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Hypersegmented airway neutrophils and its association with reduced lung function in 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 access 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) x 10^2/mL and 23.5 (11.0-46.5) %), respectively. An increased proportion of hypersegmented neutrophils in airway disease participants was significantly associated with lower FEV_1/FVC % (ρ= -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.

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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 triggers [1]. Activation results in neutrophil migration to the site of infection, where pathogens can be eliminated by phagocytosis or NETosis [2]. 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 patters) with pattern recognition receptors of airways. This facilitates the release of chemotactic stimuli such as CXCL8, IL-1β, and TNF-α, resulting in neutrophil recruitment to the airways [3], which is important for the resolution of infection and inflammation [4]. In contrast, a disproportionate or dysregulated influx or efflux of neutrophils can result in persistent neutrophilic airway inflammation and tissue damage [5].

Inflammation characterised by airway neutrophilia is reported in many cases of chronic obstructive airway disease [6]. This includes 20-30% cases of asthma [7], more than 40% of cases of chronic obstructive pulmonary disease (COPD) [8 9], and 70% of cases of non-cystic fibrosis (CF) bronchiectasis [10]. 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 corticosteroids [11 12]. In non-CF bronchiectasis treatment relies on antibiotics to control the infective nature of the disease[13]. While inhaled corticosteroids are highly effective in modifying eosinophilic inflammation in the airways[14], 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 FEV_1 in obstructive airway disease [17], little is known about variations

within the population of neutrophils in the airways. Recent studies have identified heterogeneity within circulating neutrophils. Pillay, *et al* [18] identified three subsets of neutrophils (normal, banded and hypersegmented) in the circulation following an inflammatory challenge. Each subtype had a distinct nuclear morphology and pattern of surface adhesion molecule expression, with hypersegmented neutrophils showing increased capacity for oxidative burst along with a unique ability to suppress T lymphocytes. The same morphologically distinct subsets have been identified in both bronchial lavage (BL) and blood from patients with acute respiratory distress syndrome [19] and in infants with severe viral respiratory infection [20].

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

MATERIAL AND METHODS:

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

Participants: Adults were who were undergoing bronchoscopy for airways assessment were recruited from the John Hunter Hospital. The study was approved by Hunter New England Human Research Ethics Committee (Reference No 05/08/10/3.09) and all participants provided written informed consent.

Study design: A cross sectional study was conducted in which BL samples were obtained after the assessment of clinical history including respiratory symptoms, smoking status and medication. Spirometry and bronchoscopy were performed as outlined below.

Study group: Adults (>18 years) with no history of a clinical chest or upper respiratory tract infection in the previous 6 weeks were studied. Healthy non-smokers (n=20) had normal lung function assessed by spirometry, and had no previous history of respiratory disease. Adults with asthma (n=40) had a physician's diagnosis of asthma with objective evidence of airflow variability or bronchial hyperactivity on provocation challenge. Bronchiectasis (n=20) was defined as evidence of a permanent dilation of airway segment on high resolution computed tomography scan while those with COPD (n=20) had evidence of respiratory symptoms in combination with a post bronchodilator FEV_1 of less than 80% of predicted value and/or a post bronchodilator FEV_1/FVC less than 70%. Current smokers were excluded.

Spirometry: Spirometry was performed (Easy One Spirometer, ndd Medical Technologies, Massachusetts, USA) at John Hunter Hospital. Variable obstruction defined as a post bronchodilator change in FEV_1 of 12% or 200mL after 400 mcg of salbutamol and the bronchial hyper-responsiveness defined as at least 15% decline in FEV_1 after inducing bronchial provocation with 4.5% saline solution.

Bronchoscopy: Flexible bronchoscopy was performed at John Hunter Hospital and bronchial wash was taken by wedging the bronchoscope into the right middle lobe and washing with 40

mL of sterile saline solution. A fraction of BL was sent for microbial detection while the rest was processed as described below.

BL processing: BL was filtered and total cell count (TCC) and viability assessed within one hour of collection at Hunter Medical Research Institute. The BL was centrifuged and the cell pellet was resuspended in PBS to the concentration of $1x10^6$/mL and cellular cytospins were prepared. The cytospins were stained with May-Grünwald Giemsa (Beckman Coulter, Brea, CA, USA) and a differential cell count of 400 non squamous cells was performed.

Neutrophil subtype assessment: Stained cytospins were examined under oil immersion and 100 neutrophils were enumerated into banded, normal and hypersegmented neutrophils. Banded neutrophils had a single banded lobe without any visible division; normal neutrophils had two to four lobes with every lobe having a properly visible outer boundary; and hypersegmented neutrophils had more than four lobes with every lobe having a properly visible outer boundary as shown in Figure 1.

Statistical Analysis: Data were analysed using Stata software version 11 (StataCorp, College Station, TX, USA). Results are reported as mean (SD) or median (interquartile range), unless otherwise stated. Continuous measures were analysed using the two-sample Wilcoxon's rank sum test or t-test and Kruskal-Wallis test or one way analysis of variance (ANOVA) as appropriate. Categorical data were analysed using Fisher's exact test. Spearman correlation coefficients were calculated for the association between neutrophil subsets and clinical characteristics.

RESULTS

Clinical characteristics

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

Inflammatory cell counts

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

Neutrophil subsets

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

When considering the relative distribution of neutrophil subtypes by proportion (shown in Figure 2 D-F), participants with COPD had a significantly reduced proportion of normal and banded 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	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. FEV_1: 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	0.61 (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 FEV_1% predicted (ρ = -0.278, p=0.012) and FEV_1/FVC% (ρ = -0.313, p=0.005) (Figure 3) in participants with obstructive airway disease. While the same was not observed for banded neutrophils [FEV_1% predicted (ρ = 0.151, p=0.183), FEV_1/FVC% (ρ = 0.191, p=0.090)] and normal neutrophils [FEV_1% predicted (ρ = 0.163, p=0.149), FEV_1/FVC% (ρ =0.204, p=0.069)]. There was no association between the total neutrophil proportion with either FEV_1% predicted (ρ = -0.170, p=0.132) or FEV_1/FVC% (ρ = -0.151, p=0.181).

In participants with COPD, the proportion of hypersegmented neutrophils was positively associated with proportion of eosinophils (ρ =0.597, p=0.006) (Figure 4A) and negatively associated with cell viability (ρ = -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) (≥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 µg 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 ± SD or median (interquartile range; q1- q3) unless otherwise stated. FEV_1: 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 FEV_1 and more severe airflow obstruction (FEV_1/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 emerged [21]. 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 patients[23]. The emergence of banded neutrophils in the airway can occur after depletion of mature neutrophils in bone marrow following excessive demand during acute inflammation [20].

The hypersegmented morphology of the neutrophil implies increased maturation compared with banded and normal neutrophils [18]. Maturation is thought to occur in inflamed airways due to the presence of a cytokine rich environment consisting of pro-survival mediators [24]. 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].

The ability of a chemoattractant rich milieu to change the phenotype of neutrophils was recently shown when neutrophils from the blood of healthy volunteers were incubated with the broncoalveolar lavage from a patient with ARDS. These neutrophils altered their phenotype, with an increase in those with a hypersegmented morphology [19]. It may be possible that a similar process is occurring chronically in the airways of obstructive airway disease participants, who generally have higher levels of pro-inflammatory cytokines and inflammatory mediators. Previous studies have demonstrated that hypersegmented neutrophils in the circulation demonstrate low expression of L-selectins, which may reduce their anchoring ability on endothelial cells and hence reduce their chances to egress into inflamed airways[26]. Thus, it is possible that the hypersegmented neutrophils we observed in our study have not directly come from circulation and instead may have become hypersegmented in the airways under the influence of pro-survival mediators.

Mediators that promote neutrophil survival and can be present in the airways include; GM-CSF, chemokines like CXCL-8 and lipid mediators such as serum amyloid A [2 22]. GM-CSF and CXCL-8 are known to enhance neutrophil survival by promoting the expression of anti-apoptotic proteins like survivins and by preventing TNF-α mediated apoptosis [27 28]. While serum amyloid A is known to prolong neutrophil longevity by preventing mitochondrial damage and decreasing caspase-3 (apoptotic protein) activity[29]. Our past studies have reported elevated levels of CXCL-8 in sputum samples of neutrophilic asthma, bronchiectasis[30], and COPD patients[31]. Beside this, we have also reported that elevated levels of serum amyloid A in COPD was associated with neutrophilic inflammation in airways and this was refractory to corticosteroids[32]. This suggests that the elevated presence of these markers might have played some role in enhancing the survival of neutrophils in airways and promoting the presence of hypersegmented neutrophils.

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

further elevate the level of GM-CSF due to their own production of this cytokine[33], which can further promote maturation of neutrophils. Beside this, the use of ICS to control eosinophilic inflammation may enhance neutrophil survival in the inflamed airways by increasing the activity of anti-apoptotic proteins such as Mcl-1 (induced myeloid leukaemia cell differentiation protein) and IAPs (inhibitor of apoptosis proteins) in neutrophils [34]. This increased maturity and prevention of death may result in an increased proportion of hypersegmented neutrophils.

