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Biomarkers of Rheumatoid Arthritis–Associated Interstitial Lung Disease

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Abstract

Objective—Interstitial lung disease (ILD) is a relatively common extraarticular manifestation of rheumatoid arthritis (RA) that contributes significantly to disease burden and excess mortality. The purpose of this study was to identify peripheral blood markers of RA-associated ILD that can facilitate earlier diagnosis and provide insight regarding the pathogenesis of this potentially devastating disease complication.

Methods—Patients with RA who were enrolled in a well-characterized Chinese identification cohort or a US replication cohort were subclassified as having RA–no ILD, RA–mild ILD, or RA–advanced ILD, based on high-resolution computed tomography scans of the chest. Multiplex

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ascherman had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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enzyme-linked immunosorbent assays (ELISAs) and Luminex xMAP technology were used to assess 36 cytokines/chemokines, matrix metalloproteinases (MMPs), and acute-phase proteins in the identification cohort. Unadjusted and adjusted logistic regression models were used to quantify the strength of association between RA-ILD and biomarkers of interest.

Results—MMP-7 and interferon-γ–inducible protein 10 (IP-10)/CXCL10 were identified by multiplex ELISA as potential biomarkers for RA-ILD in 133 RA patients comprising the Chinese identification cohort (50 RA–no ILD, 41 RA-ILD, 42 RA–indeterminate ILD). The findings were confirmed by standard solid-phase sandwich ELISA in the Chinese identification cohort as well as an independent cohort of US patients with RA and different stages of ILD (22 RA–no ILD, 49 RA-ILD, 15 RA–indeterminate ILD), with statistically significant associations in both unadjusted and adjusted logistic regression analyses.

Conclusion—Levels of MMP-7 and IP-10/CXCL10 are elevated in the serum of RA patients with ILD, whether mild or advanced, supporting their value as pathogenically relevant biomarkers that can contribute to noninvasive detection of this extraarticular disease complication.

Clinically evident extraarticular manifestations occur in ~40% of individuals with rheumatoid arthritis (RA) (1), adding to disease burden and leading to excess mortality. Compared with the general population, for example, the average life expectancy among patients with RA is shortened by 10–11 years (2). Among the most significant factors contributing to this excess mortality is interstitial lung disease (ILD), the most common subtype of lung involvement in RA (3–5). In fact, the risk of death among individuals with clinically evident RA-associated ILD is 3 times higher than that among RA patients without ILD. Recent studies have further demonstrated that even though overall mortality rates in RA are declining, the rate of death due to RA-ILD has increased significantly (3).

Further illustrating the scope of this problem, studies using open lung biopsy or high-resolution computed tomography (HRCT) scanning demonstrate interstitial lung involvement in >40% of the overall RA patient population (6–8). This includes clinically evident RA-ILD, which occurs in ~10% of RA patients, as well as a substantial amount of subclinical disease (9). In fact, based on the existing literature, ~30–55% of asymptomatic individuals have evidence of interstitial lung abnormalities on HRCT (6,8,10,11). From an outcomes perspective, this observation is quite significant given Gochuico and colleagues' demonstration of radiologic progression over 2 years in >50% of 21 studied patients with subclinical forms of RA-ILD (10). Consistent with these findings, another prospective study of 29 RA-ILD patients also showed that 34% had radiologic progression over a 24-month period (12).

Although the pathogenesis of RA-ILD remains poorly defined, several pieces of evidence implicate a combination of environmental, genetic, and immunologic factors that ultimately mediate inflammatory cell infiltration and eventual tissue remodeling/fibrosis. This conceptual framework mirrors pathogenic stages of idiopathic pulmonary fibrosis (IPF), a disease in which available data indicate that, if unchecked, dysregulated inflammatory cascades can elaborate a host of cytokines, chemokines, and growth factors that collectively promote epithelial and endothelial cell damage, angiogenesis, fibroblast differentiation/proliferation, and lung fibrosis (13).

Given the clinical implications of putative signaling cascades involved in these processes, biomarkers that can be used to clarify disease pathogenesis and identify factors governing disease progression are clearly needed. While biomarkers in this setting may encompass radiologic parameters and physiologic variables, molecular profiles consisting of genetic and protein markers are more directly relevant to disease pathogenesis (14). Because lung tissue itself is relatively inaccessible, however, alternative compartments, such as bronchoalveolar lavage (BAL) fluid and peripheral blood, must serve as surrogate sources for markers of disease activity. Validating this concept, Gochuico et al (10) have used multiplex enzyme-linked immunosorbent assay (ELISA) of BAL fluid to demonstrate that levels of selected proteins not only correlate with the clinical/radiographic stage of RA-ILD, but also predict disease progression—effectively illustrating the power and sensitivity of this methodologic approach.

Beyond this sentinel study of BAL fluid (10), investigators have attempted to define peripheral blood proteins associated with RA-ILD—including rheumatoid factor (RF) (15), KL-6 (16), and anti–citrullinated protein antibodies (17–19). While high-titer RF has been linked to the presence of RA-ILD and decreased diffusing capacity for carbon monoxide (DLCO) (15), KL-6 levels have been shown to correlate with the severity of CT findings (16). However, despite these intriguing observations that relate to immune activation and alveolar damage, respectively, currently known associations provide limited pathogenic insight.

Assessments of serum-derived proteins in other diseases that share final common pathways with different stages of RA-ILD have indicated that multiplex ELISA of peripheral blood represents a viable approach for detection of pathway-specific biochemical alterations in the lung microenvironment, potentially addressing the above-mentioned knowledge gaps. For example, while analysis of serum proteins in IPF has revealed statistically significant elevations in the levels of several matrix metalloproteinases (MMPs) that likely characterize advanced/fibrotic stages of RA-ILD, with histopathologic features similar to those observed in usual interstitial pneumonia (UIP) (20–22), the findings of parallel studies of patients with Jo-1 antibody–positive ILD (which includes nonspecific interstitial pneumonia as well as UIP variants) have highlighted the predominance of specific chemokines (CXCL9, interferon-γ[IFNγ]–inducible protein 10 [IP-10]/CXCL10) that likely contribute to earlier, more inflammatory stages of RA-ILD (23). Demonstration of CXCR3+ lymphocytes (typically manifesting a Th1 phenotype) in lung biopsy specimens from individuals with RA-ILD (24) provides additional support for the latter hypothesis that enhanced expression of IFNγ-inducible, CXCR3-binding chemokines such as IP-10 will mark early stages of RA-ILD.

