

Proteomic analysis identifies novel molecular signatures and immune-metabolic pathways in rheumatoid arthritis-associated interstitial lung disease

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Received May 3, 2025; Accepted September 5, 2025

DOI: 10.3892/br.2025.2065

Abstract. Rheumatoid arthritis (RA)-associated interstitial lung disease (RA-ILD) is a severe extra-articular manifestation of RA characterized by complex pathogenesis and limited therapeutic options. The present study aimed to identify circulating serum proteins that may reveal novel molecular mechanisms underlying RA-ILD and inform the development of disease-modifying strategies. A multi-center cohort study was conducted including patients with RA-ILD (n=40), patients with RA but without ILD (n=40) and healthy controls (n=7). Pooled serum samples were analyzed using a high-throughput antibody array targeting 440 proteins. Differentially expressed proteins were defined by statistical criteria ($P < 0.05$) and a fold change > 1.2 or < 0.83 . Functional enrichment and protein-protein interaction (PPI) analyses were performed to explore associated biological pathways. A total of 20 proteins that showed a stepwise increase in expression were identified: Levels were significantly higher in patients with RA compared with healthy controls and further elevated in patients with RA-ILD relative to RA alone. Hierarchical clustering and principal component analysis revealed distinct protein expression profiles across groups. Gene Ontology analysis indicated enrichment in pathways related to immune

cell activation, proliferation and cytokine production. Kyoto Encyclopedia of Genes and Genomes pathway analysis highlighted cytokine-cytokine receptor interactions and PI3K-Akt signaling. PPI network analysis identified insulin as a central hub interacting with IGF-1R, IL-7, and other profibrotic mediators. Several proteins (for example, CA9, EDA-A2, Gas1, CRTAM, IL-2Rb and IL-31) emerged as novel candidates not previously linked to RA-ILD. The present study identified a panel of 20 dysregulated serum proteins in RA-ILD, implicating immune dysregulation, fibrotic processes and metabolic signaling-particularly the insulin/IGF-1R-PI3K-Akt axis-in disease pathogenesis. These findings provide potential therapeutic targets for RA-ILD that warrant further validation.

Introduction

Rheumatoid arthritis-associated interstitial lung disease (RA-ILD) is a serious extra-articular manifestation of RA that significantly increases morbidity and mortality. Epidemiological studies indicate that 10-40% of patients with RA develop ILD, with mortality rates up to 3-fold higher than those with RA without pulmonary involvement (1,2). RA-ILD is marked by progressive pulmonary fibrosis and inflammation, ultimately leading to respiratory failure and reduced survival. It accounts for 10-20% of RA-related deaths (3,4). The high prevalence and poor prognosis highlight the urgent need for improved understanding of its pathogenesis and more effective management strategies.

The pathogenesis of RA-ILD remains poorly understood. Diagnostic delays are common due to nonspecific early symptoms and the absence of validated screening protocols, which often result in treatment initiation only after irreversible lung damage has occurred (5-7). Current evidence points to a multifactorial etiology involving genetic susceptibility (for example, *HLA-DRB1* alleles), environmental exposures (for example, smoking), and autoimmune dysregulation (8,9). Molecular mechanisms driving RA-ILD include abnormal immune activation (for example, TNF- α and IL-6), fibroblast proliferation and dysregulated extracellular matrix

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Abbreviations: RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease; HRCT, high-resolution computed tomography; PCA, principal component analysis; GO, Gene Ontology; KEGG, Kyoto encyclopedia of genes and genomes; PPI, protein-protein interaction

Key words: RA, ILD, cytokines, inflammation, fibrosis

deposition-pathways that parallel those in idiopathic pulmonary fibrosis (IPF) (10-12). Emerging evidence implicates neutrophil extracellular traps (NETs), YKL-40 and KL-6 in lung injury, while signaling pathways such as AKT/TMEM175 and JAK-STAT may contribute to fibrosis progression (13-16). However, the exact molecular drivers linking RA to ILD remain unclear, and currently available biomarkers (for example, KL-6 and YKL-40) lack sufficient specificity for broad clinical application (13). Management primarily relies on immunosuppressive agents (for example, methotrexate and rituximab) and antifibrotic therapies (for example, nintedanib and pirfenidone), but their effectiveness is limited by heterogeneous patient responses and the lack of RA-ILD-specific targeted treatments (12,17). For instance, antifibrotic agents have demonstrated benefit in IPF, their effectiveness in RA-ILD has been inconsistent, likely due to distinct underlying molecular pathways (18). These challenges highlight the urgent need for precision medicine strategies tailored to the unique pathogenesis of RA-ILD (19,20).

Elucidating the molecular mechanisms underlying RA-ILD is crucial for the discovery of novel therapeutic targets. Emerging evidence implicates that dysregulated immune pathways (such as IL-36 and NETs), aberrant fibroblast activation and interactions between the gut and lungs play a role in disease progression (11,21). For example, targeting NETs may help mitigate lung injury, while modulation of the gut microbiota could attenuate systemic inflammation (9,16). Despite these advances, our understanding of RA-ILD pathogenesis remains incomplete, limiting the development of effective targeted therapies. The present study aimed to identify novel molecular mechanisms that could inform the design of disease-modifying strategies for RA-ILD.

Materials and methods

Study design and population. The present study was conducted as part of a multi-center cohort that includes participants from Beijing Haidian Hospital and Beijing Shunyi Hospital. The study population consisted of three groups: (i) Patients diagnosed with RA-ILD, (ii) patients with RA but without ILD and (iii) healthy individuals. All patients with RA met the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA (22), while patients with RA-ILD also met the diagnostic criteria for ILD established by the American Thoracic Society/European Respiratory Society (23). Health controls were selected from individuals without inflammatory or rheumatic diseases. All participants underwent pulmonary function tests and chest high-resolution computed tomography (HRCT). Patients with RA but without ILD and healthy controls showing pulmonary symptoms or abnormal HRCT findings were excluded to ensure specificity. The study protocol was approved by the Research Ethics Committees of Beijing Haidian Hospital (approval no. 2024-006; Beijing, China) and Beijing Shunyi Hospital (approval no. 2023k-021; Beijing, China). Written informed consent was obtained from all participants prior to enrollment. Blood samples were collected for further analysis.

