

# Are sputum autoantibodies more clinically relevant in idiopathic pulmonary fibrosis than serum autoantibodies?

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**Background:** The adaptive immune system plays a role in the pathogenesis of idiopathic pulmonary fibrosis (IPF) has been reported previously. However, the association between airway and circulating autoantibodies (AABs) levels is unclear. The aim of this study is to investigate the link between the AAB levels in airway and circulation in stable patients with IPF. **Materials and Methods:** From June 2016 to March 2017, 21 stable IPF patients and 22 healthy volunteers were recruited. We established Luminex interacting AABs with bead-antigen complex to detect the immunoglobulin G antibodies levels of ten autoantigens which were matched serum (Se) and sputum (Sp) samples collected from recruited subjects, including Smith (Sm), Anti-ribosomal P antibody (P0), Sjögren syndrome type A antigen (SSA), La/Sjögren syndrome type B antigen (SSB), DNA topoisomerase (Scl-70), histidyl-tRNA synthetase (Jo-1), U1 small nuclear ribonucleoprotein (U1-SnRNP), thyroid peroxidase, Proteinase 3, and Myeloperoxidase. Spearman's rank correlation matrix was applied to explore the associations of Ab profiles between Se and Sp. **Results:** For IPF patients, Spearman's correlation matrix showed multiple intercorrelations among Sp-AABs and Sp-AABs ( $P < 0.05$ ), while only the levels of AAB against Sm and anti-La in Se were correlated with those Sp-AAB counterparts ( $P < 0.05$ ). For healthy individuals, only anti-La in Se was associated with those Sp-AAB counterparts ( $P < 0.05$ ). For IPF patients, there was a positive correlation between carbon monoxide diffusing capacity (DL<sub>CO</sub>)% predicted and Sp-anti-P0 level ( $r = 0.464$ ,  $P = 0.034$ ). Forced vital capacity% predicted was positively correlated with Sp-anti-Scl-70 level ( $r = 0.466$ ,  $P = 0.033$ ). **Conclusion:** Comparing to Se-AABs, Sp-AABs are more associated with clinical parameters in the patients with IPF. In order to better understand the role of autoimmunity in the pathogenesis of IPF, detection of Sp-AABs for local autoimmune responses may be a good choice.

**Key words:** Autoantibodies, autoimmunity, idiopathic pulmonary fibrosis, serum, sputum

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## INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive disease, characterized by irreversible pulmonary parenchyma fibrosis. It is refractory, with a median survival of approximately 3 years. The incidence of IPF is currently reported as approximately 2.8–19/100,000 in the USA and Europe.<sup>[1]</sup> Repeated injury

to the alveolar epithelium from unknown causes leads to a dysregulated tissue repair, which is considered as an important mechanism for IPF.<sup>[2]</sup>

The pathogenesis of IPF remains poorly understood, the presence of lymphoid aggregates in the lung tissue, together with autoantibodies (AABs) in the serum (Se), suggests that the immune system is likely to play a role in either initiation or progression of the disease.<sup>[3]</sup> Lymphocyte aggregates in the IPF lung

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consist of CD3+ T-lymphocytes and mature DC cells, which are generally adjacent to fibroblastic foci. A subset of these aggregates also contain CD20+ B cells that localize in cohesive focal clusters within the center of the aggregate, resulting in an organized appearance.<sup>[4]</sup> Besides innate immunity, abnormalities of humoral immunity are common in patients with IPF.<sup>[5-12]</sup> It indicates that IPF may be associated with pulmonary and systemic immunity response. Chronic obstructive pulmonary disease was put forward “spill over” theory arguing that antibodies may be from the lungs overflow to the blood.<sup>[13]</sup> Different from it, the association of IPF between pulmonary and systemic AAb level in IPF was unclear. There is no study published to date detecting simultaneous quantification of the same AAb levels of stable IPF in both pulmonary and systemic compartments. In clinical work, Se testing is convenient and can be performed multiple times. However, in our concept, the observation of the local area of the sample may be more representative of the development of the disease. So far, there has been a lack of research comparing the relationship between IPF systemic and local immune indicators.