Based on these considerations, we used multiplex ELISA as a screening tool for identification of biochemical markers of RA-ILD in a well-characterized cohort of Chinese RA patients with different clinical and radiographic stages of ILD. To confirm results of this peripheral blood analysis supporting the predicted associations between RA-ILD, IP-10, and MMP-7, we also performed standard solid-phase sandwich ELISAs for these proteins in both the Chinese cohort and an independent group of North American RA patients. Collectively, the results demonstrated that elevated levels of IP-10 and MMP-7 strongly

correlate with the presence of RA-ILD, effectively supporting the underlying hypothesis that RA-ILD represents a spectrum of pathology involving parenchymal lung inflammation and dysregulated tissue remodeling.

PATIENTS AND METHODS

Patients

Identification cohort—The identification cohort consisted of adult patients (> 18 years old) who met the American College of Rheumatology (ACR) 1987 criteria for definite RA (25) and were seen consecutively between July 2012 and March 2013 at the Rheumatology Department of the First Hospital of Xiamen University (11). Demographic information, clinical features, medication history, comorbidities, and the 28-joint Disease Activity Score (DAS28) (26) were recorded for each subject.

Replication cohort—Patients who were evaluated at the University of Pittsburgh (October 2010–June 2013) or Brigham and Women's Hospital (October 2010–June 2012) and had RA according to the ACR criteria were enrolled in the replication cohort after providing written informed consent. Medical records were subsequently reviewed to obtain information on demographic characteristics, smoking history, occupational exposures, and medications. Articular disease activity was quantified through DAS28 assessment, and dyspnea was evaluated with the University of California, San Diego Shortness of Breath Questionnaire (27).

In both cohorts, HRCT of the chest and pulmonary function testing tests (PFTs) were performed to assess radiographic and functional abnormalities indicative of ILD. All study-related procedures were approved by the appropriate institutional review committees representing participating centers in China and the US.

Imaging

Identification cohort—In the identification cohort, HRCT (Aquilion 16; Toshiba Medical Systems) of the chest without contrast was performed during end inspiration using 1–2-mm collimation at 1–2-mm intervals.

Replication cohort—In the replication cohort, conventional noncontrast HRCT scanning with 1–2-mm–thickness cuts was performed during end inspiration. For individuals with previously diagnosed ILD, the scan obtained in closest temporal proximity to the date of enrollment was chosen for review.

Scoring

Identification cohort—HRCT scans obtained from patients at the First Hospital of Xiamen University were assessed by 2 independent reviewers who were blinded with regard to the patients' clinical data. In the grading scheme used by the reviewers, a numerical score is assigned based on the type and distribution of interstitial lung abnormalities (ILA) consisting of septal lines, reticulation, traction bronchiectasis, cyst formation, and/or ground-glass attenuation (28,29), where 0 = no ILD, 1 = indeterminate ILD (focal or

unilateral groundglass attenuation, focal or unilateral reticulation, or patchy ground-glass abnormality involving <5% of the lung), 2 = mild/early ILD (changes affecting >5% of any lobar region with nondependent ground-glass or reticular abnormalities, diffuse centrilobular nodularity, nonemphysematous cysts, honeycombing, or traction bronchiectasis), 3 = advanced ILD (bilateral fibrosis in multiple lobes associated with honeycombing and traction bronchiectasis in a subpleural distribution). Discrepant readings were repeated and then resolved by consensus, with final scores of ≥2 classified as ILD.

Replication cohort—All HRCT scans from patients in the replication cohort were systematically evaluated using a previously described sequential reading method (28,29) that involves scoring based on the same imaging criteria described above (where 0 = no ILD, 1 = indeterminate ILD, 2 = mild ILD, and 3 = advanced ILD). In this approach, scans were visually inspected by a primary reader, after which 20% of the scans scored as 0 and all scans scored as 1, 2, or 3 were independently interpreted by a second reader to ensure uniformity. Scans with discrepant readings were then evaluated by a third radiologist and resolved by consensus (29).

Pulmonary function testing

PFTs were performed as part of the enrollment protocol, and the findings were assessed according to American Thoracic Society recommendations (30). Results of PFTs performed for clinical indications in individuals with known/suspected ILD were included on the basis of temporal proximity to the time of enrollment. Reference values were derived from the Third National Health and Nutrition Examination Survey (31).

Multiplex ELISA

Multiplex ELISAs were performed using Luminex xMAP technology in a 96-well microplate format according to the instructions of the manufacturers (eBioscience, Procarta), using methods that have been described previously (32). A combined 36-plex assay was used to determine serum levels of a range of cytokines/chemokines, MMPs, and acute-phase proteins.

ELISA

Quantitative sandwich ELISAs for human MMP-7 and IP-10/CXCL10 were performed according to the protocol recommended by the manufacturer (R&D Systems), using 1:4 serum dilutions and the standards provided.

Statistical analysis

The significance of differences in baseline clinical and demographic features was determined by univariate analysis using Fisher’s exact test (for binary and categorical variables) or Wilcoxon’s rank sum test (for continuous variables). In multivariate analyses, unadjusted and adjusted logistic regression models were used to assess the strength of association between RA-ILD and biomarkers of interest. Covariates used for adjustment included age, sex, smoking history, medication use, and DAS28 score. Receiver operating characteristic (ROC) curves were generated to identify numerical thresholds/cutoffs

optimizing the sensitivity and specificity of designated biomarker assays. For correlative studies involving biomarker levels and parameters of pulmonary function, Spearman's nonparametric rank correlation coefficients were calculated. All analyses were performed using SAS version 9.2 (SAS Institute). Two-tailed *P* values less than 0.05 were considered significant.

RESULTS

Cohort characteristics

Chinese identification cohort—Among 133 consecutively enrolled RA patients in the identification cohort, 41 (31%) had evidence of definite interstitial lung abnormalities on chest HRCT, scored as radiographically mild (ILA score 2) in most cases. As shown in Table 1, individuals with RA-ILD (ILA score 2 or 3) were more likely than RA patients without ILD (ILA score 0) to be older with higher RF and anti–cyclic citrullinated peptide (anti-CCP) titers and DAS28 scores; however, there was no clear association between RA-ILD and other demographic variables or environmental factors, such as sex or smoking (which was relatively uncommon in this cohort). Paralleling the modest increase in frequency of dyspnea and/or cough, the percent predicted forced vital capacity (FVC) and DLCO were reduced in patients with RA-ILD relative to RA patients without ILD.