The significant association between the proportion of hypersegmented neutrophils with FEV_1 and severe airflow obstruction suggests that where hypersegmented neutrophils are common, airway obstruction is poor. This could be a result of high oxidative burst produced by hypersegmented neutrophils as observed in previous studies, in which hypersegmented neutrophil exhibited high oxidative burst after ex-vivo stimulation [18] [19]. The generation of high oxidative burst by neutrophils may also impair their timely clearance from the airway[35] and can trigger a vicious cycle of neutrophils influx into the airways[6]. The impairment of neutrophil clearance in airway may cause necrosis of neutrophils which can spill its cytotoxic content such as reactive oxygen species and proteolytic enzymes like neutrophil elastase in the lumen of airways[36]. This can further damage airway wall and promote mucus hypersecretion which may result in significant decline in FEV_1 as earlier reported in a study on COPD patients [37]. Further research is needed to understand if hypersegmented neutrophils are common as a result of more severe disease or conversely if they influence disease severity.

The cross-sectional nature of study is a limitation in properly establishing the cause and effect of relationship of neutrophil heterogeneity in airways. A further detailed ex-vivo study of influence of pathogen, pro-survival mediators, and current medications like ICS on neutrophil subsets morphology, surface expressions, and functional behaviour is needed to provide a better understanding of the formation of hypersegmented neutrophils in the airways and subsequently in

developing a more comprehensive strategy for assessment and management of airway neutrophilia.

CONCLUSION

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

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CONTRIBUTORS: JLS developed the idea and designed the study. JLS also supervised and coordinated the study throughout. RL performed the subtype counting and wrote the manuscript which was further refined and edited by JLS, PABW, KB and DB. PABW performed the bronchoscopy, KB supervised the bronchial lavage processing and cytospin preparation and DB supervised the statistical analysis.

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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. ^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 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.*

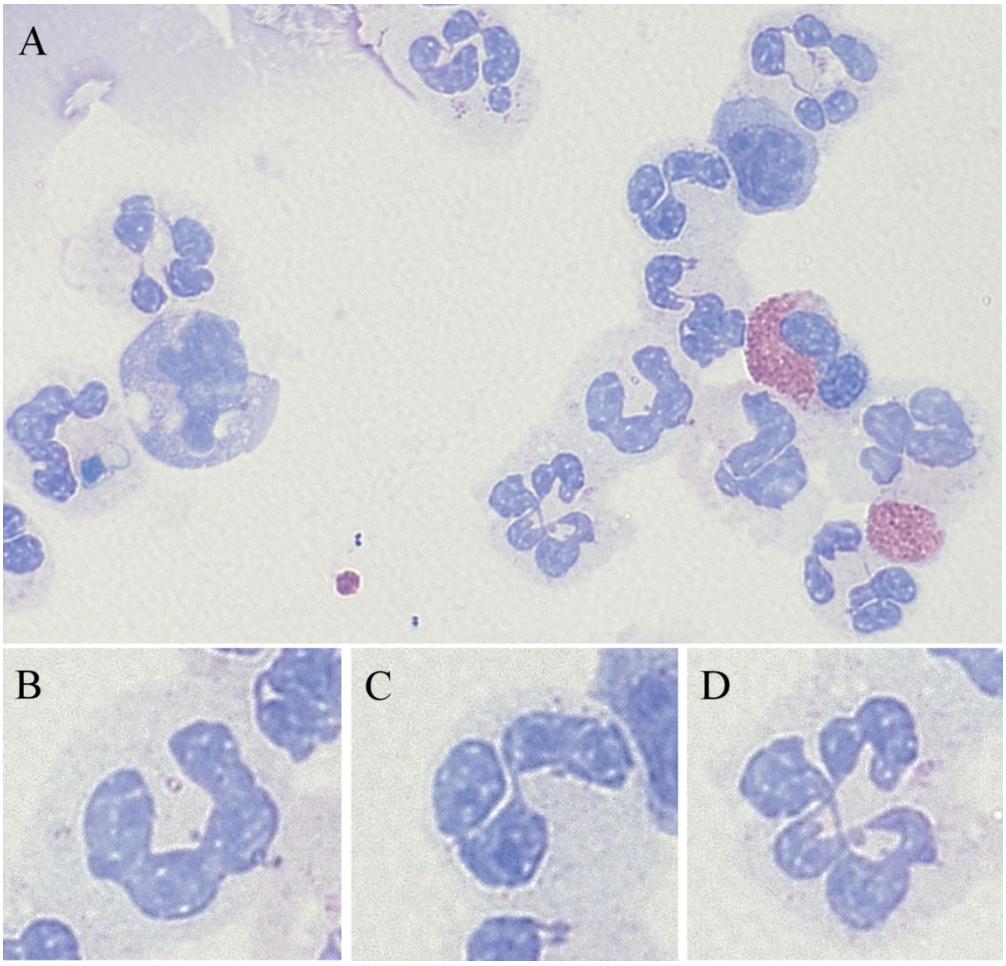
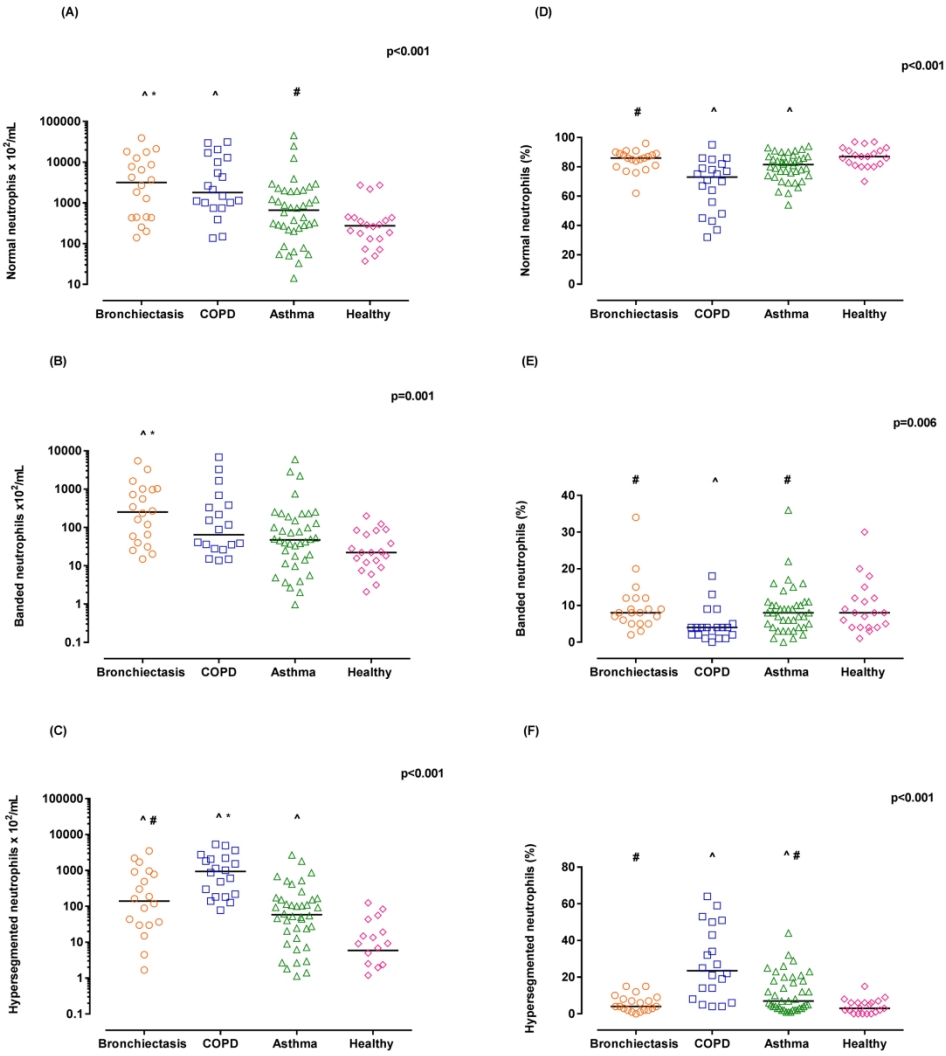


Figure 1: Subtypes of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants(X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented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

153x146mm (300 x 300 DPI)



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237x260mm (300 x 300 DPI)

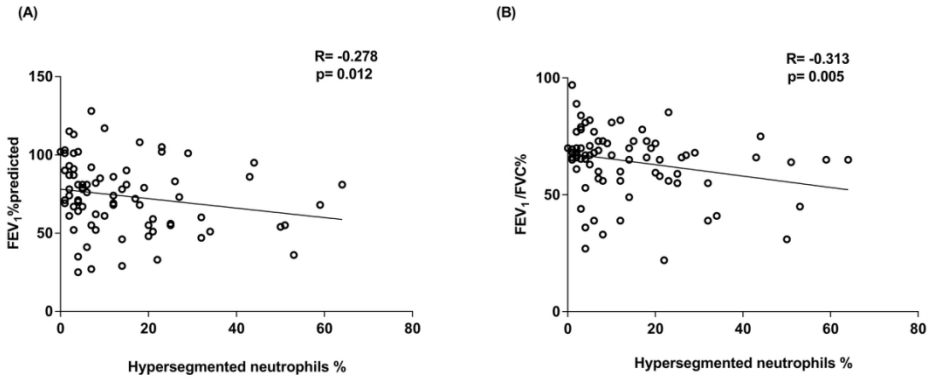

Figure 3: Scatterplot of hypersegmented neutrophil proportion vs FEV1% predicted (A) and FEV1/FVC (B) in BL of obstructive airway disease participant's.

