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Asthma and Lower Airway Disease

Cluster Analysis to Identify Distinct Asthma Phenotypes in the ATLANTIS Cohort

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ABSTRACT

Background: Previous cluster analyses have identified subgroups of asthma. However, only a few studies included parameters of small airways dysfunction (SAD), or gene expression profiles reflecting underlying disease mechanisms. We aimed to identify clinically distinct asthma phenotypes, beyond GINA asthma severity, using available data from the ATLANTIS study which focused on identifying the prevalence of SAD in asthma and its role in asthma control, exacerbations and quality of life.

Methods: The ATLANTIS study included 773 asthma patients (mean age 44 years, 58% female, 76% never-smoker, GINA 1–5). Subjects were extensively characterized, including symptoms, parameters of large and small airways dysfunction, blood and sputum differential cell counts, and genome-wide gene expression profiling from nasal brushes. Clusters were generated using the Self-Organizing Map–Ward's method.

Results: Four distinct clusters were identified: A ($N = 62$; 8%) characterized by the most frequent exacerbations, lower post-bronchodilator FEV_1 % predicted, more small airways dysfunction, higher sputum and blood eosinophils, and high expression of asthma-related genes. B ($N = 206$; 27%) consisting of atopic patients with early-onset asthma, uncontrolled symptoms, and normal lung function and bronchial hyperresponsiveness, along with a high expression of asthma-related genes in the nasal epithelium. C ($N = 277$; 36%), predominantly male former smokers, with well-controlled asthma, mild obstructive

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lung disease, and relatively high neutrophil levels. D ($N = 228$; 29%), with normal lung function and low blood and sputum eosinophils.

Conclusions: Four distinct clusters were identified, where the presence of SAD was associated with high type-2 inflammation, lower lung function, and frequent exacerbations. SAD may be a marker of poorly controlled asthma and should be considered as an important clinical trait.

1 | Introduction

Bronchial asthma is a complex and heterogeneous disease characterized by chronic airway inflammation, leading to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing [1]. The disease heterogeneity is evidenced by the variability of clinical presentation, disease severity, underlying pathophysiology, and response to treatment. This poses a challenge for effective disease management, as a one-size-fits-all approach is unlikely to be suitable for all patients [2]. With the development of targeted treatments, such as anti-IL-5 and anti-IL-4-receptor antibodies, which are effective only in specific subtypes of asthma [3], the need for adequate phenotyping and endotyping has increased.

Cluster analysis offers a method to identify distinct subtypes of asthma based on the diverse characteristics of individuals with asthma [4]. Several studies have identified a mild asthma cluster characterized by normal lung function and an earlier onset of asthma [5, 6]. Additionally, a distinct type-2 inflammation cluster with airway dysfunction and elevated peripheral blood and sputum eosinophil levels has been consistently identified [5, 7]. Furthermore, several studies have recognized an asthma cluster associated with obesity [7, 8]. However, important characteristics such as the presence of small airways dysfunction and molecular characteristics are often not included in these analyses. In the ATLANTIS study, these aspects were also assessed to gain a more comprehensive understanding of asthma subtypes.

The ATLANTIS (Assessment of Small Airways Involvement in Asthma) study offers an opportunity to expand this research scope. The primary objective of the ATLANTIS study was to investigate factors related to the small airways. Important findings from ATLANTIS to date include that small airways dysfunction can be evaluated using simple, clinically applicable tests such as spirometry and impulse oscillometry, and is significantly associated with asthma severity, symptom control, exacerbation history, and quality of life [9]. As the role of the small airways in asthma pathogenesis is increasingly recognized [10], the inclusion of these parameters in asthma subtyping may be of importance. Notably, nasal epithelial cells were collected in ATLANTIS to assess the nasal transcriptome. Previous studies have shown that the nasal epithelial transcriptome can serve as an easily accessible proxy for the bronchial epithelial transcriptome in many instances, and it can identify clinically relevant asthma endotypes [11–13]. We aimed to perform a cluster analysis using data from the ATLANTIS study to identify clinically distinct phenotypes complementing earlier cluster analyses by including small airways function and molecular profiling in the nasal transcriptome.

2 | Methods

2.1 | Study Design and Participants

From June 2014 to March 2017, the ATLANTIS study recruited 773 asthma patients across 29 clinics in nine countries worldwide. Detailed in- and exclusion criteria and methods have been published previously [NCT02123667] [9, 14]. In summary, eligible patients were between 18 and 65 years of age, with a clinical diagnosis of asthma for more than six months. Additionally, participants were required to have stable asthma and have been receiving any asthma treatment at a consistent dose for at least eight weeks prior to baseline. The primary exclusion criteria included a confirmed diagnosis of COPD by a chest physician or a smoking history of 10 or more pack-years. Patients were extensively characterized, which included assessments of large airways function by spirometry and small airways function by impulse oscillometry (IOS) and multiple breath nitrogen washout (MBNW). These methods have been previously described by Postma et al. [9]. Prior to baseline visits, a bronchodilator wash-out period was implemented to standardize lung function and oscillometry testing. Short-acting beta-agonists (SABA) were withheld for 6 h, and short-acting muscarinic antagonists (SAMA) for 12 h (8 h in patients with GINA step 4 or 5). Long-acting beta-agonists (LABA) used twice daily were withheld for 12 h, and once-daily LABA or long-acting muscarinic antagonists (LAMA) were withheld for 72 h (24 h in GINA step 4 or 5 patients). Additional assessments included airway hyperresponsiveness (AHR), measured using a methacholine provocation test, in subjects with a baseline FEV_1 greater than 55% predicted or 1.5 L, as well as blood sampling, sputum induction, computed tomography (CT) scan, and questionnaires to assess asthma control (asthma control questionnaire 6 [ACQ6]). GINA treatment steps were determined based on medication usage, according to the 2012 guidelines [15]. The occurrence of exacerbations was reviewed every three months for 1 year; twice during follow-up visits at 6 and 12 months and in between with telephone follow-up calls at 3 and 9 months. Exacerbations were defined as a deterioration in asthma symptoms that led to the use of systemic corticosteroids for at least three days, and/or an emergency room visit, and/or hospitalization within the 12 months following the baseline visits. Ethical approval was obtained from the Medical Ethics Committee of each participating center, and written informed consent was obtained from all participants.

