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Review

Autoantibodies in idiopathic inflammatory myopathies: Clinical associations and laboratory evaluation by mono- and multispecific immunoassays

Jan Damoiseaux[a,*], Jean-Baptiste Vulsteke[b,c], Chih-Wei Tseng[d], Anouk C.M. Platteel[e], Yves Piette[f], Ora Shovman[g,h], Carolien Bonroy[i,j], Dörte Hamann[k], Ellen De Langhe[l,m], Lucille Musset[n], Yi-Hsing Chen[d,o], Yehuda Shoenfeld[g,h,p], Yves Allenbach[q,r], Xavier Bossuyt[s,t,**]

[a] Central Diagnostic Laboratory, Maastricht University Medical Center, Maastricht, The Netherlands
[b] Department of General Internal Medicine, University Hospitals Leuven, Leuven, Belgium
[c] Skeletal Biology and Engineering Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium
[d] Division of Allergy, Immunology and Rheumatology, Taichung Veterans General Hospital, Taichung, Taiwan
[e] Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, The Netherlands
[f] Department of Rheumatology, Ghent University Hospital, Ghent, Belgium
[g] Zabludowitz Center for Autoimmune Diseases, Sheba Medical Center, Tel Hashomer, Israel
[h] Sackler Faculty of Medicine, Tel-Aviv University, Tel Aviv, Israel
[i] Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium
[j] Department of Diagnostic Sciences, Ghent University, Ghent, Belgium
[k] Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands
[l] Department of Development and Regeneration, Laboratory of Tissue Homeostasis and Disease, KU Leuven, Leuven, Belgium
[m] Division of Rheumatology, University Hospital Leuven, Leuven, Belgium
[n] Département d'Immunochimie, Sorbonne Universités, AP-HP, Hôpital Pitié Salpêtrière, Paris, France
[o] Faculty of Medicine, National Yang-Ming University, Taipei, Taiwan
[p] Incumbent of the Laura Schwarz-Kipp Chair for Research of Autoimmune Diseases, Sackler Faculty of Medicine, Tel-Aviv University, Tel Aviv, Israel
[q] Département de médecine Interne et Immunologie Clinique, Centre de Référence Maladies Neuro-Musculaires, Sorbonne Universités, AP-HP, Hôpital Pitié Salpêtrière, Paris, France
[r] INSERM - Centre de Recherche en Myologie, UMRS 974, Paris, France
[s] Department of Microbiology and Immunology, Experimental Laboratory Immunology, KU Leuven, Leuven, Belgium
[t] Laboratory of Medicine, University Hospitals Leuven, Leuven, Belgium

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ABSTRACT

Idiopathic inflammatory myopathies (IIM) are a group of diseases characterized by immune-mediated muscular lesions that may be associated with extra-muscular manifestations involving skin, lungs, heart or joints. Four main groups of IIM can be distinguished: dermatomyositis (DM), overlap myositis including mainly anti-synthetase syndrome (ASS), immune mediated necrotizing myopathy (IMNM), and inclusion body myositis (IBM). Myositis-specific autoantibodies (MSA) are increasingly recognized as valuable tools for diagnosis, classification and prognosis of IIM. For example, ASS is associated with anti-aminoacyl tRNA synthetase antibodies (anti-Jo-1, PL-7, PL-12, …), IMNM with anti-SRP and anti-HMGCR; IBM with anti-cytosolic 5'nucleotidase 1A (cN1A), and DM with anti-Mi-2, anti-MDA-5, anti-TIF-1γ, anti-NXP-2 and anti-SAE. Moreover, anti-MDA-5 is associated with amyopathic myositis and interstitial lung disease and anti-TIF-1γ and anti-NXP-2 with juvenile DM as well as malignancy in patients > 40 years. Most MSA have initially been discovered by immunoprecipitation. In routine laboratories, however, MSA are screened for by indirect immunofluorescence and identified by (automated) monospecific immunoassays or by multispecific immunoassays (mainly line/dot immunoassays). Validation of these (multispecific) assays is a challenge as the antibodies are rare and the assays diverse. In this review, we give an overview of the (clinical) performance characteristics of monospecific assays as well as of multispecific assays for detection of MSA. Although most assays are clinically useful, there are differences between techniques and between manufacturers. We discuss that efforts are needed to harmonize and standardize detection of MSA.

* Correspondence to: Jan Damoiseaux, Central Diagnostic Laboratory, Maastricht University Medical Center, P. Debyelaan 25, 6229HX Maastricht, The Netherlands.
** Correspondence to: Xavier Bossuyt, Department of Microbiology and Immunology, University of Leuven and Department of Laboratory Medicine, University Hospitals Leuven, Herestraat 49, 3000 Leuven, Belgium.
E-mail addresses: jan.damoiseaux@mumc.nl (J. Damoiseaux), xavier.bossuyt@uzleuven.be (X. Bossuyt).

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1. Introduction

Idiopathic inflammatory myopathies (IIM) are immune-mediated diseases centered around destruction of muscle tissue due to distinct pathophysiological mechanisms [1]. Actually, it is currently recognized that IIM may also present without myositis, with skin, lung, heart or joint manifestations or in overlap with other systemic autoimmune rheumatic diseases (SARD). These different entities of IIM each have their typical set of clinical manifestations, histopathological findings, and autoantibodies [1–5]. The expanding number of IIM/myositis-specific autoantibodies (MSA) is a challenge for laboratories specialized in clinical immunology because detection of these relatively rare antibodies involves a combination of mono- and multi-specific immunoassays. The first challenge is to select a test panel of good quality, the second challenge is to verify this panel for each distinct autoantibody, and the third challenge is to incorporate the tests within the testing algorithm for SARD [6].

Traditionally, there is a special symposium organized by the European Autoimmunity Standardization Initiative (EASI) at the biannual International Congress on Autoimmunity [7]. At the recent 11th International Congress on Autoimmunity in Lisbon (May 2018) the EASI symposium was dedicated to the role of autoantibody detection in the diagnostic work-up of IIM. Within the clinical spectrum of IIM and the increasing array of autoantibodies, the challenges of autoantibody detection have been discussed with a focus on the different approaches to verify the quality of the immunoassays currently available. This verification is a requirement for ISO 15189 accreditation, which appears more difficult to achieve for autoimmune diagnostic laboratories [8].

The current review first provides an overview of the clinical spectrum of IIM, the array of MSA, and the possible role of the indirect immunofluorescence (IIF) test for the detection of anti-nuclear antibodies (ANA). Next, the approaches that have been used to verify mono- and multi-specific immunoassays for the detection of MSA will be summarized. The discussion will focus on issues related to the need for standardization and harmonization of these immunoassays for appropriate implementation in daily clinical practice.

2. The clinical spectrum of idiopathic inflammatory myopathies

The IIM are a heterogeneous group of diseases, characterized by muscular immune-mediated lesions that may be associated with systemic inflammation involving other structures, such as joints, skin, lungs and heart.

The clinical spectrum of IIM covers a wide range of signs and symptoms and clinical features. Patients can present with symptoms related to muscle lesions, such as muscle weakness or pain. The extra-muscular manifestations may develop synchronously with muscular symptoms or may be the only clinical signs at disease onset and even during follow-up. Extramuscular manifestations include skin changes (e.g heliotrope rash, mechanic hands or Raynaud's phenomenon), respiratory complaints (dyspnea on exertion or cough), and articular signs (arthralgia or arthritis), with or without constitutional symptoms. Furthermore, some IIM patients have an underlying malignancy.

Most of these clinical features are not specific. In addition, myositis can be the only manifestation of a muscular specific autoimmune disease (*e.g.* immune mediated necrotizing myopathy), constitutive of a systemic phenotype of IIM (*e.g.* anti-synthetase syndrome), or a part of the spectrum of systemic diseases (*e.g.* systemic sclerosis or mixed connective tissue disease).

