Original article

Biological exacerbation clusters demonstrate asthma and COPD overlap with distinct mediator and microbiome profiles

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ABSTRACT

BACKGROUND: Exacerbations of asthma and chronic obstructive pulmonary disease (COPD) are heterogeneous.

OBJECTIVE: We sought to investigate the sputum cellular, mediator, and microbiome profiles of both asthma and COPD exacerbations.

METHODS: Patients with severe asthma or moderate-to-severe COPD were prospectively recruited to a single centre. Sputum mediators were available in 32 asthma and 73 COPD patients assessed at exacerbation. Biologic clusters were determined using factor and cluster analyses on a panel of sputum mediators. Patterns of clinical parameters, sputum mediators, and microbiome communities were assessed across the identified clusters.

RESULTS: The asthma and COPD patients had different clinical characteristics and inflammatory profiles, but similar microbial ecology. Three exacerbation biologic clusters were identified. Cluster 1 was COPD predominant, with 27 COPD and 7 asthma patients exhibiting elevated blood and sputum neutrophil counts, proinflammatory mediators (IL-1β, IL-6, IL-6R, TNFα, TNF-R1, TNF-R2, and VEGF), and proportion of the bacterial phylum *Proteobacteria*. Cluster 2 had 10 asthma and 17 COPD patients with elevated blood and sputum eosinophil counts, Type 2 (T2) mediators (IL-5, IL-13, CCL13, CCL17, and CCL26), and proportion of the bacterial phylum *Bacteroidetes*. Cluster 3 had 15 asthma and 29 COPD subjects with elevated Type 1 (T1) mediators (CXCL10, CXCL11, and IFN-ɣ) and proportions of phyla *Actinobacteria* and *Firmicutes*.

CONCLUSIONS: A biologic clustering approach revealed three subgroups of asthma and COPD exacerbations each with different percentages of overlapping asthma and COPD patients. The sputum mediator and microbiome profiles were distinct between clusters.

Clinical implications:

Sputum mediator and microbiome profiling can determine the distinct and overlapping asthma and COPD biologic exacerbation clusters, highlighting the heterogeneity of these exacerbations.

Capsule summary:

Biologic clustering approach to asthma and COPD exacerbations can provide further insight into the heterogeneity of their underlying immune pathophysiology and microbial dysbiosis, and aid in the development of novel biomarkers and targeted therapies.

Key words: Asthma; chronic obstructive pulmonary disease; asthma and COPD heterogeneity; inflammatory profiles; microbiome abundances; phylum and genus levels; factor and cluster analyses

Abbreviations used

COPD: Chronic obstructive pulmonary disease

INTRODUCTION

The prevalence of asthma and COPD continue to rise, exceeding 358 million and 174 million worldwide, respectively[1]. Asthma and COPD are heterogeneous for clinical characteristics, cellular sources of inflammation, etiologies of exacerbations, and responses to therapies[2-7]. They share similar features such as symptoms, airflow limitation, bronchial hyper-responsiveness and inflammatory profiles[8]. A previous examination of asthma and COPD biologic clusters during stable disease demonstrated an overlap of sputum inflammatory profiles[7]. However, understanding the distinctive and common heterogeneities of both diseases at exacerbations remains elusive.

Despite current guidelines on management strategies for asthma and COPD, many patients still experience exacerbations. Asthma exacerbations impair health-related quality of life, result in lost productivity and increase health care resource utilization. Moreover, COPD exacerbations are associated with accelerated loss of lung function, poorer health-related quality of life, comorbidities, significant mortality, and increased health care costs[9, 10]. Standard treatment for asthma and COPD exacerbations include the use of bronchodilators, corticosteroids, and antibiotics with little attention paid to the underlying heterogeneity of these exacerbations. Biologic heterogeneity of COPD exacerbations has previously been demonstrated[4], with sputum IL-1ß, serum CXCL10, and peripheral blood eosinophils best identifying bacteria-, virus-, or eosinophil-associated exacerbations, respectively. However, whether this biologic heterogeneity is similar between asthma and COPD exacerbations is unknown.

The main objective of this study was to identify the degree of overlap in biologic clusters of asthma and COPD exacerbations derived from sputum mediator profiling as a measure of airway inflammation and to determine the airway bacterial ecology in each of these clusters.

METHODS

Study population

Patients with severe asthma or moderate-to-severe COPD were recruited from a single centre at the Glenfield Hospital, Leicester, United Kingdom. Assignment to asthma or COPD was made by the patients' physicians consistent with definitions of asthma or COPD based on the Global Initiative for Asthma[9] or the Global Initiative for Chronic Obstructive Lung Disease[10] guidelines, respectively. The asthma patients had participated in a published stable study[7, 11] and those patients with COPD had participated in a published exacerbation study[4, 12]. All patients were assessed at stable state, at least 6 weeks from an exacerbation. They had assessment at exacerbation, defined as an increase in symptoms necessitating a course of oral corticosteroids and/or antibiotic therapy. All patients provided written informed consent. The studies were approved by the local Leicestershire, Northamptonshire, and Rutland ethics committee.

Measurements

Demographics, clinical and lung function data, including pre- and post-bronchodilator FEV_1 and forced vital capacity, were collected. Patients were asked to score the severity of their dyspnea and cough using the visual analogue scale (VAS). VAS is a horizontal line which is 100 mm in length and anchored by word descriptors at both ends. It uses absence of breathlessness or cough on one end, and maximum breathlessness or cough on the other end. It is scored by measuring the distance from the left end to the mark indicated by the patient. A bacterial-associated exacerbation was defined as colony-forming units greater than 10^7/mL sputum or positive culture. Microbiome communities were obtained from 16S rRNA sequencing of bacterial genomic DNA extracted from the sputum samples using the Qiagen DNA Mini kit (Qiagen, CA, USA), as described previously[13]. The sequencing reads were processed using QIIME pipeline[14]. RNA was extracted from selected

sputum plugs and an RT-PCR panel for all common respiratory viruses (rhinoviruses, other picornaviruses, respiratory syncytial virus, human parainfluenza virus 1-3, adenoviruses, influenza viruses A and B, coronavirus 229E and OC43, human metapneumovirus and human bocavirus) was undertaken for the COPD samples as described previously[13]. For the asthma samples, the same methods were used, but virus detections were limited to rhinoviruses, other picornaviruses, respiratory syncytial virus and influenza viruses A and B. A viral-associated exacerbation was defined as those exacerbations in which a virus was detected. Inflammatory mediators were measured in sputum supernatants and serum using the Meso Scale Discovery Platform (MSD; Gaithersburg, MD, USA). The mediators measured were selected to reflect cytokines, chemokines, and proinflammatory mediators implicated in airway disease. The performance of the MSD platform for recovery of spiked exogenous recombinant proteins has been described[8]. Sputum and serum inflammatory mediators below the detectable range were replaced with their corresponding halves of the lower limits of quantification. In addition, mediators below the limit of quantification for more than 60% of the patients were excluded from further analysis.