We hypothesized that there was a relatively independent immune environment between pulmonary and systemic in stable IPF. To verify this hypothesis, we established Luminex interacting AAbs with bead-antigen complex to detect the immunoglobulin G (IgG) antibody levels of ten autoantigens. Because they were derived from eukaryotic cells, the AAbs could be detected in Se and sputum (Sp) of patients with stable IPF. In addition, to exclude connective tissue-associated lung diseases, these antibodies are routinely tested clinically.<sup>[14]</sup> Se and Sp were gathered simultaneously from the same person.

## METHODS

### Study design and subjects

In this prospective, observational study, 21 patients with IPF were enrolled consecutively in our center between June 2016 and March 2017. The inclusion criteria were: (1) Age from 50 to 80 years (2) IPF was diagnosed according to the 2011 American Thoracic Society (ATS)/European Respiratory Society (ERS)/Japanese Respiratory Society/Latin American Thoracic Association guideline diagnostic criteria<sup>[15]</sup> and had negative conventional autoimmune serological tests, including rheumatoid factor, anti-cyclic citrullinated peptide, anti-nucleosome antibody, anti-centromere B protein antibody, anti-ribonucleoprotein antibody, anti-histone antibody, myeloperoxidase (MPO), PR3, anti-Peripheral neutrophil antibody (P-ANCA), neutrophil cytoplasmic antibody (cANCA), syndrome type A antigen (SSA), SSB, Scl-70, Smith (Sm), Jo-1, n-RNP, double-stranded DNA, RO-52, with a low sensitivity, semi-quantitative method.<sup>[16]</sup> Patients with one or more of

the following criteria were excluded: (1) Diagnosis of known respiratory disorders other than IPF; (2) history of significant inflammatory disease other than IPF; (3) IPF progression or using antibiotic within 4 weeks of enrolment; (4) patients received steroids and immunosuppressants; (5) history of lung surgery; (6) recent diagnosis of cancer; and (7) recipient of a blood transfusion within 4 weeks of enrollment. All the patients have signed informed consent. The study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University. (Approval no. 2016-6-9).

### Clinical parameter measurement

Before Sp induction, IPF patients' pulmonary function tests, including VC and diffusing capacity of carbon monoxide (DL<sub>CO</sub>), were quantified by using spirometry. According to the ATS/ERS guidelines, pulmonary function was performed using a spirometer (MasterScreen PFT; Jaeger™, CareFusion, Hoechberg, Germany). Spirometry was performed in accordance with the ATS guidelines. DL<sub>CO</sub> was performed by using the single-breath method.<sup>[17]</sup> Arterial blood gases were recorded.

### Sample collection and processing

Before Sp induction, venous blood samples were obtained onto ethylenediaminetetraacetic acid anticoagulation tubes. Moreover, a differential white blood cell count was performed on a Coulter instrument (Sysmex-XE2100, Kobe, Japan).

The process of Sp induction was performed according to the guidelines suggested by the Task Force of the ERS. The Sp was processed using a two-step procedure as previous report.<sup>[18]</sup> Briefly, patients received salbutamol twice (100 µg/spray) 15 min before he was inducted. Each subject was induced for 15 min by inhalation of a 3% hypertonic saline solution. Before Sp cough, the patient rinsed the mouth with 0.9% saline to reduce oral contamination and blow the nose. Discard the first Sp and continue atomization for 15 min. Add 8 volumes of PBS to the Sp and shake for 15 min in a vortex shaker at 4 degrees., and then, the Sp sample was centrifuged (3000 rpm, 10 min at 4°C). After collecting four volumes of Sp supernatant, two volumes of dithiothreitol solution were added and allowed to stand for 15 min to dissolve the mucus. The Sp sample was centrifuged and stored in -80°C. Blood samples were drawn and centrifuged (3000 rpm, 10 min at 4°C), dispensed and stored at -80°C.