Historically IIM were classified into two categories: dermatomyositis (DM), defined as myositis with characteristic skin changes, and polymyositis (PM), defined by proximal muscle weakness, elevated muscles enzymes, presence of inflammatory alterations on muscle biopsy and myopathic features on electromyography, without DM skin rash [9]. To date, based on the muscle pathology, the extramuscular manifestations and the presence of MSA, it is possible to delineate four main groups of IIM: DM, overlap myositis including mainly anti-synthetase syndrome (ASS), immune mediated necrotizing myopathy (IMNM), and inclusion body myositis (IBM) [10–14].

2.1. Subtypes of idiopathic inflammatory myopathies

- **Dermatomyositis**: this group is hallmarked by a characteristic skin rash including, Gottron's sign or papules, periungual erythema, V and shawls sign and liliaceous eyelid edema (heliotrope rash), whereas the spectrum of muscle involvement ranges from severe symmetrical proximal weakness to no muscular deficit (clinically amyopathic DM). Along that line, a third of DM patients has normal creatine kinase level. The myopathological features are characteristic, combining by definition a perifascicular atrophy with a vasculopathy [12,13]. Nevertheless, DM remains a heterogeneous group since in addition to musculocutaneous variations among the DM phenotypes, some patients may present with polyarthritis, severe and rapidly progressive interstitial lung disease (ILD) or malignancy. These DM subtypes are associated with specific MSA.
- **Anti-synthetase syndrome**: this subset is clinically characterized by the triad of myositis, ILD and arthritis, often in a symmetric polyarticular pattern. This triad can be accompanied with Raynaud's phenomenon, mechanic's hands and fever. The ASS is linked to the presence of anti-synthetase antibodies, further referred to as anti-aminoacyl-tRNA synthetase (anti-ARS) antibodies. As for DM, the myopathological features are characteristic, with the presence of a perifascicular necrotizing myositis [15]. Again, there are variations in ASS phenotype depending on the type of anti-ARS.
- **Immune mediated necrotizing myopathy**: contrary to DM and ASS, IMNM is a subgroup of IIM without clinical extra-muscular auto-immune manifestation [11]. This entity is characterized by a severe muscle weakness with a sub-acute onset. Muscle biopsy findings demonstrate characteristic myofibre necrosis with only minimal inflammation, if any. Patients often present with high creatine kinase levels resistant to immunosuppressant strategies [16–18]. Of note, some patients may have a slowly progressive form mimicking a muscular dystrophy. IMNM diagnosis is based on serological (presence of MSA) or pathological criteria [11]. Some IMNM patients have also an increased risk of malignancy and MSA are crucial to determine this risk [19].
- **Inclusion body myositis**: IBM has a phenotype limited to the muscles. One key feature is that it occurs only after the age of 40, being the most frequent IIM after the age of 50. Contrary to other IIMs, IBM is characterized by a slowly progressive course, and an asymmetrical distribution of muscle weakness, involving not only the proximal but also the distal muscles (mainly quadriceps muscles and finger flexors). The muscle pathology is also characteristic, combining muscle inflammation with CD8+ T cells invading non-necrotic muscle fibers and signs of muscle degeneration (*e.g.*: presence of rimmed vacuoles). This subtype of IIM is also unique since it does not respond to immunosuppressive drugs [20], whereas all other IIMs do.

Due to the heterogeneity of IIM, their diagnosis and classification is often challenging. Nevertheless, making a correct diagnosis is essential for choosing proper treatment (*e.g.* IMNM and IBM) and estimating prognosis (*e.g.* association of malignancy). In the context of the

Table 1
Myositis-specific autoantibodies in IIM, adapted from (1,2).

Antibody	Cellular target	Muscle	Lung	Skin	Other
Anti-ARS	Aminoacyl tRNA synthetases *attach dedicated amino acids to their cognate tRNA.*	Myositis, mostly in anti-Jo1, - PL7, - EJ Myositis may be absent in other anti-ARS	ILD common, may be presenting feature, more severe in anti-PL7, -KS, -OJ, -PL12, most commonly NSIP (but UIP as well)	Mechanic's hands	Raynaud phenomenon Non-erosive arthritis (especially anti-Jo1) Fever
Anti-Jo	*Histidyl tRNA synthetase*				
Anti-PL12	*Alanine tRNA synthetase*				
Anti-PL7	*Threonyl tRNA synthetase*				
Anti-EJ	*Glycyl tRNA synthetase*				
Anti-OJ	*Isoleucyl tRNA synthetase*				
Anti-Zo	*Phenylalanyl tRNA synthetase*				
Anti-Ha	*Tyrosyl tRNA synthetase*				
Anti-KS	*Asparaginyl tRNA synthetase*				
Anti-MDA-5 = *anti-CADM-140*[*]	Melanoma differentiation associated gene 5, RNA helicase encoded by *MDA-5* is involved in the innate immune defense against viruses.	Often amyopathic or mild myositis	ILD possible, rapid progressive ILD can occur (mostly East Asian population)	Classic DM rash Ulceration, palmar pustules	Raynaud phenomenon reported
Anti-SAE	*Small ubiquitin like modifier activating enzyme, involved in post-translational modification of numerous target proteins ("protein sumoylation").*	May be amyopathic initially	No known association	Classic DM rash	None
Anti-Mi-2 = *anti-p140/−MJ*[*]	component of the nucleosome remodeling-deacetylase (NuRD) complex, *involved in transcription regulation*	Myositis (generally mild)	No known association	Classic DM rash	None
Anti-TIF-1γ = *anti-p155/140*[*]	Transcriptional intermediary factor, *a nuclear transcription factor, overexpressed in solid tumors*	Myositis (mild or rarely amyopathic)	No known association	Classic DM rash, severe skin disease, 'red on white' lesions	Adult and juvenile dermatomyositis. Strong association with malignancy
Anti-NXP-2	Nuclear matrix protein 2, *plays roles in diverse nuclear functions, including RNA metabolism maintenance of nuclear architecture regulation of activation of tumor suppressor gene p53*	Myositis (generally severe)	No known association	Classic DM rash Calcinosis	Possible association with Adult and juvenile dermatomyositis. Association with malignancy
Anti-SRP	Signal recognition particle, *involved in the translocation of proteins across the endoplasmic reticulum*	Necrotizing myositis	Association with ILD reported	Often absent	Cardiac involvement unclear
Anti-HMGCR	HMGCoA-reductase, *rate-limiting step in cholesterol synthesis and the pharmacological target of statins.*	Necrotizing myositis	No known association	Often absent	Statin use association

ARS: aminoacyl tRNA synthetases, IBM: inclusion-body myositis, NSIP: non-specific interstitial pneumonia, UIP: usual interstitial pneumonia, ILD: interstitial lung disease, DM: dermatomyositis.

[*] Initial name before discovery of target protein.

complexity of this group of diseases, with involvement of physicians with different backgrounds, the use of MSA will definitely contribute to facilitate and refine IIM diagnosis and classification.

3. Spectrum of autoantibodies in relation to the clinical spectrum and disease criteria

Autoantibodies can nowadays be found in up to 70% of IIM patients [1]. Since the discovery of anti-Mi-2 antibodies in 1976 > 20 autoantibodies have been found in patients with myositis. They are traditionally divided into MSA and myositis-associated autoantibodies (MAA). MSA are found exclusively in patients with a clinical diagnosis of IIM and are generally mutually exclusive. They are present in up to 50% of IIM patients [3]. MSA are associated with specific clinical features in the IIM spectrum and are able to identify subsets of patients with specific phenotypes of skin, muscle and lung disease and malignancy (see Table 1). MAA can also be found in other SARD and they are frequently present in patients with overlap disease.

