Abstract word count: 238

Text word count: 3380

Interrelationships between small airways dysfunction, neutrophilic inflammation and exacerbation frequency in COPD

Short title/running head: Small airways disease and exacerbations in COPD

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Summary conflict of interest statements

KD, KJS, JC, TW, AW, CMS and DC report grants from Astrazeneca, during the conduct of the study. JC reports personal fees from Trudell Medical, outside the submitted work and TW reports personal fees and other from MyMHealth, grants from GSK, grants and personal fees from AstraZeneca, grants and personal fees from Synairgen, personal fees from BI, outside the submitted work. KO is a paid employee of Astrazeneca. Prof Thompson has nothing to disclose.

Funding information

The study was funded by AstraZeneca. AstraZeneca reviewed the publication, without influencing the opinions of the authors, to ensure medical and scientific accuracy, and the protection of intellectual property. The corresponding author had access to all data in the study, and had the final responsibility for the decision to submit the manuscript for publication

Notation of prior abstract publication/presentation

Preliminary data from this study was presented in abstract form at the ERS conference 2019, Madrid.

Abbreviation list

BAL: Bronchoalveolar lavage

BAL Neutrophil %: The average of the percentage of neutrophils from the sampling of two lobes during bronchoscopy

CT: Computed Tomography

FE: Frequent exacerbator subgroup

FOT: Forced Oscillation Technique

ICS: Inhaled Corticosteroids

IFE: Infrequent exacerbator subgroup

%LAA: Percentage Low Attenuation Area <-950HU

MBNW: Multiple Breath Nitrogen Washout

MLD E/I: The ratio of the Mean Lung Density (MLD) of expiration to inspiration (MLD E/I)

RV/TLC: The ratio of residual volume to total lung capacity

S_{acin}: Acinar ventilation heterogeneity

SAD: Small Airways Disease

TLCO: Transfer factor for carbon monoxide

Abstract

Background

Small airways disease (SAD) is a key component of COPD and is a main contributing factor to lung function decline.

Research Question

Is small airways disease a key feature of frequent COPD exacerbators and is this related to airway inflammation?

Study Design and Methods

Thirty nine COPD subjects defined as either frequent exacerbators (≥ 2 exacerbations per year, n = 17) and infrequent exacerbators (≤1 exacerbation per year, n = 22) underwent Forced Oscillation Technique (R5-R19, AX), multiple breath nitrogen washout (S_{cond}, S_{acin}), plethysmography (RV/TLC), single breath transfer factor (TLCO), spirometry (FEV_1%, FEV_1/FVC) and paired inspiratory – expiratory CT scans to ascertain small airways disease. A subpopulation underwent bronchoscopy to enable enumeration of BAL cell proportions.

Results

Acinar ventilation heterogeneity (S_{acin}) was significantly higher in COPD FE compared to IE (*P* = .027). In the FE group, markers of SAD were strongly associated with BAL neutrophil proportions, R5-R19 (*P* = .001, r = 0.795), AX (*P* = .049, rho = 0.560), RV/TLC (*P* = .004, r = 0.730) and the mean lung density of the paired CT scans (*P* = .018, r = 0.639).

Interpretation

Increased acinar ventilation heterogeneity may be a consequence of previous exacerbations or highlight a group of patients prone to exacerbations. Measures of SAD were strongly associated with neutrophilic inflammation in the small airways of FE supporting the hypothesis that frequent exacerbations are associated with small airway disease related to increased cellular inflammation.

