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IMMUNOPATHOLOGY

Autoantibodies in interstitial lung diseases

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Summary

The role of autoantibody testing for patients with interstitial lung disease is an evolving area. Recent guidelines recommend routine anti-nuclear antibodies, rheumatoid factor, and anti-citrullinated cyclic peptide antibody testing for patients undergoing diagnostic evaluation for interstitial lung disease, with further autoantibody testing reserved for selected cases guided by rheumatological features. Even this approach may miss patients with clinically significant autoantibodies when interstitial lung disease is the dominant or first manifestation of autoimmune disease.

We retrospectively performed autoimmune serology in a clinically well characterised cohort of interstitial lung disease patients. Using stored serum, additional testing was performed to ensure all patients had complete autoantibody profiles including anti-nuclear antibodies, extractable nuclear antigen antibodies, double-stranded DNA antibodies, rheumatoid factor, anti-citrullinated cyclic peptide antibodies, anti-neutrophil cytoplasmic antibodies, and myositis antibodies.

Eighty patients with interstitial lung disease, and available stored serum, were assessed. Mean age at interstitial lung disease diagnosis was 65.2 years and 42 patients were male. Positive autoimmune serology was found in 56 of 80 (70.0%) patients; the most common positive result was anti-nuclear antibodies (n=34; 42.5%). Myositis antibodies were detected in 13 of 80 (16.2%) patients. Four (5%) patients had elevated anti-citrullinated cyclic peptide antibodies, and two (2.5%) patients had detectable myeloperoxidase antibodies. Eleven (13.7%) patients with negative anti-nuclear antibodies had other significant disease associated autoantibodies.

An extended panel of autoantibody testing may detect cases of connective tissue disease associated interstitial lung disease, regardless of clinical or radiological subtype, and prior to extra-pulmonary manifestations of systemic autoimmunity.

Key words: Lung diseases; interstitial; serology; connective tissue diseases.

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INTRODUCTION

Interstitial lung diseases (ILDs) are a heterogeneous group of chronic lung disorders characterised by inflammation and/or fibrosis of the lung interstitium. ILDs may be idiopathic [such as idiopathic pulmonary fibrosis (IPF)], or due to secondary causes including environmental exposures, drugs or connective tissue disease (CTD).[1–3] The prognoses and therapeutic approaches vary considerably according to ILD subtype.[3] Novel anti-fibrotic therapies can slow IPF progression and prolong survival.[4,5] In contrast, immunosuppression in IPF has been associated with harm.[6] Connective tissue associated ILDs (CT-ILDs), however, appear to have a better prognosis and response to immunosuppression.[7] Therefore, precise, early diagnosis is crucial to tailor treatment and optimise outcomes.[5,8]

Connective tissue diseases are a group of systemic autoimmune disorders including rheumatoid arthritis (RA), systemic lupus erythematosus, vasculitis and idiopathic inflammatory myopathies (IIMs). ILD can complicate any CTD although recognition of CT-ILD can be challenging.[9] Current guidelines vary regarding patient selection and extent of serological testing in ILD. The 2011 American Thoracic Society and Latin American Thoracic Association (ATS/ERS/JRS/ALAT) guidelines recommended testing for anti-nuclear antibodies (ANA), anti-cyclic citrullinated peptide antibodies (CCP), and rheumatoid factor (RF) in ILD patients.[6] Further testing for extractable nuclear antigens antibodies (ENA), myositis antibodies, and anti-neutrophil cytoplasmic antibodies (ANCA) were recommended only in selected cases.[6,10] Exactly how to select such cases was not specified. More recent guidelines suggest a wider panel, but still reserve extensive myositis antibody and ANCA screening for selected cases, based on clinical suspicion.[8,11] There is increasing evidence that ILD can be the first or dominant manifestation of CTDs, including ANCA associated vasculitis (AAV) and IIM, both of which are frequently

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ANA negative.[7,9,12,13] Therefore, limited screening panels may fail to detect all ILD patients with an underlying immune disease.[10]

In addition to patients fulfilling CT-ILD criteria, many ILD patients have non-specific clinical features (e.g., arthralgia) or serology (e.g., ANA or RF only), which suggest but do not meet CTD diagnostic criteria. Recently the European Respiratory Society / American Thoracic Society (2015) proposed the term 'interstitial pneumonia with autoimmune features' (IPAF) to encompass such cases and stimulate future research.[14] An empiric classification of IPAF based on clinical, serological and radiological or histopathological domains has been proposed, but remains unvalidated.[14] The incidence, pathogenesis, management implications and prognosis of this group are not well understood.[15–17]

We hypothesised the rate of ILD patients with underlying autoimmunity was underestimated by current diagnostic approaches. We retrospectively performed comprehensive autoimmune serology on an unselected ILD cohort to assess the diagnostic yield of extended autoantibody screening.