3.1. Dermatomyositis-related autoantibodies

The autoantibodies targeting Mi-2, melanoma differentiation associated protein 5 (MDA-5), small ubiquitin-like modifier activating enzyme (SAE), nuclear matrix protein 2 (NXP-2) and transcription intermediary factor 1γ (TIF-1γ) are associated with classic DM rashes [1]. Other cutaneous involvement includes psoriasis-like lesions and 'red on white' lesions (anti-TIF-1γ) and calcinosis (anti-NXP-2) [21,22]. Muscle involvement varies from mild to amyopathic forms, as can be the case in anti-MDA-5, anti-SAE or anti-TIF-1γ, to severe involvement, as in anti-NXP-2.

Anti-Mi-2 antibodies were the first MSA detected [23] and are associated with DM (frequency up to 31%) with typical cutaneous lesions and relatively mild disease [24].

Anti-MDA-5-positive patients typically suffer from clinically amyopathic DM (typical DM rashes but no myositis). Rapidly progressive and often therapy-resistant and fatal ILD, is the most dreaded complication [25–27]. The antibodies were first described in an East-Asian population, but they are also found in other populations with reports of an expanded clinical spectrum (*e.g.* inflammatory arthritis) [28–30].

Anti-TIF-1γ antibodies are found in adult and juvenile DM [31]. The antibodies are associated with aggressive skin lesions. In adults above the age of 40, anti-TIF-1γ antibodies are strongly associated with malignancy (in up to 75% of anti-TIF-1γ positive patients) [32,33].

Anti-NXP-2 antibodies are found in juvenile and adult DM. The antibodies are associated with severe cutaneous lesions, including calcinosis. An increased prevalence of malignancy in anti-NXP-2 patients has repeatedly been suggested but a statistically significant association has been difficult to prove [34].

Characteristic clinical features/association among patients with anti-SAE antibodies have not yet been identified [35].

3.2. Anti-aminoacyl-tRNA synthetase autoantibodies

The ASS is the most well-known MSA-defined phenotype. It is associated with anti-ARS autoantibodies. Anti-Jo-1 was the first to be discovered [36] and is the most frequently encountered anti-ARS. Seven other anti-ARSs have been recognized: anti-PL7, anti-PL12, anti-EJ, anti-OJ, anti-Ha, anti-KS and anti-Zo autoantibodies [reviewed in 1]. Presence and severity of the ASS features as well as survival have been correlated with the anti-ARS autoantibody specificity [37–40]. For example, myositis and joint involvement was more common and ILD less frequent in anti-Jo-1 than in anti-PL7/PL-12 ASS patients [35,36]; Survival was lower in non-anti-Jo-1 than in anti-Jo-1 ASS patients [38,40]. ILD can be the sole presenting clinical feature. Detection of MSA is particularly relevant in this group, given the better prognosis of myositis-related ILD compared to idiopathic pulmonary fibrosis [41].

3.3. Immune-mediated necrotizing myopathy-related autoantibodies

IMNM is associated with antibodies to signal recognition particle (SRP) and 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) [reviewed in [16] and [42]]. The muscle pathology is by definition characterized by a diffuse necrotizing myopathy whereas muscle inflammation is only observed in the severe forms [43]. The presence of anti-SRP is associated with severe symmetric proximal muscle disease, and can be accompanied by dysphagia and ILD [44]. The presence of anti-HMGCR is associated with statin use, although this is reported in only 40–60% of patients [45–47]. Disease course is worse in younger and juvenile anti-HMGCR positive patients [48,49].

3.4. Inclusion body myositis-related autoantibodies

Anti-cytosolic 5′-nucleotidase 1A autoantibodies (anti-cN1A; previously referred to as anti-Mup44) are the only known autoantibodies associated with IBM. Anti-cN1A autoantibodies are present in 30–50% of IBM patients, but they have also been found in patients with juvenile DM [50] or other SARD, such as Sjögren's syndrome and systemic lupus erythematosus [51,52]. A recent study found an increased risk of mortality in anti-cN1A-positive patients [53].

3.5. Common myositis-associated autoantibodies

Anti-PM/Scl patients classically have an overlap disease of myositis and systemic sclerosis [54]. Anti-U1RNP patients overlap with mixed connective tissue disease. Anti-Ro52 co-occurs frequently with anti-Jo-1 and potentially identifies patients with more severe ILD [55,56]. Additional less-frequent MAA have been described [57].

3.6. Current role of autoantibodies in classification criteria and diagnosis

Autoantibodies were first included in classification criteria by Love et al. in 1991 [58] and still play a prominent role in a recent classification proposal by Senécal et al. in 2017 [57]. The European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) jointly published new classification criteria for adult and juvenile IIM in 2017 in which they discerned 4 groups (PM, DM, amyopathic DM and IBM) [13]. Of the 5 examined MSA (anti-Jo-1, anti-Mi-2, anti-SRP, anti-PL7 and anti-PL12) only the presence of anti-Jo-1 autoantibodies was retained and assigned the highest number of score points. The lack of sufficient observations of the other MSA prohibited further analyses and incorporation and this was perceived a major limitation of the long awaited new classification criteria [59]. Additional criteria by Mariampillai et al. based on unsupervised analysis and hierarchical cluster analysis of a cohort of 260 patients revealed that MSA, but not muscle biopsy findings, were crucial for classification into 4 subgroups (DM, IMNM, IBM, ASS) [10]. For IMNM, classification and diagnostic criteria based on clinical features and presence of anti-HMGCR or anti-SRP without need for muscle biopsy have been proposed, in addition to pathological criteria [11].

In current clinical practice, MSA already play a key role in diagnosis, next to CK levels, electromyography and muscle biopsy. As each autoantibody is associated with a specific clinical phenotype, as well as characteristic muscle biopsy findings [60], identification of such an autoantibody in the near future could obviate the need for an invasive muscle biopsy, as is already the case for IMNM. The high specificity of MSA allows adequate differential diagnosis with other non-IIM types of myopathy (*e.g.*, metabolic, genetic, or toxic myopathy) and between IIM subgroups. This expanding role demands reliable and practical MSA assays. Further validation of assays and implementation of autoantibody status in upcoming studies are therefore of paramount importance.

Table 2
Studies using monospecific assays in patients with IIM or overlap syndrome to evaluate performance or clinical associations.