Statistical methods

A two-stage (factor and cluster analyses) approach was performed to identify the common and distinctive biologic subgroups of asthma and COPD. First, factor analysis was applied to the panel of sputum inflammatory mediators and reduced to small independent factors. Sampling adequacy for factor analysis was assessed using Kaiser-Meyer-Olkin. The optimal factors were retained on the basis of scree plot (factors above the break in the curve) and eigenvalue above one. Subsequently, the corresponding factor scores representing each patient were generated, and used as input variables into k-means clustering algorithm to identify the clusters. The optimal number of clusters was chosen on the basis of scree plot (clusters above the break in the curve) by plotting within cluster sum of the squares against a series of sequential number of clusters and by assessing

how natural the clusters provide biologic implications, clinical meaning, and interpretability. In addition, linear discriminant analysis was performed on the sputum mediators across the clusters to validate how the identified clusters from the factor scores can be predicted using the actual mediators' measurements, and to identify the contribution of each mediator in discriminating the clusters (data not shown). Discriminant scores for individual patients were calculated and used to represent the patients' biologic cluster membership graphically. Microbiome measurements from 16S rRNA sequencing at both phylum and genus levels were performed. Thirty species at phylum and 400 species at genus levels were screened. The relative abundance of each species was calculated, and the alpha (within patient) and beta (between patients) diversities at both phylum and genus levels were estimated using Shannon-Weiner and Sorensen indices, respectively (Vegan R-package version 2.3). The patterns were compared between diseases and the identified biologic clusters. In addition, patterns of those most abundant species (median relative abundance greater than 2%) and/or those known to be major airway pathogens in asthma and COPD at both phylum and genus levels were presented graphically across the diseases and the identified biologic clusters.

The statistical summary of all the available characteristics is presented across the diseases and the identified biologic clusters, and within each cluster by disease subgroups. The clusters were interpreted according to the patterns of these characteristics. In addition, the change in clinical characteristics and mediators between stable and exacerbation states within each cluster was assessed. Parametric data were presented as mean with standard error of the mean (SEM), and log transformed data were presented as geometric mean with 95% confidence interval (CI). The chi square (χ^2) test or the Fisher exact test, and one-way analysis of variance (ANOVA) were used to compare percentages and means across groups. Nonparametric data were presented as median values, with first and third quartiles, and the Kruskal-Wallis test was used to compare these data between clusters. All statistical analyses were performed using SPSS version 24 (IBM Corp. in

Armonk, NY), STATA/IC version 14.0 for Windows (StataCorp, College Station, TX, USA) and R version 3.2 (R Foundation for statistical computing, Vienna, Austria).

RESULTS

Thirty-two asthma and 73 COPD patients with sputum mediator records at exacerbation were included in this study. Their demographics, clinical characteristics, and sputum mediators were summarized across the diseases in the Online Repository (see Table E1). Inhaled corticosteroid dose was not different between asthma and COPD. All asthma patients were receiving long-acting beta-agonist treatment (LABA) and all COPD patients except 2 were receiving LABA and/or a long-acting muscarinic antagonist (LAMA). Asthma patients were younger and more obese, and had better lung function than COPD patients. The VAS scores, cellular profiles, and bacterial-associated exacerbations were not significantly different between the two diseases.

Several sputum mediators IL-5, IL-6R, CXCL10, CXCL11, CCL5, and CCL26 were significantly elevated in asthma versus COPD patients, while IL-6, CCL3, CCL4, and TNF-R1 were significantly elevated in COPD versus asthma patients. However, the majority of mediators, such as IL-1β, IL-8, IL-10, IL-13, CCL2, CCL13, CCL17, TNFα, TNF-R2, VEGF, and IFNγ were not significantly different between asthma and COPD patients (Table E1). Similarly, the serum mediators IL-5, IL-8, CXCL10, CXCL11, CCL17, CCL26, TNFα were increased in asthma, versus IL-1β, IL-6, CCL4, TNF-R1 increased in COPD and others CCL2, CCL13, TNF-R2, VEGF were not different between groups (Table E1).

Fourteen asthma and 40 COPD patients who had sputum mediators at exacerbation also provided sufficient sputum for further microbiomic analysis. Alpha diversities at phylum and genus levels were not different between asthma and COPD groups, as provided in the Online Repository (see Figures E1 and E2). Beta diversities were 0.25 for asthma patients and 0.20 for COPD patients at the phylum level. At the genus level, beta diversities were 0.52 for asthma patients and 0.49 for COPD patients. Phyla and genera with median relative abundance greater than 2% were

Actinobacteria, Bacteroidetes, Firmicutes, and *Proteobacteria* at phylum level, and *Actinomyces* and *Rothia* (both phylum *Actinobacteria*), *Lactobacillus* and *Streptococcus* (both phylum *Firmicutes*), and *Neisseria*, *Haemophilus*, *Moraxella*, and *Pseudomonas* (all phylum *Proteobacteria*) at the genus level. The relative abundance of the most abundant phyla and/or those known to be major airway pathogens across asthma and COPD, and the *Proteobacteria*:*Firmicutes* ratio (P:F ratio) for patients with asthma versus COPD are presented (see Figure E1). At the phylum level, the airway ecology was similar between asthma and COPD with only *Bacteroidetes* significantly elevated for asthma patients compared with COPD patients (see Figure E1). The relative abundances of the most abundant and clinically relevant genera (*Streptococcus*, *Haemophilus*, and *Moraxella*) were also not significantly different between asthma and COPD patients (see Figure E2).

Asthma and COPD biologic factors at exacerbation

Factor analysis with varimax rotation, was performed using the sputum mediators at exacerbation for all 32 asthma patients and 73 COPD patients, and four factors were retained. IL-13 was below limit of detection for most of the patients, and CCL-3 was missing for several patients. Therefore, those values were excluded from factor and cluster analyses. However, their patterns were assessed for the identified biologic subgroups. The rotated factor loadings are depicted in the Online Repository (see Table E2), indicating the relationship between the factors and mediators. Proinflammatory mediators appeared to load together in factor 1, T1 mediators in factor 2, and T2 mediators in factor 3.

