Pitfalls in the detection of myositis specific antibodies by lineblot in clinically suspected idiopathic inflammatory myopathy

Y. Piette[1,2], M. De Sloovere[3], S. Vandendriessche[3], J. Dehoorne[4], J.L. De Bleecker[5], L. Van Praet[1], A.-S. Vander Mijnsbrugge[1], S. De Schepper[6], P. Jacques[1], F. De Keyser[7,8], V. Smith[1,7], C. Bonroy[3,9]

[1]Department of Rheumatology, Ghent University Hospital, Ghent, Belgium; [2]Department of Rheumatology, AZ Sint-Jan Brugge – Oostende AV, Belgium; [3]Department of Laboratory Medicine, [4]Department of Paediatric Rheumatology, [5]Department of Neurology, and [6]Department of Dermatology, Ghent University Hospital, Belgium; [7]Praktijk 10A, Maldegem, Belgium; [8]Department of Internal Medicine, [9]Department of Diagnostic Science, Ghent University, Belgium.

Abstract

Objective

Today, the contribution of myositis-specific autoantibodies (MSA) in the diagnostic workup of idiopathic inflammatory myopathies (IIM) is on the rise. The aim of this study was to document MSA frequency as detected by lineblot in a set of consecutive MSA requests and to correlate the results with clinical diagnosis, IIM subtype and indirect immunofluorescence (IIF) findings. Additionally, a comparison between two lineblots was performed.

Methods

A total of 118 consecutive samples of patients with suspicion of IIM were analysed on IIF and two lineblots. A total of 107 patients with autoimmune rheumatic diseases served as controls.

Results

MSA were detected in 55% of IIM patients (n=31) and 7.9% (n=12) of patients without clinical diagnosis of IIM or myositis overlap syndrome. All the IIM patients had a MSA-compatible clinical subtype. There was no to fair agreement between both lineblots for the individual antibodies, with most discrepancies observed for anti-TIF1γ (κ=-0.021), anti-SRP (κ=-0.006) and anti-SAE (κ=0.395). Differences between both assays were mostly observed in the non-IIM patients, also showing significantly lower blot signal intensities compared to IIM patients (p=0.0013). MSA in the non-IIM patients frequently showed an incompatible IIF pattern.

Conclusion

Lineblot seems to be an interesting tool for MSA detection in a clinical context, allowing the identification of clinical subtypes. However, considerable caution must be exercised in interpreting the results in case of low positive MSA signal intensity, discordant lineblot results and/or an incompatible IIF pattern.

Key words

myositis specific antibodies, antinuclear antibodies, Idiopathic inflammatory myopathy

Yves Piette, MD
Maxime De Sloovere, Pharm D
Stien Vandendriessche, Pharm D, PhD
Joke Dehoorne, MD, PhD
Jan L. De Bleecker, MD, PhD
Liesbet Van Praet, MD, PhD
An-Sofie Vander Mijnsbrugge, med. student
Sofie De Schepper, MD, PhD
Peggy Jacques, MD, PhD
Filip De Keyser, MD, PhD
Vanessa Smith, MD, PhD
Carolien Bonroy, Pharm D, PhD

Please address correspondence to:
Yves Piette,
University Hospital Ghent,
C. Heymanslaan 10,
9000 Gent, Belgium.
E-mail: yves.piette@ugent.be

Reprints will not be available from the authors.

Received on February 11, 2019; accepted in revised form on April 29, 2019.

Funding: this work was supported by the Fund for Scientific Research in Rheumatology and the King Baudouin Foundation (grant no. 2016-J5820590-205753).

Competing interests: none declared.

Introduction

The idiopathic inflammatory myopathies (IIM) are a heterogeneous group of acquired muscle diseases with distinct clinical, serological and histological features. The historical classification of IIM initially included 2 main subgroups: polymyositis (PM) and dermatomyositis (DM) (including juvenile DM (jDM) (1). The most recent classification strategies describe several main entities: DM (including the amyopathic subset), overlap myositis including mainly the anti-synthetase syndrome (ASS), immune mediated necrotising myopathy (IMNM), inclusion body myositis (IBM) and PM (2-4).

Making a correct diagnosis of IIM is challenging but essential because of their association with malignancy and systemic autoimmune rheumatic diseases (SARD), the possibility of (multi) organ involvement and treatment implications (5). Traditionally, the diagnostic confirmation of patients with a suspect medical history and clinical examination is based on laboratory tests (serum muscle enzymes), muscle and/or skin biopsy and instrumental investigations (electromyography and MRI) (1, 6). A newer approach includes the detection of autoantibodies, which have been identified in about 50% of the IIM patients (3, 7-9). Autoantibodies in myositis are traditionally subdivided in myositis specific antibodies (MSA) and myositis associated antibodies (MAA). MSA are predominantly disease specific and, with some exceptions, mutually exclusive, where MAA can also be found in other SARD and are frequently present in IIM overlap disease (for review see (10)). Both MSA and MAA are associated with specific clinical features and allow categorisation of IIM patients in subsets with different prognosis and treatment response (11, 12).