MATERIALS AND METHODS

We performed a single-centre retrospective study of ILD patients of any type attending the Interstitial Lung Diseases Respiratory Clinic, Sir Charles Gairdner Hospital between August 2015 and January 2017. All diagnoses of ILD were confirmed by a respiratory physician and multidisciplinary case review with pulmonologists, radiologists and, where indicated, pathologists.

Of patients identified, those with remnant serum stored in the Immunology Department, PathWest Laboratory Medicine, dating ±365 days from first respiratory clinic review, were included. Previous results were reviewed and additional testing performed to complete the autoantibody panel of ANA, ENA, double-stranded DNA (dsDNA), RF, CCP, ANCA and myositis antibodies, for each patient. Where multiple samples were available, testing was performed on serum closest in time to ILD diagnosis.

Assays were performed according to the manufacturer's instructions. ANA testing was performed by indirect immunofluorescence on Hep2000 (Immunoconcepts, USA) with levels expressed in IU/mL.[18] ENA screening was performed by ELISA (Quanta Lite ENA 6 ELISA; Inova, USA) and confirmed by immunoblot (ANA profile 3 plus DFS70; Euroimmun, Germany) for antibodies to CENPB, histones, Jo-1, PMScl, Sm, SS-A (Ro60), SS-B, Scl70, Ribosomal P and Ro52. Anti-dsDNA testing was performed by Farr radioimmunoassay (Trinity Biotech, Ireland). RF quantitation was performed by turbidimetry (Integra 800; Roche Diagnostics, USA) and CCP antibodies performed by ELIA (Thermofisher Scientific, Netherlands). ANCAs were identified by indirect immunofluorescence (NOVA Lite; Inova) with myeloperoxidase (MPO) and proteinase-3 (PR3) antibody ELIA assays (Thermofisher Scientific) only performed on samples with ANCA immunofluorescence $\geq$3 IU/mL. Myositis antibodies were performed on the Euroline Autoimmune Inflammatory Myopathies 16 antigen assay (EJ, OJ, Jo-1, Ku, MDA-5, Mi-2a, Mi-2b, NXP2, PL-7, PL-12, PMScl75, PMScl100, Ro-52, SAE1, SRP and TIF1ɣ) (Euroimmun). Reference ranges were established for the myositis assay using a population cohort from the Busselton Health Study.[19]

Ethical approval was granted by the Sir Charles Gairdner Hospital Human Research Ethics Committee (HREC EC00271).

RESULTS

A cohort of 123 consecutive ILD cases was identified from respiratory clinic records; 43 cases were excluded due to lack of stored serum. Of the remaining 80 cases, 42 (52.5%) were males and the mean age at ILD diagnosis was 65.2 years (range 26–89). The mean time of blood draw to diagnosis date was −8.7 days (interquartile range −20 to +12 days; minimum −339 days; maximum +327 days). The initial diagnoses were CT-ILD (n=18), idiopathic non-specific interstitial pneumonitis (NSIP) (n=17), IPF (n=16), hypersensitivity pneumonitis (n=5), IPAF (n=2), AAV-ILD (n=1), combined pulmonary fibrosis and emphysema (CPFE) (n=1) and respiratory bronchiolitis-ILD (n=1). In the remaining 19 cases the ILD subtype was unclassified.

Autoantibody prevalence in ILD cohort

A total of 636 tests were analysed: 364 tests had been previously performed and 272 additional tests were performed on stored serum. Overall, 120/636 (18.8%) tests were positive with 41/636 (6.4%) new findings as part of this study (Table 1). Positive serology was found in 56/80 (70.0%) cases, with ANA the most frequently positive test (n=34; 42.5%). Of note, 11/80 (13.7%) cases with a negative ANA had other significant autoantibody results, the majority being myositis antibodies. Four (5%) cases had elevated CCP antibodies: three had known RA-ILD and one had a known scleroderma-ILD with Scl-70 antibodies and a low CCP antibody titre. One patient had a borderline positive dsDNA antibody at 7 (normal <7) which was unlikely to be clinically significant. Thirteen of 80 (16.2%) cases had detectable myositis antibodies and 2/80 (2.5%) had detectable MPO antibodies. Thirty-five of 80 (43.7%) cases had no clinically significant autoantibodies [ILD diagnoses were NSIP (n=11), IPF (n=10), hypersensitivity pneumonitis (n=4), CPFE (n=2), unspecified (n=8)].