Biologic exacerbation clusters

Three biologic clusters were identified using factor scores (derived from sputum mediators) as input into the k-means clustering algorithm. The patterns of the clinical parameters and sputum mediators

253 are presented across the identified clusters in Tables 1a and 1b respectively. The serum mediators
254 across the clusters are as shown in Table E3. The summary data (clinical parameters, sputum and
255 serum mediators) for asthma and COPD subjects within each cluster are presented in the supplement
256 materials (Tables E4, E5 and E6, respectively). There were no significant differences in age, sex,
257 smoking status, pack-year history, body mass index (BMI), frequency of exacerbations,
258 corticosteroid dosage, symptom scores, and lung function between the clusters. The clusters are
259 presented graphically across the first two discriminant scores in Figure 1. Microbiome data was
260 available from 19 patients (asthma=4; COPD=15) in Cluster 1, 11 patients (asthma=4; COPD=7) in
261 Cluster 2 and 24 patients (asthma=6; COPD=18) in Cluster 3. Alpha diversity, the proportions and
262 patterns of relative abundance of the most abundant phyla, and P:F ratios are presented for each
263 cluster in Figure 2. Similarly, the alpha diversity, as well as proportions and patterns of the relative
264 abundance of the most abundant genera, are presented (see Figure 3). The change in characteristics
265 between stable and exacerbation states within each cluster are reported (clinical parameters and
266 sputum mediators in Tables 2, and serum mediators in Table E7), and further breakdown of these
267 characteristics by disease within each cluster is reported in tables E8 and E9, respectively. At
268 exacerbation, both pre- and post-bronchodilator FEV_1 decreased while the VAS scores of cough and
269 dyspnea increased significantly for all three clusters, with no significant differences between
270 clusters.
271
272 **Cluster 1**
273 Cluster 1 was a COPD-predominant group of 34 patients (asthma=7; COPD=27). Of these, 59%
274 were men, the group's mean age was 67 years, and 71% were current or ex-smokers. Patients in this
275 cluster had elevated blood and sputum neutrophil counts and sputum proinflammatory mediators
276 (IL-1β, IL-6, IL-6R, IL-8, TNFα, TNF-R1, TNF-R2, and VEGF) and greater proportions of
277 bacterial-associated exacerbations compared with Clusters 2 and 3. In addition, this group had

greater *Proteobacteria* and P:F ratios (see Figure 2), with beta diversities of 0.16 and 0.46 at the phylum and genus levels, respectively. When compared with stable state, blood and sputum neutrophil counts, and sputum total cell count, concentrations of IL-1β, IL-6R, IL-8, IL-10, CCL5, TNFα, TNF-R1, TNF-R2, VEGF, and IFNγ were significantly increased. Sputum eosinophil and macrophage counts, IL-5, CXCL10, CXCL11, CCL2, CCL13, CCL17, and CCL26 were significantly lower (see Table 2). Serum IL-6, TNF-R1 and TNF-R2 increased and CCL2, 13, 17 and 26 decreased at exacerbation compared with stable visits (Table E7).

Cluster 2

Cluster 2 consisted of 27 patients (asthma=10; COPD=17). Some 63% were men with a mean age of 63 years, and 78% were current or ex-smokers. Patients in this cluster had elevated sputum and blood eosinophil counts, sputum (IL-5, IL-13, CCL13, CCL17, and CCL26) and serum (IL-5 and CCL26) T2 mediators (see Table 1 and Table E3). This cluster exhibited significantly greater alpha diversity of the microbiome and a greater proportion of *Bacteroidetes* at phylum level compared with Cluster 1 (see Figure 2), with beta diversities of 0.24 and 0.57 at the phylum and genus levels, respectively. In the paired comparison between stable and exacerbation states, sputum IL-5, IL-6R, CCL4, CCL17, and CCL26 were significantly increased at exacerbation whereas in contrast serum IL-8 and TNFα were decreased (see Table 2 and Table E7).

Cluster 3

Cluster 3 consisted of 44 patients (asthma=15; COPD=29). Some 68% were men with a mean age of 68 years, and 73% were current or ex-smokers. Patients in this cluster had elevated T1 mediators in the sputum CXCL10, CXCL11, and IFNγ (Table 1) and serum CXCL10 (Table E3). There were greater proportions of *Actinobacteria* and *Firmicutes* at phylum level, and *Streptococcus* (phylum *Firmicutes*) at genus level, with a lesser proportion of Proteobacteria and P:F ratio (see Figures 2

and 3). The beta diversities were 0.29 at phylum and 0.62 at genus levels. Blood eosinophil count and sputum CCL13 and CCL17 concentrations were lower and IL-6, IL-10, IL-13, CXCL10, CXCL11, CCL2, CCL5, TNFα, and IFNγ concentrations were greater at exacerbation compared with stable state (see Table 2). Serum CXCL10 increased at exacerbation versus stable and serum CCL13 and CCL17 decreased (Table E7).

The changes within each cluster for the highest loading factors sputum IL-1β, IL-5 and CXCL10 between stable and exacerbation states are as shown in Figure E3. Sputum IL-1β and IL-5 significantly increased in Clusters 1 and 2 respectively with concentrations for these mediators also increased in stable state. Sputum CXCL10 increased at exacerbation in cluster 3, but in contrast this mediator was not different between the groups in stable state. The changes between stable state and exacerbations for both asthma and COPD within each cluster is as shown (Tables E8 and 9).

DISCUSSION

In this study, three exacerbation biologic clusters were identified using a combination of factor and cluster analyses. Each cluster had different percentages of asthma and COPD patients. Interestingly, the clusters seemed to demonstrate three distinct and biologically plausible inflammatory profiles. Cluster 1 was a COPD-predominant group, with evidence of neutrophilic inflammation, elevated pro-inflammatory mediators, bacterial-associated exacerbations, and proportions of *Proteobacteria* and P:F ratio at the phylum level. Patients in Cluster 2 exhibited evidence of eosinophilic inflammation, with elevated T2 mediators and proportion of *Bacteroidetes*. Patients in Cluster 3 had greater T1 mediators and greater proportions of *Actinobacteria* and *Firmicutes* at the phylum level. Importantly, comparisons with assessments performed while the patients were stable demonstrated that the pro-inflammatory, T2 and T1 mediators were increased at exacerbation in Clusters 1, 2 and

3, respectively, whereas eosinophilic inflammation and T2 mediators were decreased in Clusters 1 and 3 at exacerbation. In Cluster 1 and 2 pro-inflammatory and T2 mediators were also increased in stable state respectively. Our findings therefore indicate that three exacerbation biologic clusters are shared between asthma and COPD. In addition, the inflammatory profiles in these clusters are increased compared with stable state, and are associated with distinct airway bacterial ecologies.

The strength of our study was our ability to use statistical techniques, applied previously to asthma and COPD independently[2,13], to characterize the biologic heterogeneity of asthma and COPD combined using sputum mediators assessed with the same protocols and the same analytical platform. Similarities and differences of asthma and COPD, identified by comparing their characteristics at the disease level, have been published[8]. This simple separation approach may not reflect the underlying biologic heterogeneity and does not provide insight into the multidimensional characteristics of the diseases. Cluster analysis has uncovered biological clusters within disease and can uncover the common and distinctive meaningful subgroups of both diseases that are not evident at disease level. We have extended the examination of such derived biologic clusters to include their associations with airway bacterial ecology to aid the understanding of the pathophysiologic connection with outcomes of airway diseases towards the realisation of potentially new biomarkers and targeted therapies.

