Neutrophilic inflammation in sputum or blood does not define a clinically distinct asthma phenotype in ATLANTIS

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Shareable abstract (@ERSpublications)
This study within ATLANTIS and U-BIOPRED reveals that neither sputum nor blood neutrophilia in asthma patients is associated with disease severity. Neutrophilia within sputum or blood does not define a clinically distinct asthma phenotype. https://bit.ly/4evRuCF

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Abstract

Introduction Neutrophilic asthma has been suggested to be a clinically distinct phenotype characterised by more severe airflow obstruction and higher exacerbation risk. However, this has only been assessed in few and smaller studies, using different cut-offs to define neutrophilia, and with conflicting results. We used data from ATLANTIS, an observational longitudinal study including a large number of patients with asthma and healthy controls. The aim of the present study was to examine whether neutrophilic inflammation, either in sputum or blood, is more prevalent in asthma and whether it correlates with disease severity.

Methods ATLANTIS included 773 asthma patients, with blood collected from 767 (99%) and sputum from 228 patients (30%). Data were available from 244 healthy controls, all providing blood and 126 (52%) providing sputum. Asthma patients were characterised, including parameters of large and small airways disease at baseline and after 6 and 12 months of follow-up. Sputum and blood neutrophilia were defined as values exceeding the upper quartile in asthma patients.

Results The prevalence of sputum neutrophilia did not differ between asthma patients and healthy controls. Asthma patients with sputum neutrophilia did not display more severe symptoms, large or small airways disease or more frequent exacerbations. Blood neutrophilia was more common in asthma and was associated with higher body mass index, female sex, current smoking and systemic corticosteroid use. Patients with blood neutrophilia had a statistically significant, but small, increase in residual volume/total lung capacity. Blood neutrophilia was not associated with large or small airways disease or exacerbation risk.

Conclusion Sputum and blood neutrophilia do not define a distinct clinical phenotype in asthma.

Background

Asthma is a heterogeneous disease where the presence of type 2 inflammation is a well-recognised clinical phenotype [1–4]. It has been suggested that neutrophilic inflammation also represents a phenotype with a

distinct clinical expression of asthma. Neutrophils can migrate from the circulation to the airways when triggered by pollutants or pathogens, leading to the release of inflammatory mediators, potentially causing airway damage [5, 6].

Some studies have shown that asthma patients with high percentages of sputum neutrophils have more severe disease, lower lung function and higher exacerbation risk [4, 7–9]. However, this is subject to debate, as other studies were unable to replicate these results [10, 11]. Interpretation of studies so far has been hampered by different definitions used for neutrophilia, ranging from sputum neutrophils >40% to >76% [2, 12–14]. Most studies contained relatively small sample sizes without validation of findings in an independent cohort. Also, the studies so far have not had systematic assessment of the small airways. Therefore, we aimed to investigate whether sputum or blood neutrophilia in asthma relates to a clinically distinct phenotype in a large well-characterised asthma cohort, including the assessment of small airways.

The ATLANTIS study (Assessment of Small Airways Involvement in Asthma) included 773 asthma patients with a variety of disease severity [15, 16]. At baseline, patients were extensively assessed clinically, and subsequently observationally followed over 1 year with spirometry. In the current study, we assessed the prevalence of sputum and blood neutrophilia in patients with asthma and compared this with healthy controls. In addition, we compared the level of symptoms, large and small airways function, and the number of exacerbations between patients with and without neutrophilic asthma in the ATLANTIS cohort. We defined neutrophilic inflammation using different cut-offs and replicated our findings in U-BIOPRED (Unbiased BIOmarkers in PREDiction of respiratory disease study) [17].

Methods

Participants

The ATLANTIS study included patients with asthma from 29 centres across nine countries between June 2014 to March 2017. Inclusion and exclusion criteria have been published previously (NCT02123667) [15, 16]. Briefly, patients were between 18–65 years old, had a clinical asthma diagnosis supported by objective evidence of airway hyperresponsiveness (AHR), bronchodilator reversibility or peak expiratory flow variability. Subjects were required to have stable disease. The main exclusion criteria were a COPD diagnosis or lifetime smoking history of 10 or more pack-years. In addition, ATLANTIS included individuals who did not exhibit respiratory symptoms and had normal spirometry without AHR. To examine the prevalence of neutrophilia in healthy controls, data of three additional studies were combined to expand the healthy control group: ARMS (NCT03141814), NORM (NCT00848406) and SHERLOCK (NCT04263961). For all studies, approval from the local ethics committee was obtained, and the included participants gave their written informed consent.

Study design and procedures

Clinical characterisation was performed at baseline visits, including large and small airway function, multiple breath nitrogen washout (MBNW), AHR measurement, sputum induction, blood collection, computed tomography (CT) scan and questionnaires (Asthma Control Questionnaire 6 (ACQ6)) [16]. Sputum was collected in a subset of participants at selected sites in Europe, the United States and Canada. Sputum samples containing plugs with lower respiratory tract cells were selected. Sputum was processed as described by Hargreave *et al.* [18] and percentages of nonsquamous cells were counted; a detailed description of the sputum processing is provided in the supplement. Subsequently, participants had follow-up visits at 6 and 12 months, and telephone follow-ups at 3 and 9 months, to review exacerbation occurrence. At the 6- and 12-month visits, spirometry was performed and the ACQ6 questionnaire was completed to assess asthma control. Exacerbations were defined as a deterioration of asthma requiring a systemic course of corticosteroids (⩾3 days) and/or hospitalisation and/or emergency room attendance.

Cut-offs to define neutrophilic inflammation in sputum and blood

We chose to use a cut-off value defined by the upper quartile (25%) for neutrophilia in sputum and blood in ATLANTIS asthma patients. This corresponded to ⩾70.6% neutrophils in sputum and to ⩾$4.7\times10^{9}\cdot L^{-1}$ blood neutrophils. Since we recognise that such a cut-off is arbitrary, we also performed a subanalysis using a stricter cut-off for sputum neutrophilia defined by the upper fifth percentile (>5%) in asthma patients corresponding to ⩾89.6% sputum neutrophils. Additionally, an analysis was conducted using the absolute cell count of neutrophils in sputum, with the cut-off also defined by the upper quartile, corresponding to 2.26×10^{6} neutrophils in the sputum. Finally, to align the findings with some previous studies, subjects were classified into four groups based on the combination of sputum eosinophil and neutrophil percentages as follows: eosinophilic inflammation without neutrophilia, mixed inflammation of both eosinophilia and neutrophilia, neutrophilic inflammation without eosinophilia and paucigranulocytic inflammation consisting of neither eosinophilia nor neutrophilia. For eosinophilia a cut-off score of 2.8%

was used, defined by the upper quartile of sputum eosinophilia within ATLANTIS asthma patients, which is in line with thresholds used in previous studies [1–3, 19].