2.2 | Statistical Analysis

Clustering was performed using Viscovery-SOMine (v7.2; Viscovery Software, Vienna, Austria). The Self-Organizing Maps (SOM)–Ward method used in this program, also known as Kohonen maps, is an unsupervised hierarchical clustering

technique that groups patients into homogeneous data clusters based on their similar characteristics in the selected input variables. The clustering process was fully data-driven and not guided by clinical assumptions or outcome variables.

Further statistical analyses were conducted using Rstudio (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria). Differences between the identified clusters were tested using the "tableone" package. A p-value < 0.05 was considered statistically significant. For categorical variables, Chi-square tests were performed. For numerical variables, histograms were employed to assess the normality of the distribution of the variables, and quantile-quantile plots (QQ-plots) were utilized in cases of uncertainty. Next, normally distributed variables were analyzed using Analysis of Variance (ANOVA), while for non-normally distributed variables, the Kruskal–Wallis test was employed. Differential gene expression (DGE) analysis was performed using the edgeR package (version 4.2.2). Normalization of library sizes was carried out using the calcNormFactors function with the trimmed mean of *M*-values (TMM) to normalize for effective library sizes. A negative binomial generalized linear model was fitted, adjusting for sex, age, and smoking status (current smokers, ex-smokers, and never-smokers). The analysis was restricted to clusters that showed similar enrichment patterns for asthma-related gene modules in order to explore differences in underlying gene expression. Multiple testing correction was performed using the Benjamini and Hochberg method, taking into account 67 tests (genes from module 4–6).

2.3 | Gene Modules

The analyzed gene modules were established in a previous ATLANTIS analysis described by Karp et al. [16]. These modules were determined by clustering genes identified as differentially expressed in the nasal epithelium of asthmatic patients compared to healthy controls. In summary, gene modules 1–3 consist of genes that were downregulated in asthma patients compared to healthy controls. In contrast, gene modules 4–6 include genes that were upregulated in asthma patients compared to their healthy counterparts. Pathways associated with these modules were identified using gene ontology overrepresentation analysis. The activity of each gene module was quantified using Gene Set Variation Analysis (GSVA). GSVA calculates a single score per sample that reflects the overall expression level of a group of genes that are functionally related or co-regulated, providing a composite score that reflects the activity of a gene set in each sample. Supporting Information provides a description of the gene modules, and Table S2 represents the genes included in each gene module.

2.4 | Variable Selection for Clustering

The original ATLANTIS dataset contained over 800 variables, which had to be reduced before cluster analysis could be performed. Redundant variables were removed by selecting variables that best represented specific parameters. As a result, 36 variables were selected, relating to patient characteristics, asthma control, pulmonary traits, CT scan-derived parameters, blood and sputum inflammatory cell counts, and nasal brush gene modules. This selection is detailed in Table S2. The categorical variables included in the analysis were either ordinal or binary. There was no need to impute missing values to construct the Self-Organizing Map, as the clustering software relies solely on the existing data points to determine the optimal position for each patient.

3 | Results

A total of 773 patients with asthma (41.8% male, mean ± SD age 44.3 ± 13.0 years, 3.5% current smokers) were included in the cluster analysis (Table 1).

3.1 | Patient Profiles of the Four Different Clusters

Four distinct clusters were generated in Viscovery (Figure 1). Table 2 provides an overview of the demographic, clinical, lung, CT scan, blood, sputum, and gene module characteristics of the four identified clusters.

The smallest cluster, Cluster A, consisted of 62 (8%) patients (35.5% male, mean ± SD age 52.2 ± 8.5 years, 1.6% current smokers). Among the patients in this cluster, 95.2% were classified as GINA 4 or 5, indicating that this cluster comprised patients with the most severe treatment requirements. Cluster A, similar to Cluster B, exhibited poorer asthma control, as evidenced by significantly higher ACQ6 scores and more severe airway hyperresponsiveness compared to Clusters C and D. Patients in Cluster A experienced more frequent exacerbations during follow-up and displayed the lowest FEV_1 and FEF_{25-75} % predicted values, indicating large airway obstruction, compared to Cluster B, C, and D. Additionally, all parameters assessing small airways, including RV/TLC, R_{5-20}, and S_{COND}, were most affected within this cluster. Eosinophil counts in blood and sputum were highest in Cluster A. On chest CT, Cluster A demonstrated the most pronounced structural and quantitative abnormalities compared to the other clusters. Patients in this cluster had a significantly higher airway wall area/total area ratio, indicative of more airway remodeling. In addition, Cluster A showed the highest VI 950%, reflecting the greatest degree of emphysematous changes, as well as the highest mean lung density and lung volume ratios, reflecting air trapping. Finally, Cluster A showed, to a lesser extent than Cluster B, positive GSVA composite scores for gene modules 5 and 6, suggesting an association with an upregulation of asthma-related genes. However, this difference was only significant when compared with Cluster D and not with Cluster C.

Cluster B included 206 (27%) patients (33.5% male, mean ± SD age 34.8 ± 11.5 years, 4.4% current smokers). Most patients in Cluster B were atopic, characterized by the earliest onset of asthma. Additionally, similar to Cluster A, moderate to severe hyperresponsiveness was observed in these patients. Lung function parameters, FEV_1 and FEF_{25-75} % of predicted, RV/TLC, S_{COND}, and R_{5-20}, were relatively preserved. The significantly highest GSVA scores for gene module 4 were observed in this cluster compared to all other clusters. GSVA scores for modules 5 and 6 were higher in Cluster B than in Clusters C and D, and though not significant, they were numerically higher than

TABLE 1 | Demographics, clinical characteristics, pulmonary characteristics, CT-scan, blood, sputum and gene module status of the cohort.

