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Hypersegmented airway neutrophils and its association with reduced lung function in adults with obstructive airway disease.

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3 **Hypersegmented airway neutrophils and its association with reduced lung function in**
4 **adults with obstructive airway disease.**
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ABSTRACT:

Objectives: The significance of neutrophilic inflammation in obstructive airway disease remains controversial. Recent studies have demonstrated presence of an active neutrophil population in systemic circulation, featuring hypersegmented morphology, with high oxidative burst and functional plasticity in inflammatory conditions. The aim of this study was to characterize neutrophil subsets in obstructive airway disease participants (asthma, COPD and bronchiectasis) and healthy controls on the basis of nuclear morphology and to assess association between neutrophil subsets and clinical parameters of obstructive airway disease participants.

Design: A cross-sectional study.

Setting: John Hunter Hospital and Hunter Medical Research Institute, Australia.

Participants: 80 adults with obstructive airway disease, stable asthma (n=40) COPD (n=20), and bronchiectasis (n=20) and 20 healthy controls.

Material and Methods: Cytospins were prepared and neutrophil subtypes were classified based on the cells nuclear morphology into hypersegmented (>4 lobes), normal (2-4 lobes) and banded (1 lobe) neutrophils and enumerated.

Results: Neutrophils from each subset were identified in all participants. Numbers of hypersegmented neutrophils were elevated in participants with airway disease compared with healthy controls ($p < 0.001$). Both the number and proportion of hypersegmented neutrophils were highest in COPD participants (median (q1-q3) of 939.8 (201.1-2136) $\times 10^2/\text{mL}$ and 23.5 (11.0-46.5) %), respectively. An increased proportion of hypersegmented neutrophils in airway disease participants was significantly associated with lower FEV₁/FVC % ($\rho = -0.313$, $p = 0.005$).

Conclusion: Neutrophil heterogeneity is common in BL and is associated with more severe airflow obstruction in adults with airways disease. Further work is required to elucidate the functional consequences of hypersegmented neutrophils in the pathogenesis of disease.

Word count: 254

STRENGTHS AND LIMITATION OF STUDY

- This study characterizes three neutrophil subsets in bronchial lavage from adults with obstructive airway disease and healthy controls on the basis of nuclear morphology.
- There was an increase presence of hypersegmented neutrophils in obstructive airway disease in comparison with healthy controls.
- We show a clinical association of hypersegmented neutrophils with airway obstruction.
- The cross-sectional nature of study is a limitation in properly understanding the reason behind neutrophil heterogeneity in airways.

INTRODUCTION:

Neutrophils are phagocytic innate immune cells which patrol blood vessels and become activated in response to inflammatory triggers¹. Activation results in neutrophil migration to the site of infection, where pathogens can be eliminated by phagocytosis or NETosis². Similarly, infection or injury can result in the initiation of an innate immune response following the engagement of PAMPs (pathogen associated molecular patterns) and DAMPs (damage associated molecular patterns) with pattern recognition receptors of airways. This facilitates the release of chemotactic stimuli such as CXCL8, IL-1 β , and TNF- α , resulting in neutrophil recruitment to the airways³, which is important for the resolution of infection and inflammation⁴. In contrast, a disproportionate or dysregulated influx or efflux of neutrophils can result in persistent neutrophilic airway inflammation and tissue damage⁵.

Inflammation characterised by airway neutrophilia is reported in many cases of chronic obstructive airway disease⁶. This includes 20-30% cases of asthma⁷, more than 40% of cases of chronic obstructive pulmonary disease (COPD)^{8,9}, and 70% of cases of non-cystic fibrosis (CF) bronchiectasis¹⁰. Current therapeutic and management strategies for asthma and COPD focus on bronchodilation to overcome airflow limitation, or inhaled corticosteroids based therapies along with modification of eosinophilic airway inflammation using corticosteroids¹¹¹². In non-CF bronchiectasis treatment relies on antibiotics to control the infective nature of the disease¹³. While inhaled corticosteroids are highly effective in modifying eosinophilic inflammation in the airways¹⁴, there are no treatments that have been shown to influence neutrophil mediated inflammation. One of the primary reasons behind this is our lack of understanding about neutrophils^{15,16}.

Despite the fact that previous studies have shown an association between elevated neutrophils in airways with lower FEV₁ in obstructive airway disease¹⁷, little is known about variations

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3 within the population of neutrophils in the airways. Recent studies have identified
4 heterogeneity within circulating neutrophils. Pillay, *et al*¹⁸ identified three subsets of
5 neutrophils (normal, banded and hypersegmented) in the circulation following an inflammatory
6 challenge. Each subtype had a distinct nuclear morphology and pattern of surface adhesion
7 molecule expression, with hypersegmented neutrophils showing increased capacity for
8 oxidative burst along with a unique ability to suppress T lymphocytes. The same
9 morphologically distinct subsets have been identified in both bronchial lavage (BL) and blood
10 from patients with acute respiratory distress syndrome¹⁹ and in infants with severe viral
11 respiratory infection²⁰.

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23 The presence and characteristics of neutrophil subsets in obstructive airways disease is
24 unknown. In this study, we have characterised neutrophil subsets in BL fluid from adults with
25 asthma, COPD, non-CF bronchiectasis and healthy controls. We hypothesised that participants
26 with obstructive airway disease would have increased numbers of hypersegmented neutrophils
27 and that the presence of hypersegmented neutrophils would be associated with clinical disease
28 severity.

29 30 31 32 33 34 35 36 37 38 39 **MATERIAL AND METHODS:**

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42 **Patient and Public Involvement (PPI):** Patients and or the public were not involved in the
43 development of the research question and outcome measures of this study. The research
44 question was developed by authors (JLS and PABW). Patients were recruited if they were
45 undergoing a bronchoscopy as explained in "participants" section. The results will be
46 disseminated through publication and presentation at local, national and international research
47 meetings.

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3 **Participants:** Adults who were undergoing bronchoscopy for airways assessment were
4 recruited from the John Hunter Hospital. The study was approved by Hunter New England
5 Human Research Ethics Committee (Reference No 05/08/10/3.09) and all participants provided
6 written informed consent.
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12 **Study design:** A cross sectional study was conducted in which BL samples were obtained after
13 the assessment of clinical history including respiratory symptoms, smoking status and
14 medication. Spirometry and bronchoscopy were performed as outlined below.
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19 **Study group:** Adults (>18 years) with no history of a clinical chest or upper respiratory tract
20 infection in the previous 6 weeks were studied. Healthy non-smokers (n=20) had normal lung
21 function assessed by spirometry, and had no previous history of respiratory disease. Adults
22 with asthma (n=40) had a physician's diagnosis of asthma with objective evidence of airflow
23 variability or bronchial hyperactivity on provocation challenge. Bronchiectasis (n=20) was
24 defined as evidence of a permanent dilation of airway segment on high resolution computed
25 tomography scan while those with COPD (n=20) had evidence of respiratory symptoms in
26 combination with a post bronchodilator FEV₁ of less than 80% of predicted value and/or a post
27 bronchodilator FEV₁/FVC less than 70%. Current smokers were excluded.
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39 **Spirometry:** Spirometry was performed (Easy One Spirometer, ndd Medical Technologies,
40 Massachusetts, USA) at John Hunter Hospital. Variable obstruction defined as a post
41 bronchodilator change in FEV₁ of 12% or 200mL after 400 mcg of salbutamol and the
42 bronchial hyper-responsiveness defined as at least 15% decline in FEV₁ after inducing
43 bronchial provocation with 4.5% saline solution.
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50 **Bronchoscopy:** Flexible bronchoscopy was performed at John Hunter Hospital and bronchial
51 wash was taken by wedging the bronchoscope into the right middle lobe and washing with 40
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3 mL of sterile saline solution. A fraction of BL was sent for microbial detection while the rest
4
5 was processed as described below.
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8 **BL processing:** BL was filtered and total cell count (TCC) and viability assessed within one
9
10 hour of collection at Hunter Medical Research Institute. The BL was centrifuged and the cell
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12 pellet was resuspended in PBS to the concentration of 1×10^6 /mL and cellular cytopspins were
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14 prepared. The cytopspins were stained with May-Grünwald Giemsa (Beckman Coulter, Brea,
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16 CA, USA) and a differential cell count of 400 non squamous cells was performed.
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19 **Neutrophil subtype assessment:** Stained cytopspins were examined under oil immersion and
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21 100 neutrophils were enumerated into banded, normal and hypersegmented neutrophils.
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23 Banded neutrophils had a single banded lobe without any visible division; normal neutrophils
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25 had two to four lobes with every lobe having a properly visible outer boundary; and
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27 hypersegmented neutrophils had more than four lobes with every lobe having a properly visible
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29 outer boundary as shown in Figure 1.
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33 **Statistical Analysis:** Data were analysed using Stata software version 11 (StataCorp, College
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35 Station, TX, USA). Results are reported as mean (SD) or median (interquartile range), unless
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37 otherwise stated. Continuous measures were analysed using the two-sample Wilcoxon's rank
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39 sum test or t-test and Kruskal-Wallis test or one way analysis of variance (ANOVA) as
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41 appropriate. Categorical data were analysed using Fisher's exact test. Spearman correlation
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43 coefficients were calculated for the association between neutrophil subsets and clinical
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45 characteristics.
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51 **RESULTS**

52 **Clinical characteristics**

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3 Participants with COPD were older, more likely to be ex-smoking males with more severe
4 airflow obstruction (Table 1). Fewer participants with COPD were prescribed ICS compared
5 with the asthma group, however, the mean daily dose of ICS was significantly higher in COPD
6 participants.
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11 **Inflammatory cell counts**

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15 BL inflammatory cell counts for the participants are detailed in Table 2. Participants with
16 bronchiectasis and COPD had an increased total cell count (TCC) (Table 2). The proportion and
17 number of neutrophils was significantly higher in the bronchiectasis and COPD group
18 compared with healthy controls, while the proportion of neutrophils in asthma were
19 significantly lower in comparison with COPD. The asthma group also had significantly lower
20 number of neutrophils in comparison with bronchiectasis and COPD. The proportion of
21 eosinophils was significantly higher in COPD and asthma compared with healthy controls,
22 while the number of eosinophils was significantly higher in all three obstructive airways
23 diseases compared with healthy controls.
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37 **Neutrophil subsets**

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40 All three neutrophil subsets were identified in the BL of all participants. The numbers of
41 normal neutrophils were significantly higher in bronchiectasis and COPD group in comparison
42 to healthy and asthma (Figure 2A). Numbers of banded neutrophils were highest in those
43 participants with bronchiectasis compared with both healthy and asthma groups (Figure 2B).
44 Hypersegmented neutrophil numbers were significantly increased in all the obstructive airway
45 disease groups compared with healthy controls and increased in participants with COPD
46 compared with asthma and bronchiectasis (Figure 2C).
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3 When considering the relative distribution of neutrophil subtypes by proportion (shown in
4 Figure 2 D-F), participants with COPD had a significantly reduced proportion of normal and
5 banded neutrophils and subsequently a significantly increased proportion of hypersegmented
6 neutrophils.
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Table 1: Clinical characteristics of participants with bronchiectasis, asthma, COPD, and healthy controls.

	Bronchiectasis	COPD	Asthma	Healthy	p value
n	20	20	40	20	
Age	68.3 (7.3)	69.7 (9.8)	65.0 (7.4)	61.3 (9.7)	0.289
Males, n (%)	7 (35)	15 (75)	18 (45)	9 (45)	0.062
Ex-smoker, n (%)	0 (0)	20.00 (100.00) ^{^φ}	15.00 (37.50) ^{φ#}	2.00 (10.00)	<0.001
Smoking (pack years)	--	37.5 (20.5-60.0)	10 (4-30) [#]	(5,5)	0.005
FEV ₁ % predicted	92.0 (17.8) [#]	57.5 (17.0) [^]	72.9 (20.1) ^{^φ#}	98.6 (12.1) (n=19)	<0.001
FEV ₁ /FVC (%)	73.0 (67.5-78.5) [#]	56.5 (39.0- 65.5)	67.0 (59.8-74.0) ^{^ #}	75.0 (69.0-80.0) [#] (n=19)	<0.001
Taking ICS n (%)	--	9 (45)	38 (95) [#]	--	<0.001
BDP equivalent ICS dose μg day ⁻¹	--	1733.33 (529.15)	965.79 (400.86) [#]	--	<0.001
Bacterial pathogen, n (%)	8 (40) [^]	7 (37) [^]	12 (30) [^]	0 (0)	0.006

Data are presented as mean ± SD or median (interquartile range; q1- q3) unless otherwise stated. FEV₁: force expiratory volume in 1s; FVC: Forced vital capacity; ICS: Inhaled corticosteroids; BDP equivalent: ICS dose is calculated as beclomethasone dipropionate equivalent, where 1μg of beclomethasone =

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1µg budesonide = 0.5µg fluticasone. ^p<0.0125 compared with healthy controls, φ p<0.0125 compared with bronchiectasis and # p<0.0125 compared with COPD.

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Table 2: Inflammatory cell count of participants with bronchiectasis, asthma, COPD, and healthy controls.

	Bronchiectasis	COPD	Asthma	Healthy	p value*
Total cells x 10 ⁶ /mL	0.61 (0.17-1.67) [^]	0.51 (0.15-1.92) [^]	0.16 (0.10-0.34) ^φ	0.08 (0.05- 0.21)	<0.001
Viability, %	82.1 (75.0-90.7) [^]	87.75 (73.60-93.50) [^]	78.2 (62.4-87.2)	75.0 (72.2-85.9)	0.008
Neutrophil, %	65.00 (37.88-83.50) [^]	76.88 (69.88-85.13) [^]	56.63 (23.37-71.62) [#]	28.25 (14.75-63.50)	<0.001
Neutrophils x 10 ⁴ cells/mL	36.60 (5.11-139.46) [^]	35.17 (11.34-149.70) [^]	3.03 (7.89-23.98) ^{φ#}	3.18 (1.51-5.03)	<0.001
Eosinophils, %	1.13 (0.50-6.13)	3.75 (1.13-8.88) [^]	2.25 (1.00-11.75) [^]	1.00 (0.75-1.25)	0.039
Eosinophils x 10 ⁴ cells/mL	0.72 (0.43-2.49) [^]	1.79 (0.43-3.87) [^]	0.59 (0.11-3.07) [^]	0.09 (0.05-0.23)	<0.001
Macrophages,%	18.38 (11.00-33.25)	15.75 (9.25-21.65) [^]	25.25 (9.25-40.50)	29.25 (17.00-63.12)	0.033
Macrophages x 10 ⁴ cells/mL	11.96 (6.67-22.24) [^]	6.63 (2.62-18.90)	4.43 (2.01-7.80) ^φ	2.10 (1.42-6.43)	0.008
Lymphocytes, %	0.75 (0.13-1.62)	0.25 (0.00-1.25) [^]	0.50 (0.00-1.50)	1.5 (0.25-5.13)	0.038
Lymphocyte x 10 ⁴ cells/mL	0.30 (0.01-0.91)	0.11 (0.00-0.89)	0.08 (0.00-0.36)	0.18 (0.05-0.42)	0.383
Columnar epithelial cells, %	2.75 (0.88-10.75)	0.50 (0.00-3.38) [^]	4.75 (2.00-10.75) [#]	9.50 (4.88-23.63)	<0.001
Columnar epithelial x 10 ⁴ cells/mL	2.16 (0.54-2.80) [#]	0.28 (0.00-0.56) [^]	1.02 (0.44-2.10) [#]	0.88 (0.38-2.38)	<0.001

Data are presented as median (interquartile range; q1- q3) unless otherwise stated.

* Kruskal-Wallis test, ^ p<0.0125 compared with healthy, φ p<0.0125 compared with bronchiectasis, and # p<0.0125 compared with COPD.

Association of neutrophil subsets with clinical characteristics in obstructive airway disease

There was a significant negative correlation between the proportion of hypersegmented neutrophils with both FEV₁% predicted ($\rho = -0.278$, $p=0.012$) and FEV₁/FVC% ($\rho = -0.313$, $p=0.005$) (Figure 3) in participants with obstructive airway disease. While the same was not observed for banded neutrophils [FEV₁% predicted ($\rho = 0.151$, $p=0.183$), FEV₁/FVC% ($\rho = 0.191$, $p=0.090$)] and normal neutrophils [FEV₁% predicted ($\rho = 0.163$, $p=0.149$), FEV₁/FVC% ($\rho = 0.204$, $p=0.069$)]. There was no association between the total neutrophil proportion with either FEV₁% predicted ($\rho = -0.170$, $p=0.132$) or FEV₁/FVC% ($\rho = -0.151$, $p=0.181$).

In participants with COPD, the proportion of hypersegmented neutrophils was positively associated with proportion of eosinophils ($\rho = 0.597$, $p=0.006$) (Figure 4A) and negatively associated with cell viability ($\rho = -0.738$, $p<0.001$) (Figure 4B). This association was not observed in any other group or in the overall population (data not shown).

To explore the correlation between proportion of eosinophils and hypersegmented neutrophils further we decided to examine the COPD participants according to their inflammatory subtype categorised as eosinophilic COPD (E-COPD) ($\geq 3\%$ eosinophils) and non-eosinophilic COPD (NE-COPD) ($< 3\%$ eosinophils).

