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Autoantigenic properties of the aminoacyl tRNA synthetase family in idiopathic inflammatory myopathies

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ABSTRACT

Objectives: Autoantibodies are thought to play a key role in the pathogenesis of idiopathic inflammatory myopathies (IIM). However, up to 40% of IIM patients, even those with clinical manifestations of anti-synthetase syndrome (ASSD), test seronegative to known myositis-specific autoantibodies. We hypothesized the existence of new potential autoantigens among human cytoplasmic aminoacyl tRNA synthetases (aaRS) in patients with IIM.

Methods: Plasma samples from 217 patients with IIM according to 2017 EULAR/ACR criteria, including 50 patients with ASSD, 165 without, and two with unknown ASSD status were identified retrospectively, as well as age and gender-matched sera from 156 population controls, and 219 disease controls. Patients with previously documented ASSD had to test positive for at least one of the five most common anti-aaRS autoantibodies (anti-Jo1, -PL7, -PL12, -EJ, and -OJ) and present with one or more of the following clinical manifestations: interstitial lung disease, myositis, arthritis, Raynaud's phenomenon, fever, or mechanic's hands. Demographics, laboratory, and clinical data of the IIM cohort (ASSD and non-ASSD) were compared. Samples were screened using a multiplex bead array assay for presence of autoantibodies against a panel of 117 recombinant protein variants, representing 33 myositis-related proteins, including all nineteen cytoplasmic aaRS. Prospectively collected clinical data for the IIM cohort were retrieved and compared between groups within the IIM cohort and correlated with the results of the autoantibody screening. Principal component analysis was used to analyze clinical manifestations between ASSD, non-ASSD groups, and individuals with novel anti-aaRS autoantibodies.

Results: We identified reactivity towards 16 aaRS in 72 of the 217 IIM patients. Twelve patients displayed reactivity against nine novel aaRS. The novel autoantibody specificities were detected in four previously seronegative patients for myositis-specific autoantibodies and eight with previously detected myositis-specific autoantibodies. IIM individuals with novel anti-aaRS autoantibodies (n = 12) all had signs of myositis, and they had either muscle weakness and/or muscle enzyme elevation, 2/12 had mechanic's hands, 3/12 had interstitial lung disease, and 2/12 had arthritis. The individuals with novel anti-aaRS and a pathological muscle biopsy all presented widespread up-regulation of major histocompatibility complex class I. The reactivities against novel aaRS could be confirmed in ELISA and western blot. Using the multiplex bead array assay, we could confirm previously known reactivities to four of the most common aaRS (Jo1, PL12, PL7, and EJ (n = 45)) and identified

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patients positive for anti-Zo, -KS, and -HA (n = 10) that were not previously tested. A low frequency of anti-aaRS autoantibodies was also detected in controls.
Conclusion: Our results suggest that most, if not all, cytoplasmic aaRS may become autoantigenic. Autoantibodies against new aaRS may be found in plasma of patients previously classified as seronegative with potential high clinical relevance.

1. Introduction

Idiopathic inflammatory myopathies (IIM) are characterized by a broad spectrum of clinical manifestations with high mortality and morbidity [1,2]. Autoantibodies have been identified in more than 50% of patients with IIM, and autoimmunity is thought to play a key role in the pathogenesis of the disease. One sub-group of IIM, named anti-synthetase syndrome (ASSD), is characterized by the presence of autoantibodies targeting aminoacyl transfer(t) RNA synthetases (aaRS), together with specific clinical manifestations such as myositis, interstitial lung disease (ILD), arthritis, mechanic's hand, Raynaud's phenomenon, and fever [3,4].