### AAb detections

A multiplex bead-based assay consisted of 10 AAbs which were respectively coupled with magnetic beads (Luminex, USA), including Sm, P0, Ro/SSA, La/SSB, Scl-70, Jo-1, U1-SnRNP, thyroid peroxidase, Proteinase 3, MPO. Sp supernatant and Se samples were 1:10 and 1:180 diluted,

respectively. Diluted samples were interacted with the multiplex beads at 37°C for 1h. Beads were washed at Bio-Plex Pro Wash Station (Bio-Rad, USA). Biotin-conjugated anti-human IgG (ThermoFisher, USA) at 1:1000 dilution was applied to each reaction well and incubated at 37°C for 1h. Following incubation, the beads were washed and reacted with streptavidin-R-phycoerythrin (SAPE) (Bio-Rad, USA) at 37°C for 15 min. After wash and resuspend the microspheres, fluorescence was measured using Bio-Plex 200 (Bio-Rad, USA) and Bio-Plex Manager 6.0 software was used to generate the result files.

### Statistical analysis

Statistical analysis was performed using the SPSS software package (version 18.0; IBM Corp., Armonk, NY, USA). Data are expressed as the mean ± standard deviation of continuous variables. Variables with a skewed distribution are represented as median (interquartile range [P25-P75]). Correlation analysis was assessed using Pearson correlation or Spearman correlation. The relationship between Sp-AAb levels and Se-AAb levels was examined using the Spearman rank correlation coefficient.  $P < 0.05$  was considered statistically significant. All statistical analyses were performed using SPSS Version 19.0 (IBM Corporation, New York, NY, USA). The Heatmap plot created with GraphPad Prism 7 shows the correlation matrix of AAbs and clinical parameters.

## RESULTS

### Patients

All of the 22 healthy individuals with adequate Sp were induction by atomization with 3% hypertonic saline. Of the 21 patients with adequate Sp-, 5 individuals coughed spontaneous samples and 16 provided induced Sp-. The clinical characteristics of the 22 healthy control and 21 IPF patients are shown in Table 1. There were significantly different among FEV1 (%predicted), forced vital capacity FVC (%predicted), DL<sub>CO</sub> (%predicted) between healthy people and IPF patients.

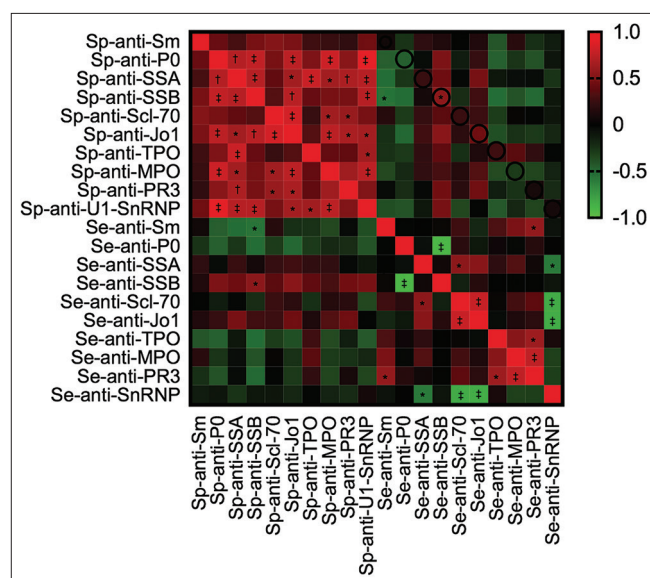
### Spearman rank correlation coefficient of AAb profiles

According to Haldun study, correlation coefficient was defined as (a) 1.0 perfect; (b) 0.7–1.0 strong correlation; (c) 0.4–0.7 moderate correlation; (d) 0.1–0.4 weak correlation; 0.0–0.1 very weak correlation or no correlation.<sup>[19]</sup> Figure 1 depicts the correlations among AAbs for healthy individual. There is multiple positive correlation between Sp-AAbs and Se-AAbs, but there is little correlation between Sp-AAbs and Se-AAbs. Notable findings include: (1) There are many high correlations among the 10 Sp-AAbs ( $P < 0.05$ ); (2) There is a large correlation between the 10 Se-AAbs compared to the Sp-AAbs' correlation ( $P < 0.05$ ); (3) There was almost no significant correlation between Sp-AAbs and Se counterparts; (4) Sp-anti-SSB was positively correlated

