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# 256th ENMC international workshop: Myositis specific and associated autoantibodies (MSA-ab): Amsterdam, The Netherlands, 8-10 October 2021

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## 1. Introduction

The organizers of this 256th European NeuroMuscular Center (ENMC) workshop welcomed 20 participants from 10 countries (Belgium, China, Czech Republic, France, Germany, Netherlands, Spain, Sweden, United Kingdom and United States of America), comprising clinicians from different disciplines, laboratory specialists, researchers and patient representatives. Due to COVID-19 restrictions about half of the participants attended the meeting on-line, while the other half gathered in Amsterdam on 8-10 October 2021 for this workshop on the harmonization of myositis specific autoantibodies (MSA) and myositis associated autoantibodies (MAA).

Idiopathic inflammatory myopathies (IIM) can be subdivided in different types: polymyositis (PM), anti-synthetase syndrome (ASyS), dermatomyositis (DM), juvenile DM (jDM), immune-mediated necrotizing myopathy (IMNM), inclusion body myositis (IBM), and overlap myositis (OM) [1,2]. Some authors consider PM and ASyS as largely overlapping entities, while others even include these entities in OM. The latter, however, is more strictly confined to IIM patients that simultaneously have another systemic autoimmune rheumatic disease (SARD), e.g. systemic sclerosis (SSc). The IIM subtypes differ in clinical manifestations, prognosis, and therapeutic options [2], but still these subgroups are heterogenous with varying prognosis and probably also with varying pathophysiology within these subgroups. In the last decades multiple autoantibodies have been discovered that support the diagnosis and add information on subtype and prognosis [3]. Detection of, in particular, MSA was originally restricted to research laboratories using immunoprecipitation (IP) techniques, but nowadays several commercial assays have become available, enabling widespread introduction of the assays in clinical laboratories [4]. At the 239th ENMC workshop about classification of DM and the importance of MSA in this classification,

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it was realized that the different aspects of autoantibody detection require more attention in terms of standardization and/or harmonization [5]. Standardization, i.e., the process of implementing a standard preparation to achieve uniformity of test-results, has been shown to be extremely difficult for autoantibody assays due to the lack of adequate standard preparations and heterogeneity of the measurand [6]. Since harmonization is one of the major goals of the European Autoimmunity Standardisation Initiative (EASI), the current 256$^{th}$ ENMC workshop was organized in close collaboration with EASI. Although MAA were included in the presentations and discussions, the workshop focused on MSA. The experts that assembled at this workshop addressed the following goals:

- Consensus regarding the clinical indications that ask for detection of MSA
- Consensus regarding the optimal testing strategy for patients suspected of IIM
- Consensus on the format for reporting results to the clinician in order to enable optimal interpretation
- Proposition of the research agenda to obtain reliable data on the test-characteristics of the immuno-assays for MSA.

In order to achieve these goals, participants presented about their area of expertise and the shared information was used in the discussions to achieve consensus.

## 2. Current positioning of myositis specific and associated autoantibodies

### *2.1. Overview of myositis specific and associated autoantibodies (Carolien Bonroy, Belgium)*

Classification and subtyping of IIM has evolved over the years (for review [7]). For many years, IIM were subdivided in three main subgroups using a clinical/pathological approach, revealing PM, DM, including jDM, and IBM. Today, we have evolved towards a clinico–seropathological classification, an evolution which was mostly driven by the discovery of the MSA, about half of them discovered since 2005. MSA, but not MAA, are a well-defined group of autoantibodies, which are generally considered highly disease-specific (except for anti-cN1A) and mutually exclusive. Moreover, the current spectrum of MSA, consisting out of ≥15 autoantibodies, reduced the serological gap up to less than 30-35%, more impacting juvenile IIM compared to adult IIM [3]. The MSA are differentially associated with the distinct IIM subgroups: (i) PM/ASyS associated with anti-synthetase antibodies (e.g. anti-Jo1, -PL7, - PL12), (ii) DM associated with antibodies to Mi2, SAE, NXP2, TIF1y and MDA5, (iii) IMNM associated with anti-HMGCR and anti-SRP, and (iv) IBM associated with anti-cN1A [1,2].

A review of historical disease criteria (<2017), some focusing on 'defining' the disease subset, while others serving subclassification, showed that MSA were gradually included, mainly following the discoveries in the field as well as the availability of the immuno-assays in the 'routine' laboratories. Nevertheless, the inclusion of MSA in these disease criteria was mostly derived empirically and was not supported by large international studies [7].

In 2017, the European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) jointly presented new EULAR/ACR classification criteria for adult and juvenile IIM in which they distinguish 4 groups: PM (including IMNM), amyopathic DM, IBM and (j)DM [8]. Unfortunately, MSA were underrepresented in these data-supported criteria (only anti-Jo1 was included as 1 of the 16 variables in the score system), contrasting the clinical utility of MSA as acknowledged by the experts in the field; a limitation linked to the fact that the major advancements in MSA knowledge/detection methods took place after the study started. The advancement in MSA knowledge/detection also lead to the revision of the mainly clinico-pathological oriented expert-based classification of the ENMC [9] Clinico-seropathological-oriented updates were published for DM and IMNM [5,10]. In these recent expert-based criteria MSA have a prominent role, and they even have been given the potential to overrule 'classic' criteria of IIM diagnosis such as biopsy. This expanding role obviously demands reliable and routinely applicable MSA/MAA assays.

Today, there are several commercial options (mostly line and dot immunoassays; LIA and DIA) for MSA/MAA detection, which represent easy accessible alternatives compared to the historically used conventional techniques. These newer assays are, however, not standardized or even harmonized as is already documented in several published studies (for review [4]), and therefore represent important challenges for routine use. Solutions suggested are further documentation of their values and limitations (ideally in multicenter prospective validation studies), creation of awareness on their limitations and harmonization initiatives on several levels (from the pre-analytical to the post-analytical phase).

### *2.2. Which clinical manifestations ask for detection of myositis specific autoantibodies (Ingrid Lundberg, Sweden)*

As discussed above, the identification of MSA has been a major breakthrough in myositis research to improve diagnosis in patients with suspicion of IIM and also to understand disease mechanisms in subgroups. However, there are several yet unanswered questions such as the diagnostic and prognostic value of the MSA in different clinical settings. Furthermore, limited information is available on how frequent the MSA are in a healthy population or in patients with, for instance, pulmonary diseases and partially overlapping clinical manifestations. As such, defining clinical manifestations that warrant testing for MSA will prevent the use of the respective multiplex immuno-assays out of the appropriate clinical context and reduce the number of "false"-positive results. Three patient categories were presented as examples where testing for the full panel of MSA could have a diagnostic and prognostic implication and support treatment decisions.

The first category was presented as a case, which was a 37 year old woman without family history of muscle or rheumatic diseases with a progressive muscle weakness, moderately elevated creatinine kinase (CK; 840 IU/L; ref: <170 IU/L) and no other organ manifestations. Electromyography (EMG) showed a severe generalized myopathy in proximal shoulder and pelvic muscles with spontaneous activity and fibrillation. Magnetic resonance imaging (MRI) revealed atrophy with fat replacement of thigh and hip muscles and mild signs of inflammation. A muscle biopsy was almost normal without inflammatory infiltrates. This woman was diagnosed as having PM and was treated with prednisolone and azathioprine, that was switched to cyclosporine due to intolerance, without effect; treatment was stopped after 6 months. The patient experienced a slowly progressive weakness despite regular exercise and one year later a second muscle biopsy showed occasional regenerating fibers, but still no inflammation and with normal dysferlin and calpain expression. Three years later a new diagnostic evaluation, including limb girdle muscular dystrophy (LGMD) and Pompeś disease, was performed but with negative genetics and enzyme investigations. The patient was re-diagnosed as having limb girdle muscle dystrophy, despite being negative for known mutations. Finally, her stored serum was tested in a research collaboration (Andy Mammen, Bethesda, USA) and was found to be positive for anti-3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) autoantibodies. Her diagnosis was changed to anti-HMGCR IMNM. She was started on treatment with high dose

intravenous immunoglobulin (IVIg) and her muscle function slowly improved, after 13 years of worsening.

The situation described above is one example of when to test for MSA: in patients with a necrotizing myopathy in biopsies, but also in individuals with suspected, but genetically unconfirmed, muscular dystrophy an IMNM is to be considered and testing for relevant autoantibodies (anti-HMGCR) is warranted (even without statin treatment). Another group of patients includes the ones that may present with arthritis in wrists, MCP and PIP joints, but are rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) negative. The presence of, for instance, anti-synthetase autoantibodies such as anti-Jo1, may lead to identification of interstitial lung disease (ILD) that demands immunosuppressive treatment.

A third group where information on MSA may have implications for treatment with immunosuppressive drugs entails the patients with primary pulmonary symptoms with isolated ILD or pulmonary fibrosis. This is probably a more controversial group of patients and we do not have any good biomarkers that could predict who is going to have MSA, like anti-synthetase autoantibodies that would indicate aggressive immunosuppressive treatment. Notably the prevalence of ILD in IIM varies between 5-65%, but is present in up to 90% in patients with anti-synthetase autoantibodies.