Historically, the number of MSA was very limited and their detection was confined to research laboratories using laborious and time-consuming conventional techniques, mostly immunoprecipitation using [^{35}S]-methionine labelled K562 cell extract and RNA immunoprecipitation with silver staining (13). Later on, several newly characterised MSA were identified in formerly 'seronegative' IIM patients (*e.g.* anti-TIF1γ, anti-NXP2, anti-MDA5, anti-SAE, anti-HMGCR). The growing awareness of the importance of MSA and MAA has led to the integration of anti-Jo1 in the 2017 EULAR/ACR classification criteria for IIM (4). The inclusion of more MSA in future revisions of these criteria is currently being discussed (2, 14, 15). Within this context, the last years, several immunoassays for the detection of these antibodies have become commercially available. We expect that the wider accessibility of MSA will allow better and earlier diagnosis, as well as characterisation of patients suspect for IIM. These assays are, however, not standardised, using variable techniques and antigen sources, and data on their routine use are scarce (8, 16-18).

The aim of this study was to document the frequency of MSA as detected by two lineblot assays in a set of consecutive patients with MSA requests and to correlate the MSA results with the clinical diagnosis, the IIM subtype and the indirect immunofluorescence (IIF) findings. Additionally, a comparison of both lineblots was performed.

Patients and methods

Samples

In total, 118 consecutive samples with an MSA request in the context of suspicion of IIM between December 2014 and March 2017 were included in this study (in the case of multiple samples per patient, only the first sample was included). Globally, 61% of patients were female and the median age was 51 years (range 3–86). Clinical diagnosis (clinical definite IIM [cDIIM], clinical probable IIM [cPIIM], immune mediated inflammatory disease (IMID) with myositis overlap not excluded or IIM excluded) was documented retrospectively by the treating physician using a standardised questionnaire in May 2017. In the case that MSA were detected, the medical records were additionally reviewed by YP or JD to determine the IIM-subtype. In the standardised questionnaire the variables for the EULAR/ACR probability score of the new IIM classification criteria were also documented (4). The EULAR/

ACR probability score was calculated for all clinically diagnosed IIM patients (for more details on the EULAR/ACR probability score see http://www.imm.ki.se/biostatistics/calculators/iim/). In addition, a set of 107 controls [50 systemic sclerosis (SSc), 29 systemic lupus erythematosus (SLE) and 28 rheumatoid arthritis (RA) patients] were also included in the study.

All serum samples were obtained as part of routine analysis for MSA. The study was performed according to the Declaration of Helsinki and approved by the Local Hospital Ethics Committee (EC UZG 20170283); no informed consent was needed for this retrospective analysis.

Laboratory analysis

Two lineblots and IIF analysis for antinuclear antibodies (ANA) were performed on all samples. The EUROLine Autoimmune inflammatory myopathies 16 Ag (AIM16, Euroimmun AG, Lübeck, Germany) and the MYOS-12DIV-24 Blue Diver dot blot (MYO12, D-Tek, Mons, Belgium) were applied according to the manufacturer's instructions using the Euroblotmaster and Blue Diver instrument, respectively. These assays contain both the following antigens: Jo1, PL7, PL12, EJ, SRP, Mi2, MDA5, TIF1γ, SSA/Ro52kD, SAE and NXP2. The OJ, Ku, PM/Scl-100 and PM/Scl75 antigens are additionally present on the AIM16 blot, the HMGCR antigen on the MYO12 blot. Results were digitised using a calibrated scanner, and absolute signal intensities were imported by a computer programme for further analysis (EUROLineScan and Dr Dot software, respectively). MSA positivity was defined based on positivity in at least one of both lineblot assays using cut-off values as proposed by the manufacturers (positive if >10 arbitrary units [AU]). Data on the MAA (anti-Ku, anti-Ro52 and anti-PM/Scl) were excluded from further analysis.

ANA IIF analysis was performed on HEp-2 cells according to the manufacturer's instructions (Inova diagnostics, San Diego, CA), using a serum dilution of 1:40 in order to obtain high sensitivity. Pattern assignment was performed according to the ICAP guidelines and compared with the theoretically expected ANA IIF pattern, based on the observed MSA (18) (see Table SI in the Supplementary data). For the anti-HMGCR antibodies, the IIF pattern as described by Musset and colleagues was evaluated (19).

Data analysis

For comparison of proportions, Chi-square testing with Yates' correction for continuity was applied. For comparison of two independent samples, Mann-Whitney was applied. Two sided *p*-values <0.05 were considered significant. Cohen's kappa (κ) statistics was used for comparison of the lineblot results (20). Statistical analysis was performed with MedCalc (Mariakerke, Belgium).

Results

Characterisation of the consecutive routine cohort

All 118 consecutive samples were retrospectively categorised for clinical diagnosis. In total, 31 patients were categorised as IIM (26%, 20 cDIIM and 11 cPIIM) and 29 as IMID with myositis overlap not excluded (25%). In 46 patients (39%) IIM was excluded at the moment of the retrospective review. No data were available for 11 patients (9%). One patient showed myopathic features without IIM (drug-induced rhabdomyolysis).

Frequency of MSA and comparison between lineblots

MSA were detected in 17 of 31 IIM-patients (55%) (positivity was defined based on a positive signal intensity on at least one lineblot), and in 25 (21%) of the global consecutive cohort. The sensitivity for the individual lineblots was 35% for AIM16 and 48% for MYO12. See Table I for an overview of the MSA detected in patients and controls. The highest frequencies in IIM patients were observed for anti-HMGCR (13%, n=4), anti-Mi-2 (13%, n=4), anti-Jo1 (10%, n=3) and anti-TIF1γ (6%, n=2). No multiple MSA reactivities were observed. Remarkably, 23% of the MSA in IIM patients were only observed in 1 lineblot system, which could not be explained by differences in the antigens present on the lineblots.