		Data available, *n*
Patients	773	
Demographics and clinical characteristics		
Female sex	450 (58%)	773
Age at inclusion, years	44.3 ± 12.99	773
BMI, kg/m^2	27.23 ± 5.88	773
Smoking status		767
Never smoker	585 (76%)	
Ex-smoker	154 (20%)	
Current smoker	28 (4%)	
Pack-years (in all subjects)	4.34 [2.00, 7.50]	767
GINA treatment step		773
1	135 (17%)	
2	85 (11%)	
3	207 (27%)	
4	300 (39%)	
5	46 (6%)	
Age of asthma diagnosis, years	24.05 [9.35, 40.89]	769
Exacerbations during follow-up	0.00 [0.00, 0.00]	752
Positive phadiatop test	454 (80.5%)	564
Airway hyperresponsiveness category		556
Very mild	141 (25.4%)	
Mild	169 (30.4%)	
Moderate	134 (24.1%)	
Severe	112 (20.1%)	
FeNO. Parts per billion	25.00 [16.00, 38.00]	627
ACQ6 score	0.80 [0.30, 1.50]	772
Pulmonary traits		
FEV_1, % predicted (post-bronchodilator)	89.3 ± 16.0	760
$FEF_{25–75}$, % predicted (post-bronchodilator)	76.8 ± 36.10	720
RV/TLC, %	33.1 ± 9.05	693
$R_{5–20}$, kPa/L/s	0.05 [0.02, 0.09]	617
S_{COND}, 1/L	0.03 [0.02, 0.04]	380
CT-scan-derived parameters		
Wall area/total area %	63.03 ± 3.47	304
VI 950%	3.64 [1.46, 7.75]	304
Mean lung density ratio, E/I	0.83 [0.77, 0.88]	292
Lung volume ratio, E/I	0.83 [0.77, 0.88]	292
Blood-derived parameters		767
Blood basophil count, 10^9/L	0.03 [0.02, 0.06]	
Blood eosinophil count, 10^9/L	0.23 [0.13, 0.38]	
Blood lymphocyte count, 10^9/L	1.87 [1.57, 2.26]	
Blood monocyte count, 10^9/L	0.46 [0.38, 0.58]	
Blood neutrophil count, 10^9/L	3.68 [2.96, 4.70]	
Sputum-derived parameters		228
Bronchial epithelial cells sputum, % of non-squamous cells	1.80 [0.80, 4.03]	
Eosinophils sputum, % of non-squamous cells	0.50 [0.10, 2.82]	
Lymphocytes sputum, % of non-squamous cells	0.60 [0.30, 1.33]	
Macrophages sputum, % of non-squamous cells	36.40 [18.45, 59.50]	
Neutrophils sputum, % of non-squamous cells	50.80 [26.42, 70.62]	
Gene profile		302
Gene module 1, GSVA score	−0.05 [−0.39, 0.29]	
Gene module 2, GSVA score	−0.11 [−0.42, 0.35]	
Gene module 3, GSVA score	−0.08 [−0.37, 0.30]	
Gene module 4, GSVA score	−0.02 [−0.26, 0.33]	

(Continues)

TABLE 1 | (Continued)

		Data available, *n*
Gene module 5, GSVA score	0.04 [−0.44, 0.43]	
Gene module 6, GSVA score	−0.03 [−0.43, 0.36]	

Note: Data are presented as *n*, *n* (%), mean ± SD or median [interquartile range]. GINA treatment steps were determined based on medication usage, according to the 2012 guidelines. Very mild airway hyperresponsiveness = $PC_{20} \geq 4$ & < 16 mg/mL, $PD_{20} \geq 0.5$ & < 2 mg. Mild airway hyperresponsiveness = $PC_{20} \geq 1$ & < 4 mg/mL, PD20 ≥ 0.13 & < 0.5 mg. Moderate airway hyperresponsiveness = $PC_{20} \geq 0.25$ & < 1 mg/mL, $PD_{20} \geq 0.03$ & < 0.13 mg. Severe airway hyperresponsiveness = $PC_{20} < 0.25$ mg/mL, PD20 < 0.03 mg.
Abbreviations: ACQ6, asthma control questionnaire 6; BMI, body mass index; E/I, expiratory/inspiratory; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; R_{5-20}, resistance at 5 Hz–resistance at 20 Hz; RV/TLC, residual volume/total lung capacity; S_{cond}, ventilation heterogeneity in the conductive zone of the lungs corrected for tidal volume; VI 950, voxel index at −950 hounsfield units.

those found in Cluster A. The high GSVA scores for these gene modules suggest an association with an upregulation of asthma-related genes.

Cluster C comprised 227 (36%) patients (63.5% male, mean ± SD age 50.9 ± 9.2 years, 3.3% current smokers). Cluster C was characterized by a predominance of males, the highest percentage of former smokers and number of pack-years, and well-controlled asthma symptoms, as measured by the ACQ6. Patients in Cluster C exhibited a significantly lower FEV_1 and FEF_{25-75} % predicted values compared with Clusters B and D, indicating more large airway obstruction. However, FEV_1 and FEF_{25-75} % predicted were significantly higher in Cluster C compared with Cluster A. Additionally, patients in Cluster C showed significantly elevated levels of sputum neutrophils compared with Clusters B and D.

Cluster D consisted of 228 (29%) patients (24.6% male, mean ± SD age 42.8 ± 13.3 years, 4.0% current smokers). Similar to patients in Cluster C, those in Cluster D exhibited remarkably well-controlled asthma, as reflected by the ACQ6 scores. This cluster was characterized by a relatively preserved lung function in both the large and small airways, as evidenced by a high FEV_1 and FEF_{25-75} % predicted values and a low RV/TLC, S_{COND}, and R_{5-20}. Significantly lower numbers of eosinophils, monocytes, and neutrophils were found in the peripheral blood of Cluster D patients compared to other clusters. Gene modules 1 and 3, consisting of genes downregulated in asthma, had positive GSVA scores in Cluster D, while gene modules 4–6 had negative GSVA scores, which suggests that these samples exhibit a nasal transcriptome profile more characteristic of healthy individuals. Table 3 provides an overview of the key findings.

3.2 | Comparison of Asthma-Associated Gene Modules Between Cluster A and B

Clusters A and B both showed associations with the asthma-related gene modules. To explore differences in asthma-related gene expression between Cluster A and Cluster B, the 67 genes from modules 4–6 were evaluated (Table S3). Differential gene expression analysis identified *NTRK2*, a gene involved in airway hyperresponsiveness, as the only gene that was significantly differentially expressed (logFC = 1.23, FDR = 0.044), with higher expression in Cluster B. The remaining genes showed no significant differences (FDR > 0.05), indicating largely similar expression profiles across both clusters.

4 | Discussion

In the current study, we show that incorporating small airways function and nasal gene expression profiles provides novel insights into asthma phenotypes and endotypes, leading to identification of four distinct asthma clusters extending previous clustering studies and revealing potentially treatable traits. The most severe cluster, Cluster A, was characterized by type-2 inflammation and exhibited not only large but also small airways dysfunction, and an upregulation of asthma-related genes in the nasal epithelium. Another notable identified cluster, Cluster B, included the subgroup of patients with early onset asthma, and besides a relatively preserved lung function, exhibited more severe bronchial hyperresponsiveness. This cluster was also associated with asthma-related genes in the nasal epithelium.