Keywords: Small airways, COPD, exacerbation, inflammation

Chronic Obstructive Pulmonary Disease (COPD) is a heterogenous disease of the lungs that can comprise of different pathophysiological entities, including emphysema, chronic bronchitis and Small Airways Disease (SAD)[1,2]. COPD is also associated with chronic inflammation and this ongoing inflammation may result in airway remodelling and excessive mucus plugging within the small airways (those defined as < 2 mm in diameter)[3,4]. This leads to a loss of the support structures keeping these airways open, resulting in airway narrowing and increased small airways resistance[5]. Increased small airways resistance has been shown to be a main contributor to airflow limitation in COPD[3,6]. In the past, COPD patients were broadly split between an emphysematous phenotype and a chronic bronchitic phenotype, but not only can these features co-exist in the same patient but it is now recognised that COPD patients exhibit multiple phenotypes and endotypes. One such phenotype are those patients who experience frequent exacerbations (≥ 2 exacerbations per year)[1,7], which appears to be a relatively stable phenotype[8]. Exacerbations are an acute worsening of symptoms resulting in additional therapy and can be classified as mild, moderate or severe[1]. Exacerbations are associated with faster lung function decline[8,9] and hospital admissions due to exacerbations have major healthcare utilization implications[10,11]. During both stable periods and exacerbations, there is increased neutrophilic inflammation in the airways of COPD subjects[12]. Furthermore, frequent exacerbators have increased neutrophilic inflammatory markers over time and this inflammation is positively associated with bacterial load[12]. Exacerbations are associated with disease progression and work is ongoing to try to understand the mechanisms related to exacerbation susceptibility[13]. It is unclear what the relationship between SAD and exacerbation frequency is and what the mechanistic links between the two features of COPD are.

Changes in the small airways can be identified through increases in ventilation heterogeneity and gas trapping, however, there is no universally agreed gold standard for the measurement of this SAD. Gas trapping, an indirect measure of SAD, can be assessed using a paired high resolution computed tomography (HRCT) scan and/or body plethysmography[14,15]. The HRCT measure gives the ratio of the Mean Lung Density (MLD) of the expiratory scan to the inspiratory scan (MLD E/I), reflecting increased low attenuation areas after expiration due to incomplete volume reduction[16]. Body plethysmography yields a residual volume to total lung capacity ratio (RV/TLC) which is also raised due to incomplete volume reduction as a result of pathology within the small airways. Although not yet adopted into routine clinical practice, measures derived from the Forced Oscillation Technique (FOT) and the Multiple Breath Nitrogen Washout (MBNW) have been shown to associate with ventilation heterogeneity attributed to SAD in asthma and COPD with MBNW recently shown to be feasible in COPD populations[17,18].

FOT uses pressure oscillations during normal breathing to examine the resultant flow pressure relationship and calculate resistance (R) and reactance (X) of the airways and lung tissue[19]. In COPD, narrowing of the small airways results in frequency dependence of resistance , denoted as R5-R19 and an increased low frequency reactance area (AX) due to oscillations being unable to access the smaller airways as peripheral lung units are derecruited[19,20]. R5-R19 may be elevated due to either upper airways shunting (especially during airways obstruction)[21,22], widespread airways constriction, or heterogeneity of constriction[23]and studies using computational modelling have demonstrated that these measures are most impacted by narrowing of the small airways[24]. Both R5-R19 and AX have been shown to reflect small airways abnormalities and will therefore be used as a marker of small airways dysfunction in this analysis[19]. The MBNW test measures ventilation heterogeneity and is able to compartmentalize that within the conducting airways (S_{cond}) and that within the acinar (S_{acin}) regions of the lung[25-27]. S_{acin} is increased in COPD[25,28] and this can be due to uneven narrowing of small airways, parenchymal destruction and/or loss of patent terminal bronchioles[27,29,30]. An advantage of FOT over MBNW is that it is quick and easy for subjects to complete compared to MBNW which takes longer and may not be as repeatable[31].

Significant small airways dysfunction has been described in COPD compared to health[2,27,28,32] but there is mixed literature about the clinical relevance of small airways dysfunction in COPD[18]. Furthermore, there is limited information about how measures of SAD may differ between exacerbation phenotypes of COPD. There are also a lack of studies examining the relationship between these physiological tests and airway inflammation with most studies using resected lung tissue or sputum[32,33]. Exploring the associations between indices derived from non-invasive measures of SAD and distal lung inflammation would provide insight into the physiological manifestations of inflammation and help in our understanding of disease processes.