111x48mm (300 x 300 DPI)

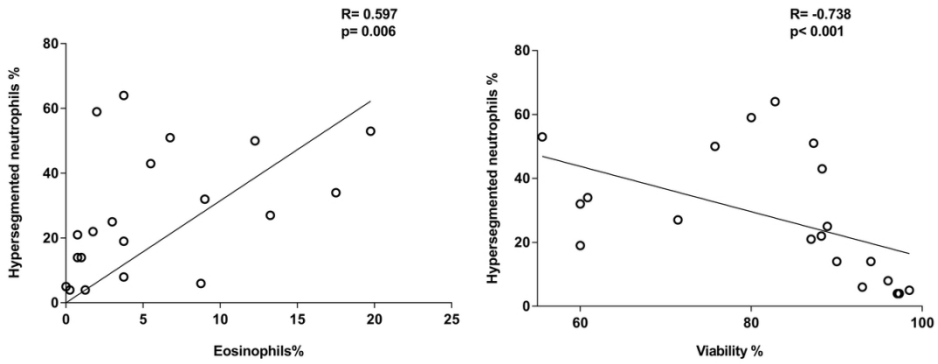

Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

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, Gotzsche 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	6-7

		recruitment, exposure, follow-up, and data collection	
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
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
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.
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.
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	8
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding	8
	#12b	Describe any methods used to examine subgroups and interactions	n/a…we examined COPD subgroups (Eosinophilic and Non-Eosinophilic) based on

			pre-defined cut off values on page 12-14.
	#12c	Explain how missing data were addressed	Missing data were excluded from analysis.
	#12d	If applicable, describe analytical methods taking account of sampling strategy	n/a. The study did not use any analytical method.
	#12e	Describe any sensitivity analyses	n/a. No sensitive analysis were performed in this study.
Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.	n/a…Since it was just a one visit study, the participant only included if they met the inclusion criteria and hence participant number were same throughout the study.
	#13b	Give reasons for non-participation at each stage	n/a…. No non-participation to report for this study.
	#13c	Consider use of a flow diagram	n/a
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
	#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).
Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	8
Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence	7-14

		interval). Make clear which confounders were adjusted for and why they were included	
	#16b	Report category boundaries when continuous variables were categorized	14
	#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.
Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	12-14
Key results	#18	Summarise key results with reference to study objectives	15
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
Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	15-17
Generalisability	#21	Discuss the generalisability (external validity) of the study results	n/a
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

BMJ Open

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

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Secondary Subject Heading:	Immunology (including allergy)
Keywords:	Immunology < THORACIC MEDICINE, Bronchoscopy < THORACIC MEDICINE, Chronic airways disease < THORACIC MEDICINE

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

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Key words: Immunology, Chronic airways disease, Bronchoscopy.

Word count: 3211

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 bronchial lavage (BL) of obstructive airway disease participants (asthma, COPD and bronchiectasis) and healthy controls on the basis of nuclear morphology and to access association between neutrophil subsets and clinical parameters of obstructive airway disease participants.

Design: An observational study.

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

Participants: Seventy-eight adults with obstructive airway disease, stable asthma (n=39) COPD (n=20), and bronchiectasis (n=19) 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 1073.6 (258.8-2742) x 10^2/mL and 24.5 (14.0-46.5) %, respectively). An increased proportion of hypersegmented neutrophils in airway disease participants was significantly associated with lower FEV_1/FVC % (spearman's Rho= -0.322, p= 0.004).

Conclusion: Neutrophil heterogeneity is common in bronchial lavage 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: 260

STRENGTHS AND LIMITATION OF STUDY

- This is the first observational study to characterize three morphologically different subset of neutrophils in the bronchial lavage of adults with obstructive airway disease and healthy controls.
- The study investigated clinical association of neutrophils subset 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 triggers [1]. Activation results in neutrophil migration to the site of infection, where pathogens can be eliminated by phagocytosis or NETosis [2]. 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 patters) with pattern recognition receptors of airways. This facilitates the release of chemotactic stimuli such as CXCL8, IL-1β, and TNF-α, resulting in neutrophil recruitment to the airways [3], which is important for the resolution of infection and inflammation [4]. In contrast, a disproportionate or dysregulated influx or efflux of neutrophils can result in persistent neutrophilic airway inflammation and tissue damage [5].

Inflammation characterised by airway neutrophilia is reported in many cases of chronic obstructive airway disease [6]. This includes 20-30% cases of asthma [7], more than 40% of cases of chronic obstructive pulmonary disease (COPD) [8] [9], and 70% of cases of non-cystic fibrosis (CF) bronchiectasis [10]. 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 corticosteroids [11] [12]. In non-CF

bronchiectasis treatment relies on antibiotics to control the infective nature of the disease[13]. While inhaled corticosteroids are highly effective in modifying eosinophilic inflammation in the airways[14], 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 FEV_1 in obstructive airway disease [17], little is known about variations within the population of neutrophils in the airways. Recent studies have identified heterogeneity within circulating neutrophils. Pillay, *et al* [18] identified three subsets of neutrophils (normal, banded and hypersegmented) in the circulation following an inflammatory challenge. Each subtype had a distinct nuclear morphology and pattern of surface adhesion molecule expression, with hypersegmented neutrophils showing increased capacity for oxidative burst along with a unique ability to suppress T lymphocytes. The same morphologically distinct subsets have been identified in both bronchial lavage (BL) and blood from patients with acute respiratory distress syndrome [19] and in infants with severe viral respiratory infection [20].

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

MATERIAL AND METHODS:

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

Participants: Adults who were undergoing bronchoscopy either for medical purposes or were undergoing a surgical procedure that involved endotracheal intubation and had spirometry results, were recruited for this study from the outpatient clinic of John Hunter Hospital. The study was approved by Hunter New England Human Research Ethics Committee (Reference No 05/08/10/3.09) and all participants provided written informed consent.

Study design: A cross sectional study was conducted in which BL samples were obtained after the assessment of clinical history including respiratory symptoms, smoking status and medication. Spirometry and bronchoscopy were performed as outlined below.

Study group: Adults (>18 years) with no history of a clinical chest or upper respiratory tract infection in the previous 6 weeks were studied. Healthy non-smokers (n=20) had normal lung function assessed by spirometry, and had no previous history of respiratory disease. Adults with asthma (n=40) had a physician's diagnosis of asthma with objective evidence of airflow variability or bronchial hyperactivity on provocation challenge. Bronchiectasis (n=20) was defined as evidence of a permanent dilation of airway segment on high resolution computed tomography scan while those with COPD (n=20) had evidence of respiratory symptoms in combination with a post bronchodilator FEV_1 of less than 80% of predicted value and/or a post bronchodilator FEV_1/FVC less than 70%. Current smokers were excluded. Since this was an exploratory study in a completely

new setting, the number of participants in each group were decided on the basis of previous exploratory studies in this area[18 19 21].

Spirometry: Spirometry was performed (Easy One Spirometer, ndd Medical Technologies, Massachusetts, USA) at John Hunter Hospital. Variable obstruction defined as a post bronchodilator change in FEV_1 of 12% or 200mL after 400 mcg of salbutamol and the bronchial hyper-responsiveness defined as at least 15% decline in FEV_1 after inducing bronchial provocation with 4.5% saline solution.

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

BL processing: BL was filtered and total cell count (TCC) and viability was assessed by using trypan blue exclusion method, within one hour of collection at Hunter Medical Research Institute. The BL was centrifuged and the cell pellet was resuspended in PBS to the concentration of $1x10^6$/mL and cellular cytospins were prepared. The cytospins were stained with May-Grünwald Giemsa (Beckman Coulter, Brea, CA, USA) and a differential cell count of 400 non squamous cells was performed.

Neutrophil subtype assessment: Stained cytospins were examined under oil immersion and 100 neutrophils were enumerated into banded, normal and hypersegmented neutrophils. Banded neutrophils had a single banded lobe without any visible division; normal neutrophils had two to four lobes with every lobe having a properly visible outer boundary; and hypersegmented

neutrophils had more than four lobes with every lobe having a properly visible outer boundary as shown in Figure 1.

Statistical Analysis: Data were analysed using Stata software version 11 (StataCorp, College Station, TX, USA). Results are reported as mean (SD) or median (interquartile range), unless otherwise stated. Continuous measures were analysed using the two-sample Wilcoxon's rank sum test or t-test and Kruskal-Wallis test or one way analysis of variance (ANOVA) as appropriate. Categorical data were analysed using Fisher's exact test. Spearman correlation coefficients were calculated for the association between neutrophil subsets and clinical characteristics.

RESULTS

Clinical characteristics

Participants with COPD were more likely to be ex-smoking males with more severe airflow obstruction (Table 1). Fewer participants with COPD were prescribed ICS compared with the asthma group, however, the mean daily dose of ICS was significantly higher in COPD participants. The number of participants with severe asthma were higher than the number with severe COPD (Table 1) according to GINA[22] and GOLD[23] severity classification, respectively. Bronchiectasis participants were generally of mild severity according to their bronchiectasis severity index[24] (Table 1). The causes of bronchiectasis is mainly idiopathic and post-infection (Table S1, supplementary data).

Inflammatory cell counts

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

Neutrophil subsets

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

When considering the relative distribution of neutrophil subtypes by proportion (shown in Figure 2 D-F), participants with COPD had a significantly reduced proportion of normal and banded 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)		

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. FEV_1: 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

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 FEV_1% predicted (spearman's Rho -0.301, p=0.007) and FEV_1/FVC% (Rho= -0.322, p=0.004, Figure 3) in participants with obstructive airway disease (n=78). While the same was not observed for banded neutrophils [FEV_1% predicted (Rho= 0.181, p=0.114), FEV_1/FVC% (Rho= 0.213, p=0.061)] and normal neutrophils [FEV_1% predicted (Rho= 0.189, p=0.097), FEV_1/FVC% (Rho= 0.213, p=0.062)]. There was no association between total hypersegmented neutrophils (x 10^2 cells/mL) with both FEV_1% predicted (Rho= -0.152, p=0.185) and FEV_1/FVC% (Rho= -0.173, p=0.131). Similarly, no association was observed between total neutrophil proportion and number with either FEV_1% predicted [Rho= -0.143, p=0.212 and Rho=-0.036, p=0.758, respectively] or FEV_1/FVC% [Rho= -0.142, p=0.214 and Rho=-0.043, p=0.707, respectively).

