Identification of clinically relevant subgroups of COPD based on airway and circulating autoantibody profiles

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Abstract. Autoimmunity may serve a role in the pathological features of a subgroup of patients with chronic obstructive pulmonary disease (COPD); however, in immunological subgroups of COPD patients, the interrelationships between airway and circulating autoantibody responses, and clinical parameters, remain unclear. The present study was undertaken to evaluate these interrelationships in various immunological subgroups of COPD patients. Sputum supernatant and serum obtained from 102 patients with stable COPD were assayed for the presence of immunoglobulin G antibodies against ten autoantigens via Luminex multiplex technology. Hierarchical clustering based on principal components was performed on autoantibody profiles to classify patients into clusters. Network-based and module analyses were conducted to explore interrelationships among autoantibodies and clinical variables in each cluster. Topological characteristics were compared between clusters. Unsupervised clustering identified four clusters: No significant differences in the majority of clinical characteristics were observed among clusters. In cluster 1, retrospective exacerbation was only positively associated with COPD assessment test score. Lung functions (predicted % of forced expiratory volume in 1 sec and maximal mid-expiratory flow) were negatively associated with exacerbation risk only in cluster 2. Sputum autoantibodies (against U1 small nuclear ribonucleoprotein, proteinase-3 and Ro/Sjögren syndrome type A antigen) were negatively associated with exacerbation risks in cluster 2, but positively associated in cluster 3. The four networks also exhibited distinct topological properties. In COPD, autoantibody responses were heterogeneous and differentially associated with exacerbation risk in certain subgroups; their dual character should be considered in future research.

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Introduction

Chronic obstructive pulmonary disease (COPD) is an important and growing cause of morbidity and mortality, and is predicted to be the third leading cause of mortality globally by 2020 (1). Patients with COPD can suffer from episodes of symptom exacerbations during the course of the disease that negatively affect their prognosis; however, COPD and its exacerbations are both heterogeneous conditions that are linked to complex and heterogeneous immune responses (2,3).

Autoimmunity has been suggested to be an influential factor in the progression of patients who have suffered from COPD for >10 years (4,5), as COPD shares numerous pathophysiological and clinical characteristics with autoimmune diseases (4). Increasing evidence indicates that autoimmune responses serve a role in the development and progression of COPD (6-10). Autoantibodies in stable COPD have been comprehensively reviewed recently (11); however, the

heterogeneity of autoimmunity should also be considered. Kim *et al* (12) reported abnormal blood T-lymphocyte subsets in a subgroup of patients with COPD. Our recent study demonstrated that sputum autoantibody levels were associated with exacerbation risk in a subgroup of COPD patients (13), suggesting that autoimmunity is highly heterogeneous in COPD.

Network-based analysis is a novel integrative research approach that is suitable for the study of complex and heterogeneous conditions, such as COPD and its exacerbations (14-17). Divo *et al* (18) used network analysis to investigate the association between multiple comorbidities in patients with stable COPD. The authors included 79 comorbidities and various demographic, clinical and functional parameters in the network analysis, and observed that the comorbidities were significantly interlinked and formed a complex network in which six sub-networks (also termed modules) were identified. Grosdidier *et al* (19) used an integrative network-based approach to investigate the biological associations between COPD, its comorbidities and the chemical products contained in tobacco smoke. They revealed that comorbidities shared genes, proteins and biological pathways with COPD. Faner *et al* (20) explored the association between comorbidities and patients with exacerbated COPD from a molecular viewpoint (also termed a molecular diseasome) using network analysis. Noell *et al* (21) explored the pathobiological mechanisms of exacerbations and biomarkers by comparing multi-level (clinical, physiological, biological, imaging and microbiological) correlation networks determined during exacerbation and convalescence in patients with COPD; however, no known study has investigated the interrelationships between airway and circulating autoantibody responses, and clinical parameters in immunological subgroups of patients with COPD. It was hypothesised that network analysis, an analytical approach that involves the comparison of clinical, functional, biological and immunological correlation networks, may provide a novel insight into the complex association between autoantibody profiles and COPD clinical parameters. To properly adjust for the redundancy of autoantibody profiles and the heterogeneity of autoantibody responses, principal component analysis (PCA) and hierarchical clustering were performed prior to network analysis. Thus, in the present proof-of-concept study, network analysis based on unsupervised classification was used to: i) Compare the network structures of different COPD subgroups identified by sputum and serum autoantibody profiles; and ii) identify a series of exacerbation risk-associated factors.

Materials and methods

Patients. This was a prospective cross-sectional study. A total of 102 patients with COPD with stable disease were enrolled at the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China) between March 2017 and October 2017. A group of 18 non-smoking healthy controls was also enrolled for comparison. Inclusion criteria for patients with COPD were: i) Aged >40 years; and ii) confirmed diagnosis of COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (22) [post-bronchodilator forced expiratory volume in 1 sec (FEV1)/forced vital capacity (FVC) ratio <0.7]. Exclusion criteria were: i) Diagnosis of known respiratory disorders other than COPD; ii) history of significant inflammatory disease other than COPD; iii) COPD exacerbation within 4 weeks of enrolment; iv) history of lung surgery and tuberculosis; v) diagnosis of cancer; vi) having undergone a blood transfusion within 4 weeks of enrolment; vii) diagnosis of autoimmune diseases; and viii) enrolment in a blinded drug trial. The clinicopathological data of the patients and healthy controls are presented in Table I.