The use of FOT and MBNW in COPD is not fully understood and there is a significant global interest and debate about the future of these two tests within respiratory medicine[34]. Markers of SAD measure different aspects of this disease process and because there is no gold standard measure, we chose to examine indices derived from lung function tests and HRCT to provide a non-biased comprehensive assessment. The use of FOT and MBNW indices in addition to gas trapping markers provides information about heterogenous small airways constriction and ventilation heterogeneity in the peripheral airways. In order to gain insight into the mechanisms leading to frequent exacerbation in COPD and the potential role of the small airways within this pathology, this study aimed to compare markers of SAD between infrequent (IFE) and frequent exacerbators (FE) to understand if SAD is a key feature of frequent exacerbators. Furthermore, it aimed to examine the relationships between these SAD markers and neutrophilic inflammation to test the hypothesis that COPD frequent exacerbators

149 have increased SAD resulting from increased lower airways inflammation. This study used a well
150 characterised cohort of COPD patients which has previously been used to compare two CT quantitative
151 analysis techniques[2]. Furthermore, cells purified from bronchoscopy of this cohort of patients, have
152 been used to model the dynamics of IFN-β responses during respiratory viral infection[35].

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Methods and Materials

COPD and healthy controls were recruited into the study as previously described[2] . As this analysis focuses on small airways disease and COPD exacerbations only the 39 COPD subjects were included. These subjects were GOLD Stage I and II former smokers with at least a 10 pack year history. Briefly, subjects were recruited from various sources including a research database, study advertisements, local healthcare facilities or contacted by clinicians involved in or aware of the study. Subjects had quit smoking at least 6 months before enrolment and non-smoking status was confirmed by urine cotinine testing. For this analysis, subjects were classified as either frequent exacerbators (defined as those with a history of frequent exacerbations (≥ 2 per year in the preceding 12 months before enrolment)[1,7], n = 17 or infrequent exacerbators (defined as with a history of infrequent exacerbations (≤1 per year in the preceding 12 months before enrolment), n = 22. Exacerbations were considered as moderate exacerbations (those requiring oral steroids and/or antibiotics) or severe exacerbations defined as those requiring steroid and/or antibiotics plus hospital admission. Subjects were free of exacerbations for a minimum of 1 month before enrolment. All subjects gave written informed consent and the study was approved by the South Central Research Ethics Committee C (REC number 15/SC/0528).

Following administration of 400 µg of salbutamol, subjects performed spirometry as per guidelines at study enrolment[36]. Subjects then underwent a visit with extensive lung function testing which has previously been described in detail[2]. Briefly, pre-bronchodilator, single breath diffusion was performed as per guidelines[37], with percent-predicted carbon monoxide transfer coefficient calculated (TLCO%). Following administration of 400 µg of salbutamol, the tidal breathing tests, MBNW (S_{cond} and $S_{acin)}$and oscillometry (R5-R19, AX) were performed before plethysmography, with subjects allowed sufficient recovery time between testing.

HRCT analysis was performed by VIDA Diagnostics with emphysema measured as the percent of voxels with attenuation values less than -950 HU on the inspiratory scan (%LAA). MLD E/I, a CT marker of gas trapping was calculated as the ratio of mean lung density on paired expiratory and inspiratory scans.

A subpopulation of subjects underwent flexible video bronchoscopy and bronchoalveolar lavage (BAL) sampling (n = 17 for IFE, n = 13 for FE). Two lobes were sampled per subject with 100 ml 0.9% (w/v) saline being instilled into each lobe and recovered by aspiration. The BAL was filtered using a 100 µm cell strainer and centrifuged at 400 g for 10 min and room temperature to isolate the cell pellet. Cytospin slides were generated and 500 cells were counted to obtain a differential cell count. BAL neutrophil proportions and eosinophil proportions were averaged from differential cell counts from both lobes as previously described[38].

