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Characterization and Peripheral Blood Biomarker Assessment of Jo-1 Antibody-Positive Interstitial Lung Disease

Thomas J. Richards, Ph.D.[1,*], **Aaron Eggebeen, M.D.**[2,*], **Kevin Gibson, M.D.**[1], **Samuel Yousem, M.D.**[3], **Carl Fuhrman, M.D.**[4], **Bernadette R. Gochuico, M.D.**[5], **Noreen Fertig, B.S.**[2], **Chester V. Oddis, M.D.**[2], **Naftali Kaminski, M.D.**[1], **Ivan O. Rosas, M.D.**[6], and **Dana P. Ascherman, M.D.**[2]

[1] Dorothy P. and Richard P. Simmons Center for Interstitial Lung Disease and Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

[2] Department of Medicine, Division of Rheumatology and Clinical Immunology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

[3] Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

[4] Department of Radiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

[5] Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD

[6] Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Abstract

Objectives—Combining clinical, radiographic, functional, and serum protein biomarker assessment, this study defines the prevalence and clinical characteristics of ILD in a large cohort of patients possessing anti-Jo-1 antibodies.

Methods—Clinical records, pulmonary function testing, and imaging studies determined the existence of ILD in anti-Jo-1 antibody positive (anti-Jo-1 Ab+) individuals accumulated in the University of Pittsburgh Myositis Database from 1982–2007. Multiplex ELISA of serum inflammatory markers, cytokines, chemokines, and matrix metalloproteinases in different patient subgroups then permitted assessment of serum proteins associated with anti-Jo-1 Ab+ ILD.

Results—Among 90 anti-Jo-1 Ab+ individuals with sufficient clinical, radiographic, and/or pulmonary function data, 77 (86%) met criteria for ILD. While computerized tomography scans revealed a variety of patterns suggestive of underlying UIP or NSIP, review of histopathologic abnormalities in a subset (n=22) of individuals undergoing open lung biopsy demonstrated a preponderance of UIP and DAD. Multiplex ELISA yielded statistically significant associations between Jo-1 Ab+ ILD and elevated serum levels of CRP, CXCL9, and CXCL10 that distinguished this subgroup from IPF and anti-SRP Ab+ myositis. Recursive partitioning further

Corresponding Authors: Dana P. Ascherman, M.D. Department of Medicine, Division of Rheumatology and Clinical Immunology, University of Pittsburgh School of Medicine, BST S707, 3500 Terrace Street, Pittsburgh, Pennsylvania 15261, Phone: (412) 383-8734, FAX: (412) 383-8864, E-mail: ascher@pitt.edu. Ivan O. Rosas, M.D., Department of Internal Medicine, Division of Pulmonary Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, Phone: (617) 278-0434, FAX: (617) 264-5133, E-mail: irosas@rics.bwh.harvard.edu.
[*] contributed equally to this work

demonstrated that combinations of these and other serum protein biomarkers can distinguish these subgroups with high sensitivity and specificity.

Conclusion—In this large cohort of anti-Jo-1 Ab+ individuals, the incidence of ILD approaches 90%. Multiplex ELISA demonstrates disease-specific associations between Jo-1 Ab+ ILD and serum levels of CRP as well as the IFN-γ-inducible chemokines CXCL9 and CXCL10, highlighting the potential of this approach to define biologically active molecules contributing to the pathogenesis of myositis-associated ILD.

INTRODUCTION

Interstitial lung disease (ILD) is an increasingly recognized complication of several systemic autoimmune disorders, most notably rheumatoid arthritis, Sjogren's syndrome, systemic sclerosis, and idiopathic inflammatory myopathy (IIM). Perhaps the most recognizable variant of connective tissue disease-associated ILD is the anti-synthetase syndrome in which antibodies directed against Jo-1 (histidyl-tRNA synthetase) and other amino-acyl tRNA synthetases are associated with stereotypical clinical features that include myositis, fever, Raynaud phenomenon, arthritis, "mechanics hands," and ILD (1). While estimates of the overall incidence of ILD associated with inflammatory myopathy typically range from 5–45% (2–9), this systemic complication is clearly more prevalent among patients possessing anti-synthetase antibodies (10–14). In fact, depending on the clinical, functional, and radiographic criteria used to define ILD, a number of small studies have revealed incidence rates as high as 80–95% in anti-synthetase antibody-positive patients (13–15).

Limited pathologic case series have shown that ILD associated with the anti-synthetase syndrome encompasses many histologic subtypes, including cellular and fibrotic NSIP (non-specific interstitial pneumonia), COP (cryptogenic organizing pneumonia), UIP (usual interstitial pneumonia), and DAD (diffuse alveolar damage) (5,10,16,17). Response to therapy and overall prognosis largely reflect the underlying lung histology (18); therefore, the relative predominance of NSIP in published case series of myositis-associated ILD (regardless of autoantibody status) likely explains the improved survival rates relative to idiopathic pulmonary fibrosis (IPF) that classically demonstrates more severe UIP histology (10,19,20).