MSA were also observed in 12 patients (7.9%) without a clinical diagnosis of IIM or myositis overlap syndrome (combined specificity 92%, AIM16 specificity 97%, MYO12 specificity 95%), 3 of them were observed in the consecutive cohort, 9 in the control group. The reactivities observed in these patients were anti-TIF1γ in 6 [3 SSc,1 SLE, 1 RA and 1 IIM excluded]; anti-SRP in 1 [RA]; anti-SAE in 3 [1 SSc, 1 RA and 1 IIM excluded]); anti-Mi2 in 1 [SLE]; anti-Jo1 in 1 [IIM excluded]); all showing reactivity in only one lineblot system. Finally, MSA (2 anti-SRP, 1 anti-PL7, 1 anti-TIF1γ, 1 anti-Jo1 and 1 anti-Mi2) were also detected in 6 patients with non-conclusive autoimmune features (IIM could not be confirmed nor excluded) (see Table I and Suppl. Table S2).

When comparing MSA lineblot results on all tested samples with clinical data available (n=214), we observed no to fair agreement between the two lineblots for the individual antibodies (more details and κ-values are shown in Suppl. Table S3). Discrepancies were seen concerning both the novel autoantibodies, as well as established autoantibodies. Most discrepancies were observed for anti-TIF1γ, anti-SRP and anti-SAE positivity in the non-IIM samples. Nevertheless, differences were also observed in IIM-patients. More details on the positive MSA results in patients are given in Table II. Details on the positive MSA results in other samples are given in a Supplementary Table S2.

MSA positivity in relation to ANA IIF pattern and lineblot signal intensities

Figure 1 shows a comparison of MSA blot signal intensities between IIM, non-IIM and IIM not excluded patients for both lineblots. In general, low antibody positivity in any of the lineblot assays (<20 arbitrary units [AU]) was significantly more detected in non-IIM versus IIM (77% *vs*. 12%, *p*=0.0013).

MSA presence in relation to the theoretically compatible ANA IIF pattern was also evaluated (18). In IIM patients, a compatible IIF pattern was observed in 88% (n=15/17). The remaining 12% showed an incompatible IIF pattern (1 anti-EJ showing a nuclear

Table I. Overview of the MSA detected in patients and controls by lineblot.

	Combined MSA positivity[1]				MSA positivity on AIM16[1]				MSA positivity on MYO12[1]			
	Global cohort (n)	Patients (n,% of total [31])	Controls IIM excluded (total=152) (n)	Controls IIM not excluded (total=31) (n)	Global cohort (n)	Patients (n,% of total [31])	Controls (n)	Controls IIM not excluded (n)	Global cohort (n)	Patients (n,% of total [31])	Controls (n)	Controls IIM not excluded (n)
Mi2	6	4 (13%)	1[a]	1	5	3	1	1	4	4		
Jo1	5	3 (10%)	1[b]	1	5	3	1	1	3	3		
EJ	1	1 (3%)			1	1			1	1		
OJ	0				0				NP			
PL7	1			1	0				1			1
PL12	0				0				0			
NXP2	1	1 (3%)			1	1			1	1		
TIF1γ	9	2 (6%)	6[c]	1	5	2	2	1	4		4	
SAE	5[g]	1 (3%)	3[d]		1	1			4	1	3	
MDA5	1	1 (3%)			0				1	1		
SRP	3		1[e]	2[f]	1			1	2		1	1
HMGCR	4	4 (13%)			NP				4	4		
Total	36[g]	17 (55%)	12	6	19	11 (35%)	4	4	25	15 (48%)	8	2

[1]Details on the 2 lineblots used: D-tek MYOS12DIV-24 Blue Diver dot blot (MYO12) and EUROLine Autoimmune inflammatory myopathies 16 Ag (AIM16), combined MSA positivity was defined based on positivity in at least one of both lineblot assays using cut-off values (positive if >10 arbitrary units [AU]) as proposed by the manufacturers; SSc: systemic sclerosis, SLE: systemic lupus erythematosus, RA: rheumatoid arthritis, IIM: idiopathic inflammatory myopathy; [a]1 SLE; [b]1 IIM excluded [c]3 SSc, 1 SLE, 1 RA and 1 IIM excluded; [d] 1 SSc, 1 RA and 1 IIM excluded; [e]1 RA; [f]1 RA patient with IIM not excluded; [g]1 MSA positive sample with missing clinical information; NP: antigen not present in the specific lineblot.

Table II. Overview of the MSA results, clinical features and EULAR score of the IIM patients.