Eosinophilic and non-eosinophilic COPD

Twelve participants were characterized as eosinophilic COPD (E-COPD) and eight participants were characterized as non-eosinophilic COPD (NE-COPD). The smoking pack-years were significantly higher in the NE-COPD group compared with E-COPD group (Table 3). The NE-

COPD group also had a significantly elevated total cell count and cell viability along with significantly elevated proportion and number of neutrophils in comparison with E-COPD. The number and proportion of eosinophils were significantly higher in ECOPD.

Table 3: Clinical characteristics and cell counts of participants with E-COPD and NE-COPD.

	E-COPD (n = 12)	NE-COPD (n = 8)	P value
Age	70.92 (9.87)	67.75 (10.02)	0.494
Males, n (%)	8 (66.67)	7 (87.50)	0.603
Ex-smoker, n (%)	12 (100.00)	8 (100.00)	1.000
Smoking pack years	33.17 (16.74)	56.38 (31.55)	0.045
FEV ₁ % Predicted	59.17 (16.45)	55.00 (18.53)	0.604
FEV ₁ /FVC (%)	51.25(14.68)	53.38 (18.65)	0.779
Taking ICS n (%)	6 (50.00)	3 (37.50)	0.465
BDP equivalent ICS dose $\mu\text{g day}^{-1}$	2000 (800-2000)	2000 (2000-2000)	0.285
Bacterial pathogen, n (%)	5 (41.67)	2 (25.00)	0.392
Total cells x 10 ⁶ / mL	0.57 (0.53)	1.94 (1.68)	0.016
Viability, %	76.66 (14.63)	91.52 (6.39)	0.015
Neutrophil, %	74.13 (65.63-77.75)	85.50 (77.00-91.75)	0.025
Neutrophils x 10 ⁴ cells/mL	43.12 (41.86)	169.43(152.59)	0.013
Eosinophils, %	8.92 (5.66)	0.97 (0.69)	0.001
Eosinophils x 10 ⁴ cells/mL	4.34 (4.10)	1.00 (1.05)	0.038
Macrophages, %	17.38 (12.00-23.15)	11.25 (7.75-16.88)	0.177
Lymphocytes, %	0.46 (0.51)	0.72 (0.65)	0.328
Columnar epithelial cells, %	1.00 (0.13-3.38)	0.13 (0.00-2.88)	0.453

Data are presented as mean \pm SD or median (interquartile range; q1- q3) unless otherwise stated. FEV₁: forced expiratory volume in 1s; FVC: Forced vital capacity; ICS: Inhaled corticosteroids; BDP equivalent:

ICS dose is calculated as beclomethasone dipropionate equivalent, where 1µg of beclomethasone = 1µg budesonide = 0.5µg fluticasone.

Neutrophil subsets in eosinophilic and non-eosinophilic COPD

While not statistically different, participants with E-COPD had half the number of banded neutrophils and ten times fewer normal neutrophils in comparison to those with NE-COPD (Table 4). The proportion of normal neutrophils were significantly reduced in E-COPD while proportion of hypersegmented neutrophils were significantly elevated.

Table 4: Numbers and proportion of neutrophil subsets in eosinophilic (E-COPD) and non-eosinophilic COPD participants.

Neutrophil type	E-COPD (n = 12)	NE-COPD (n = 8)	P value *
Normal, x 10 ² /mL	1306.24 (876.66-3484.33)	14893.66 (880.95-25103.90)	0.165
Banded, x 10 ² /mL	63.41 (27.09-241.98)	128.20 (25.50-1982.52)	0.537
Hypersegmented, x 10 ² /mL	811.59 (183.14-2136.15)	1193.40 (258.75-2282.96)	0.817
Normal, %	65.50 (46.50-74.00)	80.00 (75.00-86.00)	0.031
Banded, %	4.00 (2.00-4.50)	4.00 (2.00-6.50)	0.906
Hypersegmented, %	33.00 (22.00-50.50)	14.00 (4.50-21.50)	0.037

Data are presented as median (interquartile range; q1- q3) unless otherwise stated. * Two-sample Wilcoxon's rank sum test.

DISCUSSION

The study identified three morphologically distinct subsets of neutrophils i.e. banded, normal, and hypersegmented in the BL of participants with chronic obstructive airways disease patients and healthy controls. There were a significantly higher number of hypersegmented neutrophils in those with obstructive airway disease compared with healthy controls. The proportion of hypersegmented neutrophils was associated with lower FEV₁ and more severe airflow obstruction (FEV₁/FVC %) in obstructive airway disease participants and with the presence of eosinophilic airway inflammation in COPD.

The concept of morphological heterogeneity in neutrophil population has recently emerged²¹. We have examined neutrophil heterogeneity in the bronchial lavage of obstructive airway disease participants and healthy controls. The reason for neutrophil heterogeneity is unclear but may be attributable to the different stages of cell maturation in the bone marrow before transition to the tissue, or alternatively, neutrophils might change their morphology during the course of inflammation to adjust with the stressors in inflamed airways^{5 22}.

Banded neutrophils are also known as immature neutrophils and are deemed incompetent in anti-microbial immune functions as reported in the systemic circulation of sepsis patients²³. The emergence of banded neutrophils in the airway can occur after depletion of mature neutrophils in bone marrow following excessive demand during acute inflammation²⁰.

The hypersegmented morphology of the neutrophil implies increased maturation compared with banded and normal neutrophils¹⁸. Maturation is thought to occur in inflamed airways due to the presence of a cytokine rich environment consisting of pro-survival mediators²⁴. The mechanism behind formation of hypersegmented neutrophils are known to be linked with the life cycle of the neutrophils. The increase in survival cause the nucleus of neutrophil to develop more indentation and segmentation, and hence the hypersegmented neutrophils are also called as “old neutrophils”²⁵.

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3 The ability of a chemoattractant rich milieu to change the phenotype of neutrophils was recently
4 shown when neutrophils from the blood of healthy volunteers were incubated with the
5 broncoalveolar lavage from a patient with ARDS. These neutrophils altered their phenotype, with
6 an increase in those with a hypersegmented morphology¹⁹. It may be possible that a similar
7 process is occurring chronically in the airways of obstructive airway disease participants, who
8 generally have higher levels of pro-inflammatory cytokines and inflammatory mediators. Previous
9 studies have demonstrated that hypersegmented neutrophils in the circulation demonstrate low
10 expression of L-selectins, which may reduce their anchoring ability on endothelial cells and hence
11 reduce their chances to egress into inflamed airways²⁶. Thus, it is possible that the hypersegmented
12 neutrophils we observed in our study have not directly come from circulation and instead may
13 have become hypersegmented in the airways under the influence of pro-survival mediators.

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15 Mediators that promote neutrophil survival and can be present in the airways include; GM-CSF,
16 chemokines like CXCL-8 and lipid mediators such as serum amyloid A²². GM-CSF and CXCL-8
17 are known to enhance neutrophil survival by promoting the expression of anti-apoptotic proteins
18 like survivins and by preventing TNF- α mediated apoptosis^{27 28}. While serum amyloid A is known
19 to prolong neutrophil longevity by preventing mitochondrial damage and decreasing caspase-3
20 (apoptotic protein) activity²⁹. Our past studies have reported elevated levels of CXCL-8 in sputum
21 samples of neutrophilic asthma, bronchiectasis³⁰, and COPD patients³¹. Beside this, we have also
22 reported that elevated levels of serum amyloid A in COPD was associated with neutrophilic
23 inflammation in airways and this was refractory to corticosteroids³². This suggests that the elevated
24 presence of these markers might have played some role in enhancing the survival of neutrophils in
25 airways and promoting the presence of hypersegmented neutrophils.

26
27 In this study, we also reported a positive correlation between eosinophils and hypersegmented
28 neutrophils proportion in COPD participants along with significantly elevated proportion of
29 hypersegmented neutrophils in E-COPD participants. The presence of eosinophils in airways can

1
2
3 further elevate the level of GM-CSF due to their own production of this cytokine³³, which can
4
5 further promote maturation of neutrophils. Beside this, the use of ICS to control eosinophilic
6
7 inflammation may enhance neutrophil survival in the inflamed airways by increasing the activity of
8
9 anti-apoptotic proteins such as Mcl-1 (induced myeloid leukaemia cell differentiation protein) and
10
11 IAPs (inhibitor of apoptosis proteins) in neutrophils³⁴. This increased maturity and prevention of
12
13 death may result in an increased proportion of hypersegmented neutrophils.
14
15

16
17 The significant association between the proportion of hypersegmented neutrophils with FEV₁ and
18
19 severe airflow obstruction suggests that where hypersegmented neutrophils are common, airway
20
21 obstruction is poor. This could be a result of high oxidative burst produced by hypersegmented
22
23 neutrophils as observed in previous studies, in which hypersegmented neutrophil exhibited high
24
25 oxidative burst after ex-vivo stimulation^{18 19}. The generation of high oxidative burst by
26
27 neutrophils may also impair their timely clearance from the airway³⁵ and can trigger a vicious
28
29 cycle of neutrophils influx into the airways⁶. The impairment of neutrophil clearance in airway
30
31 may cause necrosis of neutrophils which can spill its cytotoxic content such as reactive oxygen
32
33 species and proteolytic enzymes like neutrophil elastase in the lumen of airways³⁶. This can
34
35 further damage airway wall and promote mucus hypersecretion which may result in significant
36
37 decline in FEV₁ as earlier reported in a study on COPD patients³⁷. Further research is needed to
38
39 understand if hypersegmented neutrophils are common as a result of more severe disease or
40
41 conversely if they influence disease severity.
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45
46 The cross-sectional nature of study is a limitation in properly establishing the cause and effect of
47
48 relationship of neutrophil heterogeneity in airways. A further detailed ex-vivo study of influence
49
50 of pathogen, pro-survival mediators, and current medications like ICS on neutrophil subsets
51
52 morphology, surface expressions, and functional behaviour is needed to provide a better
53
54 understanding of the formation of hypersegmented neutrophils in the airways and subsequently in
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1
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3 developing a more comprehensive strategy for assessment and management of airway
4
5 neutrophilia.
6

7 8 **CONCLUSION**

9
10 We have shown the presence of three morphologically different subsets of neutrophils in the
11
12 airways of healthy and obstructive airway disease participants i.e. asthma, COPD, and
13
14 bronchiectasis. The increased proportion of hypersegmented neutrophils in the airways of
15
16 obstructive airway disease participants was associated with reduced lung function of these
17
18 participants. The proportion of hypersegmented neutrophils was highest in COPD participants in
19
20 comparison with all other groups.
21
22
23
24
25
26

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28
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30
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32
33 Research Centre for Healthy Lungs.
34
35
36
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38
39

40 **CONTRIBUTORS:** JLS developed the idea and designed the study. JLS also supervised and
41
42 coordinated the study throughout. RL performed the subtype counting and wrote the
43
44 manuscript which was further refined and edited by JLS, PABW, KB and DB. PABW
45
46 performed the bronchoscopy, KB supervised the bronchial lavage processing and cytospin
47
48 preparation and DB supervised the statistical analysis.
49
50
51
52
53

54 **COMPETING INTERESTS:** None.
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FIGURES LEGENDS:

Figure 1: Subtypes of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants (X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented neutrophil.

Figure 2: Neutrophil subtype number (A-C) and neutrophil subtype proportion (D-F) in BL of participants with bronchiectasis, COPD, asthma, and healthy controls. $\wedge p < 0.0125$ compared with healthy controls, * $p < 0.0125$ compared with asthma and # $p < 0.0125$ compared with COPD.

Figure 3: Scatterplot of hypersegmented neutrophil proportion vs FEV₁% predicted (A) and FEV₁/FVC (B) in BL of obstructive airway disease participant's.

Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

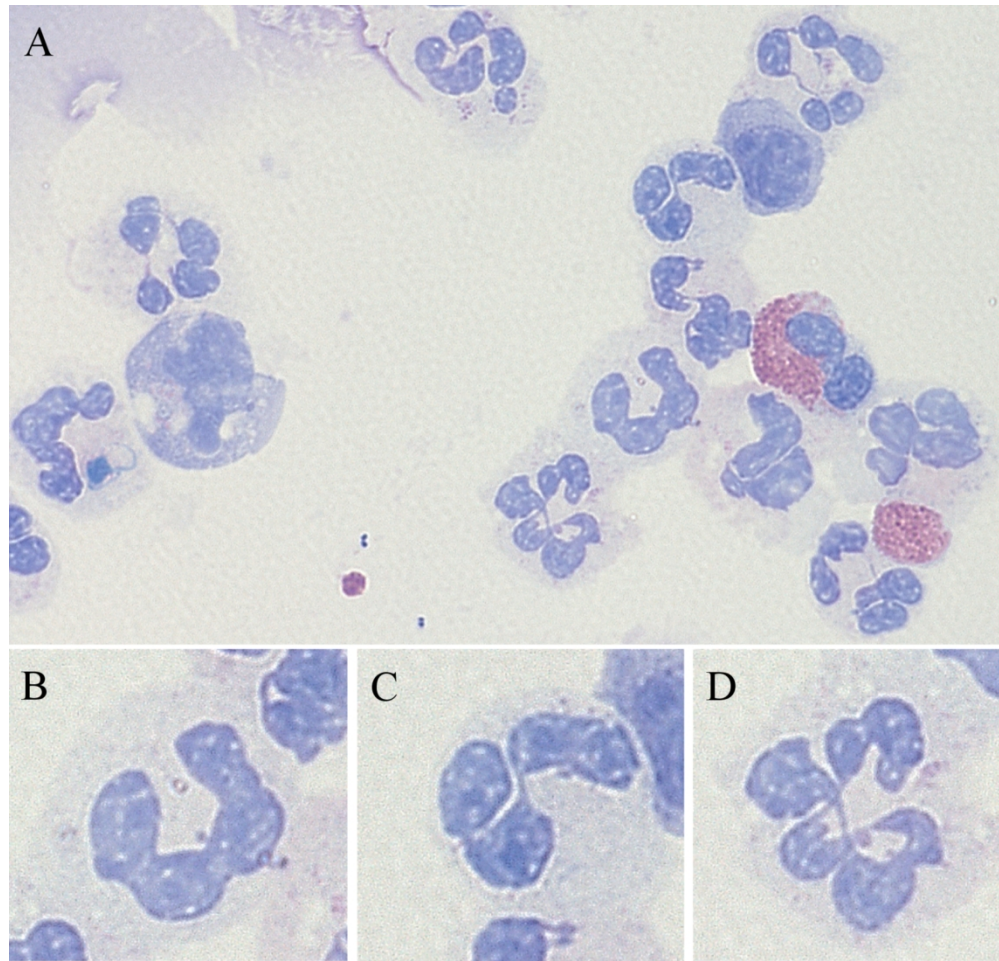


Figure 1: Subtypes of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants(X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented neutrophil.

153x146mm (300 x 300 DPI)

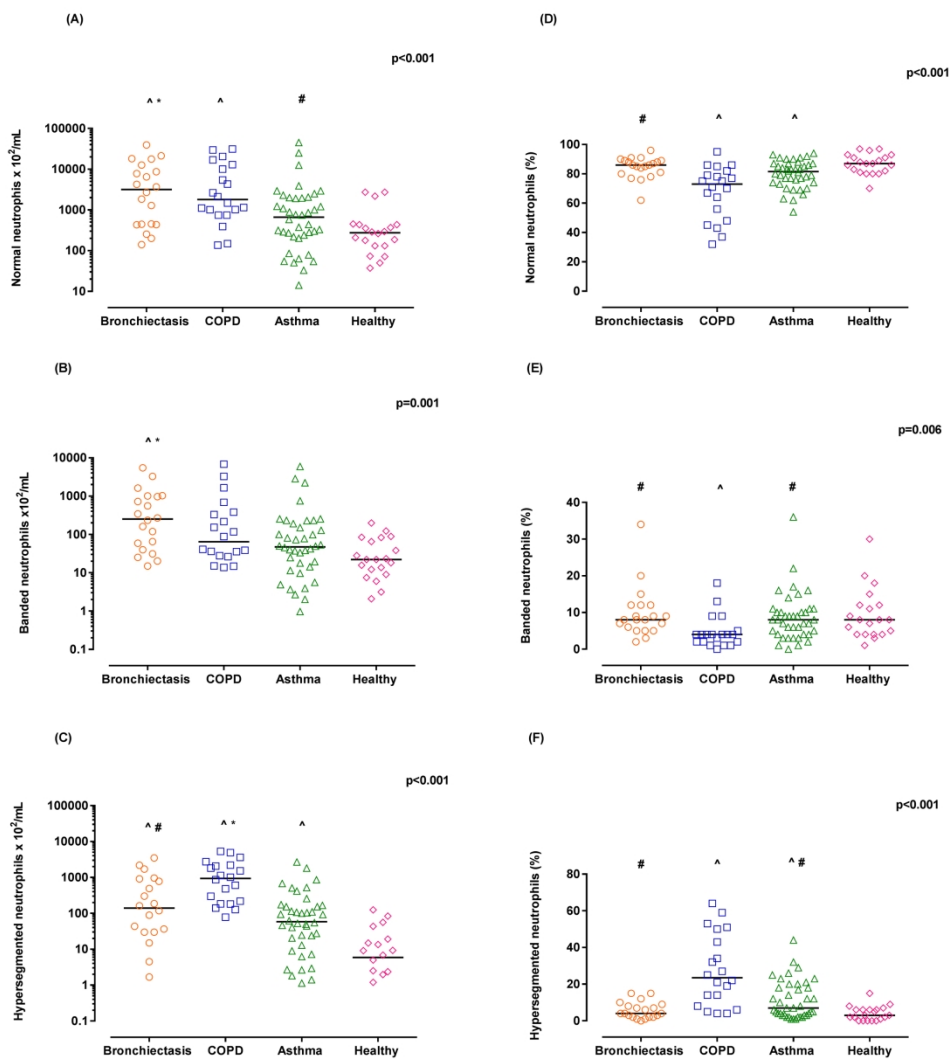


Figure 2: Neutrophil subtype number (A-C) and neutrophil subtype proportion (D-F) in BL of participants with bronchiectasis, COPD, asthma, and healthy controls. ^p<0.0125 compared with healthy controls, * p<0.0125 compared with asthma and # p<0.0125 compared with COPD.