Statistical analysis

All statistical analyses were performed using RStudio (version 3.5.1). Histograms were used to assess variables on distribution normality. Univariate differences between groups were analysed using chi-squared tests, independent t-tests and Mann–Whitney U-tests as appropriate. The package "tableone" (version 0.13.0) in R was used to create baseline tables. Non-normally distributed variables are presented as median with interquartile range (IQR). For the multivariate analysis, we used a model correcting for age, sex, body mass index (BMI), current smoking, ex-smoking, corticosteroids use and blood eosinophils. For the multivariate analysis, variables with a $p<0.1$ in the univariate analyses were added one by one. Linear mixed effect models were created using the "lmerTest" (version 3.1.3) package and run to investigate the effect of sputum or blood neutrophilia on forced expiratory volume in 1 s (FEV_1) and ACQ6 across the follow-up visits, while adjusted for age, sex, BMI, ex-smoking, corticosteroid use and blood eosinophils. A Pearson's correlation test was used to assess the correlation between absolute counts and percentage sputum neutrophils. An analysis on the occurrence of exacerbations in relation to neutrophilia was carried out using "survminer" (version 0.4.9) and "ggplot2" (version 3.3.1). Subjects were censored after their first exacerbation or after their last recorded visit. Finally, we used a Cox regression model to analyse the association between neutrophilia and exacerbations. The model was corrected for age, sex, BMI, ex-smoking, corticosteroids use and blood eosinophils.

Findings were validated in a subset of the U-BIOPRED study, comprising cohort A (adults with severe asthma and less than 5 pack-years) and cohort B (adults with severe asthma and over 5 pack-years) [17].

Results

Comparing the occurrence of sputum neutrophilia in asthma patients and healthy controls

Of 773 patients with asthma included in ATLANTIS, sputum was collected in a subset, totalling 228 patients with available sputum data. 58 (25%) patients had sputum neutrophilia (55% female, mean±SD age 45±13 years, 3% current smokers), whereas 170 (75%) did not (49% female, mean±SD age 43±14 years, 3% current smokers). The control group (Supplementary Table 1) consisted of 244 subjects and in 126 subjects, sputum was induced. 31 (25%) subjects of the healthy control group had sputum neutrophilia (55% female, mean±SD age 47±15 years, 26% current smokers), whereas 95 (75%) did not (41% female, mean±SD age 45±16 years, 30% current smokers). The occurrence of sputum neutrophilia was not more frequent in asthma patients compared with healthy controls (χ^2 0.030, p=0.86) (figure 1).

Correlation between percentage sputum neutrophils and absolute sputum neutrophil counts

Pearson's correlation analysis revealed a moderate positive relationship between percentage sputum neutrophils and absolute sputum neutrophil counts ($r=0.51$, $p<0.01$), indicating a statistically significant association, with higher percentages of sputum neutrophils being associated with higher absolute counts (Supplementary Figure 1).

Differences in clinical characteristics between asthma patients with and without sputum neutrophilia

Clinical characteristics of asthma patients with and without sputum neutrophilia are presented in table 1. The groups did not differ with respect to medication use, disease severity or lung function parameters of large and small airways function. CT scan-derived parameters were available in 316 ATLANTIS asthma subjects. In patients with sputum neutrophilia, the percentage of median lumen area, wall area and median total area were significantly larger and median airway wall area to median total area significantly lower, both in the univariate and multivariate analysis (table 1 and Supplementary Table 2). We also performed subgroup analyses, one using the stricter cut-off score of ⩾89.6% for sputum neutrophils and one only including those with more severe asthma (Global Initiative for Asthma (GINA) treatment step 4/5), which yielded similar results (Supplementary Tables 3–5). Patients with sputum neutrophilia based on the absolute sputum neutrophil count were older compared with patients without sputum neutrophilia; the other results were similar to those obtained using the percentage of sputum neutrophils as the cut-off criterion (Supplementary Table 6).

Sputum neutrophilia and its association with lung function and asthma control over time

A linear mixed model showed that sputum neutrophilia was not associated with changes in FEV_1 (visit 2×sputum neutrophilia: p=0.62, visit 3×sputum neutrophilia: p=0.43), nor with changes in ACQ6 score (visit 2×blood neutrophilia p=0.55, visit 3×blood neutrophilia p=0.30) at follow-up.

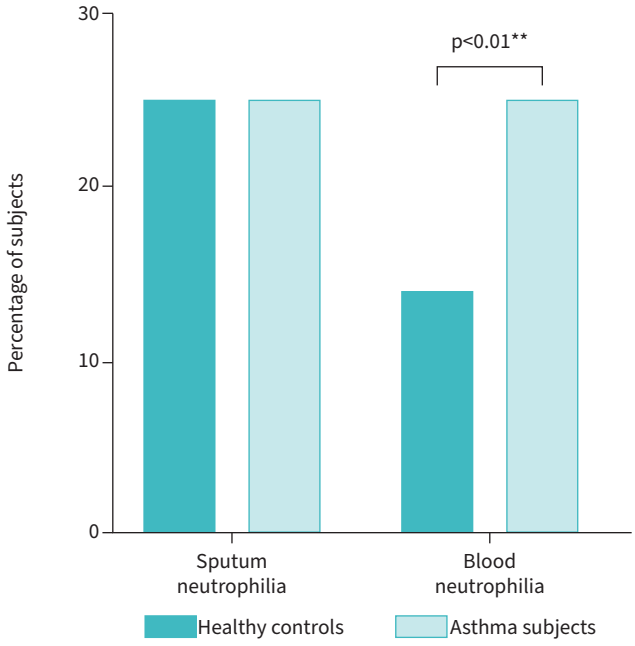

FIGURE 1 Distribution of neutrophilic inflammation in asthma and healthy controls. Blood neutrophilia, but not sputum neutrophilia, was more common in people with asthma compared with healthy controls. **: p<0.01.

Association between sputum and nonsputum neutrophilia and asthma exacerbations

Next, we assessed whether sputum neutrophilia is associated with the occurrence of asthma exacerbations. Data on both exacerbation occurrence and sputum cell counts were available for 225 asthma patients; 58 of these patients showed sputum neutrophilia, whereas 168 did not. The Kaplan–Meier curve (figure 2a) and Cox regression analysis (figure 2b) did not show a difference in time to first exacerbation between patients with and without sputum neutrophilia.