In Cluster A, a severe cluster characterized by type-2 inflammation, the affected small airways were a notable finding. While the type 2-high asthma subtype has been well described in previous literature, our analysis extends this phenotype by demonstrating that the entire bronchial tree is affected, ranging from the large airways, as assessed by FEV_1 and FEF_{25-75}, to the smaller airways, evaluated by RV/TLC, R_{5-20}, and S_{COND}. These abnormalities were further supported by structural abnormalities on chest CT, which demonstrated thicker airway walls and air trapping. Taken together, our findings show that small airway dysfunction is an integral feature of type 2-high severe asthma as well as may represent a treatable trait with leukotriene inhibitors, extra-fine formulations, or biologicals [17–19].

Unexpectedly, FeNO levels were not elevated in Cluster A, despite elevated levels of blood and sputum eosinophils, indicating type-2 inflammation. A possible explanation for this type-2 biomarker disconnect may be the higher inhaled corticosteroids use in this cluster, as FeNO is more sensitive to the effects of inhaled corticosteroids than eosinophilic inflammation in blood and sputum.

Another important finding was the presence of moderate to severe airway hyperresponsiveness in Cluster B, which consisted of early-onset, atopic asthmatics with a relatively preserved lung function in both the large and small airways. Currently, the assessment of airway hyperresponsiveness is limited and typically conducted only in secondary care settings. However, our findings suggest its potential value, particularly in patients with a relatively preserved spirometry, whose asthma may be underestimated. The finding that cluster B subjects had preserved lung function suggests that the more severe AHR was not driven by airway geometry. In addition, we found conventional type-2 inflammatory biomarkers, such as blood and sputum eosinophils