There are nineteen cytoplasmic aaRS in human cells, one for each amino acid. The bifunctional EPRS (Glu-ProRS) counts as one aaRS but can catalyze the ligation of two amino acids (Glu and Pro) [5,6]. The most common anti-aaRS autoantibody (anti-Jo1), targeting histidyl tRNA synthetase (HisRS), is present in up to 20–30% of IIM patients [3], and up to 90% of patients with IIM and ILD [7,8]. Besides HisRS, there are seven other identified autoantigens within the aaRS family in IIM/ASSD [9–12]. Of these, only five are included in the most commonly used commercial assays; anti-Jo1, -PL7, -PL12, -EJ, and -OJ (anti-HisRS, -ThrRS, -AlaRS, -GlyRS, and -IleRS, respectively) [9], indicating a possible underrepresentation of the number of positive patients with anti-aaRS autoantibodies. In addition, there is a potential presence of non-identified anti-aaRS autoantibodies targeting one of the other cytoplasmic aaRS proteins.

A few studies have mentioned additional autoantigens within the human aaRS family, including LysRS (SC), TrpRS (WARS), GlnRS (JS), and SerRS [13–16]. Currently, there is limited data available on the detection of these additional aaRS autoantigens. Moreover, anti-OJ autoantibodies targeting IleRS, one of the members of the intracellular multi-synthetase complex (MSC), have been suggested to potentially target several members of this complex [17,18], which consists of eight aaRS and three scaffold proteins; aaRS complex interaction proteins (AIMP) 1, −2 and −3 [19].

In this study, we tested the hypothesis that the entire human cytoplasmic aaRS family displays autoantigenic properties, by analyzing an IIM cohort and compare with population and disease controls. In addition, we explored the correlations between clinical manifestations and anti-aaRS autoantibody status within patients with ASSD and IIM.

2. Materials and methods

2.1. IIM patients

Plasma samples from 217 consecutive patients with IIM attending Karolinska University Hospital between 1995 and 2014 were retrospectively identified in the local biobank for this cross-sectional study. Information on laboratory and clinical data was retrieved by revising the medical charts, the Swedish Rheumatology Quality Register for IIM (Swemyonet) [20] and the EuroMyositis register [21]. Classification of IIM was according to the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) criteria (probability threshold of 55%) [22]. The 2017 European Neuromuscular Centre (ENMC) criteria were applied to classify immune-mediated necrotizing myopathies (IMNM) [23]. Patients with IIM were further sub-grouped into ASSD or non-ASSD based on Connors criteria [24]. The inclusion into the ASSD group was based on the presence of at least one positive test for any of five anti-aaRS antibodies (anti-Jo1, -PL7, -PL12, -EJ, and -OJ) ever tested by line blot (Euroimmun), immunoprecipitation or ELISA, together with one or more of the following clinical manifestations: ILD, myositis, arthritis, Raynaud's phenomenon, fever, or mechanic's hands. All 217 individuals (both with and without ASSD) were included in the analysis. Muscle involvement was based on the presence of at least one of the following features: myopathic weakness (manual muscle test-8 (MMT-8) below 80 and/or impaired muscle endurance by myositis functional index-2 [25]), muscle enzymes elevation (creatine kinase (CK), lactate dehydrogenase (LD), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT)), myopathic electromyography (EMG), and pathological muscle biopsy consistent with myositis. One of the following skin manifestations had to be present to define the skin involvement: periungual erythema, mechanic's hand, Gottron's sign, Gottron's papules, V-sign, shawl sign, erythroderma, periorbital edema, heliotrope rash, calcinosis. Diagnosis of ILD was based on the American Thoracic Society criteria [26]. High-resolution computed tomography (HRCT) and spirometry data were checked for consistent features of ILD. Cardiac involvement was considered if any of the following events occurred during the disease course: pericarditis, myocarditis, arrhythmia, sinus tachycardia. Cancer diagnosis was assigned to patients if ever confirmed during the follow-up (interval between time of diagnosis and last visit at the Rheumatology Clinic). Smoking status was defined as never/ever smoker. Ethnicity was determined at the first visit by the patient self-reporting, and then each patient's ethnicity has been classified by the responsible physician according to a fixed set of categories. Immunosuppressive treatment was recorded at the time of the plasma sampling. Human leukocyte antigen (HLA)-DRB1 genotyping data was retrieved as previously described [27] for selected patients. Myositis-specific autoantibodies (MSAs (anti-Jo1, -PL7, -PL12, -OJ, -EJ, -Mi-2, -SRP, -TIF1γ and -MDA5)) and/or myositis-associated autoantibodies (MAAs (anti-U1RNP, -Pm-Scl, -Ku, -SSA, -SSB)) were analyzed using any of the following assays: immunoprecipitation, Line Blot (Euroimmun), or ELISA. With regard to anti-SSA antibodies, information on reactivity against Ro52 (TRIM21) and Ro60 was not available for all patients and therefore was not reported. Autoantibody positivity and clinical manifestations (myositis, skin pathology, arthritis, Raynaud's phenomenon, dysphagia, ILD, cardiac involvement (any of myocarditis, pericarditis or arrythmia), cancer diagnosis) were assigned to patients if ever confirmed during the follow-up (interval time occurring between the time of diagnosis and the time of the last visit at the Rheumatology Clinic). Other autoimmune diseases included: rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis, morphea, Sjögren's syndrome, and mixed connective tissue disease.