Clinical characteristics associated with sputum inflammatory subtypes in asthma patients

To assess the different inflammatory asthma subtypes, patients were divided into four groups according to their sputum granulocyte levels. Table 2 shows the characteristics of the four inflammatory subtypes: eosinophilia without neutrophilia (n=52), neutrophilia without eosinophilia (n=52), mixed granulocytic (n=6) and paucigranulocytic inflammation (n=118). The mixed granulocytic inflammatory subtype was rare compared with the other subtypes. The eosinophilic inflammatory subtype shows higher exhaled nitric oxide fraction (F_{ENO}) levels, more airflow obstruction as reflected by a lower FEV_1 and more hyperinflation as reflected by residual volume (RV)/total lung capacity (TLC), compared with the neutrophilic and paucigranulocytic inflammatory subtype. In addition, the eosinophilic subtype has a higher prevalence of GINA 4 or 5, increased use of inhaled corticosteroids (ICSs) and more small airway disease as reflected by S_{ACIN} (ventilation homogeneity of the acinar zone of the lungs corrected for tidal volume) and S_{COND} (ventilation heterogeneity in the conductive zone of the lungs corrected for tidal volume), compared with the paucigranulocytic subtype alone. When comparing the isolated neutrophilia inflammatory subtype to the paucigranulocytic inflammatory subtype, no differences in patient characteristics, disease severity or parameters of the large and small airways were found.

Thus, our analysis reveals notable distinctions between eosinophilic and non-eosinophilic inflammatory asthma subtypes. In contrast, the neutrophilic subtype did not exhibit discernible differences when compared with the paucigranulocytic subtype in terms of patient characteristics, disease severity or parameters of large and small airways.

Comparing the occurrence of blood neutrophilia in asthma patients and healthy controls

Blood was collected in 767 ATLANTIS asthma patients and in 244 healthy controls. 194 (25%) asthma patients showed blood neutrophilia (67% female, mean±SD age 44±12, 4% current smoker), and 573 patients did not (56% female, mean±SD age 45±13 years, 2% current smokers). Within the healthy control group, 34 subjects showed blood neutrophilia (53% female, mean±SD age 41±13 years, 27% current smokers), whereas 210 subjects had no blood neutrophilia (50% female, mean±SD age 44±16 years, 17%

TABLE 1 Baseline characteristics of asthma subjects in ATLANTIS stratified for sputum neutrophilia

	No sputum neutrophilia (<70.6%)	Sputum neutrophilia (⩾70.6%)	p-value
Subjects, n	170	58	
Neutrophils in sputum, % of nonsquamous cells	39.80 (21.27–54.82)	84.25 (77.05–88.53)	
Age, years	42.85±13.65	44.62±13.07	0.39
Female sex	84 (49.4)	32 (55.2)	0.54
BMI, kg·m^{-2}	27±5.25	28±5.66	0.33
Age of asthma diagnosis, years	17.24 (5.45–34.79)	16.82 (6.77–38.54)	0.61
Smoking status			0.96
Current smoker	5 (2.9)	2 (3.4)	
Ex-smoker	36 (21.2)	13 (22.4)	
Never-smoker	129 (75.9)	43 (74.1)	
Pack-years in current and ex-smokers	0.00 (0.00–0.00)	0.00 (0.00–0.20)	0.55
Total cell count sputum, ×10^{6} U·mL^{-1}	1.98 (1.04–3.36)	3.55 (1.60–6.30)	**<0.01**
Blood neutrophil count, ×10^{9}·L^{-1}	3.40 (2.78–4.30)	3.74 (3.04–4.92)	0.06
Blood eosinophil count, ×10^{9}·L^{-1}	0.21 (0.15–0.40)	0.20 (0.11–0.30)	0.07
Sputum eosinophils, % of nonsquamous cells	0.60 (0.10–4.07)	0.40 (0.00–1.48)	0.05
Positive Phadiatop test	122 (81.9)	33 (73.3)	0.30
F_{ENO}, parts per billion	22.00 (15.00–37.00)	21.00 (16.00–38.00)	0.86
ACQ6 score	0.80 (0.33–1.33)	0.74 (0.21–1.67)	0.81
GINA classification			0.84
1	39 (22.9)	10 (17.2)	
2	20 (11.8)	9 (15.5)	
3	44 (25.9)	15 (25.9)	
4	61 (35.9)	21 (36.2)	
5	6 (3.5)	3 (5.2)	
Systemic corticosteroid use	4 (2.4)	2 (3.4)	1.00
Systemic corticosteroid dose in those on systemic corticosteroids, mg	5.00 (3.12–17.50)	12.50 (8.75–16.25)	0.77
Use of ICS or ICS/LABA	130 (76.5)	47 (81.0)	0.59
Daily ICS dose (beclomethasone equivalent) in those on ICS or ICS/LABA, µg	800 (400–1000)	600 (400–1000)	0.34
Airway hyperresponsiveness category			0.69
Very mild (PC_{20} ⩾4 and <16 mg·mL^{-1}, PD_{20} ⩾0.5 and <2 mg)	39 (30.2)	15 (35.7)	
Mild (PC_{20} ⩾1 and <4 mg·mL^{-1}, PD_{20} ⩾0.13 and <0.5 mg)	40 (31.0)	12 (28.6)	
Moderate (PC_{20} ⩾0.25 and <1 mg·mL^{-1}, PD_{20} ⩾0.03 and <0.13 mg)	33 (25.6)	12 (28.6)	
Severe (PC_{20} <0.25 mg·mL^{-1}, PD_{20} <0.03 mg)	17 (13.2)	3 (7.1)	
Severe/moderate airway hyperresponsiveness (PC_{20} <1, PD_{20} <0.13 mg)	50 (38.8)	15 (35.7)	0.87
FEV_1 % pred (post-bronchodilator)	90.23±10.97	89.15±10.73	0.52
FEV_1/FVC, % (post-bronchodilator)	73.48±9.68	72.33±9.76	0.44
Variables on SAD			
RV/TLC, %	32.00±7.74	31.00±8.92	0.46
S_{ACIN}, L^{-1}	0.09 (0.06–0.15)	0.10 (0.06–0.17)	0.54
S_{COND}, L^{-1}	0.03 (0.02–0.05)	0.03 (0.02–0.05)	0.64
$R_{5–20}$, kPa·L^{-1}·s^{-1}	0.04 (0.02–0.07)	0.04 (0.01–0.08)	0.92
R_5, kPa·L^{-1}·s^{-1}	0.34 (0.27–0.42)	0.35 (0.28–0.42)	0.72
R_{20}, kPa·L^{-1}·s^{-1}	0.31 (0.25–0.35)	0.30 (0.25–0.36)	0.69
X5, kPa·L^{-1}·s^{-1}	−0.10 (−0.14– −0.07)	−0.11 (−0.13– −0.08)	0.64
AX, Hz×kPa·L^{-1}·s^{-1}	0.30 (0.14–0.52)	0.29 (0.18–0.59)	0.62
CT scan-derived parameters			
Median lumen area, mm^2	19.99±4.21	24.55±4.50	**<0.01**
Median wall area, mm^2	33.75±5.74	37.59±5.40	**<0.01**
Median total area, mm^2	54.27±9.10	62.34 ±9.29	**<0.01**
Wall area/total area %	62.71±3.41	60.51±2.95	**<0.01**
Pi10	7.10±1.17	7.20±1.15	0.70
VI 856%	7.99 (2.65–18.35)	8.38 (2.36–17.16)	0.94
VI 950%	3.95 (2.11–8.30)	4.06 (2.50–8.36)	0.71
Lung volume ratio, E/I	0.49±0.14	0.49±0.13	0.90
Mean lung density ratio, E/I	0.81±0.08	0.81±0.08	0.92