For instance, Cluster 1, with its neutrophilic inflammation, elevated proinflammatory mediators, and bacterial-associated exacerbations, represents the group that would most likely respond to antibiotics. There is some role of antibiotics for the prevention of COPD exacerbations [15, 16]. Antibiotics for patients admitted to intensive care for COPD exacerbations also appeared to be beneficial[17, 18]. There is still continued uncertainty in asthma, with macrolide antibiotics providing some benefit in stable disease[19, 20] while in acute exacerbations some benefit was demonstrated

with telithromycin[21] but not azithromycin[22]. In the latter study, almost half of those screened had already received antibiotics in primary care and the study was underpowered. The lack of response observed might have been a consequence of failure to include those most likely to respond to antibiotics. With advances in culture independent techniques, we can now study the role of lung microbiome in respiratory diseases to a better extent. Of interest, Wang and colleagues have found lung microbiome dynamics were associated with COPD exacerbations[23]. Identification of biomarkers and a biologic cluster with evidence of microbial dysbiosis might therefore enable future targeted antibiotic trials. The ratio of the *proteobacteria:firmicutes* (P:F) ratio is also emerging as a possible simple measure that reflects the bacterial composition and whether this can be applied in future intervention studies to guide therapies needs to be tested.[24, 25].

Cluster 2 would seem likely to respond to therapies targeting eosinophilic and T2 inflammation. Targeting eosinophilic inflammation in both asthma and COPD with corticosteroids reduces exacerbation frequency[12, 26, 27]. Likewise monoclonal antibody therapies targeting IL-5 cytokine and its receptor are effective for decreasing the risk of exacerbation in eosinophilic asthma[28-35] with evidence of some benefit, albeit less consistent, in eosinophilic COPD[36, 37]. Findings from two pivotal Phase III studies evaluating the efficacy and safety of mepolizumab, an anti-IL-5 monoclonal antibody in addition to standard-of-care in COPD, demonstrated reductions in the frequency of moderate-to-severe exacerbations in those with higher blood eosinophil count[38]. Our study underscores the importance of an eosinophilic phenotype in asthma and COPD but future studies need to consider whether the underlying mechanisms are common in asthma and COPD. One limitation was that IgE was not assessed at exacerbation and the role of allergy could be explored further in future studies.

The interferon-inducible chemokines CXCL10 and 11 in sputum and serum were not only elevated

in Cluster 3, they were also significantly elevated at exacerbation compared with stable state. This biologic cluster with high interferon-inducible chemokines was not previously observed in stable asthma and COPD patients[7]. As these chemokines have been identified as biomarkers of viral-associated exacerbations[4, 39, 40], it would seem likely that viral infections are possible triggers and that this cluster would be most amenable to future anti-viral interventions. Consistently the proportion of viral-associated exacerbations were higher in cluster 3 but this did not reach statistical significance between clusters with asthma and COPD combined or independently. Viral identification is challenging especially as sputum viral load may peak before the peak of lower respiratory symptoms[41]. Therefore our findings likely under-represented the proportion of viral-associated exacerbations.

One major limitation of this study is that the number of patients with asthma was small and the percentage of both asthma and COPD patients who provided sufficient samples to study the microbial ecology was approximately half of the patients. The remaining patients were unable to produce sufficient sputum for analysis. The clinical characteristics between those who did versus those who did not provide samples for microbiological assessment were similar, suggesting these groups were comparable. We cannot exclude the possibility of an acquisition bias toward a microbial ecology associated with more sputum production. Notwithstanding this limitation, we did find consistent differences in the microbial ecology between the clusters. Secondly, we cannot demonstrate the stability of these exacerbation biologic clusters based on assessment of a single exacerbation. Therefore, these findings need to be explored in larger, multi-centre studies. Another limitation of the size of the study is that we could not analyse the patients according to the severities of their asthma or COPD, nor the severities of exacerbations. We did not control for baseline therapy between asthma and COPD patients however the corticosteroid dosages were very similar between the groups, which indicates this was unlikely to have had a differential impact on the underlying

inflammatory or microbial profile. A limitation of cluster analysis is that it is specific to the dataset studied and therefore the asthma and COPD groups nor the stable visits can be independently analysed to generate the proportions of the same clusters. This means cluster stability cannot be simply determined. However, we found that the patterns of the inflammatory profiles were similar for those subjects with asthma or COPD between clusters; the patterns were similar between stable and exacerbation state for the pro-inflammatory (Cluster 1) and eosinophilic (Cluster 2) phenotypes with amplification of an underlying inflammatory profile at the exacerbation event. Whereas the T1 mediators that were increased at exacerbation in Cluster 3 were not increased in stable state. Whether the stable state mediator profiles can identify subjects most likely to respond to specific anti-inflammatory or antibiotic therapy to reduce future exacerbation risk requires further study. We included a large number of cytokines and chemokines in the analysis but this still only reflects a minority of the number of mediators present in the sputum and serum samples. Due to limitations of available sample we could not extend the study to include other important mediators such as eicosanoids and beta-interferons. Metabolomic approaches such as Somologics might provide more insights into the inflammatory mediator network and should be considered in future studies.

In conclusion, we used a biologic clustering approach to look at asthma and COPD exacerbations. We identified a COPD-predominant cluster with evidence of neutrophilic inflammation, elevated proinflammatory mediators, and bacterial-associated exacerbations, a cluster with evidence of eosinophilic inflammation and elevated T2 mediators and a cluster with elevated T1 mediators. Our study aids in the understanding of the heterogeneity of asthma and COPD exacerbations and suggests that endotype may be more important than an asthma or COPD diagnosis. It highlights the need for further research in developing novel biomarkers to predict disease outcome and guide targeted therapies.

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Authors' contribution

MAG, PHP and SD undertook the data analysis and statistical analysis. DD and MBaf undertook patient recruitment, data collection, and were involved in data analysis. KH was involved in microbiologic assessment. SC, PN, PR, LR, and JW were involved in sputum mediator assessment and analysis. IDP was co-supervisor for the COPD patients. TK and SLJ performed the viral analyses. MBar was involved in microbiologic assessment. RDM and CEB led the design of the study, data collection, data interpretation, data analysis and had full access to the data and are responsible for the integrity of the data and final decision to submit. All authors contributed to the study design, writing of the manuscript and have approved the final version for submission.