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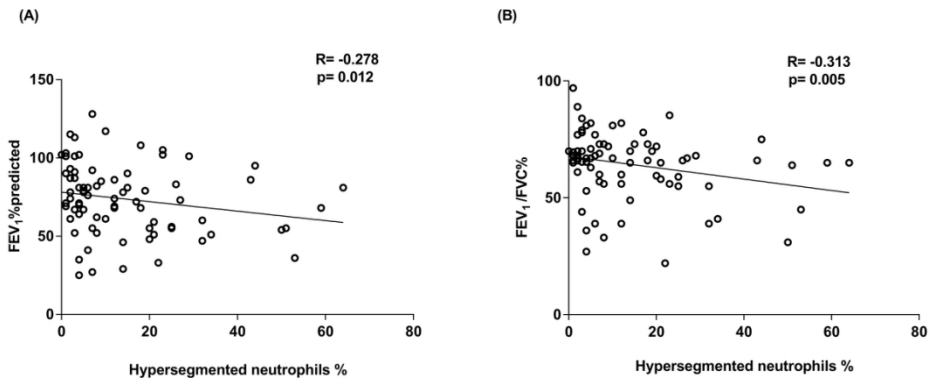


Figure 3: Scatterplot of hypersegmented neutrophil proportion vs FEV₁% predicted (A) and FEV₁/FVC (B) in BL of obstructive airway disease participant's.

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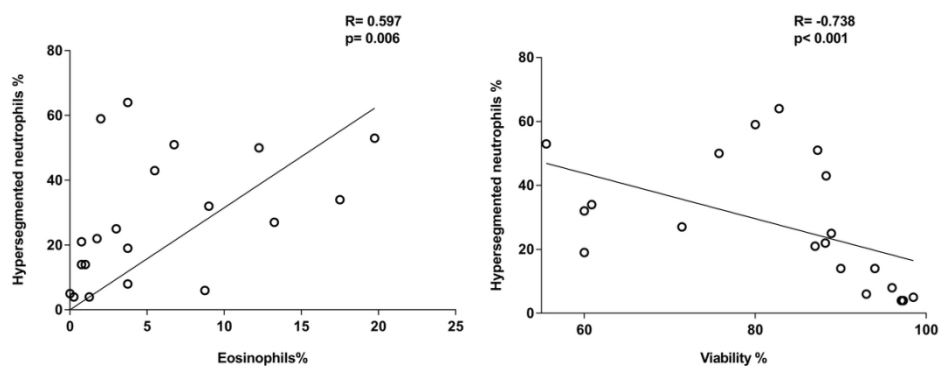


Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

108x44mm (300 x 300 DPI)

Reporting checklist for cross sectional study.

Based on the STROBE cross sectional guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the STROBE cross sectional reporting guidelines, and cite them as:

von Elm E, Altman DG, Egger M, Pocock SJ, Gotsche PC, Vandembroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.

		Reporting Item	Page Number
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	3
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	3
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	#3	State specific objectives, including any prespecified hypotheses	6
Study design	#4	Present key elements of study design early in the paper	6
Setting	#5	Describe the setting, locations, and relevant dates, including periods of	6-7

		recruitment, exposure, follow-up, and data collection	
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4	Eligibility criteria	#6a Give the eligibility criteria, and the sources and methods of selection of participants.	6-7
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8		#7 Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-7
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14	Data sources / measurement	#8 For each variable of interest give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group. Give information separately for for exposed and unexposed groups if applicable.	6-7
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26	Bias	#9 Describe any efforts to address potential sources of bias	6-7, study utilized standard guidelines to formulate exclusion and inclusion criteria for every group to limit the selection bias.
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34	Study size	#10 Explain how the study size was arrived at	n/a... The size of study was decided after considering previous studies on neutrophil subsets eg Juss et al, on neutrophil subset in acute respiratory distress syndrome.
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44	Quantitative variables	#11 Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	8
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51	Statistical methods	#12a Describe all statistical methods, including those used to control for confounding	8
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55		#12b Describe any methods used to examine subgroups and interactions	n/a...we examined COPD subgroups (Eosinophilic and Non-Eosinophilic) based on
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1			pre-defined cut off values on
2			page 12-14.
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4		#12c	Explain how missing data were addressed
5			Missing data were excluded
6			from analysis.
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8		#12d	If applicable, describe analytical methods
9			n/a. The study did not use any
10			analytical method.
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12		#12e	Describe any sensitivity analyses
13			n/a. No sensitive analysis
14			were performed in this study.
15	Participants	#13a	Report numbers of individuals at each
16			stage of study—eg numbers potentially
17			eligible, examined for eligibility, confirmed
18			eligible, included in the study, completing
19			follow-up, and analysed. Give information
20			separately for for exposed and unexposed
21			groups if applicable.
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27		#13b	Give reasons for non-participation at each
28			stage
29			n/a.... No non-participation to
30			report for this study.
31		#13c	Consider use of a flow diagram
32			n/a
33	Descriptive data	#14a	Give characteristics of study participants
34			(eg demographic, clinical, social) and
35			information on exposures and potential
36			confounders. Give information separately
37			for exposed and unexposed groups if
38			applicable.
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43		#14b	Indicate number of participants with
44			missing data for each variable of interest
45			10 (Table 1...spirometry data
46			for only 19 healthy
47			participants out of 20).
48	Outcome data	#15	Report numbers of outcome events or
49			summary measures. Give information
50			separately for exposed and unexposed
51			groups if applicable.
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55	Main results	#16a	Give unadjusted estimates and, if
56			applicable, confounder-adjusted estimates
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interval). Make clear which confounders were adjusted for and why they were included

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6		#16b	Report category boundaries when 14
7			continuous variables were categorized
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9		#16c	If relevant, consider translating estimates n/a....was not relevant in this
10			of relative risk into absolute risk for a study.
11			meaningful time period
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14	Other analyses	#17	Report other analyses done—e.g., 12-14
15			analyses of subgroups and interactions,
16			and sensitivity analyses
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20	Key results	#18	Summarise key results with reference to 15
21			study objectives
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24	Limitations	#19	Discuss limitations of the study, taking into 17
25			account sources of potential bias or
26			imprecision. Discuss both direction and
27			magnitude of any potential bias.
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30	Interpretation	#20	Give a cautious overall interpretation 15-17
31			considering objectives, limitations,
32			multiplicity of analyses, results from similar
33			studies, and other relevant evidence.
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37	Generalisability	#21	Discuss the generalisability (external n/a
38			validity) of the study results
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41	Funding	#22	Give the source of funding and the role of 2
42			the funders for the present study and, if
43			applicable, for the original study on which
44			the present article is based
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BMJ Open

Hypersegmented airway neutrophils and its association with reduced lung function in adults with obstructive airway disease: An observational study.

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-024330.R1
Article Type:	Research
Date Submitted by the Author:	19-Oct-2018
Complete List of Authors:	Lokwani, Ravi; University of Newcastle School of Medicine and Public Health, Priority Research Centre for Healthy Lungs, Faculty of Health and Medicine, Wark, Peter; Centre for Asthma and Respiratory Disease University of Newcastle, Respiratory and Sleep Medicine Baines, Katherine; University of Newcastle, Respiratory and Sleep Medicine Barker, Daniel; University of Newcastle School of Medicine and Public Health, Faculty of Health and Medicine Simpson, Jodie; The University of Newcastle, Respiratory and Sleep Medicine
Primary Subject Heading:	Respiratory medicine
Secondary Subject Heading:	Immunology (including allergy)
Keywords:	Immunology < THORACIC MEDICINE, Bronchoscopy < THORACIC MEDICINE, Chronic airways disease < THORACIC MEDICINE

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Manuscripts

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3 **Hypersegmented airway neutrophils and its association with reduced lung function in adults**
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5 **with obstructive airway disease: An observational study.**
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8 **Authors:** Ravi Lokwani ^{1, 3}, Peter AB Wark ¹⁻³, Katherine J Baines ^{1, 3}, Daniel Barker ³, Jodie L
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10 Simpson ¹⁻³
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50 **Key words:** Immunology, Chronic airways disease, Bronchoscopy.
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55 **Word**

count:

3211

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3 **1 ABSTRACT:**
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6 **2 Objectives:** The significance of neutrophilic inflammation in obstructive airway disease remains
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8 **3** controversial. Recent studies have demonstrated presence of an active neutrophil population in systemic
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10 **4** circulation, featuring hypersegmented morphology, with high oxidative burst and functional plasticity in
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12 **5** inflammatory conditions. The aim of this study was to characterize neutrophil subsets in bronchial lavage
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14 **6** (BL) of obstructive airway disease participants (asthma, COPD and bronchiectasis) and healthy controls on
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16 **7** the basis of nuclear morphology and to assess association between neutrophil subsets and clinical parameters
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18 **8** of obstructive airway disease participants.
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21 **9 Design:** An observational study.
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24 **10 Setting:** John Hunter Hospital and Hunter Medical Research Institute, Australia.
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27 **11 Participants:** Seventy-eight adults with obstructive airway disease, stable asthma (n=39) COPD (n=20), and
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29 **12** bronchiectasis (n=19) and 20 healthy controls.
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32 **13 Material and Methods:** Cytospins were prepared and neutrophil subtypes were classified based on the cells
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34 **14** nuclear morphology into hypersegmented (>4 lobes), normal (2-4 lobes) and banded (1 lobe) neutrophils and
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36 **15** enumerated.
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39 **16 Results:** Neutrophils from each subset were identified in all participants. Numbers of hypersegmented
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41 **17** neutrophils were elevated in participants with airway disease compared with healthy controls ($p < 0.001$). Both
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43 **18** the number and proportion of hypersegmented neutrophils were highest in COPD participants (median (q1-
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45 **19** q3) of 1073.6 (258.8-2742) $\times 10^2/\text{mL}$ and 24.5 (14.0-46.5) %, respectively). An increased proportion of
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47 **20** hypersegmented neutrophils in airway disease participants was significantly associated with lower
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49 **21** FEV₁/FVC % (spearman's Rho= -0.322, $p = 0.004$).
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3 22 **Conclusion:** Neutrophil heterogeneity is common in bronchial lavage and is associated with more severe
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5 23 airflow obstruction in adults with airways disease. Further work is required to elucidate the functional
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7 24 consequences of hypersegmented neutrophils in the pathogenesis of disease.
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10 25 **Word count:** 260
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13 26 **STRENGTHS AND LIMITATION OF STUDY**

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- 16 27 ➤ This is the first observational study to characterize three morphologically different subset of
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18 28 neutrophils in the bronchial lavage of adults with obstructive airway disease and healthy
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20 29 controls.
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22 30 ➤ The study investigated clinical association of neutrophils subset with airway obstruction.
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24 31 ➤ The cross-sectional nature of study is a limitation in properly understanding the reason
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26 32 behind neutrophil heterogeneity in airways.
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1718 47 **INTRODUCTION:**
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21 48 Neutrophils are phagocytic innate immune cells which patrol blood vessels and become activated in
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23 49 response to inflammatory triggers¹. Activation results in neutrophil migration to the site of infection,
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25 50 where pathogens can be eliminated by phagocytosis or NETosis². Similarly, infection or injury can
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27 51 result in the initiation of an innate immune response following the engagement of PAMPs (pathogen
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29 52 associated molecular patterns) and DAMPs (damage associated molecular patterns) with pattern
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31 53 recognition receptors of airways. This facilitates the release of chemotactic stimuli such as CXCL8,
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33 54 IL-1 β , and TNF- α , resulting in neutrophil recruitment to the airways³, which is important for the
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35 55 resolution of infection and inflammation⁴. In contrast, a disproportionate or dysregulated influx or
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37 56 efflux of neutrophils can result in persistent neutrophilic airway inflammation and tissue damage⁵.
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42 57 Inflammation characterised by airway neutrophilia is reported in many cases of chronic obstructive
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44 58 airway disease⁶. This includes 20-30% cases of asthma⁷, more than 40% of cases of chronic
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46 59 obstructive pulmonary disease (COPD)^{8 9}, and 70% of cases of non-cystic fibrosis (CF)
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48 60 bronchiectasis¹⁰. Current therapeutic and management strategies for asthma and COPD focus on
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50 61 bronchodilation to overcome airflow limitation, or inhaled corticosteroids based therapies along
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52 62 with modification of eosinophilic airway inflammation using corticosteroids^{11 12}. In non-CF
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3 63 bronchiectasis treatment relies on antibiotics to control the infective nature of the disease¹³. While
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5 64 inhaled corticosteroids are highly effective in modifying eosinophilic inflammation in the airways¹⁴,
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8 65 there are no treatments that have been shown to influence neutrophil mediated inflammation. One
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10 66 of the primary reasons behind this is our lack of understanding about neutrophils ^{15 16}.

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13 67 Despite the fact that previous studies have shown an association between elevated neutrophils in
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15 68 airways with lower FEV₁ in obstructive airway disease ¹⁷, little is known about variations within the
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18 69 population of neutrophils in the airways. Recent studies have identified heterogeneity within
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20 70 circulating neutrophils. Pillay, *et al* ¹⁸ identified three subsets of neutrophils (normal, banded and
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22 71 hypersegmented) in the circulation following an inflammatory challenge. Each subtype had a
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24
25 72 distinct nuclear morphology and pattern of surface adhesion molecule expression, with
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27 73 hypersegmented neutrophils showing increased capacity for oxidative burst along with a unique
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29 74 ability to suppress T lymphocytes. The same morphologically distinct subsets have been identified
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31 75 in both bronchial lavage (BL) and blood from patients with acute respiratory distress syndrome ¹⁹
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34 76 and in infants with severe viral respiratory infection ²⁰.

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37 77 The presence and characteristics of neutrophil subsets in obstructive airways disease is unknown. In
38
39 78 this study, we have characterised neutrophil subsets in BL fluid from adults with asthma, COPD,
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41 79 non-CF bronchiectasis and healthy controls. We hypothesised that participants with obstructive
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44 80 airway disease would have increased numbers of hypersegmented neutrophils and that the presence
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46 81 of hypersegmented neutrophils would be associated with clinical disease severity.

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52 83 **MATERIAL AND METHODS:**

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3 84 **Patient and Public Involvement (PPI):** Patients and or the public were not involved in the
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5 85 development of the research question and outcome measures of this study. The research
6
7 86 question was developed by authors (JLS and PABW). Patients were recruited if they were
8
9 87 undergoing a bronchoscopy as explained in "participants" section. The results will be disseminated
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11 88 through publication and presentation at local, national and international research meetings.
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15 89 **Participants:** Adults who were undergoing bronchoscopy either for medical purposes or were
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17 90 undergoing a surgical procedure that involved endotracheal intubation and had spirometry results,
18
19 91 were recruited for this study from the outpatient clinic of John Hunter Hospital. The study was
20
21 92 approved by Hunter New England Human Research Ethics Committee (Reference No
22
23 93 05/08/10/3.09) and all participants provided written informed consent.
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27 94 **Study design:** A cross sectional study was conducted in which BL samples were obtained after the
28
29 95 assessment of clinical history including respiratory symptoms, smoking status and medication.
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31 96 Spirometry and bronchoscopy were performed as outlined below.
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35 97 **Study group:** Adults (>18 years) with no history of a clinical chest or upper respiratory tract
36
37 98 infection in the previous 6 weeks were studied. Healthy non-smokers (n=20) had normal lung
38
39 99 function assessed by spirometry, and had no previous history of respiratory disease. Adults with
40
41 100 asthma (n=40) had a physician's diagnosis of asthma with objective evidence of airflow variability
42
43 101 or bronchial hyperactivity on provocation challenge. Bronchiectasis (n=20) was defined as evidence
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45 102 of a permanent dilation of airway segment on high resolution computed tomography scan while
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47 103 those with COPD (n=20) had evidence of respiratory symptoms in combination with a post
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49 104 bronchodilator FEV₁ of less than 80% of predicted value and/or a post bronchodilator FEV₁/FVC
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51 105 less than 70%. Current smokers were excluded. Since this was an exploratory study in a completely
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3 106 new setting, the number of participants in each group were decided on the basis of previous
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5 107 exploratory studies in this area^{18 19 21}.

7
8 108 **Spirometry:** Spirometry was performed (Easy One Spirometer, ndd Medical Technologies,
9
10 109 Massachusetts, USA) at John Hunter Hospital. Variable obstruction defined as a post bronchodilator
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12 110 change in FEV₁ of 12% or 200mL after 400 mcg of salbutamol and the bronchial hyper-
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14 111 responsiveness defined as at least 15% decline in FEV₁ after inducing bronchial provocation with
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16 112 4.5% saline solution.

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20 113 **Bronchoscopy:** Flexible bronchoscopy was performed at John Hunter Hospital bronchial wash was
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22 114 taken by wedging the bronchoscope into the right middle lobe and washing with 40 mL of sterile
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24 115 saline solution. A fraction of BL was sent for microbial detection while the rest was processed as
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26 116 described below.