Antibody array assay. Serum proteins were analyzed in three cohorts: Healthy controls (n=7), RA (n=40, with 10 subjects

pooled), and RA-ILD (n=40, with 10 subjects pooled). The clinical characteristics of the participants are summarized in Table I. Serum cytokine profiling was performed using the Human Cytokine Antibody Array (cat. no. GSH-CAA-440; RayBiotech, Inc.), a high-throughput platform with 11 non-overlapping arrays for simultaneous detection of 440 cytokines. Peripheral blood serum samples were diluted 1:2 with blocking buffer and incubated overnight in array chambers coated with cytokine-specific capture antibodies. After washing away unbound proteins, a biotin-conjugated anti-cytokine antibody cocktail was added to form antibody-cytokine-antibody sandwich complexes. Cy3-conjugated streptavidin was then applied to amplify fluorescent signals via biotin-streptavidin binding. All incubation steps were conducted with 100 μ l of reagents per well. Fluorescence intensity was measured with an InnoScan 300 Microarray Scanner (Innopsys) at optimized 532 nm excitation/emission wavelengths for Cy3. Raw signal values were normalized against internal positive and negative controls for assay reproducibility.

Enzyme-linked immunosorbent assays (ELISAs). Insulin and IL-31 were validated by ELISAs using commercially available kits (Human Insulin ELISA Kit; cat. no. ELH-Insulin; Human IL-31 ELISA Kit; cat. no. ELH-IL31; RayBiotech, Inc.) with an expanded cohort consisting of 64 patients with RA-ILD, 64 patients with RA and 40 healthy controls (demographics provided in Table II). Briefly, serum samples were incubated in antibody-precoated wells overnight at 4°C. Following washing steps, biotin-conjugated detection antibodies were added and incubated for 2 h. Horseradish peroxidase-conjugated streptavidin was then applied to bind the biotinylated complexes for 45 min. The enzymatic reaction was developed using tetramethylbenzidine substrate form 30 min. After the reaction was stopped, absorbance was measured at 450 nm using an ELx800NB microplate reader (BioTek; Agilent Technologies, Inc.).

Statistical analysis. Statistical comparisons between experimental groups were conducted using a one-way ANOVA followed by Bonferroni's post hoc test in SPSS version 20.0 (IBM Corp.). Differences were considered statistically significant when meeting two criteria: (i) $P < 0.05$; (ii) a fold change threshold of < 0.83 (downregulation) or > 1.2 (upregulation). Continuous data were expressed as the mean $\pm$ standard deviation (SD). Correlation analyses between serum biomarkers (IL-31 and insulin) and clinical parameters of RA-ILD severity were performed using GraphPad Prism 9.0 (GraphPad Software; Dotmatics). Pearson's correlation coefficient (r) was calculated to assess linear relationships between biomarkers and clinical parameters. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Proteomic screening identifies 20 proteins associated with RA and RA-ILD progression. A panel of 20 proteins demonstrated sequential upregulation, significantly elevated in patients with RA compared with healthy controls ($P < 0.05$), and showing even greater increases in patients with RA-ILD compared with patients with RA ($P < 0.05$) (Fig. 1). These proteins

Table I. Clinical information of subjects for the antibody array assay.

Characteristics	RA-ILD	RA	Healthy	P-value (RA-ILD vs. RA)
n	40	40	7	
Age, years	62.5±9.5	59.2±6.9	60.1±2.1	0.074
Sex (F/M)	30/10	30/10	4/3	
KL-6 (U/ml)	647.0±221.4	178.6±42.2	181.6±55.0	<0.001
DAS28	4.2±1.5	3.3±7.9	-	0.007
CRP (mg/l)	2.3±2.2	1.0±1.6	-	0.004
VAS	34.8±31.9	11.9±16.7	-	<0.001
ESR (mm/h)	45.4±22.3	31.6±19.1	-	0.004
D-dimer (mg/l)	1.3±0.7	0.4±0.4	0.16±0.1	<0.001
FVC (%)	60.3±13.4	93.2±9.4	-	<0.001
DLCO (%)	57.2±15.7	91.9±7.3	-	<0.001
FEV1 (%)	59.4±14.0	90.2±6.3	-	<0.001
SLS I	2.6±1.1	0±0	-	<0.001

RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease; DAS28, disease activity score in 28 joints; CRP, C-reactive protein; VAS, visual analogue scale; ESR, erythrocyte sedimentation rate; FVC, forced vital capacity; FEV1, forced expiratory volume in the first second; DLCO, diffusing capacity of the lung for carbon monoxide; SLS I, scleroderma lung study I.

Table II. Clinical information of subjects for ELISA.

Characteristics	RA-ILD	RA	Healthy	P-value (RA-ILD vs. RA)
n	64	64	40	
Age, years	65.6±9.6	62.1±8.0	57.8±9.9	0.081
Sex (F/M)	40/24	51/13	23/17	
KL-6 (U/ml)	457.3±260.2	208.0±127.6	-	<0.001
DAS28	4.1±1.5	3.2±1.3	-	<0.001
CRP (mg/l)	2.2±2.0	0.9±1.5	-	<0.001
VAS	32.4±29.1	12.9±16.5	-	<0.001
ESR (mm/h)	43.3±19.9	33.1±20.9	-	<0.001
D-dimer (mg/l)	1.2±0.8	0.4±0.4	-	<0.001
FVC (%)	63.3±13.5	92.1±7.7	-	<0.001
DLCO (%)	61.2±15.1	91.5±6.8	-	<0.001
FEV1 (%)	62.7±13.2	90.9±6.0	-	<0.001
SLS I	6.5±5.6	0±0	-	<0.001

RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease; DAS28, Disease activity score in 28 joints; CRP, C-reactive protein; VAS, visual analogue scale; ESR, erythrocyte sedimentation rate; FVC, forced vital capacity; FEV1, forced expiratory volume in the first second; DLCO, diffusing capacity of the lung for carbon monoxide; SLS I, scleroderma lung study I.

included carbonic anhydrase IX (CA9), IL-1F9, ectodysplasin A2 (EDA-A2), growth arrest-specific gene 1 (Gas1), IL-2Rb, IL-7, amphiregulin (AR), sonic hedgehog N-terminal (Shh-N), IGF-1R, MMP-7, insulin, Eotaxin, EpCAM, tissue factor pathway inhibitor (TFPI), MIP-3b, CRTAM, IL-31, tissue factor (TF), syndecan-1 and cathepsin L. The information of these 20 proteins is summarized in Table III.

