consistent with other published results4,45 that sputum neutrophils increase in response to corticosteroid treatment. In addition, conversion of PGA to EA would more likely represent an aggravating state of airway inflammation. The possible reasons for this result were medication compliance and allergen exposure history. We thus concluded that a small proportion of patients with PGA in our study were derived from other phenotypes after treatment, which confirmed the "transitional phenotype." The "transitional phenotype" may result from changes in the percentage of neutrophils and eosinophils, which is influenced by external factors including environmental exposures such as diet, infection, and medication use such as corticosteroids.13 The strength of this study was that we performed it in a realworld setting, which has considerable external validity. To ensure that adequate information truly represents the characteristics in a real-world setting, we collected a range of demographic, clinical, functional, and inflammatory information as well as performing multidimensional assessment, which would help us adjust for confounding factors that may impact the outcomes. Furthermore, all subjects with asthma were diagnosed, treated, and managed by physicians having received standardized training to ensure the reliability of the real-world study. In addition, we validated our results in another separate population. However, this study had some limitations that need to be acknowledged. First, we could not absolutely avoid bias due to several subjective issues addressed in the cluster analysis including the variable selection and optimal number of clusters. However, we used factor analysis and statistical indices such as the CCC, the pseudo F-statistic, and the pseudo T2-statistic, to ensure objectivity as much as possible. Second, although no sample size guidelines exist for cluster analysis as indicated by Siddiqui48 and other data science academics,49 we identified 3 clusters or phenotypes with potential implications for clinical practice. Furthermore, in part II, the differences in sociodemographic, clinical characteristics, and future risk of asthma exacerbation between clusters significantly indicate their clinical implications. The wide CI in part II may be associated with a large standard deviation of asthma exacerbations and the small sample size,50 although it would be powered to differentiate these identified clusters. Third, we validated the identified clusters in an independent population in a real-world setting. It is still a single-centered study in which results should be extrapolated carefully, although, to some extent, findings based on a real-world study are characterized by good external validity and generalizability.51 Accordingly, further studies are needed to validate these findings in the future. Fourth, the observation

of PGA and inflammatory phenotype variability was conducted within a short period, which needs to be further explored in future studies.

CONCLUSIONS

In conclusion, this was the first study to identify the subtypes of PGA using cluster analysis in a real-world setting. As a result, we confirmed that PGA is heterogeneous, and identified 3 subtypes of PGA, which are “mild PGA,” “PGA with psychological dysfunction and rhinoconjunctivitis and other allergic diseases,” and “smoking-associated PGA.” These clusters could be reliably detected using 2 clinical variables, and cluster 3 had the highest risk of severe exacerbations within the 12-month follow-up period. We recommend further studies to validate our results, and that patients with PGA require high-quality health care and novel targeted interventions to reduce their exacerbations.

Acknowledgments

We wish to thank Xiao Fei Liu and Michelle Gleeson for their work in the sputum processing. GW, PGG, XZ, and KD designed the framework for the study. LZ, GW, XZ, and KD assisted with acquisition, and analysis of data. GW, YL, MF, BGO, LW, PMH, LQ, JZ, and KD contributed to interpretation of results. GW, XZ, and KD contributed to study design, data analysis, and interpretation of results. WML, GW, PGG, JS, ZHC, MX, XZ, and KD contributed to interpretation of results, and drafted the manuscript.

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