Data were analysed using IBM SPSS Statistics 24 and Graphpad prism 8.2.0. Each variable was checked for normality by plotting histograms and either mean and standard deviation or median and interquartile range were reported, as appropriate. A *P* value of < .05 was considered statistically significant. A two sample t-test or Mann-Whitney U test was used to test for differences between the infrequent and frequent exacerbator groups, as appropriate. Due to the categorical nature of gender and of ICS usage, chi square tests were used to test for any differences between the groups. Bivariate associations were determined using either Pearson's correlation or Spearman's rank correlation analyses, as appropriate.

Results

Table 1 shows the demographic, lung function and emphysema scores for the COPD subjects included in this analysis and has some overlap with previously published work[2,35] The use of ICS was higher in FE vs IFE, however there was no difference in any of the other demographic, spirometry or emphysema scores between the infrequent and frequent exacerbator groups (Table 1).

To understand if small airways disease is a key feature of frequent COPD exacerbators, physiological and CT parameters were compared between the IFE and FE groups. Of the six parameters investigated, only S_{acin} was significantly different between infrequent and frequent exacerbators, with FE having higher median values than IFE (Table 2).

We next investigated the association between exacerbation phenotype and neutrophilic inflammation. There were more BAL neutrophils in FE (median 9.40, IQR 29.40) compared to IFE (median 3.10, IQR 7.50, one tailed P = .036) (Figure 1). For comparison of other BAL cell types and for total BAL cell count see supplement- e-Appendix 1. Figure 1 indicates a sub-cluster of FE with excessive neutrophilic inflammation (values above the median of the FE group), n = 6. However, no differences in small airways measures between this sub-cluster and other FE was found except for MLD E/I which was significantly higher in the excessive neutrophilic group compared to other FE (e-Table 3). In order to understand how markers of small airways dysfunction relate to BAL neutrophilic inflammation, bivariate correlations with BAL neutrophil proportions were then conducted. When all COPD subjects were analysed, only R5-R19 and RV/TLC were significantly associated with BAL neutrophils (Table 3). Regarding eosinophilic inflammation, there was no difference in BAL eosinophil proportions between IFE and FE and no significant correlations between any markers of SAD and BAL eosinophil proportions (e-Table 2 and e-Appendix 1).

Bivariate correlations were next analysed in the infrequent and frequent exacerbator groups separately to determine if associations between markers of SAD and BAL neutrophil proportions differed by exacerbation phenotype. There were no significant associations between any markers of SAD and BAL neutrophil proportions in the infrequent group (e-Table 1). For the FE group, scatterplots were visualised (Figure 2A-D) if there were significant associations between markers of SAD and BAL neutrophil proportions. In frequent exacerbators, there were significant moderate to very strong associations between R5-R19, AX, MLD E/I, RV/TLC and BAL neutrophil proportions. There was a trend towards an association between S_{acin} and BAL neutrophil proportions (P = .067). There were no significant associations between S_{cond} and BAL neutrophil proportions in this subgroup (all P > .05 – data not shown). For eosinophil proportions, there were no significant correlations with markers of SAD in the infrequent or frequent exacerbator subgroups except for S_{cond} in the FE group (e-Table 2).

Sub-group analyses of only subjects on ICS revealed similar results as described when COPD subjects irrespective of ICS usage were analysed (see e-Appendix 1 for full results of this sub-analysis).

Discussion

To our knowledge this is the first study using both physiological and CT measures of SAD to demonstrate small airways dysfunction is strongly associated with BAL neutrophil not eosinophil proportions in frequent but not in infrequent COPD exacerbators. These data highlight the important interrelationship between neutrophilic inflammation, exacerbation frequency and small airways disease in COPD. Furthermore, it is the first to describe increased acinar ventilation heterogeneity in COPD patients who are frequent exacerbators. This is not purely driven by airflow limitation or disease severity as there was no significant difference in FEV_1/FVC or FEV_1%, as determined by spirometry, between the two exacerbation groups. SAD may be either a cause or consequence of frequent exacerbations and associated neutrophilic inflammation and the measurement of acinar ventilation heterogeneity may help in identifying subjects who experience frequent exacerbations as a guide to patient management.