Classification capacity of the 20 proteins. To assess the discriminatory value of the 20 proteins, hierarchical clustering and principal component analysis (PCA) were performed.

Using normalized expression data from the pooled analysis, hierarchical clustering of these proteins unveiled unique expression patterns and demonstrated 100% accuracy in classifying RA-ILD from RA and the healthy groups (Fig. 2). PCA effectively segregated RA, RA-ILD, and healthy controls into separate clusters within the reduced-dimensional space (Fig. 3).

Functional enrichment analysis. Gene Ontology (GO) enrichment analysis revealed critical biological processes associated with the 20 proteins, including T cell differentiation,

Table III. Information on the 20 proteins.

Protein	Uniport ID	RA/Con	RA-ILD/RA	Known functions	Novelty in RA-ILD
CA9	Q16790	1.4	1.8	Response to hypoxia	Induces hypoxic fibrosis
IL-1F9	Q9NZH8	4.5	1.7	Interleukin-1 receptor binding	A proinflammatory cytokine in the lung disease
EDA-A2	Q92838	3.5	1.5	A ligand activating the DEATH-domain containing receptors EDAR and EDA2R	Activates the inflammatory pathway NF- κ B signaling
Gas1	P54826	4.4	1.8	Specific growth arrest protein involved in growth suppression	Gas1/Axl pathway
IL-2Rb	P14784	2.5	1.8	Receptor for interleukin-2	Contributes to immune microenvironment disorder
IL-7	P13232	2.1	1.4	Regulates B cell proliferation	Exacerbates IPF
AR	P15514	1.7	2.3	Ligand of the EGF receptor/EGFR	Involves into inflammatory lung disease
Shh-N	Q15465	3.0	2.6	Displays an autoprolysis and a cholesterol transferase activity	Promotes pulmonary fibrosis
IGF-1R	P08069	3.9	2.2	Receptor tyrosine kinase which mediates actions of insulin-like growth factor 1	Promotes IPF
MMP-7	P09237	4.5	1.6	Activates pro-collagenase	A mediator of extracellular matrix remodeling in IPF
Insulin	P01308	4.1	1.8	Decreases blood glucose concentration	Participates in PI3K-Akt signaling
Eotaxin	P51671	7.0	2.0	Promotes the accumulation of eosinophils	Produces profibrotic cytokines contributing to pulmonary fibrosis
EpCAM	P16422	14.0	2.6	Plays a role in embryonic stem cells proliferation and differentiation	Involves into IPF
TFPI	P10646	11.3	1.6	Inhibits VIIa/tissue factor activity	Involves into IPF
MIP-3b	Q99731	5.9	2.2	Chemokine activity	Involves into IPF
CRTAM	O95727	8.8	2.0	Mediates heterophilic cell-cell adhesion	Activates STAT signaling
IL-31	Q6EBC2	18.3	2.2	Activates STAT3	Activates JAK-STAT signaling
TF	P13726	57.2	2.3	Initiates blood coagulation	Involves into IPF
Syndecan-1	P18827	1.9	1.8	Cell surface proteoglycan	Exacerbates inflammation-to-fibrosis transitions in pulmonary fibrosis
Cathepsin L	P07711	4.7	2.7	Regulates CD4 ⁺ T cell positive selection	Involves into IPF

RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease; IPF, idiopathic pulmonary fibrosis; TFPI, tissue factor pathway inhibitor; CA9, carbonic anhydrase IX.

T cell activation, lymphocyte differentiation, mononuclear cell proliferation, leukocyte proliferation and positive regulation of cytokine production. Further analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway implicated two key pathways cytokine-cytokine receptor interaction and the PI3K-Akt signaling pathway (Fig. 4).

Protein-protein interaction (PPI) analysis. PPI network analysis identified insulin as the highest-degree hub protein

(node degree=6), showing direct interactions with multiple profibrotic and immunomodulatory mediators: IGF-1R, Shh-N, AR, TF, IL-7 and EpCAM (Fig. 5). This indicates its central role in coordinating molecular crosstalk within the RA-ILD proteomic landscape.

ELISA validation and clinical value analysis. The independent validation of insulin and IL-31 with an expanded cohort by ELISA demonstrated strong concordance with the initial