Although the histopathologic subtypes of myositis-associated ILD have been relatively well characterized, much less is known regarding patterns of altered gene and protein expression that predispose to ILD in the context of the anti-synthetase syndrome. A related question is the pathogenic relationship between myositis-associated ILD and IPF, particularly because both entities can involve UIP and DAD histology. Pertinent to these issues, recent work in sporadic and familial forms of IPF has employed a proteomics approach involving multiplex ELISA to define peripheral blood markers representative of changes in the alveolar microenvironment of IPF patients. These studies have shown that expression levels of matrix metalloproteinases 1 and 7 (MMP1, MMP7) are increased in IPF, corroborating previous gene expression profiles and providing pathogenic insight relevant to this disease (21).

Whether such findings apply to other forms of ILD remains unclear, but validation of this proteomics approach suggests similar utility for assessment of ILD stemming from the anti-synthetase syndrome. Because screening measures for ILD have traditionally relied on physical examination features, pulmonary function tests (PFTs), and imaging studies that have limited capacity to define etiologic subtypes of ILD or distinguish pulmonary disease activity from damage, multiplex ELISA represents a valuable tool for defining much needed biomarkers that predict the presence and course of disease. Equally important, this approach can potentially elucidate pathogenic processes responsible for generating different

histopathologic patterns of myositis- or autoimmune-associated ILD, supplementing existing markers such as KL-6 (mucin-like glycoprotein) that reflect alveolar damage but provide limited mechanistic insight.

Given the power and sensitivity of multiplex ELISA, we have applied this methodology in a relatively large cohort of well-characterized anti-Jo-1 antibody-positive (anti-Jo-1 Ab+) patients to define relevant peripheral blood biomarkers of ILD associated with the anti-synthetase syndrome. Compared to a control group of anti-signal recognition particle antibody-positive (anti-SRP Ab+) polymyositis patients lacking pulmonary involvement, this analysis shows that among patients possessing anti-Jo-1 antibodies, ILD is associated with elevations of CRP as well as the IFN-γ-inducible chemokines CXCL9 and CXCL10. Along with detailed clinical, functional, histological, and radiographic characterization of the largest individual anti-Jo-1 Ab+ cohort ever reported, these findings distinguish our analysis from previous studies and establish the foundation for peripheral blood molecular phenotyping of ILD associated with the anti-synthetase syndrome.

METHODS

Inclusion criteria and patient samples

Patients possessing anti-Jo-1 antibodies (anti-Jo-1 Ab) were derived from the University of Pittsburgh Myositis Database and included patients seen at the University of Pittsburgh Medical Center from 1982 through 2007 who consented to registry enrollment. Sera obtained at the time of clinical evaluation were tested for the presence of anti-Jo-1 Ab using immunodiffusion, with subsequent confirmation by ELISA. Inclusion in the current study required the presence of anti-Jo-1 Ab as well as appropriate clinical data, pulmonary function tests (PFTs), and imaging studies permitting the ascertainment of ILD (n=90). Diagnosis of ILD required a) chest x-ray abnormalities indicative of fibrosis with or without restrictive PFTs (some combination involving <80% predicted FEV1, FVC, TLC, D_LCO; >80% predicted FEV1/FVC) *or* b) an abnormal high resolution computerized tomography (HRCT) scan showing at least one of the following features (with or without restrictive physiology on PFTs): i) reticulation and fibrosis, ii) traction bronchiectasis, iii) honeycombing, or iv) ground glass opacification. In anti-Jo-1 Ab+ patients having imaging studies *and* PFTs within 90 days of serum sampling (n=42), we performed multiplex ELISA analysis of the serum protein markers detailed below.

Control sera were derived from normal subjects (n=7), individuals with anti-SRP antibody-positive (anti-SRP Ab+) myositis (in which anti-Jo-1 Ab is not present and lung involvement is rare; n=10), and patients with IPF (n=7). While subjects with IPF evaluated at the Warren Grant Magnussen Clinical Center of the National Institutes of Health (NIH) had mild to moderately severe disease based on radiographic and PFT abnormalities (22), each of the anti-SRP Ab+ myositis patients from the University of Pittsburgh Myositis Database satisfied Bohan and Peter criteria (23) for IIM with clinical evidence of active myositis at the time of serum sampling (as assessed by the muscle MITAX component of the MDAAT, a semi-quantitative index of clinical disease activity (24)). All subjects were recruited using institutional review board approved protocols.

Pulmonary Function Tests (PFTs)

Assessments of pulmonary function were performed according to ATS guidelines using standard equipment. Forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), total lung capacity (TLC), and diffusion capacity for carbon monoxide (D_LCO) were expressed as percentages of predicted values as denoted above.