Data are presented as n (%), mean±SD or median (IQR) unless otherwise specified. BMI: body mass index; IQR: interquartile range; F_{ENO}: fractional exhaled nitric oxide; ACQ6: asthma control questionnaire 6; GINA: Global Initiative for Asthma; ICS: inhaled corticosteroids; LABA: long acting β2 agonist; PC_{20}: provocative concentration causing a 20% fall in FEV_1; PD_{20}: provocative dose causing a 20% fall in FEV_1; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; SAD: small airways disease; RV: residual volume; TLC: total lung capacity; S_{ACIN}: ventilation homogeneity of the acinar zone of the lungs corrected for tidal volume; S_{COND}: ventilation heterogeneity in the conductive zone of the lungs corrected for tidal volume; $R_{5–20}$: resistance at 5 Hz − resistance at 20 Hz; R_5: resistance at 5 Hz; R_{20}: resistance at 20 Hz; X5: reactance at 5 Hz; AX: area of reactance; CT: computed tomography; Pi10: 10-mm internal luminal perimeter; VI 856%: voxel index at −856 HU; VI 950%: voxel index at −950 HU; E/I: expiratory/inspiratory. Bold values indicate statistical significance (p<0.05).

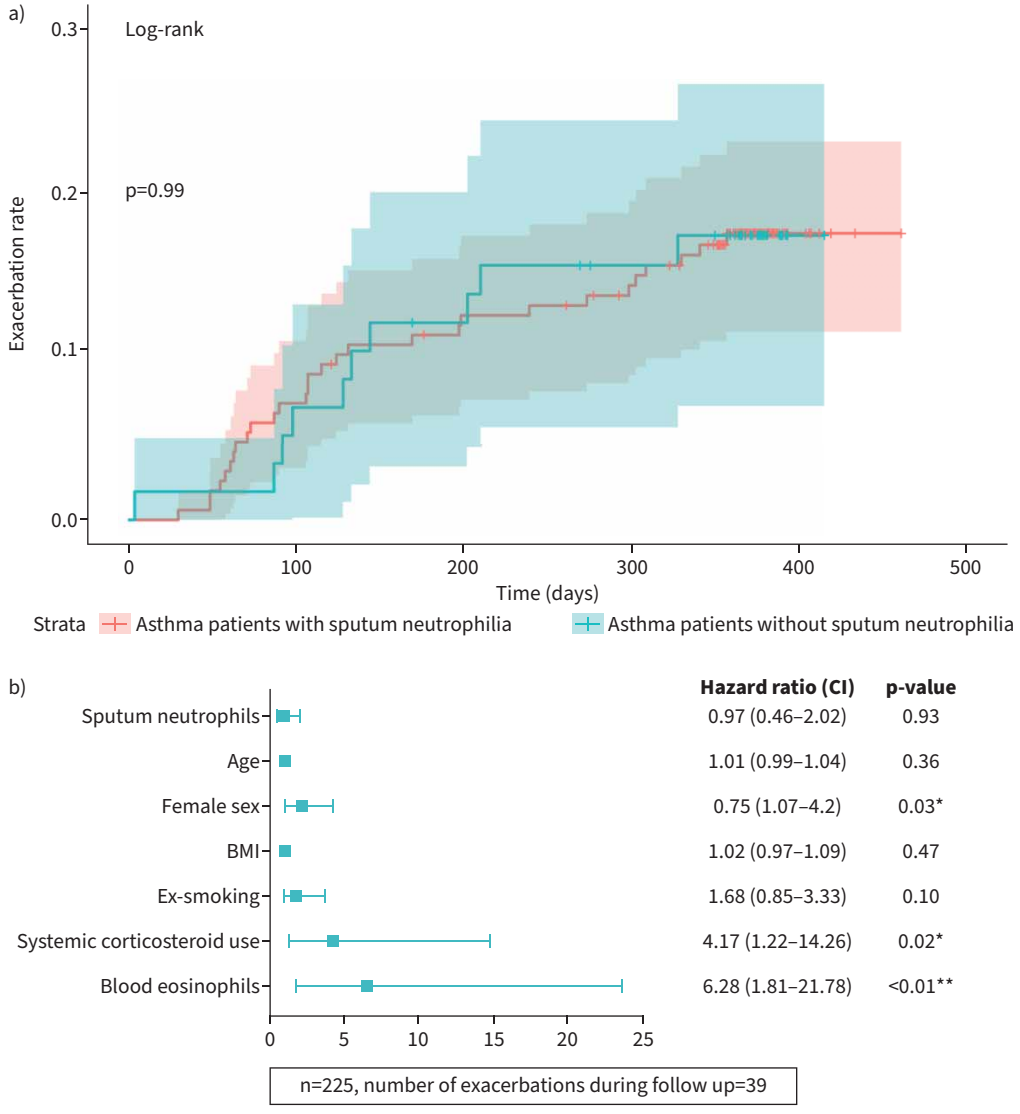

FIGURE 2 a) Time to first asthma exacerbations during 1 year of follow-up among asthma patients stratified by sputum neutrophilia. No significant difference in time to first exacerbation was found between patients with and without sputum neutrophilia. b) Forest plot showing independent predictors of exacerbations in asthma patients. Female sex, systemic corticosteroid use and blood eosinophils were independent predictors of exacerbations. Sputum neutrophilia, age, body mass index (BMI) and ex-smoking did not independently predict exacerbation occurrence. *: p<0.05; **: p<0.01.

current smokers). Comparing the existence of blood neutrophilia in asthma patients to healthy controls, blood neutrophilia was significantly more frequently present in asthmatic subjects than in healthy controls ($\chi^2$13.67, p<0.001) (figure 1).

Differences in clinical characteristics of asthma patients with and without blood neutrophilia

Characteristics stratified for patients with or without blood neutrophilia are shown in table 3. Patients with blood neutrophilia were more often female and had a higher BMI. Furthermore, the use of systemic corticosteroids was higher in the blood neutrophilia group. Blood neutrophilia was associated with a higher RV/TLC. Patients with blood neutrophilia had a significant higher R_{5-20}, R_5, R_{20} and X5, which are parameters of small airways disease. Regarding CT scan findings, a significantly higher 10-mm internal luminal perimeter (Pi10) was found in the blood neutrophilia group (table 3). In multivariate analysis, female sex, higher BMI, more blood eosinophils, current smoking and systemic corticosteroid use, but not dose, were independent predictors for blood neutrophilia. Furthermore, a higher RV/TLC and higher Pi10 were independently associated with blood neutrophilia (Supplementary Table 7).