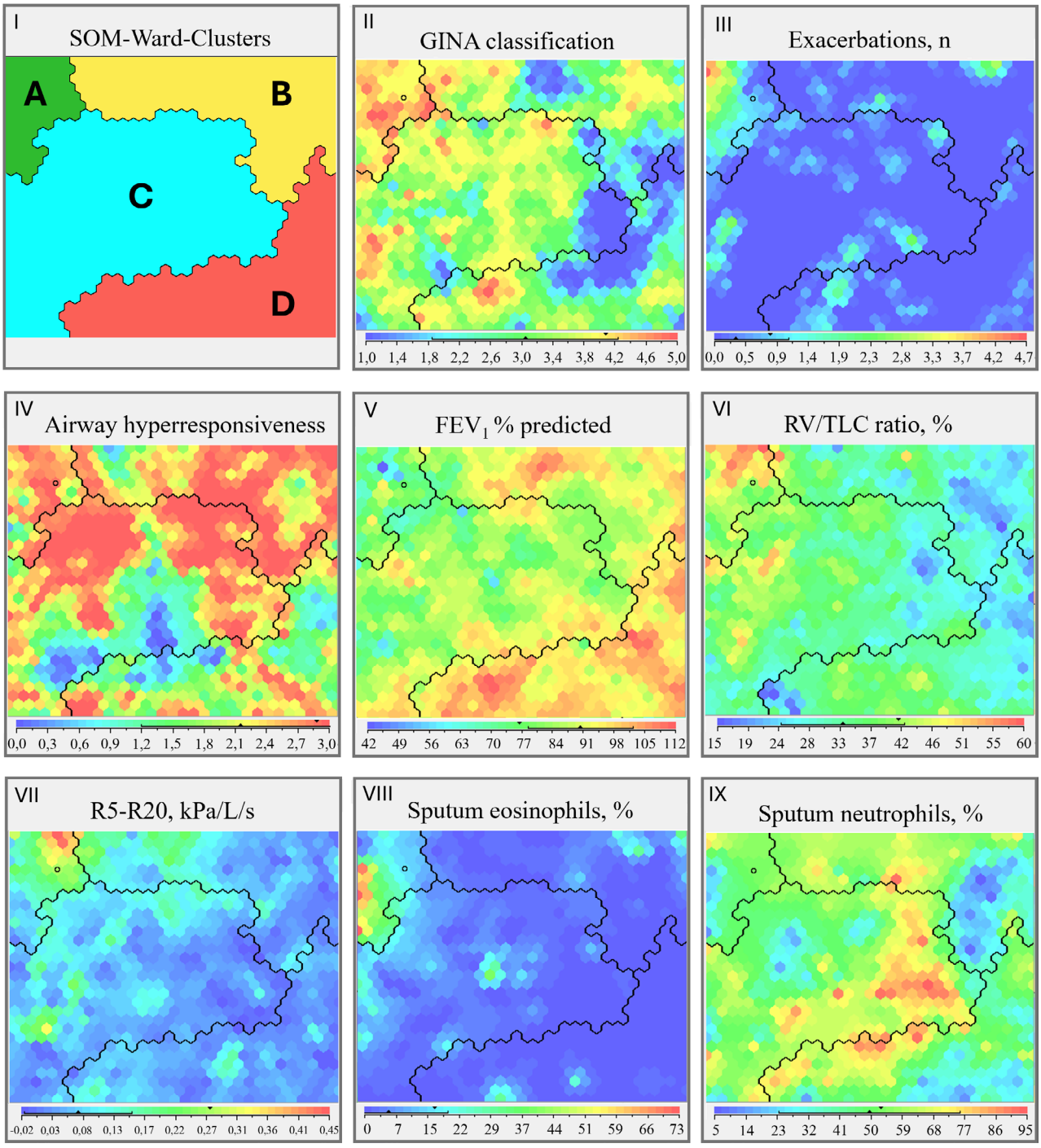

FIGURE 1 | Visual representation of the four ATLANTIS asthma clusters. (I) Four clusters were identified, A depicted in green ($n = 62$), B depicted in yellow ($n = 206$), C depicted in blue ($n = 277$), and D depicted in red ($n = 228$). The method mapped all patients according to their characteristics, placing similar subjects in proximity, and those with greater differences are positioned further apart. Clusters were then defined by the program. The colors in each map represent the size of the attribute, with blue indicating low values and red high values. (II) GINA 2012 classification 1–5; (III) Asthma exacerbations during the one year follow-up; (IV) Airway hyperresponsiveness, 0 = very mild ($PC_{20} \geq 4$ & < 16 mg/mL, $PD_{20} \geq 0.5$ & < 2 mg), 1 = mild ($PC_{20} \geq 1$ & < 4 mg/mL, PD20 ≥ 0.13 & < 0.5 mg), 2 = moderate ($PC_{20} \geq 0.25$ & < 1 mg/mL, $PD_{20} \geq 0.03$ & < 0.13 mg), 3 = severe ($PC_{20} < 0.25$ mg/mL, PD20 < 0.03 mg); (V) Forced expiratory volume in 1 s (FEV_1), % of predicted; (VI) Residual volume/total lung capacity (RV/TLC); (VII) Resistance at 5 Hz–resistance at 20 Hz (R5-R20); (VIII) Eosinophils sputum, % of non-squamous cells; (IX) Neutrophils sputum, % of non-squamous cells. I–VIII represent a selection of the variables, the full representation of all variables are provided in the Supporting Information.

or FeNO, not to be elevated in this group. However, of interest, we did find increased expression of asthma-associated genes in the nasal epithelium, similar to that observed in Cluster A. Among the upregulated well-known genes were *CLCA1* and *POSTN*, genes previously linked to airway inflammation and corticosteroid responsiveness. The observed lower inhaled corticosteroid use in this cluster may reflect potential undertreatment. Although patients in Cluster B had relatively preserved

TABLE 2 | Demographics, clinical, pulmonary, CT-scan, blood, sputum and gene module/profile characteristics of the four different clusters.

	Cluster A: Type 2-high inflammation with small airways dysfunction	Cluster B: Earlier-onset asthma with more severe hyperreactivity	Cluster C: Mild obstructive, ex-smokers with relatively high neutrophil levels	Cluster D: Mild asthma with low inflammation	*p*
Patients	62 (8)	206 (27)	277 (36)	228 (29)	
Demographics and clinical characteristics					
Female sex	40 (64.5)^{C}	137 (66.5)^{C}	101 (36.5)^{A,B,D}	172 (75.4)^{C}	<0.001
Age, years	52.18 ± 8.46 ^{B,D}	34.84 ± 11.52^{A,C,D}	50.92 ± 9.24^{B,D}	42.79 ± 13.31^{A,B,C}	<0.001
BMI, kg/m^2	29.09 ± 6.80^{C,D}	28.86 ± 7.67^{C,D}	26.43 (±3.69)^{A,B}	26.23 (±5.51)^{A,B}	<0.001
Smoking status[a]					
Never smoker	52 (83.9)^{C}	173 (84.4)^{C}	176 (64.0)^{A,B,D}	184 (81.8)^{C}	<0.001
Ex-smoker	9 (14.5)^{C}	23 (11.2)^{C}	90 (32.7)^{A,B,D}	32 (14.2)^{C}	
Current smoker	1 (1.6)	9 (4.4)	9 (3.3)	9 (4.0)	
Pack-years in former and current smokers	2.55 [2.12, 3.38]^{C}	1.00 [0.50, 2.98]^{C,B}	6.00 [4.00, 9.00]^{A,B,D}	3.70 [1.90, 5.00]^{C,B}	<0.001
Patients with GINA classification	^{B,C,D}	^{A,D,C}	^{A,B,D}	^{A,B,C}	<0.001
1	0 (0.0%)	41 (19.9%)	33 (11.9%)	61 (26.8%)	
2	1 (1.6%)	25 (12.1%)	26 (9.4%)	33 (14.5%)	
3	2 (3.2%)	50 (24.3%)	86 (31.0%)	69 (30.3%)	
4	44 (71.0%)	84 (40.8%)	114 (41.2%)	58 (25.4%)	
5	15 (24.2%)	6 (2.9%)	18 (6.5%)	7 (3.1%)	
Severe asthma (GINA 4 or 5)[a]	59 (95.2)^{B,C,D}	90 (43.7)^{A,D}	132 (47.7)^{A,D}	65 (28.5)^{A,B,C}	<0.001
ACQ6 score	1.17 [0.67, 1.83]^{C,D}	1.25 [0.50, 2.30]^{C,D}	0.50 [0.00, 1.16]^{A,B}	0.66 [0.17, 1.33]^{A,B}	<0.001
Age of asthma diagnosis, years	23.99 [6.60, 41.69]^{B,C,D}	10.00 [4.22, 23.87]^{A,C,D}	33.74 [13.25, 44.57]^{A,B}	32.00 [14.97, 45.05]^{A,B}	<0.001
Duration of asthma, years[a]	27.77 [15.17, 42.13]^{B,C,D}	21.05 [11.11, 29.06]^{A,C}	16.4 [6.4, 34.8]^{A,B,D}	7.90 [3.19, 19.27]^{A,C}	<0.001
Use of ICS or ICS/LABA[a]	62 (100)^{B,C,D}	162 (79.0)^{A}	243 (88.4)^{A,D}	159 (71)^{A,C}	<0.001
Daily ICS dose (beclomethasone equivalent) in those on ICS or ICS/LABA, in μg[a]	1000.00 [800.00, 1600.00]^{B,C,D}	800.00 [400.00, 1000.00]^{A}	800.00 [400.00, 1000.00]^{A,D}	500.00 [400.00, 1000.00]^{A,C}	<0.001

(Continues)

TABLE 2 | (Continued)

