PEER REVIEW HISTORY

This paper was submitted to a another journal from BMJ but declined for publication following peer review. The authors addressed the reviewers' comments and submitted the revised paper to BMJ Open. The paper was subsequently accepted for publication at BMJ Open.

(This paper received three reviews from its previous journal but only two reviewers agreed to published their review.)

ARTICLE DETAILS

TITLE (PROVISIONAL)	Hypersegmented airway neutrophils and its association with reduced lung function in adults with obstructive airway disease: An exploratory study.
AUTHORS	Lokwani, Ravi; Wark, Peter; Baines, Katherine; Barker, Daniel; Simpson, Jodie

VERSION 1 – REVIEW

REVIEWER	Elizabeth Sapey University of Birmingham UK
REVIEW RETURNED	08-Jun-2018

GENERAL COMMENTS	There is significant interest in identifying and understanding neutrophil subtypes and their potential role in inflammation and resolution of inflammation in chronic diseases including those with airflow obstruction. This paper is clearly written and studies airway neutrophils, which is a real strength. The authors have tried to match patients and controls by age, which is commendable, but the COPD patients have smoked more and have worse lung function, which would be expected. I have some comments which I would like the authors to consider: 1. Methodology - how did the authors check the chosen 100 cell counted were representative and did they blind the process of cell counting to ensure this did not influence results? 2. Hypersegmented neutrophils can be a feature of megaloblastic anaemias, uraemia and moderately hypersegmented neutrophils (as shown here) are not infrequently seen in iron deficiency. Did the authors account for this? 3. COPD, asthma and BE can share airflow obstruction, but are very different diseases in terms of underlying causes. Do the authors feel that the hypersegmented phenotype is therefore specific to obstructive lung disease, or could this be a response to any chronic inflammatory lung or indeed any chronic inflammatory condition? 4. I can see that the data has been very carefully collated and interpreted but the authors only studied nucleus appearance without any specific cell surface staining, and I worry that

	functionally different cells might be put in one group due to the simplicity of this approach. Indeed, there is some debate about whether all "hypersegmented" neutrophils are the same. Hypersegmented neutrophils are often considered mature but have been described as pro-inflammatory and cytotoxic in one study (J Immunol. 2017 Mar 1; 198(5): 1793–1797. doi: 10.4049/jimmunol.1601292) and anti-inflammatory or immune-suppressive in other studies (for example, Pillay, JCI, 2012) although crucially the surface markers differed between publications. Could the authors comment on how they might interpret potential differences in cell subtype even within the hypersegmented group, and do they believe that counting nucleus lobes is enough to tell you something about the cell? What do they think this data means in terms of neutrophil function? 5. I worry that the eosinophil versus non-eosinophil work may be under powered, and that is why some of the data parameters are not different, but this is interesting data 6. The causes of bronchiectasis (BE) are variable and this might impact on inflammatory phenotype. Could the authors provide (in a supplement?) the causes of BE in this group? 7. Patients with COPD were on higher doses of BDP equivalent than the asthma patients - could this have had an impact? In total, I found this a very interesting and well written paper, but ideally I would have liked further evidence that looking at nuclear structure is enough to tell us something about cellular function.

REVIEWER	Mona Bafadhel University of Oxford, UK About to start a collaboration with the Senior Author
REVIEW RETURNED	10-Sep-2018

GENERAL COMMENTS	In this manuscript the authors have investigated neutrophil phenotype in the BAL of patients with obstructive airway disease and healthy controls. I have a few comments: 1. It is not clear from the abstract that the samples studied were from lavage. Please add 2. BL is abbreviated in the abstract – but this again has not been clarified. Please change to lavage. 3. What is β relate to in the final line of the results section of the abstract? 4. Could the authors add if there was any exclusion criteria – I am unsure what the lung function criteria for bronchoscopy would be and this may be additional bias. 5. Why were current smokers excluded? 6. There is little detail on the severity of disease; could GOLD/GINA categorisation be added. What do the authors think of the clinical severity of the patients with bronchiectasis? The mean lung function would make it appear that this is a very milld group of patients. 7. Some of the ‘normal’ population appear to have airflow obstruction (the lower 25th centile is below 70). I am not sure these patient are true ‘normal/healthy controls’ and may actually