Inclusion criteria for non-smoking healthy controls were: i) Aged >40 years; and ii) without any known respiratory disorders and significant inflammatory diseases. Subjects with one or more of the following criteria were excluded: i) Diagnosis of known respiratory diseases; ii) history of significant inflammatory disease; iii) diagnosis of cancer; iv) blood transfusion within 4 weeks of enrolment; v) inability to walk; or vi) current participation in an intervention trial.

Written informed consent was obtained from all patients. The study was approved by the ethics committee of the First Affiliated Hospital of Guangzhou Medical University (permit no. 2017-22) and was registered with www.clinicaltrials.gov (NCT 03240315).

Clinical and functional parameters. Data collected at enrolment included demographic characteristics, lung function, COPD assessment test (CAT), and modified Medical Research Council Dyspnea Scale (mMRC) of subjects prior to sputum induction. Spirometry was performed according to the American Thoracic Society guidelines (23).

Blood samples, sputum collection and processing. Peripheral venous blood samples (4 ml per subject) were collected into a vacuum tube, and serum was obtained by centrifuging whole blood at 1,057 x g (3,000 rpm) for 10 min at room temperature. Sputum induction was performed according to guidelines suggested by the Task Force of the European Respiratory Society (24). A two-step procedure was conducted to process the sputum as previously described (25). Sputum supernatant and serum were stored at -80˚C.

Autoantibody detection. Based on a literature search, ten autoantigens with known or putative links to COPD were selected (26-29), including Smith antigen (Sm), ribosomal phosphoprotein P0 (P0), Ro/Sjögren syndrome type A antigen (SS-A), La/Sjögren syndrome type B antigen (SS-B), DNA topoisomerase I (Scl70), histidyl-tRNA synthetase (Jo1), U1 small nuclear ribonucleoprotein (U1-SnRNP), thyroid peroxidase (TPO), proteinase-3 (PR3) and myeloperoxidase (MPO). Autoantigens (DIARECT AG) were coupled with multiplex magnetic beads (Bio-Rad Laboratories, Inc.) and incubated with sputum supernatant and serum samples diluted 1:10 and 1:180, respectively, at 37˚C for 1 h. The beads were washed using the Bio-Plex Pro™ wash station (Bio-Rad Laboratories, Inc.), and then incubated at 37˚C for 1 h with biotin-conjugated anti-human IgG (1:1,000; cat. no. A24474; Thermo Fisher Scientific, Inc.). Subsequently, they were washed and then reacted for 15 min at 37˚C with streptavidin-R-phycoerythrin (Bio-Rad Laboratories, Inc.). After the microspheres were washed and resuspended, the median fluorescence intensity of each encoded microsphere was measured using Bio-Plex 200 with an excitation wavelength at 532 nm and emission

Table I. Subject demographics and clinical characteristics.

Characteristic	Non-smoking healthy controls, n=18	COPD patients, n=102
Age, years	58.33±7.67	66.46±8.10
Sex (M/F)	10/8	98/4
BMI, kg/m^2	25.26±3.65	21.86±4.11
Smoking, n (never/ex/current)	18/0/0	10/73/19
Pre-BD FEV1, litres	2.51±0.79	1.27±0.57
Pre-BD FEV1pred%	96.94±16.79	49.02±21.40
Pre-BD FVC, litres	3.15±0.98	2.57±0.73
Pre-BD FEV1/FVC	0.80±0.06	0.49±0.13
Post-BD FEV1, litres	ND	1.40±0.59
Post-BD FEV1pred%	ND	53.34±22.41
Post-BD FVC, litres	ND	2.74±0.73
Post-BD FEV1/FVC	ND	0.51±0.14
CAT score	NA	11.67±6.44
mMRC	NA	2 (1-2)
Respiratory medications		
ICS	NA	66 (64.7%)
LABA	NA	66 (64.7%)
LAMA	NA	41 (40.2%)

Data are presented as n (%), mean ± standard deviation or median (interquartile range) unless otherwise stated. COPD, chronic obstructive pulmonary disease; pre-BD, pre-bronchodilator; post-BD, post-bronchodilator; BMI, body mass index; FEV1, forced expiratory volume in 1 sec; FVC, forced vital capacity; FEV1pred%, forced expiratory volume in 1 sec as percentage of predicted; FVCpred%, forced vital capacity as percentage of predicted; CAT, COPD assessment test; mMRC, Modified Medical Research Council Dyspnea Scale; ICS, inhaled corticosteroids; LABA, long-acting β agonist; LAMA, long-acting muscarinic antagonist; ND, no data; NA, not applicable.

wavelength at 575 nm (Bio-Rad Laboratories, Inc.). Bio-Plex Manager™ 6.0 software (Bio-Rad Laboratories, Inc.) was used to generate the result files.

Statistical analysis. All statistical analyses were performed using SPSS software (version 19.0; IBM Corp.). PCA was performed on autoantibody profiles in sputum and serum, and components with eigenvalues >1 were extracted. Unsupervised agglomerative hierarchical clustering was performed on the above components, using the un-centred correlation as the similarity metric (Cluster version 3.0) (30). The dendrogram and resulting heatmap were visualised using TreeView (version 1.60) (31). Shapiro-Wilk test was performed to access the normality of distribution of each continuous variable, and depending on the distribution of the data, ANOVA or Kruskal-Wallis test were used to compare the clusters. Then, Fisher's Least Significant Difference (LSD) test or the Nemenyi test was performed to analyse the differences between clusters. Correlation networks integrating 45 clinical and molecular parameters were then established using Gephi software (version 0.9.1) (32). Networks integrating clinical and autoantibody parameters in each group were constructed using Spearman's correlation test. Correlation coefficients with P>0.05 were excluded. Network clustering was conducted using the 'fast unfolding' algorithm within the Gephi software.