US replication cohort—Of the 86 RA patients in the replication cohort, 49 (57%) had evidence of interstitial lung abnormalities based on a parallel HRCT classification scheme and the above-described sequential method of interpretation. The high proportion of RA-ILD reflects the inclusion of 38 patients (44% of the overall cohort) with established, symptomatic ILD at the time of enrollment. Among the 48 patients without clinically evident lung disease, 13 (27%) had HRCT abnormalities indicative of ILD (ILA score 2 or 3). While 22 (46%) of the remaining patients had no radiographic markers of ILD, 13 (27%) had indeterminate CT findings (ILA score 1) and were excluded from the primary analyses (except where indicated).

Similar to the Chinese identification cohort, individuals with RA-ILD in the US replication cohort (Table 2) were more likely to be older with a trend toward higher RF titers and increased articular disease activity (relative to patients with RA–no ILD). In contrast, however, CCP titers did not correlate with the presence of ILD, which was more closely associated with male sex and extent of smoking history among the US patients. Duration of articular disease was also significantly longer among US patients compared to Chinese patients, particularly among those with RA-ILD. Dyspnea scores in the US cohort increased predictably with radiographic severity of ILD, corresponding to uniform reductions in forced expiratory volume in 1 second, FVC, and DLCO. Overall, comparative analysis of these functional parameters between subgroups indicated a greater level of relative respiratory compromise in RAILD patients in the US cohort.

Multiplex ELISA–based screening for serum proteins correlating with RA-ILD in the Chinese identification cohort

To investigate the hypothesized association of CXCR3-binding chemokines and selected MMPs with RA-ILD, we first performed multiplex ELISA–based screening of the Chinese identification cohort (detailed results available upon request from the corresponding author). MMP-7 and IP-10/CXCL10 were among the serum biomarkers most strongly linked to the presence of ILD in an initial subset of this RA cohort (with MMP-7 showing the top-ranked statistical association by unadjusted logistic regression). Based on these results, we restricted the subsequent analysis of remaining (non-overlapping) cohort members to 8 serum proteins that included MMP-7, IP-10, IFNγ, interleukin-8 (IL-8), IL-10, IL-15, IL-22, and IL-2 receptor α-chain (a panel of markers collectively representing different T cell and innate immune activation pathways). In this subset of patients, MMP-7 and IP-10 again emerged as two of the most highly significant markers of RA-ILD by unadjusted logistic regression (with IP-10 showing the strongest statistical association), providing the rationale for confirmatory ELISA analysis of MMP-7 and IP-10 in the entire cohort.

Relationship between stage of RA-ILD and serum levels of MMP-7 and IP-10 in the Chinese identification cohort

Supporting the results of our initial multiplex ELISA screen, standard solid-phase ELISAs revealed strong correlations between the grade of interstitial lung abnormality and mean serum levels of both MMP-7 and IP-10 in the overall Chinese identification cohort. The mean ± SD serum concentration of MMP-7 increased from 3.06 ± 1.73 ng/ml in RA–no ILD (ILA score 0) to 5.35 ± 4.55 ng/ml in RA-ILD (ILA score 2 or 3) ($P = 0.005$), and the corresponding level of IP-10 increased from 173.8 ± 96.1 pg/ml in RA–no ILD to 308.6 ± 235.8 pg/ml in RA-ILD ($P = 0.0004$) (Figure 1A and Table 3). Statistically significant elevations of both MMP-7 and IP-10 were observed even in the RA–indeterminate ILD category (ILA score 1), strengthening the apparent dose-response relationship between these biomarkers and severity of radiographically defined interstitial lung abnormalities. Consistent with this dose-response effect, clinical stratification of RA-ILD patients based on the presence of cough and/or dyspnea identified a small group of patients (n = 9) with symptomatic/clinically evident ILD who had particularly high levels of both MMP-7 (mean ± SD 8.32 ± 5.95 ng/ml) and IP-10 (464.0 ± 280.0 pg/ml) (Figure 1A) that were clearly distinguishable from those in the RA–no ILD category ($P = 0.0003$ and $P = 0.0002$ for MMP-7 and IP-10, respectively).

ELISA analysis of serum MMP-7 and IP-10 levels in the US replication cohort

To substantiate these findings supporting our original hypotheses, we performed similar ELISA-based assessment of serum MMP-7 and IP-10 levels in an independent replication cohort of 86 RA patients with different stages of ILD that was derived from 2 academic centers in the US. As shown in Figure 1B and Table 3, serum MMP-7 levels were strongly associated with the presence of ILD, increasing from a mean ± SD of 2.98 ± 1.32 ng/ml in RA–no ILD to 6.16 ± 3.37 ng/ml in RA-ILD (ILA score 2 or 3) ($P < 0.0001$). Similarly, serum levels of IP-10 increased from 83.3 ± 79.3 pg/ml in RA–no ILD to 209.7 ± 288.2 pg/ml in RA-ILD ($P = 0.001$). Even when RA patients with radiographically defined

interstitial lung abnormalities were subclassified into groups with subclinical versus clinically evident ILD based on the presence/absence of cough and/or dyspnea, these strong correlations persisted—particularly for MMP-7, with respective mean values for RA-subclinical ILD (no cough or dyspnea) and RA-clinically evident ILD of 5.94 ± 3.46 ng/ml and 6.24 ± 3.38 ng/ml ($P = 0.0006$ and $P < 0.0001$, respectively, versus no ILD) (Figure 1B).

Given the range of potential confounders in this analysis, we assessed the statistical effect of age, sex, smoking, medication usage, and DAS28 on the associations between biomarker levels and RA-ILD. In both the Chinese and the US patient cohorts, the only variable that significantly correlated with MMP-7 level was age (US cohort only; $\rho = 0.54$, $P < 0.0001$); conversely, none of these factors exhibited significant covariation (defined as $\rho > 0.5$) with IP-10. Based on this analysis, we performed logistic regression with adjustment for age as well as DAS28 (to fully ensure that the observed biomarker associations were not confounded by articular disease activity). As shown in Table 3, both MMP-7 and IP-10 retained statistically significant associations with RA-ILD in the Chinese and US cohorts following these adjustments, indicating that both biomarkers were independently linked to the organ-specific manifestation of ILD. While the overall sample sizes of the cohorts precluded more detailed combinatorial analysis of potential confounders, adjusted logistic regression for the remaining individual variables did not significantly alter the predictive relationship between serum MMP-7/IP-10 levels and the presence of RA-ILD (data not shown).