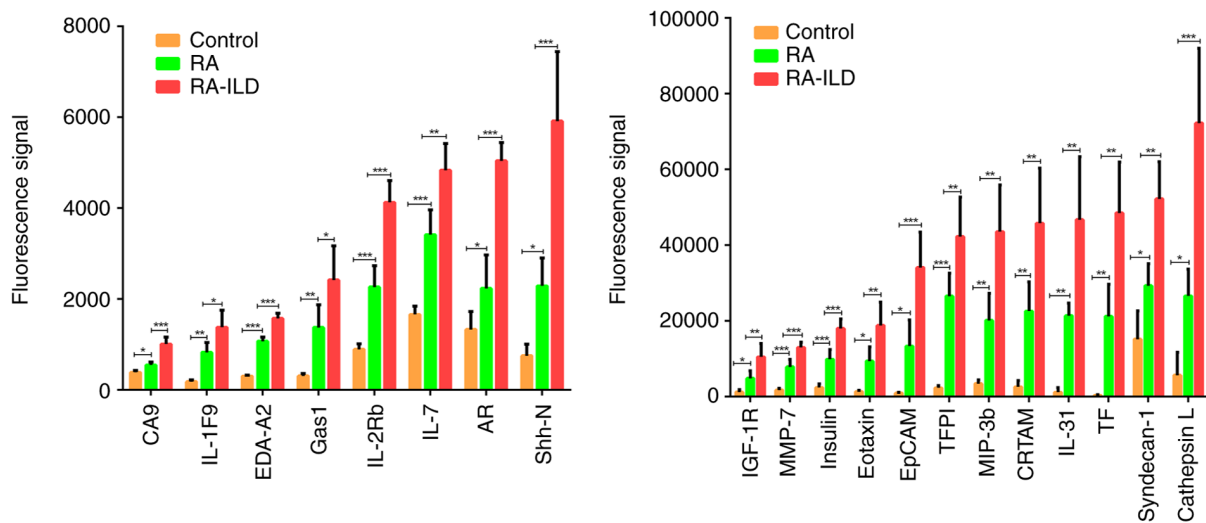


Figure 1. Histogram. Serum protein levels were significantly higher in patients with RA compared with healthy controls, and even more elevated in patients with RA-ILD compared with patients with RA. The bar graph illustrates the relative expression levels of 20 differentially expressed proteins among healthy controls, patients with RA and patients with RA-ILD. The data are presented as mean $\pm$ SD. The data from patients with RA and patients with RA-ILD represent pooled samples. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease; TFPI, tissue factor pathway inhibitor; CA9, carbonic anhydrase IX; AR, amphiregulin; Shh-N, sonic hedgehog N-terminal; Gas1, growth arrest-specific gene 1; EDA-A2, ectodysplasin A2; TF, tissue factor.

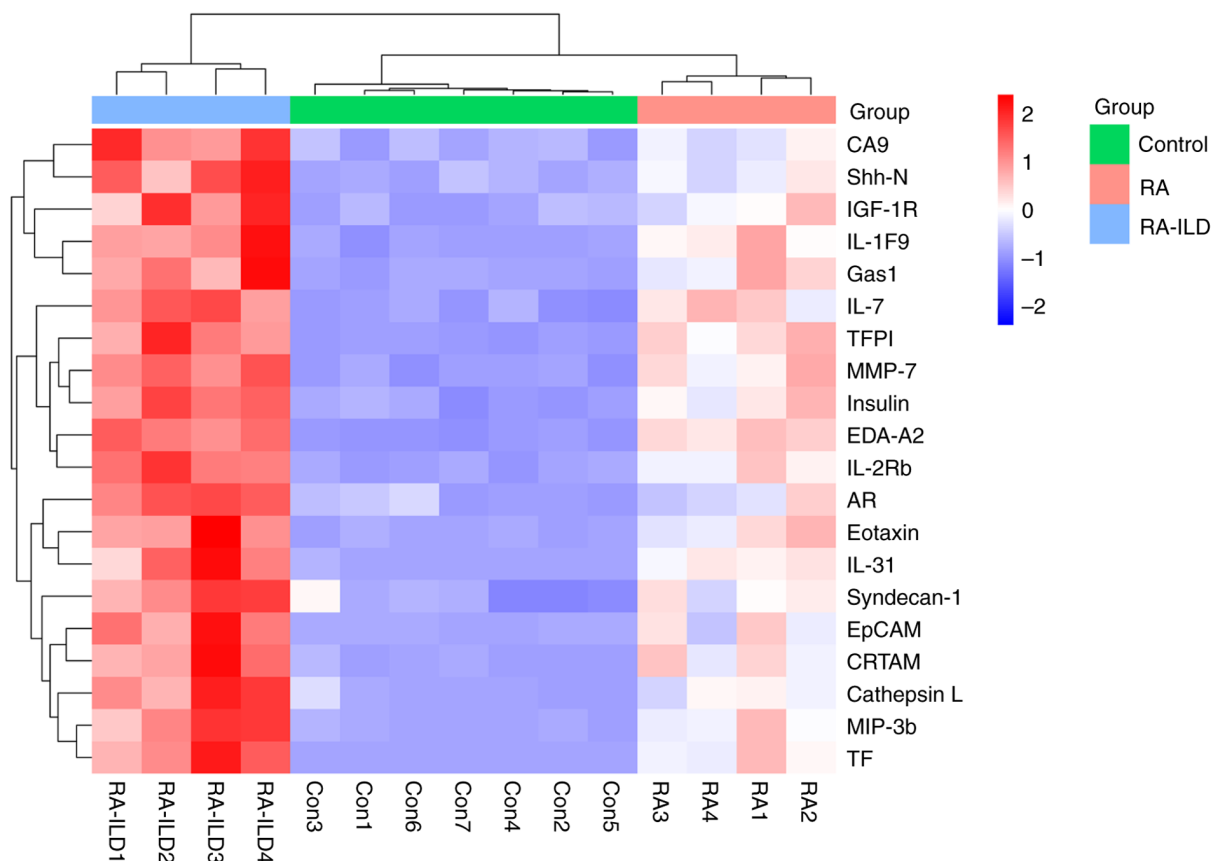


Figure 2. Heatmap. This heatmap represents hierarchical clustering analysis based on the expression patterns of 20 proteins, showing distinct molecular signatures among the three groups. The color scale indicates normalized protein expression levels (blue: low; red: high). The data from patients with RA and patients with RA-ILD represent pooled samples. RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease; TFPI, tissue factor pathway inhibitor; CA9, carbonic anhydrase IX; AR, amphiregulin; Shh-N, sonic hedgehog N-terminal; Gas1, growth arrest-specific gene 1; EDA-A2, ectodysplasin A2; TF, tissue factor.