In summary, early identification of individuals at high risk for myositis is important to aim for early treatment and even prevention, similar to what is ongoing for patients with rheumatoid arthritis (RA). Subgroups where we can use serology as a tool to identify early cases with IIM are: (a) patients with clinical suspicion of myositis/necrotizing myopathy, (b) patients with progressive, proximal muscle weakness, elevated CK, and suspicion of LGMD but negative genetics (anti-HMGCR or anti-SRP) [11], (c) patients with seronegative RA-like arthritis, and (d) patients with ILD of unknown cause. For the latter two groups of patients testing for MSA should in particular be considered in patients with some signs of autoimmune disease. Of utmost importance is the availability of an immuno-assay covering the whole spectrum of MSA with high validity. For an appropriate gating strategy for autoantibody testing, physicians seeing patients with musculoskeletal, pulmonary and/or dermal manifestations associated with IIM are advised to interact with a multidisciplinary team to accomplish early diagnosis of emerging myositis.

## 3. Myositis specific and associated autoantibodies in relation to clinical/pathological phenotypes

### *3.1. Four groups of myositis specific autoantibodies and four groups of myositis (Olivier Benveniste, France)*

Based on the clinical and pathological manifestations, the French research group of Benveniste currently distinguishes four groups of IIM, i.e., OM (including ASyS), DM, IMNM and IBM, and these groups are differentially associated with subsets of MSA [1]. The distinction of four groups of IIM and four groups of MSA was confirmed in a retrospective cohort of IIM patients by unsupervised multiple correspondence analysis and hierarchical clustering analysis to aggregate patients in subgroups [12]. This cohort study aimed to assess the weight of MSA in diagnosis and subtyping of IIM. A selection was made of 47 discriminant variables according to their relevance for distinguishing historical entities (PM, DM and IBM) and agreement with clinical practice from the point of view of expert physicians. These variables cover different domains, such as the clinical phenotype with muscle strength evaluation and extra-muscular involvements (skin, joints, lung, malignancy), muscle biopsy, and laboratory parameters (CK and MSA).

Among 260 participants (163 [62.7%] women; mean age, 59.7 years), 4 clusters of patients emerged. Cluster 1 (n = 77) included patients who were male, Caucasian, and older than 60 years manifesting finger flexor and quadriceps weakness and findings of vacuolated fibers and mitochondrial abnormalities. Cluster 1 grouped patients who had IBM (72 of 77 patients [93.5%]; 95%CI, 85.5%-97.8%; P < 0.001). Importantly, presence of anti-cN1A was not evaluated in this study. Cluster 2 (n = 91) grouped patients who were women and had high CK levels, necrosis without inflammation, and anti-SRP or anti-HMGCR antibodies corresponding to IMNM (53 of 91 [58.2%]; 95%CI, 47.4%-68.5%; P < 0.001). Cluster 3 (n = 52) grouped patients who had DM rash and anti-Mi2, anti-MDA5, or anti-TIF1$\gamma$ antibodies, mainly corresponding with patients who had DM (43 of 52 [82.7%]; 95%CI, 69.7%-91.8%; P < 0.001). Cluster 4 (n = 40) was defined by the presence of anti-Jo1 or anti-PL7 antibodies corresponding to ASyS (36 of 40 [90.0%]; 95%CI, 76.3%-97.2%; P < 0.001).

These four subgroups of IIM were also identified by Dr. Mammen's team by a totally different approach. The authors started from muscle biopsies of 119 IIM patients (well characterised as having IBM, ASyS, DM or IMNM) and 20 healthy donors. They performed a transcriptomic analysis of the whole biopsies. The RNA sequencing analysis with an appropriate machine learning algorithm classified the muscle biopsies with >90% accuracy among the four categories. They actually found unique gene expression profiles by these subgroups of IIM suggesting that different pathological mechanisms underly muscle damage in each of these diseases [13].

These findings underscore a classification of patients with IIM with detectable autoantibodies into 4 subgroups: DM, IBM, IMNM and ASyS. In addition, there are seronegative patients accounting for up to 50% in some cohorts. This classification system suggests that a targeted clinical-serologic approach for identifying IIM may be warranted and outline the importance of MSA testing.

### *3.2. The anti-synthetase syndrome and myositis specific autoantibodies (Jirí Vencovský, Czech Republic)*

There are no approved diagnostic criteria for ASyS. The diagnosis is usually established when an antibody to aminoacyl-tRNA synthetase (ARS) is detected together with a single or combination of typical clinical manifestations [2]. ARS are enzymes that charge individual tRNAs with their cognate amino acids [14]. Eight different ARS for histidine, tyrosine, phenylalanine, glycine, threonine, isoleucine, asparagine and alanine are targets for autoantibodies (anti-Jo1, anti-Ha/YRS, anti-Zo, anti-EJ, anti-PL7, anti-OJ, anti-KS, anti-PL12, respectively) and all are associated with ASyS. Anti-Jo1 is found most frequently in about 15-30% of myositis cases, followed by anti-PL7 (5-10%), anti-PL12 (< 5%), with all the others occurring rarely (each < 1%). The main clinical manifestations include myositis, ILD, arthritis, Raynaud's phenomenon, mechanic's hands, and fever [15]. However, symptomatology is frequently incomplete, particularly at presentation, when isolated ILD is seen in 32%, isolated myositis in 27% and polyarthritis in 18% [16]. Even after 78 months of follow-up, isolated ILD, myositis or polyarthritis persist in 15%, 16% or 2% of patients, respectively. Arthritis is frequently present at disease onset and it precedes muscle symptoms in more than 50% of cases [17]. Occasionally some patients are initially misdiagnosed as RA. Arthritis is more frequent in anti-Jo1 positivity than with other ARS [18]. ILD is common in anti-Jo1 positive myositis occurring in 72.5% of patients [19]. Progressive onset is more common than the acute start of the disease. Almost 30% are clinically asymptomatic although abnormalities consistent with ILD in functional tests or high-resolution computer tomography (HRCT) are often present. Nonspecific interstitial pneumonia (NSIP) is the

most frequent pattern. When a usual interstitial pneumonia (UIP) pattern is present, it carries a poorer prognosis and this is the case for respiratory muscle involvement and higher age too. Some ARS are associated mainly with ILD, such as in anti- PL12 and anti-OJ positive patients, in whom myositis can be lacking [18], although it is not always reported. Pericarditis is usually not a manifestation of myositis but was described in 53% of patients with anti-PL7 antibodies in one study [20]. Most analyses show that patients with ARS have a significantly lower risk of cancer than other patients with IIM. Patients with ASyS have unique features in muscle biopsy showing oedematous and/or fragmented perimysium that stains with alkaline phosphatase, perifascicular myofibre necrosis, scattered perimysial CD68, CD4, CD8 cells, increased MHC I expression with perifascicular predominance, membrane attack complex (MAC) of complement depositions in fibres adjacent to the perimysium, myonuclear actin filament inclusions in electron microscopy, and a normal capillary density and absent staining for MxA [21]. In the rituximab in myositis (RIM) study patients with anti-Jo1 (and anti-Mi2) had a shorter time to improve than patients without these antibodies [22]. Anti-Jo1 levels decreased after rituximab treatment and correlated with improvement in all core set measures, particularly with CK levels. Similarly, the effect of anti-Jo1 and BAFF levels on CK was observed in a cross-sectional study in which a disappearance of anti-Jo1 activity was seen in patients with a decrease in disease activity. Estimated survival rates in anti-Jo1 ASyS patients are 88% after 5 years and 75% after 10 years, which is 4-times lower compared with the general population [23].

### 3.3. Dermatomyositis and myositis specific autoantibodies (Yves Allenbach, France)

Dermatomyositis is defined by characteristic skin and muscle features [5]. Some patients may also have extra-dermatomuscular involvement that can be life-threatening. Similarly, the presence of a cancer is associated with a poor prognosis. It also appears that the heterogeneity of the phenotypes does not only concern the extra-dermatomuscular manifestations. Variations in the severity of the muscular involvement, which can sometimes even be absent, are also observed. In addition to the typical DM skin rash, some patients may present other features, such as ulcers and/or calcifications.

Growing sets of data show that DM patients can be subclassified in more homogenous subsets of patients according to the presence of DM specific autoantibodies [5]. Five DM specific autoantibodies have been described: anti-Mi2, anti-NXP2; anti-SAE, anti-TIF1$\gamma$ and anti-MDA5 antibodies. They are mutually exclusive, and they are now considered to be diagnostic biomarkers for the DM subsets. The importance of MSA in DM diagnosis and sub-classification led to revisit DM classification criteria in a recent ENMC workshop [5].

Anti-MDA5 antibodies are associated with a phenotype characterized by the predominance of extra-cutaneous signs, while muscle signs are usually mild or absent [24]. The large majority (>85%) of patients harbor an ILD which can be rapidly progressive. Frequently, patients have polyarthralgia/arthritis. These pulmonary and rheumatic signs are absent in DM without anti-MDA5 antibodies. Malignancy occurs mainly in anti-TIF1$\gamma$ patients [5], whereas the association seems less clear in patients with anti-NXP2 antibody [5] and has not been clearly demonstrated for the other DM specific autoantibodies. Anti-NXP2, anti-Mi2 and anti-SAE patients harbor mainly cutaneous and muscle signs. Anti-NXP2 positive patients are characterized by a more severe vasculopathy including limb oedema [5], whereas anti-Mi2 positive patients display more frequently pathological features with muscle fibers necrosis [25]. Anti-SAE positive patients seem to have a less severe muscle weakness (personal data). Finally, it was concluded that DM specific autoantibodies are crucial for both diagnosis and sub-classification, but it should be noted that some DM patients do not have MSA [26].