TABLE 2 Baseline characteristics of asthma subjects in ATLANTIS stratified for sputum inflammatory cell dominance

	Sputum eosinophilic asthma		Sputum non-eosinophilic asthma		p-value
	Eosinophilic (eosinophils ⩾2.8%, neutrophils <70.6%)	Mixed granulocytic (eosinophils ⩾2.8%, neutrophils ⩾70.6%)	Neutrophilic (neutrophils ⩾70.6%, eosinophils <2.8%)	Paucigranulocytic (eosinophils <2.8%, neutrophils <70.6%)	
Subjects, n	52	6	52	118	
Age, years	45.27±13.07	38.67±13.88	45.31±12.94	41.79±13.82	0.22
Female sex	23 (44.2)	2 (33.3)	30 (57.7)	61 (51.7)	0.45
BMI, kg·m^{-2}	25±3.82*+	25±4.00	28±5.78¶	27±5.67¶	0.05
Age of asthma diagnosis, years	15.56 (4.50–36.07)	9.81 (5.66–24.00)	19.82 (6.92–39.15)	17.90 (6.06–34.12)	0.81
Smoking status					0.99
Current smoker	2 (3.8)	0 (0.0)	2 (3.8)	3 (2.5)	
Ex-smoker	11 (21.2)	1 (16.7)	12 (23.1)	25 (21.2)	
Never-smoker	39 (75.0)	5 (83.3)	38 (73.1)	90 (76.3)	
Pack-years in current and ex-smokers	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.20)	0.00 (0.00–0.00)	0.88
Total cell count sputum, ×10^6 U·mL^{-1}	2.57 (1.04–4.85)*	4.17 (2.75–5.04)	3.45 (1.56–6.11)*	1.91 (1.07–2.85)¶+	**<0.01**
Positive Phadiatop test	37 (84.1)	5 (83.3)	28 (71.8)	85 (81.0)	0.53
F_{ENO}, parts per billion	36.00 (19.50–49.25)*+	29.00 (20.00–36.50)	21.00 (15.50–37.00)¶	19.00 (14.00–29.00)¶	**0.01**
ACQ6 score	0.83 (0.50–1.21)	0.50 (0.12–0.63)	0.83 (0.29–1.70)	0.67 (0.31–1.50)	0.44
GINA 4 or 5	29 (55.8)*	3 (50.0)	21 (40.4)	38 (32.2)¶	**0.04**
Systemic corticosteroids use	3 (5.8)	1 (16.7)	1 (1.9)	1 (0.8)	**0.04**
Systemic corticosteroids dose, in those on systemic corticosteroids, mg	3.12 (2.19–4.06)	20.00 (20.00–20.00)	5.00 (5.00–5.00)	30.00 (30.00–30.00)	0.32
Use of ICS or ICS/LABA	47 (90.4)*	5 (83.3)	42 (80.8)	83 (70.3)¶	**0.03**
Daily ICS dose (beclomethasone equivalent) in those on ICS or ICS/LABA, in µg	800 (400–1000)	1000 (600–1000)	500 (400–1000)	800 (400–1000)	0.70
Severe/moderate airway hyperresponsiveness (PC_{20} <1, PD_{20} <0.13 mg)	19 (50.0)	3 (60.0)	12 (32.4)	31 (34.1)	0.22
FEV_1 % pred (post)	84.36±11.72*+	85.88±7.67	89.53±11.02¶	92.84±9.57¶	**<0.01**
FEV_1/FVC % (post-bronchodilator)	68.40±10.43+*	70.44±7.74	72.55±10.01¶*	75.74±8.43¶+	<0.01
Variables on SAD					
RV/TLC, %	34.76±7.73*+	31.55±8.20	31.15±9.08¶	30.94±7.49¶	**0.04**
S_{ACIN}, L^{-1}	0.14 (0.08–0.16)*	0.09 (0.06–0.17)	0.10 (0.06–0.16)	0.08 (0.06–0.12)¶	0.05
S_{COND}, L^{-1}	0.05 (0.03–0.06)*	0.05 (0.03–0.06)	0.03 (0.02–0.05)	0.02 (0.02–0.04)¶	**0.01**
$R_{5–20}$, kPa·L^{-1}·s^{-1}	0.04 (0.01–0.07)	0.01 (-0.01–0.03)	0.04 (0.02–0.08)	0.04 (0.02–0.08)	0.29
R_5, kPa·L^{-1}·s^{-1}	0.34 (0.30–0.43)	0.30 (0.25–0.34)	0.36 (0.28–0.42)	0.33 (0.27–0.41)	0.31
R_{20}, kPa·L^{-1}·s^{-1}	0.32 (0.27–0.36)	0.28 (0.24–0.29)	0.32 (0.25–0.37)	0.30 (0.25–0.34)	0.25
X5, kPa·L^{-1}·s^{-1}	−0.11 (−0.15– −0.08)	−0.08 (−0.10– −0.07)	−0.11 (−0.14– −0.08)	−0.10 (−0.13– −0.07)	0.39
AX, Hz×kPa·L^{-1}·s^{-1}	0.30 (0.16–0.58)	0.18 (0.15–0.20)	0.31 (0.18–0.68)	0.30 (0.13–0.47)	0.25
CT scan parameters					
Median lumen area, mm^2	20.87±4.06+	24.88±4.45	22.43±5.18*¶	19.50±4.25+	**<0.01**
Median wall area, mm^2	35.56±6.09*	38.04±5.24	34.71±6.73*	32.77±5.34+¶	**<0.01**
Median total area, mm^2	56.56±9.23+	63.14±8.98	57.25±11.68*¶	53.01±8.87+	**<0.01**
Pi10	7.03±1.18	6.59±0.88	7.30±1.17	7.13±1.18	0.74
Wall area/total area %	63.05±3.52+	60.79±1.06)	60.47±3.16*¶	62.53±3.36+	0.05
VI 856%	17.82 (4.14–25.33)*#	0.48 (0.45–3.82)¶	10.43 (3.24–19.72)	5.79 (2.47–12.85)¶	**0.02**
VI 950%	4.26 (2.26–9.49)	1.31 (1.15–1.92)	6.15 (2.67–9.04)	3.64 (2.06–7.86)	0.16
Lung volume ratio, E/I	0.50±0.11	0.56±0.15	0.48±0.12	0.48±0.15	0.70
Mean lung density ratio, E/I	0.83±0.08	0.84±0.08	0.80±0.08	0.80±0.08	0.45