antibody array data (Fig. 6). The diagnostic potential of these two biomarkers showed excellent discriminatory capacity

with area under the curve values of $>75\%$ (Fig. 7). Notably, the correlation analysis revealed a strong positive correlation

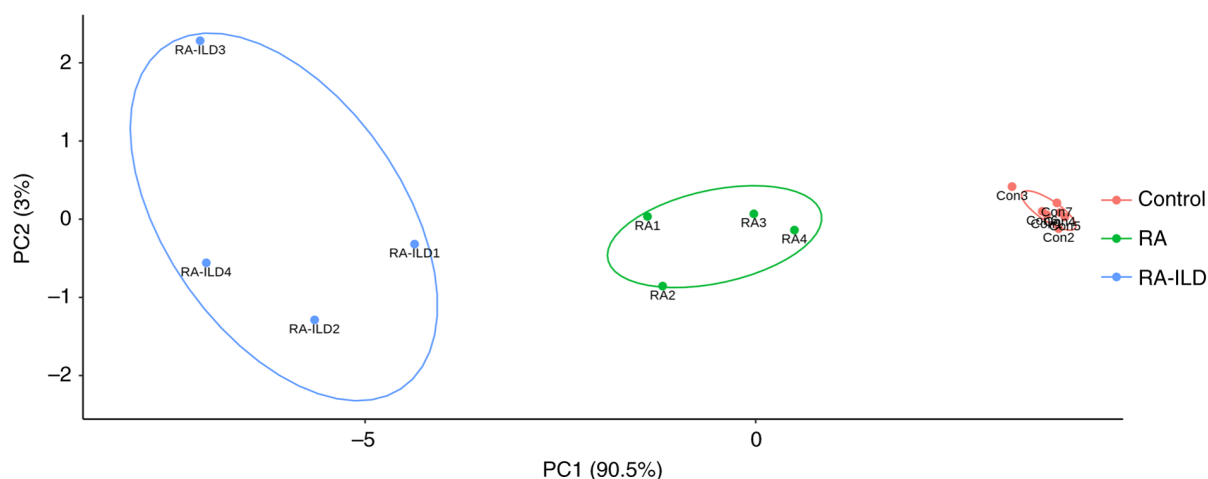


Figure 3. PCA. The two-dimensional PCA score plot demonstrates clear clustering of healthy controls, patients with RA, and patients with RA-ILD based on the expression patterns of 20 differentially expressed proteins. Principal components 1 and 2 (PC1 and PC2) account for 90.5 and 3% of the total variance, respectively. The ellipses on the plot represent 95% confidence intervals for each group. The data from patients with RA and patients with RA-ILD are from pooled samples. PCA, principal component analysis; RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease.

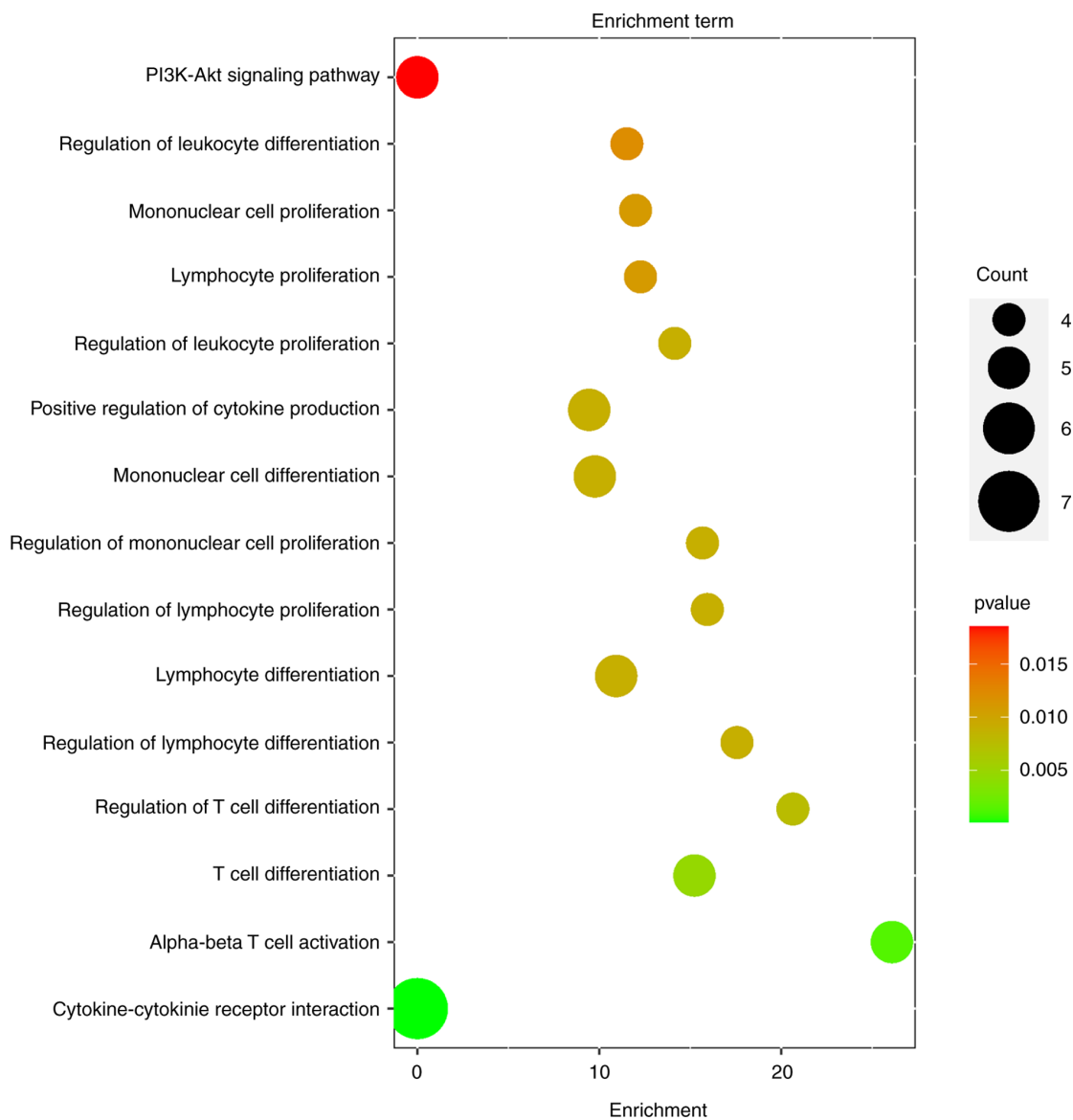


Figure 4. Bioinformatic analysis. The bubble plot displays significantly enriched Gene Ontology terms and Kyoto encyclopedia of genes and genomes pathways. The size of bubble represents the number of enriched proteins in each term, while the color intensity indicates the $-\log_{10}(P\text{-value})$.