Reference and type of study	Antibody	Samples	Techniques	Results	Clinical associations and comments
Nakashima et al. [79] Cohort study on IIM and IIP patients and controls with validation against conventional technique (RNA-IP)	Anti-ARS	***N* = 724** - 250 IIM, - 276 CTD controls (91 SLE, 70 SSc, 75 RA, 27 SS, 13 other CTDs) - 168 IIP - 30 HI	ELISA Plate: Maxisorp; Nunc, Rochester, NY, USA Recombinant ARS antigens including Jo-1, PL-7, PL-12, EJ, and KS except OJ RNA-IP Comparison method	ELISA detected Anti-ARS in 30.8% of IIM, 2.5% of other CTDs and 10.7% of IIP. No anti-ARS was detected in HI. ELISA detected anti-ARS in 100% anti-ARS positive samples by RNA-IP (excluding positive anti-OJ by RNA-IP patients) ELISA detected anti-ARS in one patient negative for anti-ARS by RNA-IP (sensitivity: 100%, specificity: 99.8%)	Anti-ARS can be detected not only in IIM but also in IIP patients. As interstitial pneumonia can precede myositis, autoimmune features including anti-ARS should be evaluated.
Fujimoto et al. [80] Cohort study on IIM and IIP patients and controls with validation against conventional technique (IP)	Anti-TIF-1γ & Anti-Mi-2β	***N* = 709** - 70 PM, 104 DM (Peter and Bohan classified), 68 CADM (Sontheimer classified), - 190 CTD controls (45 RA, 67 SLE, 43 SSc, 20 MCTD, 8 SS, 7 other CTD), - 154 IIP, - 123 HI	ELISA Anti-TIF-1γ (MBL; Nagoya, Japan) ELISA Anti-Mi-2β (MBL; Nagoya, Japan) IP Comparison method	ELISA detected anti-TIF-1γ in 100% anti-TIF1γ positive samples by IP (28.8% DM and 5.9% CADM) but also in 1.1% of CTD controls and 0.6% of IIP (though anti-TIF-1γ was negative by IP in CTD controls and IIP) ELISA detected anti-Mi-2β Abs in 100% anti-Mi-2β positive samples by IP (13.5% DM) but also in 1.1% of CTD controls and in 0.6% of IIP (though anti-Mi-2β was negative by IP in CTD controls and IIP) ELISA didn't detect Anti-TIF-1γ & Anti-Mi-2β in PM and HI	These anti-TIF-1γ & anti-Mi-2β ELISAs showed high concordance with the IP assay. But samples positive for anti-TIF-1γ at low titres need a reconfirmation for a negative anti-Mi-2β due to concerns about cross-reactivity.
Sato et al. [81] Cohort study on IIM and IIP patients and controls with validation against conventional technique (IP)	Anti-MDA5	**N = 709** - 70 PM, 104 DM (Peter and Bohan classified), 68 CADM (Sontheimer classified), - 190 CTD controls (45 RA, 67 SLE, 43 SSc, 20 MCTD, 8 SS, 7 other CTD) - 154 IIP - 123 HI	ELISA Plate: Maxisorp; Nunc, Rochester, NY, USA Recombinant GST-MDA5 fusion protein ELISA Anti-ARS (MBL; Nagoya, Japan) IP and RNA-IP Comparison method	ELISA detected anti-MDA5 antibodies in 98.2% of anti-MDA5 positive samples by IP ELISA didn't detect anti-MDA5 antibodies in anti-MDA5 negative samples by IP	Levels of anti-MDA5 antibodies were important for prognosis and follow up
Aggarwal R et al. [84] Cohort study on anti-SRP positive and negative patients and validation against conventional technique (IP)	Anti-SRP	***N* = 104** - 26 anti-SRP positive patients by IP (25 PM, 1 UCTD; 24/26 fulfilled criteria for IMNM) - 78 anti-SRP negative patients by IP (52 PM, 15 DM, 7 SSc, 4 SLE)	ELISA Plate: Corning, NY, USA Recombinant, purified, full-length human SRP54 (Origene Technologies, Rockville, MD, USA) IP Comparison method	ELISA detected anti-SRP antibodies in 88% of anti-SRP positive samples by IP ELISA didn't detect anti-SRP antibodies in anti-SRP negative samples by IP	Over time, decrease in anti-SRP levels was correlated with decrease in CK levels and increase in manual muscle strength (following treatment).
Musset L et al. [68] Validation of ELISA and CLIA against different technique (ALBIA)	Anti-HMGCR	***N* = 864** - 20 myositis patients positive for anti-HMGCR by ALBIA - 20 age/sex matched controls negative for anti-HMGCR by ALBIA - 824 controls (49 Celiac, 47 RA, 117 SS, 28 HCV, 20 HBV, 14 HSV, 14 CMV, 7 Toxo, 6 HIV, 5 Rubella, 517 HI)	ELISA (QUANTA Lite HMGCR, Inova Diagnostics Inc., San Diego, CA, USA) CLIA (QUANTA Flash HMGCR, Inova Diagnostics Inc., San Diego, CA, USA) ALBIA (ALBIA, Rouen, France) – comparison method	ELISA detected anti-HMGCR antibodies in 100% of positive samples by ALBIA ELISA detected anti-HMGCR antibodies in 0.7% of controls (specificity of 99.3%) CLIA detected anti-HMGCR antibodies in 100% of positive samples by ALBIA	Significant correlation was found between the levels of anti-HMGCR using different methods: ELISA *versus* ALBIA ($\rho = 0.84$), ALBIA *versus* CLIA, ($\rho = 0.89$), and ELISA *versus* CLIA ($\rho = 0.86$). 45% of anti-HMGCR positive patients were statin-exposed.
Shovman O et al. [88] Comparison between two techniques (ELISA and CLIA)	Anti-HMGCR	***N* = 80** - 13 statin-exposed suspected IMNM patients - 67 controls (29 HI, 12 SLE, 10 RA, 8 AS, 2 SSc, 2 PM, 4 APS)	ELISA (QUANTA Lite HMGCR, Inova Diagnostics Inc., San Diego, CA, USA) CLIA	ELISA detected anti-HMGCR antibodies in 100% of the study group CLIA detected anti-HMGCR antibodies in 92% of study group	12/13 of the study group were confirmed as having IMNM by biopsy

(*continued on next page*)

Table 2 (*continued*)

Reference and type of study	Antibody	Samples	Techniques	Results	Clinical associations and comments
			(QUANTA Flash HMGCR, Inova Diagnostics Inc., San Diego, CA, USA)	No anti-HMGCR antibodies were detected in the control group by both methods	
Kramp SL et al. [90] Cohort study on sIBM patients and controls and validation against conventional technique (IP)	cN-1A (Mup44, NT5C1A)	Lab A (USA cohort): ***N* = 286** - 17 definite sIBM patients - 14 suspected sIBM patients - 110 myositis controls (7 PM, 4 DM, 94 unspecified myositis without sIBM, 1 muscle atrophy, 4 myonecrosis) - 93 other autoimmune disease controls (33 SLE, 20 SS, 20 RA) - 52 HI Lab B (Dutch cohort, German controls): ***N* = 253** - 51 definite sIBM patients - 202 HI	ELISA (Euroimmun, IgG) using recombinant full-length cN-1A IP Comparison method	Lab A: ELISA detected anti-CN-1A antibodies in 47% of definite sIBM patients and 21% of suspected sIBM patients, but also in 4% of myositis controls, 5% of other autoimmune diseases controls, and 1% of HI sensitivity: 35.5%, specificity: 96.1% Lab B: ELISA detected anti-CN-1A antibodies in 39% of definite sIBM patients, but also in 4% of HI controls sensitivity: 39.2% specificity: 96.5% Significant correlation was found between anti-CN-1A and IP ($r = 0.8320$, $P < 0.0001$, 30 patients)	Anti-cN-1A reactivity was absent in biopsy-proven classic polymyositis or dermatomyositis
Mahler et. al. [92] Cohort study on PM/Scl overlap syndrome patients and controls and validation against conventional technique (IB and/or IIF)	Anti-PM1-α	*N* = 567 40 PM/SSc overlap 452 Rheumatic disease controls including 205 SSc, 40 PM, 69 RA, 6 MCTD, 10 UCTD, 114 SLE, 8 others	ELISA Plate: maxisorb; Nunc, Rosilke, Denmark A novel antigen “PM1-α peptide” comprising alpha-helical structure of the major PM/Scl-100 epitope ELISA with recombinant PM/Scl-100 (Diarect AG, Freiburg, Germany) IB and/or IIF Comparison method	ELISA detected anti-PM1-α in 55% PM/SSc, 13.2% SSc and 7.5% PM patients. ELISA using PM1-α peptide detected 32 (97%) anti-PM/Scl in 33 sera identified by IB and/or IIF while ELISA using recombinant PM/Scl-100 detected 26 (78.8%) in 33.	anti-PM1-α antibodies are identified mainly in PM/SSc overlap syndrome, SSc or PM. PM1-α antigen is more sensitive than recombinant PM/Scl-100 to detect anti-PM/Scl.