Diagnostic implications of autoantibody results

Following additional testing, the ILD diagnosis could be reclassified in 15/80 (18.7%) cases (Fig. 1). Of these, 6/80 (7.5%) could be reclassified as CT-ILD (5 myositis and 1 AAV), and 9/80 (11.2%) as IPAF based on serological and radiological domains. A further 9/80 (11.2%) cases fulfilled the serological domain for IPAF; an additional clinical or morphological domain would be required for IPAF diagnosis. Further assessment of these cases was beyond the scope of this study. Diagnostic reclassification was most common in cases with unspecified ILD (10/19), IPF (6/16) and NSIP (6/17).

Cases with detectable myositis antibodies

Myositis antibodies were detected in 13/80 (16.2%) cases, of which 10/80 (12.5%) were new findings in this study (Table 2). In one additional case (Case 3 in Table 2), the treating team was unaware of a Jo-1 antibody previously detected during the original ILD work-up. Jo-1 and PM-SCl antibody detection was concordant between the ENA and myositis assay. Only 5/13 (38.5%) had clinical features of a CTD at ILD diagnosis and only 3/13 (23.1%) had a significantly elevated ANA. Myositis results were discordant with the working diagnosis for 8/13 (61.5%) cases.

Chart reviews were performed in 10 cases where new myositis antibodies were identified (Table 2). Interestingly, two of these had a diagnosis of underlying CTD in the absence of the disease-defining autoantibodies. Patient 2 was diagnosed with RA-ILD in the absence of a significantly elevated RF or CCP antibody: retrospective testing uncovered a strong Mi-2b antibody. Patient 13 was diagnosed with mixed connective tissue disease associated ILD in the absence of the pathognomonic U1-RNP autoantibodies, but presence of strong PL-7 antibodies and features of antisynthetase syndrome.

Table 1 Total number of test results performed at time of initial ILD work-up, and number of additional tests completed retrospectively as part of this study

	ANA	RF	CCP	ENA	dsDNA	ANCA	MPO	PR3	Myositis antibody
Result available, *n*	74	60	34	10	49	45	13	14	11
Additional tests performed, *n*	6	20	46	5	31	35	17	17	69
Positive results, *n* (% total tests performed)	34 (42.5%)	19 (23.7%)	4 (5.0%)	9 (60.0%)	1 (1.2%)	25 (31.2%)	2 (6.7%)	0 (0%)	13 (16.2)[a]
New findings, *n* (% total cohort)	3 (3.7%)	3 (3.7%)	2 (2.5%)	1 (1.2%)	1 (1.2%)	15 (18.7%)	1 (1.2%)	0 (0%)	10 (12.5%)[a]

ANA, anti-nuclear antibody; ANCA, anti-neutrophil cytoplasmic antibody screen; CCP, citrullinated cyclic peptide antibody; dsDNA, double stranded DNA antibody; ENA, extractable nuclear antigen; MPO, myeloperoxidase antibody; PR3, proteinase-3 antibody; RF, rheumatoid factor.
[a] In one case, two myositis antibodies were detected in one patient and this has been counted only once.