Results

Patient information. The clinical characteristics of 102 patients with COPD and 18 non-smoking healthy controls are presented in Table I. The mean ages of the patients and controls were 66.46±8.10 and 58.33±7.67 years, respectively.

Hierarchical clustering based on PCA. Sputum and serum autoantibody profile data were processed with PCA: The eight largest principal components extracted were able to account for 69.54% of the variability contained in the original data (Fig. S1; Table SI), suggesting that these eight components alone contributed to the majority of the information among the groups. Components and coefficient sets used in the analysis are presented in Table SII. Using hierarchical cluster analysis, four clusters of patients with COPD were identified based on the above components (Fig. 1).

Clinical characteristics of the four clusters. To determine whether the patients within these clusters represented clinically distinct subgroups of COPD, the clinical parameters of the four clusters were analysed (Table II). The average CAT score and mMRC of individuals in Cluster 2 were significantly increased compared with those in Cluster 4. Conversely, there were no significant differences in various other clinical characteristics [age, number of exacerbations in the previous year (AE), FEV1, FEV1 as a percentage of the predicted value (FEV1pred%), maximal mid-expiratory flow (MMEF), and body mass index (BMI)] among clusters. Autoantibody levels of the four subtypes were also analysed (Figs. S2 and S3; Table SIII).

Differential network analysis. Fig. 2 presents the Spearman correlation networks integrating clinical and autoantibody parameters in healthy controls (Fig. 2A) and the aforementioned four clusters (Fig. 2B-E). Table III presents the comparisons of the topological properties of the five groups. Notable observations included: i) The five networks exhibited different topological properties (the cluster 2 network displayed high density, whereas the cluster 1 network displayed low density); ii) there were seven modules in cluster 1, five modules in clusters 2 and 3, and six modules in cluster 4, but all modules appeared markedly heterogeneous in their clinical and biological content, as the majority contained nodes of distinct functional and immunological categories (Fig. 2); and iii) the retrospective exacerbation-associated factors (AE-nodes in Fig. 2) were significantly different among the four clusters. In cluster 1 (Fig. 2B), the AE was only positively associated with the CAT score. In cluster 2 (Fig. 2C), the AE was negatively associated with age, lung function (FEV1pred% and MMEF), sputum autoantibodies (P0, Scl70, Sm, U1-SnRNP, PR3 and Ro/SSA) and serum globulin (Glb), and positively associated with blood cell counts (peripheral