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30 117 **BL processing:** BL was filtered and total cell count (TCC) and viability was assessed by using
31
32 118 trypan blue exclusion method, within one hour of collection at Hunter Medical Research Institute.
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34 119 The BL was centrifuged and the cell pellet was resuspended in PBS to the concentration of 1×10^6 /mL
35
36 120 and cellular cytopins were prepared. The cytopins were stained with May-Grünwald Giemsa
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38 121 (Beckman Coulter, Brea, CA, USA) and a differential cell count of 400 non squamous cells was
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40 122 performed.

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44 123 **Neutrophil subtype assessment:** Stained cytopins were examined under oil immersion and 100
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46 124 neutrophils were enumerated into banded, normal and hypersegmented neutrophils. Banded
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48 125 neutrophils had a single banded lobe without any visible division; normal neutrophils had two to
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50 126 four lobes with every lobe having a properly visible outer boundary; and hypersegmented
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3 127 neutrophils had more than four lobes with every lobe having a properly visible outer boundary as
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5 128 shown in Figure 1.
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8 129 **Statistical Analysis:** Data were analysed using Stata software version 11 (StataCorp, College
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10 130 Station, TX, USA). Results are reported as mean (SD) or median (interquartile range), unless
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12
13 131 otherwise stated. Continuous measures were analysed using the two-sample Wilcoxon's rank sum
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15 132 test or t-test and Kruskal-Wallis test or one way analysis of variance (ANOVA) as appropriate.
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18 133 Categorical data were analysed using Fisher's exact test. Spearman correlation coefficients were
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20 134 calculated for the association between neutrophil subsets and clinical characteristics.
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32 138 **RESULTS**

33 34 35 139 **Clinical characteristics**

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38 140 Participants with COPD were more likely to be ex-smoking males with more severe airflow
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40 141 obstruction (Table 1). Fewer participants with COPD were prescribed ICS compared with the asthma
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42 142 group, however, the mean daily dose of ICS was significantly higher in COPD participants. The
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45 143 number of participants with severe asthma were higher than the number with severe COPD (Table
46
47 144 1) according to GINA²² and GOLD²³ severity classification, respectively. Bronchiectasis
48
49 145 participants were generally of mild severity according to their bronchiectasis severity index²⁴ (Table
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51 146 1). The causes of bronchiectasis is mainly idiopathic and post-infection (Table S1, supplementary
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53
54 147 data).
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148 **Inflammatory cell counts**

149 BL inflammatory cell counts for the participants are detailed in Table 2. Participants with
150 bronchiectasis and COPD had an increased total cell count (TCC) (Table 2). The proportion and
151 number of neutrophils was significantly higher in the bronchiectasis and COPD group compared
152 with healthy controls, while the proportion of neutrophils in asthma were significantly lower in
153 comparison with COPD. The asthma group also had significantly lower number of neutrophils in
154 comparison with bronchiectasis and COPD. The proportion of eosinophils was significantly higher
155 in COPD and asthma compared with healthy controls, while the number of eosinophils was
156 significantly higher in all three obstructive airways diseases compared with healthy controls.

158 **Neutrophil subsets**

159 All three neutrophil subsets were identified in the BL of all participants. The numbers of normal
160 neutrophils were significantly higher in bronchiectasis and COPD group in comparison to healthy
161 and asthma (Figure 2A). Numbers of banded neutrophils were highest in those participants with
162 bronchiectasis compared with both healthy and asthma groups, while in COPD banded neutrophils
163 numbers were higher in comparison with healthy participants only (Figure 2B). Hypersegmented
164 neutrophil numbers were significantly increased in all the obstructive airway disease groups
165 compared with healthy controls and increased in participants with COPD compared with asthma and
166 bronchiectasis (Figure 2C).

167 When considering the relative distribution of neutrophil subtypes by proportion (shown in Figure 2
168 D-F), participants with COPD had a significantly reduced proportion of normal and banded
169 neutrophils and subsequently a significantly increased proportion of hypersegmented neutrophils.

Table 1: Clinical characteristics of participants with bronchiectasis, asthma, COPD, and healthy controls.

	Bronchiectasis	COPD	Asthma	Healthy	p value
n	19	20	39	20	
Age	67.8 (7.1)	68.8 (10.2)	64.8 (7.3)	61.3 (9.7)	0.024
Males, n (%)	7 (36.8)	14 (70.0)	18 (46.2)	9 (45.0)	0.184
Ex-smoker, n (%)	0 (0.0)	20 (100.0) ^{^ φ}	15 (38.5) ^{φ #}	2 (10.0)	<0.001
Smoking (pack years)	--	35.0 (20.0-55.0)	10.0 (4.0-30.0) [#]	(5.0,5.0)	0.007
FEV ₁ % predicted	91.9 (18.3) [#]	57.4 (16.9) [^]	72.3 (20.1) ^{^ φ}	98.6 (12.1), n=19	<0.001
FEV ₁ /FVC (%)	73.0 (67.0-78.0) [#]	59.5 (39.0- 65.0)	66.0 (59.0-72.0) ^{^ #}	75.0 (72.0-80.0) [#] , n=19	<0.001
Taking ICS n (%)	0 (0.0)	8 (40.0)	37 (94.9) [#]	0 (0.0)	<0.001
BDP equivalent ICS dose μg day ⁻¹	--	1700.00 (555.49)	978.37 (398.70) [#]	--	<0.001
Bacterial pathogen, n (%)	8 (42.1) [^]	8 (40.0) [^]	12 (30.8) [^]	0 (0)	0.003
Bronchiectasis severity index	4 (2.0-7.0), n=18	--	--	--	--
GINA stages of asthma severity, n (%)					
Intermittent			1 (2.6)		
Mild persistent			6 (15.8)		
Moderate persistent			9 (23.7)		
Severe persistent			22 (56.4)		

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GOLD stages of COPD severity, n (%)				
GOLD stage 1 (mild)		2 (10.0)		
GOLD stage 2 (moderate)		11 (55.0)		
GOLD stage 3 (severe)		6 (30.0)		
GOLD stage 4 (very severe)		1 (5.0)		

Data are presented as mean ± SD or median (interquartile range; q1- q3) unless otherwise stated. FEV₁: force expiratory volume in 1s; FVC: Forced vital capacity; ICS: Inhaled corticosteroids; BDP equivalent: ICS dose is calculated as beclomethasone dipropionate equivalent, where 1µg of beclomethasone = 1µg budesonide = 0.5µg fluticasone. ^p<0.0125 compared with healthy controls, φ p<0.0125 compared with bronchiectasis and # p<0.0125 compared with COPD.

Table 2: Inflammatory cell count of participants with bronchiectasis, asthma, COPD, and healthy controls.

	Bronchiectasis	COPD	Asthma	Healthy	p value*
Total cells x 10 ⁶ / mL	0.62 (0.19-1.74) [^]	0.83 (0.16-1.88) [^]	0.16 (0.09-0.34) ^{#φ}	0.08 (0.05- 0.21)	<0.001
Viability, %	82.26 (75.00-91.67) [^]	87.75 (73.60-92.95) [^]	77.78 (62.30-88.00)	72.22 (50.00-75.00)	0.005
Neutrophil, %	67.50 (41.00-84.25) [^]	77.25 (73.00-85.13) [^]	58.00 (24.50-72.50) [#]	28.25 (14.75-63.50)	<0.001
Neutrophils x 10 ⁴ cells/mL	43.20 (5.21-164.43) [^]	60.35 (13.31-149.70) [^]	8.24 (3.12-25.01) ^{φ#}	3.18 (1.51-5.03)	<0.001
Eosinophils, %	1.00 (0.50-6.50)	3.75 (1.13-8.88) [^]	2.25 (1.00-11.75) [^]	1.00 (0.75-1.25)	0.016
Eosinophils x 10 ⁴ cells/mL	0.75 (0.40-2.76) [^]	1.89 (1.03-4.03) [^]	0.63 (0.14-3.07) [^]	0.09 (0.05-0.23)	<0.001
Macrophages,%	18.75 (11.00-34.75)	15.50 (8.50-20.03) [^]	25.00 (9.25-39.25)	29.25 (17.00-63.12)	0.025
Macrophages x 10 ⁴ cells/mL	12.40 (5.94-24.42) [^]	9.66 (2.91-18.24)	4.24 (2.00-7.77) ^φ	2.10 (1.42-6.43)	0.002
Lymphocytes, %	0.75 (0.00-1.50)	0.38 (0.00-1.25)	0.50 (0.00-1.50)	1.5 (0.25-5.13)	0.058
Lymphocyte x 10 ⁴ cells/mL	0.30 (0.00-1.02)	0.18 (0.00-0.89)	0.09 (0.00-0.37)	0.18 (0.05-0.42)	0.459
Columnar epithelial cells, %	1.75 (0.75-10.50)	0.25 (0.00-2.50) [^]	4.50 (1.50-10.75) [#]	9.50 (4.88-23.63)	<0.001
Columnar epithelial x 10 ⁴ cells/mL	1.99 (0.48-2.67) [#]	0.28 (0.00-0.59) [^]	1.00 (0.35-1.98) [#]	0.88 (0.38-2.38)	<0.001

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Data are presented as median (interquartile range; q1- q3) unless otherwise stated.

* Kruskal-Wallis test, ^ p<0.0125 compared with healthy, φ p<0.0125 compared with bronchiectasis, and # p<0.0125 compared with COPD.

170 Association of neutrophil subsets with clinical characteristics in obstructive airway disease

171 There was a significant negative correlation between the proportion of hypersegmented neutrophils
172 with both FEV₁% predicted (spearman's Rho -0.301, p=0.007) and FEV₁/FVC% (Rho= -0.322, p=0.004,
173 Figure 3) in participants with obstructive airway disease (n=78). While the same was not observed
174 for banded neutrophils [FEV₁% predicted (Rho= 0.181, p=0.114), FEV₁/FVC% (Rho= 0.213,
175 p=0.061)] and normal neutrophils [FEV₁% predicted (Rho= 0.189, p=0.097), FEV₁/FVC% (Rho=
176 0.213, p=0.062)]. There was no association between total hypersegmented neutrophils (x 10² cells/mL)
177 with both FEV₁% predicted (Rho= -0.152, p=0.185) and FEV₁/FVC% (Rho= -0.173, p=0.131).
178 Similarly, no association was observed between total neutrophil proportion and number with either
179 FEV₁% predicted [Rho= -0.143, p=0.212 and Rho=-0.036, p=0.758, respectively] or FEV₁/FVC%
180 [Rho= -0.142, p=0.214 and Rho=-0.043, p=0.707, respectively).

181 In participants with COPD, the proportion of hypersegmented neutrophils was positively
182 associated with proportion of eosinophils (Rho=0.535, p=0.015) (Figure 4A) and negatively
183 associated with cell viability (Rho= -0.697, p<0.001) (Figure 4B). This association was not
184 observed in any other group or in the overall population (data not shown).

185
186 To explore the correlation between proportion of eosinophils and hypersegmented neutrophils
187 further we decided to examine the COPD participants according to their inflammatory subtype
188 categorised as eosinophilic COPD (E-COPD) ($\geq 3\%$ eosinophils) and non-eosinophilic COPD (NE-
189 COPD) ($< 3\%$ eosinophils).

191 Eosinophilic and non-eosinophilic COPD

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3 192 Twelve participants were characterized as eosinophilic COPD (E-COPD) and eight participants
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5 193 were characterized as non-eosinophilic COPD (NE-COPD). The NE-COPD group had a
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7 194 significantly elevated total cell count [NE COPD, 1.71 (1.47); E COPD, 0.67 (0.55), p=0.037] and
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9 195 cell viability [NE COPD, 90.82 (5.80); E COPD, 76.67 (14.64), p=0.019] along with significantly
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11 196 elevated neutrophil proportion [NE COPD, 85.50 (77.00-92.38); E COPD, 75.75 (69.88-77.75),
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13 197 p=0.037] and number [NE COPD, 148.37 (132.16); E COPD, 50.93 (42.95), p=0.028] in
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15 198 comparison with E-COPD. The number and proportion of eosinophils were significantly higher in
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17 199 ECOPD i.e. [NE COPD, 1.14 (1.05); E COPD, 4.71 (4.09), p=0.040] and [NE COPD, 1.09 (0.57);
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19 200 E COPD, 9.08 (5.50), p<0.001], respectively. Besides this, no significant difference were observed
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21 201 between these groups for other clinical parameters such as age, sex, lung function, ICS dose etc.
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30 203 **Neutrophil subsets in eosinophilic and non-eosinophilic COPD**

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32 204 The proportion of normal neutrophils were significantly reduced while the proportion of
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34 205 hypersegmented neutrophils were elevated (Figure 5 A & C, respectively) in E-COPD compared
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36 206 with NE-COPD. While no significant differences were observed for the number of any individual
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38 207 subset (Figure 5 D-F) between E and NE-COPD. There was also no significant difference for any
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40 208 individual subset proportion or number in total eosinophilic (n=33) vs non eosinophils (n=45)
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42 209 obstructive airway disease participants (data not shown).
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55 213 **DISCUSSION**

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3 214 The study identified three morphologically distinct subsets of neutrophils i.e. banded, normal, and
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5 215 hypersegmented in the BL of participants with chronic obstructive airways disease patients and healthy
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8 216 controls. There were a significantly higher number of hypersegmented neutrophils in those with
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10 217 obstructive airway disease compared with healthy controls. The proportion of hypersegmented
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12 218 neutrophils was associated with lower FEV₁ and more severe airflow obstruction (FEV₁/FVC %) in
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14 219 obstructive airway disease participants and with the presence of eosinophilic airway inflammation in
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17 220 COPD.
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20 221 The concept of morphological heterogeneity in neutrophil population has recently emerged²⁵. We have
21
22 222 examined neutrophil heterogeneity in the bronchial lavage of obstructive airway disease participants
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24 223 and healthy controls. The reason for neutrophil heterogeneity is unclear but may be attributable to the
25
26 224 different stages of cell maturation in the bone marrow before transition to the tissue, or alternatively,
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28 225 neutrophils might change their morphology during the course of inflammation to adjust with the
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31 226 stressors in inflamed airways^{5 26}.
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34 227 Banded neutrophils are also known as immature neutrophils and are deemed incompetent in anti-
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36 228 microbial immune functions as reported in the systemic circulation of sepsis patients²⁷. The emergence
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38 229 of banded neutrophils in the airway can occur after depletion of mature neutrophils in bone marrow
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41 230 following excessive demand during acute inflammation²⁰.
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44 231 The presence of hypersegmented neutrophils in airways could be an attribute of inflammation as the
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46 232 hypersegmented neutrophils have also been reported in other inflammatory conditions such as trauma¹⁸
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48 233 and in chronic inflammatory lung diseases such as ARDS¹⁹.
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52 234 The hypersegmented morphology of the neutrophil implies increased maturation compared with banded
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54 235 and normal neutrophils¹⁸. Maturation is thought to occur in inflamed airways due to the presence of a
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3 236 cytokine rich environment consisting of pro-survival mediators²⁸. The mechanism behind formation of
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5 237 hypersegmented neutrophils are known to be linked with the life cycle of the neutrophils. The increase
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8 238 in survival cause the nucleus of neutrophil to develop more indentation and segmentation, and hence the
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10 239 hypersegmented neutrophils are also called as “old neutrophils”²⁹.

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13 240 The ability of a chemoattractant rich milieu to change the phenotype of neutrophils was recently shown
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15 241 when neutrophils from the blood of healthy volunteers were incubated with the broncoalveolar lavage
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17 242 from a patient with ARDS. These neutrophils altered their phenotype, with an increase in those with a
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19 243 hypersegmented morphology¹⁹. It may be possible that a similar process is occurring chronically in the
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21 244 airways of obstructive airway disease participants, who generally have higher levels of pro-
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23 245 inflammatory cytokines and inflammatory mediators. Previous studies have demonstrated that
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25 246 hypersegmented neutrophils in the circulation demonstrate low expression of L-selectins, which may
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27 247 reduce their anchoring ability on endothelial cells and hence reduce their chances to egress into inflamed
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29 248 airways³⁰. Thus, it is possible that the hypersegmented neutrophils we observed in our study have not
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31 249 directly come from circulation and instead may have become hypersegmented in the airways under the
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33 250 influence of pro-survival mediators.