Abbreviations: ALBIA addressable laser bead immunoassay; APS antiphospholipid syndrome; ARS aminoacyl-tRNA synthetases; AS ankylosing spondylitis; CADM clinically amyopathic dermatomyositis; CK creatine kinase; CLIA chemiluminescent immunoassay; CMV cytomegalovirus; cN-1A (Mup44 NT5C1A) cytosolic 5′-nucleotidase 1A (muscle protein of 44 kDa); CTD Connective tissue disease; DM dermatomyositis; ELISA enzyme-linked immunosorbent assay; HBV hepatitis B virus; HCV hepatitis C virus; HI healthy individuals; HIV human immunodeficiency virus; HMGCR 3-hydroxy-3-methylglutaryl-CoA reductase; HSV herpes simplex virus; IB immunoblot; IIF indirect immunofluorescence; IIM idiopathic inflammatory myopathy; IIP idiopathic interstitial pneumonia; IMNM immune mediated necrotizing myopathy; IP immunoprecipitation; MCTD mixed connective tissue disease; PM polymyositis; sIBM sporadic inclusion body myositis; RA rheumatoid arthritis; r Pearson's correlation coefficients; ρ Spearman's rho; RNA-IP RNA immunoprecipitation; SLE systemic lupus erythematosus; SS Sjögren syndrome; SSc systemic sclerosis; SRP signal recognition particle; Toxo toxoplasmosis; UCTD undifferentiated connective tissue disease.

Table 3
Studies using multiplex assays in the context of IIM to evaluate performance characteristics or clinical associations.

Reference and type of study	Samples	Techniques	Results	Clinical associations and Comments
Rönnelid J et al. [97] Cohort study on IIM patients and controls	$N = 230$ 153 IIM (50 DM, 89 PM, 4 JDM [Peter and Bohan classified],10 IBM) 77 disease controls (26 pSS, 26 SLE, 25 SSc)	LIA (Euroimmun, myositis profile)[a] LIA (Euroimmun, separate SRP-strip)	LIA detected MSA/MAA (anti-SRP excluded) in 43% (20% with exclusion of anti-SSA/Ro52) of IIM patients and 38% (4% with exclusion of anti-SSA/Ro52) of controls. LIA detected single anti-SRP reactivity in 5% of IIM patients and 3% of controls.	LIA showed temperature-dependent reactivity (temperature control necessary)
Ghirardello A et al. [98] Cohort study on IIM patients and controls and validation against conventional techniques (IP and IB)	$N = 438$ 208 IIM (100 PM, 63 DM, 2 JDM [Peter and Bohan classified], 27 overlap myositis, 9 CAM and 7 ASS) 50 age/sex-matched HC 180 disease controls (11 non-autoimmune myopathy, 23 sporadic or genetic muscular dystrophies, 11 UCTD, 68 SLE, 36 SSc, 22 pSS, 9 arthropathy)	LIA (Euroimmun, myositis profile)[a] IP (for ASS-associated antibodies), anti-SRP) IB (for Mi-2 and MAA)	LIA detected MSA/MAA in 47% (38% with exclusion of anti-SSA/Ro52) of IIM patients and 31% (8% with exclusion of anti-SSA/Ro52) of controls. Conventional techniques detected MSA/MAA in 51% (43% with exclusion of anti-SSA/Ro52) of IIM patients and 23% (5% with exclusion of anti-SSA/Ro52) of controls. Concordance rate between assays 91%.	Anti-SRP not included in the line blot
Cruellas MG et al. [99] Cohort study on IIM patients	$N = 222$ 127 DM, 95 PM [Peter and Bohan classified]	LIA (Euroimmun, myositis profile)[a]	LIA detected MAA in 41% and MSA in 34%.	Anti-SRP not included in the line blot Anti-Mi-2 in DM (12%): photosensitivity ($p = .002$); Shawl's sign ($p = 0.020$) Anti-Jo-1 in PM (32%): articular manifestations ($p = 0.010$) and pulmonary disorders ($p < 0.001$) Anti-SRP (3.2%): no correlations with signs of myositis severity or heart disease
Cavazzana I et al. [75] Cohort study on IIM patients and validation against conventional techniques (IP)	$N = 66$ 66 IIM (23 PM, 29 DM [Peter and Bohan classified], 8 ASS, 6 overlap syndromes)	LIA (Euroimmun, Autoimmune Inflammatory Myopathies) [b] IP (57 samples)	IP detected MSA in 63% LIA detected MSA in 59% Good agreement between LIA and IP for anti-TIF1-γ, anti-MDA5 and anti-NXP-2, moderate agreement for anti-Mi-2 and anti-EJ, poor agreement for anti-Jo1	Multiple reactivities in LIA (17%), none on RNA-IP
Tampoia M et al. [100] Cohort study on IIM patients and controls	$N = 122$ 53 IIM (33 PM, 20 DM) 20 HC 49 diseased controls (19 RA, 3 SSc, 3 UCTD, 22 SLE, 1 SjS, 1 MCTD)	2 DIA: - Alphadia Synthetase 10 IgG [c] - Sclero-Poly-Synthetase Profile 12 Ag IgG [d]	DIA detected MAA/MSA in 55% of IIM patients. The specificity of the DIA ranged from 98 to 100% (for the individual antibodies).	Comparison with IIF (lower sensitivity for IIF) and CLIA for anti-Jo1 93% of patients affected with ILD had anti-Jo-1 Anti-Mi-2 and -SAE positive patients had heliotrope rash and Gottron papules. Anti-TIF-1γ positive patient ($n = 1$) had no neoplasia
Tan TC et al. [101] Retrospective study on consecutive routine samples	$N = 96$ Consecutive patients with MSA/MAA request (1 year) Retrospectively clinically documented: 21 IIM, 18 ILD, 57 other diseases	LIA (Euroimmun, myositis profile 3) [e]	LIA detected MSA/MAA in 86% of IIM and 56% of ILD patients and 25% of other disease controls	
Bundell C et al. [102] Cohort study on IIM patients and controls	$N = 321$ 124 IIM (biopsy proven) (27 DM, 11 PM, 51 IBM, 10 IMNM, 25 overlap myositis) 197 HC	LIA (Euroimmun, myositis profile 3) [e]	LIA detected MSA/MAA (except anti SSA/Ro52) in 34% of IIM and 17% of HC. Definition of cut-off for individual antibodies based on 99th percentile of HC resulted in detection of MSA/MAA (except anti-SSA/Ro52) in 21% of IIM and 4.6% of HC.	Suggests that adjusting cut-off levels might increase specificity
Infantino M et al. [103] Retrospective study on consecutive routine samples, further preselected on ANA IIF pattern	$N = 493$ Consecutive patients with ANA request and presence of a fine dense speckled cytoplasmic pattern ($n = 254$) Control sera with negative cytoplasmic pattern ($n = 239$)	DIA (Alphadia Synthetase 10 IgG)[c]	DIA detected ASS-related antibodies in 7.1% of sera with cytoplasmic pattern and 1.7% in the controls. Of the 22 ASS-related antibody positive patients: 8 had compatible clinical symptoms, 10 undefined connective tissue disease, 1 RA and 3 a diagnosis not associated with ASS.	Only ASS-related antibodies taken into account. Retrospective clinical documentation of antibody positive samples only.