Data are presented as n (%), mean±SD or median (IQR) unless otherwise specified. BMI: body mass index; IQR: interquartile range; F_{ENO}: fractional exhaled nitric oxide; ACQ6: asthma control questionnaire 6; GINA: Global Initiative for Asthma; ICS: inhaled corticosteroids; LABA: long acting β_2 agonist; PC_{20}: provocative concentration causing a 20% fall in FEV_1; PD_{20}: provocative dose causing a 20% fall in FEV_1; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; SAD: small airways disease; RV: residual volume; TLC: total lung capacity; S_{ACIN}: ventilation homogeneity of the acinar zone of the lungs corrected for tidal volume; S_{COND}: ventilation heterogeneity in the conductive zone of the lungs corrected for tidal volume; $R_{5–20}$: resistance at 5 Hz – resistance at 20 Hz; R_5: resistance at 5 Hz; R_{20}: resistance at 20 Hz; X5: reactance at 5 Hz; AX: area of reactance; CT: computed tomography; Pi10: 10-mm internal luminal perimeter; VI 856%: voxel index at −856 HU; VI 950%: voxel index at −950 HU; E/I: expiratory/inspiratory. Bold values indicate statistical significance (p<0.05); *: p<0.05 compared with paucigranulocytic; #: p<0.05 compared with mixed granulocytic; ¶: p<0.05 compared with eosinophilic; +: p<0.05 compared with neutrophilic.

TABLE 3 Baseline characteristics of asthma subjects in ATLANTIS stratified for blood neutrophilia

	No blood neutrophilia ($<4.7\times10^9$ U·L^{-1})	Blood neutrophilia ($\geq4.7\times10^9$ U·L^{-1})	p-value
Subjects, n	573	194	
Blood neutrophil count, $\times10^9$·L^{-1}	3.29 (2.78–3.86)	5.49 (5.02–6.25)	
Age, years	44.45±13.30	43.89±12.00	0.61
Female sex	321 (56.0)	129 (66.5)	**0.01**
BMI, kg·m^{-2}	27±5.08	29±7.38	**<0.01**
Age of asthma diagnosis, years	24.00 (9.00–41.50)	24.38 (9.70–40.81)	0.81
Smoking status			0.06
Current smoker	16 (2.8)	11 (5.7)	
Ex-smoker	109 (19.0)	45 (23.2)	
Never-smoker	448 (78.2)	128 (71.1)	
Pack-years in current and ex-smokers	0.00 (0.00–0.00)	0.00 (0.00–1.00)	**0.04**
Neutrophils sputum, % of nonsquamous cells	48.35 (26.10–70.00)	54.30 (36.65–82.40)	0.13
Blood eosinophil count, $\times10^9$·L^{-1}	0.21 (0.13–0.36)	0.26 (0.16–0.41)	**<0.01**
Eosinophils sputum, % of nonsquamous cells	0.50 (0.08–3.50)	0.30 (0.10–1.35)	0.23
Positive Phadiatop test	341 (82.4)	112 (75.2)	0.08
F_{ENO}, parts per billion	25.00 (16.00–37.00)	23.00 (14.25–38.75)	0.42
ACQ6 score	0.67 (0.17–1.50)	0.83 (0.33–1.77)	0.15
GINA classification			0.09
1	108 (18.8)	25 (12.9)	
2	57 (9.9)	28 (14.4)	
3	155 (27.1)	51 (26.3)	
4	224 (39.1)	74 (38.1)	
5	29 (5.1)	16 (8.2)	
Systemic corticosteroids use	8 (1.4)	14 (7.2)	**<0.01**
Systemic corticosteroids dose in those on systemic corticosteroids, mg	5.00 (5.00–16.25)	10.00 (5.00–17.50)	0.50
Use of ICS or ICS/LABA	460 (80.3)	165 (85.1)	0.17
Daily ICS dose (beclomethasone equivalent) in those on ICS or ICS/LABA, in µg	800 (400–1000)	750 (400–1000)	0.84
Airway hyperresponsiveness category			0.17
Very mild (PC_{20} ⩾4 and <16 mg·mL^{-1}, PD_{20} ⩾0.5 and <2 mg)	109 (26.5)	36 (25.2)	
Mild (PC_{20} ⩾1 and <4 mg·mL^{-1}, PD_{20} ⩾0.13 and <0.5 mg)	133 (32.3)	34 (23.8)	
Moderate (PC_{20} ⩾0.25 and <1 mg·mL^{-1}, PD_{20} ⩾0.03 and <0.13 mg)	94 (22.8)	39 (27.3)	
Severe (PC_{20} <0.25 mg·mL^{-1}, PD_{20} <0.03 mg)	76 (18.4)	34 (23.8)	
Severe/moderate airway hyperresponsiveness (PC_{20} <1, PD_{20} <0.13 mg)	170 (41.3)	73 (51.0)	0.05
FEV_1 predicted (post-bronchodilator)	90.55±11.89	90.04±12.18	0.61
FEV_1/FVC % (post-bronchodilator)	73.74±10.54	73.53±10.74	0.81
Variables on SAD			
RV/TLC, %	0.33±0.09	0.34±0.10	**0.03**
S_{ACIN}, L^{-1}	0.10 (0.06–0.15)	0.12 (0.07–0.19)	0.10
S_{COND}, L^{-1}	0.03 (0.01–0.04)	0.03 (0.02–0.05)	0.05
$R_{5–20}$, kPa·L^{-1}·s^{-1}	0.04 (0.02–0.09)	0.06 (0.03–0.12)	**<0.01**
R_5, kPa·L^{-1}·s^{-1}	0.35 (0.28–0.44)	0.41 (0.32–0.49)	**<0.01**
R_{20}, kPa·L^{-1}·s^{-1}	0.31 (0.26–0.37)	0.34 (0.28–0.39)	**<0.01**
X5, kPa·L^{-1}·s^{-1}	−0.11 (−0.14– −0.07)	−0.12 (−0.16–−0.09)	**<0.01**
AX, Hz×kPa·L^{-1}·s^{-1}	0.31 (0.15–0.72)	0.35 (0.20–0.80)	0.10
CT scan-derived parameters			
Median lumen area, mm^2	19.91±5.32	19.12±4.72	0.24
Median wall area, mm^2	33.52±6.53	33.03±5.73	0.55
Median total area, mm^2	53.86±11.13	52.25±9.71	0.26
Wall area/total area %	62.85±3.40	63.56±3.64	0.12
Pi10	7.13±0.96	7.40±0.98	0.04
VI 856%	8.25 (2.26–19.21)	7.17 (2.96–20.23)	0.78
VI 950%	3.76 (1.60–7.24)	3.25 (1.31–9.38)	0.99
Lung volume ratio, E/I	0.51±0.13	0.54±0.14	0.14
Mean lung density ratio, E/I	0.82±0.08	0.83±0.08	0.33

Data are presented as n (%), mean±SD or median (IQR) unless otherwise specified. BMI: body mass index; IQR: interquartile range; F_{ENO}: fractional exhaled nitric oxide; ACQ6: asthma control questionnaire 6; GINA: Global Initiative for Asthma; ICS: inhaled corticosteroids; LABA: long acting β_2 agonist; PC_{20}: provocative concentration causing a 20% fall in FEV_1; PD_{20}: provocative dose causing a 20% fall in FEV_1; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; SAD: small airways disease; RV: residual volume; TLC: total lung capacity; S_{ACIN}: ventilation homogeneity of the acinar zone of the lungs corrected for tidal volume; S_{COND}: ventilation heterogeneity in the conductive zone of the lungs corrected for tidal volume; $R_{5–20}$: resistance at 5 Hz − resistance at 20 Hz; R_5: resistance at 5 Hz; R_{20}: resistance at 20 Hz; X5: reactance at 5 Hz; AX: area of reactance; CT: computed tomography; Pi10: 10-mm internal luminal perimeter; VI 856%: voxel index at −856 HU; VI 950%: voxel index at −950 HU; E/I: expiratory/inspiratory. Bold values indicate statistical significance.