### 3.4. Immune-mediated necrotizing myopathy and myositis specific autoantibodies (Andy Mammen, USA)

At the 224$^{th}$ ENMC International Workshop three subtypes of IMNM were defined [10]. These include (a) anti-HMGCR myopathy, which is defined by high CK and proximal muscle weakness along with anti-HMGCR autoantibodies, (b) anti-SRP myopathy which is defined by high CK and proximal muscle weakness along with anti-SRP autoantibodies, and (c) antibody-negative IMNM, which is defined by high CK, proximal muscle weakness, and a necrotizing muscle biopsy in the absence of an MSA. Given the importance of autoantibodies for defining the different subtypes of IMNM, techniques for detecting these autoantibodies were discussed. These techniques included IP from radiolabeled cell extracts, IP of the protein products obtained by *in vitro* transcription and translation, and enzyme-linked immunosorbent assays (ELISA). Data on validation of the anti-HMGCR ELISA revealed high sensitivity (99.4%) and specificity (99.3%) [27].

The clinical presentations of patients with each subtype of IMNM were reviewed. Anti-SRP patients typically present with severe proximal muscle weakness, very high CK levels, necrotizing muscle biopsies and occasional extra-muscular manifestations including cardiomyopathy and/or ILD [28]. Anti-HMGCR patients often have a very similar clinical presentation, except that extra-muscular manifestations are rare. Furthermore, as already illustrated by Ingrid Lundberg (Sweden) anti-HMGCR IMNM can also present in younger patients as a slowly progressive myopathy with scapular winging that can mimic a LGMD [29], emphasizing that younger patients with high CK levels and proximal muscle weakness should be tested for anti-HMGCR and anti-SRP autoantibodies unless they already have a genetically proven LGMD.

Next, risk factors for developing IMNM were discussed. Data revealed that the class II HLA allele DRB1*11:01 is strongly associated with developing anti-HMGCR myopathy [30]. Furthermore, statin-triggered anti-HMGCR myopathy seems to be especially common in American Indians, suggesting that this population may have additional genetic risk factors [31]. Finally, while environmental triggers for anti-SRP myopathy have not been defined, statins are a well-established risk factor for developing anti-HMGCR myopathy.

In line with data presented by Olivier Benveniste (France), machine learning models were trained to analyze the transcriptomic profiles of muscle biopsy specimens from IMNM patients with either anti-SRP or anti-HMGCR antibodies. These models could distinguish these IMNM muscle biopsies from other types of myositis muscle biopsies (i.e., IBM, ASyS, and DM) with 92% accuracy [13]. Moreover, using a computational method called recursive feature elimination, *APOA4* was identified as the single gene that is expressed in anti-HMGCR muscle biopsies, but not in muscle biopsies from healthy controls or other types of myositis, including anti-SRP myopathy. Interestingly, APOA4 plays a role in the reverse cholesterol pathway and may, like statins, upregulate HMGCR protein levels.

Recommended treatment strategies from the 224$^{th}$ ENMC International Workshop on IMNM were reviewed, highlighting the early use of rituximab in anti-SRP myopathy and the early use of IVIG in anti-HMGCR myopathy [10]. Although these strategies may be successful for some patients, it should be emphasized that many IMNM patients continue to have active muscle

disease despite aggressive therapy with immunomodulatory agents. Considering emerging evidence that complement activation might play a role in damaging muscle fibers in IMNM, a clinical trial was recently conducted using the subcutaneously administered C5 inhibitor, zilucoplan, to treat patients with refractory autoantibody positive IMNM. Unfortunately, complement inhibition was ineffective in treating these patients. Thus, future work is needed to elucidate pathophysiologic mechanisms and subsequently define better treatments for patients with anti-SRP and anti-HMGCR myopathies.

### *3.5. Inclusion body myositis and myositis specific autoantibodies (not presented)*

Inclusion body myositis (IBM) has a specific pattern of muscle involvement, which is distinct from other forms of IIM. IBM occurs in patients over 50 years of age and presents with asymmetric muscle weakness and atrophy of proximal and distal muscle groups, with a predilection for wrist and finger flexors and knee extensors [32]. Dysphagia is present in up to two thirds of patients and may have a serious impact on quality of life. IBM has an insidious onset and is usually refractory to immunosuppressive therapies, leading to speculation whether IBM is a primary inflammatory myopathy with secondary degeneration, or rather a primary degenerative myopathy with secondary autoimmune response [32].

Anti-cytosolic 5′-nucleotidase 1A autoantibodies (anti-cN1A) are the only known autoantibodies associated with IBM. Anti-cN1A autoantibodies are present in 30 to 70% of IBM patients, but they have also been found in some patients with DM/PM and other systemic autoimmune rheumatic diseases, such as Sjögren's syndrome and systemic lupus erythematosus (SLE), frequencies, however, varying between different detection methods [33,34]. Whether anti-cN1A positive and negative patients differ in clinical features and/or disease severity is actually unclear [35].

### *3.6. Myositis associated autoantibodies and idiopathic inflammatory myopathies (Yves Allenbach, France)*

By definition, MAA can also be detected in selected other conditions, such as SLE, SSc or Sjögren's syndrome. MAA are present in 20% of IIM patients and anti-U1RNP, anti-PM/Scl, anti-Ku, anti-Ro52 and anti-SSA/Ro60 are the most frequent MAA. They are not specific for IIM and thus have a less important diagnostic value than the MSA. Obviously, specificity of MSA is also not perfect, but so-called false-positive results for MSA seem not to be related to distinct disease entities. When MAA are present, there is usually no association with any MSA. However, among the MAA anti-Ro52 is the only autoantibody frequently found associated with MSA. Several studies showed that the presence of anti-Ro52 in IIM patients is associated with anti-Jo1 and more severe ILD [36]. Nevertheless, the pathological character of anti-Ro52 is still a matter of debate in the field of autoimmunity.

Presence of MSA allows to create homogeneous patients subgroups [1], but this is less well established for patients with MAA, although they have distinct phenotypes from patients with MSA. Anti-U1RNP positive patients with myositis are characterized by the presence of necrotic muscle fibers and the frequent association with extra-muscular signs including ILD, Raynaud phenomenon and/or puffy hands, and/or arthralgia [37,38]. Anti-PM/Scl myositis patients are also characterized by more extensive extra-muscular manifestations including ILD and skin changes as compared to the IIM patients with MSA [39]. To the contrary, there is no frequent skin change in anti-Ku myositis. In addition, the muscle phenotype of anti-Ku patients is characterized by a more frequent distal weakness compared to the other myositis subtypes (except IBM) and by the presence of necrotic muscle fibers [40]. As for anti-U1RNP and anti-PM/Scl, anti-Ku is also associated with extra-muscular involvement including ILD [40].

### *3.7. Myositis specific and associated autoantibodies and muscle pathology (Werner Stenzel, Germany)*

Autoantibodies and muscle biopsy findings in IIM are closely related. Similarly to the fact that autoantibodies in myositis have the potential to delineate certain patient subgroups, help to predict certain complications during the course of disease and may in the future also help to decide on therapeutic strategies in an individualized manner, it has been investigated whether morphological alterations in muscle biopsies can be found in different autoantibody subgroups as well. In addition, the study of skeletal muscle tissues offers the unique possibility to analyze and understand underlying pathogenic mechanisms of certain subgroups and helps to answer the question whether the autoantibodies have a pathogenic significance.

With this focus, the essential characteristics were described of the five DM subtypes related to the autoantibodies anti-Mi2, anti-MDA5, anti-TIF1$\gamma$, anti-NXP2 and anti-SAE. The distribution of complement deposits varies between sarcolemmal (in anti-Mi2 DM) and capillary (in -TIF1$\gamma$ and -NXP2) localizations, which may reflect the differences in the target that is relevant for the ongoing immune-reaction, i.e., the destruction of myofibers vs the destruction of capillaries. Conversely the unifying immunological pattern may be the activation of Type I interferon (IFN) in all subtypes reflected by similar and strong MxA or ISG15 positivity of myofibers as well as the proof of tubule-reticular inclusions in capillary endothelial cells.

In IMNM the essential morphological features reveal (i) presence of diffuse and individualized myofiber necrosis, myophagocytosis and regeneration at different stages (in comparison to areas of myofiber necrosis which can occur as a consequence of ischemia), (ii) variable sarcolemmal staining with MHC class I and constant absence of sarcolemmal MHC class II and MxA or ISG15, (iii) mild lymphocytic endomysial infiltrate but absence of T cell cytotoxicity or myofiber invasion, (iv) variably intense sarcolemmal complement deposition with complement on capillaries being the exception, and (v) a fine granular sarcoplasmic positivity for p62 reflecting an activation of autophagolysosomal compartments in IMNM.

Biopsies of patients with ASyS, being related to the major autoantibodies anti-Jo1, anti-PL12, -PL7, -EJ and -OJ, are typically showing perifascicular pathology with necrotic myofibres and non-necrotic fibres that are sarcolemmally decorated by complement (MAC; C5b-9), MHC class I and II positivity as well as actin inclusions in myofibres, and a substantial oedema and fragmentation of the perimysium where different kinds of mononuclear leukocytes accumulate. The characteristics of IBM on the morphological level comprise inflammatory features with presence of dysfunctional, KLRG1+ T cells [41] or effector memory T cells re-expressing CD45RA ($T_{EMRA}$), degenerative features with presence of vacuoles containing autophagolysosomal large deposits of debris. Of note, IBM biopsies regularly comprise mitochondrial abnormalities that have been described in detail recently [42].