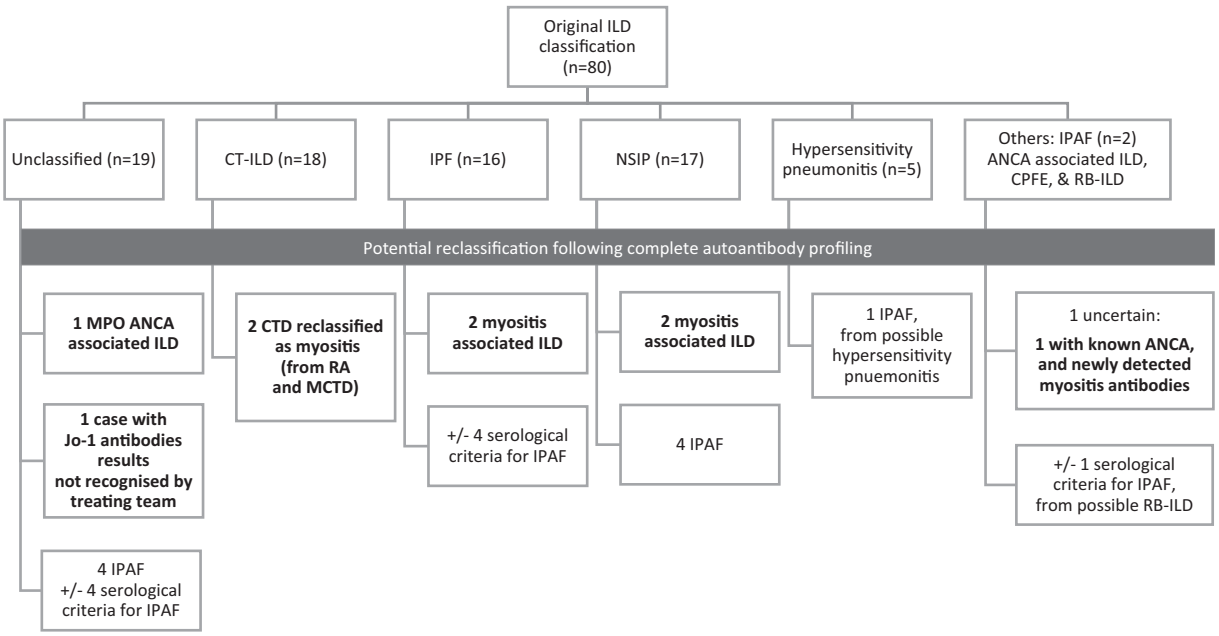

Fig. 1 Potential reclassifications of ILD subtype following complete autoantibody profiling.
ANCA, anti-neutrophil cytoplasmic antibody; CPFE, combined pulmonary fibrosis and emphysema; CT, connective tissue disease; ILD, interstitial lung disease; IPAF, idiopathic pulmonary fibrosis with autoimmune features; IPF, interstitial pulmonary fibrosis; MPO, myeloperoxidase antibody; MCTD, mixed connective tissue disease; NSIP, non-specific interstitial pneumonitis; RA, rheumatoid arthritis; RB, respiratory bronchiolitis.

Table 2 Myositis antibodies detected in patients with ILD

Case	Age (Sex)	Respiratory diagnosis	Radiology	CTD features at ILD diagnosis	ANA	RF (<14 kU/L)	ENA antibody	Myositis antibody	Significance of myositis antibody
1	69 (M)	AAV-ILD	UIP	No	Negative	Negative	–	**NXP2**	Uncertain
2	60 (M)	RA-ILD	NSIP	Yes	Negative	17	–	**Mi-2b**	Possible IIM-ILD
3	77 (F)	Unclassified (possible RB-ILD)	Not specified	No	Negative	Negative	Jo-1	**Jo-1**	Possible IIM-ILD
4	66 (M)	IPF	UIP	No	Negative	14	–	**Mi-2b**	Possible IIM-ILD
5	79 (M)	IPF	Not specified	No	Negative	Negative	–	**SAE1**	Possible IIM-ILD
6	64 (M)	NSIP	NSIP	No	Positive	77	–	**SAE1**	Possible IIM-ILD
7	77 (F)	NSIP	NSIP	No	Negative	Negative	–	**SAE1, MDA5**	Possible IIM-ILD
8	26 (F)	ASS CT-ILD	NSIP	Yes	Positive	**19.2**	Jo-1, Ro-52, Ro-60	Jo-1, Ro-52	–
9	58 (F)	DM CT-ILD	NSIP	No	Negative	Negative	–	**MDA5**	Confirms DM CT-ILD
10	61 (M)	MCTD / myositis CT-ILD	NSIP	Yes	Positive	66	U1-RNP	**MDA5**	Consistent with diagnosis
11	72 (M)	Myositis CT-ILD	NSIP	Yes	Positive	Negative	–	Ku	–
12	42 (F)	Scleroderma / myositis CT-ILD	NSIP	No	Positive	37	–	PMScl75, PMScl100	–
13	41 (F)	MCTD CT-ILD	NSIP	Yes	Negative	**Negative**	Ro-52	**Ro52, PL-7**	Possible IIM-ILD

New findings as part of this study that were not known at diagnosis are in bold.
AAV, ANCA associated vasculitis; ANA, anti-nuclear antibody; ASS, anti-synthetase syndrome; CCP, citrullinated cyclic peptide antibody; CTD, connective tissue disease; DM, dermatomyositis; ENA, extractable nuclear antigen; IIM, idiopathic inflammatory myopathy; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; MCTD, mixed connective tissue disease; NSIP, non-specific interstitial pneumonitis; RA, rheumatoid arthritis; RB, respiratory bronchiolitis; RF, rheumatoid factor; UIP, usual interstitial pneumonia.