Further demonstrating the predictive potential of these biomarkers for RA-ILD, ROC assessment (Figure 2) revealed strong performance characteristics for both MMP-7 and IP-10 in each of the cohorts, with area under the curve [AUC] values ranging from 0.68 to 0.86. Of note, analysis of AUC values indicated that levels of MMP-7 and IP-10 compared favorably with anti-CCP antibody levels as well as RF titers, suggesting that MMP-7 and IP-10 might add predictive value to existing laboratory parameters that have been previously linked to the development of RA-ILD. While the overall trends were quite similar between the Chinese and US cohorts, the AUCs showed stronger *relative* performance of MMP-7 (versus IP-10) in the US patient group, high-lighting potential differences in disease phenotype and/or severity between the two cohorts.

Correlation between serum biomarker levels and parameters of pulmonary function

As a complement to this correlative analysis, we assessed the relationship between serum levels of MMP-7/IP-10 and various measures of pulmonary function. Although the correlations between these biomarkers and selected PFT parameters (percent predicted FVC, DLCO) were relatively modest with rho values of <0.4, the plots depicted in Figure 3 indicate a general trend toward inverse relationships between mean serum levels of these proteins and pulmonary function. Intriguingly, however, the relative strength of these correlations was somewhat different between the US and Chinese cohorts, favoring MMP-7 in the former and IP-10 in the latter. Coupled with the ROC analysis (Figure 2), this observation again suggested that differences in disease severity (or phenotype) might influence biomarker profiles—a conclusion that was further substantiated by the

predominant correlation between MMP-7 and dyspnea score in the US cohort of RA patients (Figure 3).

DISCUSSION

Through multiplex as well as standard ELISA-based approaches, we have demonstrated that serum levels of MMP-7 and IP-10/CXCL10 are elevated in various stages of RA-ILD relative to RA occurring in the absence of parenchymal lung disease. Consistent observations in ethnically distinct identification and replication cohorts support the validity of these associations, which persist in multivariate analysis even after adjustment for potential covariates including age, sex, smoking history, and articular disease activity. Moreover, correlations between functional/radiographic parameters of pulmonary disease activity and serum levels of MMP-7 and IP-10 provide additional compelling evidence that these serum proteins can serve as effective biomarkers for organ-specific pathology in the context of a systemic inflammatory disorder. While the relative distribution of radiographically mild and more advanced ILD in these cohorts precludes definitive subset analysis, the clear link between IP-10, MMP-7, and the composite category of RA-ILD supports the underlying hypothesis that this extraarticular complication represents a combination of dysregulated immunity and overexuberant tissue remodeling.

From the standpoint of molecular profiling, the emergence of MMP-7 and IP-10 as potential biomarkers for RA-ILD complements work in IPF and other connective tissue diseases associated with ILD. With regard to IPF, for example, MMP-7 levels are increased in the peripheral blood of patients with subclinical disease as well as those with advanced disease, demonstrating an inverse correlation with functional parameters that include FVC and DLCO (20). Beyond MMP-7, increased serum levels of surfactant protein A (SP-A), SP-D, and KL-6 have been found in IPF and other forms of idiopathic ILD in which the blood–alveolar barrier is likely disrupted. Predictive models underscore the significance of these associations, as increased levels of SP-A, SP-D, KL-6, and MMP-7 have been linked to radiographic progression and/or increased mortality rates (21).

Paralleling some of these findings, analysis of peripheral blood proteins in systemic sclerosis (SSc)–associated ILD has demonstrated elevated levels of KL-6, SP-A, SP-D, CXCL4 (33), and CCL18 (34–37). Complementary assessment of BAL fluid from patients with SSc-associated ILD indicates that IL-8 and monocyte chemotactic protein 1 are also linked to the development of lung fibrosis and poor outcome, extending observations from peripheral blood and reinforcing the prognostic potential of selected cytokines, chemokines, and lung-derived proteins. Equally important, however, differences in peripheral blood and BAL fluid protein expression between SSc-associated ILD, IPF, and RA-associated ILD suggest that comparative biomarker profiling may provide critical insight regarding divergent disease pathways that contribute to the underlying pathogenesis of specific diseases.

As noted above, studies of protein biomarkers in RA-ILD have focused on both peripheral blood and BAL fluid. Gochuico and colleagues' assessment of BAL fluid showed that platelet-derived growth factor isoforms AB and BB could reliably distinguish RA patients with subclinical ILD from those without ILD (10). More detailed examination also indicated

that elevated levels of transforming growth factor β1 and IFNγ in BAL fluid were associated with radiographic progression in a subset of individuals with early/subclinical ILD. The latter observation is consistent with unpublished data from the same analysis showing elevated BAL fluid levels of the IFNγ-inducible chemokine IP-10 in patients with subclinical RA-ILD, highlighting similarities between fluid compartments that may reflect overlapping mechanisms influencing the breach of alveoli/respiratory epithelium and vascular endothelium.

In terms of pathogenesis, the predominant association of serum levels of MMP-7 with RA-ILD suggests a mechanistic overlap with IPF that is consistent with emerging clinicoepidemiologic data linking outcome/survival in RA-UIP and IPF. However, in RA-ILD, the association with MMP-7 is not limited to subsets with advanced/fibrotic lung disease, as MMP-7 levels clearly distinguished RA patients with ILD from those without ILD in the Chinese identification cohort that was composed primarily of patients with subclinical, radiographically mild ILD (ILA score 2). Whether the correlation between MMP-7 and a broader spectrum of RA-ILD reflects true differences in pathogenesis versus observation bias in IPF remains unclear, but the previously demonstrated association between subclinical stages of familial IPF and elevated MMP-7 levels (20) indicates that augmented MMP-7 expression likely occurs early in the disease process of IPF as well as RA-ILD. The relationship between MMP-7 and ILD is not universal, however, as shown by molecular profiling studies in Jo-1 antibody–positive ILD, where levels of C-reactive protein, CXCL9, and IP-10/CXCL10 yield the strongest univariate associations with ILD—including cases with UIP histopathology (23).