Finally, detailed alterations in other entities where described that do not harbor MSA, such as Shulman Syndrome, overlap myositis with anti-Ku, -PM/Scl and -U1RNP autoantibodies, as well as neuromuscular sarcoidosis, all of them being highly characteristic and recognizable on skeletal muscle biopsies. The latter have to be explored with more emphasis in the future and their relevance with respect to certain disease characteristics as well as therapeutic decisions.

## 4. Myositis specific and associated autoantibodies and prognosis

### *4.1. Myositis specific antibodies as biomarker for disease activity (Hector Chinoy, United Kingdom)*

There are specific autoantibodies in SARD that can act as useful biomarkers for diagnosis and/or as part of particular clinical manifestations. Well known examples are anti-dsDNA in SLE and ANCA levels in granulomatosis with polyangiitis and microscopic polyangiitis. Currently, antibody testing in IIM is largely used for confirmation of diagnosis. The frequency of seropositivity in IIM remains in excess of 70% in adult- and juvenile-onset disease [3]. Autoantibodies are increasingly being used to help compartmentalize clinical and pathological components of disease [1]. Knowledge of specific autoantibodies can help avoid the need for biopsy, define extra-muscular manifestations, and predict future response to treatment.

One of the original descriptions of autoantibody levels was in 1990 where Miller and colleagues noted that IgM and IgG1 anti-Jo1 correlated with clinical activity [43]. More recent studies have shown that autoantibody levels alter with clinical disease within other specificities, including TIF1$\gamma$, MDA5, Mi2 and SRP. Coexistent markers can also point towards particular phenotypes. For example, co-existent anti-Ro52 antibody is associated with a particular phenotype of the ASyS, leading to more severe myositis and joint impairment. Anti-Ro52 can also be used as a biomarker for detection of connective tissue disease associated ILD in the absence of other antibodies [36]. A further recently described autoantibody is anti-cortactin, increased in frequency in adult DM patients with co-existing anti-Mi2 or anti-NXP2 autoantibodies, and associated with dysphagia and ILD [44].

In the future, measuring MSA levels to monitor disease activity may become part of routine management. For now, larger scale observational validation studies are required and it is recommended to incorporate antibody level testing as exploratory endpoints in future interventional clinical trials.

### *4.2. Myositis specific autoantibodies and malignancy; the example of anti-TIF1$\gamma$ (Olivier Boyer, France)*

Both autoimmunity and cancer are associated with a dysregulated immune system and either may be a risk factor for the other. Patients with an autoimmune disease may develop malignancy on the long term. These associations may be inherent to the dysregulated immune system, as is probably the case in Sjögren's syndrome, but may also be due to long-term immunosuppressive therapy. On the other hand, cancer may also give rise to autoimmunity. This is often with a short delay and is recognized as paraneoplastic disease, but can also be induced upon therapy with checkpoint inhibitors. Anti-TIF1$\gamma$ is typically associated with the occurrence (22 to 84%) of malignancies in adult, but not juvenile, DM patients [45]. The meta-analysis indicates a 9.4 diagnosis odds ratio of cancer in the presence of anti-TIF1$\gamma$ antibodies [45]. Cancer onset occurs early (within 1-2 years before or after DM diagnosis). In 2019, it was reported that the detection of anti-TIF1$\gamma$ IgG2 was significantly associated with mortality and occurrence of cancer during the follow-up [46]. To confirm this observation, it was decided during the 239$^{th}$ ENMC meeting [5] to conduct an international confirmatory study. Unpublished data of this retrospective study were presented. More than 130 adult patients with anti-TIF1$\gamma$ positive DM were included from 6 different centres (Sweden, Canada, Spain, USA, Czech Republic and UK). Roughly half of these patients developed cancer (half of which before or concurrent to the diagnosis of DM). Study results confirmed (i) that the level of anti-TIF1$\gamma$ antibodies determined by ELISA was higher in patients with cancer ($p<0.05$), (ii) that the presence of IgG2 anti-TIF1$\gamma$ isotype was more frequent in patients with cancer ($p<0.05$) and (iii) that the level of these anti-TIF1$\gamma$ IgG2 autoantibodies was higher in patient with cancer than without cancer ($p<0.05$). It was discussed why cancer-associated DM has special immunological features. A possible hypothesis is that carcinogenesis generates mutated TIF1$\gamma$ proteins in tumours that are phenotypically different from the native molecule, leading to a breakdown in tolerance and thus to a specific immune response. Along those lines, Pinal-Fernandez *et al.* found loss of heterozygosity at the *TIF1(TRIM33)* locus in a series of 7 tumours from DM patients [47]. Recently, 14 tumours from anti-TIF1$\gamma$ antibody-positive DM individuals were analysed, with additionally 2 tumours from non-DM controls. Fourteen somatic mutations were identified from 4 tumours in the *TIF(TRIM33)* gene [48]. It was agreed that such results support that *TIF(TRIM33)* somatic gene mutations in tumours may concur to the pathophysiology of anti-TIF1$\gamma$ autoantibody-positive DM.

### *4.3. Myositis specific autoantibodies and (rapidly progressive) interstitial lung disease (Ghuochun Wang, China)*

ILD is one of the most frequent extra-muscular manifestations in IIM patients, but it reveals various clinical courses and therapeutic responsiveness according to clinical and serological subsets. MSA are important immunological markers for the classification of ILD and provide valuable information for predicting prognosis and determining treatment in patients with IIM. Accordingly, IIM associated ILD can be divided into three major groups: ASyS-ILD, DM-ILD (MDA5-ILD and non MDA5-ILD), and IMNM-ILD based on the different MSA.

ASyS-ILD more often occurs in middle-aged women. Anti-Jo-1, anti-PL7, anti-PL12 and anti- EJ are the common types of ASyS specific MSA; less common types are anti-OJ and anti-KS autoantibodies. The clinical features are similar among the ASyS MSA. NSIP is the most common HRCT image pattern, followed by organizing pneumonia (OP). About 9% of ASyS-ILD have a rapidly progressive course (RP-ILD), and half of the RP-ILD came from the anti-PL7 positive group. 19% of the patients with anti-PL7 developed RP-ILD, which was significantly higher than that in other type of ASyS [49]. ASyS-ILD is usually well responding to steroid plus immunosuppressant treatment. However, relapse after steroid tapering is common. Thus, prevention of the relapse is an important issue for the ASyS-ILD management.

RP-ILD is one of the unique features of anti-MDA5 positive patients. Cohort data show that about 80% of the anti-MDA5 positive patients had RP-ILD [50]. The radiological feature of the anti-MDA5 related RP-ILD usually presents as dynamic process: from perilobular opacity developing into wide consolidation in a short period of time. However, the pathological features of the RP-ILD are unclear: only few cases reported so far from the literature showing the diffuse alveolar damage (DAD) or OP pattern. The prognosis of anti-MDA5 patients with RP-ILD is the worst within the subtypes of DM, of which the 5-year survival rate was reported to be only 50.2% [51].

All types in the non-MDA5 DM group, which includes anti-Mi2, anti-NXP2, anti-SAE, anti-TIF1$\gamma$, and MSA-negative DM, could have ILD. However, the main clinical feature for this group is not lung involvement. The prevalence of ILD is relatively lower with 12.5%-50% of the patients from this group having ILD [52]. In this group the severity of ILD was mild and RP-ILD was rare.

Both anti-SRP and anti-HMGCR are specific autoantibodies for IMNM. The predominant feature of IMNM is muscle weakness, but the lung could be involved as well. In general, the prevalence of ILD was reported to be 49.5%, being more common in anti-SRP myopathy than in anti-HMGCR myopathy (64.4% vs 34.8%).

However, compared to DM-ILD or ASyS-ILD, most of the IMNM-ILD is not severe: 80% of the patients have no respiratory symptoms, and 20% of the patients have only dry cough and shortness of breath after activity. Pulmonary function (PF) tests showed that more than 90% of the patients had mild or moderately decreased PF, and only less than 10% of the patients showed severely decreased PF. No RP-ILD case was observed in the IMNM patients [53].

## 5. Detection of myositis specific and associated autoantibodies

### *5.1. Anti-synthetase syndrome specific autoantibodies: test-comparison and reliability (Neil McHugh, United Kingdom)*

Since the discovery of anti-Jo1 autoantibodies in 1980 there are now 8 anti-ARS autoantibodies known to be present in the myositis spectrum of disorders, commonly referred to as the ASyS. The most commonly found anti-ARS is anti-Jo1 with other specificities less common. As well, each individual ARS associates with different features within the syndrome, e.g. anti-Jo1 with arthritis versus anti-PL12 with ILD such that each feature may be the sole manifestation respectively. Indeed, the absence of myositis in such cases challenges the concept of an ARS being referred to as a MSA.

The reference technique for the identification of an ARS is IP of either the protein autoantigen or its cognate tRNA from radio-labelled cell lines, methods used in the original discovery of each autoantibody system. However, these techniques are not routinely available apart from in a few specialized research laboratories. Hence the detection of ARS in routine practice relies on the use of commercial assays, the most common of which are line or dot blot methods or ELISA as recently reported from a survey of members of the International Myositis Assessment and Clinical Studies Group (IMACS) involving 111 centers world-wide [54]. The survey also highlighted concern about the reliability of testing for MSA.

One major concern with the detection of an ARS is the reliance on screening by HEp-2 indirect immunofluorescent assays (IFA) as typically an ARS may yield a weak or negative nuclear staining but instead demonstrate a stronger cytoplasmic pattern. If the latter is ignored, the serum may be termed anti-nuclear antibody (ANA) negative and further testing not pursued. The other concern is the reliability and comprehensiveness of commercial assays for detecting ARS, that may not contain the full array of ARS. To address the latter concern an IMACS study group led by Dr Takahisa Gono as part of the myositis autoantibody special interest group is conducting a systematic literature review of the accuracy of commercial myositis autoantibody testing evaluated against the IP assay as a reference standard.