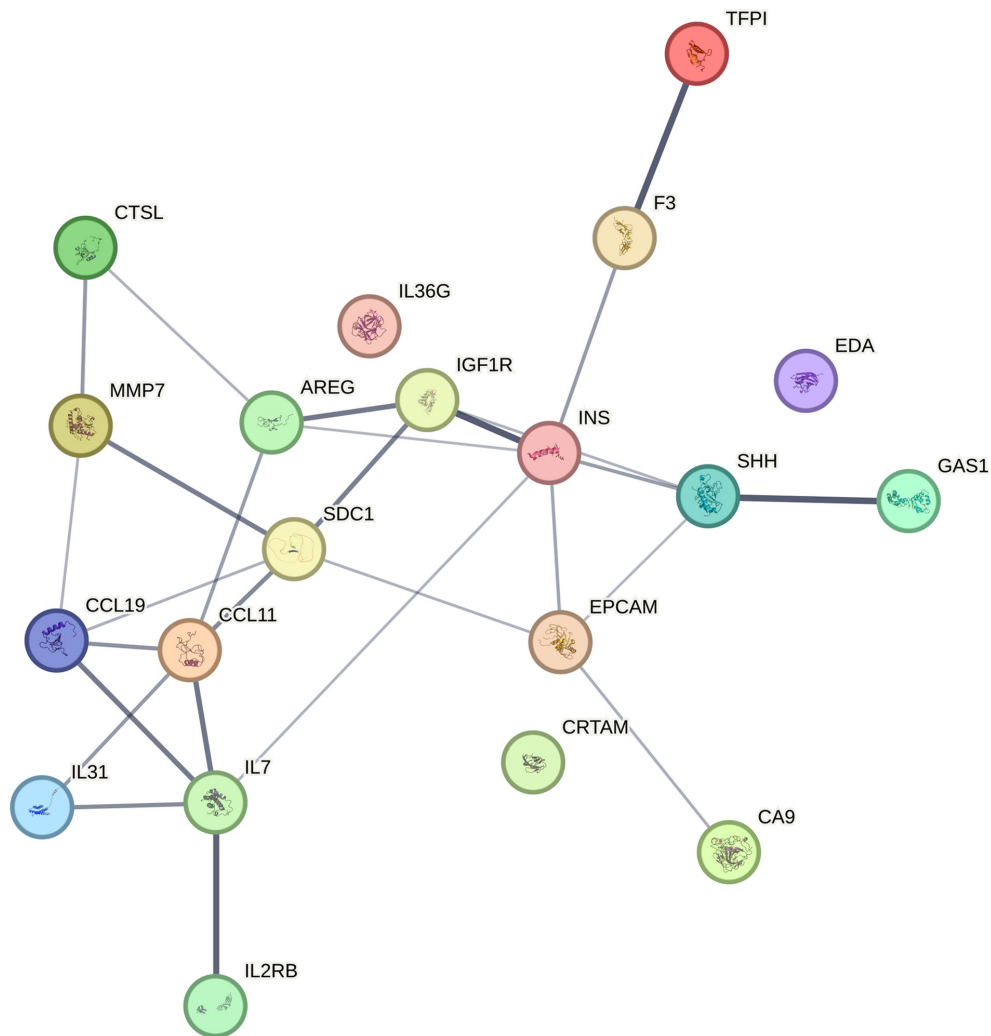


Figure 5. STRING-based protein-protein interaction network of the 20 differentially expressed proteins. Lines connecting two proteins represent a biological functional correlation. The thickness of the edges indicates the combined interaction scores, which exceeded the 0.7 confidence threshold. TFPI, tissue factor pathway inhibitor; CA9, carbonic anhydrase IX; Gas1, growth arrest-specific gene 1; EDA, ectodysplasin.

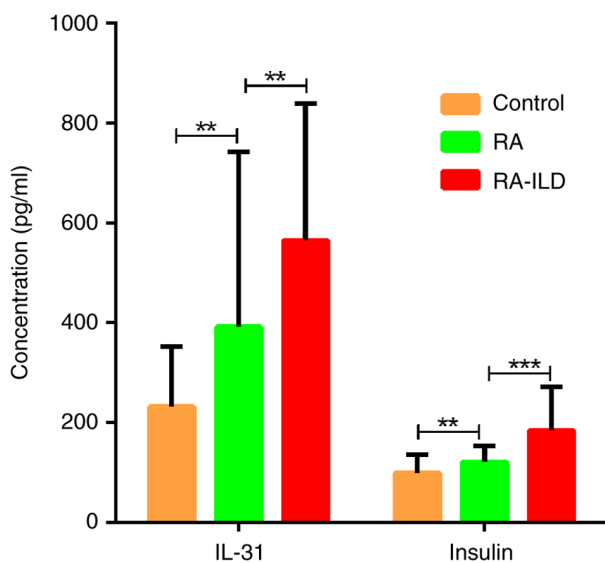


Figure 6. Showing serum concentrations of IL-31 and insulin measured by ELISA. The bar graph compares the mean serum levels of IL-31 and insulin with error bars representing the standard deviation of the measurements. ** $P < 0.01$ and *** $P < 0.001$. RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease.