Patient nr.	MSA	MSA blot signal intensity (AU) [and method][a]	Clinical Diagnosis	Minimal EULAR/ACR score	Clinical associations
1 2 3 4	Mi2 Mi2 Mi2 Mi2	100 [DT], 31 [EI] 61 [DT], 28 [EI] 63 [DT] 69 [DT], 35 [EI]	4/4 cDIIM	3/4 DIIM (99-100%) 1/4 PIIM (68%)	4/4 typical DM skin lesions, 4/4 proximal muscle weakness with CK elevation, no ILD, 1/4 RP, no malignancies
5	EJ	36 [DT], 37 [EI]	cPIIM	6%	Suggestive muscle biopsy, no proximal muscle weakness and no CK elevation, no ILD, no arthritis, no RP
6	MDA5	12 [DT]	cDIIM	DIIM (100%)	Proximal muscle weakness without CK elevation, typical DM skin lesions, polyarthritis and ILD
7	NXP2	56 [DT], 78 [EI]	cDIIM	DIIM (100%)	Child with muscle weakness and CK elevation, typical DM skin lesions, extensive calcinosis
8 9 10	Jo1 Jo1 Jo1	74 [DT], 136 [EI] 73 [DT], 98 [EI] 86 [DT], 22 [EI]	2/3 cDIIM 1/3 pDIIM	2/3 DIIM (99-100%) 1/3 PIIM (66%)	2/3 proximal muscle weakness with CK elevation, 2/3 ILD, 2/3 RP, no arthritis, no skin lesions
11 12 13 14	HMGCR HMGCR HMGCR HMGCR	91 [DT] 55 [DT] 33 [DT] 70 [DT]	3/4 cDIIM 1/4 cPIIM	4/4 PIIM (56-60%)	4/4 proximal muscle weakness with CK elevation, no ILD, no arthritis, 1/4 malignancy, 4/4 statin exposure
15	SAE	95 [DT], 24 [EI]	cDIIM	DIIM (99%)	Typical DM skin lesions, no proximal muscle weakness, no CK elevation, no ILD, no RP, no malignancies
16 17	TIF1γ TIF1γ	15 [EI] 86 [EI]	2/2 cDIIM	DIIM (97%-100%)	2/2 typical DM skin lesions (1 jDM), 2/2 proximal muscle weakness without CK elevation, 1/2 arthritis, 1/2 RP, no ILD, no malignancies
18 to 31	No MSA	NA	7/14 cDIIM 7/14 cPIIM	2/14 DIIM (96-99%) 1/14 PIIM (85%) 11/14 (9-45%)	11/14 proximal muscle weakness, 11/14 CK elevations, 2/14 ILD, 4/14 RP, 2/14 arthritis, no calcinosis, 1/14 malignancies, 5/14 typical DM skin lesions

DT: D-tek MYOS12DIV-24 Blue Diver dot blot; EI: EUROLine Autoimmune inflammatory myopathies 16 Ag; cDIIM: definite clinical diagnosis of IIM; cPIIM: probable clinical diagnosis of IIM; DIIM: definite diagnosis of IIM according to the 2017 EULAR/ACR classification criteria; PIIM: probable diagnosis of IIM according to the 2017 EULAR/ACR classification criteria; NA: not applicable; ILD: interstitial lung disease; RP: Raynaud's phenomenon; CK: creatine kinase; (j)DM: (juvenile) dermatomyositis ; [a] cut-off values as proposed by the manufacturers (positive if >10 AU) are applied.

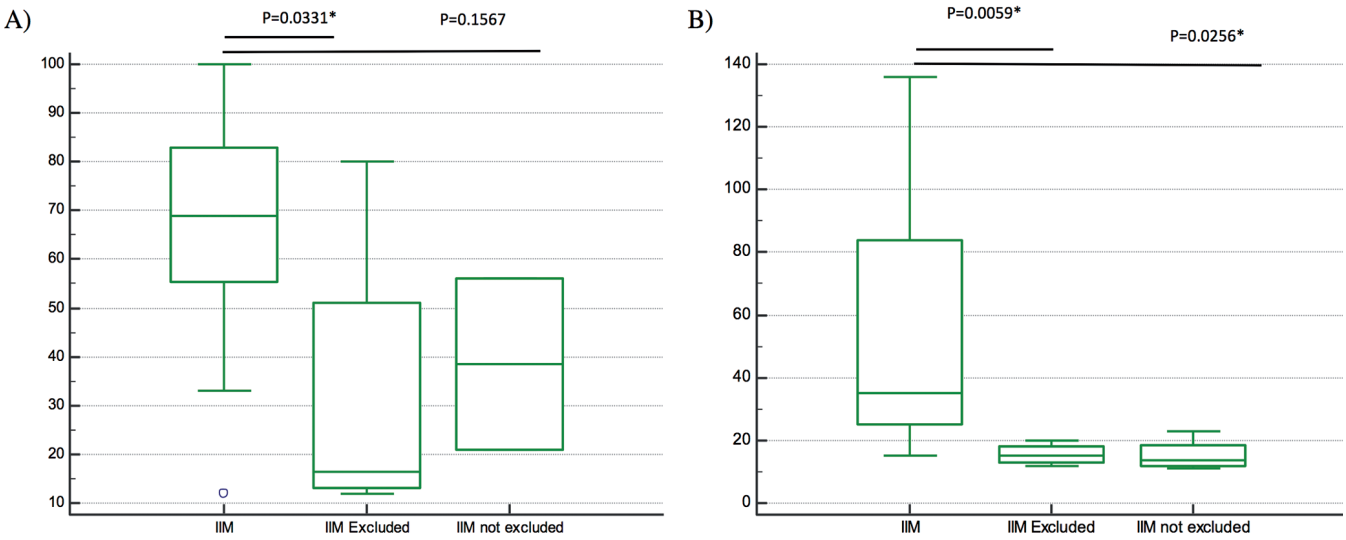

Fig. 1. Comparison of the MSA positive signal intensities between IIM, non-IIM, IIM not excluded patients for both lineblots. **A**: D-tek lineblot, **B**: Euroimmun lineblot. Mann-Whitney testing was performed to compare signal intensities between subsets. Two sided *p*-values <0.05 were considered significant*.