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Table 1a. Summary statistics across the three identified exacerbation biologic clusters

	Cluster 1	Cluster 2	Cluster 3	P-value			
	Asthma=7; COPD=27	**Asthma=10; COPD=17**	**Asthma=15; COPD=29**	**C1 vs. C2**	**C1 vs. C3**	**C2 vs. C3**	**ANOVA**
Male [n (%)]	20 (58.8)	17 (63.0)	30 (68.2)	0.74	0.39	0.65	0.69
Current or ex-smokers [n (%)]	24 (70.6)	21 (77.8)	32 (72.7)	0.53	0.84	0.64	0.81
Pack-year history[a $]	45.5 (22.0 to 51.0)	37.0 (25.8 to 48.0)	37.5 (22.9 to 62.7)	0.84	0.80	0.66	0.90
Age (years)[±]	67.2 (1.7)	62.9 (2.2)	68.0 (5.2)	0.49	0.90	0.39	0.67
BMI (kg/m^2)[±]	26.9 (1.0)	27.9 (1.2)	27.9 (0.9)	0.53	0.47	0.99	0.74
Exacerbations in last year[a]	3.0 (1 to 6)	4.5 (2 to 6)	3.0 (2 to 5)	0.35	0.42	0.65	0.58
Maintenance prednisolone [n (%)]	10 (29.4)	7 (25.9)	11 (25.0)	0.76	0.66	0.93	0.90
Daily prednisolone dose (mg)[a §]	5 (5 to 15)	10 (7.5 to 10)	10 (7.5 to 15)	0.44	0.26	0.47	0.44
Daily ICS dose (µg/d)[a ¤]	2000 (800 to 2000)	1600 (800 to 2000)	1300 (800 to 2000)	0.74	0.47	0.61	0.73
Pre FEV_1 (L)[±]	1.21 (0.10)	1.35 (0.20)	1.37 (0.11)	0.49	0.29	0.91	0.62
Post FEV_1 (L)[±]	1.30 (0.09)	1.43 (0.24)	1.33 (0.11)	0.52	0.82	0.62	0.81
Pre FEV_1 predicted (%)[±]	49.98 (4.12)	45.97 (5.05)	44.96 (4.59)	0.57	0.42	0.88	0.70
Post FEV_1 predicted (%)[±]	53.54 (4.01)	48.12 (6.06)	44.02 (4.81)	0.48	0.14	0.58	0.33
Pre FEV_1/FVC ratio (%)[±]	56.06 (2.88)	54.91 (2.99)	55.72 (2.25)	0.78	0.93	0.84	0.96
VAS-cough (mm)[±]	69.36 (2.88)	61.23 (4.51)	64.27 (3.36)	0.14	0.27	0.56	0.31
VAS-dyspnea (mm)[±]	68.76 (3.77)	69.31 (4.67)	66.73 (2.98)	0.92	0.67	0.63	0.86
Blood neutrophil x10^9/L[±]	7.98 (0.58)	5.91 (0.49)	6.54 (0.43)	**0.008**	**0.044**	0.39	**0.02**
Blood eosinophil x10^9/L	0.13 (0.10 to 0.17)	0.30 (0.20 to 0.45)	0.12 (0.09 to 0.16)	**0.002**	0.59	**<0.0001**	**0.0004**
TCC (x 10^6 cells/g sputum)	16.48 (11.19 to 24.29)	3.00 (1.92 to 4.69)	3.06 (1.95 to 4.81)	**<0.0001**	**<0.0001**	0.94	**<0.0001**
Sputum neutrophil count (%)[±]	89.17 (2.71)	58.44 (4.22)	64.78 (3.59)	**<0.0001**	**<0.0001**	0.21	**<0.0001**
Sputum eosinophil count (%)	0.34 (0.27 to 0.43)	6.35 (3.13 to 12.88)	0.83 (0.49 to 1.39)	**<0.0001**	**0.004**	**<0.0001**	**<0.0001**
Bacterial load (Log 10 CFU/ml)	7.0 (1.0)	5.7 (0.9)	6.3 (1.0)	**0.0007**	**0.047**	**0.147**	**0.001**
Bacterial-associated exacerbation (%)	78.8	23.1	21.1	**<0.0001**	**<0.0001**	0.85	**<0.0001**
Viral (%)	29.0	30.0	47.1	0.94	0.14	0.22	0.25

Geometric mean with 95% confidence interval unless otherwise stated; [a]median (first and third quartiles); [±]mean (standard error of mean); [$]Pack-year history of current and ex-smokers; [§]Dose for only those patients prescribed daily prednisolone; [¤]Beclomethasone dipropionate equivalent. Abbreviations: BMI=Body Mass Index; ICS=inhaled corticosteroid; FEV_1=Forced Expiratory Volume in the First Second; FVC=Forced Vital Capacity; VAS=Visual Analogue Scale; TCC=Total sputum cell count; C=cluster.

Table 1b. Summary statistics across the three identified exacerbation biologic clusters