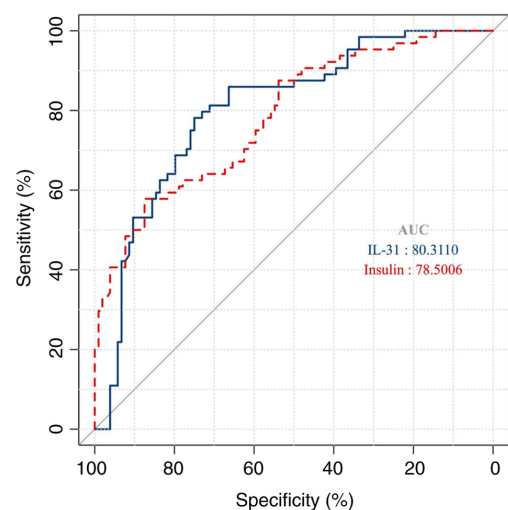


Figure 7. Receiver Operating Characteristic curve analysis for IL-31 and insulin. The figure compares the diagnostic performance of IL-31 (AUC=80.31%) and insulin (AUC=78.50%) in distinguishing between non-RA-ILD and RA-ILD. Higher AUC values indicate better predictive accuracy, with IL-31 demonstrating slightly superior performance over insulin in this analysis. AUC, area under the curve; RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease.

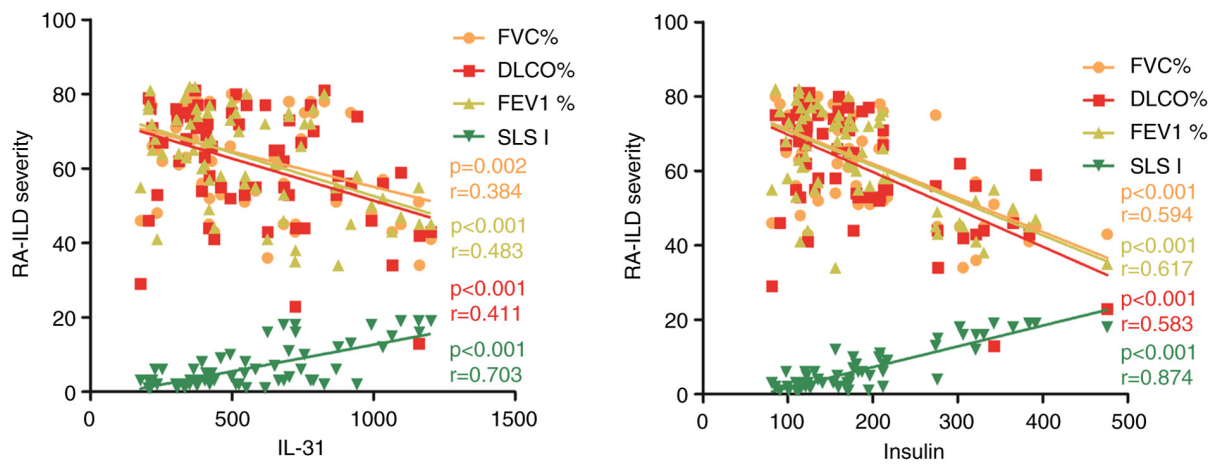


Figure 8. Correlation between serum IL-31 and insulin levels with disease severity markers in patients with RA-ILD. The clinical parameters used to assess disease severity in RA-ILD for the correlation analysis with IL-31 and insulin levels include percentage of FVC, DLCO and FEV, and SLS I. RA, rheumatoid arthritis; RA-ILD, RA-associated interstitial lung disease; FVC, forced vital capacity; DLCO, diffusing capacity of the lung for carbon monoxide; FEV1, forced expiratory volume in the first second; SLS I, scleroderma lung study I.

between these two biomarkers and the Scleroderma Lung Study I score of HRCT images (Fig. 8, $r>0.6$). This finding suggests that insulin and IL-31 may play a role in the disease activity of RA-ILD.

Discussion

RA-ILD is a severe extra-articular manifestation of RA, characterized by progressive pulmonary fibrosis and high morbidity. Its etiology is multifactorial and complex, involving genetic predisposition (for example, *MUC5B* promoter variants), autoimmune-driven inflammation, aberrant fibroblast activation, and environmental exposures such as smoking. The interplay of these factors leads to dysregulated immune responses and irreversible lung remodeling (10). However, the precise molecular mechanisms triggering these immune responses remain elusive, limiting the development of effective targeted therapies for RA-ILD. In the present study, 20 proteins including CA9, IL-1F9, EDA-A2, Gas1, IL-2Rb, IL-7, AR, Shh-N, IGF-1R, MMP-7, insulin, Eotaxin, EpCAM, TFPI, MIP-3b, CRTAM, IL-31, TF, Syndecan-1 and Cathepsin L were identified, which were significantly elevated in patients with RA compared with healthy controls, with further upregulation in RA-ILD relative to RA alone. The stepwise increase in these proteins suggests their contribution to RA pathogenesis and progression to ILD, the most common and serious pulmonary complication of RA.

GO analysis revealed that these 20 proteins were enriched in immune-related processes, including T cell differentiation, T cell activation and cytokine production. These findings align with prior studies implicating aberrant T cell responses and cytokine dysregulation in RA-ILD pathogenesis (24-26). KEGG pathway analysis highlighted the cytokine-cytokine receptor interaction pathway and PI3K-Akt signaling pathway, both of which play key roles in immune cell proliferation and differentiation in ILD (27). The PI3K-Akt pathway is known to drive fibroblast activation and fibrosis in ILD (28). Moreover, cytokine-receptor interactions -such as IL-7/IL-7R, IGF-1R/insulin-may perpetuate chronic

inflammation and fibrogenesis (29,30). Notably, Wu *et al* (31) reported that the AhR/IGF1R axis contributes to the development of IPF through activation of the TGF- β /Smad/STAT signaling cascade. In the present study, KEGG pathway analysis revealed that IGF-1R and insulin were involved in the PI3K-Akt signaling pathway, suggesting a potential role for the insulin/IGF-1R axis in the progression of RA to ILD through this pathway. It was hypothesized that the insulin/IGF1R axis could be a promising therapeutic target for the treatment of RA-ILD. PPI analysis revealed that insulin is a central hub protein that interacts with IGF-1R, Shh-N, AR, TF, IL-7 and EpCAM. These interactions suggest that insulin and its network partners may collectively regulate immune and metabolic pathways in RA-ILD, potentially exacerbating disease progression. While no previous studies have directly linked insulin to RA-ILD, its network prominence and functional interactions generate a strong interest in investigating the modulation of the insulin pathway as a potential therapeutic strategy. Further validation is needed to confirm the potential involvement of insulin in RA-ILD.