In participants with COPD, the proportion of hypersegmented neutrophils was positively associated with proportion of eosinophils (Rho=0.535, p=0.015) (Figure 4A) and negatively associated with cell viability (Rho= -0.697, 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) (≥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 NE-COPD group had a significantly elevated total cell count [NE COPD, 1.71 (1.47); E COPD, 0.67 (0.55), p=0.037] and cell viability [NE COPD, 90.82 (5.80); E COPD, 76.67 (14.64), p=0.019] along with significantly elevated neutrophil proportion [NE COPD, 85.50 (77.00-92.38); E COPD, 75.75 (69.88-77.75), p=0.037] and number [NE COPD, 148.37 (132.16); E COPD, 50.93 (42.95), p=0.028] in comparison with E-COPD. The number and proportion of eosinophils were significantly higher in 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); E COPD, 9.08 (5.50), p<0.001], respectively. Besides this, no significant difference were observed between these groups for other clinical parameters such as age, sex, lung function, ICS dose etc.

Neutrophil subsets in eosinophilic and non-eosinophilic COPD

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

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 FEV_1 and more severe airflow obstruction (FEV_1/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 emerged [25]. 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 26].

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 patients[27]. The emergence of banded neutrophils in the airway can occur after depletion of mature neutrophils in bone marrow following excessive demand during acute inflammation [20].

The presence of hypersegmented neutrophils in airways could be an attribute of inflammation as the hypersegmented neutrophils have also been reported in other inflammatory conditions such as trauma[18] and in chronic inflammatory lung diseases such as ARDS[19].

The hypersegmented morphology of the neutrophil implies increased maturation compared with banded and normal neutrophils [18]. Maturation is thought to occur in inflamed airways due to the presence of a

cytokine rich environment consisting of pro-survival mediators [28]. 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"[29].

The ability of a chemoattractant rich milieu to change the phenotype of neutrophils was recently shown when neutrophils from the blood of healthy volunteers were incubated with the broncoalveolar lavage from a patient with ARDS. These neutrophils altered their phenotype, with an increase in those with a hypersegmented morphology [19]. It may be possible that a similar process is occurring chronically in the airways of obstructive airway disease participants, who generally have higher levels of pro-inflammatory cytokines and inflammatory mediators. Previous studies have demonstrated that hypersegmented neutrophils in the circulation demonstrate low expression of L-selectins, which may reduce their anchoring ability on endothelial cells and hence reduce their chances to egress into inflamed airways[30]. Thus, it is possible that the hypersegmented neutrophils we observed in our study have not directly come from circulation and instead may have become hypersegmented in the airways under the influence of pro-survival mediators.

Mediators that promote neutrophil survival and can be present in the airways include; GM-CSF, chemokines like CXCL-8 and lipid mediators such as serum amyloid A [2 26]. GM-CSF and CXCL-8 are known to enhance neutrophil survival by promoting the expression of anti-apoptotic proteins like survivins and by preventing TNF-α mediated apoptosis [31 32]. While serum amyloid A is known to prolong neutrophil longevity by preventing mitochondrial damage and decreasing caspase-3 (apoptotic protein) activity[33]. Our past studies have reported elevated levels of CXCL-8 in sputum samples of neutrophilic asthma, bronchiectasis[34], and COPD patients[35]. Beside this, we have also reported that elevated levels of serum amyloid A in COPD was associated with neutrophilic inflammation in airways and this was

refractory to corticosteroids[36]. This suggests that the elevated presence of these markers might have played some role in enhancing the survival of neutrophils in airways and promoting the presence of hypersegmented neutrophils.

In this study, we also reported a positive correlation between eosinophils and hypersegmented neutrophils proportion in COPD participants along with elevated proportion of hypersegmented neutrophils in E-COPD participants. The presence of eosinophils in airways can further elevate the level of GM-CSF due to their own production of this cytokine[37], which can further promote maturation of neutrophils. Beside this, the use of ICS to control eosinophilic inflammation may enhance neutrophil survival in the inflamed airways by increasing the activity of anti-apoptotic proteins such as Mcl-1 (induced myeloid leukaemia cell differentiation protein) and IAPs (inhibitor of apoptosis proteins) in neutrophils [38]. This increased maturity and prevention of death may result in an increased proportion of hypersegmented neutrophils.

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

The significant association between the proportion of hypersegmented neutrophils with FEV_1 and severe airflow obstruction in our study suggests that where hypersegmented neutrophils are common,

airway obstruction is poor. This could be a result of high oxidative burst produced by hypersegmented neutrophils as observed in previous studies, in which hypersegmented neutrophil exhibited high oxidative burst after ex-vivo stimulation [18 19]. The generation of high oxidative burst by neutrophils may also impair their timely clearance from the airway[40] and can trigger a vicious cycle of neutrophils influx into the airways[6]. The impairment of neutrophil clearance in airway may cause necrosis of neutrophils which can spill its cytotoxic content such as reactive oxygen species and proteolytic enzymes like neutrophil elastase in the lumen of airways[41]. This can further damage airway wall and promote mucus hypersecretion which may result in significant decline in FEV_1 as earlier reported in a study on COPD patients [42]. Interestingly, we did not observe this correlation with other neutrophil subsets or with total neutrophil proportion or number. Further research is needed to understand if hypersegmented neutrophils are common as a result of more severe disease or conversely if they influence disease severity.

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

CONCLUSION

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

participants was associated with reduced lung function of these participants. The proportion of hypersegmented neutrophils was highest in COPD participants in comparison with all other groups.

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CONTRIBUTORS: JLS developed the idea and designed the study. JLS also supervised and coordinated the study throughout. RL performed the subtype counting and wrote the manuscript which was further refined and edited by JLS, PABW, KB and DB. PABW performed the bronchoscopy, KB supervised the bronchial lavage processing and cytospin preparation and DB supervised the statistical analysis.

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DATA SHARING STATEMENT: Raw data can be obtained by contacting the corresponding 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. ^$p<0.0125$ 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.*

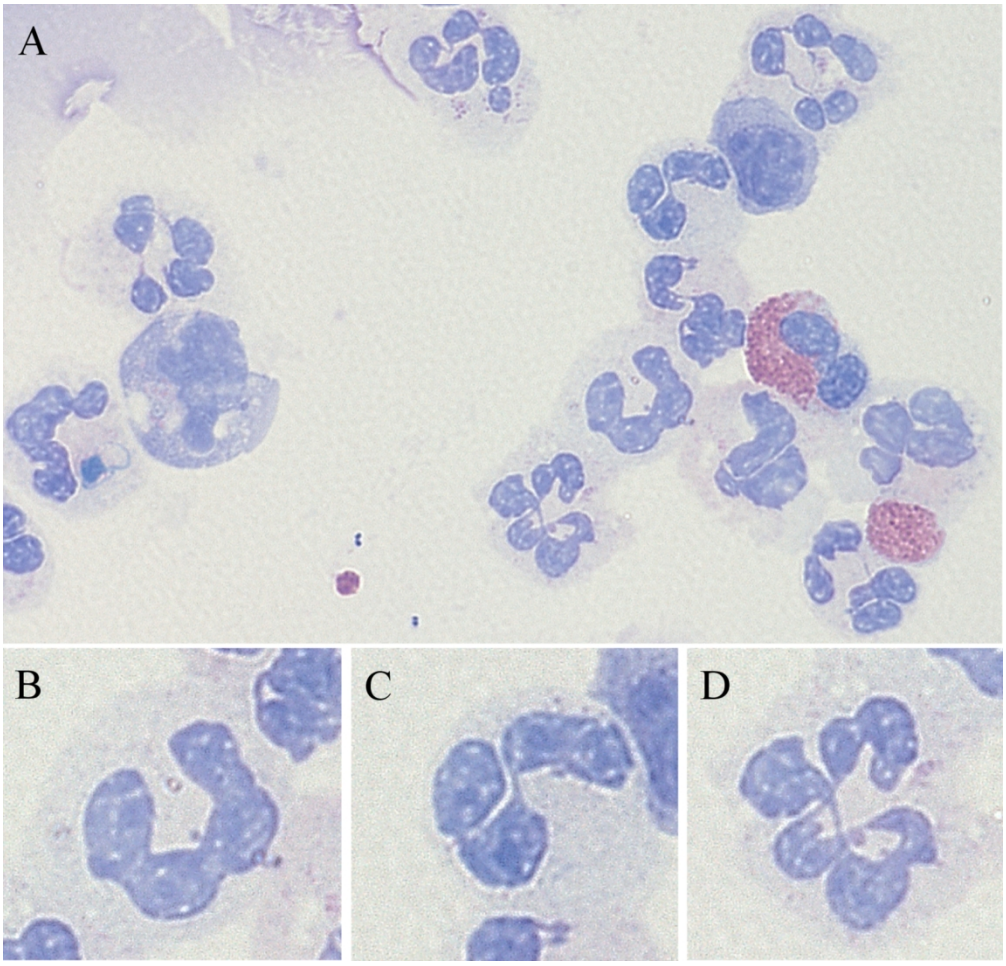
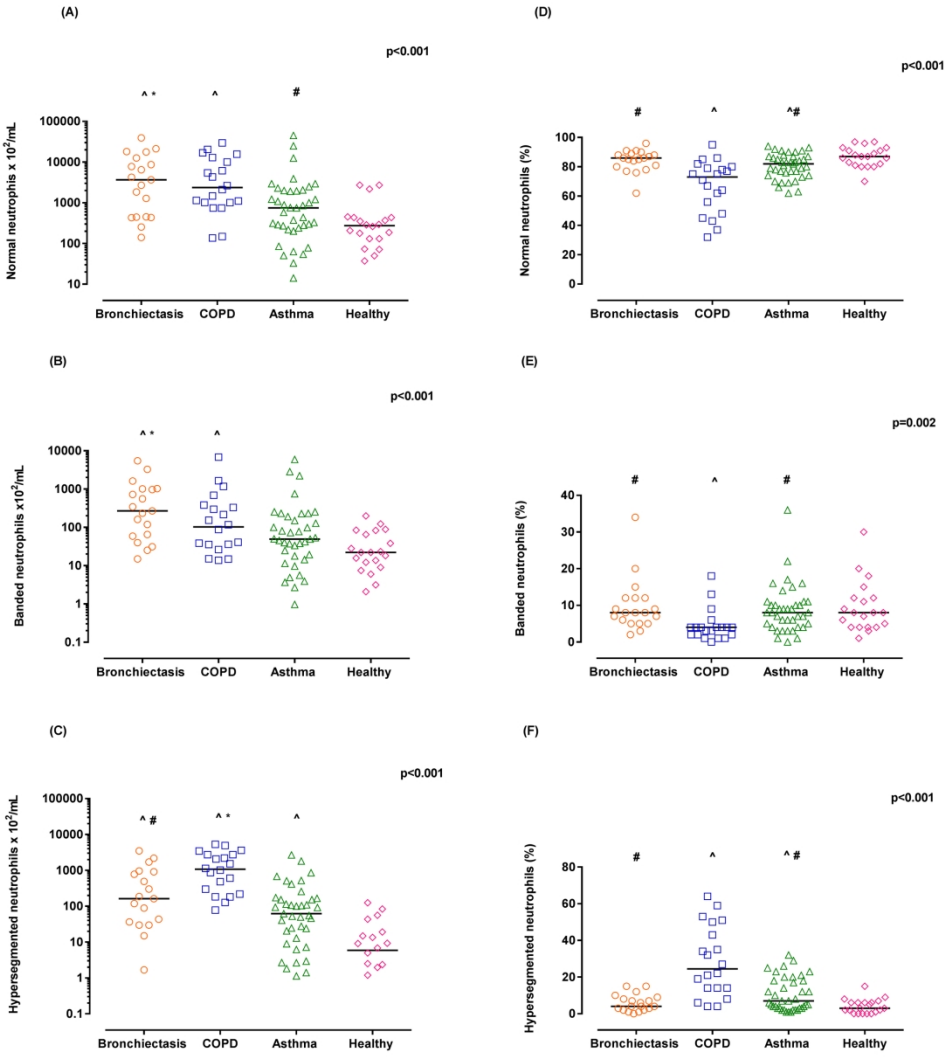