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36 251 Mediators that promote neutrophil survival and can be present in the airways include; GM-CSF,
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38 252 chemokines like CXCL-8 and lipid mediators such as serum amyloid A^{2,26}. GM-CSF and CXCL-8 are
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40 253 known to enhance neutrophil survival by promoting the expression of anti-apoptotic proteins like
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42 254 survivins and by preventing TNF- α mediated apoptosis^{31,32}. While serum amyloid A is known to prolong
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44 255 neutrophil longevity by preventing mitochondrial damage and decreasing caspase-3 (apoptotic protein)
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46 256 activity³³. Our past studies have reported elevated levels of CXCL-8 in sputum samples of neutrophilic
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48 257 asthma, bronchiectasis³⁴, and COPD patients³⁵. Beside this, we have also reported that elevated levels of
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50 258 serum amyloid A in COPD was associated with neutrophilic inflammation in airways and this was
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3 259 refractory to corticosteroids³⁶. This suggests that the elevated presence of these markers might have
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5 260 played some role in enhancing the survival of neutrophils in airways and promoting the presence of
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8 261 hypersegmented neutrophils.
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11 262 In this study, we also reported a positive correlation between eosinophils and hypersegmented
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13 263 neutrophils proportion in COPD participants along with elevated proportion of hypersegmented
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15 264 neutrophils in E-COPD participants. The presence of eosinophils in airways can further elevate the level
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17
18 265 of GM-CSF due to their own production of this cytokine³⁷, which can further promote maturation of
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20 266 neutrophils. Beside this, the use of ICS to control eosinophilic inflammation may enhance neutrophil
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22 267 survival in the inflamed airways by increasing the activity of anti-apoptotic proteins such as Mcl-1
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24 268 (induced myeloid leukaemia cell differentiation protein) and IAPs (inhibitor of apoptosis proteins) in
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27 269 neutrophils³⁸. This increased maturity and prevention of death may result in an increased proportion of
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29 270 hypersegmented neutrophils.
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31
32 271 There is also a debate about whether all hypersegmented neutrophils have same functional
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34 272 characteristics. Pillay *et al*¹⁸ observed that hypersegmented neutrophils obtained after inducing acute
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37 273 systemic inflammation were exhibiting immunosuppressive effect on T lymphocyte in an *in vitro* co-
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39 274 culture. While in another study by Whitmore *et al*³⁹, observed that neutrophils changed into a
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41 275 hypersegmented phenotype following incubation with *H. Pylori*, which could then exhibit cytotoxic
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44 276 activity on stomach epithelial cells. But interestingly in both these study, hypersegmented neutrophils
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46 277 exhibited their respective response by same mechanism i.e. by administering high amount of ROS
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48 278 (reactive oxygen species) in respective cells, and also had similar pattern of adhesion molecules
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51 279 expression on their surface.
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54 280 The significant association between the proportion of hypersegmented neutrophils with FEV₁ and
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56 281 severe airflow obstruction in our study suggests that where hypersegmented neutrophils are common,
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3 282 airway obstruction is poor. This could be a result of high oxidative burst produced by hypersegmented
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5 283 neutrophils as observed in previous studies, in which hypersegmented neutrophil exhibited high
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8 284 oxidative burst after ex-vivo stimulation^{18 19}. The generation of high oxidative burst by neutrophils
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10 285 may also impair their timely clearance from the airway⁴⁰ and can trigger a vicious cycle of neutrophils
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12 286 influx into the airways⁶. The impairment of neutrophil clearance in airway may cause necrosis of
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14 287 neutrophils which can spill its cytotoxic content such as reactive oxygen species and proteolytic
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16 288 enzymes like neutrophil elastase in the lumen of airways⁴¹. This can further damage airway wall and
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19 289 promote mucus hypersecretion which may result in significant decline in FEV₁ as earlier reported in a
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21 290 study on COPD patients⁴². Interestingly, we did not observe this correlation with other neutrophil
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23 291 subsets or with total neutrophil proportion or number. Further research is needed to understand if
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25 292 hypersegmented neutrophils are common as a result of more severe disease or conversely if they
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27
28 293 influence disease severity.

30
31 294 The cross-sectional nature of study is a limitation in properly establishing the cause and effect of
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33 295 relationship of neutrophil heterogeneity in airways. A further detailed ex-vivo study of influence of
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35 296 pathogen, pro-survival mediators, and current medications like ICS on neutrophil subsets morphology,
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37 297 surface expressions, and functional behaviour is needed to provide a better understanding of the
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39 298 formation of hypersegmented neutrophils in the airways and subsequently in developing a more
40
41 299 comprehensive strategy for assessment and management of airway neutrophilia.

42 43 44 45 300 **CONCLUSION**

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49 301 We have shown the presence of three morphologically different subsets of neutrophils in the airways
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51 302 of healthy and obstructive airway disease participants i.e. asthma, COPD, and bronchiectasis. The
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53 303 increased proportion of hypersegmented neutrophils in the airways of obstructive airway disease

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3 304 participants was associated with reduced lung function of these participants. The proportion of
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5 305 hypersegmented neutrophils was highest in COPD participants in comparison with all other groups.
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11 307 **ACKNOWLEDGEMENTS**
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13

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15
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17
18 310 for Healthy Lungs.
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23
24 312 **CONTRIBUTORS:** JLS developed the idea and designed the study. JLS also supervised and
25
26 313 coordinated the study throughout. RL performed the subtype counting and wrote the manuscript
27
28 314 which was further refined and edited by JLS, PABW, KB and DB. PABW performed the
29
30 315 bronchoscopy, KB supervised the bronchial lavage processing and cytospin preparation and DB
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32 316 supervised the statistical analysis.
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6 **COMPETING INTERESTS:** None.
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12 **FUNDING:** This research received no specific grant from any funding agency in the public,
13 commercial or not for profit sector.
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20 **DATA SHARING STATEMENT:** Raw data can be obtained by contacting the corresponding
21 author.
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FIGURES LEGENDS:

Figure 1: Subsets of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants (X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented neutrophil.

Figure 2: Neutrophil subset number (A-C) and neutrophil subset proportion (D-F) in BL of participants with bronchiectasis, COPD, asthma, and healthy controls. The line in dot plots of each group represents the median. $\wedge p < 0.0125$ compared with healthy controls, * $p < 0.0125$ compared with asthma and # $p < 0.0125$ compared with COPD, as per Kruskal-Wallis test.

Figure 3: Scatterplot of hypersegmented neutrophil proportion vs $FEV_1\%$ predicted (A) and FEV_1/FVC (B) in BL of obstructive airway disease participant's.

Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

Figure 5: Neutrophil subsets proportion (A-C) and neutrophil subsets number (D-F) in bronchial lavage of eosinophilic (E-COPD) and non-eosinophilic COPD (NE-COPD) participants. The line in dot plots of each group represents the median and the p value in each graph is an outcome of Wilcoxon rank-sum test.

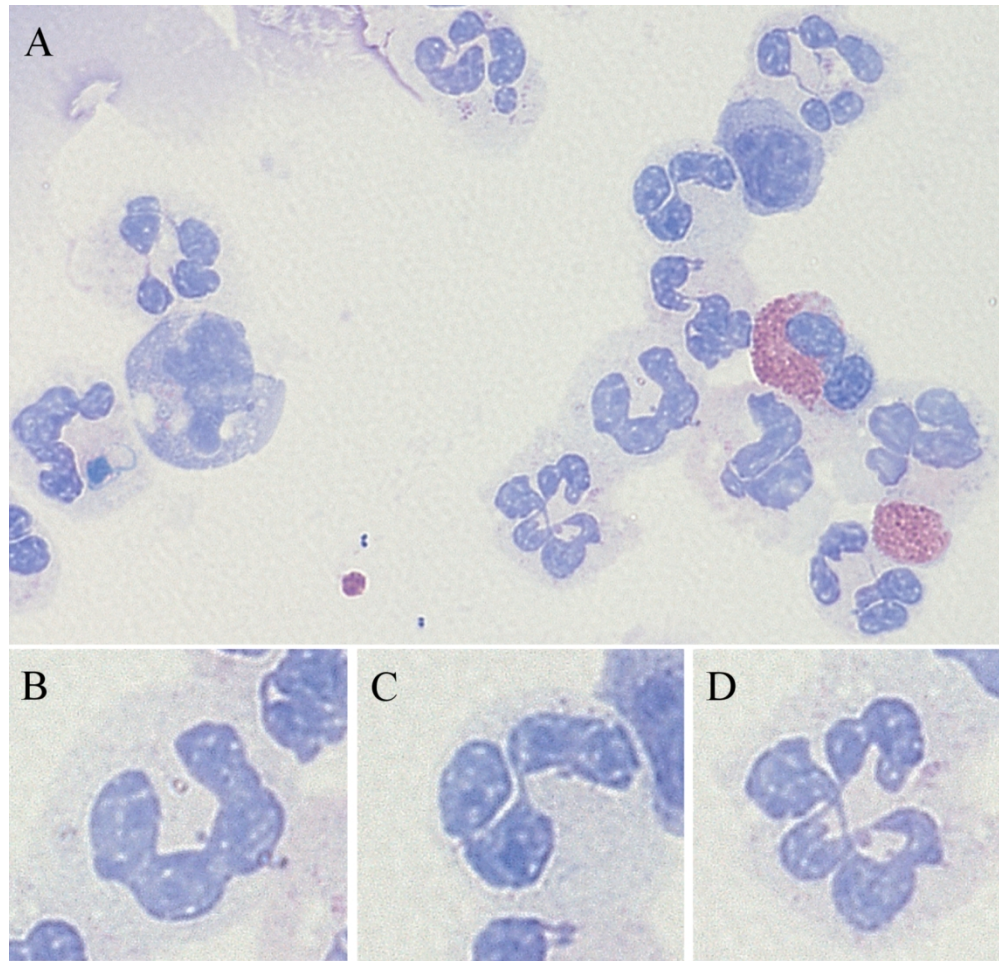


Figure 1: Subtypes of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants(X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented neutrophil.

153x146mm (300 x 300 DPI)

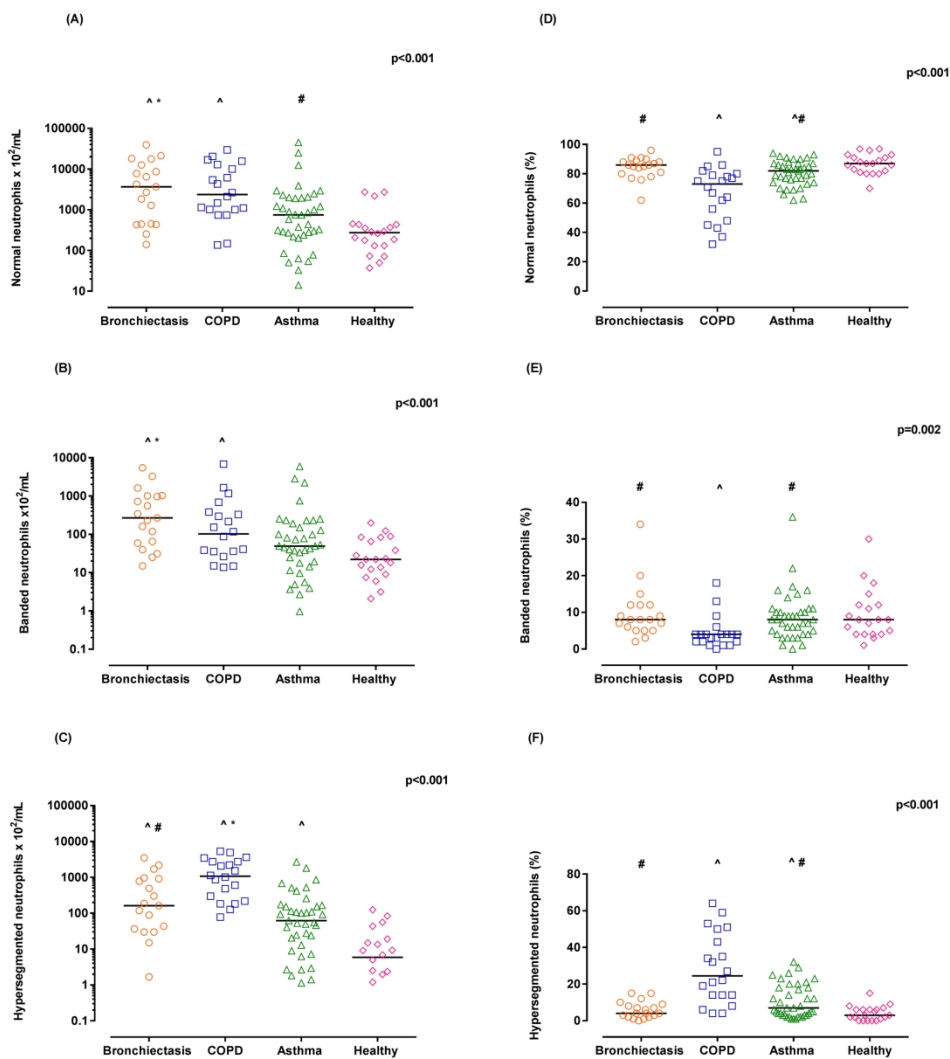


Figure 2: Neutrophil subset number (A-C) and neutrophil subset proportion (D-F) in BL of participants with bronchiectasis, COPD, asthma, and healthy controls. The line in dot plots of each group represents the median. ^p < 0.0125 compared with healthy controls, * p < 0.0125 compared with asthma and # p < 0.0125 compared with COPD, as per Kruskal-Wallis test.

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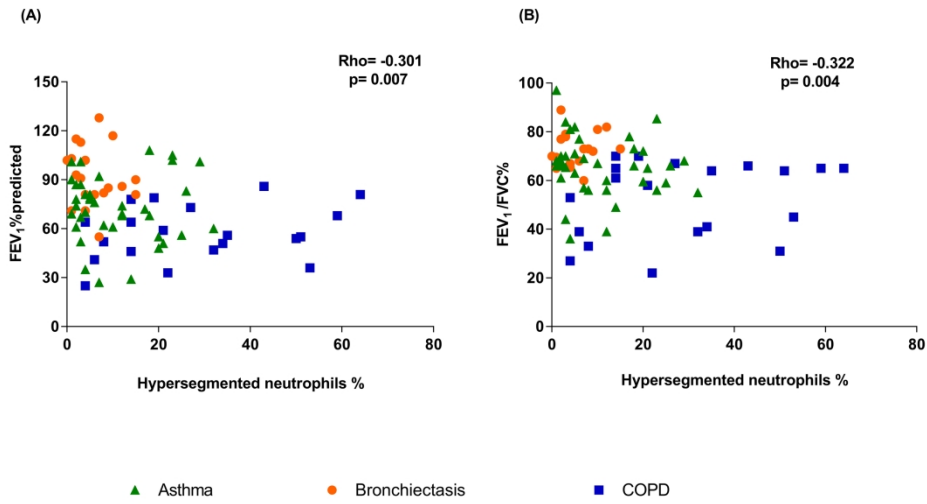


Figure 3: Scatterplot of hypersegmented neutrophil proportion vs FEV₁% predicted (A) and FEV₁/FVC (B) in BL of obstructive airway disease participant's.

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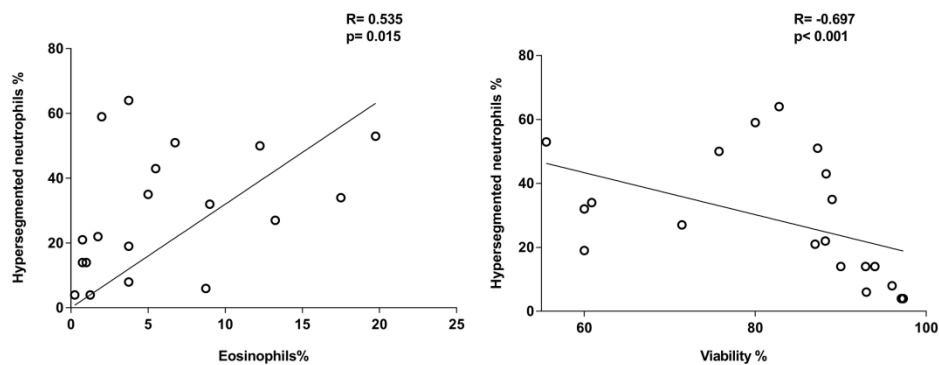


Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

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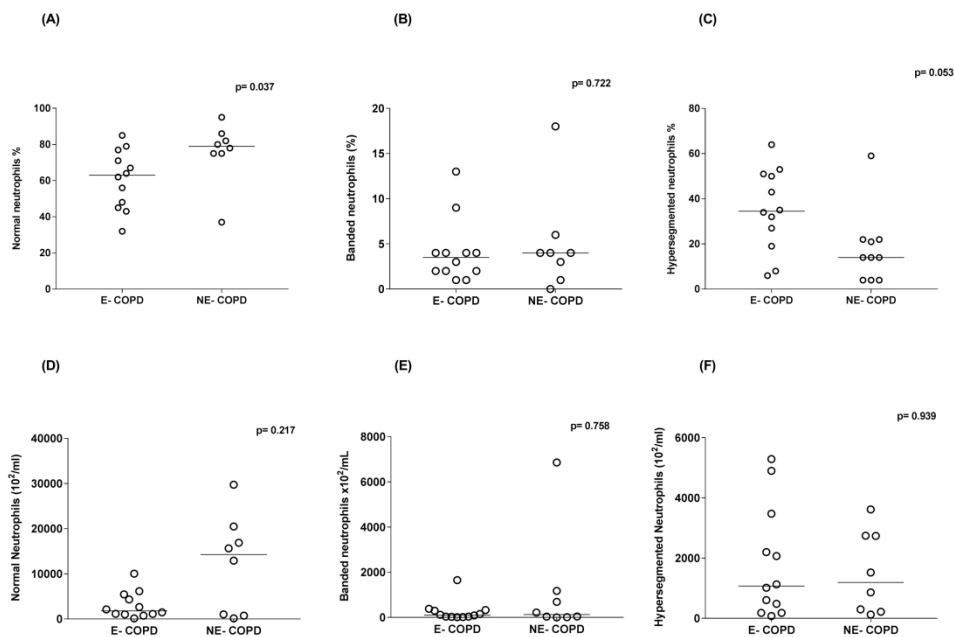


Figure 5: Neutrophil subsets proportion (A-C) and neutrophil subsets number (D-F) in bronchial lavage of eosinophilic (E-COPD) and non-eosinophilic COPD (NE-COPD) participants. The line in dot plots of each group represents the median and the p value in each graph is an outcome of Wilcoxon rank-sum test.