Cases with positive MPO antibodies

In two cases positive MPO antibodies were detected at moderate-to-high titres (15 and 33 U/mL, reference range <3.5 U/mL) with strong perinuclear ANCA immunofluorescence. In one case, the diagnosis of AAV-ILD [with usual interstitial pneumonitis (UIP) pattern], had been made prior to this study, but years after the patient's original presentation. The second case had been diagnosed with RA-ILD based on a high RF but negative CCP antibodies. This patient had progressive UIP pattern fibrosis, treated with tocilizumab, rituximab and pirfenidone, and clinical features compatible with AAV (livedo reticularis, arthralgias, neuropathy, peripheral eosinophilia).

Cases meeting IPAF classification criteria

Excluding 25 cases that met a diagnosis of CT-ILD, or had a positive myositis antibody or ANCA, 20/80 (25.0%) ILD cases met the serological domain for IPAF (Fig. 1). The majority (n=16; 76.2%) met the ANA criteria. Three cases met the RF criteria and one had a U1RNP antibody in the absence of CTD features. Of these 20 cases, 10/80 (12.5%) could be diagnosed with IPAF based on radiological categorisation (n=6) or histopathology results (n=3).

Autoantibody profiles in CT-ILD cases

Of the 18 cases who had an initial clinical diagnosis of CT-ILD, five (27.8%) presented with isolated ILD without rheumatological symptoms. In two of these five, a complete autoantibody panel at the time of ILD review would have aided in the diagnosis of CTD (2.5% of total ILD cohort).

DISCUSSION

This study is the first, to our knowledge, to retrospectively complete a broad autoantibody profile in patients with a range of ILD subtypes, without reference to clinical or radiological features. Our data identified a significant number of positive tests supporting broad autoantibody testing in the routine ILD diagnostic work-up. Although the significance of some of these antibodies in ILD remains unclear, a clear alternate diagnosis could be made in a number of cases. Comprehensive testing may help in future to better understand ILD subtypes.

The most striking finding was the high rate of myositis antibodies in this cohort (16.2%), most of which were new findings as part of this study. In the majority of cases, clinical features of myositis or anti-synthetase syndrome were absent, and ANA and RF screening tests were negative, leading to a diagnosis of idiopathic ILD. The high positivity rate is consistent with previous studies, with myositis autoantibodies detected in 6.6–38% of ILD cases according to method of detection and patient population.[20–22] Song *et al.* reported 12 of 32 (37.5%) patients diagnosed with idiopathic ILD had detectable myositis autoantibodies. The authors reported clinical characteristics (mechanic's hands, arthralgia, non-specific autoantibodies) were useful for prompting myositis autoantibody testing. However, myositis autoantibodies also frequently occur in the absence of other clinical symptoms or signs of underlying CTD, and in the absence of an ANA.[12,21,23–25] Therefore, clinicians following current guidelines are likely to overlook myositis antibody associated ILD.[6,11] Further research is required to establish the best therapeutic approach for myositis antibody associated ILD without cutaneous or muscle involvement.

Two patients with MPO antibody-associated ILD were detected in this study. In both cases, there were no classical features of AAV at diagnosis. The clinical significance of MPO antibodies in ILD patients without AAV is an area of research interest.[26–29] In a cohort of 305 Japanese ILD patients, 5.2% were MPO antibody positive at the time of diagnosis.[27] The higher rate of MPO antibody-associated ILD in this Japanese cohort compared with our cohort is consistent with known ethnic variability.[30] The 5-year cumulative incidence of microscopic polyangiitis in the Japanese MPO seropositive ILD patients was 24.3%.[27] This suggests that detection of MPO antibodies is clinically relevant, as ILD may precede AAV. Unlike AAV, there are no well-established treatment guidelines for MPO antibody positive ILD without systemic vasculitis.[31] Prospective studies will be required to determine whether immunosuppression may be beneficial in this group.