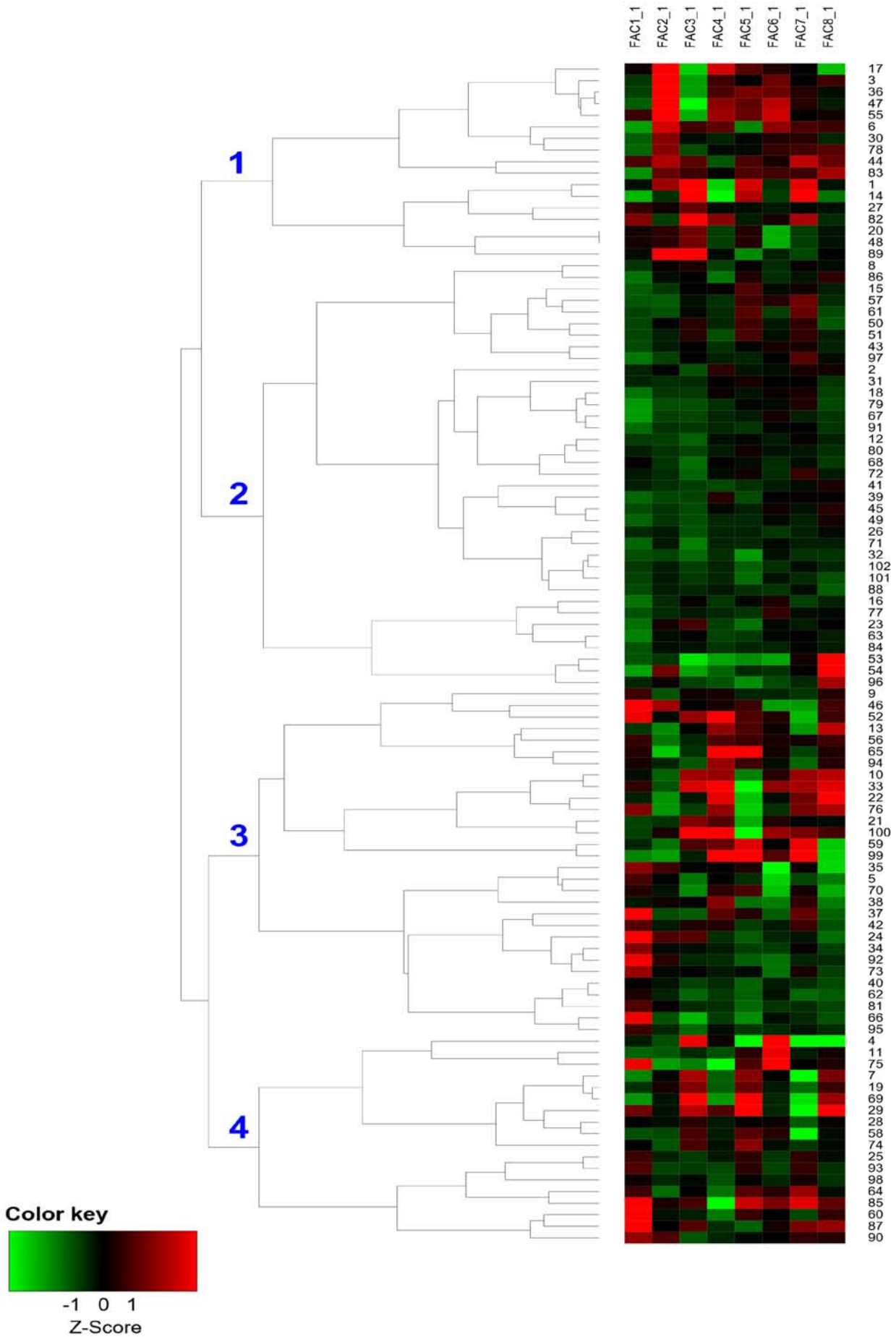

Figure 1. Hierarchical clustering based on principal component analysis. Each column is a component; each row is an individual patient. Numbers at the right side of the heat map indicate the patient number. Left, dendrogram presenting the similarity of groups; four clusters are indicated.

Table II. Comparison of clinical parameters among clusters.

Clinical parameter	Cluster 1, n=17	Cluster 2, n=37	Cluster 3, n=30	Cluster 4, n=18	P-value
Age, years	66.65±8.21	66.95±8.58	67.03±8.40	64.33±6.67	0.681
BMI, kg/m^2	21.76±5.62	22.47±3.63	20.79±4.07	22.49±3.37	0.355
Number of exacerbations in the previous year	0 (0-1)	1 (0-1.5)	1 (0-2)	0 (0-1)	0.109
Blood neutrophil count, x10^9/l	4.4±1.11	4.76±2.45	4.04±1.71	4.46±1.69	0.527
FEV1pred%	45.16±18.72	43.65±17.25	53.26±24.11	56.63±24.46	0.095
MMEF	0.43±0.25	0.46±0.30	0.62±0.49	0.68±0.46	0.105
CAT	9.88±4.85	13.84±6.90	11.80±5.46	8.67±7.01	0.022[a]
mMRC	1 (1-2)	2 (1-2)	1 (1-2.25)	1 (0-2)	0.029[a]

[a]P<0.05; cluster 2 vs. cluster 4. BMI, body mass index; CAT, chronic obstructive pulmonary disease assessment test score; FEV1pred%, forced expiratory volume in 1 sec as percentage of predicted; MMEF, maximal mid-expiratory flow; mMRC, modified Medical Research Council Dyspnea Scale.

white blood cell, neutrophil and monocyte) and blood haemoglobin. In cluster 3 (Fig. 2D), the AE was positively associated with the CAT score and sputum autoantibodies (U1-SnRNP, PR3, MPO and Ro/SSA), and in cluster 4 (Fig. 2E), the AE was negatively associated with serum uric acid and blood neutrophil count (Table III). Additionally, sputum anti-PR3, sputum anti-Ro/SSA, and sputum anti-U1-SnRNP were significantly negatively correlated with AE in cluster 2 (Fig. 2C), but were positively correlated with AE in cluster 3 (Fig. 2D; Table III). The network of non-smoking controls had a lower density, lower average path length, lower average degree and longer diameter than those in COPD groups, reflecting normal immunological condition (Fig. 2A; Table III).

Exacerbation-related module analysis. In each cluster, network clustering yielded modules, and the module containing retrospective exacerbation (AE-node) was extracted for further analysis (Fig. 3). Table IV presents the topological properties of the four AE modules of the corresponding clusters. These modules exhibited significant heterogeneity in terms of their biological and clinical contents, in addition to their topological properties. Module 2 demonstrated very high network density, whereas module 4 demonstrated low density. Module 2 contained 13 nodes with a high average degree, whereas module 1 contained only three nodes with a low average degree. In module 1, AE was only positively associated with the CAT score. In module 2, AE was negatively associated with sputum autoantibodies (P0, Scl70, Sm, U1-SnRNP, PR3 and Ro/SSA) and Glb, and positively associated with neutrophil counts. In module 3, AE was positively associated with the CAT score and sputum autoantibodies (U1-SnRNP, PR3, MPO and Ro/SSA). In module 4, AE was negatively associated with serum uric acid (Fig. 3, Table IV). Sputum anti-PR3, sputum anti-Ro/SSA and sputum anti-U1-SnRNP were significantly negatively correlated with AE in module 2, but were positively correlated with AE in module 3.

Discussion

Autoimmune components in COPD have received increasing attention, as COPD shares various pathophysiological and clinical characteristics with autoimmune diseases (4,8,10,11); however, there remains a lack of medical literature regarding the relationship between airway/circulating autoantibody responses and clinical parameters in COPD, particularly in different heterogenous subgroups. In the present proof-of-concept study, three methods were employed to investigate the interrelationships among autoantibody profiles and clinical variables in various COPD subgroups. First, a highly sensitive detection method was used to simultaneously investigate autoantibody profiles in sputum and serum. Second, unsupervised clustering was performed on the PCA-transformed autoantibody profile data, independent of clinical parameters, to identify immunological subgroups of COPD. Third, a network-based analysis was applied to investigate the association between immunological and clinical parameters, and COPD exacerbation risks, in each cluster, followed by comparison of the networks and module properties of these clusters. The following main findings were reported: i) Four stable COPD subgroups with distinguished immunological features were identified, although there were no significant differences among subgroups for the majority of clinical characteristics; ii) the networks of the four subgroups exhibited distinct topological properties; iii) the exacerbation risk-associated factors were significantly different among the four clusters; and iv) sputum anti-PR3, sputum anti-Ro/SSA and sputum anti-U1-SnRNP were significantly negatively associated with exacerbation risk in cluster 2, but positively associated in cluster 3, suggesting the heterogeneity and dual nature of the airway autoantibody responses in COPD.