fine speckled pattern (AC-4) instead of the expected cytoplasmic speckled pattern (AC-19/20), 1 anti-SAE showing a nuclear coarse speckled pattern (AC-5) instead of the expected nuclear fine speckled pattern (AC-4)). The same results were also observed using a 1 in 80 serum dilution. In non-IIM patients, a compatible pattern was shown in 54% (n=7/13), while 15% (n=2/13) showed an incompatible pattern. Using a serum dilution of 1 in 80, an incompatible pattern was observed in 38% of samples (n=5/13). In four non-IIM samples, the ANA IIF analysis was not conclusive. In three of them other autoantibodies generated a strongly positive ANA IIF pattern (centromere pattern), possibly masking the MSA-related pattern.

Clinical features in IIM patients

A clinical diagnosis of IIM was made by the treating clinician in 31 patients (26%) (male: female 11:20, median age 54 [range 11–86]). An MSA was present in 17 (55%) of them. All but one of these patients had a MSA-compatible clinical IIM subtype. See Table II for an overview of the clinical features of the MSA+ patients (more details available in Suppl. Table S4). All four patients with anti-Mi2 positivity had proximal weakness, CK elevation and pathognomonic skin lesions, consistent with DM (Gottron sign/papules, shawl/holster sign). One patient positive for anti-TIF1γ had typical skin lesions (Gottron sign/papules, shawl sign), with clinical signs of muscle involvement. The other anti-TIF1γ positive patient equally showed typical skin lesions (heliotrope erythema of the eyelids, Gottron sign/papules, periungual erythema), with Raynaud's phenomenon, arthritis, calcinosis and clinical muscle involvement, consistent with juvenile DM. Neither of these two patients had a history of malignancy, nor was malignancy detected during work-up. The patient that was positive for anti-MDA5, suffered from muscle weakness, skin lesions, polyarthritis and interstitial lung disease, although not rapidly progressive. The anti-NXP2 positive patient was a young girl with muscle weakness, skin lesions and extensive calcinosis. The three patients that were anti-Jo1 positive, all had a clinical phenotype of ASS, although the classic triad of polyarthritis, interstitial lung disease and muscle involvement was not always present. Finally, all four patients with anti-HMGCR positivity were diagnosed with a necrotising inflammatory myopathy and had previous statin exposure. Only the anti-EJ positive patient had a less compatible clinical phenotype, with myalgia and IIM suggestive muscle biopsy.

EULAR/ACR probability score calculation

The EULAR/ACR probability score of the new classification criteria for IIM was calculated for all IIM patients. Patients in the MSA+ IIM group had a significantly higher EULAR/ACR probability score compared to patients in the MSA negative (MSA-) IIM group (median 99% *vs*. median 24%, *p*=0,0010). See Table II for an overview of the EULAR/ACR probability score of the patients (more details available in Suppl. Table S4). In the MSA+ IIM group, 10 out of 17 patients (59%) had a probability score of ≥90%, and could thus be EULAR/ACR classified as definite IIM (DIIM). Additionally, six of 17 patients (35%) had a score between 55% and <90%, which is required for the EULAR/ACR classification as probable IIM (PIIM). In the MSA- IIM group, in contrast, only two out of 14 patients (14%) had a probability score compatible with DIIM and one out of 14 (7%) with PIIM.

Discussion

Historically, the diagnosis and classification of IIM was based on clinical examination, muscle enzymes, electromyography and muscle biopsy (1, 6). Newer diagnostic/classification strategies include the evaluation of MSA. Consequently, anti-Jo1 was integrated in the new EULAR/ACR classification criteria (2, 4, 22-25) and it is likely that more MSA will be incorporated in future updates (26).

Recently, several lineblots for the detection of MSA have become commercially available (7-9, 17, 27). These

assays have the advantage of being fast and easy methods and allow simultaneous routine testing of multiple autoantibodies. In contrast, they are only validated to a limited extent in different disease cohorts as well as unselected patients (28-30).

In our study, we applied two lineblot assays on a consecutive cohort of patients with suspicion for IIM, representing the real life routine diagnostic context in which these assays will be used. Globally, MSA were detected in 21% of our unselected routine cohort (based on positivity in at least one lineblot), with 68% detected in the clinically diagnosed IIM patients, 20% in the patients where IIM could not be excluded nor confirmed, but also 12% in the non-IIM patients (combined specificity of 92%). The latter two groups illustrate the challenges of using lineblot in a diagnostic context of low to moderate clinical suspicion. Moreover, the presence of MSA in non-IIM patients was also confirmed in our control groups. In these controls, we mostly observed anti-TIF1γ and anti-SAE (reactivity in only one lineblot, mostly with low signal intensity), with several anti-TIF1γ within our SSc cohort (6%). The latter observation was also recently described by others, with up to 5% in their cohort using the same assay (8). Considering the strong relation between anti-TIF1γ positive DM and malignancy, the importance of the detection of this MSA by lineblot in a non-IIM population needs further clarification to prevent unnecessary investigations and anxiety (31). Moreover, larger studies with more positive samples for each MSA will be interesting to see whether adapting cut-off values for these two antibodies will increase specificity without losing sensitivity.