Figure 1: Subtypes of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants(X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented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

153x146mm (300 x 300 DPI)



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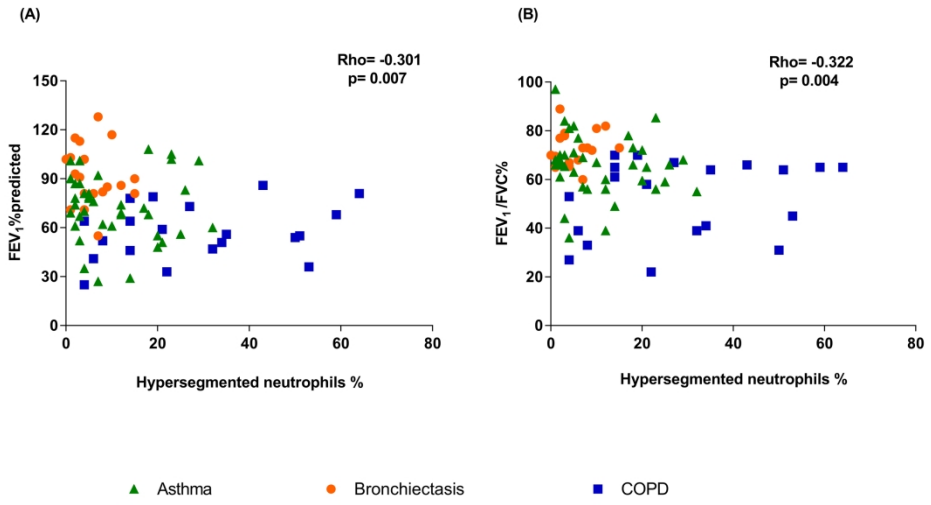

Figure 3: Scatterplot of hypersegmented neutrophil proportion vs FEV1% predicted (A) and FEV1/FVC (B) in BL of obstructive airway disease participant's.

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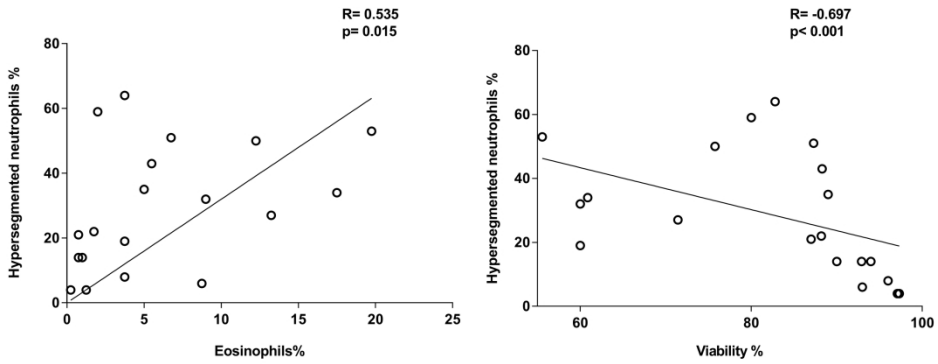

Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

268x108mm (300 x 300 DPI)

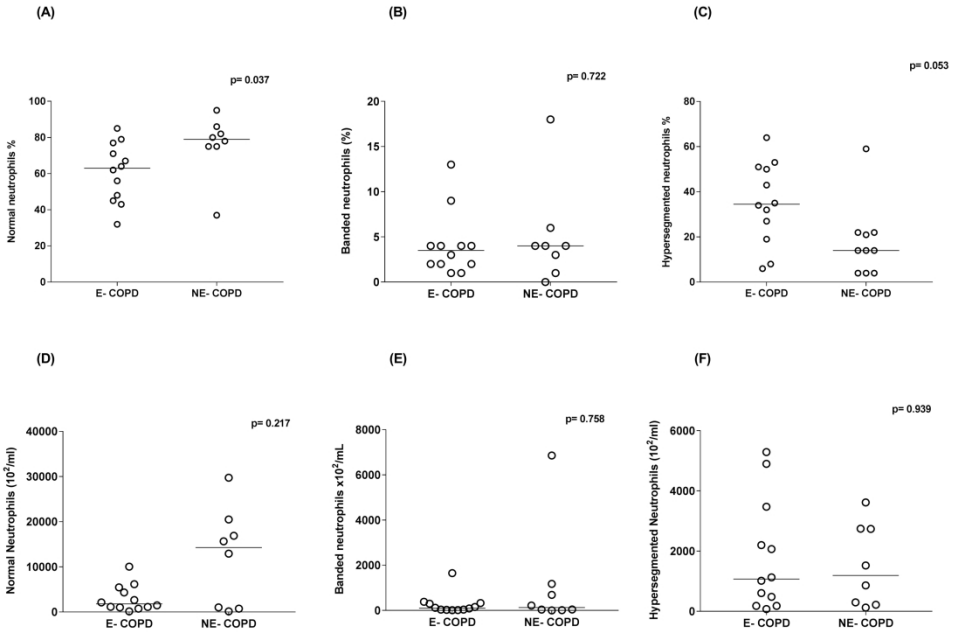

Figure 5: Neutrophil subsets proportion (A-C) and neutrophil subsets number (D-F) in bronchial lavage of eosinophilic (E-COPD) and non-eosinophilic COPD (NE-COPD) participants. The line in dot plots of each group represents the median and the p value in each graph is an outcome of Wilcoxon rank-sum test.

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, Gotzsche 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

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
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.
Study size	#10	Explain how the study size was arrived at	6, (described in study group section")
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	8
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding	8
	#12b	Describe any methods used to examine subgroups and interactions	n/a…we examined COPD subgroups (Eosinophilic and Non-Eosinophilic) based on pre-defined cut off values on page 12-14.
	#12c	Explain how missing data were addressed	Missing data were excluded from analysis.
	#12d	If applicable, describe analytical methods taking account of sampling strategy	n/a. The study did not use any analytical method.
	#12e	Describe any sensitivity analyses	n/a. No sensitive analysis were performed in this study.
Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.	n/a…Since it was just a one visit study, the participant only included if they met the inclusion criteria and hence participant number were same throughout the study.
	#13b	Give reasons for non-participation at each stage	n/a…. No non-participation to report for this study.
	#13c	Consider use of a flow diagram	n/a

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
	#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).
Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	8
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
	#16b	Report category boundaries when continuous variables were categorized	14
	#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.
Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	12-14
Key results	#18	Summarise key results with reference to study objectives	15
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
Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	15-17
Generalisability	#21	Discuss the generalisability (external validity) of the study results	n/a
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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Hypersegmented airway neutrophils and its association with reduced lung function in adults with obstructive airway disease: An exploratory study.