**Table 1: Demographic and clinical characteristics of healthy controls and patients with idiopathic pulmonary fibrosis**

Characteristic	Mean±SD		P
	IPF patients (n=21)	Healthy control (n=21)	
Age (years)	66.10±7.13	65.18±8.42	0.582
Men, n (%)	21 (100)	8 (36.36)	-
BMI (kg/m <sup>2</sup> )	24.11±3.28	23.83±5.17	0.843
FEV1 (% predicted)	78.83±12.48	93.10±11.31	0.002**
FVC (% predicted)	76.41±15.55	98.00±5.69	0.001**
DL <sub>CO</sub> (% predicted)	49.62±15.21	92.57±3.31	0.001**

\*\* $P < 0.01$ . Data are presented as n (%), mean±SD. SD=Standard deviation, IPF=Idiopathic pulmonary fibrosis, BMI=Body mass index, FVC (% predicted)=Forced vital capacity in percentage of predicted; FEV1 (% predicted)=Forced expiratory volume in 1 s in percentage of predicted; DL<sub>CO</sub> (% predicted)=Carbon monoxide diffusing capacity in percentage of predicted



**Figure 1:** The correlation matrix of AAbs for healthy control. Sp = Sputum; Se = Serum; AAb = Autoantibody; Sm = Smith; P0 = Anti-ribosomal p antibody; SSA = Sjögren syndrome type A antigen; SSB = La/Sjögren syndrome type B antigen; DNA topoisomerase (Scl-70), histidyl-tRNA synthetase (Jo-1), U1-SnRNP = U1 small nuclear ribonucleoprotein; TPO = thyroid peroxidase; PR-3 = Proteinase 3; MPO = Myeloperoxidase. \* $P < 0.05$ , † $P < 0.01$ , ‡ $P < 0.001$ , a circle represents a correlation between Sp-AAbs and Se-AAbs regarding 1 type of AAb

with Se-anti-SSB ( $r = 0.576$ ,  $P = 0.031$ ). Figure 2 shows the correlation between AAbs in IPF patients. (1) There were many high correlations among the 10 Sp-AAbs ( $P < 0.05$ ); (2) There was a large correlation between the 10 Se-AAbs compared with the Sp-AAbs' correlation ( $P < 0.05$ ). There was little significant correlation between Sp-AAbs and Se counterparts; (4) Sp-anti-SSB was positively correlated with Se-anti-SSB ( $r = 0.619$ ,  $P = 0.003$ ). Sp-anti-Sm was positively correlated with Se-anti-Sm ( $r = 0.754$ ,  $P = 0.0001$ ).

### Relationship between AAb profiles and clinical parameters

Among all the detected AAbs, there was a positive correlation between the predicted carbon monoxide

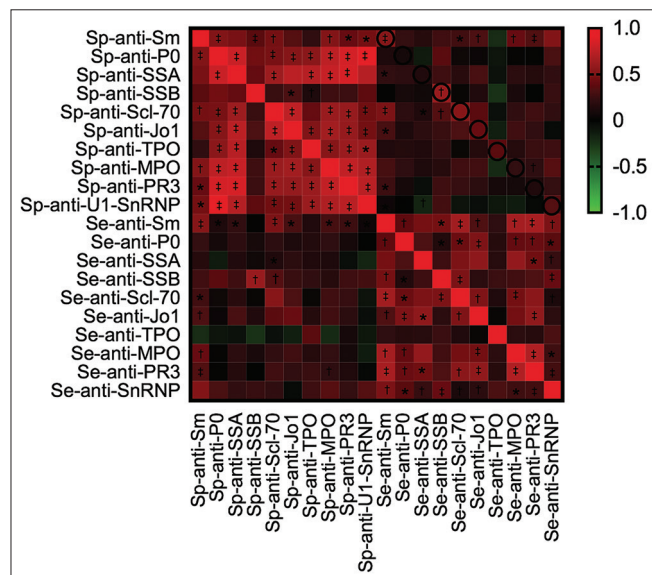
diffusion ( $DL_{CO}$ )% and the Sp-anti-P0 level ( $r = 0.464$ ,  $P = 0.034$ ), as shown in Figure 3. FVC% was positively correlated with Sp-anti-Scl-70 levels ( $r = 0.466$ ,  $P = 0.033$ ), as shown in Figure 4.