Radiology

Conventional HRCT scans of the chest were performed without intravenous contrast during end-inspiration. Classification of HRCT abnormalities was based on independent readings of an expert chest radiologist (CF) and pulmonologist (KG), both of whom were blinded to histopathologic diagnoses and clinical features. Discrepant readings were re-reviewed by both experts to determine consensus classification.

Histopathology

Lung tissue derived from open biopsies, explants, or autopsy specimens was paraffin embedded, cut, and stained with hematoxylin and eosin according to standard protocols employed in the University of Pittsburgh Department of Surgical Pathology. Histological designations (using ATS criteria) were based on review by an experienced lung pathologist (SY) blinded to clinical data; none of the specimens were derived from patients with documented pulmonary infection

Bead-based multianalyte LabMAP™ profiling (Luminex)

Multiplex ELISAs were performed in designated Jo-1 Ab+ subjects as well as previously defined control groups using Luminex xMAP technology (Luminex Corp., Austin, TX) in 96-well microplate format according to manufacturers' protocols (Invitrogen, Camarillo, CA, and R&D Systems, Minneapolis, MN), as previously described (21,25). Samples were batch analyzed with the BioPlex suspension array system (Bio-Rad Laboratories, Hercules, CA). Fluorescence measures were converted to protein concentrations with a five-parameter logistic curve (5-PL). Assessed markers included 36 cytokines/chemokines, 5 matrix metalloproteinases (MMPs), and 3 acute phase proteins.

Sources of bead-based immunoassays—A 34-plex assay was performed for IL1α, IL1RA, IL1β, IL2, IL2R, IL4, IL5, IL6, IL7, IL8, IL10, IL12β, IL13, IL15, IL17, TNFα, IFNα, IFNγ, GMCSF, EGF, VEGF, GCSF, FGF2, HGF, CXCL9, CXCL10, CCL2, CCL3, CCL4, CCL5, CCL11, TNFRS1α, TNFRS1β and TRAIL-R2 (Invitrogen). An 8-plex assay was performed for MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP12, and MMP13 (R&D Systems). Assays for Fas, EGFR, FASL, Cyfra 21-1 (CKRT19 fragment), IGFBP1, KLK10, were developed in our Pittsburgh Luminex Core Facility. The assays were validated as previously described (21).

Statistical Analysis

ANOVA and non-parametric rank sum analysis were used to identify associations between indicated serum protein markers and defined cohorts with ILD. Spearman's rank correlation analysis determined the relationship between serum protein markers and pulmonary function parameters including percent predicted FVC (forced vital capacity) and D_LCO (diffusion capacity of carbon monoxide).

Data were analyzed using the R language for statistical computing (http://www.r-project.org/). Classification and regression trees (CART) methodology delineated potential combinations of peripheral blood biomarkers distinguishing anti-Jo-1 Ab+ ILD patients from individuals with anti-SRP Ab+ polymyositis, IPF, and no disease. CART was performed using the part package for recursive partitioning.

Kaplan-Meier curves depict survival differences between patients with biopsy-proven DAD and those individuals without histological evidence of DAD *or* clinical manifestations of Acute Respiratory Distress Syndrome (ARDS). This analysis commenced with the onset of pulmonary symptoms, and survival times were censored at the end of calendar year 2007.

For all patients who developed DAD, survival time prior to the biopsy date was attributed to the non-DAD category. Cox hazard regression models incorporating a time-dependent DAD status indicator variable were used to adjust for potential confounding factors including gender, race, and age at first visit.

RESULTS

Demographic and clinical characteristics of anti-Jo-1 antibody positive myositis patients with/without ILD

Between 1982 and 2007, 90 patients possessing anti-Jo-1 antibodies and sufficient clinical, functional, and/or radiographic data to determine the presence of ILD were enrolled in the University of Pittsburgh myositis registry (representing 90/683=13% of total registry enrollment and 90/106=85% of Jo-1 antibody positive patients accrued during this time period). The mean age at disease presentation was 43 years, with a preponderance of Caucasian females that reflects the known gender predilection of this disease as well as regional demographic characteristics (data not shown). Comparison of clinical features between anti-Jo-1 Ab+ patients with (n=77) and without (n=13) accompanying ILD (Table 1) revealed no significant differences in the frequency of current/past smoking or the occurrence of fever, arthritis, myositis, and Raynaud phenomenon (known extra-pulmonary manifestations of the anti-synthetase syndrome). Overall, 77/90 (86%; 95% C.I.=76.6–92.1%) anti-Jo-1 Ab+ patients with evaluable pulmonary data listed in Table 1 met defined criteria for ILD, and in the subset of patients with available CT data, 59/64 (92%) had evidence of parenchymal lung abnormalities.