	Cluster A: Type 2-high inflammation with small airways dysfunction	Cluster B: Earlier-onset asthma with more severe hyperreactivity	Cluster C: Mild obstructive, ex-smokers with relatively high neutrophil levels	Cluster D: Mild asthma with low inflammation	*p*
Use of biologicals	11 (18)	5 (2)	12 (4)	4 (2)	< 0.001
Number of exacerbations during follow up	1.00 [0.00, 3.00]^{B,C,D}	0.00 [0.00, 0.00]^{A}	0.00 [0.00, 0.00]^{A}	0.00 [0.00, 0.00]^{A}	< 0.001
Patients with at least one exacerbation during follow up[a]	45 (73.8)^{B,C,D}	25 (12.6)^{A}	41 (15.3)^{A}	32 (14.5)^{A}	< 0.001
Positive phadiatop test	33 (71.7)^{B}	139 (89.7)^{A,C,D}	161 (79.3)^{B}	121 (75.6)^{B}	0.004
Airway hyperresponsiveness category					< 0.001
Very mild	7 (22.6)	17 (11.3)^{C,D}	58 (29.3)^{B}	59 (33.5)^{B}	
Mild	5 (16.1)^{D}	45 (29.8)	54 (27.3)	65 (36.9)^{A}	
Moderate	7 (22.6)	43 (28.5)	47 (23.7)	37 (21.0)	
Severe	12 (38.7)^{C,D}	46 (30.5)^{C,D}	39 (19.7)^{A,B,D}	15 (8.5)^{A,B,C}	
FeNO. Parts per billion	28.00 [20.50, 65.50]^{D}	25.00 [14.00, 42.50]^{D}	26.00 [18.00, 37.00]^{D}	21.00 [14.00, 33.00]^{A,B,C}	0.002
Pulmonary physiology					
FEV_1, % predicted (post-bronchodilator)	75.66 (±13.00) ^{B,C,D}	92.09 (±9.40)^{A,C,D}	85.71 (±10.73)^{A,B,D}	98.84 (±7.67)^{A,B,C}	< 0.001
FEF_{25-75}, % predicted (post-bronchodilator)	43.62 (±26.85)^{B,C,D}	78.35 (±31.57)^{A,C,D}	63.29 (±26.97)^{A,B,D}	102.59 (±36.05)^{A,B,C}	< 0.001
RV/TLC, %	45.04 (±9.56)^{B,C,D}	29.93 (±8.00)^{A,C,D}	34.61 (±7.89)^{A,B,D}	31.12 (±8.22)^{A,B,C}	< 0.001
R_{5-20}, kPa/L/s	0.18 [0.07, 0.27]^{B,C,D}	0.06 [0.02, 0.11]^{A,D}	0.04 [0.02, 0.09]^{A,D}	0.03 [0.01, 0.06]^{A,B,C}	< 0.001
S_{COND}, 1/L	0.06 [0.04, 0.09]^{B,C,D}	0.03 [0.02, 0.05]^{A,D}	0.03 [0.02, 0.05]^{A,D}	0.02 [0.01, 0.03]^{A,B,C}	< 0.001
CT-scan-derived parameters					
Wall area/total area %	65.61 (±3.42)^{B,C,D}	64.15 (±2.78)^{A,C,D}	62.40 (±3.59)^{A,B}	61.57 (±3.11)^{A,B}	< 0.001
VI 950%	4.81 [2.49, 7.16]^{B}	3.13 [0.95, 6.84]^{A,C}	4.38 [1.90, 9.32]^{B}	3.00 [1.55, 6.86]	0.112
Mean lung density ratio, E/I	0.88 [0.86, 0.92]^{B,D}	0.78 [0.74, 0.83]^{A,C}	0.87 [0.82, 0.91]^{B,D}	0.81 [0.75, 0.86]^{A,C}	< 0.001
Lung volume ratio, E/I	0.61 [0.54, 0.73]^{B,C,D}	0.45 [0.39, 0.50]^{A,C,D}	0.53 [0.47, 0.64]^{A,B,D}	0.49 [0.40, 0.57]^{A,B,C}	< 0.001
Blood-derived parameters					

(Continues)

TABLE 2 | (Continued)

	Cluster A: Type 2-high inflammation with small airways dysfunction	Cluster B: Earlier-onset asthma with more severe hyperreactivity	Cluster C: Mild obstructive, ex-smokers with relatively high neutrophil levels	Cluster D: Mild asthma with low inflammation	*p*
Blood basophil count, 10^9/L	0.06 [0.03, 0.09]^{B,C,D}	0.04 [0.02, 0.06]^{A,D}	0.03 [0.02, 0.05]^{A}	0.03 [0.02, 0.05]^{A,B}	< 0.001
Blood eosinophil count, 10^9/L	0.50 [0.29, 0.72]^{B,C,D}	0.26 [0.18, 0.40]^{A,C,D}	0.24 [0.14, 0.35]^{A,B,D}	0.17 [0.10, 0.29]^{A,B,C}	< 0.001
Blood lymphocyte count, 10^9/L	2.06 [1.57, 2.40]^{C}	2.09 [1.63, 2.58]^{C,D}	1.79 [1.45, 2.10]^{A,B}	1.83 [1.58, 2.12]^{B}	< 0.001
Blood monocyte count, 10^9/L	0.50 [0.43, 0.67]^{B,C,D}	0.49 [0.39, 0.60]^{A,D}	0.46 [0.38, 0.58]^{A,D}	0.43 [0.34, 0.52]^{A,B,C}	< 0.001
Blood neutrophil count, 10^9/L	4.16 [3.27, 5.40]^{C,D}	3.90 [2.90, 4.95]^{D}	3.69 [2.99, 4.59]^{A,D}	3.40 [2.87, 4.28]^{A,B,C}	0.001
Sputum-derived parameters					
Bronchial epithelial cells sputum, % of non-squamous cells	1.50 [0.70, 2.00]	1.70 [0.85, 5.05]	2.05 [1.00, 3.88]	1.55 [0.45, 3.62]	0.281
Eosinophils sputum, % of non-squamous cells	29.40 [1.90, 55.70]^{B,C,D}	0.50 [0.10, 2.50]^{A,D}	0.75 [0.10, 5.10]^{A,D}	0.10 [0.00, 0.58]^{A,B,C}	< 0.001
Lymphocytes sputum, % of non-squamous cells	0.40 [0.10, 0.60]^{D}	0.70 [0.30, 1.25]	0.50 [0.20, 1.10]	0.80 [0.30, 1.80]^{A}	0.053
Macrophages sputum, % of non-squamous cells	8.40 [7.00, 16.50]^{B,C,D}	48.20 [25.00, 69.20]^{A,C}	28.10 [15.03, 44.90]^{A,B,D}	45.95 [23.95, 64.40]^{A,C}	< 0.001
Neutrophils sputum, % of non-squamous cells	50.80 [26.50, 61.10]	40.00 [20.35, 60.25]^{C}	61.25 [43.35, 78.83]^{B,D}	47.25 [25.52, 69.20]^{C}	0.001
Gene profile					
Gene module 1, GSVA score	−0.34 [−0.49, −0.01]^{C,D}	−0.31 [−0.53, 0.04]^{C,D}	0.00 [−0.37, 0.25]^{A,B,D}	0.12 [−0.21, 0.50]^{A,B,C}	< 0.001
Gene module 2, GSVA score	−0.23 [−0.50, 0.02]^{C}	−0.29 [−0.51, 0.11]^{C,D}	0.00 [−0.37, 0.37]^{A,B}	0.04 [−0.36, 0.44]^{B}	< 0.001
Gene module 3, GSVA score	−0.40 [−0.51, −0.17]^{C,D}	−0.32 [−0.47, −0.11]^{C,D}	−0.05 [−0.30, 0.25]^{A,B,D}	0.29 [−0.11, 0.53]^{A,B,C}	< 0.001
Gene module 4, GSVA score	0.05 [−0.20, 0.26]^{B,D}	0.27 [0.02, 0.56]^{A,C,D}	0.13 [−0.24, 0.44]^{B,D}	−0.22 [−0.38, −0.12]^{A,B,C}	< 0.001
Gene module 5, GSVA score	0.24 [−0.02, 0.46]^{D}	0.43 [0.11, 0.57]^{C,D}	0.05 [−0.31, 0.34]^{B,D}	−0.45 [−0.58, −0.17]^{A,B,C}	< 0.001
Gene module 6, GSVA score	0.19 [−0.27, 0.54]^{D}	0.20 [−0.14, 0.41]^{D}	−0.03 [−0.37, 0.41]^{D}	−0.38 [−0.58, 0.15]^{A,B,C}	< 0.001