## DISCUSSION

Using high sensitive detection, it is the first study to investigate AAb levels of 22 healthy control and 21 stable patients with IPF in two compartments (airway and circulation) simultaneously. The main observation of the current study is Sp-AAbs are more clinically relevant in IPF than Se-AAbs, suggesting a relatively independent immune environment in local lung. There were correlations between Sp- AAb levels and pulmonary function index, but not in Se.

### Previous study

Our previous study on asthma and AAbs observed that Sp-AAbs is more associated with clinical parameters and the severity of disease in asthma compared to Se-AAbs.<sup>[20]</sup> Several previous studies have proved that the cytokines of IPF patients between airway and circulating are different, by detecting the same mediators in different compartments simultaneously. Especially for acute exacerbation IPF, local and systemic inflammation levels are very different because of changes in the airway environment.<sup>[21-23]</sup> Cytokines released by cells, from tissue fluid into the airways and blood. Comparing with circulation, the concentration of inflammation mediators in airway was higher.<sup>[24,25]</sup>



**Figure 2:** The correlation matrix of AABs for IPF patients. Sp = Sputum; Se = Serum; AAb = Autoantibody; Sm = Smith; P0 = Anti-ribosomal p antibody; SSA = Sjögren syndrome type A antigen; SSB = La/Sjögren syndrome type B antigen; DNA topoisomerase (Scl-70), histidyl-tRNA synthetase (Jo-1), U1-SnRNP = U1 small nuclear ribonucleoprotein; TPO = Thyroid peroxidase; PR-3 = Proteinase 3; MPO = Myeloperoxidase. \* $P < 0.05$ , † $P < 0.01$ , ‡ $P < 0.001$ , a circle represents a correlation between Sp- AABs and Se-AABs regarding 1 type of AAb

However, these studies did not show the correlation of mediators between the two compartments. Besides, no previous study has evaluated the relationships between airway and circulating autoimmunity in IPF.

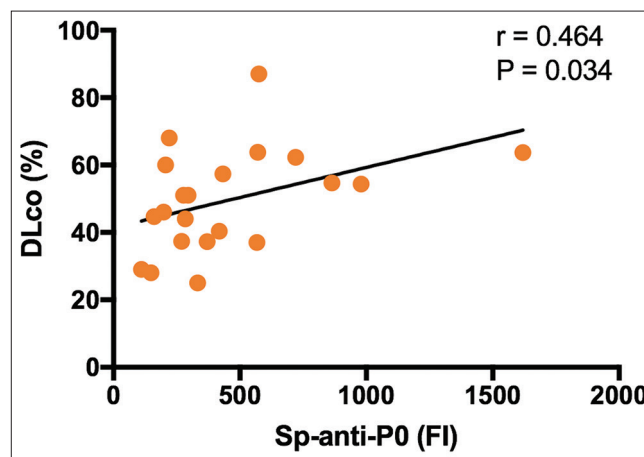
### Interpretation of main findings

It is widely accepted that the injury of IPF is limited to the lung, but little we knew about the relationships between the local and systemic autoimmune response. Therefore, we evaluated the possible relationship between airway and circulating AAbs response.

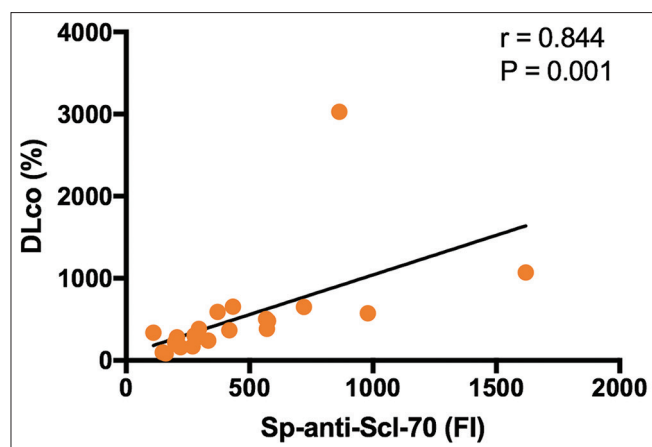
It is known that Se-AABs are associated with immune aging with increasing age in healthy subjects, resulting in increased exposure to autoantigens. It is worth noting that, the IPF patients and healthy control were age-matched. Besides, neither the Sp-AABs nor Se-AABs were significant correlated with the age. The average age of the recruiters in our study was 66 years old, which made the result more representative.