280x185mm (300 x 300 DPI)

Supplementary data:*Table S1: Possible causes of bronchiectasis in bronchiectasis group (n=18).*

Causes of Bronchiectasis	Number of participants (n), (%)
Idiopathic	10 (55.55)
Post-infection	6 (33.33)
Immune deficient	1 (5.56)
Ciliary dyskinesia	0 (0)
COPD	0 (0)
Asthma	0 (0)
Others	1 (5.56)

Reporting checklist for cross sectional study.

Based on the STROBE cross sectional guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the STROBE cross sectional reporting guidelines, and cite them as:

von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.

		Reporting Item	Page Number
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	3
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	3
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	#3	State specific objectives, including any prespecified hypotheses	6
Study design	#4	Present key elements of study design early in the paper	6
Setting	#5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6-7
Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of selection of participants.	6-7
	#7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-7

1	Data sources /	#8	For each variable of interest give sources of data and details of	6-7
2	measurement		methods of assessment (measurement). Describe	
3			comparability of assessment methods if there is more than one	
4			group. Give information separately for for exposed and	
5			unexposed groups if applicable.	
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9	Bias	#9	Describe any efforts to address potential sources of bias	6-7, study utilized standard guidelines to
10				formulate exclusion and inclusion criteria
11				for every group to limit the selection bias.
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15	Study size	#10	Explain how the study size was arrived at	6, (described in study group section")
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17	Quantitative	#11	Explain how quantitative variables were handled in the	8
18	variables		analyses. If applicable, describe which groupings were chosen,	
19			and why	
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22	Statistical	#12a	Describe all statistical methods, including those used to	8
23	methods		control for confounding	
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26		#12b	Describe any methods used to examine subgroups and	n/a...we examined COPD subgroups
27			interactions	(Eosinophilic and Non-Eosinophilic)
28				based on pre-defined cut off values on
29				page 12-14.
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32				
33		#12c	Explain how missing data were addressed	Missing data were excluded from
34				analysis.
35				
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37		#12d	If applicable, describe analytical methods taking account of	n/a. The study did not use any analytical
38			sampling strategy	method.
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41		#12e	Describe any sensitivity analyses	n/a. No sensitive analysis were performed
42				in this study.
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45	Participants	#13a	Report numbers of individuals at each stage of study—eg	n/a...Since it was just a one visit study,
46			numbers potentially eligible, examined for eligibility,	the participant only included if they met
47			confirmed eligible, included in the study, completing follow-	the inclusion criteria and hence
48			up, and analysed. Give information separately for for exposed	participant number were same throughout
49			and unexposed groups if applicable.	the study.
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54		#13b	Give reasons for non-participation at each stage	n/a... No non-participation to report for
55				this study.
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58		#13c	Consider use of a flow diagram	n/a
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1	Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	10
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8		#14b	Indicate number of participants with missing data for each variable of interest	10 (Table 1...spirometry data for only 19 healthy participants out of 20).
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11	Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	8
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16	Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7-14
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20		#16b	Report category boundaries when continuous variables were categorized	14
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24		#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a...was not relevant in this study.
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28	Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	12-14
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32	Key results	#18	Summarise key results with reference to study objectives	15
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36	Limitations	#19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.	17
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43	Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	15-17
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49	Generalisability	#21	Discuss the generalisability (external validity) of the study results	n/a
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53	Funding	#22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2
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1 The STROBE checklist is distributed under the terms of the Creative Commons Attribution License CC-BY. This checklist can be
2 completed online using <https://www.goodreports.org/>, a tool made by the [EQUATOR Network](#) in collaboration with [Penelope.ai](#)
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For peer review only

BMJ Open

Hypersegmented airway neutrophils and its association with reduced lung function in adults with obstructive airway disease: An exploratory study.

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-024330.R2
Article Type:	Research
Date Submitted by the Author:	29-Nov-2018
Complete List of Authors:	Lokwani, Ravi; University of Newcastle School of Medicine and Public Health, Priority Research Centre for Healthy Lungs, Faculty of Health and Medicine, Wark, Peter; Centre for Asthma and Respiratory Disease University of Newcastle, Respiratory and Sleep Medicine Baines, Katherine; University of Newcastle, Respiratory and Sleep Medicine Barker, Daniel; University of Newcastle School of Medicine and Public Health, Faculty of Health and Medicine Simpson, Jodie; The University of Newcastle, Respiratory and Sleep Medicine
Primary Subject Heading:	Respiratory medicine
Secondary Subject Heading:	Immunology (including allergy)
Keywords:	Immunology < THORACIC MEDICINE, Bronchoscopy < THORACIC MEDICINE, Chronic airways disease < THORACIC MEDICINE

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Manuscripts

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3 **Hypersegmented airway neutrophils and its association with reduced lung function in**
4 **adults with obstructive airway disease: An exploratory study.**
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8 **Authors:** Ravi Lokwani ^{1,3}, Peter AB Wark ¹⁻³, Katherine J Baines ^{1,3}, Daniel Barker ³, Jodie L
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50 **Key words:** Immunology, Chronic airways disease, Bronchoscopy.
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55 **Word**

count:

3230

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3 **1 ABSTRACT:**
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6 **2 Objectives:** The significance of neutrophilic inflammation in obstructive airway disease remains
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8 **3** controversial. Recent studies have demonstrated presence of an active neutrophil population in systemic
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10 **4** circulation, featuring hypersegmented morphology, with high oxidative burst and functional plasticity in
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12 **5** inflammatory conditions. The aim of this study was to characterize neutrophil subsets in bronchial lavage
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14 **6** (BL) of obstructive airway disease participants (asthma, COPD and bronchiectasis) and healthy controls
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16 **7** on the basis of nuclear morphology and to assess the association between neutrophil subsets and the
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18 **8** clinical parameters of the obstructive airway disease participants.
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21 **9 Design:** A cross-sectional exploratory study.
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24 **10 Setting:** John Hunter Hospital and Hunter Medical Research Institute, Australia.
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27 **11 Participants:** Seventy-eight adults with obstructive airway disease comprised of those with stable asthma
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29 **12** (n=39) COPD (n=20), and bronchiectasis (n=19) and 20 healthy controls.
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32 **13 Material and Methods:** Cytospins were prepared and neutrophil subsets were classified based on
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34 **14** nuclear morphology into hypersegmented (>4 lobes), normal (2-4 lobes) and banded (1 lobe) neutrophils
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36 **15** and enumerated.
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39 **16 Results:** Neutrophils from each subset were identified in all participants. Numbers of hypersegmented
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41 **17** neutrophils were elevated in participants with airway disease compared with healthy controls ($p < 0.001$).
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43 **18** Both the number and proportion of hypersegmented neutrophils were highest in COPD participants
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45 **19** (median (q1-q3) of 1073.6 (258.8-2742) $\times 10^2/\text{mL}$ and 24.5 (14.0-46.5) %, respectively). An increased
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47 **20** proportion of hypersegmented neutrophils in airway disease participants was significantly associated with
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49 **21** lower FEV₁/FVC % (spearman's Rho= -0.322, $p = 0.004$).
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52 **22 Conclusion:** Neutrophil heterogeneity is common in bronchial lavage and is associated with more severe
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54 **23** airflow obstruction in adults with airways disease. Further work is required to elucidate the functional
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56 **24** consequences of hypersegmented neutrophils in the pathogenesis of disease.
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59 **25 Word count:** 266
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26 STRENGTHS AND LIMITATION OF STUDY

- 27 ➤ This is the first exploratory study to characterize three morphologically different subsets
28 of neutrophils in bronchial lavage of adults with obstructive airway disease and healthy
29 controls.
30 ➤ The study investigated clinical association of neutrophils subset with airway obstruction.
31 ➤ The cross-sectional nature of study is a limitation in properly understanding the reason
32 behind neutrophil heterogeneity in airways.

47 INTRODUCTION:

48 Neutrophils are phagocytic innate immune cells which patrol the blood vessels and become
49 activated in response to inflammatory triggers¹. Activation results in neutrophil migration to the
50 site of infection, where pathogens can be eliminated by phagocytosis or NETosis². Similarly,
51 infection or injury can result in the initiation of an innate immune response following the
52 engagement of PAMPs (pathogen associated molecular patterns) and DAMPs (damage
53 associated molecular patterns) with pattern recognition receptors of airways. This facilitates the
54 release of chemotactic stimuli such as CXCL8, IL-1 β , and TNF- α , resulting in neutrophil
55 recruitment to the airways³, which is important for the resolution of infection and inflammation
56⁴. In contrast, a disproportionate or dysregulated influx or efflux of neutrophils can result in
57 persistent neutrophilic airway inflammation and tissue damage⁵.

58 Inflammation characterised by airway neutrophilia is reported in many cases of chronic
59 obstructive airway disease⁶. This includes 20-30% cases of asthma⁷, more than 40% cases of
60 chronic obstructive pulmonary disease (COPD)^{8,9}, and 70% cases of non-cystic fibrosis (CF)
61 bronchiectasis¹⁰. Current therapeutic and management strategies for asthma and COPD focus
62 on bronchodilation to overcome airflow limitation, or inhaled corticosteroids based therapies for
63 the modification of eosinophilic airway inflammation.^{11,12} In non-CF bronchiectasis, treatment
64 relies on antibiotics to control the infective nature of the disease¹³. While inhaled corticosteroids
65 are highly effective in modifying eosinophilic inflammation in the airways¹⁴, there are no
66 treatments that have been shown to influence neutrophil mediated inflammation. One of the
67 primary reasons behind this is our lack of understanding about neutrophils^{15,16}.

68 Despite the fact that previous studies have shown an association between elevated neutrophils in
69 airways with lower FEV₁ in obstructive airway disease¹⁷, little is known about variations within
70 the population of neutrophils in the airways. Recent studies have identified heterogeneity within

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3 71 circulating neutrophils. Pillay, *et al*¹⁸ identified three subsets of neutrophils (normal, banded and
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5 72 hypersegmented) in the circulation following an inflammatory challenge. Each subset had a
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7 73 distinct nuclear morphology and pattern of surface adhesion molecule expression, with
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10 74 hypersegmented neutrophils showing increased capacity for oxidative burst along with a unique
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12 75 ability to suppress T lymphocytes activation. The same morphologically distinct subsets have
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14 76 been identified in both bronchial lavage (BL) and blood from patients with acute respiratory
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16 77 distress syndrome¹⁹ and in infants with severe viral respiratory infection²⁰.

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20 78 The presence and characteristics of neutrophil subsets in obstructive airways disease is unknown.
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22 79 In this exploratory study, we have characterised and estimated neutrophil subsets in BL fluid
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24 80 from adults with asthma, COPD, non-CF bronchiectasis and healthy controls. In addition we
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26 81 have explored the association of these subsets with the clinical characteristics of obstructive
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28 82 airway disease participants.
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31 32 83 33 34 35 84 **MATERIAL AND METHODS:**

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38 85 **Patient and Public Involvement (PPI):** Patients and or the public were not involved in the
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40 86 development of the research question and outcome measures of this study. The research
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42 87 question was developed by authors (JLS and PABW). Patients were recruited if they were
43
44 88 undergoing a bronchoscopy as explained in "participants" section. The results will be
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46 89 disseminated through publication and presentation at local, national and international research
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48 90 meetings.
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52 91 **Participants:** Adults who were undergoing bronchoscopy either for medical purposes or were
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54 92 undergoing a surgical procedure that involved endotracheal intubation and had spirometry
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56 93 results, were recruited for this study from the outpatient clinic of John Hunter Hospital. The study
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3 94 was approved by Hunter New England Human Research Ethics Committee (Reference No
4
5 95 05/08/10/3.09) and all participants provided written informed consent.
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8 96 **Study design:** A cross sectional exploratory study was conducted in which BL samples were
9
10 97 obtained after an assessment of clinical history including respiratory symptoms, smoking status
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12
13 98 and medication. Spirometry and bronchoscopy were performed as outlined below.
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15

16 99 **Study group:** Adults (>18 years) with no history of a clinical chest or upper respiratory tract
17
18 100 infection in the previous 6 weeks were studied. Healthy non-smokers (n=20) had normal lung
19
20 101 function assessed by spirometry, and had no previous history of respiratory disease. Adults with
21
22 102 asthma (n=39) had a physician's diagnosis of asthma with objective evidence of airflow
23
24 103 variability or bronchial hyperactivity on provocation challenge. Bronchiectasis (n=19) was
25
26 104 defined as evidence of a permanent dilation of airway segment on high resolution computed
27
28 105 tomography scan while those with COPD (n=20) had evidence of respiratory symptoms in
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30 106 combination with a post bronchodilator FEV₁ of less than 80% of predicted value and/or a post
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32 107 bronchodilator FEV₁/FVC less than 70%. Current smokers were excluded. Since this was an
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34 108 exploratory study in a completely new setting, the number of participants in each group were
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36 109 decided on the basis of previous exploratory studies in this area^{18 19 21}.
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42 110 **Spirometry:** Spirometry was performed (Easy One Spirometer, ndd Medical Technologies,
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44 111 Massachusetts, USA) at John Hunter Hospital. Variable obstruction defined as a post
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46 112 bronchodilator change in FEV₁ of 12% or 200mL after 400 mcg of salbutamol and the bronchial
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48 113 hyper-responsiveness defined as at least 15% decline in FEV₁ after inducing bronchial
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50 114 provocation with 4.5% saline solution.
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54 115 **Bronchoscopy:** Flexible bronchoscopy was performed at John Hunter Hospital, bronchial wash
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56 116 was taken by wedging the bronchoscope into the right middle lobe and washing with 40 mL of
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3 117 sterile saline solution. A fraction of BL was sent for microbial detection while the rest was
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5 118 processed as described below.
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8 119 **BL processing:** BL was filtered and total cell count (TCC) and viability was assessed by using
9
10 120 trypan blue exclusion method, within one hour of collection at Hunter Medical Research
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12 121 Institute. The BL was centrifuged and the cell pellet was re-suspended in PBS to the
13
14 122 concentration of $1 \times 10^6/\text{mL}$ and cellular cytopspins were prepared. The cytopspins were stained
15
16 123 with May-Grünwald Giemsa (Beckman Coulter, Brea, CA, USA) and a differential cell count of
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18 124 400 non squamous cells was performed.
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23 125 **Neutrophil subtype assessment:** Stained cytopspins were examined under oil immersion and 100
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25 126 neutrophils were enumerated into banded, normal and hypersegmented neutrophils. Banded
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27 127 neutrophils had a single banded lobe without any visible division; normal neutrophils had two to
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29 128 four lobes with every lobe having a properly visible outer boundary; and hypersegmented
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31 129 neutrophils had more than four lobes with every lobe having a properly visible outer boundary
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33 130 as shown in Figure 1.
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37 131 **Statistical Analysis:** Data were analysed using Stata software version 11 (StataCorp, College
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39 132 Station, TX, USA). Results are reported as mean (SD) or median (interquartile range), unless
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41 133 otherwise stated. Continuous measures were analysed using the two-sample Wilcoxon's rank
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43 134 sum test or t-test and Kruskal-Wallis test or one way analysis of variance (ANOVA) as
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45 135 appropriate. Categorical data were analysed using Fisher's exact test. Spearman correlation
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47 136 coefficients were calculated for the association between neutrophil subsets and clinical
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49 137 characteristics.
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57 139 **RESULTS**

60 140 **Clinical characteristics**

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3 141 Participants with COPD were more likely to be ex-smoking males with more severe airflow
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5 142 obstruction (Table 1). Fewer participants with COPD were prescribed ICS compared with the
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8 143 asthma group, however, the mean daily dose of ICS was significantly higher in COPD
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10 144 participants. The number of participants with severe asthma were higher than the number with
11
12 145 severe COPD (Table 1) according to GINA²² and GOLD²³ severity classification, respectively.
13
14
15 146 Bronchiectasis participants were generally of mild severity according to their bronchiectasis
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17 147 severity index²⁴ (Table 1). The causes of bronchiectasis is mainly idiopathic and post-infection
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19 148 (Table S1, supplementary data).

22 149 **Inflammatory cell counts**

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25 150 BL inflammatory cell counts for the participants are detailed in Table 2. Participants with
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27 151 bronchiectasis and COPD had an increased TCC (Table 2). The proportion and number of
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30 152 neutrophils was significantly higher in the bronchiectasis and COPD group compared with
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32 153 healthy controls, while the proportion of neutrophils in asthma were significantly lower in
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34 154 comparison with COPD. The asthma group also had a significantly lower number of
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37 155 neutrophils in comparison with bronchiectasis and COPD. The proportion of eosinophils was
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39 156 significantly higher in COPD and asthma compared with healthy controls, while the number of
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41 157 eosinophils was significantly higher in all three obstructive airways diseases compared with
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44 158 healthy controls.

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49 160 **Neutrophil subsets**

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52 161 All three neutrophil subsets were identified in the BL of all participants. The numbers of normal
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54 162 neutrophils were significantly higher in bronchiectasis and COPD group in comparison to healthy
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56 163 and asthma (Figure 2A). Numbers of banded neutrophils were highest in those participants with
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58
59 164 bronchiectasis compared with both healthy and asthma groups, while in COPD banded
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3 165 neutrophils numbers were higher in comparison with healthy participants only (Figure 2B).
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5 166 Hypersegmented neutrophil numbers were significantly increased in all the obstructive airway
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8 167 disease groups compared with healthy controls and increased in participants with COPD
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10 168 compared with asthma and bronchiectasis (Figure 2C).
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13 169 When considering the relative distribution of neutrophil subsets by proportion (shown in Figure
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15 170 2 D-F), participants with COPD had a significantly reduced proportion of normal and banded
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18 171 neutrophils and subsequently a significantly increased proportion of hypersegmented
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20 172 neutrophils.
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Table 1: Clinical characteristics of participants with bronchiectasis, asthma, COPD, and healthy controls.