In total, combined MSA sensitivity was 55%, with a lower sensitivity for the individual lineblots. This observation is compatible with other studies evaluating lineblot sensitivity (47-63%) in established IIM patients (7, 9). The highest MSA frequencies in patients were observed for anti-HMGCR (13%, n=4), anti-Mi-2 (13%, n=4) and anti-Jo1 (10%, n=3). These observations are in line with the frequencies described in literature, although the frequency of anti-HMGCR and anti-Jo1 in our cohort compared to others seems to be slightly higher (5%) and lower (16–24%), respectively (7-9, 27, 32, 33). In contrast to Cavazzana and colleagues, we did not observe multiple MSA reactivities (7).

Remarkably, nearly half of the MSA in IIM patients (47%) were only observed in one lineblot system. Even though both lineblots differ in antigen composition (*e.g.* anti-OJ and anti-HMGCR only being present on one lineblot), this element can only partially explain these discrepancies (after correction, 23% remains single positive). Vulsteke and colleagues also demonstrated differences in performance characteristics of three lineblot methods in a cohort of well-defined IIM cases as well as controls (8). Our data showed no to fair agreement between the two lineblot for the individual antibodies, with not only discrepancies concerning the novel autoantibodies, but also concerning well-established autoantibodies. Most discrepancies were observed for anti-TIF1γ (mostly low signal intensity [n=6/7], but in both assays), anti-SRP (low to moderate signal intensity in one assay) and anti-SAE (mostly low signal intensities [n=2/3] in one assay) positivity in the non-IIM samples. Nevertheless, differences were also observed in IIM-patients (including high signal intensity samples). Awareness on the performance characteristics of these lineblot assays is of particular importance in the context of the increasing use of these assays in daily clinical practice in patients with low to moderate clinical suspicion of IIM and its potential inclusion in future diagnostic and classification criteria. We agree with others that further work is needed to harmonise and optimise these assays (8, 28, 29).

Very recently, Lecouffe-Desprets and colleagues described lower MSA titres in non-IIM patients, compared to IIM patients (17). In addition, Infantino and colleagues observed, in a study focusing on anti-synthetase antibodies (ARS), that the cytoplasmic speckled pattern was associated with higher blot positivity and that blot signal intensities were higher in samples with a concordant cytoplasmic staining (34). Our results are in line with these observations, with significantly more low signal intensities (<20 AU) in non-IIM *vs.* IIM patients (77% *vs.* 12%, p=0.0013). Furthermore, we correlated the lineblot results with the theoretically expected ANA IIF pattern (see Suppl. Table S1) and the observed pattern. We found 38% incompatible MSA IIF patterns in non-IIM patients (n=5/13) *vs.* 12% (n=2/17) in IIM patients (35, 36). Therefore, we suggest that, despite the limitations of ANA IIF for MSA screening, IIF should be preserved in the diagnostic work-up of IIM as it may guide the identification of false-positive results.

In addition to the diagnostic potential of MSA, these autoantibodies play an increasing role in the subclassification of IIM. There is growing evidence that each MSA, or group of MSA, is predictive of a specific phenotype of IIM. For example, the ARS, with anti-Jo1 as most prominent antibody, are linked to the ASS, characterised by polyarthritis, interstitial lung disease and muscle involvement, often together with accompanying findings such as cutaneous lesions (mechanic hands), Raynaud's phenomenon and fever (37, 38). Other correlations are, among others, anti-Mi2 antibodies with classical DM with favourable prognosis, anti-TIF1γ with CAM, anti-MDA5 with clinically aDM and rapidly progressive ILD and anti-SRP and anti-HMGCR with IMNM (10, 36). In our study, clinical symptoms and phenotype corresponded with those expected based on the MSA positivity. However, the patient positive for anti-EJ, did not fulfil the classical triad of ASS. A potential explanation is that this may be related to the heterogeneity in clinical phenotype observed at time of diagnosis in ASS patients, not excluding that additional characteristic clinical features may appear during the follow-up (37-40).

Recently, in a joint effort between the European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR), new classification criteria for adult and juvenile IIM and their major subgroups were proposed (4). Despite the fact that our data were collected before the publication of the new EULAR/ACR classification

criteria, clinical judgment in our MSA+ IIM group, correlates well with the calculated EULAR/ACR probability score and we therefore believe that these new EULAR/ACR criteria could be a helpful tool in daily clinical practice. On the other hand, in the MSA- IIM group, clinical judgment correlates less with the EULAR/ACR probability score. Moreover, we documented a statistically higher probability score in MSA+ IIM patients, compared to MSA- patients, possibly reflecting different phenotypes, less covered by these criteria, or improper diagnosis of patients with myopathies of non-inflammatory origin within the MSA- IIM group.

Our study has some limitations. First of all, as we investigated a consecutive routine cohort reflecting the real life routine situation, the total number of IIM patients is limited, resulting in low numbers of patients in the different MSA/IIM subtypes. In addition, this real life context implies that clinical diagnosis was made by different physicians not based on any standardised diagnostic protocol (*e.g.* in some cases of clear DM no muscle biopsy was performed). Moreover, the physicians were not blinded for the MSA results of one lineblot. However, given the high EULAR/ACR probability scores in the MSA+ IIM group, versus very low scores in the MSA+ IIM excluded group (data not shown), we believe that the treating physicians were mostly guided by the clinical presentation in combination with other technical investigations, rather than the MSA results. Thirdly, this was a cross-sectional study, without longitudinal evaluation. We found MSA in patients with IMID or myopathic features, in which IIM could not be diagnosed, nor excluded at the time of retrospective review of the clinical data. Longitudinal follow-up studies are needed to clarify if this concerns false positive results, or alternatively, an early disease phase, preceding the onset of IIM.