Functional, radiographic, and histopathologic abnormalities in anti-Jo-1 Ab+ patients with ILD

Analysis of PFTs in a subset of anti-Jo-1 Ab+ patients (n=62) with clinical and/or radiographic evidence of ILD demonstrated classic restrictive physiology, with reductions in mean values for percent predicted FEV1 (67.7 +/− 17.6%), FVC (61.0 +/−17.9%), and D_LCO (50.8 +/− 19.5%) (additional data not shown). Although PFT data from anti-Jo-1 Ab + patients without ILD were not uniformly available for comparison, these changes were consistent with gas exchange abnormalities typically observed in mild to moderate pulmonary fibrosis. Corresponding HRCT findings in n=30 anti-Jo-1 Ab+ individuals (representing patients studied at the University of Pittsburgh following conversion to digital imaging) included ground glass opacities (n=20; 67%), architectural distortion (n=12; 40%), and in some cases, traction bronchiectasis (n=15; 50%) or early honeycombing (n=6; 20%) suggestive of UIP (Figure 1). Of note, however, several CT scans (n=4; 13%) showed abnormalities in patients with no overt clinical symptoms of dyspnea or cough, highlighting the sensitivity of HRCT in detecting subclinical ILD (even in cases with normal PFTs, n=2/4).

Among 22 anti-Jo-1 Ab+ patients undergoing open lung biopsy (n=14), transplant (n=4), or autopsy (n=4), histopathologic findings indicated an overwhelming preponderance of UIP (n=10; 45%) and DAD (n=12; 55%) relative to NSIP (n=3; 14%) (Figure 1C)—contrasting with previously published reports suggesting that NSIP is the most common histopathologic subtype in myositis-associated ILD. Several biopsy specimens did show pleomorphic features combining DAD and fibrotic NSIP (n=1) as well as DAD and UIP (n=2). As shown in Figure 1D, the concordance rate between biopsy findings and HRCT interpretation in n=13 individuals undergoing both studies was quite high, reflecting the predominance of traction bronchiectasis in UIP and ground glass opacification in DAD. More importantly, survival analysis in this biopsy cohort demonstrates that DAD portends a poor prognosis, with a significant 7.4 year reduction in median survival compared to patients without

histopathologic or clinical evidence of DAD ($p<0.0001$, Figure 2). After adjusting for potential confounding variables such as age, gender, and race, the estimated hazard ratio of DAD versus non-DAD in anti-Jo-1 Ab+ ILD patients is 6.3 +/− 0.5. Given the apparent reduction in survival associated with development of DAD, identification of early biomarkers for this complication as well as other forms of ILD is of obvious importance.

Multiplex ELISA assessment of serum proteins

To establish serum protein markers correlating with the development of ILD in anti-Jo-1 Ab + individuals (using a subset possessing PFTs and/or CT imaging within 90 days of serum sampling; n=42), we employed multiplex ELISA for measurement of various serum proteins including matrix metalloproteinases, cytokines, chemokines, and acute phase reactants. Control groups included healthy volunteers, individuals with mild to moderately severe IPF (mean D_LCO=83.9 +/− 24.5%; mean FEV_1/FVC=81.1 +/− 6.3%), and anti-SRP Ab+ patients with clinically active myositis (mean muscle MITAX= 6 +/− 3.16 vs. 1.2 +/− 1.3 in anti-Jo-1 Ab+ ILD subset), but no ILD.

Relative to healthy controls and subjects with IPF, anti-Jo-1 Ab+ ILD patients demonstrated significant serum elevations of the general inflammatory markers C-reactive protein (CRP), serum amyloid A (SAA), and serum amyloid protein (SAP); matrix metalloproteinases MMP3 and MMP8; and the IFNγ-inducible chemokines CXCL9 and CXCL10 (Table 2). While levels of SAA, SAP, MMP3, and MMP8 did not distinguish anti-Jo-1 Ab+ ILD and anti-SRP Ab+ (ILD negative) cohorts, CXCL9 and CXCL10 were clearly elevated in anti-Jo-1 Ab+ ILD patients compared to controls with anti-SRP Ab+ myositis or SIPF (Table 2 and Figure 3). The small number of anti-Jo-1 Ab+ individuals without evidence of ILD (n=5) limited meaningful biomarker comparisons with anti-Jo-1 Ab+ ILD patients, reaching statistical significance only for SAP (data not shown). Interestingly, subgroup analysis of anti-Jo-1 Ab+ ILD individuals with/without histologically proven DAD indicated statistically significant differences in serum levels of CXCL9 (MIG) and CXCL10 (IP10), with higher levels of both IFNγ-inducible chemokines in DAD (Figure 3C).