Our first observation was of increased S_{acin} in the FE subjects. No differences in S_{cond} were noted between the two groups suggesting the increased ventilation heterogeneity is in the acinar region and not in the more proximal conducting airways. Increased ventilation heterogeneity occurs due to non-uniform emptying of the lungs potentially as a result of some areas being less ventilated than others[39] and therefore an increased S_{acin} may arise due to structural changes in the acinar region leading to acinar ventilation heterogeneity[26]. Such changes could be due to emphysema[40]. However, in our cohort, there is no difference in either %LAA or TLCO, both indicative of emphysema. This lack of difference between IE or FE subjects suggests that destruction of the lung parenchyma is not the sole reason for the increased acinar ventilation heterogeneity found in the FE phenotype. Verbanck *et al* has recently shown through simulation studies that reduction in the number of patent terminal bronchioles in COPD can increase acinar ventilation heterogeneity, however such analysis was not in the scope of our study[30]. Another cause for the increased S_{acin} may be uneven narrowing of respiratory bronchioles[29,41], due to small airway lumen obstruction related to increased airway inflammation and/or mucus secretions. In addition, structural alterations as a result of either fibrosis/remodelling in the small airways may contribute to bronchiole narrowing[42]. Although, S_{acin} was higher in frequent exacerbators, it is not significantly associated with BAL neutrophil proportions although a positive trend was noted. One reason for this may be that the BAL sampled specific lobes and may not be reflective of the acinar ventilation heterogeneity throughout the lung. However, this data could also suggest that neutrophilic inflammation in the distal airways is a contributing factor, but not the only explanation for an increased acinar ventilation heterogeneity in frequent exacerbators.

In other diseases like Cystic Fibrosis (CF), measures of ventilation heterogeneity are predictors of pulmonary exacerbation and have been linked to changes in the microbiome of the airways[43,44]. Alterations in the microbiome of COPD frequent exacerbators have been described[13] and there is a possibility that such alterations may lead to increased airway wall inflammation and mucus exudate in the distal lung causing the increased S_{acin} in frequent compared to infrequent COPD exacerbators. In asthma, gas trapping, R5-R20 and S_{acin} are also associated with increased exacerbations[45].

In contrast to the increased acinar ventilation heterogeneity observed in FE, there were no differences observed in gas trapping or FOT indices of small airways dysfunction between the IE and FE groups. Such disconcordance between MBNW and FOT has been previously described [39,46]. The R5-R19 may be thought of as more a measure of widespread/diffuse small airways constriction and may not reflect more localised small airways obstruction which can result in increased ventilation heterogeneity[39]. In addition, differences between the two techniques exist with FOT potentially being confounded by upper and larger airways shunts, an issue which does not affect MBNW[22]. The lack of standardisation in measuring SAD creates further complexity in the interpretation of such data and it is likely that such proposed markers of SAD measure a facet of a multifaceted dysfunction.