	Bronchiectasis	COPD	Asthma	Healthy	p value
n	19	20	39	20	
Age	67.8 (7.1)	68.8 (10.2)	64.8 (7.3)	61.3 (9.7)	0.024
Males, n (%)	7 (36.8)	14 (70.0)	18 (46.2)	9 (45.0)	0.184
Ex-smoker, n (%)	0 (0.0)	20 (100.0) [^] ϕ	15 (38.5) ^{ϕ} [#]	2 (10.0)	<0.001
Smoking (pack years)	--	35.0 (20.0-55.0)	10.0 (4.0-30.0) [#]	(5.0,5.0)	0.007
FEV ₁ % predicted	91.9 (18.3) [#]	57.4 (16.9) [^]	72.3 (20.1) [^] ϕ	98.6 (12.1), n=19	<0.001
FEV ₁ /FVC (%)	73.0 (67.0-78.0) [#]	59.5 (39.0- 65.0)	66.0 (59.0-72.0) [^] [#]	75.0 (72.0-80.0) [#] , n=19	<0.001
Taking ICS, n (%)	0 (0.0)	8 (40.0)	37 (94.9) [#]	0 (0.0)	<0.001
BDP equivalent ICS dose μ g day ⁻¹	--	1700.00 (555.49)	978.37 (398.70) [#]	--	<0.001
Bacterial pathogen, n (%)	8 (42.1) [^]	8 (40.0) [^]	12 (30.8) [^]	0 (0)	0.003
Bronchiectasis severity index	4 (2.0-7.0), n=18	--	--	--	--
GINA stages of asthma severity, n (%)					
Intermittent			1 (2.6)		
Mild persistent			6 (15.8)		
Moderate persistent			9 (23.7)		

Severe persistent			22 (56.4)		
GOLD stages of COPD severity, n (%)					
GOLD stage 1 (mild)		2 (10.0)			
GOLD stage 2 (moderate)		11 (55.0)			
GOLD stage 3 (severe)		6 (30.0)			
GOLD stage 4 (very severe)		1 (5.0)			

Data are presented as mean ± SD or median (interquartile range; q1- q3) unless otherwise stated. FEV₁: force expiratory volume in 1s; FVC: Forced vital capacity; ICS: Inhaled corticosteroids; BDP equivalent: ICS dose is calculated as beclomethasone dipropionate equivalent, where 1µg of beclomethasone = 1µg budesonide = 0.5µg fluticasone. ^p<0.0125 compared with healthy controls, φ p<0.0125 compared with bronchiectasis and # p<0.0125 compared with COPD.

Table 2: Inflammatory cell count of participants with bronchiectasis, asthma, COPD, and healthy controls.

	Bronchiectasis	COPD	Asthma	Healthy	p value*
Total cells x 10 ⁶ / mL	0.62 (0.19-1.74) [^]	0.83 (0.16-1.88) [^]	0.16 (0.09-0.34) ^{# φ}	0.08 (0.05- 0.21)	<0.001
Viability, %	82.26 (75.00-91.67) [^]	87.75 (73.60-92.95) [^]	77.78 (62.30-88.00)	72.22 (50.00-75.00)	0.005
Neutrophils, %	67.50 (41.00-84.25) [^]	77.25 (73.00-85.13) [^]	58.00 (24.50-72.50) [#]	28.25 (14.75-63.50)	<0.001
Neutrophils x 10 ⁴ /mL	43.20 (5.21-164.43) [^]	60.35 (13.31-149.70) [^]	8.24 (3.12-25.01) ^{φ#}	3.18 (1.51-5.03)	<0.001
Eosinophils, %	1.00 (0.50-6.50)	3.75 (1.13-8.88) [^]	2.25 (1.00-11.75) [^]	1.00 (0.75-1.25)	0.016
Eosinophils x 10 ⁴ /mL	0.75 (0.40-2.76) [^]	1.89 (1.03-4.03) [^]	0.63 (0.14-3.07) [^]	0.09 (0.05-0.23)	<0.001
Macrophages,%	18.75 (11.00-34.75)	15.50 (8.50-20.03) [^]	25.00 (9.25-39.25)	29.25 (17.00-63.12)	0.025
Macrophages x 10 ⁴ /mL	12.40 (5.94-24.42) [^]	9.66 (2.91-18.24)	4.24 (2.00-7.77) ^φ	2.10 (1.42-6.43)	0.002
Lymphocytes, %	0.75 (0.00-1.50)	0.38 (0.00-1.25)	0.50 (0.00-1.50)	1.5 (0.25-5.13)	0.058
Lymphocytes x 10 ⁴ cells/mL	0.30 (0.00-1.02)	0.18 (0.00-0.89)	0.09 (0.00-0.37)	0.18 (0.05-0.42)	0.459
Columnar epithelial cells, %	1.75 (0.75-10.50)	0.25 (0.00-2.50) [^]	4.50 (1.50-10.75) [#]	9.50 (4.88-23.63)	<0.001
Columnar epithelial cells x 10 ⁴ /mL	1.99 (0.48-2.67) [#]	0.28 (0.00-0.59) [^]	1.00 (0.35-1.98) [#]	0.88 (0.38-2.38)	<0.001

Data are presented as median (interquartile range; q1- q3) unless otherwise stated.

* Kruskal-Wallis test, [^] p<0.0125 compared with healthy, ^φ p<0.0125 compared with bronchiectasis, and [#] p<0.0125 compared with COPD.

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3 173 **Association of neutrophil subsets with clinical characteristics in obstructive airway**
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5 174 **disease**

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7 175 There was a significant negative correlation between the proportion of hypersegmented
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9 176 neutrophils with both FEV₁% predicted (spearman's Rho -0.301, p=0.007) and FEV₁/FVC% (Rho=
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11 -0.322, p=0.004, Figure 3) in participants with obstructive airway disease (n=78). While the same
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13 177 -0.322, p=0.004, Figure 3) in participants with obstructive airway disease (n=78). While the same
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15 178 was not observed for banded neutrophils [FEV₁% predicted (Rho= 0.181, p=0.114), FEV₁/FVC%
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17 179 (Rho= 0.213, p=0.061)] and normal neutrophils [FEV₁% predicted (Rho= 0.189, p=0.097),
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19 180 FEV₁/FVC% (Rho= 0.213, p=0.062)]. There was no association between the total number of
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21 181 hypersegmented neutrophils (x 10² cells/mL) with both FEV₁% predicted (Rho= -0.152, p=0.185)
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23 182 and FEV₁/FVC% (Rho= -0.173, p=0.131). Similarly, no association was observed between total
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25 183 neutrophil proportion and number with either FEV₁% predicted [Rho= -0.143, p=0.212 and Rho=-
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27 184 0.036, p=0.758, respectively] or with FEV₁/FVC% [Rho= -0.142, p=0.214 and Rho=-0.043,
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29 185 p=0.707, respectively].

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32 186 In participants with COPD, the proportion of hypersegmented neutrophils was positively
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34 187 associated with proportion of eosinophils (Rho=0.535, p=0.015) (Figure 4A) and negatively
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36 188 associated with cell viability (Rho= -0.697, p<0.001) (Figure 4B). This association was not
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38 189 observed in any other clinical group or in the overall population (data not shown).
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44 191 To explore the correlation between the proportions of eosinophils and hypersegmented
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46 192 neutrophils further, we decided to examine the COPD participants according to their
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48 193 inflammatory subtype categorised as eosinophilic COPD (E-COPD) ($\geq 3\%$ eosinophils) and non-
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50 194 eosinophilic COPD (NE-COPD) ($< 3\%$ eosinophils).
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57 196 **Eosinophilic and non-eosinophilic COPD**
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3 197 Twelve participants were characterized as eosinophilic COPD (E-COPD) and eight participants
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5 198 were characterized as non-eosinophilic COPD (NE-COPD). The NE-COPD group had a
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7 199 significantly elevated total cell count [NE-COPD, 1.71 (1.47) $\times 10^6/\text{mL}$; E-COPD, 0.67 (0.55)
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9 200 $\times 10^6/\text{mL}$, $p=0.037$] and cell viability [NE-COPD, 90.82 (5.80)% ; E-COPD, 76.67 (14.64)%,
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11 201 $p=0.019$] along with a significantly elevated neutrophil proportion [NE-COPD, 85.50 (77.00-
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13 202 92.38)%; E-COPD, 75.75 (69.88-77.75)% , $p=0.037$] and neutrophil number [NE-COPD, 148.37
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15 203 (132.16) $\times 10^4/\text{mL}$; E-COPD, 50.93 (42.95) $\times 10^4/\text{mL}$, $p=0.028$] in comparison with E-COPD.
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17 204 The number and proportion of eosinophils were significantly higher in E-COPD i.e. [NE-COPD,
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19 205 1.14 (1.05) $\times 10^4/\text{mL}$; E-COPD, 4.71 (4.09) $\times 10^4/\text{mL}$, $p=0.040$] and [NE-COPD, 1.09 (0.57)%;
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21 206 E-COPD, 9.08 (5.50)% , $p<0.001$], respectively. Besides this, no significant differences were
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23 207 observed between these groups for any other clinical parameters.
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209 **Neutrophil subsets in eosinophilic and non-eosinophilic COPD**

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35 210 The proportion of normal neutrophils were significantly reduced while the proportion of
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37 211 hypersegmented neutrophils were elevated (Figure 5 A & C, respectively) in E-COPD compared
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39 212 with NE-COPD. While no significant differences were observed for the number of any individual
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41 213 subset (Figure 5 D-F) between E-COPD and NE-COPD.
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217 **DISCUSSION**

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55 218 The study identified three morphologically distinct subsets of neutrophils i.e. banded, normal, and
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57 219 hypersegmented in the BL of participants with chronic obstructive airways disease and healthy
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3 220 controls. There were a significantly higher number of hypersegmented neutrophils in those with
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5 221 obstructive airway disease compared with healthy controls. The proportion of hypersegmented
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7 222 neutrophils was associated with lower FEV₁ and more severe airflow obstruction (FEV₁/FVC %) in
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9
10 223 obstructive airway disease participants and with the presence of eosinophilic airway inflammation
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12 224 in COPD.

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14
15 225 The concept of morphological heterogeneity in neutrophil population has recently emerged²⁵. We
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17 226 have examined neutrophil heterogeneity in the bronchial lavage of obstructive airway disease
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19 227 participants and healthy controls. The reason for neutrophil heterogeneity is unclear but may be
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21 228 attributable to the different stages of cell maturation in the bone marrow before transition to the
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23 229 tissue, or alternatively, neutrophils might change their morphology during the course of
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25 230 inflammation to adjust with the stressors in inflamed airways^{5 26}.

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30 231 Banded neutrophils are also known as immature neutrophils and are deemed incompetent in anti-
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32 232 microbial immune functions as reported in the systemic circulation of sepsis patients²⁷. The
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34 233 emergence of banded neutrophils in the airway can occur after depletion of mature neutrophils in
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36 234 bone marrow following excessive demand during acute inflammation²⁰.

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40 235 The presence of hypersegmented neutrophils in airways could be an attribute of inflammation as the
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42 236 hypersegmented neutrophils have also been reported in other inflammatory conditions such as
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44 237 trauma¹⁸ and in chronic inflammatory lung diseases such as ARDS¹⁹.

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47 238 The hypersegmented morphology of the neutrophil implies increased maturation compared with
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49 239 banded and normal neutrophils¹⁸. Maturation is thought to occur in inflamed airways due to the
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51 240 presence of a cytokine rich environment consisting of pro-survival mediators²⁸. The mechanism
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53 241 behind formation of hypersegmented neutrophils are known to be linked with the life cycle of the
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55 242 neutrophils. The increase in survival cause the nucleus of neutrophil to develop more indentation and
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57 243 segmentation, and hence the hypersegmented neutrophils are also called as “old neutrophils”²⁹.

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3 244 The ability of a chemoattractant rich milieu to change the phenotype of neutrophils was recently
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5 245 shown when neutrophils from the blood of healthy volunteers were incubated with the
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7 246 broncoalveolar lavage from a patient with ARDS. These neutrophils altered their phenotype, with
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9 247 an increase in those with a hypersegmented morphology¹⁹. It may be possible that a similar process
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11 248 is occurring chronically in the airways of obstructive airway disease participants, who generally
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13 249 have higher levels of pro-inflammatory cytokines and inflammatory mediators. Previous studies
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15 250 have demonstrated that hypersegmented neutrophils in the circulation demonstrate low expression
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17 251 of L-selectins, which may reduce their anchoring ability on endothelial cells and hence reduce their
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19 252 chances to egress into inflamed airways³⁰. Thus, it is possible that the hypersegmented neutrophils
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21 253 we observed in our study have not directly come from circulation and instead may have become
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23 254 hypersegmented in the airways under the influence of pro-survival mediators.

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29 255 Mediators that promote neutrophil survival and can be present in the airways include; GM-CSF,
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31 256 chemokines like CXCL-8 and lipid mediators such as serum amyloid A²⁶. GM-CSF and CXCL-8
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33 257 are known to enhance neutrophil survival by promoting the expression of anti-apoptotic proteins like
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35 258 survivins and by preventing TNF- α mediated apoptosis^{31 32}. While serum amyloid A is known to
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37 259 prolong neutrophil longevity by preventing mitochondrial damage and decreasing caspase-3
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39 260 (apoptotic protein) activity³³. Our past studies have reported elevated levels of CXCL-8 in sputum
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41 261 samples of neutrophilic asthma, bronchiectasis³⁴, and COPD patients³⁵. Beside this, we have also
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43 262 reported that elevated levels of serum amyloid A in COPD was associated with neutrophilic
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45 263 inflammation in airways and this was refractory to corticosteroids³⁶. This suggests that the elevated
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47 264 presence of these markers might have played some role in enhancing the survival of neutrophils in
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49 265 airways and promoting the presence of hypersegmented neutrophils.

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55 266 In this study, we also reported a positive correlation between eosinophils and hypersegmented
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57 267 neutrophils proportion in COPD participants along with elevated proportion of hypersegmented
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59 268 neutrophils in E-COPD participants. The presence of eosinophils in airways can further elevate the

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3 269 level of GM-CSF due to their own production of this cytokine³⁷, which can further promote
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5 270 maturation of neutrophils. Beside this, the use of ICS to control eosinophilic inflammation may
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7 271 enhance neutrophil survival in the inflamed airways by increasing the activity of anti-apoptotic
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9 272 proteins such as Mcl-1 (induced myeloid leukaemia cell differentiation protein) and IAPs (inhibitor
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11 273 of apoptosis proteins) in neutrophils³⁸. This increased maturity and prevention of death may result in
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14 274 an increased proportion of hypersegmented neutrophils.

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16
17 275 There is also a debate about whether all hypersegmented neutrophils have same functional
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19 276 characteristics. Pillay *et al*¹⁸ observed that hypersegmented neutrophils obtained after inducing
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21 277 acute systemic inflammation were exhibiting immunosuppressive effect on T lymphocyte in an *in*
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23 278 *vitro* co-culture. While in another study by Whitmore *et al*³⁹, observed that neutrophils changed into
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25 279 a hypersegmented phenotype following incubation with *H .Pylori*, which could then exhibit
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27 280 cytotoxic activity on stomach epithelial cells. But interestingly in both these studies,
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29 281 hypersegmented neutrophils exhibited their respective response by same mechanism i.e. by
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31 282 administering high amount of ROS (reactive oxygen species) in respective cells, and also had similar
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33 283 pattern of adhesion molecules expression on their surface.

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36 284 The significant association between the proportion of hypersegmented neutrophils with FEV₁ and
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38 285 severe airflow obstruction in our study suggests that where hypersegmented neutrophils are
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40 286 common, airway obstruction is most severe. This could be a result of high oxidative burst produced
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42 287 by hypersegmented neutrophils as observed in previous studies, in which hypersegmented
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44 288 neutrophil exhibited high oxidative burst after ex-vivo stimulation^{18 19}. The generation of high
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46 289 oxidative burst by neutrophils may also impair their timely clearance from the airway⁴⁰ and can
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48 290 trigger a vicious cycle of neutrophils influx into the airways⁶. The impairment of neutrophil
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50 291 clearance in airway may cause necrosis of neutrophils which can spill its cytotoxic content such as
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52 292 reactive oxygen species and proteolytic enzymes like neutrophil elastase in the lumen of airways⁴¹.
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54 293 This can further damage airway wall and promote mucus hypersecretion which may result in

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3 294 significant decline in FEV₁ as earlier reported in COPD⁴². Interestingly, we did not observe this
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5 295 correlation with other neutrophil subsets or with total neutrophil proportion or number. Further
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8 296 research is needed to understand if hypersegmented neutrophils are common as a result of more
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10 297 severe disease or conversely if they influence disease severity.

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13 298 The cross-sectional nature of study is a limitation in properly establishing the cause and effect of
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15 299 relationship of neutrophil heterogeneity in airways. Besides that, small sample sizes is another
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17 300 limitation of this study. Hence, further confirmatory studies are needed with large sample sizes to
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19 301 validate the finding of this study. Additionally, a detailed ex-vivo study of influence of pathogen,
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21 302 pro-survival mediators, and current medications like ICS on neutrophil subsets morphology, surface
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23 303 expressions, and functional behaviour is also needed to provide a better understanding of the
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25 304 formation of hypersegmented neutrophils in the airways and subsequently in developing a more
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27 305 comprehensive strategy for assessment and management of airway neutrophilia.