	Cluster 1	Cluster 2	Cluster 3	P-value			
	Asthma=7; COPD=27	**Asthma=10; COPD=17**	**Asthma=15; COPD=29**	**C1 vs. C2**	**C1 vs. C3**	**C2 vs. C3**	**ANOVA**
IL-1β (pg/ml)	2167.3 (1441.1 to 3259.2)	42.6 (22.4 to 81.2)	72.1 (42.1 to 123.5)	**<0.0001**	**<0.0001**	0.17	**<0.0001**
IL-5 (pg/ml)	0.6 (0.4 to 0.9)	8.4 (5.7 to 12.4)	1.2 (0.8 to 1.9)	**<0.0001**	**0.03**	**<0.0001**	**<0.0001**
IL-6 (pg/ml)	620.2 (374.8 to 1026.3)	171.5 (96 to 306.3)	374 (201.1 to 695.7)	**0.005**	0.22	0.068	**0.018**
IL-6R (pg/ml)	828.0 (596.4 to 1149.5)	213.8 (154.5 to 295.8)	169.9 (119.1 to 242.3)	**<0.0001**	**<0.0001**	0.36	**<0.0001**
IL-8 (pg/ml)	13195.3 (10356.0 to 16813.1)	3192.5 (2252.4 to 4524.9)	2967.8 (2010.5 to 4380.8)	**<0.0001**	**<0.0001**	0.77	**<0.0001**
IL-10 (pg/ml)	17.1 (10.1 to 29.0)	1.9 (1.6 to 2.2)	7.7 (4.2 to 14.2)	**<0.0001**	0.057	**<0.0001**	**<0.0001**
IL-13 (pg/ml)	8.7 (7.7 to 9.7)	12.8 (9.6 to 17.1)	9.8 (8.5 to 11.4)	**0.005**	0.20	**0.043**	**0.017**
CXCL10 (pg/ml)	163.0 (94.6 to 280.7)	406.3 (253.0 to 652.4)	1542.4 (820.1 to 2901.1)	**0.042**	**<0.0001**	**0.002**	**<0.0001**
CXCL11 (pg/ml)	4.2 (2.5 to 7.0)	24.8 (13.1 to 47.0)	149.6 (58.5 to 382.6)	**0.004**	**<0.0001**	**0.002**	**<0.0001**
CCL2 (pg/ml)	312.3 (221.0 to 441.4)	383.4 (262.0 to 561.1)	695.5 (454.3 to 1064.8)	0.50	**0.006**	**0.041**	**0.009**
CCL3 (pg/ml)	90.0 (55.1 to 147.1)	49.1 (29.4 to 81.9)	61.6 (36.6 to 103.8)	0.12	0.30	0.54	0.28
CCL4 (pg/ml)	1087.3 (656.7 to 1800.2)	1366.7 (910.5 to 2051.5)	898.9 (542.2 to 1490.5)	0.54	0.60	0.24	0.50
CCL5 (pg/ml)	14.2 (9.5 to 21.0)	4.6 (2.9 to 7.3)	10.8 (6.5 to 17.7)	**0.002**	0.41	**0.013**	**0.006**
CCL13 (pg/ml)	10.8 (8.9 to 13.1)	30.4 (22.3 to 41.4)	17.6 (12.9 to 24)	**<0.0001**	**0.015**	**0.008**	**<0.0001**
CCL17 (pg/ml)	4.8 (3.0 to 7.6)	71.0 (47.1 to 107.0)	10.7 (7.8 to 14.5)	**<0.0001**	**0.003**	**<0.0001**	**<0.0001**
CCL26 (pg/ml)	2.3 (1.8 to 3.0)	26.1 (18.3 to 37.2)	4.0 (2.8 to 5.7)	**<0.0001**	**0.02**	**<0.0001**	**<0.0001**
TNFα (pg/ml)	133.4 (84.3 to 211.2)	2.5 (1.5 to 4.4)	12.7 (6.2 to 26.1)	**<0.0001**	**<0.0001**	**0.001**	**<0.0001**
TNF-R1 (pg/ml)	5598.7 (4499.7 to 6966.1)	770.5 (566.6 to 1047.8)	760.7 (557.9 to 1037.2)	**<0.0001**	**<0.0001**	0.95	**<0.0001**
TNF-R2 (pg/ml)	1950.9 (1439.8 to 2643.4)	345.2 (246.3 to 483.9)	418.6 (258.7 to 677.3)	**<0.0001**	**<0.0001**	0.52	**<0.0001**
VEGF (pg/ml)	2428.1 (1947.3 to 3027.6)	1177.2 (946.9 to 1463.7)	1071.2 (909.7 to 1261.4)	**<0.0001**	**<0.0001**	0.50	**<0.0001**
IFNγ (pg/ml)	1.0 (0.6 to 1.8)	0.3 (0.3 to 0.4)	3.3 (1.4 to 7.7)	**0.034**	**0.03**	**<0.0001**	**<0.0001**

Data presented as geometric mean with 95% confidence interval. Abbreviation: C = Cluster.

Table 2. Change of clinical characteristics and mediators between stable and exacerbation states within each identified exacerbation biologic cluster

	Cluster 1 (n=34)		Cluster 2 (n=27)		Cluster 3 (n=44)	
		P-value		P-value		P-value
Pre-FEV_1 (L)±	-0.14 (0.04)	**0.004**↓	-0.19 (0.06)	**0.006**↓	-0.12 (0.04)	**0.003**↓
Post-FEV_1 (L) ±	-0.14 (0.05)	**0.004**↓	-0.10 (0.04)	**0.038**↓	-0.24 (0.04)	**<0.0001**↓
Pre-FEV_1 predicted (%)±	-6.22 (1.81)	**0.002**↓	-7.80 (2.41)	**0.004**↓	-4.37 (1.39)	**0.003**↓
Post-FEV_1 predicted (%)±	-6.15 (1.84)	**0.002**↓	-3.49 (1.45)	**0.028**↓	-8.24 (1.36)	**<0.0001**↓
Pre-FEV_1/FVC ratio (%)±	1.22 (1.86)	0.52	3.59 (1.47)	**0.024**↑	1.80 (1.83)	0.33
VAS score-cough (mm)±	17.38 (5.00)	**0.002**↑	33.92 (5.97)	**<0.0001**↑	27.19 (4.14)	**<0.0001**↑
VAS score-dyspnea (mm)±	14.52 (5.35)	**0.011**↑	37.15 (6.12)	**<0.0001**↑	23.65 (3.63)	**<0.0001**↑
Blood neutrophil $x10^9/L$±	2.03 (0.52)	**0.0004**↑	0.77 (0.40)	0.062	0.60 (0.37)	0.11
Blood eosinophil $x10^9/L$	0.69 (0.47 to 1.02)	0.06	1.10 (0.77 to 1.58)	0.58	0.60 (0.42 to 0.88)	**0.009**↓
TCC (x 10^6 cells/g sputum)	3.49 (2.10 to 5.79)	**<0.0001**↑	1.27 (0.74 to 2.16)	0.37	1.67 (0.94 to 2.99)	0.08
Sputum neutrophil count (%)±	15.22 (5.29)	**0.008**↑	-2.77 (4.63)	0.56	-3.18 (3.92)	0.42
Sputum eosinophil count (%)	0.51 (0.33 to 0.79)	**0.004**↓	2.14 (0.93 to 4.94)	0.074	0.68 (0.40 to 1.16)	0.15
IL-1β (pg/ml)	10.4 (5.2 to 20.9)	**<0.0001**↑	1.42 (0.59 to 3.38)	0.42	1.20 (0.72 to 2.01)	0.47
IL-5 (pg/ml)	0.40 (0.23 to 0.69)	**0.002**↓	2.41 (1.33 to 4.37)	**0.006**↑	0.92 (0.56 to 1.52)	0.74
IL-6 (pg/ml)	1.48 (0.90 to 2.42)	0.12	1.06 (0.59 to 1.90)	0.83	2.16 (1.11 to 4.19)	**0.025**↑
IL-6R (pg/ml)	2.98 (1.90 to 4.68)	**<0.0001**↑	1.60 (1.09 to 2.34)	**0.02**↑	1.06 (0.72 to 1.56)	0.77
IL-8 (pg/ml)	1.67 (1.19 to 2.32)	**0.004**↑	1.34 (0.80 to 2.26)	0.25	0.82 (0.55 to 1.23)	0.33
IL-10 (pg/ml)	3.34 (1.96 to 5.70)	**0.0001**↑	0.86 (0.59 to 1.26)	0.43	4.09 (2.23 to 7.49)	**<0.0001**↑
IL-13 (pg/ml)	0.82 (0.64 to 1.07)	0.14	1.05 (0.76 to 1.44)	0.76	1.23 (1.06 to 1.42)	**0.008**↑
CXCL10 (pg/ml)	0.45 (0.24 to 0.87)	**0.019**↓	1.00 (0.53 to 1.90)	0.99	3.78 (2.01 to 7.10)	**0.0001**↑
CXCL11 (pg/ml)	0.23 (0.10 to 0.51)	**0.0008**↓	0.89 (0.39 to 2.06)	0.78	7.60 (2.68 to 21.56)	**0.0003**↑
CCL2 (pg/ml)	0.46 (0.28 to 0.77)	**0.004**↓	0.87 (0.58 to 1.32)	0.49	1.71 (1.07 to 2.74)	**0.025**↑
CCL3 (pg/ml)	1.07 (0.65 to 1.77)	0.78	0.89 (0.42 to 1.92)	0.77	1.52 (0.88 to 2.62)	0.13
CCL4 (pg/ml)	1.06 (0.69 to 1.64)	0.77	1.84 (1.04 to 3.26)	**0.037**↑	1.56 (0.89 to 2.73)	0.12
CCL5 (pg/ml)	1.96 (1.30 to 2.94)	**0.002**↑	1.15 (0.67 to 1.99)	0.59	3.01 (1.88 to 4.82)	**<0.0001**↑
CCL13 (pg/ml)	0.36 (0.26 to 0.51)	**<0.0001**↓	0.85 (0.57 to 1.26)	0.40	0.65 (0.48 to 0.88)	**0.007**↓
CCL17 (pg/ml)	0.23 (0.13 to 0.41)	**<0.0001**↓	1.80 (1.08 to 3.01)	**0.026**↑	0.58 (0.40 to 0.85)	**0.007**↓
CCL26 (pg/ml)	0.54 (0.34 to 0.85)	**0.01**↓	2.48 (1.57 to 3.92)	**0.0004**↑	1.01 (0.68 to 1.52)	0.95
TNFα (pg/ml)	7.92 (4.01 to 15.62)	**<0.0001**↑	1.22 (0.56 to 2.66)	0.60	3.92 (1.82 to 8.48)	**0.0009**↑
TNF-R1 (pg/ml)	2.95 (2.02 to 4.30)	**<0.0001**↑	1.26 (0.85 to 1.87)	0.24	0.99 (0.70 to 1.39)	0.93
TNF-R2 (pg/ml)	2.93 (1.94 to 4.41)	**<0.0001**↑	1.61 (1.00 to 2.59)	0.05	1.54 (0.89 to 2.67)	0.12
VEGF (pg/ml)	1.44 (1.10 to 1.88)	**0.01**↑	1.03 (0.76 to 1.40)	0.85	0.84 (0.67 to 1.04)	0.10
IFN_Υ (pg/ml)	1.76 (1.02 to 3.03)	**0.042**↑	0.70 (0.39 to 1.25)	0.22	9.33 (3.85 to 22.60)	**<0.0001**↑