(*continued on next page*)

Table 3 (*continued*)

Reference and type of study	Samples	Techniques	Results	Clinical associations and Comments
Lecouffe-Desprets M et al. [104] Retrospective study on consecutive routine samples	$N = 237$ Consecutive patients with MSA/MAA request (1 year) Retrospectively clinically documented: 39 IIM, 10 non-IIM AID, 188 other diseases)	LIA (Euroimmun, Autoimmune Inflammatory Myopathies) [b]	LIA detected MSA/MAA (except anti-SSA/Ro52) in 56% of IIM patients, 100% of AID patients and 6.9% of patients with other diagnosis. Strong antibody reactivity significantly higher in IIM (82%) *versus* non-IIM (35%)	Suggests that adjusting cut-off levels might increase specificity.
Vulsteke JB et al. [105] Cohort study on IIM patients and controls	$N = 384$ 144 IIM 240 controls (blood donors, chronic inflammatory demyelinating polyneuropathy, RA, SSc, pSS and SLE; 40 of each)	Comparison of a DIA and 2 LIA: - Alphadia, Myositis Profile 12 IgG[f] - Euroimmun, Autoimmune Inflammatory Myopathies [b] - Trinity biotech, Immunostripe LIA [g]	Considerable differences in specificity and sensitivity between different line/dot blot assays and for different autoantibodies	Comparison of three different line/dot blot assays, demonstrates need for harmonization and standardization. Anti-Jo-1 (17–21%): highly significant association with arthritis, ILD, Raynaud's phenomenon ($p < 0.0001$ for all LIAs) Weaker association for anti-TIF-1γ and –NXP-2 with malignancy, anti-Mi-2 with DM, anti-NXP-2 with calcinosis and MDA-5 with amyopathic IIM (with differences between assays)
Yang H et al. [107] Cohort study on IIM patients	$N = 617$ 70 PM, 378 DM, 33 IMNM, 136 ASS (Peter and Bohan, ENMC or Sontheimer classified)	LIA (Euroimmun, Autoimmune Inflammatory Myopathies) [b] ELISA (Inova, for anti-HMGCR)	MSA detected in 72.4% of IIM patients Presence of anti-TIF1-y, anti-NXP2 and anti-SAE1 significantly associated with CAM	10 patients of initial 627 patients cohort excluded based on multiple reactivity
Best M et al. [107] Prospective multicentre cohort study DM patients	$N = 117$ 117 DM (Peter and Bohan or Gerami classified)	2 DIA: - Alphadia Synthetase 10 IgG [c] - Sclero-Poly-Synthetase Profile 12 Ag IgG [d]	MSA detected at time of inclusion in 47.1% of patients.	In DM patients: Anti-MDA-5 (14%) significantly associated with: palmar erythema ($p = 0.0002$ mechanic's hands ($p = 0.0014$)), cutaneous necrosis ($p = 0.0433$), higher risk of ILD ($p = 0.003$) and arthralgia and/or arthritis ($p < 0.0001$)), lower risk of elevated CPK ($p = 0.0087$) Anti-TIF-1y (11%) significantly associated with: poikiloderma ($p = 0.036$) Anti-NXP-2 (6.8%) significantly associated with: calcinosis ($p = 0.032$) and a lower risk of Gottron's sign/papules on hands ($p = 0.0087$); Anti-ARS (5.1%) significantly associated with: arthralgia and/or arthritis ($p = 0.0039$) and a lower risk of eyelid involvement ($p = 0.022$); anti-Mi-2 (8.5%) and anti-SAE (5.1%): no associations with specific skin features or muscular disease
De Sadeleer et al. [108] Cohort study on IIP patients	$N = 68$ 68 IIP with suggestive morphological interstitial lung disease patterns	DIA Alphadia, Myositis Profile 12 IgG[f]	MSA detected in 18% IIP patients (7 anti-Jo-1, 2 anti-NXP2, 1 anti-PL-7, 1 anti-TIF1-y, 1 anti-SRP)	High prevalence ofMSA antibodies without presence of ANA in a cohort of IIP patients

Abbreviations: ANA, anti-nuclear antibodies; AID, autoimmune disease; ASS, anti-synthetase syndrome; CAM, cancer associated myositis; CPK, blood creatine phosphokinase; CLIA, chemiluminescence immunoassay; DIA, dot immunoassay; DM, dermatomyositis; HC, healthy controls; IB, immunoblot; IBM, inclusion body myositis; IIF, indirect immunofluorescence; IIM, Idiopathic inflammatory myositis; IIP, idiopathic interstitial pneumonia; IMNM, immune mediated necrotizing myopathy; ILD, interstitial lung disease; IP, immunoprecipitation; JDM, juvenile dermatomyositis; LIA, line immunoassay; MCTD, Mixed connective tissue disease; PM, polymyositis; RA, rheumatoid arthritis; pSS, primary Sjögren's Syndrome; SjS, Sjögren's Syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; UCTD, undifferentiated connective tissue disease.

[a] The Myositis Profile lineblot of Euroimmun contains the following antigens Mi-2, Ku, PM/Scl, Jo-1, PL-7, PL-12, Ro-52 (and SRP in some studies); (2) The Inflammatory Myopathies lineblot of Euroimmun contains Mi-2 alpha, Mi-2 beta, TIF1-y, MDA5, NXP2, SAE1, Ku, PM/Scl-100, PM/Scl75, Jo-1, SRP, PL-7, PL-12, EJ, OJ, Ro-52;

[b] The Alphadia Synthetase 10 IgG dotblot contains OJ, KS, HA, ZO, NXP2, SAE-1, SAE-2, EIF-3, HMGCR, RNAP-III;

[c] The Alphadia Sclero-Poly-Synthetase Profile 12 Ag IgG immunodot contains Jo1, PL-7, PL-12, EJ, SRP, Mi2, MDA-5, TIF1-y, Ku, PM/Scl-100, Scl70, Ro-52);

[d] The Euroimmuun, myositis profile 3 contains Mi-2, Ku, PM/Scl-100, PM/Scl-75, Jo-1, SRP, PL-7, PL-12, EJ, OJ, Ro-52;

[f] The Alphadia, Myositis Profile 12 IgG immunodot contains Jo1, PL7, PL12, EJ, SRP, Mi-2, MDA-5, TIF1-y, Ro- 52, HMGCR, SAE-1, SAE-2, NXP2;

[g] The Trinity biotech LIA contains PM/Scl-100, PM/Scl-75, Ro-52, Jo-1, Mi-2, Ku, PL-7, PL-12, SRP54, U1RNP68, U1RNP A, U1RNP C, EJ and OJ.

4. The role of ANA in the diagnostic work-up of idiopathic inflammatory myopathies

Detection of anti-nuclear antibodies (ANA), as detected by an indirect immunofluorescence assay (IIFA) on HEp-2 cells, is considered the most optimal screening assay for autoantibodies in the SARD [61]. Whether this also holds for the IIM is more than questionable for several reasons. First, in the international recommendations on ANA testing, as issued by a combined effort of the International Union of Immunological Societies (IUIS) and the EASI, it is recognized that the ANA IIFA is not sufficiently sensitive for the spectrum of MSA, in particular for the anti-ARS autoantibodies [62]. This has recently been confirmed in a large dataset obtained in a routine setting [63]. Second, there is no consensus on whether autoantibodies to cytoplasmic components of the HEp-2 cells are to be considered as ANA positive [64]. Taking into account cytoplasmic staining in the HEp-2 IIFA strongly increases the sensitivity for the ASS [65] and asks for adequate reflex-testing. Third, in the widely used algorithm for reflex-testing upon a positive ANA result, often only anti-Jo-1 antibodies are included. Therefore, it has been advocated that upon clinical suspicion of IIM, the first test of choice is a multispecific immunoassay for the whole spectrum of MSA [6,66]. However, these tests still await approval by the US Food and Drug Administration (FDA) [67]. Furthermore, some autoantibodies, like anti-HMGCR and anti-cN1A autoantibodies, are not included in the currently available multispecific immunoassays, they have to be tested for in monospecific immunoassays as far as warranted by the clinical manifestations associated with the respective MSA.