	represent an early COPD smoking cohort. Could the authors address this or remove this population from this category? 8. In table 2 the COPD population seem to have the highest degree of eosinophilic inflammation. Was this corrected for ICS? Could the authors comment on this? 9. The authors found that the neutrophil phenotype in eosinophilic and non-eosinophilic COPD patients was different. This is an interesting finding. Is this true for eosinophilic versus non-eosinophilic obstructive disease (ie. Eosinophilic asthma+Eosinophilic COPD vs. non eosinophilic asthma+non-eosinophilic COPD)? This may yield some further information related to whether it is inflammatory cell specific or disease specific. 10. I think table 3 is repetitive. Please just address any findings in the text and remove. 11. Please comment if any standardisation for repeated measures was made.

REVIEWER	Leigh M Marsh Ludwig Boltzmann Institute for Lung Vascular Research, Austria
REVIEW RETURNED	13-Sep-2018

GENERAL COMMENTS	In their manuscript Lokwani and coauthors have examined the association of hypersegmented airway neutrophils with reduced lung function in obstructive airway disease patients. In this cross-sectional study the authors observe that hypersegmented neutrophils were highest in COPD participants and correlated with reduced lung function in patients with obstructive airway disease (asthma, Bronchiectasis and COPD patients). The manuscript is very clear and well written, however, I have a few points that require addressing. 1) In Figure 2, please confirm in the figure legend whether the line on each dot plot represents the median or mean. Please also include which statistical test was performed in the legend. There is a very large difference in the % and total number of hypersegmented neutrophils in bronchiectasis patients, could the authors comment on this. 2) It appears that the entire obstructive airway disease cohort is presented in the spearman correlation (Figure 3). If this is the case please colour-code each point based on their disease status (using the same colour scheme as Figure 2). It might be more informative to graph the individual group correlations to demonstrate which disease subtypes drive the observed correlations. Finally, please state and discuss whether the observed correlation between proportion of hypersegmented neutrophils with FEV1% predicted and FEV1/FVC% was also observed with total numbers. 3) In table 4, there is a very high variation in the number of different neutrophils, which probably explains the lack of statistical significance between the groups. However, the authors make the strong statement that “E-COPD had half the number of banded neutrophils and ten times fewer normal neutrophils in comparison to those with NE-COPD”. Based on the presented data this statement is unfounded, I would recommend presenting the data from this table as a dot-plot so that the reader can greater appreciate the similarities and differences between the groups.

	4) The authors speculate that the increase in hypersegmented neutrophils may arise either from being recruited from the circulation or becoming hypersegmented in the airways. They also list a number of factors (e.g. GM-CSF, CXCL-8 or serum amyloid A) that potentially have a role in this process. Please quantify these three proteins in the BALF of COPD patients and correlate with the number of hypersegmented neutrophils. Minor Please include details on the assessment of cell viability.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Reviewer Name: Elizabeth Sapey

1. Methodology - how did the authors check the chosen 100 cell counted were representative and did they blind the process of cell counting to ensure this did not influence results?

   We used a method according to published work in this area (Pillay et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. J Clin Invest 2012;122(1):327-36). The counter was blinded for clinical characteristics and inflammatory phenotype of the participants.

2. Hypersegmented neutrophils can be a feature of megaloblastic anaemias, uraemia and moderately hypersegmented neutrophils (as shown here) are not infrequently seen in iron deficiency. Did the authors account for this?

Thanks to the reviewer for raising such an important point. We have now checked the clinical history of all our participants and found a total four participants i.e. one in bronchiectasis group, one in asthma group and two in COPD group that were uremic with renal failure, we have now excluded these participants from the study. Since our COPD subgroups i.e. NE and E-COPD already had low number of participants so we added two new participants in COPD group, that were otherwise healthy. We did not find any participant with anaemia or with folate or B12 deficiency.

3. COPD, asthma and BE can share airflow obstruction, but are very different diseases in terms of underlying causes. Do the authors feel that the hypersegmented phenotype is therefore specific to obstructive lung disease, or could this be a response to any chronic inflammatory lung or indeed any chronic inflammatory condition?