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570 Data presented as geometric mean with 95% confidence interval unless otherwise stated; Fold changes in mediators are shown; ±Mean (standard error of

571 mean). Abbreviations: FEV_1=Forced Expiratory Volume in the First Second; FVC=Forced Vital Capacity; VAS= Visual Analog Scale; TCC=Total

572 sputum cell count.

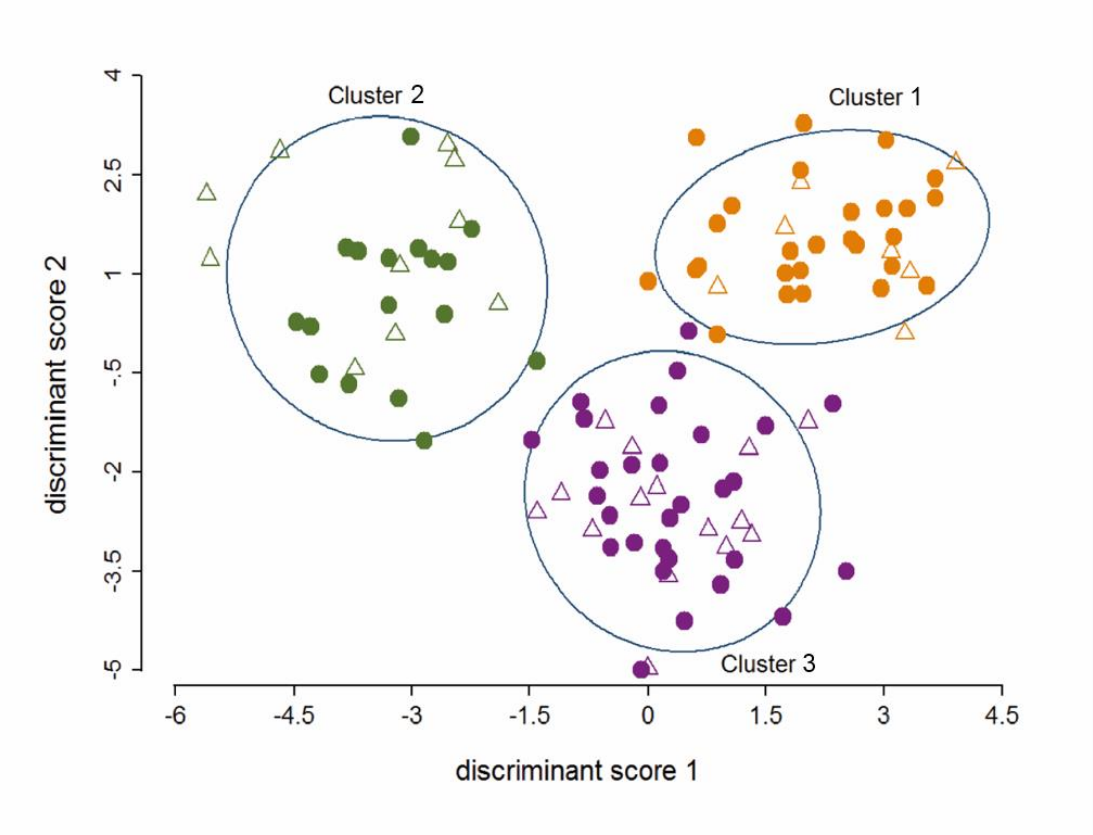

Figure 1. The three identified exacerbation biologic clusters presented using the subjects' discriminant scores. Hollow triangles indicate asthma patients and bold circles indicate COPD patients. Orange, green and purple colours represent clusters 1, 2 and 3 respectively.