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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 bronchial lavage (BL) of obstructive airway disease participants (asthma, COPD and bronchiectasis) and healthy controls on the basis of nuclear morphology and to assess the association between neutrophil subsets and the clinical parameters of the obstructive airway disease participants.

Design: A cross-sectional exploratory study.

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

Participants: Seventy-eight adults with obstructive airway disease comprised of those with stable asthma (n=39) COPD (n=20), and bronchiectasis (n=19) and 20 healthy controls.

Material and Methods: Cytospins were prepared and neutrophil subsets were classified based on 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 1073.6 (258.8-2742) x 10^2/mL and 24.5 (14.0-46.5) %, respectively). An increased proportion of hypersegmented neutrophils in airway disease participants was significantly associated with lower FEV_1/FVC % (spearman's Rho= -0.322, p= 0.004).

Conclusion: Neutrophil heterogeneity is common in bronchial lavage 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.

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STRENGTHS AND LIMITATION OF STUDY

- This is the first exploratory study to characterize three morphologically different subsets of neutrophils in bronchial lavage of adults with obstructive airway disease and healthy controls.
- The study investigated clinical association of neutrophils subset 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 the blood vessels and become activated in response to inflammatory triggers [1]. Activation results in neutrophil migration to the site of infection, where pathogens can be eliminated by phagocytosis or NETosis [2]. 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 patters) with pattern recognition receptors of airways. This facilitates the release of chemotactic stimuli such as CXCL8, IL-1β, and TNF-α, resulting in neutrophil recruitment to the airways [3], which is important for the resolution of infection and inflammation [4]. In contrast, a disproportionate or dysregulated influx or efflux of neutrophils can result in persistent neutrophilic airway inflammation and tissue damage [5].

Inflammation characterised by airway neutrophilia is reported in many cases of chronic obstructive airway disease [6]. This includes 20-30% cases of asthma [7], more than 40% cases of chronic obstructive pulmonary disease (COPD) [8 9], and 70% cases of non-cystic fibrosis (CF) bronchiectasis [10]. Current therapeutic and management strategies for asthma and COPD focus on bronchodilation to overcome airflow limitation, or inhaled corticosteroids based therapies for the modification of eosinophilic airway inflammation. [11 12]. In non-CF bronchiectasis, treatment relies on antibiotics to control the infective nature of the disease[13]. While inhaled corticosteroids are highly effective in modifying eosinophilic inflammation in the airways[14], 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 FEV_1 in obstructive airway disease [17], little is known about variations within the population of neutrophils in the airways. Recent studies have identified heterogeneity within

circulating neutrophils. Pillay, *et al* [18] identified three subsets of neutrophils (normal, banded and hypersegmented) in the circulation following an inflammatory challenge. Each subset had a distinct nuclear morphology and pattern of surface adhesion molecule expression, with hypersegmented neutrophils showing increased capacity for oxidative burst along with a unique ability to suppress T lymphocytes activation. The same morphologically distinct subsets have been identified in both bronchial lavage (BL) and blood from patients with acute respiratory distress syndrome [19] and in infants with severe viral respiratory infection [20].

The presence and characteristics of neutrophil subsets in obstructive airways disease is unknown. In this exploratory study, we have characterised and estimated neutrophil subsets in BL fluid from adults with asthma, COPD, non-CF bronchiectasis and healthy controls. In addition we have explored the association of these subsets with the clinical characteristics of obstructive airway disease participants.

MATERIAL AND METHODS:

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

Participants: Adults who were undergoing bronchoscopy either for medical purposes or were undergoing a surgical procedure that involved endotracheal intubation and had spirometry results, were recruited for this study from the outpatient clinic of John Hunter Hospital. The study

was approved by Hunter New England Human Research Ethics Committee (Reference No 05/08/10/3.09) and all participants provided written informed consent.

Study design: A cross sectional exploratory study was conducted in which BL samples were obtained after an assessment of clinical history including respiratory symptoms, smoking status and medication. Spirometry and bronchoscopy were performed as outlined below.

Study group: Adults (>18 years) with no history of a clinical chest or upper respiratory tract infection in the previous 6 weeks were studied. Healthy non-smokers (n=20) had normal lung function assessed by spirometry, and had no previous history of respiratory disease. Adults with asthma (n=39) had a physician's diagnosis of asthma with objective evidence of airflow variability or bronchial hyperactivity on provocation challenge. Bronchiectasis (n=19) was defined as evidence of a permanent dilation of airway segment on high resolution computed tomography scan while those with COPD (n=20) had evidence of respiratory symptoms in combination with a post bronchodilator FEV_1 of less than 80% of predicted value and/or a post bronchodilator FEV_1/FVC less than 70%. Current smokers were excluded. Since this was an exploratory study in a completely new setting, the number of participants in each group were decided on the basis of previous exploratory studies in this area[18 19 21].

Spirometry: Spirometry was performed (Easy One Spirometer, ndd Medical Technologies, Massachusetts, USA) at John Hunter Hospital. Variable obstruction defined as a post bronchodilator change in FEV_1 of 12% or 200mL after 400 mcg of salbutamol and the bronchial hyper-responsiveness defined as at least 15% decline in FEV_1 after inducing bronchial provocation with 4.5% saline solution.

Bronchoscopy: Flexible bronchoscopy was performed at John Hunter Hospital, bronchial wash was taken by wedging the bronchoscope into the right middle lobe and washing with 40 mL of

sterile saline solution. A fraction of BL was sent for microbial detection while the rest was processed as described below.

BL processing: BL was filtered and total cell count (TCC) and viability was assessed by using trypan blue exclusion method, within one hour of collection at Hunter Medical Research Institute. The BL was centrifuged and the cell pellet was re-suspended in PBS to the concentration of $1x10^6$/mL and cellular cytospins were prepared. The cytospins were stained with May-Grünwald Giemsa (Beckman Coulter, Brea, CA, USA) and a differential cell count of 400 non squamous cells was performed.

Neutrophil subtype assessment: Stained cytospins were examined under oil immersion and 100 neutrophils were enumerated into banded, normal and hypersegmented neutrophils. Banded neutrophils had a single banded lobe without any visible division; normal neutrophils had two to four lobes with every lobe having a properly visible outer boundary; and hypersegmented neutrophils had more than four lobes with every lobe having a properly visible outer boundary as shown in Figure 1.

Statistical Analysis: Data were analysed using Stata software version 11 (StataCorp, College Station, TX, USA). Results are reported as mean (SD) or median (interquartile range), unless otherwise stated. Continuous measures were analysed using the two-sample Wilcoxon's rank sum test or t-test and Kruskal-Wallis test or one way analysis of variance (ANOVA) as appropriate. Categorical data were analysed using Fisher's exact test. Spearman correlation coefficients were calculated for the association between neutrophil subsets and clinical characteristics.

RESULTS

Clinical characteristics

Participants with COPD were more likely to be ex-smoking males with more severe airflow obstruction (Table 1). Fewer participants with COPD were prescribed ICS compared with the asthma group, however, the mean daily dose of ICS was significantly higher in COPD participants. The number of participants with severe asthma were higher than the number with severe COPD (Table 1) according to GINA[22] and GOLD[23] severity classification, respectively. Bronchiectasis participants were generally of mild severity according to their bronchiectasis severity index[24] (Table 1). The causes of bronchiectasis is mainly idiopathic and post-infection (Table S1, supplementary data).

Inflammatory cell counts

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

Neutrophil subsets

All three neutrophil subsets were identified in the BL of all participants. The numbers of normal neutrophils were significantly higher in bronchiectasis and COPD group in comparison to healthy and asthma (Figure 2A). Numbers of banded neutrophils were highest in those participants with bronchiectasis compared with both healthy and asthma groups, while in COPD banded

neutrophils numbers were higher in comparison with healthy participants only (Figure 2B). Hypersegmented neutrophil numbers were significantly increased in all the obstructive airway disease groups compared with healthy controls and increased in participants with COPD compared with asthma and bronchiectasis (Figure 2C).

When considering the relative distribution of neutrophil subsets by proportion (shown in Figure 2 D-F), participants with COPD had a significantly reduced proportion of normal and banded 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 Mild persistent Moderate persistent			 1 (2.6) 6 (15.8) 9 (23.7)		

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

Data are presented as mean ± SD or median (interquartile range; q1- q3) unless otherwise stated. FEV_1: 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.

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 FEV_1% predicted (spearman's Rho -0.301, p=0.007) and FEV_1/FVC% (Rho= -0.322, p=0.004, Figure 3) in participants with obstructive airway disease (n=78). While the same was not observed for banded neutrophils [FEV_1% predicted (Rho= 0.181, p=0.114), FEV_1/FVC% (Rho= 0.213, p=0.061)] and normal neutrophils [FEV_1% predicted (Rho= 0.189, p=0.097), FEV_1/FVC% (Rho= 0.213, p=0.062)]. There was no association between the total number of hypersegmented neutrophils (x 10^2 cells/mL) with both FEV_1% predicted (Rho= -0.152, p=0.185) and FEV_1/FVC% (Rho= -0.173, p=0.131). Similarly, no association was observed between total neutrophil proportion and number with either FEV_1% predicted [Rho= -0.143, p=0.212 and Rho=-0.036, p=0.758, respectively] or with FEV_1/FVC% [Rho= -0.142, p=0.214 and Rho=-0.043, p=0.707, respectively).