Available data have shown reasonable agreement between line-blot assay (LIA), dot-blot assay (DIA) and IP (kappa values 0.7 – 0.9) for anti-Jo1, anti-PL7, anti-PL12 and anti-EJ [55–57] except for one study where concordance was low [58]. However anti-OJ is not reliably picked up by LIA most likely as conformational epitopes are needed for its detection [59]. Other assays including a multi-analyte ELISA [60] and a particle-based multi-analyte platform [61] perform well, but do not currently capture the full repertoire of ARS specificities.

### *5.2. Dermatomyositis specific autoantibodies: test-comparison and reliability (Livia Casciola Rosen, United States)*

The assays to detect the 5 well-defined DM specific autoantibodies (TIF1$\gamma$, MDA5, NXP2, Mi2 and SAE1) include IP, ELISA, LIA, and IP/blot. The assays were reviewed briefly, with the remark that observations presented arise from research data generated in the laboratory of Casciola Rosen, and that the assays are not all necessarily amenable to clinical implementation. IP – often referred to as the "gold standard" assay – is one in which the antibody recognizes full-length non-denatured antigen. Input for this can be radiolabeled cell lysate, or $^{35}$S-methionine-labeled protein generated by in vitro transcription and translation from the relevant DNA ("IVTT-IP"). Summarized below are the key findings that were highlighted. The LIA platform referred to below is from EUROIMMUN (Germany), using the myositis 16 antigen panel. The commercially available ELISA kits used for MDA5, Mi2 and TIF1$\gamma$ antibodies are all manufactured by MBL (Japan).

SAE1 antibody assays: the findings from 2 assays were compared (LIA and IVTT-IP), and gave good agreement (kappa value 0.88). There were 5 LIA+/IP- sera, and all were in the low positive LIA score range (15-24 U) [62]. These findings have been extended and validated in a recent paper published by the Hopkins team [63]. Data showed good agreement between LIA and IVTT-IP at moderate (>36 U) or strong (>71 U) LIA cutoff antibody levels (kappa values 0.86 and 0.9, respectively).

Mi-2 antibody assays: data from LIA, IVTT-IP and an ELISA were compared [62]. The kappa value for IVTT-IP vs LIA was 0.9, and that for IVTT-IP vs ELISA was 0.86. Findings published in a very recent paper [64] concluded that different antibody specificities may require different thresholds to define a positive result. In contrast to the anti-SAE1 assays, for anti-Mi2a and anti-Mi2b, the lowest threshold (>15 U) gave the best kappa statistic.

MDA5 antibody assays: Agreement between the 3 assays tested (LIA, IVTT-IP and ELISA was excellent. Kappa values for IVTT-IP compared to LIA and IVTT-IP compared to ELISA were 0.94 and 0.99, respectively.

NXP2 antibody assays: IVTT-IP and LIA were compared, and gave a kappa value of 0.71 (at the time of writing, there are no commercially available ELISA assays to detect NXP2 antibodies).

TIF1$\gamma$ antibody assays: accurate readouts of this antibody-specificity are high priority because this specificity is associated with cancer [65]. Data from IP/blot, LIA and ELISA assays were compared. Of the 259 sera tested, 16% were anti-TIF1$\gamma$-positive by LIA, 41% by IP/blot and 50% by ELISA. The kappa value for IP/blot versus LIA was 0.39, and for IP/blot versus ELISA it was 0.79. Several other publications have reported poor agreement of assays to detect anti-TIF1$\gamma$ antibodies using LIA compared to other platforms [4,7].

Taken together, our experience with antibody assays shows that (i) for some DM antibodies, readouts obtained using different platforms may differ substantially; and (ii) on the LIA platform, different cutoffs may be needed for defining positive status for different antibodies. To ensure consistency and reproducibility across laboratories in all parts of the world, universal use of meticulously validated platforms should be standard.

### *5.3. Immune-mediated necrotizing myopathy specific autoantibodies: test-comparison and reliability (Olivier Boyer, France)*

Comparison of distinct solid-phase assays for detection of anti-HMGCR autoantibodies (commercial ELISA and CLIA, in-house ALBIA) in a cohort of 193 consecutive sera from patients suspected of IMNM revealed a high total agreement, not only with respect to qualitative results but also for quantitative results. The antigen is nowadays also available on DIA and LIA, but extended data on clinical validity is lacking. Anti-HMGCR may give a fine granular cytoplasmic pattern on a minority of HEp-2 cells. However, this seems to be dependent on the brand of the substrate. Liver tissue has been suggested to be a better substrate to possibly confirm anti-HMGCR reactivity.

The other IMNM-associated autoantibody, anti-SRP, was evaluated by LIA and ALBIA. Results revealed good kappa-

agreement (0.87), but the blot had some false-positives. Out of 7 false-positives, 4 patients had no clinical signs of IMNM, none revealed the typical speckled cytoplasmic HEp-2 IFA pattern, and they all were only low positive. This suggests that the cut-off needs to be increased for this antigen on the LIA, as also concluded in an independent study [66].

### 5.4. Inclusion body myositis specific autoantibodies: test-comparison and reliability (Ger Pruijn, The Netherlands)

The discovery of serum autoantibodies targeting a 44 kDa skeletal muscle protein in the serum of many sporadic IBM patients has aroused new interest in the role for autoimmunity in the pathogenesis of IBM. The identification of the target autoantigen for these autoantibodies as the cytosolic 5'-nucleotidase 1A (cN1A, also indicated with cN-1A, cN-IA, NT5C1A and Mup44) facilitated the development of various tests to detect these autoantibodies [67].

ELISA studies consistently demonstrated that anti-cN1A autoantibodies are more prevalent in IBM compared with PM or DM, other neuromuscular disorders and most other autoimmune diseases. In light of these findings, anti-cN1A autoantibodies provide utility in distinguishing IBM from PM and DM [34], an important distinction when determining therapy regimes, considering that PM and DM, but not IBM, are typically responsive to immunosuppressive medications.

Methods that have been applied to analyse the presence of anti-cN1A autoantibodies in patient sera include (*a*) immunoblotting with lysates from human skeletal muscle tissue and lysates from transfected HEK293 cells expressing cN1A, and a line blot with bacterially expressed recombinant cN1A, (*b*) immunoprecipitation of *in vitro* translated recombinant cN1A, (*c*) ELISA with synthetic cN1A-derived peptides or with recombinant cN1A expressed in bacterial or eukaryotic cells, (*d*) addressable laser bead-based assays with recombinant cN1A or fragments thereof, and (*e*) immunofluorescence with transfected, cN1A-expressing COS and HEK293 cells. These are non-standardized tests developed in research laboratories. The only commercially available anti-cN1A tests that are currently available are the ELISA cN1A (Mup44, NT5C1A) and the EUROLINE Autoimmune Inflammatory Myopathies 16 Ag et cN1A tests of EUROIMMUN (containing cN1A in combination with the 16 antigens on the myositis LIA of EUROIMMUN) [68].

Although the available tests for cN1A detection in serum samples have not been systematically compared, a number of observations indicate that discordant results can be obtained with different test-formats, with full-length cN1A expressed in different systems, and with different parts of cN1A. IBM sera display heterogeneity in the reactivity with full-length cN1A in different assays and in the recognition of different linear epitopes. At least in part this will be due to the denaturation/renaturation status of full-length cN1A and differences in the accessibility of different epitopes. Also the influence of differences in the isotype specificity of secondary antibodies is not clear yet and needs to be explored in more detail.

The relatively large variation in the results obtained in different assays for anti-cN1A autoantibody detection hampers its use in clinical practice. To establish an international gold standard for anti-cN1A autoantibody testing a collaborative effort of multiple researchers is required, in which not only the same samples are tested, but in which also the only commercially available standardized ELISA test is used as a reference test.

Irrespective of the current lack of a gold standard for anti-cN1A testing and although anti-cN1A autoantibodies are rare in other forms of myositis and in other rheumatic and neuromuscular diseases, it has been demonstrated that they are not specific for IBM. For example, in various studies anti-cN1A autoantibodies have been detected in patients with Sjögren's syndrome and SLE [34,69].

## 6. Harmonization of autoantibody detection in idiopathic inflammatory myopathies

### 6.1. Options for harmonization in testing and reporting of myositis specific autoantibodies (Jan Damoiseaux, The Netherlands)

Due to the heterogeneity in autoantibody composition between patients, harmonization of autoantibody results is the best achievable option to better align results between different assays [6]. The first level of harmonization is to determine which clinical manifestations warrant the request for MSA. Although this could be established for the distinct IIM subtypes, overlap in clinical manifestations between these subtypes will complicate the establishment of an effective gating strategy to prevent requests for patients with a low pre-test probability and consequent high risk for false-positive results.

The second level of harmonization involves the choice of test and testing algorithm to be performed. Clinical laboratories are restricted by (inter)national regulations as issued by, for instance, the food and drug administration (FDA) and the European Union (EU IVD-R 2017/7460). This implies that most laboratory-developed tests, such as different methods for immunoprecipitation that are used in research laboratories, are not readily available in clinical laboratories, even if specialized in autoantibody diagnostics. As will be discussed, screening by HEp-2 IFA has insufficient sensitivity, while solid-phase immuno-assays, often referred to as ANA- or CTD-screen, have different compositions of autoantigens and lack most MSA. Alternative assays predominantly include DIA and LIA that enable to simultaneously test for a wide array of MSA [4]. However, the antigen-composition of these assays is different and none includes all identified MSA. Based on clinical information, additional single-antigen assays may be required. As discussed in other parts of this paper, these assays may give rise to discordant results. Furthermore, for the laboratories there is a challenge in terms of verification and quality control because several MSA have a very low prevalence.