For those markers distinguishing anti-Jo-1 Ab+ ILD from anti-SRP Ab+ myositis (no ILD) and IPF, we assessed their relationship to functional parameters of restrictive lung disease. Review of Figure 3B demonstrates moderate, but statistically significant, correlations between percent predicted FVC (%FVC) and the serum proteins CRP ($r=-0.60$; $p<0.001$) and CXCL10 ($r=0.43$; $p<0.01$). CXCL9, on the other hand, showed a much weaker correlation with %FVC that did not reach statistical significance ($r=0.26$; $p=0.13$). Although the functional significance and interpretation of these associations require further definition, this collective analysis suggests that CRP and CXCL10 may be particularly helpful in monitoring pulmonary disease activity and/or damage.

A combination of serum proteins distinguishes anti-Jo-1 Ab+ ILD from IPF

To more fully determine whether selected serum proteins correctly classify anti-Jo-1 Ab+ ILD patients, we applied recursive partitioning to the entire set of 48 markers and found that the combination of CXCL10, MMP-7, and IL-12 can distinguish Jo-1 Ab+ ILD from IPF with 100% sensitivity and 100% specificity (Figure 4A). All of the patients with CXCL10 concentrations of at least 5.1 ng/ml (n=29) had anti-Jo-1 Ab+ ILD; among patients with CXCL10 concentrations below 5.1 ng/ml, each of the 6 patients with MMP-7 concentrations of at least 11.7 ng/ml had anti-Jo-1 Ab+ ILD. On the other hand, in patients with CXCL10 concentrations lower than 5.1 ng/ml and MMP-7 levels less than 11.7 ng/ml, an IL-12 concentration > 6.5 ng/ml accurately predicted IPF, rather than anti-Jo-1 Ab+ ILD.

As a complement to this analysis, we repeated the recursive partitioning algorithm (using all 48 protein markers) with a data set including an additional 5 patients possessing anti-Jo-1 antibodies, but lacking ILD. This expanded analysis involving a combined cohort of Jo-1 Ab + patients resulted in a similar predictive algorithm with respect to CXCL10 and IL-12 concentrations; however, MMP-7 levels failed to discriminate disease subsets in this larger cohort containing non-ILD patients, highlighting the potential role of MMP-7 as marker of lung inflammation and/or fibrosis associated with Jo-1 antibody positivity.

A combination of serum proteins distinguishes anti-Jo-1 Ab+ ILD from anti-SRP Ab+ polymyositis

Paralleling these results, application of recursive partitioning with the same 48 markers effectively distinguished anti-Jo-1 Ab+ ILD from anti-SRP Ab+ polymyositis. In this case, however, the classifier consisted of only CXCL10 and MMP-8 (Figure 4B). Among 35 patients with CXCL10 concentration of at least 4.4 ng/ml, 33 had anti-Jo-1 Ab+ ILD; the remaining 4 anti-Jo-1 Ab+ ILD patients and 8 anti-SRP Ab+ patients separated at a threshold MMP-8 concentration of 13.5 ng/ml, yielding high overall sensitivity (100%) and specificity (80%) for the CXCL10/MMP-8 classifier.

DISCUSSION

Among the most striking features of this study is the extraordinarily high incidence of ILD in anti-Jo-1 Ab+ individuals that approaches 90%. Although the literature reports variable rates of ILD in IIM cohorts not segregated by antibody status (5,10–14), our findings substantiate Schmidt's composite analysis of 231 anti-Jo-1 Ab+ patients (derived from multiple sources) in which 75% of individuals with this antibody specificity meet radiographic and/or histopathologic criteria for ILD (26). While the characteristics of our anti-Jo-1 Ab+ cohort may be impacted by referral bias as well as criteria used for defining ILD, the observed frequency of this pulmonary complication in our database (77/90=86%, a conservative value given the lack of HRCT data for some of the individuals included in the "no ILD" category) indicates that the presence of anti-Jo-1 antibodies alone is a highly predictive biomarker for ILD. This conclusion is fully supported by our previous work demonstrating a correlation between Jo-1 antibody titers and clinical indices of pulmonary disease activity (27). Given the previously defined association between other anti-synthetase antibodies and ILD (often in the absence of myositis or other non-pulmonary features of the anti-synthetase syndrome) (14,28–36), our findings provide a compelling case for screening all patients with these antibodies for "subclinical" ILD through PFTs and HRCT scanning—particularly because the histopathologic abnormalities found in early synthetase-associated ILD are more likely to include treatment responsive lesions such as COP and NSIP (5,10,16,17) rather than the devastating variants characteristic of IPF.

Beyond these provocative observations solidifying the robust relationship between anti-Jo-1 antibodies and ILD, novel findings emerging from our analysis include the preponderance of UIP and DAD detected in biopsy specimens of patients with this antibody specificity. Although this apparent discrepancy from previously published studies reporting the predominance of NSIP may reflect procedural bias (i.e., sicker patients requiring biopsy are more likely to have severe histopathologic abnormalities encompassed by DAD and UIP), these findings indicate that subsets of Jo-1 Ab-associated ILD share final common pathways of tissue damage with IPF and may therefore serve as a disease model for the latter disorder. Further supporting the potential pathophysiologic overlap between anti-Jo-1 Ab+ ILD and IPF, recent reports have clearly demonstrated that acute exacerbations due to DAD are an important cause of increased mortality in IPF (37,38)—an observation that parallels the survival analysis depicted in Figure 2.