First, there were abnormalities of adapted immunity in patients with IPF.<sup>[5,8-12,16]</sup> However, clinical AAb profiles in Se of patients with IPF was always negative, the reason may be that it just increased locally in the lung, which was consistent with our finding. IPF injury in addition to the pulmonary interstitial, large and small airways can also produce lesions. Microbial flora in the IPF airway also affects airway immunity. A study by Shijubo showed that there was an interesting discrepancy between soluble ICAM- I levels in the circulation and BALF in patients with IPF,<sup>[26]</sup> suggesting discriminate between local and systemic.

Second, we found that Sp but not Se AAb levels correlated with clinical parameters. Studies showed that IPF patients with circulating AABs positive had a better prognosis.<sup>[27,28]</sup> However, we do not know the correlation between local



**Figure 3:** The scatter plots of the correlations between Sp-anti-P0 and  $DL_{CO}$ . Sp = Sputum; P0 = Anti-ribosomal p antibody;  $DL_{CO}$  = Carbon monoxide diffusing capacity; FI = Fluorescence intensity



**Figure 4:** The scatter plots of the correlations between Sp-anti-Scl-70 and FVC (%). Sp = Sputum; Scl-70, DNA topoisomerase; FVC = Forced vital capacity; FI = Fluorescence intensity

AABs and prognosis of the IPF patients. IPF Patients with anti-HSP70 AABs have more near-term lung function deterioration and mortality.<sup>[16]</sup> However, anti-ENA AAb profiles of Se showed no correlation with clinical parameters. It may indicate patients with IPF existed phenotypic heterogeneity or Sp-AABs could better reflect the progression of the disease. Therefore, from a clinical point of view, the current study also indicates a poor correlation between local and systemic autoimmunity. Besides, some patients with IPF are difficult to cooperate with lung function tests. From our research, we can find that Sp examination is non-invasive and more representative and more advantageous than blood.

### Strengths and limitations

We investigate the relationships of two compartments by detecting AAb profiles in high throughput and sensitivity between Se and Sp-. Ultrasonic aspiration of saline was used to collect deep viscera in patients with stable IPF, and the same patient Sp was collected within 6 days to verify the reproducibility of Sp.<sup>[29]</sup> However, there were some limitations should be addressed. First, IPF is a rare disease, the sample patient group is a little bit small (21 patients) to assess a correlation between AAb and clinical parameters. Second, because it is an observational study, we cannot draw conclusions about the causal relationship between the Sp-AAb levels and lung function. Third, because the severe IPF patients cannot withstand induced Sp, the IPF patients' lung function were mild to moderate. Besides, 40% patients couldn't be induced Sp- and all the enrolled patients were male, which may lead to select bias. Fourth, as a preliminary study, only 10 AABs have been detected in our study. Fifth, validation cohort is needed to verify this finding. Moreover, more AABs should be detected in the further study. Besides, IgM and IgA could also reflect airway situation of IPF, which we have not been detect. From the research, IPF is an interstitial disease, and the

detection of alveolar lavage fluid and lung tissue is more representative. However, Sp testing is more advantageous in terms of noninvasiveness and repeatability.

### CONCLUSION

We observed that Sp-AABs and Se-AABs differed significantly in their characteristics and their association with IPF. Sp-AABs are more relevant to clinical parameters and disease severity than Se-AABs. Based on the above data, Sp-AABs, a marker of local autoimmune phenomena, may help us better understand the role of autoimmunity in the development of IPF disease and provide new ideas for effective and novel treatment. Therefore, we hypothesize that IPF patients mainly exist pathological autoimmune components in the airway (local) rather than in circulation (system), which need to be confirmed by further research.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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