The third level of harmonization is the way test-results are reported to the clinic. This can be done in a dichotomous manner, i.e., negative and positive, based on the cut-off value provided in the insert of the assay. While these cut-off values are the same for the distinct autoantibodies, this might not be optimal for all antigens [70]. For many autoantibodies it is well established that the higher the autoantibody level, the higher the clinical relevance. Therefore, positive MSA results should differentiate between low, medium and high positive. Optimally, the cut-off values for discriminating these levels need to be strictly defined, for instance by the level of specificity. Eventually, results should be translated into likelihood ratio's for test-result intervals, or even single test-results, for optimal clinical interpretation [71]. In the context of reporting test-results it is also important to harmonize reporting of results that may be represented by two distinct antigens (Mi2$\alpha$ and Mi2$\beta$, SAE1 and SAE2, PM/Scl75 and PM/Scl100) [7]. Finally, it can be discussed whether in case of clinical suspicion of a certain IIM subtype, only the subtype related autoantibodies are reported. As mentioned above, there can be substantial overlap in the clinical manifestations and, therefore, it is warranted to report the results of all MSA that have been tested.

Finally, the fourth level of harmonization involves the interpretation of the laboratory results in the context of the clinical manifestations. This would strongly benefit from a computer algorithm that can calculate first the pre-test probability as defined by the clinical manifestations and second the likelihood

ratio related to the test-result obtained in the assay of choice. Combining these two input values can automatically generate the post-test probability which enables the clinician to make further decisions required for ascertaining the diagnosis and/or installing the appropriate therapy.

Altogether, first, a list of clinical manifestations is to be defined for an adequate gating strategy. Next, it is evident that sufficient data are lacking for optimal interpretation of test-results and that a large multi-center study is required to establish optimal cut-off values and/or likelihood ratios for test-result intervals or single test-results for the distinct MSA. This will be a major challenge, in particular for the low prevalent MSA.

### *6.2. Application of commercial assays in a real life idiopathic inflammatory myopathy cohort: do we need guidelines for requesting myositis specific and associated autoantibody assays? (Yves Piette, Belgium)*

Due to the recognition of the importance of MSA/MAA in diagnosis and subtyping of IIM [4], the number of requests for these antibodies has multiplied in daily clinical practice. However, only limited data on test performance characteristics of the assays are available, especially in situations of low pre-test probability, implicating a risk of false positive results, with potentially wrong diagnosis, excessive investigations and overtreatment as a consequence [72]. Hence, the data of a study was presented aiming to calculate pre- and post-test probabilities [73] for having IIM in different clinical situations, in order to explore in which groups of patients the detection of MSA is most useful.

Clinical symptoms (retrospectively collected with standardized questionnaires) and laboratory markers (including MSA/MAA on one immunoblot; EUROIMMUN) were documented in a consecutive cohort with clinical suspicion of IIM (December 2014-September 2020, n=282) and an additional set of known IIM patients (n=12). Consecutive patients were subclassified in IIM (n=64) and diseased controls (n=218, including also a subset of other autoimmune rheumatic diseases; n=76). Based on this dataset, we calculated the prevalence of clinical symptoms and laboratory markers (including MSA/MAA). Second, we evaluated pre-test probabilities and calculated post-test probabilities for having IIM in different clinical situations, taking into account the observed performance characteristics of the immunoblot [73].

Pre-test probabilities differed substantially depending on the clinical symptoms at presentation, with low pre-test probability in case of myalgia, arthritis and Raynaud's phenomenon, with moderate probability in case of muscle weakness, ILD and skin lesions, and with increasing probabilities in case of a combination of these features.

Positive likelihood ratio of the MSA/MAA test (MSA and/or MAA positive; anti-Ro52 excluded) was 2.8 (sensitivity 44.7%, specificity 89%), but improved towards 7.7 when only MSA were taken into account (sensitivity 42.1%, specificity 94.5%). Taken together, the data of pre-test probability of the different clinical situations and the likelihood ratio of the test, post-test probabilities in case of positive MSA/MAA results could be calculated and allowed the identification of situations with the highest impact of MSA/MAA assessment. Highest impact could be observed in situations with moderate pre-test probability. In patients with low pre-test probability, a positive test-result still left substantial diagnostic uncertainty.

Using commercial assays for MSA/MAA in a context of low pre-test probability implicates the risk of false positive results. In anticipation of more performant serological tests, the implementation of a gating strategy to increase pre-test probability would improve the diagnostic utility of the actual commercial immunoblots in daily clinical care.

### *6.3. Positivity for myositis specific autoantibodies without compatible phenotype: false positive or real positivity prior myositis onset? (Ingrid Lundberg, Sweden)*

To address this question it is important to discuss when the autoimmune disease myositis starts. Lessons could be learned from recent studies in RA. It is well established that anti-citrullinated peptide antibodies (ACPA) are present up to 10 years before onset of RA [74]. Furthermore, patients with RA often have a long history of pain and fatigue and high level of sick leave a couple of years before RA diagnosis [75]. There is also increasing information suggesting that the immune reactivity that leads to ACPA production may start in the lungs and later hit the joints [76]. For IIM there are also reports suggesting that the autoantibodies may predict development of clinical manifestations compatible with myositis [77]. This has been confirmed in a few case reports and was also found in a longitudinal study of patients with anti-Jo1 autoantibodies [78]. In this study it was also demonstrated that anti-Jo1 autoantibodies were of high affinity at time of diagnosis. In addition, patients with IIM have an increasing demand on health care and increasing costs for sick leave one year before diagnosis, suggesting that the disease may start long before the characteristic symptoms leading to diagnosis appear [79].

We still need to know the predictive value of a positive MSA without clinical manifestations and of a positive MSA with unspecific clinical manifestations. What is the likelihood that an individual with MSA will develop IIM? Are there factors that influence the risk to develop disease? We are facing a clinical dilemma in that we want to diagnose patients with IIM early to prevent chronic disease. Which tools do we have to identify patients at high risk to develop disease (tools to capture clinical manifestations, screening assays)? A positive MSA without specific manifestations - how shall we manage this individual? To answer these questions, we need to perform longitudinal follow up and as these are rare diseases we need to form multicentre studies. In parallel we also need to validate the autoantibody assays to assure that they have a good validity

### *6.4. HEp-2 indirect immunofluorescence and myositis specific autoantibodies: screening and/or confirmation (Jan Damoiseaux, The Netherlands)*

Autoantibody screening for the SARD, including IIM, traditionally starts with the search for anti-nuclear antibodies (ANA) by indirect immunofluorescent assays (IFA) with HEp-2 cells as substrate. The result is reported as negative or positive and, if positive, with information about the pattern and titer. A positive result also is followed by reflex testing for antigen-specificity, i.e., extractable nuclear antigens (ENA). Although most MSA may give a positive result with a defined pattern in the HEp-2 IFA [80,81], this is not evident for anti-MDA5 and anti-HMGCR antibodies. Moreover, the technical sensitivity of HEp-2 IFA for many MSA is limited [82]. Additional caveats are that laboratories may not report cytoplasmic patterns, as considered not to be true ANA, while multiple MSA reveal such a cytoplasmic pattern, and that reflex testing for antigen-specificity is most often restricted to Jo-1, but not includes the whole spectrum of MSA.

Alternatively, since there is discussion about the specificity of the multiplex immuno-assays for MSA detection, in particular if only low-positive results are obtained, the HEp-2 IFA might have added value for confirmation if the expected pattern is observed. However, in a study of Infantino et al. only 59% of the IIM patients with MSA revealed the corresponding pattern [83]. Furthermore, in particular the low-positive results have been reported to cause the most diagnostic challenges [70,84], and these samples, most likely, are negative by HEp-2 IFA.

Altogether, it can be concluded that the added value of HEp-2 IFA is very limited in the diagnostic work-up of IIM. Considering the restrictions of screening for MSA by HEp-2 IFA, clinical suspicion of IIM is best to be followed directly by testing for MSA panels. Suspicion of an IIM-overlap syndrome, however, should result in direct assessment of MSA/MAA in combination with HEp-2 IFA followed by an adequate algorithm for reflex testing.

### *6.5. The way to report results (qualitative/quantitative) and verify assays (Olivier Boyer, France)*

Depending on the type of assay used, a numerical value for the autoantibody level may be obtained by scanning the intensity of the staining on the DIA/LIA, or by applying a calibration curve in the immuno-assay. Results are generally expressed as arbitrary units because international standard preparations do not exist for the MSA. There is, however, a reference preparation from the Autoantibody Standardization Committee (reagent IS2187) which contains anti-Jo1, but lacks an internationally accepted unitage. As already discussed, standardization does not seem to be an option for quantifying levels of autoantibodies [6]. Therefore, (semi-)quantitative results should always be accompanied by the reference values of the respective assay. In this respect, it should be noticed that the same immuno-assays may use distinct cut-off values, depending on the type of analyzer used, i.e., analysis of strips either in the wet or dry phase.