In a subgroup analysis in patients with more severe asthma (GINA treatment steps 4 and 5), blood neutrophilia was also associated with more systemic corticosteroid use but not female sex (Supplementary Table 8). Furthermore, blood neutrophilia in more severe asthma patients was associated with more severe AHR and higher median wall area/median total area (%), but not with a higher RV/TLC. In the multivariate analysis, significance was lost for median wall area/median total area (%) (Supplementary Table 9).

Blood neutrophilia and its association with lung function and asthma control over time

A linear mixed model showed that blood neutrophilia was not associated with changes in FEV_1 (visit 2×blood neutrophilia, p=0.80, visit 3×blood neutrophilia, p=0.87), nor with changes in ACQ6 score (visit 2×blood neutrophilia, p=0.37, visit 3×blood neutrophilia, p=0.41) at follow-up.

Association between blood and nonblood neutrophilia and asthma exacerbations

In addition, the association of blood neutrophilia with the occurrence of asthma exacerbations was evaluated. For 750 patients, both blood leukocyte counts and data on exacerbation during follow-up were available; 189 of these patients showed blood neutrophilia, whereas 561 did not. The Kaplan–Meier curve (figure 3a) and Cox regression analysis (figure 3b) did not show a difference in time to first exacerbation between patients with and without blood neutrophilia.

Validation in U-BIOPRED

To validate our findings, separate analyses within the independent U-BIOPRED cohort were performed. Within the U-BIOPRED severe asthma cohort sputum induction was successful in 181 patients; 52 out of these (29%) showed sputum neutrophilia. On baseline data, in ATLANTIS, patients with sputum neutrophilia showed a slightly lower BMI, but this finding did not differ significantly between both groups in U-BIOPRED. Patients with sputum neutrophilia used a significantly lower systemic corticosteroids dose compared with patients with no sputum neutrophilia, this finding was not observed in ATLANTIS. Contrary to ATLANTIS asthma patients and ATLANTIS severe asthma patients, a trend for a lower FEV_1 in patients with sputum neutrophilia was seen. CT scan parameters were not obtained and thus not investigated (Supplementary Tables 10 and 11).

Discussion

In the current study, we found that sputum neutrophilia was not more frequently present in asthma patients compared with healthy controls. In addition, we did not find that either a higher percentage or a higher absolute count of sputum neutrophils was associated with more severe asthma. These observations remained similar when using different cut-off levels to define sputum neutrophilia, or when analysing more severe asthma patients, either as a subgroup within ATLANTIS or in a separate cohort, *i.e.* U-BIOPRED. Interestingly, blood neutrophilia was observed more frequently in asthma compared with healthy controls. It was associated with more hyperinflation as reflected by RV/TLC, but the association was relatively weak and blood neutrophilia did not associate with other parameters, reflecting a more severe clinical expression of asthma. Taken together, our findings show that defining neutrophilia in sputum or blood is not helpful in the clinical phenotyping of asthma.

When comparing sputum neutrophilic and non-neutrophilic asthma patients, no differences in severity of the disease, as reflected by symptoms, large and small airway disease, and exacerbation frequency were found. This study shows that the presence of sputum neutrophilia at baseline is not associated with more frequent exacerbations during 1 year of follow-up. We found that patients with sputum neutrophilia, as defined by absolute sputum neutrophil counts, tended to be older. This finding aligns with a previous study by Beech *et al*. [20], which showed that sputum neutrophil counts increase with healthy ageing. The observation of sputum neutrophilia in ATLANTIS being associated with a lower wall area/total area and larger airway wall area may suggest a relatively larger airway size as can be seen in bronchiectasis. The latter may not be surprising as bronchiectasis is related to bacterial colonisation, which may lead to sputum neutrophilia [5, 21]. Although our findings show that neutrophil ratios in sputum were not associated with more severe disease overall, we do not rule out the possibility that a subgroup of asthma patients may exist in whom infection or colonisation-driven neutrophilic inflammation does play a role.

Previous studies have often stratified asthma subjects into four different subgroups based on inflammatory cell dominance in sputum as follows: eosinophilic, mixed granulocytic, neutrophilic and paucigranulocytic subgroups [10, 14, 22–24]. The Severe Asthma Research Program [14] defined a percentage of sputum neutrophils of more than 40% of total nonsquamous cells, which was lower than the median of sputum neutrophils in healthy controls (50.8%) in our study. We maintained a cut-off of 70.6% and by using this classification, a clear eosinophilic phenotype was observed, that was associated with higher F_{ENO} levels, more exacerbations, a higher disease severity, more airflow obstruction and more hyperinflation.