Furthermore, among these 20 proteins, several have well-established roles in RA or pulmonary fibrosis. For example, IL-7 promotes T cell survival and Th17 differentiation (29), and its elevation has been linked to the exacerbation of IPF (32). MMP-7 serves as a predictive biomarker of disease progression and mediates extracellular matrix remodeling in IPF (33). IL-1F9 acts as a proinflammatory cytokine in lung disease by enhancing chemokine production and inflammatory cell recruitment (34). Eotaxin is associated with increased pulmonary infiltration of eosinophils and neutrophils, as well as the production of profibrotic cytokines contributing to pulmonary fibrosis (35). Both cathepsin L and tissue factor have been implicated in the pathogenesis of IPF and ILD (36,37). AR is elevated in inflammatory lung disease associated with RA (38), and EpCAM, TFPI and MIP-3b are upregulated in IPF (39-41). Shh-N promotes pulmonary fibrosis through the hedgehog signaling pathway (42), and Syndecan-1 shedding exacerbates the transition from inflammation to fibrosis by releasing heparan sulfate-bound growth factors (43).

Notably, CA9, EDA-A2, Gas1, CRTAM, IL-2Rb and IL-31 have emerged as novel candidates, with no prior studies linking them to RA-ILD or fibrotic diseases. However, chronic inflammation is a well-established precursor to fibrotic tissue remodeling (44), suggesting that these proteins may contribute indirectly to fibrosis through sustained inflammatory signaling. Among these novel targets, EDA-A2 activates the inflammatory responses through NF- κ B signaling by binding to the EDA receptor (45). CRTAM promotes STAT signaling via STAT1 phosphorylation (46). IL-2Rb contributes to immune microenvironment disorder by disrupting the Th1/Th2 cell differentiation balance (47), and IL-31 drives inflammation primarily through JAK-STAT pathway activation (48). Gas1 may play a critical role in fibrotic diseases, including RA-ILD through the Gas1/Axl axis, analogous to the pro-fibrotic Gas6/Axl signaling pathway (49,50). In addition, CA9, a hypoxia-inducible protein, may promote hypoxic-associated fibrosis (51). These findings indicate the potential of these novel candidates to drive RA-ILD progression through their roles in inflammatory and hypoxic signaling, warranting further investigation.

However, the present study has several limitations. First, the cohort included a relatively small number of healthy controls (n=7), which limited the statistical power to detect biologically relevant differences between healthy individuals and patient groups. Second, the use of pooled samples for the antibody array analysis-implemented due to cost constraints associated with high-throughput proteomic screening-represents a significant methodological limitation. Third, the present study only validated insulin (the highest-degree hub protein) and IL-31 (a novel candidate) using independent methods due to budget limitations. Fourth, the functional roles of the identified protein signatures were not experimentally validated. Future studies should prioritize individual sample analysis, larger cohort sizes, and rigorous validation of all these candidate proteins as potential biomarkers. In addition, *in vitro* and *in vivo* studies are warranted to elucidate the functional relevance of these proteins, particularly those with novel associations, and to explore their potential as therapeutic targets for RA-ILD.

In conclusion, our study highlights the complex molecular interplay underlying RA-ILD pathogenesis. A total of 20 dysregulated proteins that collectively drive immune dysregulation and fibrotic progression were identified. These proteins are functionally enriched in critical pathways, including T cell differentiation, cytokine-cytokine receptor interactions (such as IL-7/IL-7R and IGF-1R/Insulin), and the PI3K-Akt signaling pathway. The PPI network identifies insulin as a central hub, interacting with multiple profibrotic mediators (IGF-1R, Shh-N and AR) and immune modulators (IL-7, EpCAM), suggesting its pivotal role in orchestrating profibrotic and inflammatory responses in RA-ILD. While several proteins (MMP-7, IL-1F9 and Cathepsin L) have established roles in pulmonary fibrosis, our identification of novel candidates points to additional mechanisms involving sustained inflammatory signaling and hypoxia-responsive pathways. These findings provide new insight into RA-ILD pathogenesis and suggest that targeting the insulin/IGF1R-PI3K-Akt axis may represent a promising therapeutic strategy to disrupt the immune-fibrotic cascade in this disease. Further validation and mechanistic studies are warranted to explore these potential targets.

Acknowledgements

Not applicable.

Funding

The present study was supported by the 2024 Haidian Health Development Research, Cultivation Plan Project (grant no. HP2024-30-101004) and the Beijing Shunyi District Hospital Research and Development Special Fund (grant no. 2025Y01).

Availability of data and materials

The data generated in the present study may be found in zenodo database under accession number 15852072 or at the following URL: (<https://zenodo.org/records/15852072>).

Authors' contributions

WC conducted all experiments and wrote the first draft of the manuscript. YZ and QY contributed to sample collection and processing. BY and QY conducted statistical analyses. GZ contributed to conception and design of the present study, and revised the manuscript. WC and GZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Approval was obtained from the Research Ethics Committees of Beijing Haidian Hospital (approval no. 2024-006; Beijing, China) and Beijing Shunyi Hospital (approval no. 2023-k-021; Beijing, China). All participants provided informed consent to participate in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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