Our data found increased neutrophil proportions in the distal airways of frequent compared to infrequent exacerbators, confirming previous studies[33]. There is only one other study in COPD by Lapperre *et al,* which showed using physiological tests, such as single breath nitrogen washout, that markers of SAD were associated with neutrophilic inflammation in BAL[47]. Our data adds to the findings of the Lapperre study by using FOT, MBNW and HRCT markers of SAD to demonstrate the strong association between SAD by each of these measures and neutrophilic inflammation. Furthermore, it supports the study by Ostridge *et al*, who found associations between CT defined gas trapping (MLD E/I) and neutrophilic inflammatory markers (IL-8) and neutrophil-derived MMPs in BAL[38,48]. Although there was increased use of ICS in frequent compared to infrequent exacerbators, similar results and trends were noted when only subjects on ICS were analysed. This suggests ICS usage is unlikely to be a significant contributing factor to our findings and that SAD measures are associated with neutrophilic inflammation regardless of ICS use. However, the association between neutrophil proportions and small airways dysfunction in FE does not prove causation. Frequent exacerbations may cause small airway disease through increased inflammatory cell numbers and associated cytokines, leading to mucus production and airway thickening and occlusion[3,8]. Indeed, in our study, the sub-cluster of frequent exacerbators with excessive neutrophilic inflammation had significantly greater CT defined SAD than other frequent exacerbators. In addition, although not statistically significant, these subjects also showed a trend towards increased small airways dysfunction as measured by FOT and plethysmography defined gas trapping. These data do not prove causation but may further support

the role of neutrophilic inflammation in small airways disease, especially in frequent exacerbators. However, the sample size in this present study was small and such findings should be confirmed in a larger population. Conversely, it is possible that SAD predisposes subjects to frequent exacerbations because of associated hyperinflation and dyspnea, resulting in exacerbations being more easily triggered in these subjects[8].

We recognise that the main limitation of this study was the small sample size and that, with more power, other significant differences between frequent and infrequent exacerbators, or associations between markers of SAD and inflammation, may have been noted. Despite this, we have shown that both physiological and HRCT markers of SAD have moderate to strong associations with BAL neutrophil proportions in frequent exacerbators. Multiple comparisons between the frequent and infrequent exacerbator groups have been made and the chance of a Type I error is acknowledged. We compared 6 markers of SAD between infrequent and frequent exacerbator groups and tested 6 associations between physiology and CT measures of SAD and BAL neutrophil proportions in the frequent exacerbator group. At the 5% level, < 1 variable would be expected to be significantly different between the two groups and < 1 significant association would be expected just by chance. However, we found S_{acin} to be different between groups and 4 significant associations between physiological and CT measures of SAD and BAL neutrophil proportions. This is more than would be expected by chance alone. Our study subjects had mild or moderate disease and were not current smokers. Therefore, our results may not be generalizable as they may not reflect more severe disease or findings in smoking populations. In addition, patient reported retrospective exacerbation data was used which may have recall bias but these exacerbation groupings were based on accepted guidelines[1,7].

Interpretation

Our study integrates three key features; physiology, imaging and inflammometry, to highlight the importance of neutrophils in small airways disease in frequent COPD exacerbators. The strong associations between neutrophilic inflammation and increased heterogeneous small airways resistance and gas trapping suggest these measures may provide useful insights into disease mechanisms, especially in targeting treatment and identifying mechanisms of susceptibility to frequent exacerbations. Increased ventilation heterogeneity (S_{acin}) may be a consequence of previous exacerbations or highlight a group of patients prone to exacerbations and results should be confirmed in a larger prospective study. This data both supports the hypothesis that COPD patients with frequent exacerbations are more likely to suffer from concomitant small airway disease as a result of chronic

inflammation and encourages the measurement of physiological markers of SAD in clinical practice to help gain insight into disease phenotypes.

Acknowledgements

Guarantor statement

KD had full access to the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Author's contributions

KD, KO, KJS and TW contributed substantially to the study design and all authors contributed to the writing of the manuscript. KD, KO, KJS, AW, CMS, DC and TW collected or generated the data. All authors analysed or interpreted the data.

Financial/nonfinancial disclosures

KD, KJS, JC, TW, AW, CMS and DC report grants from Astrazeneca, during the conduct of the study. JC reports personal fees from Trudell Medical, outside the submitted work and TW reports personal fees and other from MyMHealth, grants from GSK, grants and personal fees from AstraZeneca, grants and personal fees from Synairgen, personal fees from BI, outside the submitted work. KO is a paid employee of Astrazeneca. Prof Thompson has nothing to disclose.