Note: Data are presented as *n*, *n* (%), mean ± SD or median [interquartile range]. Differences between groups were tested using ANOVA for normally distributed variables, Kruskal–Wallis for non-normally distributed variables, and Chi-square tests for categorical variables. GINA treatment steps were determined based on medication usage, according to the 2012 guidelines. Very mild airway hyperresponsiveness = $PC_{20} \geq 4$ & < 16 mg/mL, $PD_{20} \geq 0.5$ & < 2 mg. Mild airway hyperresponsiveness = $PC_{20} \geq 1$ & < 4 mg/mL, PD20 ≥ 0.13 & < 0.5 mg. Moderate airway hyperresponsiveness = $PC_{20} \geq 0.25$ & < 1 mg/mL, $PD_{20} \geq 0.03$ & < 0.13 mg. Severe airway hyperresponsiveness = $PC_{20} < 0.25$ mg/mL, PD20 < 0.03 mg. ^{ABCD}Statistically significant differences ($p < 0.05$) between clusters are indicated with the corresponding superscript.

Abbreviations: ACQ6, asthma control questionnaire 6; BMI, body mass index; E/I, expiratory/inspiratory; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; R_{5-20}, resistance at 5 Hz–resistance at 20 Hz; RV/TLC, residual volume/total lung capacity; S_{cond}, ventilation heterogeneity in the conductive zone of the lungs corrected for tidal volume; VI 950, voxel index at −950 Hounsfield units.

[a]Variables were not included to generate the clusters.

TABLE 3 | Qualitative summary of the key characteristics for each ATLANTIS asthma cluster.

	Sex differences	Relation with (former) smoking	Severe asthma (reflected by GINA classification)	Control of asthma symptoms	Atopic constitution	Airway hyperresponsiveness	Large airway involvement	Small airway involvement	Sputum eosinophils	Sputum neutrophils	Gene cluster
Cluster A	F > M	No	+++	–	+	++++	++	+++	++	–	4–6
Cluster B	F > M	No	++	–	++	+++	–	+	–	–	4–6
Cluster C	M > F	Yes	++	+	+	++	+	+	–	++	1–3
Cluster D	F > M	No	+	+	+	+	–	–	–	–	1–3

lung function and did not show elevated conventional type-2 biomarkers, the presence of a more severe airway hyperresponsiveness combined with a higher level of nasal epithelial inflammatory gene expression suggests ongoing disease activity that is not captured by routine clinical markers. Airway hyperresponsiveness has been associated with future risk of asthma progression and lung function decline [20]. Therefore, cluster B patients with apparently mild disease may still be at risk in the longer term if insufficiently treated. Future studies are needed to establish whether this subgroup may benefit from intensified anti-inflammatory treatment strategies to prevent future risk.

The transcriptomic profile in Cluster B aligns with expectations, as this cluster represents patients with atopy, which has previously been described to be related to these genes [21]. Earlier asthma onset, as found in Cluster B patients, is also associated with a higher genetic predisposition compared to late-onset adult asthma, which is more influenced by environmental risk factors [22]. Asthma-related genes play a crucial role in regulating immune responses, airway inflammation, and airway remodeling, which are key features of asthma pathogenesis [23]. In addition, Cluster B shows similarities to Cluster 1 identified in the Severe Asthma Research Program (SARP) cluster analysis [6], as both clusters are characterized by early-onset disease, atopy, and a relatively preserved lung function, suggesting consistency of this phenotype in different study populations.

Cluster C exhibited the highest levels of sputum neutrophils and pack-years of smoking compared to the other clusters, along with a poorer lung function compared to Clusters B and D. At first glance, this finding regarding neutrophils may seem contradictory to previous analyses in ATLANTIS [24], which indicated that sputum neutrophilia was not associated with a distinct clinical phenotype. However, it is important to consider that different methodologies were used. In the previous analysis, neutrophilia was treated as a dichotomous variable, while in the current analysis, neutrophil counts were analyzed as a continuous variable. Even though the previous analysis found no association between clinical characteristics and sputum neutrophilia, we do not rule out the possibility that a subgroup of asthma patients exists with poorer lung function and higher pack-years, in which neutrophils can play a role. The latter can be supported by findings from the SARP analysis, which identified an asthma cluster associated with worse lung function and elevated sputum neutrophils [25]. Nevertheless, the role of neutrophilia in asthma remains a topic of debate.

Cluster D was identified as a mild cluster with well-controlled asthma, normal lung function, and low inflammatory cell counts in both blood and sputum. This mild asthma cluster with normal lung function has been consistently observed in previous cluster analyses, where eosinophils were also shown to be low [5, 7]. In line with the low levels of type-2 inflammatory cells observed in blood and sputum, gene modules 4–6 were also downregulated. Previously, it was shown that the expression levels of these genes associate with blood and sputum eosinophil levels.