Although the overall results seemingly implicate both IP-10 and MMP-7 in the pathogenesis of RA-ILD, this study does have several limitations. For example, despite the construction of 2 relatively large, independent cohorts of RA patients, the uneven distribution of subclinical versus clinically evident ILD within individual cohorts precludes more direct comparisons. Moreover, relatively restricted sample sizes make it difficult to precisely control for confounding factors such as medication usage that may directly impact cytokine profiles, though statistical comparison did not reveal significant differences in the use of methotrexate or biologic agents between RA patient subsets within either cohort. Beyond these issues, the lack of uniformity in demographic features and environmental exposures likely to impact the development of ILD (e.g., smoking) further complicates between-cohort comparisons. Ultimately, however, the persistence of statistically significant associations between IP-10, MMP-7, and RA-ILD in the setting of such population differences represents a major strength of the study that further supports the validity (and generalizability) of our findings. Equally important, the strength of these findings provides clear rationale for future analysis of additional biomarkers potentially captured via the initial multiplex ELISA screening protocol (data available upon request from the corresponding author).

One of the central questions emerging from this study is the relationship between mild/subclinical and more advanced forms of RA-ILD (as defined by clinical and radiographic criteria), particularly given the potential overlap in biomarker profiles that is discrepant from epidemiologic data suggesting very different prevalence rates among RA-ILD subtypes. Adequately addressing this issue and identifying factors that govern the putative transition

between subclinical/radiographically mild and more advanced/fibrotic forms of RA-ILD will require longitudinal studies involving larger, independent cohorts of patients with subclinical RA-ILD (in whom biomarker profiling should further clarify the capacity of MMP-7 and/or IP-10 to identify early-stage disease). Longitudinal analysis will also be needed to determine the prognostic value of MMP-7 and IP-10 in RA patients with clinically/radiographically established disease, potentially enabling clinicians to identify patients who are most likely to develop progressive fibrosis and an IPF-like clinical course. Detailed characterization of these longitudinal validation cohorts should, in turn, facilitate the development of more powerful predictive models incorporating additional biomarkers (including alternative serum proteins identified by preliminary multiplex screening in this study) as well as relevant clinical, radiographic, and functional variables.

In the future, successful implementation of this strategic approach to RA-ILD should promote the identification of at-risk patients in whom early treatment intervention has the most potential for success. At the same time, data emerging from such analysis should help to elucidate the pathogenesis of this potentially devastating extraarticular complication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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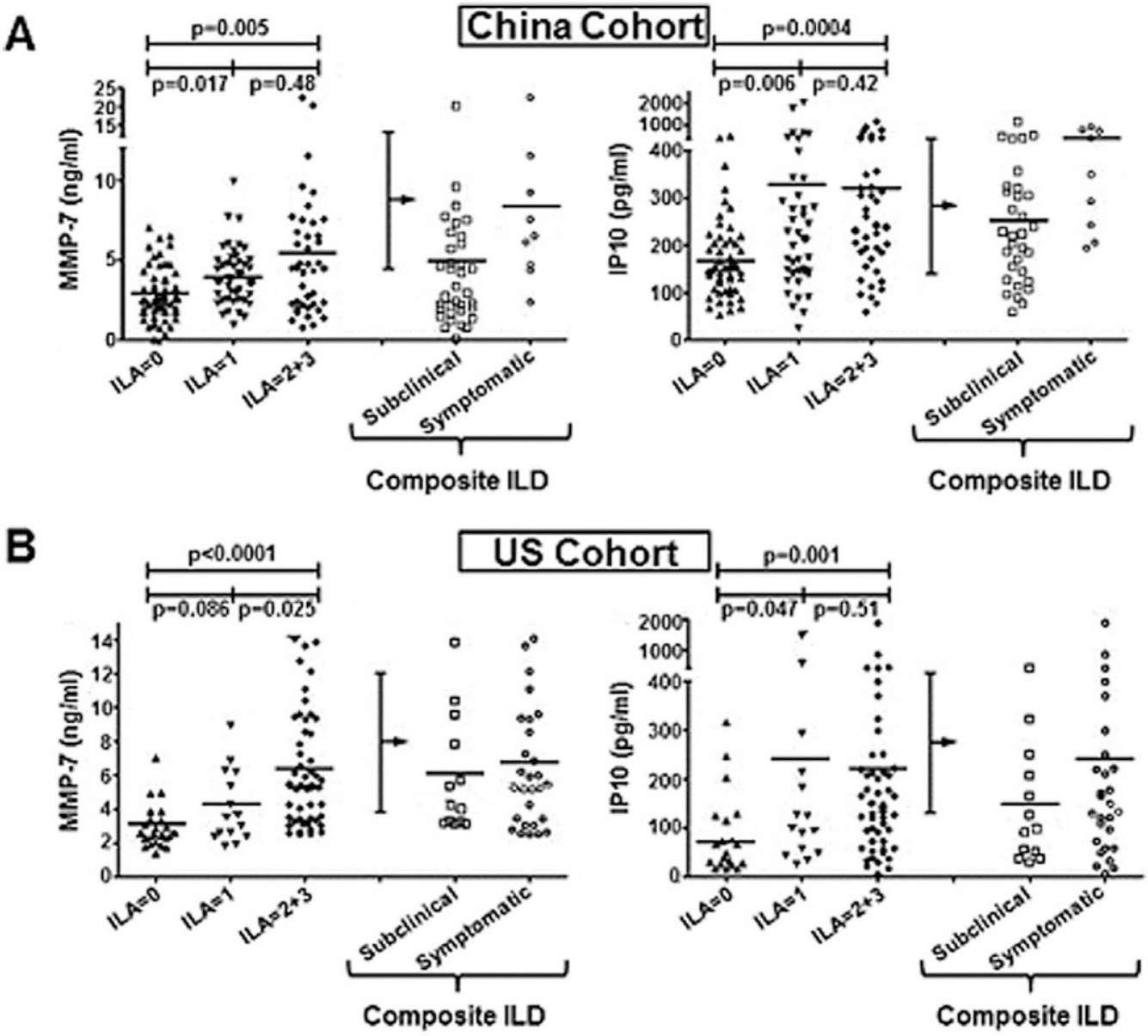

Figure 1.

Serum levels of matrix metalloproteinase 7 (MMP-7) and interferon-γ–inducible protein 10 (IP-10) in rheumatoid arthritis (RA) patients in the Chinese identification cohort (**A**) and the US replication cohort (**B**) without interstitial lung disease (ILD) or with different stages of ILD. Dot plots depict levels (measured by standard solid-phase enzyme-linked immunosorbent assay) of MMP-7 or IP-10 in individual serum samples from RA patients without ILD (interstitial lung abnormality [ILA] score 0), indeterminate ILD (ILA score 1), mild ILD (ILA score 2), or advanced ILD (ILA score 3). Samples from patients with ILA scores of 2 or 3 were further stratified into groups of subclinical ILD versus clinically evident/symptomatic ILD (based on the presence of cough and/or dyspnea). Each symbol represents an individual patient; horizontal lines show the mean. *P* values were determined by Mann-Whitney U test.