### *6.6. Myositis specific autoantibodies: test characteristics, quality of tests and cut-off decisions (Xavier Bossuyt, Belgium)*

MSA can be detected by different methods (reviewed in [4]). MSA have typically been discovered by IP, a technique which is generally considered the reference method or gold standard. LIA/DIA allow to simultaneously detect the most important MSA and are widely used in clinical immunology laboratories. ELISAs and automated solid phase assays are also used for MSA detection.

Several studies have shown that results obtained with LIA may differ from results obtained with DIA [72,85]. The differences are dependent on the antibody type and are pronounced for anti-TIF1$\gamma$ antibodies [72,85]. DIA detects less anti-TIF1$\gamma$ antibodies than LIA [72]. Non-specific reactivity in non-myositis patients (controls) differs between assays and is reported to be higher with LIA than with DIA [72]. The higher the MSA antibody level, the higher the association with IIM [72].

Several studies have evaluated the agreement between IP and LIA/DIA [56,58,84]. The agreement is dependent on the MSA and the assays compared (reviewed in [86]). When compared to IP, LIA/DIA do not perform well for all MSAs and false positives are relatively common with LIA/DIA [56]. Tansley et al. [55] showed that the sensitivity of LIA/DIA for anti-TIF1$\gamma$ antibodies was clearly lower than the sensitivity of IP and confirmed the above-mentioned differences between LIA and DIA for anti-TIF1$\gamma$ antibodies.

In conclusion, there is a need for improving the test accuracy (of LIA/DIA) and for harmonizing test-result interpretation (cut-offs). The latter can be improved by reporting test-result interval specific likelihood ratios (see next paragraph).

### *6.7. Likelihood ratios as a means to improve interpretation of myositis specific autoantibodies (Xavier Bossuyt, Belgium)*

For interpretation of autoantibody results one usually applies a single cut-off value. Such dichotomous interpretation distinguishes between positive and negative results. A disadvantage of such approach is that it overlooks the fact that for many autoantibodies, the likelihood that a particular result is associated with disease increases with increasing antibody level [71]. Applying a Bayesian approach and reporting likelihood ratios is a way to improve interpretation of test-results [71,73,82,87]. Likelihood ratios can be used for estimating the post-test probability for disease [71,73,82,87]. Likelihood ratios can be given for individual test-results or for test-result intervals, thereby giving information that is associated with the antibody level. Likelihood ratios have been shown useful for anti-neutrophil cytoplasmic antibodies, antinuclear antibodies, rheumatoid factor and anti-citrullinated protein antibody, anti-tissue transglutaminase, and specific IgE (reviewed in [71,87]). The 2017 revised international consensus on ANCA testing recommends to report test-result (interval) specific likelihood ratios for proteinase-3 (PR3)-ANCA and myeloperoxidase (MPO)-ANCA [88,89].

As it is known that solid phase assays and LIA/DIA for detection of myositis specific antibodies may suffer from non-specific reactivity and false weak positive results, reporting likelihood ratios for test-result intervals may help with the interpretation. With the current approach there is the disadvantage of over-interpretation of weak positive results, even though some manufacturers propose an “equivalent” result zone. Using test-result interval-specific likelihood ratios will objectify the diagnostic value of a particular test-result [6,71]. Therefore, we suggest that future studies should determine test-result specific likelihood ratios for myositis specific antibodies.

## 7. Consensus guidelines

The multiple discussions during the workshop resulted in a first decision to focus the consensus guidelines on the MSA. Additional inclusion of MAA would further complicate the discussions and eventual consensus guidelines. **The MSA should include the following specificities: HMGCR, SRP, TIF1$\gamma$, NXP2, Mi2, MDA5, SAE, Jo1 and other anti-ARS (as far as available).** Positioning of anti-cN1A was decided not to be within the MSA, but testing for these antibodies should be considered if IBM is suspected. Modifications in autoantibody panel to be tested may be made depending on local circumstances but should be clearly communicated between clinical and laboratory specialists.

### *7.1. Clinical indications that ask for detection of myositis specific autoantibodies*

Considering the prevalence of IIM and the complexity of the interpretation of the test-results of multiplex MSA-assays it is evident that the test should not be requested by general practitioners. Depending on the dominant clinical manifestations, patients should be referred to clinical immunologists, rheumatologists, neurologists, dermatologists, or pulmonologists. Within these disciplines it is even important to consult clinical specialists with expertise in IIM.

**Testing for MSA should be included:**

- **during initial clinical work-up of patients presenting with myositis syndrome features (Table 1), preferentially prior to treatment with immunosuppression,**
- **if a patient has diagnosis IIM but MSA have not been previously tested (for subclassification and prognostic value),**
- **if a patient presents with interstitial pneumonia with autoimmune features (IPAF) according to the criteria of the American Thoracic Society/European Respiratory Society [90], in particular if the patient presents with isolated ILD of unknown etiology.**

There is no need to test for the whole spectrum of MSA if any MSA (e.g., anti-Jo1) is known to be positive and is consistent with

**Table 1A**
Myositis syndrome features that ask for detection of myositis specific autoantibodies.

| |
|---|
| The triad of myositis, interstitial lung disease, and arthritis, possibly accompanied by Raynaud's phenomenon, mechanic's hands, and fever |
| Characteristic skin rash, including Gottron's papules, shawl sign, and heliotrope rash, most often in combination with symmetrical proximal muscle weakness |
| Severe proximal muscle weakness with a sub-acute onset and without clinical extra-muscular manifestations |
| Slowly progressive muscle weakness with an asymmetrical distribution involving both proximal and distal muscles, typically presenting after the age of 40 (only anti-cN1A*) |

*Formally, anti-cN1A is considered not to be a myositis specific autoantibody.

**Table 1B**
Myositis syndrome features for which detection of myositis specific autoantibodies should be considered after excluding other, more common, diagnoses.

| |
|---|
| Isolated seronegative and non-erosive polyarthritis* |
| Interstitial Lung Disease of unknown cause |
| Isolated high CK level on repeated samples |
| LGMD-like disease with no known molecular diagnosis nor familial history** |

*For interpretation the focus should be only on the anti-synthetase antibodies. Anti-Jo1 detection, most often, is already included in the anti-ENA analysis, but may go unnoticed in case of negativeHEp-2 IFA results.
**For interpretation the focus should be only on the anti-SRP and anti-HMGCR antibodies.

the clinical phenotype and/or muscle biopsy. Also, currently there is no need for repeated testing during follow-up of the patient, although this may change in the future if a relation between individual MSA levels and prognosis or disease activity would become apparent. However, if new clinical features develop that are not consistent with the initial MSA test-result, repeated testing is indicated. Furthermore, when the initial test-result for MSA is negative, but the patient has convincing clinical features of the myositis syndrome, repeated testing may be warranted for patients on immunosuppression during the initial evaluation, if new MSA are discovered or more sensitive techniques have become available. Finally, testing for MSA with different methods could be considered if multiple MSA are detected (MSA are considered to be mutually exclusive) or if clinical features are not consistent with initial positive MSA results. In particular anti-TIF1$\gamma$ results obtained by DIA/LIA require some further attention due to possible false-negative and false-positive results. Confirmation by a different method of the result obtained could be considered if a patient with a definite diagnosis DM is negative for anti-TIF1$\gamma$, or in case of a positive anti-TIF1y result in the absence of DM features. The association of anti-TIF1y and malignancy is documented in adult DM patients (>40 years), but unknown in the absence of DM features.

### 7.2. Optimal testing strategy for myositis specific antibodies

Although most MSA may reveal either nuclear or cytoplasmic staining of HEp-2 cells by IFA, the sensitivity of this method is rather low. As such, HEp-2 IFA is also of limited value as a second-line confirmation assay. Alternative solid-phase immuno-assays for screening of autoantibodies to the so-called extractable nuclear antigens (ENA; CTD-screen) is also of limited value since these assays often include anti-Jo1 as the only IIM autoantigen. Although this is the most prevalent MSA, such screening may give the false impression that IIM is serologically excluded. **Therefore, clinical indications as summarized in the previous paragraph should be followed by testing the full spectrum of MSA. In case an IIM-overlap syndrome is suspected, additional screening with HEp-2 IFA and/or solid-phase CTD-screen immuno-assays is mandatory (Fig 1).**