Conclusion

Lineblot seems to be an interesting tool in the diagnostic work-up of patients with a clinical suspicion of IIM, allowing MSA detection within the formerly 'seronegative' IIM subgroup and the identification of clinical subtypes. However, when applied in a context of low to moderate clinical suspicion, considerable caution must be exercised in interpreting results with low positive MSA signal intensities, discordant lineblot results or/and an incompatible IIF pattern.

Constructive interaction between laboratory experts and clinicians may contribute in overcoming these difficulties. Further studies, preferably using multicentre longitudinal cohorts to obtain larger groups within each MSA subset, are needed to clarify the meaning of (low) MSA positivity obtained in the different commercial immunoblot tests as well as in a non-IIM population.

Acknowledgements

The technical assistance of Bada Tandir, Vicky Mortier and the other members of the technical staff is greatly acknowledged. We thank all colleagues involved in the data collection.

References

1. BOHAN A, PETER JB: Polymyositis and dermatomyositis (first of two parts). *N Engl J Med* 1975; 292: 344-7.
2. MARIAMPILLAI K, GRANGER B, AMELIN D *et al*.: Development of a new classification system for idiopathic inflammatory myopathies based on clinical manifestations and myositis-specific autoantibodies. *JAMA Neurol* 2018; 75: 1528-37.
3. ALLENBACH Y, MAMMEN AL, BENVENISTE O, STENZEL W; IMMUNE-MEDIATED NECROTIZING MYOPATHIES WORKING GROUP: 224th ENMC International Workshop: Clinico-sero-pathological classification of immune-mediated necrotizing myopathies, Zandvoort, the Netherlands, 14-16 October 2016. *Neuromuscul Disord* 2018; 28: 87-99.
4. LUNDBERG IE, TJARNLUND A, BOTTAI M *et al*.: 2017 European League Against Rheumatism/American College of Rheumatology classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups. *Ann Rheum Dis* 2017; 76: 1955-64.
5. NIRMALANANTHAN N, HOLTON JL, HANNA MG: Is it really myositis? A consideration of the differential diagnosis. *Curr Opin Rheumatol* 2004; 16: 684-91.
6. DALAKAS MC, HOHLFELD R: Polymyositis and dermatomyositis. *Lancet* 2003; 362: 971-82.
7. CAVAZZANA I, FREDI M, CERIBELLI A *et al*.: Testing for myositis specific autoantibodies: Comparison between line blot and immunoprecipitation assays in 57 myositis sera. *J Immunol Methods* 2016; 433: 1-5.
8. VULSTEKE JB, DE LANGHE E, CLAEYS KG *et al*.: Detection of myositis-specific antibodies. *Ann Rheum Dis* 2019; 78: e7.
9. TAMPOIA M, NOTARNICOLA A, ABBRACCIAVENTO L *et al*.: A new immunodot assay for multiplex detection of autoantibodies in a cohort of Italian patients with idiopathic inflammatory myopathies. *J Clin Lab Anal* 2016; 30: 859-866.
10. BETTERIDGE Z, MCHUGH N: Myositis-specific autoantibodies: an important tool to support diagnosis of myositis. *J Intern Med* 2016; 280: 8-23.
11. GHIRARDELLO A, BORELLA E, BEGGIO M, FRANCESCHINI F, FREDI M, DORIA A: Myositis autoantibodies and clinical phenotypes. *Auto Immun Highlights* 2014; 5: 69-75.
12. MARASCO E, CIOFFI E, COMETI L *et al*.: One year in review 2018: idiopathic inflammatory myopathies. *Clin Exp Rheumatol* 2018; 36: 937-47.
13. BETTERIDGE ZE, GUNAWARDENA H, MCHUGH NJ: Novel autoantibodies and clinical phenotypes in adult and juvenile myositis. *Arthritis Res Ther* 2011; 13: 209.
14. LUNDBERG IE, TJARNLUND A: Response to: 2017 EULAR/ACR classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups: Little emphasis on autoantibodies, why? by Malaviya. *Ann Rheum Dis* 2017; 77: e78.
15. MALAVIYA AN: 2017 EULAR/ACR classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups: little emphasis on autoantibodies, why? *Ann Rheum Dis* 2018; 77: e77.
16. TAN TC, WIENHOLT L, ADELSTEIN S: Test performance of a myositis panel in a clinical immunology laboratory in New South Wales, Australia. *Int J Rheum Dis* 2016; 19: 996-1001.
17. LECOUFFE-DESPRETS M, HEMONT C, NEEL A *et al*.: Clinical contribution of myositis-related antibodies detected by immunoblot to idiopathic inflammatory myositis: a one-year retrospective study. *Autoimmunity* 2018; 51: 89-95.
18. RONNELID J, BARBASSO HELMERS S, STORFORS H *et al*.: Use of a commercial line blot assay as a screening test for autoantibodies in inflammatory myopathies. *Autoimmun Rev* 2009; 9: 58-61.
19. CHAN EK, DAMOISEAUX J, CARBALLO OG *et al*.: Report of the first international consensus on standardized nomenclature of antinuclear antibody HEp-2 cell patterns 2014-2015. *Front Immunol* 2015; 6: 412.
20. MUSSET L, MIYARA M, BENVENISTE O *et al*.: Analysis of autoantibodies to 3-hydroxy-3-methylglutaryl-coenzyme a reductase using different technologies. *J Immunol Res* 2014; 2014: 405956.
21. MCHUGH M: Interrater reliability: the kappa statistic. *Biochemia Medica* 2012; 22: 276-82.
22. TROYANOV Y, TARGOFF IN, TREMBLAY JL, GOULET JR, RAYMOND Y, SENECAL JL: Novel classification of idiopathic inflammatory myopathies based on overlap syndrome features and autoantibodies: analysis of 100 French Canadian patients. *Medicine* 2005; 84: 231-49.