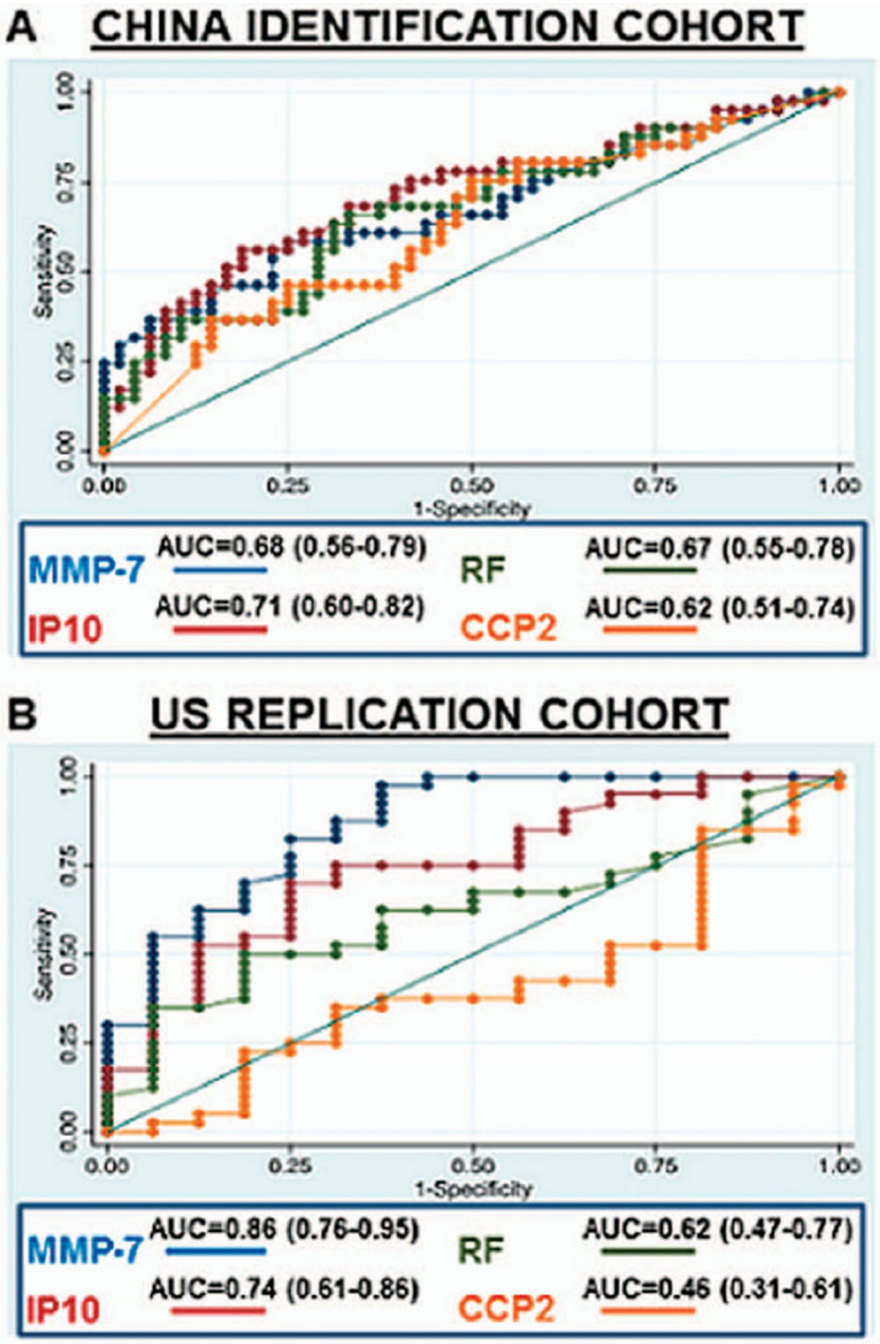

Figure 2.

Performance characteristics of biomarker levels in RA-ILD. For both the Chinese identification cohort (**A**) and the US replication cohort (**B**), receiver operating characteristic curves (shown as area under the curve [AUC]) demonstrate the predictive capacity of MMP-7 level, IP-10 level, rheumatoid factor (RF) titer, and anti–cyclic citrullinated peptide (anti-CCP) titer for the presence of RA-ILD. Ninety-five percent confidence intervals are shown in parentheses. See Figure 1 for other definitions.