A number of previous studies have investigated autoantibodies in COPD from a clinical point of view. For example, Cheng *et al* (33) detected circulating IgG, IgA and IgM against human bronchial epithelial cells (anti-HBEC) in stable patients with COPD using indirect immunofluorescence, and observed an increased positive rate of anti-HBEC expression in patients with COPD compared with in healthy controls. Sigari *et al* (34) reported increased serum levels of anti-cyclic citrullinated peptide antibody levels in wood-smoke-induced COPD compared with in tobacco-induced COPD and controls. Xiong *et al* (35) reported that the plasma autoantibody levels of

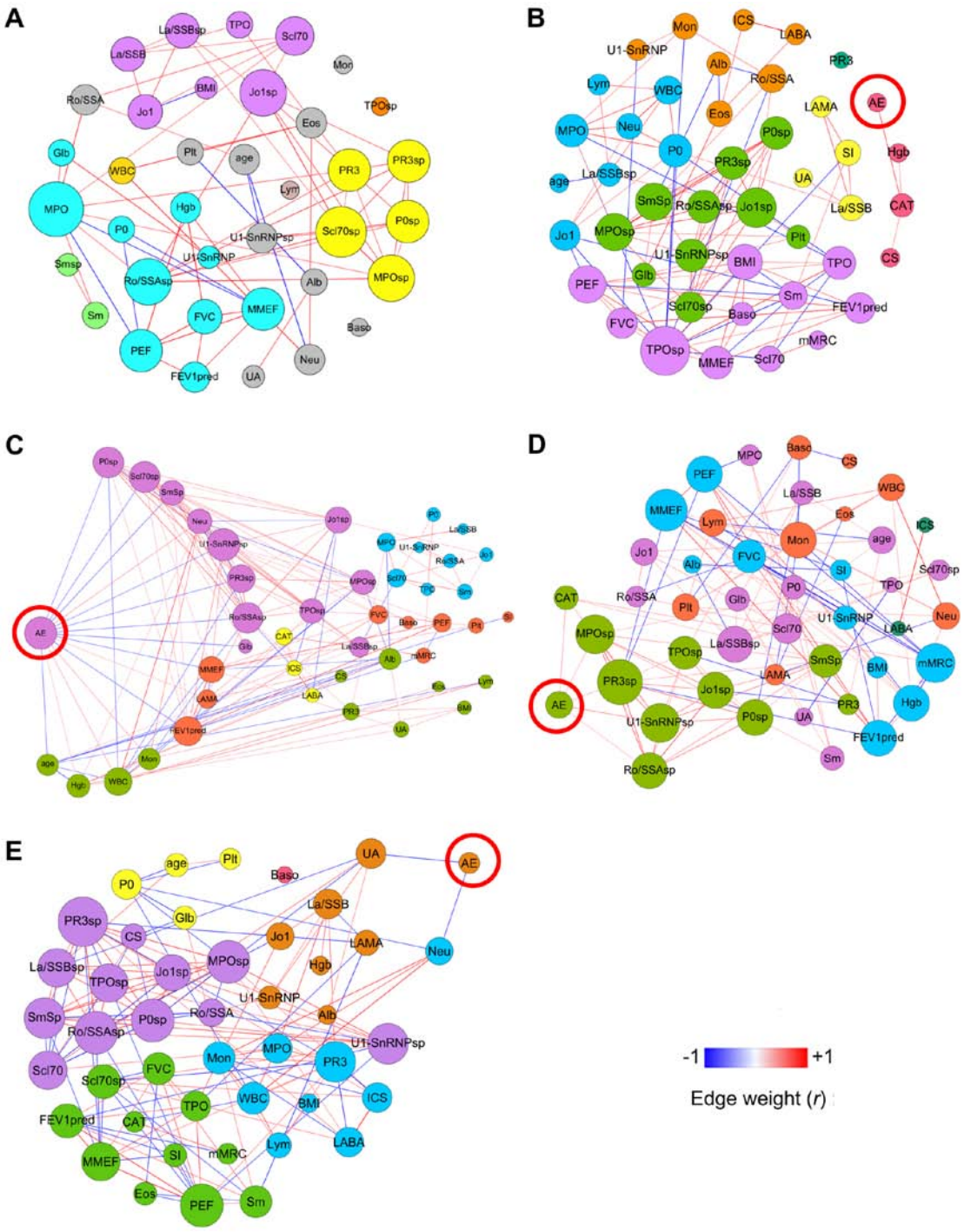

Figure 2. Network analysis of non-smoking healthy controls and the four clusters. (A) Non-smoking healthy controls and clusters (B) 1, (C) 2, (D) 3 and (E) 4. The size of each node is proportional to its weighted degree value. The colour of each node represents the corresponding module. Correlation coefficients with P>0.05 were filtered out. The colour of each edge indicates the correlation coefficient (edge weight) between two nodes. AE, number of exacerbations in the previous year; BMI, body mass index; Alb, serum albumin; CAT, chronic obstructive pulmonary disease assessment test score; FEV1pred, forced expiratory volume in 1 sec as percentage of predicted; CS, current smoker; Hgb, haemoglobin; Glb, serum globulin; MMEF, maximal mid-expiratory flow; mMRC, modified Medical Research Council Dyspnea Scale; Mon, peripheral blood monocyte count; Neu, peripheral blood neutrophil count; sp, sputum; UA, serum uric acid; WBC, peripheral white blood cell count; Sm, Smith antigen; P0, ribosomal phosphoprotein P0; Ro/SSA, Ro/Sjögren syndrome type A antigen; La/SSB, La/Sjögren syndrome type B antigen; Scl70, DNA Topoisomerase I; Jo1, histidyl-tRNA synthetase; U1-SnRNP, U1 small nuclear ribonucleoprotein; TPO, thyroid peroxidase; PR3, proteinase-3; MPO, myeloperoxidase.