Based on the situation described in the previous paragraph, it could be concluded that there is no role for ANA in the diagnostic work-up of IIM. However, this is not true. First, several of the MSA do reveal an HEp-2 IIFA pattern. Therefore, this test can be used for confirmation upon finding an antigen-specific antibody in the multispecific immunoassay. With respect to HEp-2 IIFA patterns, recently a consensus on nomenclature and description has been published by the International Consensus on ANA Patterns (ICAP) initiative [68]. The nuclear patterns associated with the MSA include the fine speckled patterns (AC-4) for anti-Mi-2 and anti-TIF-1γ autoantibodies, and the multiple nuclear dots patterns (AC-6) for anti-NXP-2 autoantibodies. However, many autoantibody specificities can give a fine speckled nuclear pattern and multiple nuclear dots are not consistently found in patients with anti-NXP-2 antibodies. With respect to the cytoplasmic patterns, antibodies to SRP and the ARS, other than Jo-1, reveal the dense fine speckled patterns (AC-19), while anti-Jo-1 is linked to the fine speckled pattern (AC-20). Whether the distinct anti-ARS antibodies really reveal two different patterns is a matter of discussion; this may also depend on the HEp-2 substrate used for the IIFA. It has been suggested that anti-HMGCR antibodies are associated with a cytoplasmic staining [69]. However, this is not consistently found and may be substrate dependent. For some autoantibodies, finding a specific HEp-2 IIFA pattern in conjunction with a positive finding in a multispecific immunoassay may confer specificity. Such added-value of IIF compared to dot immunoassay (DIA) has been shown for anti-SRP antibodies [70]. In 60 patients that tested positive by DIA, the presence of the typical anti-SRP pattern (AC-19) by IIF was strongly associated with IIM: IIM was present in 30/33 (91%) patients with the typical IIF pattern compared to 6/27 (22%) of patients without the typical IIF pattern [70].

The second reason for including ANA IIF testing in the diagnostic work-up of IIM is because this disease can occur in overlap with other SARD, in particular systemic sclerosis. Although the autoantibodies associated with the overlap syndrome, *i.e.* antibodies to Ku, PM-Scl and U1RNP, might be included in some of the IIM multispecific assays, the clinical manifestations of IIM may not yet be very apparent at the clinical onset of the disease. For systemic sclerosis, however, the ANA IIFA is still the best choice as a screening assay [71–74]. Importantly, the autoantibodies related to the overlap syndrome can also be detected in the systemic sclerosis multispecific immunoassay and, as such, should direct the clinician to search for IIM related clinical manifestations. Again, the HEp-2 IIFA pattern may give a clue to the antigen-specificity. All three antibodies are associated with nuclear patterns: anti-Ku with the fine speckled pattern (AC-4), anti-U1RNP with the large/coarse speckled pattern (AC-5), and anti-PM-Scl with the homogeneous nucleolar pattern (AC-8).

Altogether, for some MSA, the HEp-2 IIFA may be of added-value as a kind of confirmation assay for antigen-specific antibodies identified in the IIM multispecific immunoassay, or as a screening assay for the myositis overlap syndromes. Moreover, some IIM patients test positive by IIF but negative for any of the known MSA, indicating that not all antibodies have yet been identified/characterized. In addition, some MSA give a specific staining on tissue substrates. For example, anti-SRP antibodies give a staining of the gastric chief cells [70] and anti-HMGCR antibodies give a distinct IIF staining pattern on rat liver sections (cytoplasmic staining in distinct scattered hepatocytes) [75].

5. Immunoassays for the detection of myositis-specific autoantibodies

A scala of techniques is available to detect autoantibodies in myositis. Conventional technologies such as IIF and immunoprecipitation (IP) use native antigens, are laborious and require a high level of expertise. In routine diagnostics, enzyme-linked immuno-sorbent assays (ELISA), and variants thereof, are widely used. These assays use purified native and recombinant antigens or synthetic peptides, can be automated, require less expertise and have a better reproducibility. Conventional technologies employing native antigens generally allow better detection of antibodies directed against conformational-dependent epitopes as well as epitopes originating from post-translational modifications. IP is considered the gold standard assay for the detection of most MSA, with a high sensitivity and specificity.

In recent decades, novel MSA and their clinical associations have been identified by IP. Yet, different methods for MSA detection have revealed conflicting results [76]. As more and more commercial assays are introduced in clinical practice, it is important to compare the performance of the assays to gold standard IP [77]. There are currently several commercial monospecific and multispecific immunoassays available to detect MSA. The clinical evaluation of these assays is summarized in Tables 2 and 3.

5.1. Monospecific immunoassays

Within the spectrum of MSA, anti-Jo-1 is the most widely known autoantibody. Line immunoassay (LIA) or IP is often used to confirm the presence of anti-Jo-1, but also ELISA or comparable methods such as fluoroenzyme immunoassay (FEIA) are commonly used methods to confirm anti-Jo-1 antibodies. Most manufacturers provide in their kit inserts information on diagnostic sensitivity and specificity of the assay, but few have comparative data to IP.

As the presence of anti-Jo-1 has recently been included in the classification criteria of IIM [13], it is crucial to have reliable methods for anti-Jo-1 antibody detection. For example, a novel Zenit RA chemiluminescent immunoassay (CLIA) was found to have a good Kappa agreement with IP [77]. However, when compared with ELISA, inconsistent results were still found. More comparative data between assays are needed [78].

A screening ELISA (MESACUP anti-ARS test, MBL, Nagoya, Japan) has been developed for the detection of anti-ARS autoantibodies. In addition to Jo-1, also PL-7, PL-12, EJ, and KS are included as autoantigen. This assay is not to be considered a multispecific immunoassay because the antigen-specificity is not unequivocally determined. This ELISA kit has a good sensitivity and specificity when compared to IP. Besides, the assay has also been validated both in patients with IIM as well as in patients with ILD [79].

Commercial ELISAs are also available for anti-TIF-1γ, anti-Mi-2 and

anti-MDA-5 for DM, with almost 100% sensitivity and specificity when compared to IP [80,81]. Anti-Mi-2 can also be detected by FEIA, but no comparative data to IP is available.

With the introduction of anti-MDA-5 ELISA, patients with rapid progression of ILD can now be more easily identified. Serial follow-up of anti-MDA-5 provides prognostic information during the treatment [82].

The IMNM-specific autoantibodies anti-SRP and anti-HMGCR are currently part of the diagnostic criteria for IMNM [11]. Anti-SRP antibodies were initially identified by RNA IP. These antibodies were directed against conformational epitopes of SRP, including 7SL RNA and six polypeptides (molecular weights 72, 68, 54, 19, 14 and 9 kDa) [83]. Recently, an ELISA has been developed and validated for the measurement of anti-SRP antibodies directed against the 54-kDa subunit (SRP54), which is considered the main antigenic target of anti-SRP antibodies [84]. The comparison of anti-SRP-54 ELISA and IP showed a high level of inter method-agreement, with ELISA having both high specificity (100%) and high sensitivity (88%). However, other studies indicated that anti-SRP54 antibodies may not be detected by ELISA in samples containing autoantibodies to 7S RNA of SRP [18,85]. Hence, there is a risk of false negative results with SRP54-specific immunoassays.

Different technologies may be used to detect anti-HMGCR antibodies, including IP, addressable laser-bead immunoassay (ALBIA), ELISA, and CLIA [45,68,86–88]. In 2012, a novel anti-HMGCR ELISA exhibited high sensitivity (94.4%) and specificity (99.3%) when compared to IP [45]. Testing of anti-HMGCR positive sera and negative controls by ALBIA, ELISA, and CLIA in one study [68] and by ELISA and CLIA in another study [88] demonstrated a very good inter-method agreement of 100%, with a significant correlation between the levels of anti-HMGCR antibodies as determined by these methods.

Anti-cN1A autoantibodies were initially detected by immunoblotting from purified skeletal muscle extracts [89]. A novel ELISA is available for the detection of anti-cN1A IgG autoantibodies with a diagnostic specificity of > 96%, and a sensitivity that varied between 35.5% and 39.2% in patients with suspected and definite IBM [90]. As already mentioned, the high specificity is challenged by the finding that also patients with systemic lupus erythematosus and Sjögren's syndrome may have these autoantibodies. Further commercial assays are required for combined Ig isotype detection (IgM, IgA, and IgG) that may increase the diagnostic sensitivity of anti-cN1A autoantibodies.