We don't think that hypersegmented neutrophils are specific to obstructive airways disease and perhaps it is more likely to be an attribute of inflammation as the hypersegmented cells were also observed in a trauma patient (Pillay et al, 2012), in chronic inflammatory lung disease like ARDS (Juss et al, 2016) and after inducing acute systemic inflammation (Pillay et al,2012, Tak et al, 2017). However the pattern of neutrophil phenotype has not yet been investigated in obstructive airways disease. We have now added this in our discussion section from line number 241-243.

4. I can see that the data has been very carefully collated and interpreted but the authors only studied nucleus appearance without any specific cell surface staining, and I worry that functionally different cells might be put in one group due to the simplicity of this approach. Indeed, there is some debate about whether all "hypersegmented" neutrophils are the same. Hypersegmented neutrophils are often considered mature but have been described as pro-inflammatory and cytotoxic in one study (J Immunol. 2017 Mar 1; 198(5): 1793–1797. doi: 10.4049/jimmunol.1601292) and anti-inflammatory or immune-suppressive in other studies (for example, Pillay, JCI, 2012) although crucially the surface markers differed between publications.

Could the authors comment on how they might interpret potential differences in cell subtype even within the hypersegmented group, and do they believe that counting nucleus lobes is enough to tell you something about the cell? What do they think this data means in terms of neutrophil function?

We agree that further work is needed to now explore the functional differences of these neutrophils and we are thankful for the reviewer for raising such a valid point. Both of the mentioned studies (Pillay et al and Whitemore et al, cited by the reviewer) performed different assays and so it is difficult to compare them directly, and we agree that this is a very important area of further research. We have addressed this point in detail in our discussion (line number 281-289).

5. I worry that the eosinophil versus non-eosinophil work may be under powered, and that is why some of the data parameters are not different, but this is interesting data

We agree it may be underpowered and it was a post-hoc analysis based on our unexpected finding of the correlation between hypersegmented neutrophils and eosinophils in COPD participants (Rho=0.535, p=0.015). This will certainly be something of interest for us to explore in future work.

6. The causes of bronchiectasis (BE) are variable and this might impact on inflammatory phenotype. Could the authors provide (in a supplement?) the causes of BE in this group?

We have provided further detail in a supplementary data table S1.

7. Patients with COPD were on higher doses of BDP equivalent than the asthma patients - could this have had an impact?

There was no correlation between ICS dose and hypersegmented neutrophils in the cohort. However, we cannot exclude the hypothesis that ICS may be influencing neutrophil phenotype, ICS are known to increase the survival of neutrophils and we have discussed this in “discussion” (line number 276-280).

Reviewer: 2

Reviewer Name: Mona Bafadhel

1. It is not clear from the abstract that the samples studied were from lavage. Please add

Added in the abstract (line number 5-6)

2. BL is abbreviated in the abstract – but this again has not been clarified. Please change to lavage.

Changed (line number 22)

3. What is β relate to in the final line of the results section of the abstract?

This represents rho or the Spearman correlation co-efficient, now it has been changed to Spearmans’s Rho in result section (line number 181-193).

4. Could the authors add if there was any exclusion criteria – I am unsure what the lung function criteria for bronchoscopy would be and this may be additional bias.

Lung function was not an exclusion criteria for bronchoscopy in this study. We only included those who were undergoing bronchoscopy either for medical purpose or were undergoing a surgical procedure that requires endotracheal intubation. This has now been included in participant section (line number 93-95).

5. Why were current smokers excluded?

Current smoking is well established to impact on neutrophil function, we did not have enough numbers to a current smoking group in any single disease group.

6. There is little detail on the severity of disease; could GOLD/GINA categorisation be added. What do the authors think of the clinical severity of the patients with bronchiectasis? The mean lung function would make it appear that this is a very mild group of patients.

The severity for both COPD (GOLD criteria) and asthma (GINA criteria) is added in table 1 and it has been now mentioned in clinical characteristics of result section (148-149). The bronchiectasis participants were generally mild and their median (IQR) bronchiectasis severity score has been added in clinical characteristic table (Table 1) and mentioned in text in clinical characteristic section (line number 148-152).

7. Some of the 'normal' population appear to have airflow obstruction (the lower 25th centile is below 70). I am not sure these patient are true 'normal/healthy controls' and may actually represent an early COPD smoking cohort. Could the authors address this or remove this population from this category?

We thank the reviewer for this careful observation which has alerted us to a mistake in data entry and we apologize for that, it has been corrected now, all healthy controls are within normal range, i.e. <70% FEV_1/FVC. The table number 1 is corrected.