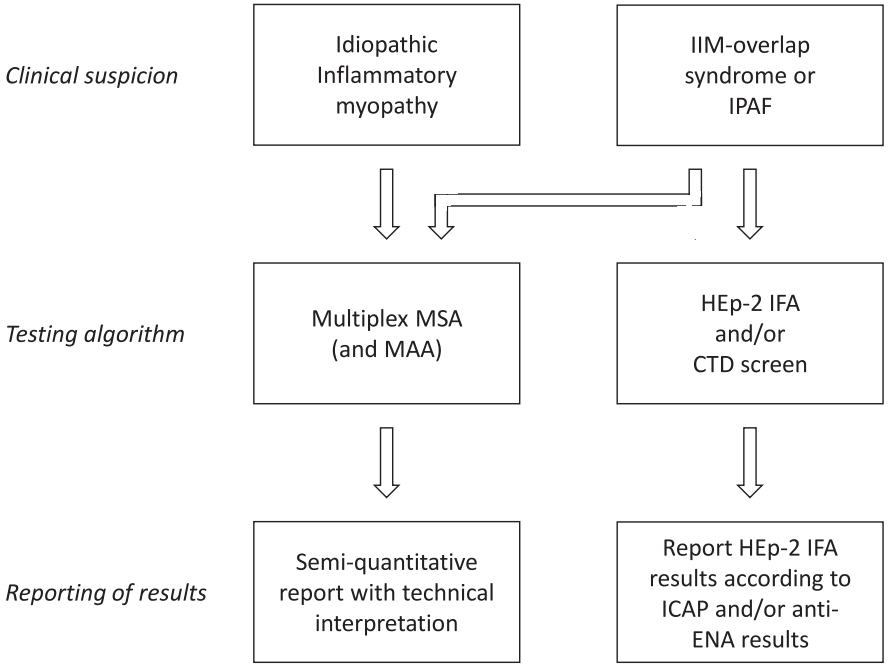


**Fig. 1.** Harmonization of autoantibody detection in IIM: from requesting to reporting.
Clinical suspicion of IIM, as defined in table 1, should directly be followed by testing for the whole spectrum of MSA; results are to be reported in a semi-quantitative manner accompanied by a technical interpretation. If there is clinical suspicion of an IIM-overlap syndrome and/or IPAF testing should include both the whole spectrum of MSA as well as screening (and follow-up) by HEp-2 IFA and/or solid-phase CTD-screen assays. Results of HEp-2 IFA should be reported according to ICAP.
Abbreviations: CTD, connective tissue diseases; ICAP, international consensus on ANA patterns; ENA, extractable nuclear antigens; IFA, indirect immunofluorescence assay; IIM, idiopathic inflammatory myopathy; IPAF, interstitial pneumonia with autoimmune features; MAA, myositis associated autoantibodies; MSA, myositis specific autoantibodies.

**Table 2**
Availability and quality of antigens in distinct immuno-assays.

| Antigen | MBL ELISA | Thermo Fisher FEIA | Euroimmun LIA | D-tek DIA | Trinity LIA | Werfen Quanta-Lite | Werfen Quanta-Flash |
|---|---|---|---|---|---|---|---|
| HMGCR | | NA | 1 | | NA | | |
| SRP | NA | NA | | | | NA | NA |
| | | | | | | | |
| TIF1γ | | NA | 2 | 3 | 2 & 4 | NA | NA |
| MDA5 | 5 | NA | 5 | 5 | 5 | NA | NA |
| Mi2 | NA | | NA | NA | | NA | NA |
| *Mi2α* | NA | NA | 6 | NA | NA | NA | NA |
| *Mi2β* | | NA | 6 | | NA | NA | NA |
| SAE | NA | NA | NA | NA | NA | NA | NA |
| *SAE1* | NA | NA | 7 | | | NA | NA |
| *SAE2* | NA | NA | NA | | NA | NA | NA |
| NXP2 | NA | NA | 8 | | NA | NA | NA |
| | | | | | | | |
| Jo1 | 9 | | | | | | |
| EJ | 9 | NA | | 7 | | NA | NA |
| OJ | NA | NA | 10 | NA | 10 | NA | NA |
| PL7 | 9 | NA | | | | NA | NA |
| PL12 | 9 | NA | | | | NA | NA |
| Ha | NA | NA | NA | NA | NA | NA | NA |
| KS | 9 | NA | NA | NA | NA | NA | NA |
| Zo | NA | NA | NA | NA | NA | NA | NA |

1. Antigen has recently been added to the assay, but will not be available in all countries due to patent restrictions; comparative studies with gold standard assays have not yet been published.
2. Detection of anti-TIF1$\gamma$ autoantibodies lacks optimal sensitivity and specificity. In patients diagnosed with dermatomyositis (DM) and negative for all DM-associated autoantibodies an alternative technology (MBL ELISA) for detection of anti-TIF1$\gamma$ autoantibodies should be considered. Also, in adult patients with a positive result, an alternative technology for confirmation of anti-TIF1$\gamma$ autoantibodies should be considered, especially in absence of DM. The relevance of finding anti-TIF1γ in absence of DM features is unknown.
3. Technical sensitivity for anti-TIF1$\gamma$ autoantibodies is very limited. Negative results should not be reported; positive results may be reported as a remark.
4. Assay also contains TIF1$\alpha$.
5. Clinicians should be contacted because of urgent medical attention
6. Optimal test characteristics are achieved if both Mi2$\alpha$ and Mi2$\beta$ are positive.
7. Low positive results are unspecific and should be reported as negative. According to manufacturer SAE1 comes either alone or in combination with SAE2; single-positivity for SAE2 is very rare.
8. While in general low-positive results are less specific, this seems not to hold for anti-NXP2 autoantibodies.
9. Besides ELISA's for individual antigens, there also is a screening ELISA for 5 ARS autoantibodies (Jo1, PL7, PL12, EJ and KS).
10. Technical sensitivity for anti-OJ autoantibodies is very limited. Negative results should not be reported; positive results may be reported as a remark.

Colors indicate the consensus of the workshop participants with respect to the quality of the assays for the respective antibodies as based on personal experience and published studies: green, acceptable; orange, awareness of limitations is indicated; red, serious concerns.

Confirmation of the obtained results with a different method is to be considered if multiple MSA are detected (MSA are considered to be mutually exclusive) or if clinical features are not consistent with initial positive MSA results. In addition, if a patient with a definite diagnosis DM is negative for MSA, testing for anti-TIF1$\gamma$ by a method other than DIA/LIA is advised (these assays are hampered by false-negative results; Table 2).

Results for MSA should be available within 7 days. In case patients present with RP-ILD, a fast diagnosis is necessary and the laboratory should prioritize the analysis and reporting for MSA (in particular anti-MDA5; ideally within 24 hours).

### *7.3. format for reporting results to the clinician in order to enable optimal interpretation*

Although IP is often referred to as "gold standard", it should be noted that IP is not a uniform assay. More importantly, IP is only available in clinical research laboratories. Since DIA and LIA are the assays most used in routine clinical practice, the current consensus is primarily focused on these types of multiplex assays, while taking into account the limitations of these assay (Table 2). One major limitation is that large clinical evaluations of these assays, in particular for the rare autoantibody specificities, are lacking. Smaller studies have hinted at the inadequate cut-off setting for

the distinct autoantibodies [66,70], but at least it is evident that, in general, higher levels of autoantibodies are associated with increased specificity for IIM. There is consensus that reporting results as likelihood ratio's for test-result intervals or individual test-results is the best for optimal clinical interpretation, but reliable likelihood ratios for MSA are not yet available.

**The consensus on reporting entails:**

- **Report should specify what test method was used to detect each MSA,**
- **Report should define which MSA were tested,**
- **Report should provide positive results semi-quantitative (+, ++, +++; Fig. 1),**
- **Report should contain technical interpretation if multiple MSA are detected, if there is a discrepancy between test methods (screening and confirmation), or in case of negative results of anti-OJ (negative results are not reliable; Table 2),**
- **Clinicians should be contacted directly about results that demand urgent medical attention (positive anti-MDA5; Table 2).**

Obviously, each laboratory needs to verify and control the performance of the MSA assay in accordance to accreditation guidelines. This is quite a challenge, especially for the rare autoantibodies. For verification of the assay a multi-centre approach as suggested by the Dutch College of Medical Immunology seems to be most feasible [91]. Also for internal and external quality control it is mandatory to monitor the performance of all individual autoantibody specificities. The DIA/LIA kits do not include controls for each antibody specificity. For internal quality control it is advised to prepare low-to-medium range mixed positive controls for multiple MSA. For external quality control there are several organisations that organize such EQC programmes, but most of them only cover a limited number of autoantibodies. Alternatively, laboratories involved could collaborate by sending around samples with distinct specificities on a regular basis.

### *7.4. Consensus on the research agenda*

From the previous paragraphs it is evident that solid data on the test performance for the individual autoantibodies are lacking. For some autoantibodies it is anticipated that there are serious concerns about the sensitivity (anti-OJ), or even about both sensitivity and specificity (anti-TIF1$\gamma$). Moreover, multiplex DIA/LIA differentially include variants of an autoantigen, i.e., Mi2$\alpha$ and Mi2$\beta$; SAE1 and SAE2, further hampering harmonization of autoantibody diagnostics [7].

With respect to the test characteristics the research agenda includes a large multi-center study with high numbers of diagnostic samples from IIM patients and relevant disease controls. Such a cohort will enable to:

- Determine optimal cut-off values for the distinct autoantibodies,
- Determine likelihood ratios for test-result intervals and/or individual test-results,
- Compare test characteristics between currently existing, and possibly novel immuno-assays based on the same cohort of patients and controls.

The challenge will be to include samples on a consecutive basis covering a representative IIM cohort with sufficient samples for the rare MSA. This is critical for establishing reliable likelihood ratios. Eventually, positive results obtained in the disease controls could be used to determine the significance of MSA without evident symptoms/signs of IIM at the time of blood sampling. Relevant questions are if these patients will develop IIM over time or if they have other clinical manifestations in common.

## 8. Conclusions

The 256$^{th}$ ENMC workshop was a hybrid meeting about the role of MSA in IIM. Since standardization of autoantibody assays seems to be hardly impossible, the focus was on harmonization. Consensus was achieved on the clinical manifestations that warrant testing for MSA (Table 1). Applying such a gating strategy will prevent from testing patients with a low pre-test probability and reduce the number of false-positive results. Next, consensus was defined for the testing algorithm and the way test-results should be reported to the clinician (Fig. 1). Finally, there was agreement on the research agenda in terms of the explicit need for a collaborative multicenter study to better define the test characteristics of the MSA in different immune-assays currently in clinical practice or under development. Optimal interpretation of the test-results, eventually, will benefit the patient in terms of appropriate diagnosis and treatment.

## Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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