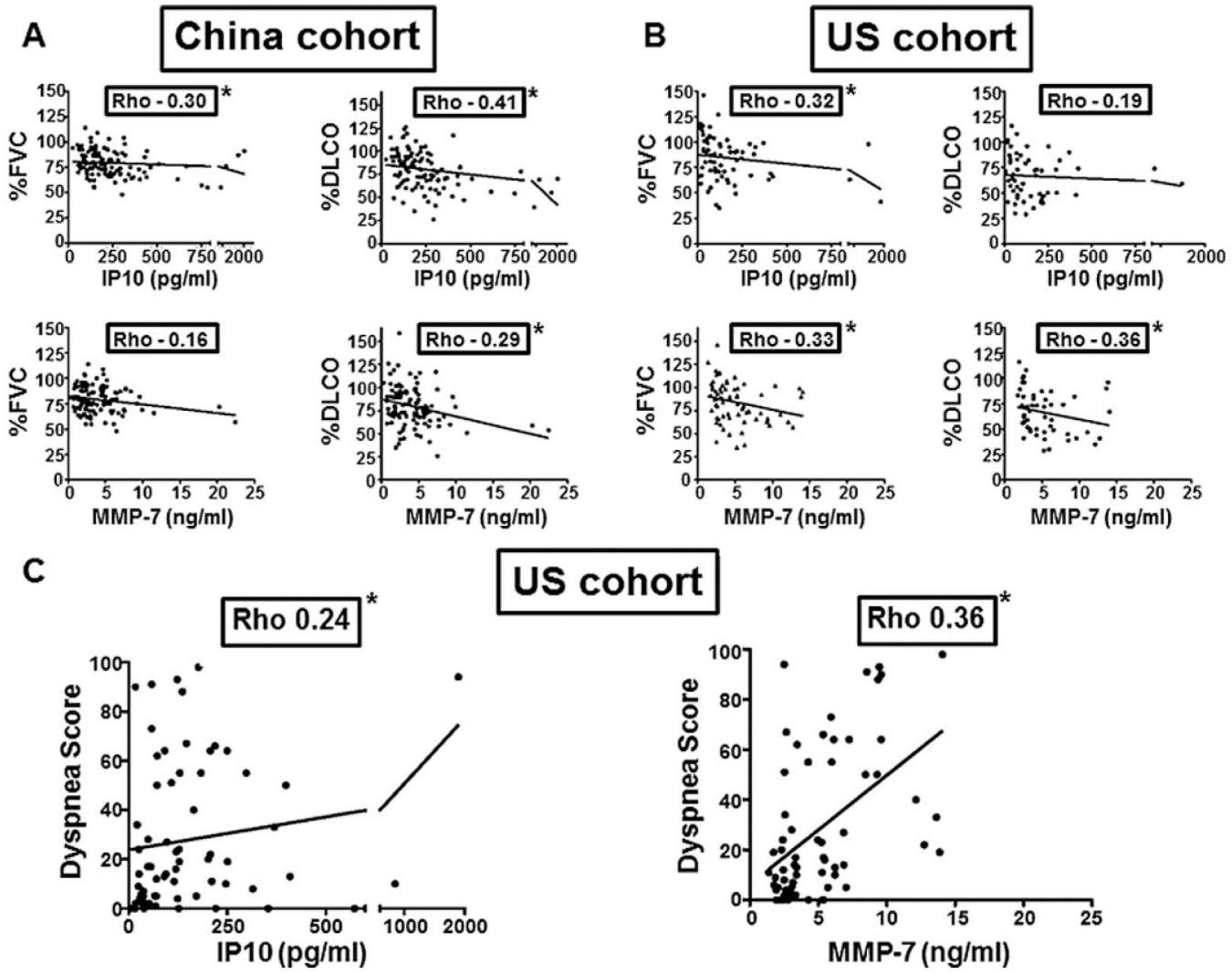

Figure 3.

Relationship between biomarker levels and pulmonary function parameters. **A** and **B**, Relationship between levels of IP-10 or MMP-7 and pulmonary function parameters (forced vital capacity [FVC] and diffusing capacity for carbon monoxide [DL_{CO}] [both measured as the percent predicted]) in the Chinese identification cohort (**A**) and the US replication cohort (**B**). **C**, Relationship between mean serum levels of IP-10 or MMP-7 and the dyspnea score in the US cohort. For all analyses, Spearman's rank correlation coefficients (rho values) indicate the relative magnitude and direction of covariation between biomarker levels and pulmonary function parameters or dyspnea score. * = $P < 0.05$. See Figure 1 for other definitions.

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Table 1

Baseline characteristics of the patients in the Chinese RA cohort, by degree of ILD*

	RA–no ILD (ILA score 0) (n = 50)	RA–indeterminate ILD (ILA score 1) (n = 42)	RA–mild ILD (ILA score 2) (n = 38)	RA–advanced ILD (ILA score 3) (n = 3)	RA-ILD (ILA score 2 or 3) (n = 41)
Demographic parameters					
Age, years	43.4 ± 15.54	57.07 ± 9.40†	51.74 ± 13.80†	69.33 ± 8.96†	53.02 ± 14.20†
Female, no. (%)	41 (82)	34 (81)	29 (76)	0 (0)†	29 (71)
Ever smoker, no. (%)	6 (12)	3 (7)	2 (5)	3 (100)†	5 (12)
Pack-years of smoking	15.50 ± 14.26	7.50 ± 6.61	21.25 ± 12.37	11.33 ± 8.08	15.30 ± 10.02
RA parameters					
RF, IU/ml	180.63 ± 228.61	319.53 ± 474.89	744.48 ± 1,987.64†	1,435.33 ± 1,891.62†	795.03 ± 1,966.34†
Anti-CCP, units/ml	179.25 ± 169.33	232.29 ± 185.99	252.64 ± 187.54	287.90 ± 216.79	255.22 ± 187.01†
DAS28	4.06 ± 1.49	4.45 ± 1.30	4.92 ± 1.25†	4.40 ± 1.53	4.88 ± 1.26†
Duration of RA, years	4.31 ± 4.34	6.26 ± 8.68	5.90 ± 5.29	1.67 ± 0.58	5.59 ± 5.21
Medication use (current), no. (%)					
Prednisone	3 (16)	12 (29)†	11 (29)†	2 (67)†	13 (32)†
Methotrexate	49 (100)	40 (98)	37 (97)	2 (67)	39 (95)
Leflunomide	21 (42)	16 (39)	15 (39)	0 (0)	15 (37)
TNFα inhibitor	3 (6)	1 (2)	3 (8)	0 (0)	3 (7)
Respiratory parameters, no. (%)					
Cough	0 (0)	0 (0)	3 (8)	2 (67)†	5 (12)†
Dyspnea	1 (2)	1 (2)	5 (13)	3 (100)†	8 (20)†
Spirometric parameters					
FEV_1, percent of predicted	83.56 ± 14.25	76.91 ± 13.81†	79.73 ± 15.17	75.00 ± 26.66	79.33 ± 15.90
FVC, percent of predicted	83.77 ± 12.84	75.55 ± 10.36†	77.15 ± 13.42	72.33 ± 14.19	76.76 ± 13.35†
DL_{CO}, percent of predicted	90.4 ± 19.34	74.06 ± 18.89†	74.53 ± 22.53†	59.33 ± 5.03†	73.30 ± 22.01†

*Data in some categories were missing for some patients, as follows: prednisone, leflunomide, or tumor necrosis factor α (TNFα) use (1 patient), methotrexate use (2 patients), forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) (20 patients), and diffusing capacity for carbon monoxide (DLCO) (26 patients).

Except where indicated otherwise, values are the mean ± SD.

ILA = interstitial lung abnormality; RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; DAS28 = 28-joint Disease Activity Score.

† $P < 0.05$ versus the group of rheumatoid arthritis (RA) patients without interstitial lung disease (ILD).