IgG, IgA and IgM against cytokeratin-18 and -19 were elevated in patients with COPD compared with healthy controls. Luo *et al* (36) investigated the presence of anti-CD80 autoantibodies in the serum of patients with stable COPD and controls, and observed that serum levels of anti-CD80 were increased in patients with COPD compared with those in controls and were positively correlated with serum levels of interleukin (IL)-6 and IL-8. Shindi *et al* (37) detected serum IgM and IgG autoantibodies in patients with COPD and controls using an antigen microarray, and reported significant differences in the

Table III. Topological properties of the four correlation networks.

A, Network properties

Factor	Non-smoking healthy controls	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Number of nodes	37	45	45	45	45
Average degree	3.459	4.711	6.711	5.156	6.311
Number of edges	64	106	151	116	142
Network diameter	9	7	6	8	6
Graph density	0.096	0.107	0.153	0.117	0.143
Average path length	3.64	3.043	2.555	2.908	2.552
Average Clustering coefficient	0.45	0.533	0.46	0.441	0.45
Modularity	0.535	0.56	0.367	0.582	0.447
Module number	10	7	5	5	6
Hubs (nodes with degree within top 10%)	Serum anti-MPO, sputum anti-Scl70, sputum anti-MPO, sputum anti-Jo1, sputum anti-Ro/SSA	Sputum anti-TPO, sputum anti-Jo1, sputum anti-Sm, sputum anti-MPO, PEF	Sputum anti-U1-SnRNP, AE, sputum anti-P0, sputum anti-Ro/SSA, sputum anti-Scl70	Sputum anti-PR3, MMEF, mMRC, sputum anti-MPO, sputum anti-U1-SnRNP	Sputum anti-PR3, sputum anti-P0, sputum anti-Ro/SSA, sputum anti-MPO, PEF

B, AE-node properties

Factor	Non-smoking healthy controls	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Degree	NA	1	15	5	2
Betweenness centrality	NA	0	70.59	3.59	7.47
Eccentricity	NA	2.0	5	5	5.0
Closeness centrality	NA	0.6	0.49	0.35	0.32
Clustering coefficient	NA	0	0.41	0.7	0
Correlated nodes	NA	Positive: CAT Negative: none	Positive: Neu, Mon, WBC, Hgb Negative: FEV1pred%, MMEF, sputum anti-P0, sputum anti-Scl70, sputum anti-Sm, sputum anti-U1-SnRNP, sputum anti-PR3, sputum anti-Ro/SSA, Glb, age	Positive: CAT, sputum anti-PR3, sputum anti-MPO, sputum anti-Ro/SSA, sputum anti-U1-SnRNP Negative: none	Positive: none Negative: UA, Neu

AE, number of exacerbations in the previous year; FEV1pred%, forced expiratory volume in 1 sec as percentage of predicted; MMEF, maximal mid-expiratory flow; Neu, peripheral blood neutrophil count; CAT, chronic obstructive pulmonary disease assessment test score; WBC, peripheral white blood cell count; Glb, serum globulin; Mon, peripheral blood monocyte count; Hgb, haemoglobin; UA, serum uric acid; Sm, Smith antigen; P0, ribosomal phosphoprotein P0; Ro/SSA, Ro/Sjögren syndrome type A antigen; Scl70, DNA topoisomerase I; Jo1, histidyl-tRNA synthetase; U1-SnRNP, U1 small nuclear ribonucleoprotein; TPO, thyroid peroxidase; PR3, proteinase-3; MPO, myeloperoxidase.

autoantigenic specificities of IgM autoantibodies compared with IgG autoantibodies in COPD serum. Conversely, none of these studies reported airway autoantibody responses in COPD, and no studies have investigated the autoantibody responses in different immunological subgroups of COPD. Therefore, the present study was conducted to investigate the