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

To explore the correlation between the proportions of eosinophils and hypersegmented neutrophils further, we decided to examine the COPD participants according to their inflammatory subtype categorised as eosinophilic COPD (E-COPD) (≥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 NE-COPD group had a significantly elevated total cell count [NE-COPD, 1.71 (1.47) x10^6/mL; E-COPD, 0.67 (0.55) x10^6/mL, p=0.037] and cell viability [NE-COPD, 90.82 (5.80)% ; E-COPD, 76.67 (14.64)%, p=0.019] along with a significantly elevated neutrophil proportion [NE-COPD, 85.50 (77.00-92.38)%; E-COPD, 75.75 (69.88-77.75)%, p=0.037] and neutrophil number [NE-COPD, 148.37 (132.16) x10^4/mL; E-COPD, 50.93 (42.95) x10^4/mL, p=0.028] in comparison with E-COPD. The number and proportion of eosinophils were significantly higher in E-COPD i.e. [NE-COPD, 1.14 (1.05) x10^4/mL; E-COPD, 4.71 (4.09) x10^4/mL, p=0.040] and [NE-COPD, 1.09 (0.57)%; E-COPD, 9.08 (5.50)%, p<0.001], respectively. Besides this, no significant differences were observed between these groups for any other clinical parameters.

Neutrophil subsets in eosinophilic and non-eosinophilic COPD

The proportion of normal neutrophils were significantly reduced while the proportion of hypersegmented neutrophils were elevated (Figure 5 A & C, respectively) in E-COPD compared with NE-COPD. While no significant differences were observed for the number of any individual subset (Figure 5 D-F) between E-COPD and NE-COPD.

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 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 FEV_1 and more severe airflow obstruction (FEV_1/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 emerged [25]. 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 26].

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 patients[27]. The emergence of banded neutrophils in the airway can occur after depletion of mature neutrophils in bone marrow following excessive demand during acute inflammation [20].

The presence of hypersegmented neutrophils in airways could be an attribute of inflammation as the hypersegmented neutrophils have also been reported in other inflammatory conditions such as trauma[18] and in chronic inflammatory lung diseases such as ARDS[19].

The hypersegmented morphology of the neutrophil implies increased maturation compared with banded and normal neutrophils [18]. Maturation is thought to occur in inflamed airways due to the presence of a cytokine rich environment consisting of pro-survival mediators [28]. 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”[29].

The ability of a chemoattractant rich milieu to change the phenotype of neutrophils was recently shown when neutrophils from the blood of healthy volunteers were incubated with the broncoalveolar lavage from a patient with ARDS. These neutrophils altered their phenotype, with an increase in those with a hypersegmented morphology [19]. It may be possible that a similar process is occurring chronically in the airways of obstructive airway disease participants, who generally have higher levels of pro-inflammatory cytokines and inflammatory mediators. Previous studies have demonstrated that hypersegmented neutrophils in the circulation demonstrate low expression of L-selectins, which may reduce their anchoring ability on endothelial cells and hence reduce their chances to egress into inflamed airways[30]. Thus, it is possible that the hypersegmented neutrophils we observed in our study have not directly come from circulation and instead may have become hypersegmented in the airways under the influence of pro-survival mediators.

Mediators that promote neutrophil survival and can be present in the airways include; GM-CSF, chemokines like CXCL-8 and lipid mediators such as serum amyloid A [2 26]. GM-CSF and CXCL-8 are known to enhance neutrophil survival by promoting the expression of anti-apoptotic proteins like survivins and by preventing TNF-α mediated apoptosis [31 32]. While serum amyloid A is known to prolong neutrophil longevity by preventing mitochondrial damage and decreasing caspase-3 (apoptotic protein) activity[33]. Our past studies have reported elevated levels of CXCL-8 in sputum samples of neutrophilic asthma, bronchiectasis[34], and COPD patients[35]. Beside this, we have also reported that elevated levels of serum amyloid A in COPD was associated with neutrophilic inflammation in airways and this was refractory to corticosteroids[36]. This suggests that the elevated presence of these markers might have played some role in enhancing the survival of neutrophils in airways and promoting the presence of hypersegmented neutrophils.

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

level of GM-CSF due to their own production of this cytokine[37], which can further promote maturation of neutrophils. Beside this, the use of ICS to control eosinophilic inflammation may enhance neutrophil survival in the inflamed airways by increasing the activity of anti-apoptotic proteins such as Mcl-1 (induced myeloid leukaemia cell differentiation protein) and IAPs (inhibitor of apoptosis proteins) in neutrophils [38]. This increased maturity and prevention of death may result in an increased proportion of hypersegmented neutrophils.

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

The significant association between the proportion of hypersegmented neutrophils with FEV_1 and severe airflow obstruction in our study suggests that where hypersegmented neutrophils are common, airway obstruction is most severe. This could be a result of high oxidative burst produced by hypersegmented neutrophils as observed in previous studies, in which hypersegmented neutrophil exhibited high oxidative burst after ex-vivo stimulation [18 19]. The generation of high oxidative burst by neutrophils may also impair their timely clearance from the airway[40] and can trigger a vicious cycle of neutrophils influx into the airways[6]. The impairment of neutrophil clearance in airway may cause necrosis of neutrophils which can spill its cytotoxic content such as reactive oxygen species and proteolytic enzymes like neutrophil elastase in the lumen of airways[41]. This can further damage airway wall and promote mucus hypersecretion which may result in

significant decline in FEV_1 as earlier reported in COPD [42]. Interestingly, we did not observe this correlation with other neutrophil subsets or with total neutrophil proportion or number. Further research is needed to understand if hypersegmented neutrophils are common as a result of more severe disease or conversely if they influence disease severity.

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

CONCLUSION

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

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CONTRIBUTORS: JLS developed the idea and designed the study. JLS also supervised and coordinated the study throughout. RL performed the subtype counting and wrote the manuscript which was further refined and edited by JLS, PABW, KB and DB. PABW performed the bronchoscopy, KB supervised the bronchial lavage processing and cytospin preparation and DB supervised the statistical analysis.

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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. ^$p<0.0125$ 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.*

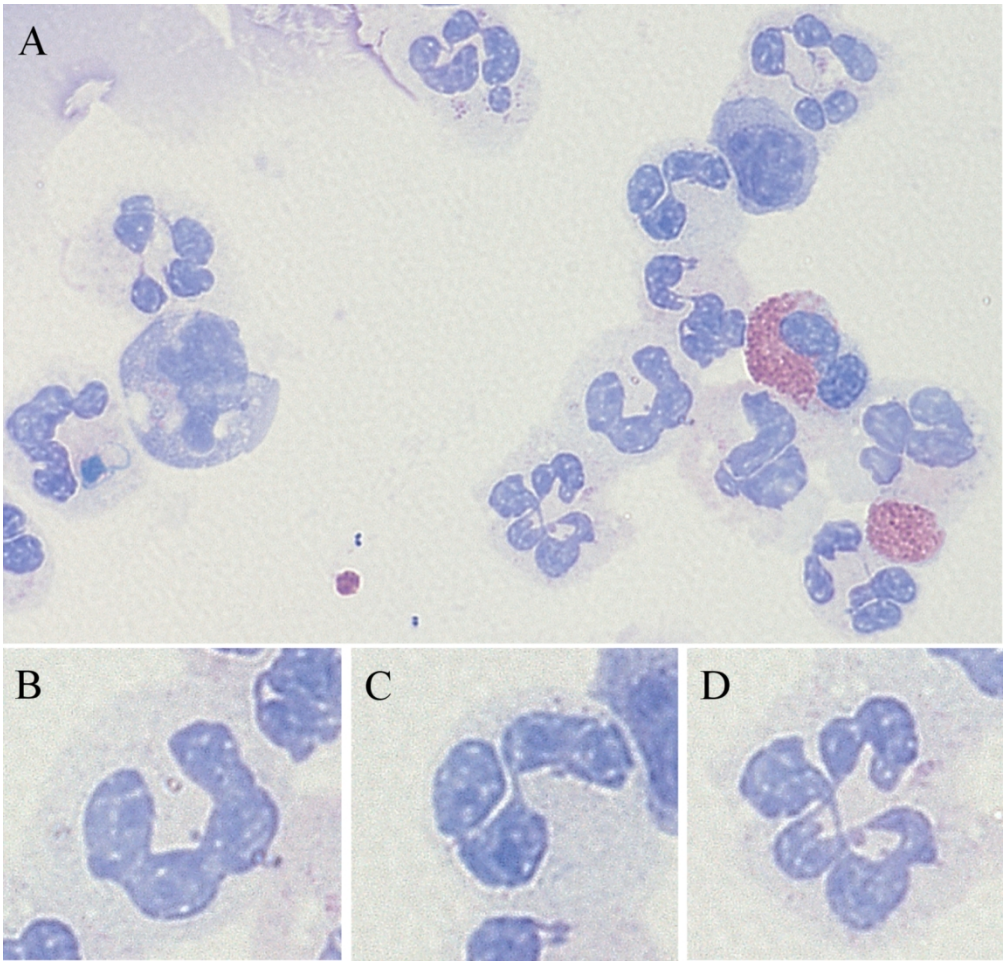
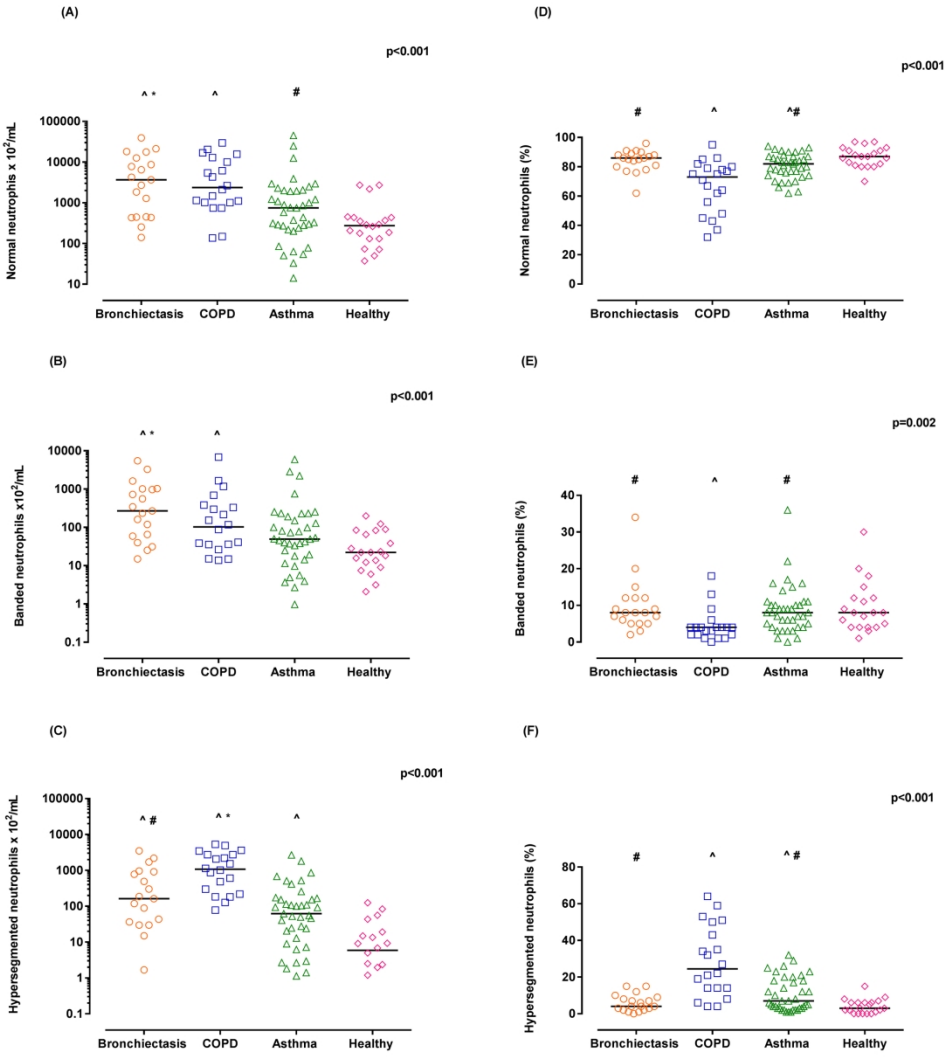