MAA, like anti-PM/Scl antibodies can also be detected by monospecific immunoassays [91–94]. Especially FEIA [91] and ELISA [92,93] have shown good diagnostic performance compared to conventional methods, including IP.

5.2. Multispecific immunoassays for myositis-specific autoantibodies

In the context of the expanding spectrum of MSA, companies and laboratories move forward in the direction of multispecific immunoassays to safe time, material and labour costs. These multispecific immunoassays include dot or line immunoassays (DIA/LIA), based on spotting antigens on nitrocellulose membranes as dot (DIA) or line (LIA), ALBIA, and solid-phase antigen microarrays. Theoretically, results for single antibody specificities obtained with multispecific assays should be comparable to results obtained with conventional techniques, and in particular with the gold standard IP, which in itself is also a multispecific technique. In this review, however, we focus on data from commercially available assays. One might expect even better performance characteristics due to increased purity of antigens, selection of immunodominant antigens as well as more sensitive detection methods. On the other hand, combining different antigens in one assay may lead to less optimal assay conditions for each antigen and interference in read out.

Due to these reasons, validation of multispecific techniques by comparing results to conventional techniques and with clinically well-defined myositis patients is a prerequisite for correct interpretation of results and translation into clinical use and to comply with ISO 15189 guidelines [95]. However, clinical evaluation of IIM multispecific assays is challenging due to several reasons.

First, MSA and MAA were originally discovered using conventional techniques and therefore clinical associations with MSA/MAA were first described using these techniques [5]. Although IP is often considered the gold standard, differences between this and newer techniques do not necessarily reflect superiority of one technique, which makes comparison more difficult.

Second, if one desires to clinically validate multispecific immunoassays, the heterogeneity of the disease should be taken into account. One approach to tackle this, makes use of samples of clinically defined patient- and disease control cohorts. Ideally, samples should be collected at time of diagnosis. While use of clinically defined patients is superior when antibody frequencies are considerably high, the approach fails for a disease context with multiple antibodies in very low frequencies unless the cohort is very large. In addition, patient cohorts may show a selection bias since different classification criteria were used. Collaborative studies like the international, multidisciplinary myositis register, EuroMyositis, currently including > 4000 patients with IIM and involving 23 clinics worldwide might overcome these problems [96]. Another approach for evaluating multispecific immunoassays are studies including consecutive patients with a request for MAA/MSA testing. Such studies reflect daily diagnostic practice but often miss a comparison with data obtained with conventional techniques. The studied population is often too small to draw conclusions for low frequency antibodies and clinical data are more difficult to gather retrospectively. In addition, patients without a clear diagnosis of IIM at the time of blood draw might develop IIM later in time. Inclusion of such patients into the non-IIM control group will negatively influence the reported specificity. Also, association of MSA with specific clinical features such as lung disease or malignancy might be underestimated. Prospective studies that circumvent these problems have not been performed yet. Ideally, clinical evaluation is combined with comparison to different existing techniques. Since multispecific assays for the detection of MSA/MAA have only become recently available not many studies have been published yet. Table 3 summarizes the current relevant literature on multispecific assays with details on study design, assays used, frequencies of MSA/MAA detected and clinical associations described [99–109].

In conclusion, multispecific DIA/LIA show an increased sensitivity compared to immunofluorescence-based techniques, especially evident for anti-ARS autoantibodies [100]. Only two studies describe a comparison between the conventional techniques (IP and immunoblot) and the multispecific LIA [75,98]. In both studies, sensitivity and specificity (the latter only evaluated in Ghirardello) were higher for the conventional techniques compared to LIA for individual MSA. Two studies suggest that adjusting cut-off levels might increase specificity of LIA [102,104]. In general, reported sensitivities and specificities largely differ. Differences could be explained by differences in the number of detected antigens, low and varying numbers of patients in the different IIM subtypes and controls included and also by differences in inclusion criteria. New EULAR/ACR classification criteria for IIM have recently been published and should improve comparability of results in future studies [13]. Since presence of only a single autoantibody (anti-Jo-1) is included in the classification criteria, future studies should be carefully designed to avoid a selection bias.

The recent discussion following the study of Vulsteke et al. that compared three different multispecific assays and revealed considerable differences in specificity and sensitivity between different DIA/LIA and for different autoantibodies, nicely demonstrates the current state of clinical validation of multispecific assays and shows the need for harmonizing and standardization efforts in multicentre approaches [105,109,110]. Despite of these shortcomings, use of multispecific assays reveals clinically relevant data and facilitates better diagnosis in

IIM.

6. Discussion

Historically, most of the MSA have been identified by IP and clinical associations were established based on IP. Accordingly, it is important that mono- and multispecific assays that are used in clinical laboratories are compared with IP. Such comparisons have been performed for several autoantibodies and some (mainly monospecific) assays. But, for the newer technologies (such as the multispecific DIA/LIA) such studies are scarce and have revealed poor agreement [75,98]. There is also poor agreement between different DIA and/or LIA [105]. Thus, standardization of DIA/LIA remains poor with substantial variability between assays. It should be noted, however, that also IP has disadvantages and that considering IP as the gold standard may be disputed.

HEp-2 IIFA can reveal MSA. However, for several MSA sensitivity is limited (not 100%). Inversely, the association of HEp-2 IIFA patterns involved with the presence of MSA is limited. The strongest association concerns the speckled cytoplasmic patterns with anti-SRP and anti-ARS antibodies. As HEp-2 IIFA is used to screen for SARD, it is important that technicians recognize these patterns. Also the nucleolar pattern is important as it is associated with antibodies found in systemic sclerosis or scleroderma-overlap syndromes. Multiple nuclear dots can suggest anti-NXP-2 and the fine speckled nuclear pattern can suggest anti-Mi-2 antibodies, however, these associations are not strong. Anti-HMGCR gives a distinct pattern on liver tissue and possibly also on HEp-2 cells [68,74]. Thus, as HEp-2 IIFA is an important technique for SARD screening, it is important to appreciate its value, but also to recognize its limitations for MSA detection. Overall, HEp-2 IIFA has a low specificity and suffers from variability between laboratories.

There is a growing interest in MSA and in daily clinical practice many clinicians (in rheumatology, neurology, pulmonology or dermatology) that take care of patients suspected to have an inflammatory myopathy request MSA panels (LIA or DIA). In many cases, multispecific analysis for MSA is beneficial and allows to diagnose IIM and/or to provide prognostic information. Therefore, a multispecific approach for MSA detection is expected to become the standard to evaluate patients suspected for IIM. However, as reviewed in this manuscript, there is variability between assays and efforts are needed to harmonize/standardize the assays and test interpretation. An important issue in this respect is to safeguard a high specificity. This is especially important given the low prevalence of MSA and the clinical relevance. As previously proposed [102,104,111], a practical way to address this issue is to use/define several (*e.g.* 2) cutoff values: a high cutoff that corresponds to a high specificity and a high likelihood ratio and a second, lower cutoff to exclude the presence of the antibody. Another, complementary way to ensure the specificity of a test result obtained by multispecific immunoassay is to correlate this result with another monospecific assay or with the HEp-2 IIFA result. The presence of a corresponding HEp-2 IIFA pattern suggests a specific reaction. This can be further optimized by including clinical information. Obviously, this requires good communication between the clinician and the laboratory specialist: one of the goals of EASI [7].

In conclusion, there is an increasing interest in MSA and MSA are being included in (clinico-serological) classification criteria [2,4,11,13]. It is the shared responsibility of the industry, laboratory professionals and clinicians to offer autoantibody assays that have clinical value [112]. Multicenter initiatives and (prospective) studies will be needed to harmonize MSA test results and to further validate and ensure clinical usefulness of the antibodies [113].

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