31 306 **CONCLUSION**

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35 307 We have shown the presence of three morphologically different subsets of neutrophils in the airways
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37 308 of healthy and obstructive airway disease participants i.e. asthma, COPD, and bronchiectasis. The
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39 309 increased proportion of hypersegmented neutrophils in the airways of obstructive airway disease
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41 310 participants was associated with reduced lung function of these participants. The proportion of
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43 311 hypersegmented neutrophils was highest in COPD participants in comparison with all other groups.

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52
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56
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6 318 **CONTRIBUTORS:** JLS developed the idea and designed the study. JLS also supervised and
7
8 319 coordinated the study throughout. RL performed the subtype counting and wrote the manuscript
9
10 320 which was further refined and edited by JLS, PABW, KB and DB. PABW performed the
11
12 321 bronchoscopy, KB supervised the bronchial lavage processing and cytospin preparation and DB
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15 322 supervised the statistical analysis.
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For peer review only

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6 **COMPETING INTERESTS:** None.
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13 commercial or not for profit sector.
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20 **DATA SHARING STATEMENT:** Raw data can be obtained by contacting the corresponding
21 author.
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25 26 27 28 29 **REFERENCES**

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FIGURES LEGENDS:

Figure 1: Subsets of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants (X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented neutrophil.

Figure 2: Neutrophil subset number (A-C) and neutrophil subset proportion (D-F) in BL of participants with bronchiectasis, COPD, asthma, and healthy controls. The line in dot plots of each group represents the median. $\wedge p < 0.0125$ compared with healthy controls, * $p < 0.0125$ compared with asthma and # $p < 0.0125$ compared with COPD, as per Kruskal-Wallis test.

Figure 3: Scatterplot of hypersegmented neutrophil proportion vs FEV₁% predicted (A) and FEV₁/FVC (B) in BL of obstructive airway disease participant's.

Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

Figure 5: Neutrophil subsets proportion (A-C) and neutrophil subsets number (D-F) in bronchial lavage of eosinophilic (E-COPD) and non-eosinophilic COPD (NE-COPD) participants. The line in dot plots of each group represents the median and the p value in each graph is an outcome of Wilcoxon rank-sum test.

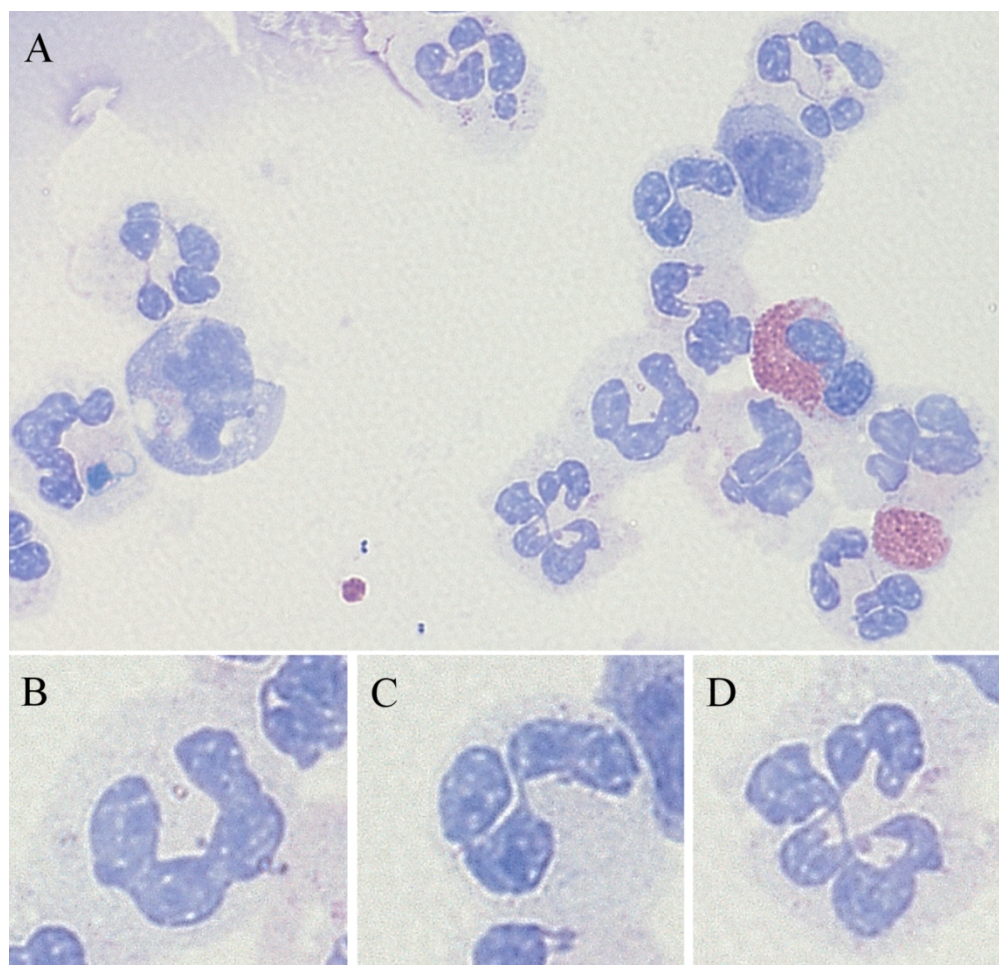


Figure 1: Subtypes of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants(X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented neutrophil.

153x146mm (300 x 300 DPI)

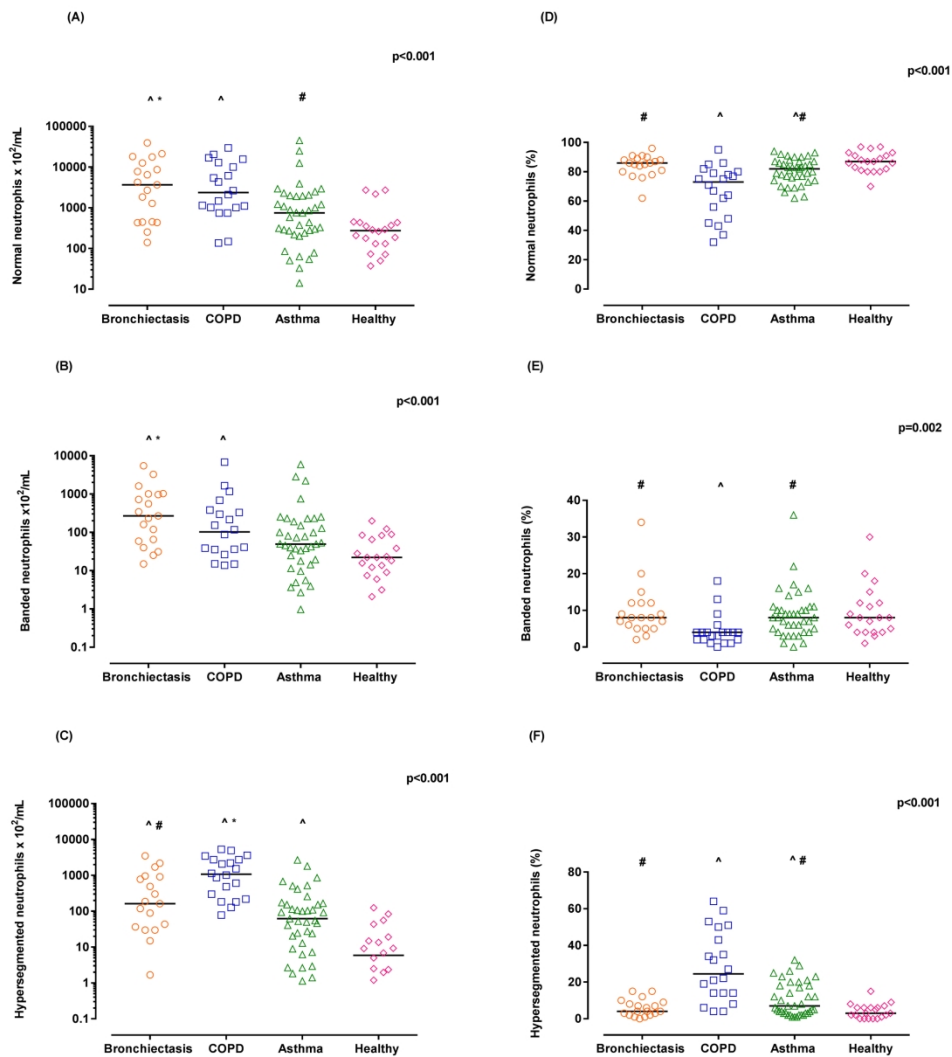


Figure 2: Neutrophil subset number (A-C) and neutrophil subset proportion (D-F) in BL of participants with bronchiectasis, COPD, asthma, and healthy controls. The line in dot plots of each group represents the median. ^p<0.0125 compared with healthy controls, * p<0.0125 compared with asthma and # p<0.0125 compared with COPD, as per Kruskal-Wallis test.

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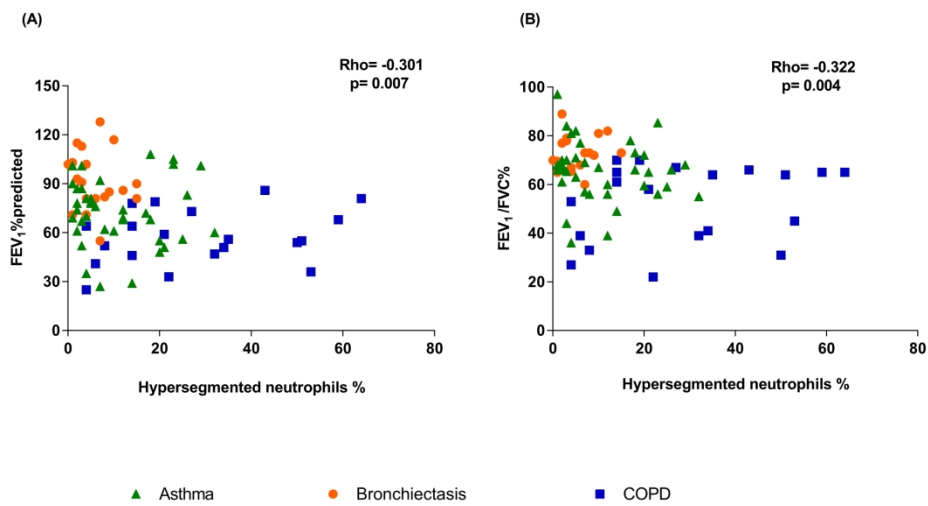


Figure 3: Scatterplot of hypersegmented neutrophil proportion vs FEV₁% predicted (A) and FEV₁/FVC (B) in BL of obstructive airway disease participant's.

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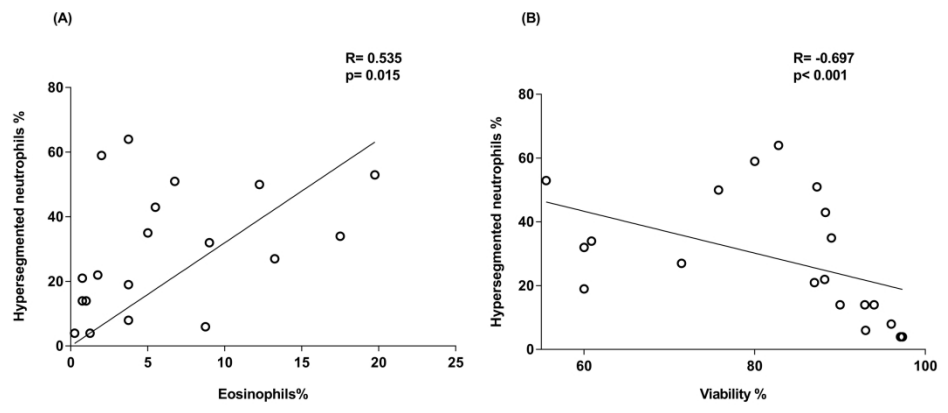


Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

268x134mm (300 x 300 DPI)

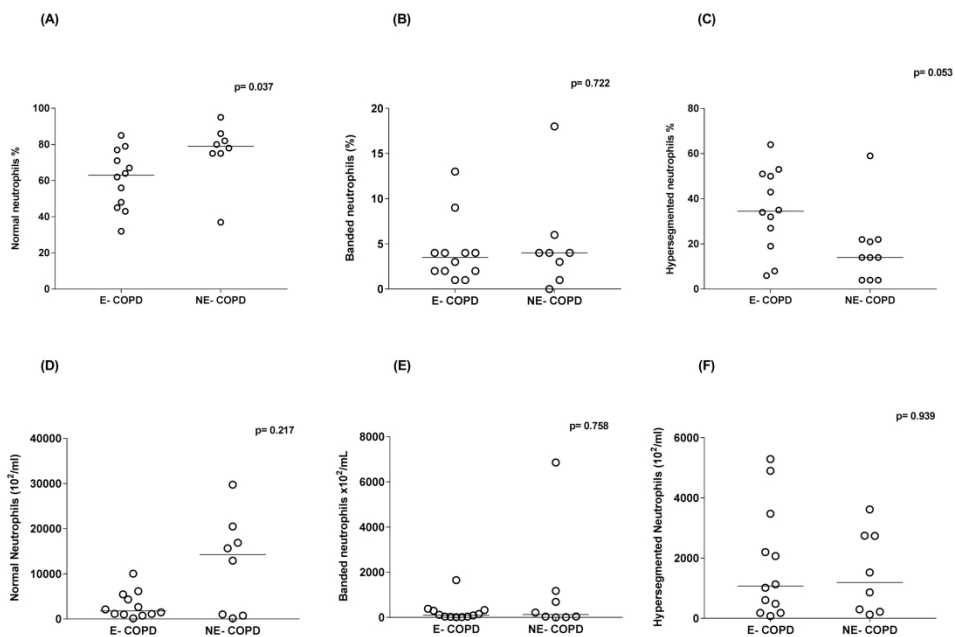


Figure 5: Neutrophil subsets proportion (A-C) and neutrophil subsets number (D-F) in bronchial lavage of eosinophilic (E-COPD) and non-eosinophilic COPD (NE-COPD) participants. The line in dot plots of each group represents the median and the p value in each graph is an outcome of Wilcoxon rank-sum test.

280x185mm (300 x 300 DPI)

Supplementary data:*Table S1: Possible causes of bronchiectasis in bronchiectasis group (n=18).*

Causes of Bronchiectasis	Number of participants (n), (%)
Idiopathic	10 (55.55)
Post-infection	6 (33.33)
Immune deficient	1 (5.56)
Ciliary dyskinesia	0 (0)
COPD	0 (0)
Asthma	0 (0)
Others	1 (5.56)

Reporting checklist for cross sectional study.

Based on the STROBE cross sectional guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the STROBE cross sectional reporting guidelines, and cite them as:

von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.

		Reporting Item	Page Number
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	3
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	3
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	#3	State specific objectives, including any prespecified hypotheses	6
Study design	#4	Present key elements of study design early in the paper	6
Setting	#5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6-7
Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of selection of participants.	6-7
	#7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-7

1	Data sources /	#8	For each variable of interest give sources of data and details of	6-7
2	measurement		methods of assessment (measurement). Describe	
3			comparability of assessment methods if there is more than one	
4			group. Give information separately for for exposed and	
5			unexposed groups if applicable.	
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9	Bias	#9	Describe any efforts to address potential sources of bias	6-7, study utilized standard guidelines to
10				formulate exclusion and inclusion criteria
11				for every group to limit the selection bias.
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14				
15	Study size	#10	Explain how the study size was arrived at	6, (described in study group section")
16				
17	Quantitative	#11	Explain how quantitative variables were handled in the	8
18	variables		analyses. If applicable, describe which groupings were chosen,	
19			and why	
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22	Statistical	#12a	Describe all statistical methods, including those used to	8
23	methods		control for confounding	
24				
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26		#12b	Describe any methods used to examine subgroups and	n/a...we examined COPD subgroups
27			interactions	(Eosinophilic and Non-Eosinophilic)
28				based on pre-defined cut off values on
29				page 12-14.
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33		#12c	Explain how missing data were addressed	Missing data were excluded from
34				analysis.
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37		#12d	If applicable, describe analytical methods taking account of	n/a. The study did not use any analytical
38			sampling strategy	method.
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41		#12e	Describe any sensitivity analyses	n/a. No sensitive analysis were performed
42				in this study.
43				
44				
45	Participants	#13a	Report numbers of individuals at each stage of study—eg	n/a...Since it was just a one visit study,
46			numbers potentially eligible, examined for eligibility,	the participant only included if they met
47			confirmed eligible, included in the study, completing follow-	the inclusion criteria and hence
48			up, and analysed. Give information separately for for exposed	participant number were same throughout
49			and unexposed groups if applicable.	the study.
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54		#13b	Give reasons for non-participation at each stage	n/a... No non-participation to report for
55				this study.
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58		#13c	Consider use of a flow diagram	n/a
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1	Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	10	
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8		#14b	Indicate number of participants with missing data for each variable of interest	10 (Table 1...spirometry data for only 19 healthy participants out of 20).	
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11	Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	8	
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14	Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7-14	
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20		#16b	Report category boundaries when continuous variables were categorized	14	
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24		#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a...was not relevant in this study.	
25					
26	Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	12-14	
27					
28	Key results	#18	Summarise key results with reference to study objectives	15	
29	Limitations	#19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.	17	
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33	Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	15-17	
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36	Generalisability	#21	Discuss the generalisability (external validity) of the study results	n/a	
37					
38	Funding	#22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2	
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