Role of the sponsors

The study was funded by AstraZeneca. AstraZeneca reviewed the publication, without influencing the opinions of the authors, to ensure medical and scientific accuracy, and the protection of intellectual property. The corresponding author had access to all data in the study, and had the final responsibility for the decision to submit the manuscript for publication.

Other Contributions

The authors thank all the study volunteers for their contribution towards furthering knowledge about chronic obstructive pulmonary disease. They also thank the nursing staff in the Southampton Centre for Biomedical Research. The authors thank VIDA for the image analysis which formed part of an academic collaboration.

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Tables

Table 1: Demographics, lung function and CT emphysema scores in infrequent and frequent COPD exacerbators

	Infrequent (N = 22)	Frequent (N = 17)	*P* value
Age	69.1 [8.2]	69.7 [7.9]	.974
Gender (% Male)	77.3	76.5	.953
% of subjects using ICS	42.9	88.2	**.004**
Pack Years	48.0 [20.9]	41.0 [29.3]	.574
BMI	29.48 [5.35]	28.36 [4.21]	.486
FEV_1%	73.8 [18.2]	67.2 [12.7]	.406
FEV_1/FVC	56.1 [10.0]	54.1 [9.3]	.751
TLCO%	72.7 [13.7]	68.9 [19.4]	.509
Emphysema (%LAA)	13.08 (9.97)	10.53 (9.30)	.714

Values are given as mean values [SD] or median (IQR). For ICS, n = 21 for IFE, n = 17 for FE. For pack years and %LAA, n = 21 for IFE, n = 17 for FE, for TLCO% n = 19 for IFE and n = 16 for FE. Chi-square tests to test for gender differences and differences in proportions of IFE and FE taking ICS . Either a t-test or Mann–Whitney U test for all other variables, as appropriate.*$P < .05$

Table 2: Markers of SAD in infrequent and frequent COPD exacerbators

	Infrequent (N = 22)	Frequent (N = 17)	*P* value
R5-R19	0.95 [0.61]	1.15 [1.05]	.687
AX	12.09 (13.91)	8.95 (29.1)	.869
S_{cond}	0.022 (0.036)	0.024 (0.034)	.927
S_{acin}	0.246 (0.209)	0.459 (0.320)	**.027**
RV/TLC	42.1 [7.4]	42.9 [9.9]	.956
MLD E/I	0.86 [0.05]	0.85 [0.06]	.783

Values are given as mean [SD] or median (IQR). For R5-R19 and AX, n = 18 for IFE, n = 17 for FE. For S_{acin}, n = 14 for IFE and for FE. For RV/TLC, n = 17 for IFE and for FE. For MLD E/I and %LAA, n = 21 for IFE, n = 17 for FE. Either a t-test or Mann–Whitney U test for all variables

Table 3: Correlation analysis between markers of SAD and BAL neutrophil proportions in all COPD subjects

Index	BAL Neutrophil %	*P* value

R5-R19	0.388	**.038**
AX	0.167	.387
S_{cond}	0.134	.541
S_{acin}	0.356	.095
RV/TLC	0.488	**.010**
MLD E/I	0.279	.135

For R5-R19, RV/TLC and MLD E/I, Pearson's r values reported. For AX and S_{acin}, Spearman's rho reported. n = 29 for R5-R19 and AX, n = 23 for S_{acin} , n = 27 for RV/TLC, n = 30 for MLD E/I.

Figure Legends

Figure 1: Bronchoalaveolar lavage (BAL) neutrophil proportions in infrequent (IE) and frequent (FE) COPD exacerbators. Data represents median. Each dot represents the average neutrophil percentage for an individual patient, N = 17 (IFE), N = 13 (FE). Statistical analysis by Mann Whitney U test.

Figure 2: Scatterplots of COPD FE subjects showing indices of SAD vs BAL neutrophil proportions (A) R5-R19,(B) AX, (C) MLD E/I, (D) RV/TLC . All (Pearson's r reported) except Spearman's rho reported for AX. N = 13.