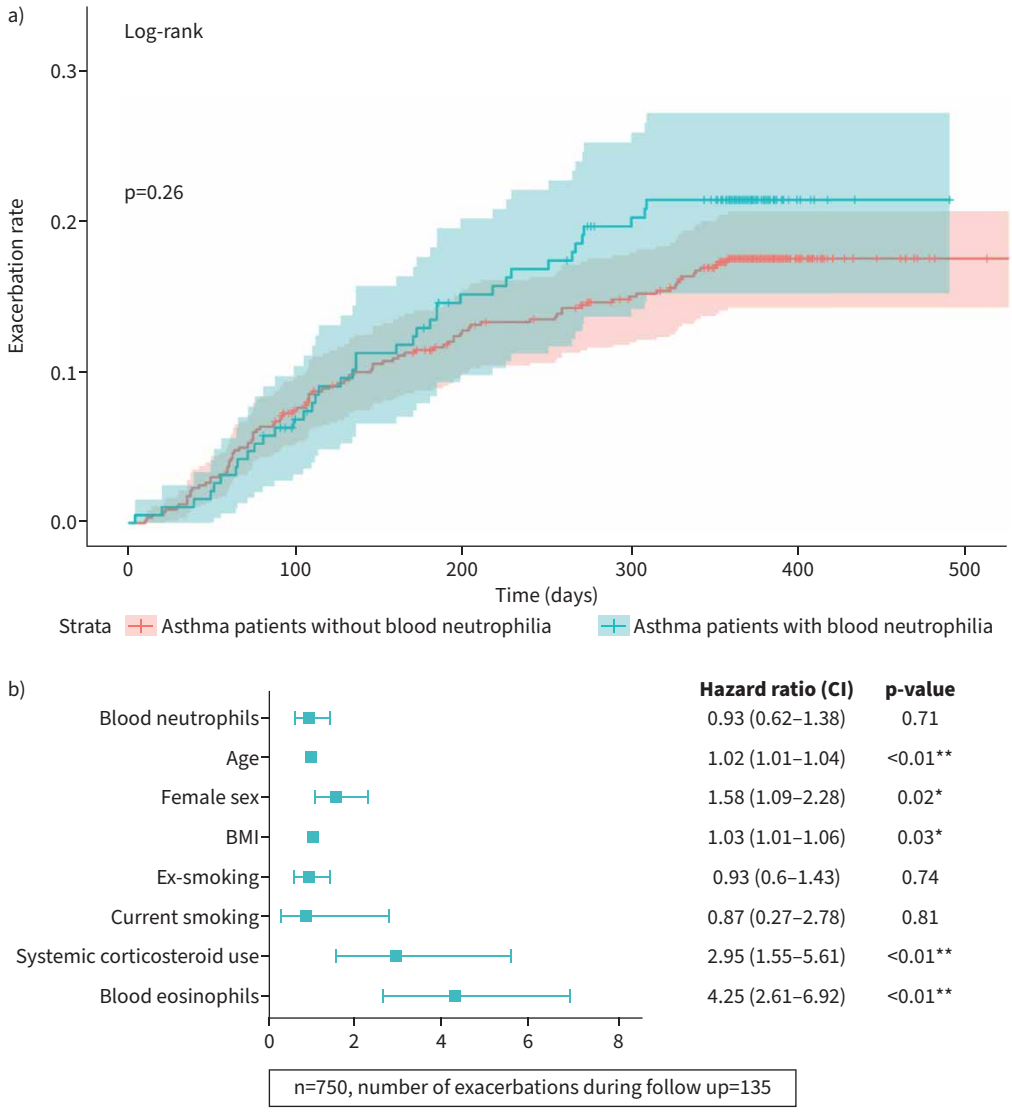

FIGURE 3 a) Time to first asthma exacerbations during 1 year of follow-up among asthma patients stratified by blood neutrophilia. No significant difference in time to first exacerbation was found between patients with and without blood neutrophilia. b) Forest plot showing independent predictors of exacerbations in asthma patients. Age, female sex, body mass index (BMI), corticosteroid use and blood eosinophils are independent predictors for exacerbations, but blood neutrophilia was not independently associated with exacerbations in asthma. *: p<0.05; **: p<0.01.

By contrast, the neutrophilic and paucigranulocytic phenotypes did not differ with respect to severity of airflow obstruction, symptoms or exacerbation risk, whereas the mixed granulocytic phenotype was found to occur rarely. Thus, the classification of four different inflammatory subtypes does not change the role of neutrophilic inflammation in clinical phenotyping asthma.

A novel observation from our study was the higher prevalence of blood neutrophilia in asthma patients compared with healthy controls. We also found blood neutrophilia to be associated with more severe hyperinflation, as reflected by RV/TLC. Although statistically significant, the clinical relevance of this finding is doubtful, since the effect was only small and other parameters of disease severity were unaffected by the presence of blood neutrophilia. Particularly, we did not find blood neutrophils to be independently related to more frequent exacerbations. Our findings are in contrast with those of Vedel-Krogh *et al.* [25] who did find elevated levels of blood neutrophils in patients experiencing moderate asthma exacerbations compared with those who had stable disease. The study by Vedel-Krogh *et al.* [25] was conducted in a much larger cohort, including almost 5000 asthma patients with a longer

follow-up of up to 8 years. This study design enables the detection of smaller signals that might have been missed in our study. Therefore, we do not rule out that elevated blood neutrophil counts may be associated with a worse clinical expression of asthma, *i.e.* more hyperinflation and higher exacerbation risk, but the effect size being small and/or only present in a subgroup of patients.

Interestingly, blood neutrophilia was associated with a higher BMI and female sex. The association between blood neutrophils and obesity has been previously described [26]. Adipose tissue produces several pro-inflammatory mediators, such as leptin and tumour necrosis factor-α, resulting in low-grade systemic inflammation followed by a rise in neutrophil numbers. This process is more common in females because they generally have more subcutaneous adipose tissue, which is more metabolically active than intra-abdominal adipose tissue [26–28].

The ATLANTIS study also found that the use of oral corticosteroids was associated with higher neutrophil levels in blood. This may be explained by the fact that corticosteroids prolong the lifespan of neutrophils by warding off apoptosis [29]. In this context, the use of corticosteroids could have been expected to also lead to higher numbers of neutrophils in induced sputum. However, we did not find this to be the case, neither in ATLANTIS nor in U-BIOPRED. A possible explanation for the observation that corticosteroids are more closely associated with blood neutrophils than with sputum neutrophils, may be that corticosteroids have a direct effect on the release of neutrophils into the systemic circulation as has been described by Nakagawa *et al.* [30].

The main strength of the current study is the large number of asthma patients across all severities, their extensive clinical characterisation and the availability of longitudinal data on exacerbations, lung function and asthma control. However, a limitation is that only patients up to the age of 65 years were included. Previous studies have shown that ageing may affect sputum neutrophilia [20]. As we excluded subjects aged 65 years and older, we cannot rule out the possibility that sputum or blood neutrophilia may be relevant in a subset of patients older than 65 years. Another limitation is that the cut-off used to define neutrophilia remains arbitrary. As there is no consensus in the literature regarding the exact cut-off, we have attempted to determine a reasonable cut-off defined by the upper quartile and additionally stringent upper fifth percentile of neutrophils in asthma patients. However, we acknowledge that there are alternative approaches to define the cut-off. Furthermore, this study did not include sputum data during the follow-up or at the time of an exacerbation. As a result, we cannot determine whether patients remain neutrophilic over time or whether neutrophilia during exacerbations exhibits distinct characteristics compared with those without. Lastly, a limitation was to only be able to investigate the role of neutrophilic inflammation in sputum and blood. It cannot be ruled out that the level of neutrophilic inflammation as assessed in other compartments, such as in bronchoalveolar lavage or bronchial biopsies, would yield different results. For future research, we suggest investigating the neutrophil activation status. Activated neutrophils release inflammatory mediators, which contribute to tissue damage and disease pathogenesis. Therefore, activation status may be more relevant than counts.

In conclusion, our results in ATLANTIS show that neutrophilic inflammation in sputum or blood is not related to asthma severity, and that blood neutrophilia coexists with asthma in association with female sex, BMI, smoking and corticosteroids use.

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