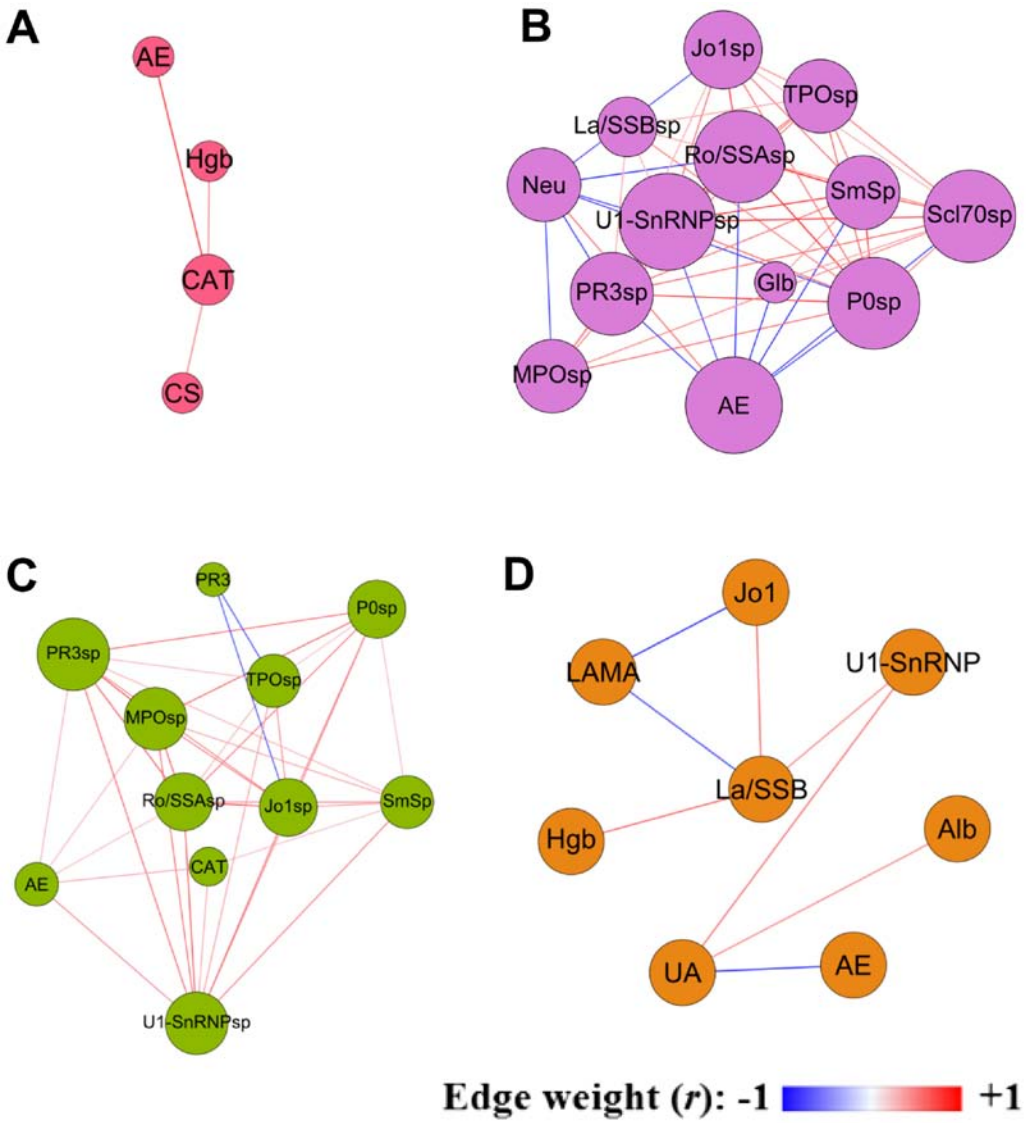

Figure 3. Exacerbation-associated modules. (A) Cluster 1; (B) cluster 2; (C) cluster 3; and (D) cluster 4. The size of each node is proportional to its weighted degree value. Correlation coefficients with P>0.05 were filtered out. The colour of each edge indicates the correlation coefficient (edge weight) between two nodes. AE, number of exacerbations in the previous year; Alb, serum albumin; CAT, chronic obstructive pulmonary disease assessment test score; CS, current smoker; Hgb, haemoglobin; Glb, serum globulin; Neu, peripheral blood neutrophil count; sp, sputum; UA, serum uric acid; Sm, Smith antigen; P0, ribosomal phosphoprotein P0; Ro/SSA, Ro/Sjögren syndrome type A antigen; La/SSB, La/Sjögren syndrome type B antigen; Scl70, DNA Topoisomerase I; LAMA, long-acting muscarinic antagonist; Jo1, histidyl-tRNA synthetase; U1-SnRNP, U1 small nuclear ribonucleoprotein; TPO, thyroid peroxidase; PR3, proteinase-3; MPO, myeloperoxidase.

airway/circulating autoantibody responses in heterogenous subgroups of COPD.

A number of previous studies have explored the associations among clinical, functional and biological parameters, and exacerbation risk, in COPD. For example, it was reported that the CAT score can assist in the prediction of COPD exacerbations (38); in the present study, it was observed that the CAT score was significantly associated with retrospective exacerbations only in two subgroups (cluster 1 and cluster 3), suggesting clinical heterogeneity in patients with COPD. Additionally, it was demonstrated that deteriorating airflow limitation is associated with an increasing prevalence of exacerbations (39); however, FEV1 lacks sufficient precision (wide variation) to be used clinically as a predictor of exacerbation in patients with COPD (40). The present study reported that airflow limitations were associated with retrospective exacerbations only in one COPD subgroup, which suggested the heterogeneity of exacerbation risks and was consistent with previous reports. Peripheral neutrophil count represents low-grade systemic inflammation in a number of chronic conditions (41); Hong *et al* (42) reported that the blood neutrophil count was significantly correlated with main clinical outcomes in patients with COPD. Of note, it was observed in the present study that blood neutrophil count was positively associated with retrospective exacerbation only in one subgroup of COPD patients (cluster 2) and was negatively associated with retrospective exacerbation in another subgroup (cluster 4). These results indicated that the existence of systemic inflammation is also heterogeneous; however, verification of this requires further research. Finally, a previous study reported that serum uric acid was associated with an increased risk of COPD exacerbation (43);

Table IV. Topological properties of the exacerbation-related module of the four clusters.