Figure 1: Subtypes of neutrophils characterized as per number of lobes in their nucleus. (A) A representative pictomicrograph of BL cytospin of obstructive airway disease participants(X 100 original magnification, MGG stain) consist of (B) Banded neutrophil, (c) Normal neutrophil, and (D) Hypersegmented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

153x146mm (300 x 300 DPI)



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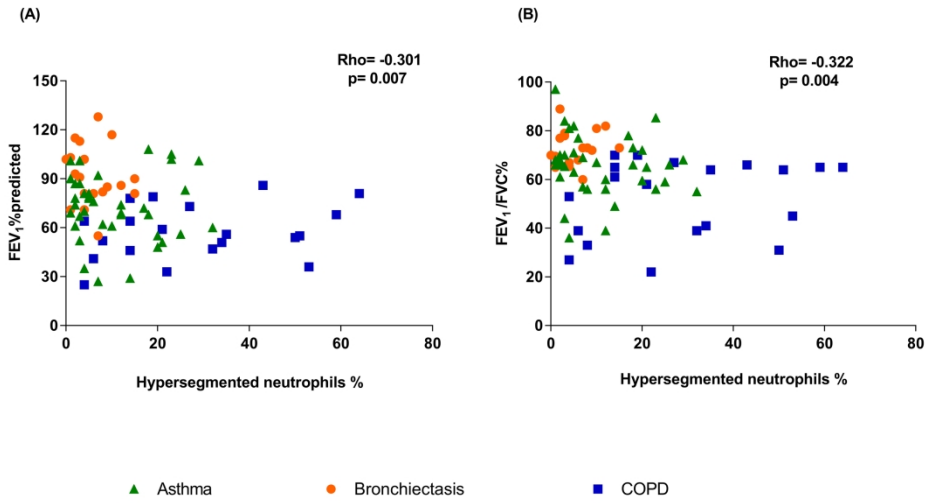

Figure 3: Scatterplot of hypersegmented neutrophil proportion vs FEV1% predicted (A) and FEV1/FVC (B) in BL of obstructive airway disease participant's.

248x134mm (300 x 300 DPI)

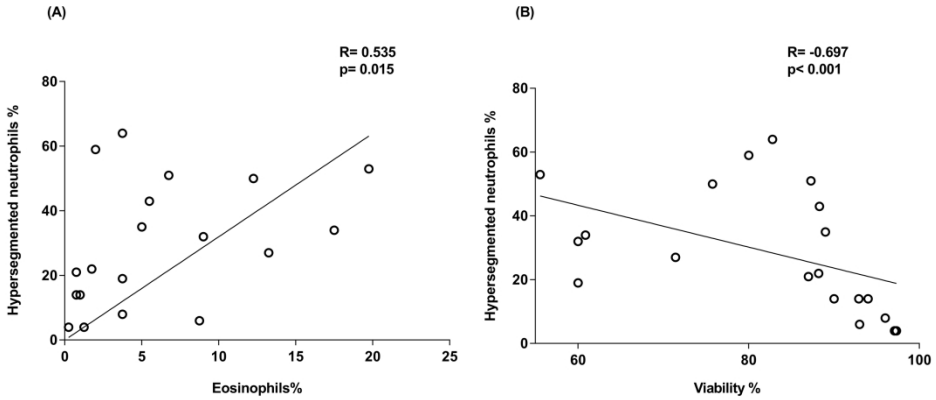

Figure 4: (A) Scatterplot of hypersegmented neutrophils proportion vs eosinophils proportion, (B) viability of total cells in BL of COPD participants.

268x134mm (300 x 300 DPI)

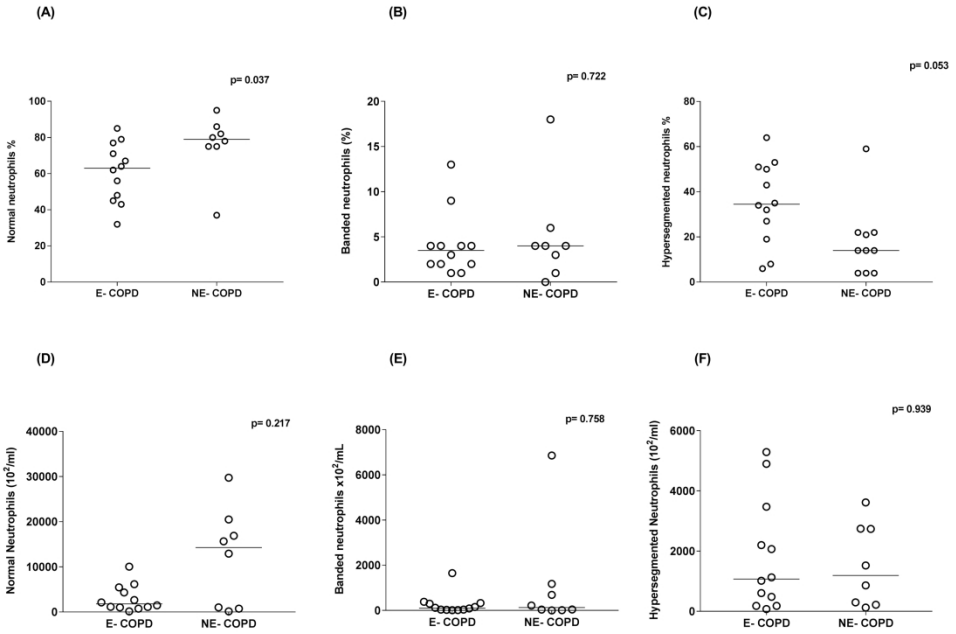

Figure 5: Neutrophil subsets proportion (A-C) and neutrophil subsets number (D-F) in bronchial lavage of eosinophilic (E-COPD) and non-eosinophilic COPD (NE-COPD) participants. The line in dot plots of each group represents the median and the p value in each graph is an outcome of Wilcoxon rank-sum test.

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, Gotzsche 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

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
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.
Study size	#10	Explain how the study size was arrived at	6, (described in study group section")
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	8
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding	8
	#12b	Describe any methods used to examine subgroups and interactions	n/a…we examined COPD subgroups (Eosinophilic and Non-Eosinophilic) based on pre-defined cut off values on page 12-14.
	#12c	Explain how missing data were addressed	Missing data were excluded from analysis.
	#12d	If applicable, describe analytical methods taking account of sampling strategy	n/a. The study did not use any analytical method.
	#12e	Describe any sensitivity analyses	n/a. No sensitive analysis were performed in this study.
Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.	n/a…Since it was just a one visit study, the participant only included if they met the inclusion criteria and hence participant number were same throughout the study.
	#13b	Give reasons for non-participation at each stage	n/a…. No non-participation to report for this study.
	#13c	Consider use of a flow diagram	n/a

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
	#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).
Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	8
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
	#16b	Report category boundaries when continuous variables were categorized	14
	#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.
Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	12-14
Key results	#18	Summarise key results with reference to study objectives	15
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
Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	15-17
Generalisability	#21	Discuss the generalisability (external validity) of the study results	n/a
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