In view of these findings, the need for non-invasive biomarkers that can distinguish histopathologic subtypes with different biological behaviors in advance of clinical decompensation is clear. Figure 3 suggests that the IFN-γ-inducible chemokines CXCL9 (MIG) and CXCL10 (IP10) are candidate biomarkers differentiating DAD from UIP in the context of Jo-1 Ab+ ILD, though greater numbers of patients with biopsy proven DAD, UIP, NSIP, and COP will be necessary to confirm these findings and to examine the relationship of these chemokines to existing markers of alveolar/epithelial damage that include KL-6, CK-19, and surfactant D (39). As reflected by the survival analysis in Figure 2 showing worse outcome in patients with DAD, however, this issue is not trivial and could significantly impact therapeutic decision making.

While these correlations involving antibody specificity, histopathology, and clinical outcome are significant, multiplex analysis of serum biomarkers may also provide equally important pathogenic insight relevant to the development of ILD. In these studies, for example, multiplex ELISA profiling revealed significant serum elevations in systemic inflammatory indices, several matrix metalloproteinases, and most interestingly, IFNγ-inducible chemokines (CXCL9, CXCL10) involved in recruitment of activated T cells that have been implicated in the pathogenesis of myositis-associated ILD (40,41). Of these markers, only CRP, CXCL9, and CXCL10 distinguished anti-Jo-1 Ab+ ILD from anti-SRP Ab+ myositis cohorts. These differences further support a possible role for the latter chemokines in ILD, though their relative absence in IPF indicates divergent pathways of pulmonary inflammation/fibrosis (that might explain the apparent survival discrepancy between IPF and UIP-associated anti-Jo-1 Ab+ ILD) or linkage to non-pulmonary features of the anti-synthetase syndrome. Interestingly, work in a bleomycin-induced model of IPF indicates that CXCL10 plays a critical role in inhibiting fibroblast migration, potentially explaining the positive correlation between levels of this chemokine and pulmonary function in the context of anti-Jo-1 Ab+ ILD. Although provocative, these results must be interpreted with caution given inherent limitations in cross-sectional assessment of pulmonary disease activity (versus damage) that are based on pulmonary function testing and imaging studies.

From a methodologic perspective, the use of recursive partitioning represents a powerful tool highlighting the predictive potential of protein biomarker combinations. For example, while CXCL10, IL-12, and MMP-7 clearly distinguish anti-Jo-1 Ab+ ILD from SIPF with great sensitivity and specificity, the combination of CXCL10 and MMP-8 effectively segregates patients with anti-Jo-1 Ab+ ILD and anti-SRP Ab+ myositis. Compared to univariate analysis, this combinatorial approach identifies additional biological molecules including IL-12, MMP-7, and MMP-8 that may be relevant to the pathogenesis of ILD occurring in the context of the anti-synthetase syndrome. Again, however, the relatively small numbers of patients in the second and third tiers of this analysis warrant cautious interpretation of these results, highlighting the need for larger, prospective studies combining clinical, radiologic, histopathologic, and functional data with serum biomarker assessment.

Analysis of additional anti-Jo-1 Ab+ patients as well as assessment of other anti-synthetase cohorts with/without ILD will therefore be critical to substantiate these collective findings. Longitudinal studies and *in situ* staining of lung specimens will also be necessary to further define the relationship between candidate biomarkers and lung inflammation in IIM, but these results clearly demonstrate that multiplex ELISA represents a powerful tool for identification of markers relevant to the pathophysiology of IIM-associated ILD. By extension, establishing a unique serologic and protein biomarker signature of autoimmune-associated ILD (which can occur in the absence of defining extra-pulmonary features) will ultimately facilitate selection of more treatment-responsive subsets of “idiopathic”

pulmonary fibrosis, further highlighting the clinical and therapeutic ramifications of this methodologic approach.

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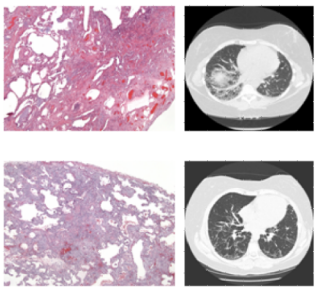
A



B

HRCT Patterns--Jo-1 Ab + ILD

Predicted Diagnosis	GG[a]	HC[b]	AD[c]	TB[d]
UIP (n=9)	2	6	6	8
NSIP (n=15)	14	0	4	4
Unclassifiable (n=6)	4	0	3	3

[a]GG=ground glass opacity
[b]HC=honeycombing
[c]AD=architectual distortion
[d]TB=traction bronchiectasis