23. TARGOFF IN, MILLER FW, MEDSGER TA JR, ODDIS CV: Classification criteria for the idiopathic inflammatory myopathies. *Curr Opin Rheumatol* 1997; 9: 527-35.
24. LOVE LA, LEFF RL, FRASER DD *et al*.: A new approach to the classification of idiopathic inflammatory myopathy: myositis-specific autoantibodies define useful homogeneous patient groups. *Medicine* 1991; 70: 360-74.
25. HOOGENDIJK JE, AMATO AA, LECKY BR *et al*.: 119th ENMC International Workshop: Trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis, 10-12 October 2003, Naarden, the Netherlands. *Neuromuscul Disord* 2004; 14: 337-45.
26. LECLAIR V, LUNDBERG IE: New myositis classification criteria-what we have learned since bohan and peter. *Curr Rheumatol Rep* 2018; 20: 18.
27. GHIRARDELLO A, RAMPUDDA M, EKHOLM L *et al*.: Diagnostic performance and validation of autoantibody testing in myositis by a commercial line blot assay. *Rheumatology* 2010; 49: 2370-4.
28. TJARNLUND A, LUNDBERG IE, RONNELID J: Response to: Detection of myositis-specific antibodies: additional notes by Infantino *et al*. *Ann Rheum Dis* 2019; 78: e30.
29. INFANTINO M, MANFREDI M, GROSSI V, BENUCCI M: Detection of myositis-specific antibodies: additional notes. *Ann Rheum Dis* 2019; 78: e29.
30. O'CONNOR A, MULHALL J, HARNEY SMJ *et al*.: idiopathic inflammatory myopathy; initial cross speciality experience with use of the extended myositis antibody panel. *Clin Pract* 2017; 7: 922.
31. TRALLERO-ARAGUAS E, RODRIGO-PENDAS JA, SELVA-O'CALLAGHAN A *et al*.: Usefulness of anti-p155 autoantibody for diagnosing cancer-associated dermatomyositis: A systematic review and meta-analysis. *Arthritis Rheum* 2012; 64: 523-32.
32. KLEIN M, MANN H, PLESTILOVA L *et al*.: Increasing incidence of immune-mediated necrotizing myopathy: single-centre experience. *Rheumatology* 2015; 54: 2010-4.
33. GHIRARDELLO A, BASSI N, PALMA L *et al*.: Autoantibodies in polymyositis and dermatomyositis. *Curr Rheumatol Rep* 2013; 15: 335.
34. INFANTINO M, PALTERER B, BIAGIOTTI R *et al*.: Reflex testing of speckled cytoplasmic patterns observed in routine ANA HEp-2 indirect immunofluorescence with a multiplex anti-synthetase dot-blot assay: A multicentric pilot study. *Immunol Res* 2018; 66: 74-8.
35. PICARD C, VINCENT T, LEGA JC *et al*.: Heterogeneous clinical spectrum of anti-srp myositis and importance of the methods of detection of anti-srp autoantibodies: A multicentric study. *Immunol Res* 2016; 64: 677-86.
36. SATOH M, TANAKA S, CERIBELLI A, CALISE SJ, CHAN EK: A comprehensive overview on myositis-specific antibodies: New and old biomarkers in idiopathic inflammatory myopathy. *Clin Rev Allergy Immunol* 2017; 52: 1-19.
37. CAVAGNA L, NUNO L, SCIRE CA *et al*.: Clinical spectrum time course in anti Jo-1 positive antisynthetase syndrome: Results from an international retrospective multicenter study. *Medicine* 2015; 94: e1144.
38. IMBERT-MASSEAU A, HAMIDOU M, AGARD C, GROLLEAU JY, CHERIN P: Antisynthetase syndrome. *Joint Bone Spine* 2003; 70: 161-8.
39. GIANNINI M, NOTARNICOLA A, DASTMALCHI M, LUNDBERG IE, LOPALCO G, IANNONE F: Heterogeneous clinical spectrum of interstitial lung disease in patients with anti-EJ anti-synthetase syndrome: A case series. *Clin Rheumatol* 2016; 35: 2363-7.
40. GONZALEZ-GAY MA, MONTECUCCO C, SELVA-O'CALLAGHAN A *et al*.: Timing of onset affects arthritis presentation pattern in antisyntethase syndrome. *Clin Exp Rheumatol* 2018; 36: 44-9.