Factor	Module 1 (Cluster 1)	Module 2 (Cluster 2)	Module 3 (Cluster 3)	Module 4 (Cluster 4)
Number of nodes	4	13	11	8
Average degree	1.5	8.308	6.364	2
Number of edges	3	54	35	8
Network diameter	2	2	3	4
Graph density	0.5	0.692	0.636	0.286
AE-node degree	1	8	5	1
AE-correlated nodes	Positive: CAT Negative: none	Positive: Neu Negative: sputum anti-P0 sputum anti-Scl70 sputum anti-Sm sputum anti-U1-SnRNP sputum anti-PR3 sputum anti-Ro/SSA Glb	Positive: CAT sputum anti-PR3 sputum anti-MPO sputum anti-Ro/SSA sputum anti-U1-SnRNP Negative: none	Positive: none Negative: UA

AE, number of exacerbations in the previous year; Neu, peripheral blood neutrophil count; CAT, chronic obstructive pulmonary disease assessment test score; Glb, serum globulin; UA, serum uric acid; Sm, Smith antigen; P0, ribosomal phosphoprotein P0; Ro/SSA, Ro/Sjögren syndrome type A antigen; Scl70, DNA topoisomerase I; U1-SnRNP, U1 small nuclear ribonucleoprotein; PR3, proteinase-3; MPO, myeloperoxidase.

however, the present study reported that serum uric acid was negatively associated with retrospective exacerbation in a subgroup of patients, which may be connected to population heterogeneity and/or recall bias during the collection of retrospective information.

Differential module analysis also provided further insight into COPD by demonstrating relationships between autoantibody modules and clinical variables in the various subgroups. Module 1 demonstrated a simple structure where retrospective exacerbations were only associated with CAT score, suggesting that it may be easier to prevent exacerbation in this subgroup (cluster 1). Modules 2 and 3 exhibited complex structures, high network density and high degrees of their respective AE-nodes, pointing towards the complexity and difficulty of preventing exacerbation in these subgroups. Of note, sputum autoantibodies (U1-SnRNP, PR3, and Ro/SSA) were negatively associated with exacerbation risk in module 2, but were positively related to exacerbation risk in module 3, implying the dual character and heterogeneity of airway autoantibody responses. Thus, these autoantibodies may mediate tissue injury, but may also serve a protective role by removing senescent cells and maintaining immune homeostasis.

The present study had two main strengths. First, autoantibody profiles were detected in sputum and serum simultaneously, whereas the majority of previous clinical studies have detected autoantibodies only in serum or plasma (34,44-47). In this study and a previous preliminary study (13), it was observed that sputum autoantibodies were more clinically relevant than serum autoantibodies, suggesting that studies solely focused on circulating autoantibodies may provide limited information. Second, due to the heterogeneity and complexity of autoantibody responses in cases of COPD, an integrative method was applied based on unsupervised classification. This method differs from previously published COPD autoantibody studies in that it provides the capacity to visualise a wide range of autoantibodies in heterogeneous subgroups, rather than focusing on a single or small number of autoantibodies and viewing all patients as homogenous. Without dividing patients into heterogeneous subgroups, those prior studies may have generated inconsistent findings (46,47).

A number of limitations of the current study should be discussed. First, this was a cross-sectional study, so causal relationships could not be drawn, meaning that exacerbation-associated factors identified in this study should be validated using longitudinal cohort data; however, previous studies reported that the type of inflammatory responses observed during exacerbation may depend on patient phenotype in stable disease (48-52), suggesting that patient parameters in stable disease and exacerbation are closely associated (3,53). Second, as this preliminary study was performed to investigate the heterogeneities of airway/circulating autoantibody responses in patients with COPD, the autoantibody profile data provide limited clinical information to accurately discriminate the COPD subgroups. Third, this study was preliminary and was limited to the analysis of autoantibodies against ten autoantigens. The inclusion of an increased number of diverse autoantibodies may be more clinically informative. Furthermore, autoreactive B cells, which are the source of autoantibodies, should be studied in the future. Finally, the patients recruited into the present study were predominantly male, which may have been related to their risk factors. According to the China Global Adults Tobacco Survey of 2010, 52.9% of

males and 2.4% of females were current smokers (54). In China, cigarette smoking is the main risk factor for COPD, although in rural Southern China it has been replaced by exposure to biomass fuel (55). The patients in the cohort were admitted to a university teaching hospital in Guangzhou (the largest city in Southern China). Thus, cigarette smoking would have been the main risk factor for COPD but would have resulted in a sexual bias, as more males than females are smokers.

In conclusion, using unsupervised clustering and network analysis, it was demonstrated that: i) Pulmonary autoantibody responses were heterogeneous and associated with exacerbation risk in certain subgroups, and therefore their dual character should be taken into consideration in future research; and ii) airway and circulating autoantibody profiles can identify COPD subgroups with various factors associated with exacerbation risk and distinct network topologies. The present study also provides support for future strategies involving personalised predictive biomarker identification and precision management. Further clinical research should focus on local (airway) autoimmune responses.

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Availability of data and materials

All the data generated and analysed in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZL, FL, FW, TP and RC designed the study and drafted the manuscript. ZL, FW, YY, WJ, LZ, JZ, KD, JXX and RC recruited patients and collected clinical data. FL, JX and TP conducted Luminex detection. ZL, FW and YY analyzed microarray data. ZL, XC, WJ, WG, JXX and JZ conducted quality control on the clinical data. ZL and MJ performed data mining. WG and YY processed biological samples.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The First Affiliated Hospital of Guangzhou Medical University (no. 2017-22). Informed consent was obtained from all patients. The trial registration number for the study was NCT03240315.

Patient consent for publication

Written informed consent was obtained from the patients for publication of the data included in the present manuscript.

Competing interests

The authors declare that they have no competing interests.

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