A number of studies have reported autoantibody seropositivity in ILD. However, most have focused on specific ILD subtypes such as IPF, and non-specific autoantibodies such as ANA and RF.[32,33] Rates of ANA positivity in IPF have varied between 1% and 34% at titres varying from 1:40 to ≥1:320.[32,33] A 2013 study reported similar rates of autoantibody positivity between IPF patients and healthy matched controls, when limiting the ANA and RF positives to titres ≥1:320 and ≥60 IU/mL, respectively.[32] Interestingly, this group also identified cases with disease specific autoantibodies (CCP, dsDNA, RNP, Scl70, PR3). The authors concluded that autoantibodies in IPF patients are unlikely to be pathological based on overall low rates of high titre ANA and ANCAs in both groups, although the potential significance of the disease specific antibodies was not examined, and myositis autoantibodies were not tested. We noted high rates of ANA (42.5%) positivity among our ILD patients, compared with a local population rate of 8.6%.[34] There were also high rates of RF positivity in our cohort (23.7%). However, the low specificity of ANA and RF means these tests have limited diagnostic value in the ILD cohort.

Based on ANA and RF results, with clinical or radiological features, a number of patients in our cohort met the criteria for the new entity 'IPAF', a term created to form a uniform cohort for further study. Where this entity sits in the classification of ILD remains unclear. One recent retrospective study reported that 12.2% of 98 patients meeting criteria for IPAF developed a CTD in 4.5 years of follow-up.[35] In our study, one-quarter of ILD patients met the serological domain for IPAF, of which half could be classified as IPAF. Exactly how clinicians should be managing such patients remains unclear, and further investigations will determine whether this sub-classification is clinically valuable.[7] At present, IPAF classification should lead to ongoing monitoring for autoimmune diseases.

Limitations of this study include the relatively small sample size and subtypes of idiopathic, CT-ILD and other ILDs. However, heterogeneity in study subjects reflects real-world experience of clinicians diagnosing and classifying undifferentiated ILD patients. Selection bias may have been introduced by limiting analysis to tertiary hospital patients who may have more advanced or refractory lung disease. The

exclusion of 43 patients without stored sera is unlikely to represent a bias on the part of clinicians; these patients likely attended private phlebotomy centres, or did not complete testing. We did not collect data on ILD treatment but this is unlikely to affect our results as most blood samples pre-dated the first ILD clinic review. Whilst we implemented a broad autoantibody testing panel, this did not include all potential scleroderma-specific antibodies (Th/To, U3-RNP, U11/U12), which may occasionally be relevant.[36] We cannot exclude the possibility that some results are false positives. However, previous studies have shown high (>97.5%) specificities for both myositis associated antibodies and MPO- and PR3-ANCA assays.[37,38] Specificity increases further with increasing levels and the majority of myositis and MPO antibodies detected in this study were high titre, making it less likely these represent false positives. Furthermore, myositis antibody reference ranges used in this study were established using a local healthy population cohort based on the 99th percentile cut-off (generally above the manufacturer's reference ranges). This strengthens the findings in this study. However, wider application of myositis antibody testing by laboratories using less stringently validated assays increases the risk of false positive results. To this end, myositis antibodies should not be requested in work up of other lung pathologies, prior to confirmation of ILD. Clinicians should also be aware of the potential variability in autoantibody results by specific immunoassay (e.g., different ENA assay methodologies), which is a limitation of immunopathology diagnostics in general. Finally, our retrospective design meant that autoantibodies detected on stored serum did not influence clinical decision-making, and serial testing was not performed. Prospective studies will be required to assess the therapeutic and health economic implications of extended autoantibody testing.

In conclusion, we have demonstrated that an extended panel of autoantibodies including ANA, dsDNA, ENA, RF, CCP, ANCA (where positive followed by MPO and PR3) and myositis antibodies has a high yield in ILD. In particular, myositis antibodies and ANCA positivity cannot be accurately predicted based on clinical features and basic autoantibody testing. We recommend clinicians consider incorporating this panel into their routine ILD work-up, with autoantibody positivity prompting further multidisciplinary assessment. We also propose this broader serological panel should be applied in future research, to allow for better understanding of ILD subtypes. While there remain uncertainties around optimal treatment approaches, the first step towards choosing correct therapy is establishing the correct diagnosis which requires more thorough serological profiling than has previously been recommended.

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