A strength of our study was the inclusion of a large cohort of asthma patients with extensive characterization at both clinical and molecular levels. However, in the current study, patients

were only followed up for a period of 1 year. A longer and more comprehensive follow-up would not only allow for a better assessment of lung function changes but also enable the investigation of the stability of the clusters over time. In addition, not all key variables contributing to cluster formation, such as airway hyperresponsiveness and nasal gene expression, were reassessed at follow-up. As a result, we were unable to repeat the clustering analysis over time. Another limitation is the lack of external validation, as no independent cohort with comparable data on small airway function and nasal gene expression was available. In addition, information on comorbidities was not systematically collected; therefore, we could not assess their potential impact on the asthma phenotypes. Finally, we acknowledge that inter-individual variation within clusters is an inherent limitation of cluster analysis. This variation should be considered when interpreting the findings, as generalizations may not apply to all individuals within a cluster.

In conclusion, this study identified four clinically distinct asthma clusters: (A) Type 2-high inflammation with small airways dysfunction; (B) Earlier-onset asthma with more severe hyperreactivity; (C) Mild obstructive, ex-smokers with relatively high neutrophil levels; (D) Mild asthma with low inflammation. Our findings emphasize the importance of assessing small airways dysfunction in patients with type 2-high inflammation and airway hyperresponsiveness in earlier-onset asthma patients, despite relatively preserved lung function. The identification of transcriptomic involvement in the earlier-onset asthma cluster may provide insights into underlying disease mechanisms. These results underscore the importance of integrating clinical and molecular characteristics in asthma phenotyping, potentially leading to more personalized and effective treatment strategies.

Author Contributions

P.J.M.K., T.K., J.E.H. contributed to data curation and formal analysis. P.J.M.K., T.K., and M.v.d.B. had access to and verified the data and were responsible for the decision to submit. M.K., S.S., L.M.F., B.B., K.F.R., A.P., C.E.B., D.S., J.W.H.K., L.M.F., and M.v.d.B. were responsible for the conceptualization of the study. The specific conceptualization and design of the manuscript were carried out by D.-J.S. and M.v.d.B. P.J.M.K. wrote the first draft of the manuscript. H.A.M.K., I.H.H., S.D.P., and D.-J.S. supervised. All authors had access to the raw data, contributed to the interpretation of results, and reviewed and edited the manuscript.

Conflicts of Interest

M.K. reports grants paid to their institution from the National Institutes of Health, American Lung Association, Areteia, AstraZeneca, and Sanofi (funds paid to the University of Arizona through June 2022 and now to the Icahn School of Medicine at Mount Sinai). They report personal fees for consultancies from AstraZeneca, Sanofi, Chiesi, GSK, Kinaset, and Genentech; speaker fees from Chiesi and Regeneron; travel support from the European Respiratory Society; participation on the ALung Data Safety Monitoring Board (funds paid to them); equity in RaeSedo Inc. (a company developing therapeutics for asthma in the preclinical phase, with no human trials, IND, or revenue); one issued and two pending patents for the development of therapeutics for inflammatory lung disease (no funding); leadership roles in the National Heart, Lung and Blood Advisory Council (completed in 2022, funds paid to them) and the Association of Professors of Medicine (unpaid); and personal fees as a Section Editor for UpToDate. L.M.F. reports consulting fees from Chiesi, GSK, AstraZeneca, Novartis, Verona Pharma, and ICON; speaker fees from Chiesi, GSK, Lusofarmaco, and Sanofi; participation on advisory boards for Chiesi, ICON, and Novartis. K.F.R. reports payments for lectures from AstraZeneca, Boehringer Ingelheim, Chiesi Pharmaceuticals, Novartis, Sanofi & Regeneron, GlaxoSmithKline, Berlin Chemie, and Roche Pharma; participation on advisory boards for AstraZeneca, Boehringer Ingelheim, and Sanofi & Regeneron; and leadership roles in the German Center for Lung Research (DZL), German Chest Society (DGP), and American Thoracic Society (ATS). A.P. reports grants to their institution from Chiesi, AstraZeneca, GlaxoSmithKline, and Sanofi; consulting fees from Chiesi, AstraZeneca, GlaxoSmithKline, Novartis, Sanofi, Iqvia, Avillion, Elpen Pharmaceuticals, Moderna, and Roche; honoraria for lectures from Chiesi, AstraZeneca, GlaxoSmithKline, Menarini, Zambon, Mundipharma, Sanofi, Edmond Pharma, Iqvia, Avillion, Sanofi, and Regeneron; participation on advisory boards for Chiesi, AstraZeneca, GlaxoSmithKline, Novartis, Sanofi, Iqvia, Avillion, Elpen Pharmaceuticals, and Moderna; and receipt of materials from Consorzio Futuro in Ricerca. C.B. reports grants and consultancy fees paid to their institution from 4D Pharma, Areteia, AstraZeneca, Chiesi, Genentech, GlaxoSmithKline, Mologic, Novartis, Regeneron Pharmaceuticals, Roche, and Sanofi. D.S. reports consulting fees received from Aerogen, AstraZeneca, Boehringer Ingelheim, Chiesi, Cipla, CSL Behring, EpiEndo, Genentech, GlaxoSmithKline, Glenmark, Gossamer Bio, Kinaset Therapeutics, Menarini, Novartis, Orion, Pulmatrix, Sanofi, Synairgen, Teva, Theravance Biopharma, Verona Pharma. J.W.H.K. reports grants to their institution from AstraZeneca, Boehringer Ingelheim, Chiesi, GSK, and Valneva; consulting fees from AstraZeneca, Boehringer Ingelheim, Chiesi, GSK, TEVA, MSD, COVIS Pharma, and Jansen; honoraria for lectures from Mundi Pharma and ALK-Albello; leadership roles as ERS group chair, President/board member of the International Primary Care Respiratory Group, member of the CAHAG scientific committee, and board member of the Inhalation Institute Netherlands; stocks as Director of the General Practitioners Research Institute and $<5\%$ in Lothar Medtec. H.A.M.K. reports grants from GlaxoSmithKline and Novartis, and participation on advisory boards with GlaxoSmithKline, Novartis, AstraZeneca, and Sanofi. I.H.H. reports receiving research grants from Roche, Boehringer Ingelheim, Health Holland, Netherlands Lung Foundation, and NWO, outside the submitted work. M.v.d.B. reports receiving research grants paid to their institution from GlaxoSmithKline, Chiesi, AstraZeneca, Novartis, Genentech, and Roche. All other authors declare no competing interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** all70127-sup-0001-DataS1.docx.