C Histopathology--Jo-1 Ab + ILD

Histopathology	Number (percent)[a]
NSIP	3 (14)
UIP	10 (45)
DAD	12 (55)
COP	0 (0)

[a]1/22 biopsies with evidence of DAD and fibrotic NSIP
[a]2/22 biopsies with evidence of DAD and UIP

D HRCT vs. Biopsy--Jo-1 Ab + ILD

Histopathology	HRCT Pattern		
	UIP	NSIP	Unclassified
UIP (n=4)	3	0	1
f-NSIP[a] (n=1)	1	0	0
DAD (n=8)	1[b]	4[c]	3[c]

[a]f-NSIP=fibrotic NSIP
[b]background fibrosis on biopsy
[c]ground glass opacity

Figure 1. Correlation between histopathology and HRCT abnormalities in anti-Jo-1 Ab+ patients with ILD

A) Anti-Jo-1 Ab+ patients with UIP (top panels) or DAD (lower panels) display extensive parenchymal tissue damage and marked HRCT abnormalities. The tables in panels B) and C) summarize radiographic patterns detected by HRCT and histopathologic subtypes observed in lung biopsy specimens, respectively. Panel D) demonstrates the direct correlation between histopathologic abnormalities and HRCT subclassification in a subset of anti-Jo-1 Ab+ individuals (n=13) from panel B) undergoing lung biopsy.

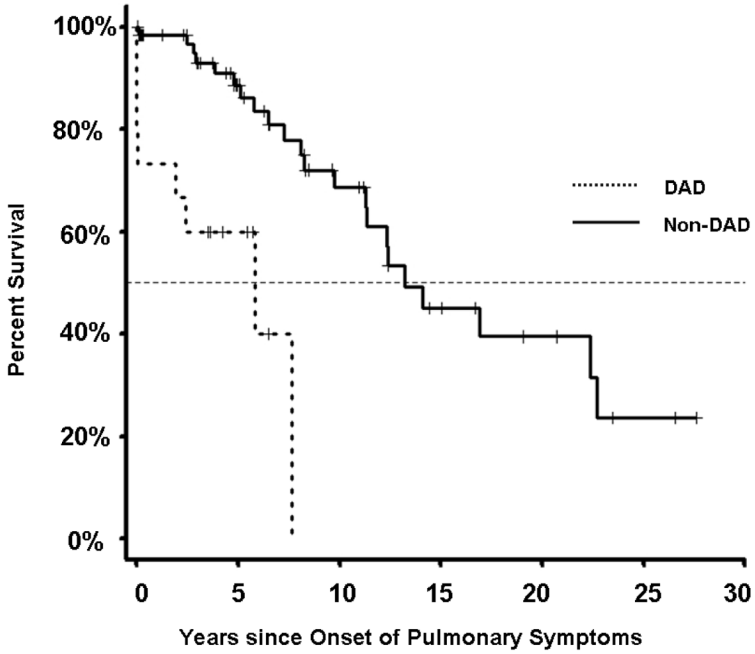


Figure 2. Survival difference between anti-Jo-1 Ab+ patients with and without DAD

Kaplan-Meier curves plot the survival of patients with biopsy-proven DAD (n=14, dotted line) relative to the survival of patients without histopathologic evidence of DAD or clinical manifestations of ARDS (n=59, solid line). The time origin reflects the onset of pulmonary symptoms or functional/radiographic demonstration of ILD, while vertical hash marks indicate censoring. Median survival estimates are 5.8 and 13.2 years for DAD and non-DAD, respectively ($p<0.0001$).

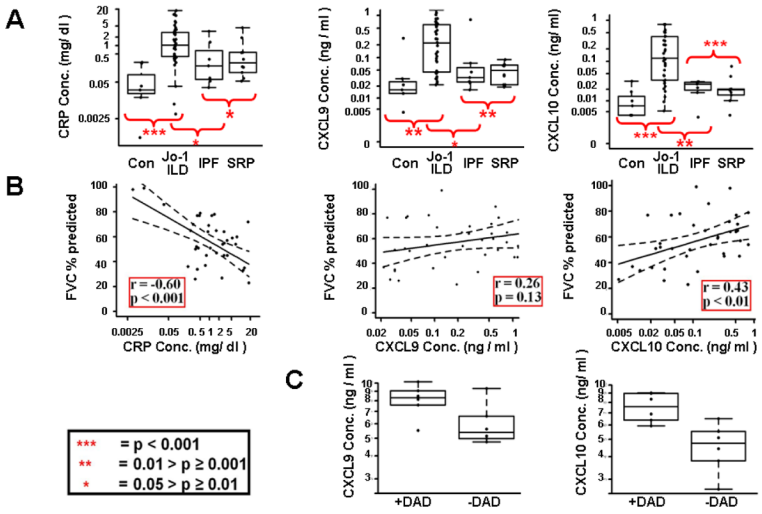


Figure 3. Comparison of serum protein biomarkers in patient subgroups and correlation with pulmonary function