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Table 2

Baseline characteristics of the US RA cohort, by degree of ILD*

	RA–no ILD (ILA score 0) (n = 22)	RA–indeterminate ILD (ILA score 1) (n = 15)	RA–mild ILD (ILA score 2) (n = 34)	RA–advanced ILD (ILA score 3) (n = 7)	RA-ILD (ILA score 2 or 3) (n = 49)†
Demographic parameters					
Age, years	50.32 ± 7.82	54.33 ± 12.24	63.38 ± 10.36‡	70.57 ± 14.29‡	65.27 ± 10.80‡
Female, no. (%)	16 (76)	15 (100)	12 (35)	2 (29)	18 (37)
White race, no. (%)	15 (71)	10 (67)	25 (74)	5 (71)	37 (76)
Ever smoker, no. (%)	8 (42)	4 (29)	18 (53)	3 (43)	27 (55)
Pack-years of smoking	11.08 ± 13.66	4.81 ± 5.94	26.08 ± 27.51	40.00 ± 26.46	25.25 ± 24.96
RA parameters					
RF, IU/ml	143.75 ± 357.00	221.27 ± 220.04	390.07 ± 765.12	439.60 ± 519.81	409.65 ± 708.20
Anti-CCP, units/ml	154.59 ± 151.36	143.98 ± 146.99	122.20 ± 146.37	73.23 ± 97.50	142.58 ± 151.91
3-variable DAS28	3.29 ± 1.68	3.47 ± 1.36	3.73 ± 1.15	3.28 ± 0.77	3.74 ± 1.16
No. of swollen joints	3.45 ± 4.06	1.93 ± 2.62	2.88 ± 3.29	1.86 ± 1.86	2.89 ± 3.20
Duration of RA, years	8.38 ± 8.14	12.00 ± 9.33	12.94 ± 10.09	16.14 ± 13.74	12.80 ± 10.28
Medication use (ever), no. (%)					
Prednisone	15 (68)	7 (47)	20 (59)	3 (43)	28 (57)
Methotrexate	14 (67)	8 (53)	21 (64)	4 (57)	30 (63)
Leflunomide	2 (9)	3 (20)	11 (32)	0 (0)	14 (29)
TNFα inhibitor	2 (9)	4 (27)	4 (12)	3 (43)	9 (18)
Dyspnea score (respiratory parameter)§	12.14 ± 12.61	20.07 ± 19.46	31.16 ± 29.64‡	58 ± 26.43‡	36.96 ± 29.77‡
Spirometric parameters					
FEV_1, percent of predicted	100.17 ± 14.49	98.6 ± 11.60	79.82 ± 21.43‡	73.86 ± 23.42‡	77.73 ± 20.70‡
FVC, percent of predicted	101.44 ± 14.49	89.9 ± 19.53	78.03 ± 20.19‡	68.14 ± 22.18‡	74.83 ± 19.99‡
DL_{CO}, percent of predicted	84.07 ± 18.71	75.88 ± 15.40	58.81 ± 15.86‡	54.00 ± 29.61‡	56.03 ± 17.58‡

*Data in some categories were missing for some patients, as follows: sex (1 patient), race (1 patient), ever smoker (4 patients), pack-years of smoking (6 patients), RF (19 patients), DAS28 (28 patients), number of swollen joints (3 patients), duration of RA (3 patients), methotrexate use (2 patients), dyspnea score (5 patients), FEV_1 (18 patients), FVC (18 patients), and DLCO (33 patients). Except where indicated otherwise, values are the mean ± SD. See Table 1 for definitions.

†Includes 8 individuals with definite RA-ILD in whom a score of 2 versus 3 could not be distinguished.

‡ $P < 0.05$ versus RA–no ILD.

§ Measured with the University of California, San Diego Shortness of Breath Questionnaire.

Table 3

Levels of MMP-7 and IP-10 among patients in the RA cohorts, by degree of ILD*

	MMP-7		IP-10	
	Mean ± SD ng/ml	***P* vs. RA–no ILD**	**Mean ± SD ng/ml**	***P* vs. RA–no ILD**
Chinese identification cohort†				
RA–no ILD (ILA score 0) (n = 49)	3.06 ± 1.73	–	173.8 ± 96.1	0.01
RA–indeterminate ILD (ILA score 1) (n = 42)	4.00 ± 1.88	0.02	322.5 ± 388.7	0.002
RA–mild ILD (ILA score 2) (n = 38)	4.77 ± 3.70	0.02	292.7 ± 232.7	NA
RA–advanced ILD (ILA score 3) (n = 3)	12.73 ± 8.52	NA	510.4 ± 211.5	0.0004
RA-ILD (ILA score 2 or 3) (n = 41)	5.35 ± 4.55	0.005	308.6 ± 235.8	0.01
US replication cohort‡				
RA–no ILD (ILA score 0) (n = 22)	2.98 ± 1.32	–	83.3 ± 79.3	0.05
RA–indeterminate ILD (ILA score 1) (n = 15)	4.13 ± 2.13	0.09	233.3 ± 372.1	0.009
RA–mild ILD (ILA score 2) (n = 34)	5.82 ± 3.37	±0.0001	220.0 ± 337.5	0.05
RA–advanced ILD (ILA score 3) (n = 7)	8.01 ± 3.24	0.0003	177.6 ± 144.4	0.001
RA-ILD (ILA score 2 or 3) (n = 49)§	6.16 ± 3.37	±0.0001	209.7 ± 288.2	0.05

*NA = not available (see Table 1 for other definitions).

†Results of logistic regression analysis for matrix metalloproteinase 7 (MMP-7) were as follows: unadjusted odds ratio (OR) 1.0003 (95% confidence interval [95% CI] 1.000–1.001), *P* = 0.003; age-adjusted OR 1.0002 (95% CI 1.000–1.000), *P* = 0.03; 28-joint DAS (3-variable)–adjusted OR 1.0003 (95% CI 1.000–1.001), *P* = 0.005. Results of logistic regression analysis for interferon-γ–inducible protein 10 (IP-10) were as follows: unadjusted OR 1.006 (95% CI 1.002–1.010), *P* = 0.003; age-adjusted OR 1.006 (95% CI 1.002–1.010), *P* = 0.006; 28-joint DAS–adjusted OR 1.005 (95% CI 1.001–1.009), *P* = 0.02.

‡Results of logistic regression analysis for MMP-7 were as follows: unadjusted OR 1.0007 (95% CI 1.000–1.001), *P* = 0.001; age-adjusted OR 1.0006 (95% CI 1.000–1.001), *P* = 0.04; 28-joint DAS–adjusted OR 1.0009 (95% CI 1.000–1.002), *P* = 0.02. Results of logistic regression analysis for IP-10 were as follows: unadjusted OR 1.009 (95% CI 1.002–1.016), *P* = 0.01; age-adjusted OR 1.006 (95% CI 0.998–1.014), *P* = 0.15; 28-joint DAS–adjusted OR 1.014 (95% CI 1.002–1.026), *P* = 0.02.

§Includes 8 individuals with definite RA-ILD in whom a score of 2 versus 3 could not be distinguished.