8. In table 2 the COPD population seem to have the highest degree of eosinophilic inflammation. Was this corrected for ICS? Could the authors comment on this?

The participants with COPD do have the highest degree of eosinophilic inflammation, we did not undertake any correction for ICS dose. This was unexpected and we have no measure of adherence to treatment which could account for eosinophilia in this group.

9. The authors found that the neutrophil phenotype in eosinophilic and non-eosinophilic COPD patients was different. This is an interesting finding. Is this true for eosinophilic versus non-eosinophilic obstructive disease (ie. Eosinophilic asthma+Eosinophilic COPD vs. non eosinophilic asthma+non-eosinophilic COPD)? This may yield some further information related to whether it is inflammatory cell specific or disease specific.

We did not find a similar difference in eosinophilic vs. non eosinophilic obstructive airways disease, it seemed to be only present in COPD. Added in line number 217 to 219.

10. I think table 3 is repetitive. Please just address any findings in the text and remove.

Table 3 removed and significant finding added in text (line number 201-211).

11. Please comment if any standardisation for repeated measures was made.

We not had any repeated measures in the analysis.

Reviewer: 3

Reviewer Name: Leigh M Marsh

1) In Figure 2, please confirm in the figure legend whether the line on each dot plot represents the median or mean. Please also include which statistical test was performed in the legend. There is a very large difference in the % and total number of hypersegmented neutrophils in bronchiectasis patients, could the authors comment on this.

The line represents the median and data was analysed using a Kruskal-Wallis test, which has been added in the legend. The large difference in bronchiectasis is related to the relatively large increase in neutrophil numbers found in those participants. Those with bronchiectasis has a relatively low proportion of hypersegmented neutrophils but the increase in number of hypersegmented neutrophil is actually a cumulative effect of increase in overall neutrophil number.

2) It appears that the entire obstructive airway disease cohort is presented in the spearman correlation (Figure 3). If this is the case please colour-code each point based on their disease status (using the same colour scheme as Figure 2). It might be more informative to graph the individual group correlations to demonstrate which disease subtypes drive the observed correlations. Finally, please state and discuss whether the observed correlation between proportion of hypersegmented neutrophils with FEV_1% predicted and FEV_1/FVC% was also observed with total numbers.

The figure 3 is amended and now it has different colour and shape code for different diseases just like figure 2. We not have power to access this association for individual group.

There was no association between FEV_1% predicted or FEV_1/FVC% with total numbers of hypersegmented neutrophils nor with total number of neutrophils, we have now amended this in result and in discussion section (line number 185 -189 and 300-301).

3) In table 4, there is a very high variation in the number of different neutrophils, which probably explains the lack of statistical significance between the groups. However, the authors make the strong statement that "E-COPD had half the number of banded neutrophils and ten times fewer normal neutrophils in comparison to those with NE-COPD". Based on the presented data this statement is unfounded, I would recommend presenting the data from this table as a dot-plot so that the reader can greater appreciate the similarities and differences between the groups.

The table has been removed and data has been plotted in the form of dot plot as figure 5.

4) The authors speculate that the increase in hypersegmented neutrophils may arise either from being recruited from the circulation or becoming hypersegmented in the airways. They also list a number of factors (e.g. GM-CSF, CXCL-8 or serum amyloid A) that potentially have a role in this process. Please quantify these three proteins in the BALF of COPD patients and correlate with the number of hypersegmented neutrophils.

We thank the author for this suggestion and will explore these inflammatory proteins in sputum supernatant which is much more concentrated rather than our very dilute bronchial lavage samples. This will be part of our future research.

Minor

Please include details on the assessment of cell viability.

Added (line 121-122)

VERSION 2 – REVIEW

REVIEWER	Elizabeth Sapey University of Birmingham, United Kingdom
REVIEW RETURNED	24-Oct-2018

GENERAL COMMENTS	The authors have addressed all my questions and comments and I now fully support the publication of this manuscript in its current form.

REVIEWER	Leigh M Marsh Ludwig Boltzmann Institute for Lung Vascular Research Stiftingtalstrasse 24 8010 Graz Austria
REVIEW RETURNED	04-Nov-2018

GENERAL COMMENTS	The authors have satisfactory addressed my previous concerns.