A) Box plots reflect levels of the indicated serum proteins (CRP, CXCL9, and CXCL10) determined by multiplex ELISA in healthy controls as well as individuals with anti-Jo-1 Ab + ILD, IPF, or anti-SRP Ab+ myositis without ILD. Statistical significance of pair wise contrasts was calculated using an omnibus test in ANOVA followed by Bonferroni correction for multiple comparisons. B) Regression lines bracketed by 95% confidence intervals (dotted lines) demonstrate the relationship between serum levels of CRP, CXCL9, or CXCL10 and percent predicted forced vital capacity (FVC% predicted) in patients with anti-Jo-1 Ab+ ILD. Underlying scatter plots represent individual data points from n=34 individuals with anti-Jo-1 Ab+ ILD. While p values are derived from linear regression analysis, r values reflect Pearson correlation coefficients. C) Box plots encompass serum levels of CXCL9 and CXCL10 in anti-Jo-1 Ab+ patients with (n=7) or without (n=6) DAD. Median levels of CXCL9 in subjects with DAD exceed those of non-DAD (8.4 ng/ml vs. 5.4 ng/ml, p=0.005); similarly, median levels of CXCL10 are greater in DAD compared to non-DAD individuals (8.0 ng/ml vs. 4.8 ng/ml, p=0.05).

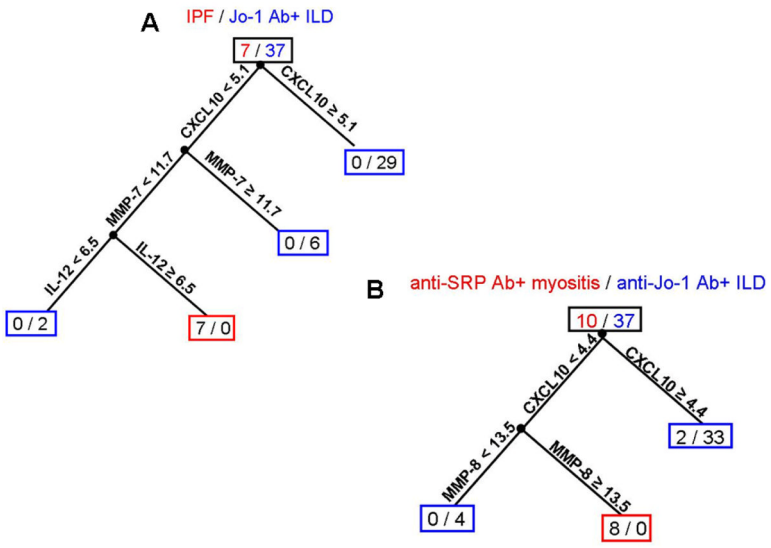


Figure 4. Combinatorial analysis of serum protein markers in patient subgroups with and without ILD

Based on levels of the indicated serum protein biomarkers, recursive partitioning methods establish a diagnostic algorithm distinguishing anti-Jo-1 Ab+ ILD from A) IPF and B) anti-SRP Ab+ myositis.

Table 1

Clinical Characteristics and Diagnostic Studies in Jo-1 Ab+ Cohort

Variable	ILD present (n=77)	ILD absent (n=13)	p-value
Fever	26 (34%)	3 (23%)	0.54
Arthritis	54 (70%)	11 (85%)	0.50
Raynaud	32 (42%)	5 (42%)	1
Myositis	72 (94%)	12 (92%)	1
Ever Smoker	30 (39%)	7 (54%)	0.37
Mean follow up (mos.)	50 +/− 50	38 +/− 44	0.18
Diagnostic Studies Completed			
Biopsy[a]	27 (35%)	0 (0%)	NA
PFTs	62 (81%)	5 (38%)	NA
Chest CT	59 (77%)	5 (38%)	NA
+biopsy	21 (27%)	0 (0%)	NA
+PFTs	56 (73%)	3 (23%)	NA
+biopsy and PFTs	19 (25%)	0 (0%)	NA

[a] includes n=5 trans-bronchial biopsies

NA=not assessed

Table 2

Luminex Comparisons (p-values)

Serum Marker	Jo-1 Ab+ ILD vs. Normal	Jo-1 Ab+ ILD vs. IPF	Jo-1 Ab+ ILD vs. SRP Ab+ Myositis
SAP	0.00002	0.0136	0.159
SAA	0.00008	0.0400	0.370
CRP	0.00037	0.0380	0.0326
MMP3	0.00349	0.0167	0.434
MMP8	0.00085	0.0070	0.765
TNFR2	0.00062	0.0320	0.286
IL-8	0.00771	0.0415	0.948
CXCL9	0.00106	0.0303	0.00344
CXCL10	0.00022	0.0035	0.00013