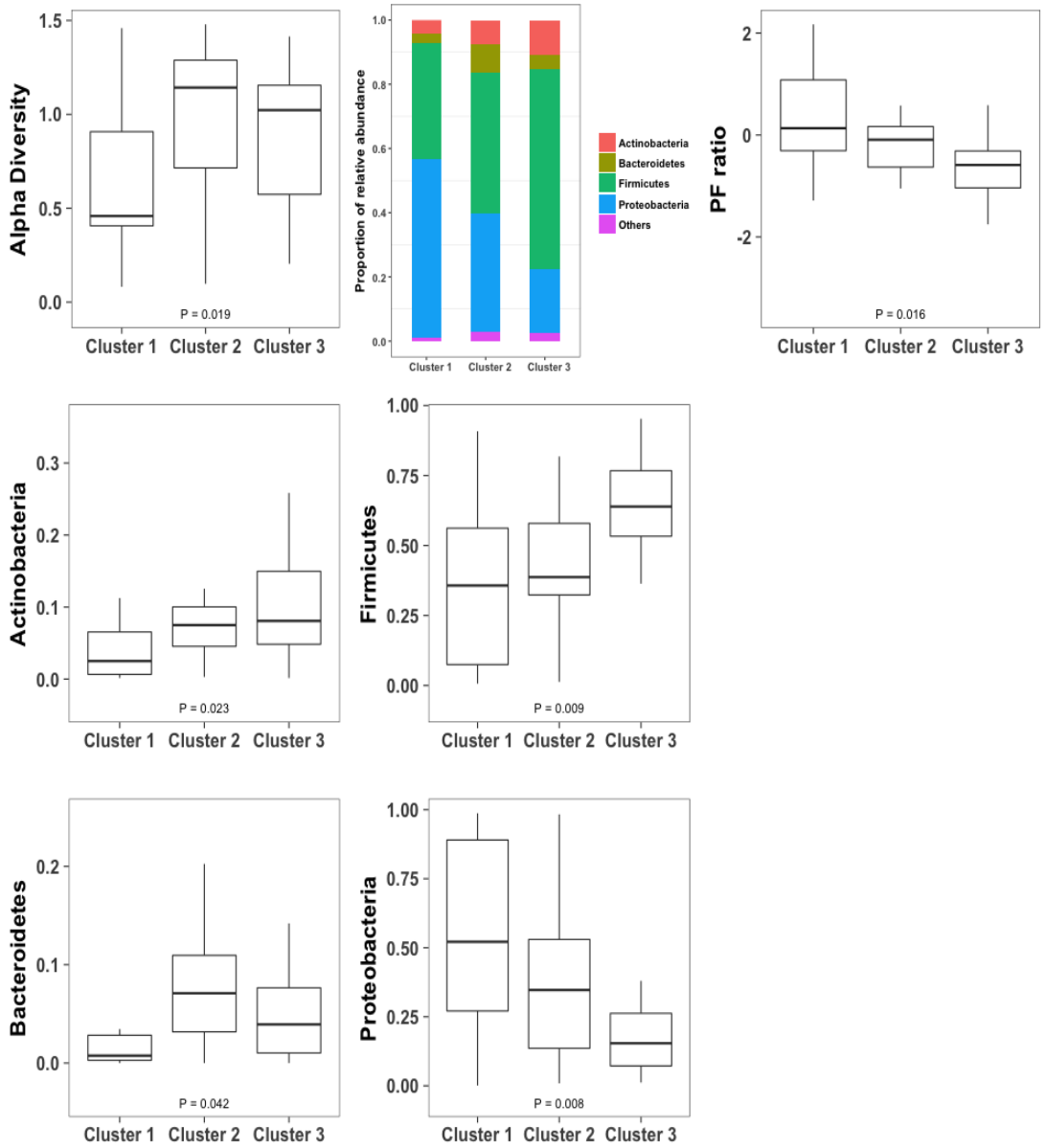

Figure 2. Alpha diversity at phylum level (using Shannon-Weiner index); proportion and patterns of relative abundance of the most abundant phyla, and *Proteobacteria* to *Firmicutes* (P:F) ratio in log format (base 10) across the identified exacerbation biologic clusters.

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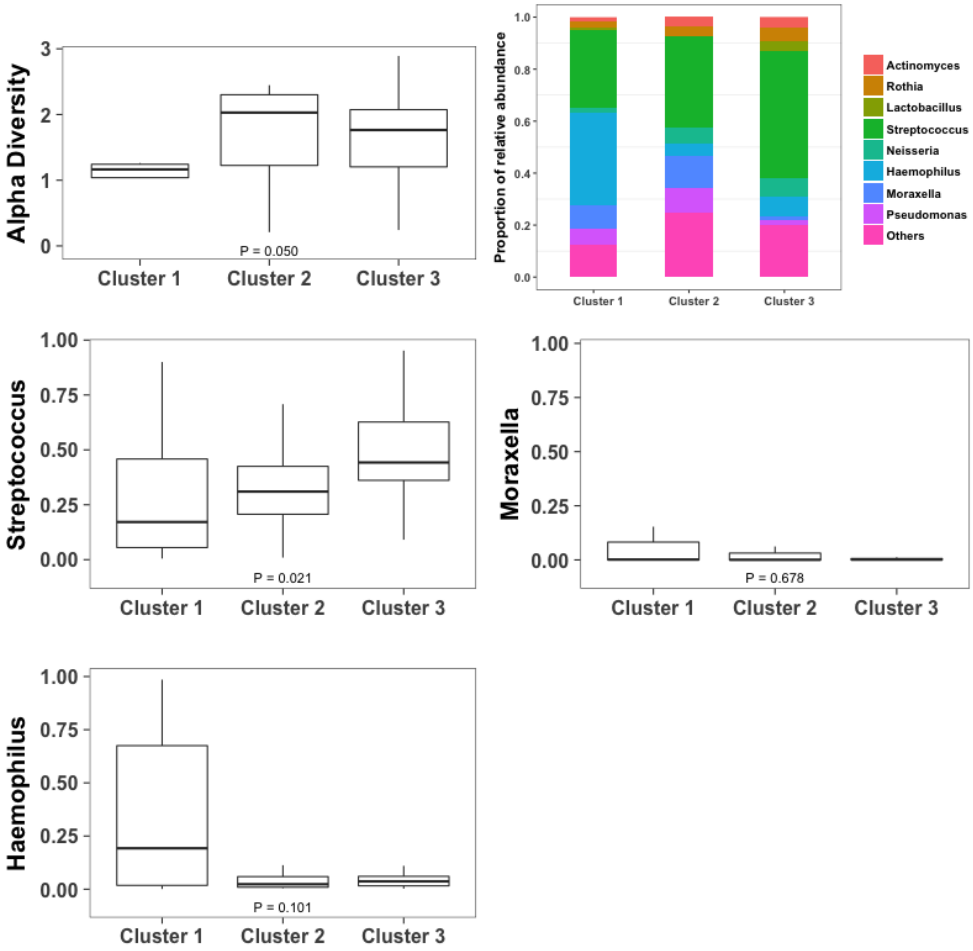

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586

587 **Figure 3.** Alpha diversity at genus level (using Shannon-Weiner index); proportion
588 and patterns of relative abundance of the most abundant genera or those known to be
589 important airway pathogens across the identified exacerbation biologic clusters.

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