2.2. Control cohorts

Sera from 156 population controls were retrospectively identified from a local biobank. The population controls were individuals not affected by rheumatoid arthritis or IIM. To control for sample differences between sera and plasma, we compared available sera and plasma from 151 patients with IIM (Fig. 1). Sera from 219 individuals with Sjögren's syndrome, RA, or SLE (n = 99, n = 50, and n = 70, respectively) retrospectively identified from a local biobank were included as disease controls. Patients with SLE fulfilled four of the American College of Rheumatology (ACR) 1982 revised classification criteria for SLE [28]. Patients with Sjögren's syndrome were classified according to the

American-European consensus group (AECG) [29] and 2016 EULAR/-ACR classification criteria for primary Sjögren's syndrome [30]. Patients with RA fulfilled EULAR/ACR 1987 [31] or 2010 criteria [32]. The controls were age and sex-matched with the 217 IIM patients on group level (Supplementary Table 1).

2.3. Ethics

This study was approved by the Ethics Committee at Karolinska Institutet, Sweden, protocol numbers (Dnr) 2005/792–31/4, 2011-1374-32, 2015/1052–31, 2018/1198–32, 2010/935–31/1, 98–367, and 03–556). All patients gave written informed consent.

2.4. Recombinant proteins

Two sets of proteins were used. The first set consisted of 74 protein epitope signature tags (PrESTs) with a size between 25 and 150 amino acids long (median of 100 amino acids) and were generated within the Human Protein Atlas (www.proteinatlas.org). The PrESTs are produced in *Escherichia coli* (*E. coli*), have an affinity tag consisting of a hexahistidine tag and an albumin binding protein domain from streptococcal protein G (His_6ABP), are purified with immobilized metal ion affinity chromatography (IMAC) and quality assured with by SDS-PAGE and MS (MALDI or ESI as previously described [33]. All PrESTs represent a protein sequence with low homology to other human proteins [34]. The second set consisted of 43 proteins produced in *E. coli* with an Avi-tag for site-specific biotinylation as previously described [35]. This set of proteins was purified in a two-step procedure, and quality was assured using SDS-PAGE as previously described [35]. The amino acid coverage was based on solved crystal structures. Different versions of the same protein were included, either as full-length versions, truncated versions, or both. All 117 recombinant proteins are listed in Supplementary Data, including the amino acid coverage. The selection of antigens used in this study was based on covering the complete human cytoplasmic aaRS protein family, in combination with other known and available myositis-specific autoantigens.

2.5. Multiplex bead array assay

Neutravidin or PrESTs were amine coupled onto color-coded magnetic beads (Magplex Luminex Corp.) as previously described [36,37]. The next day, biotinylated proteins were added to the neutravidin-coupled beads and incubated for 1 h at room temperature. Each of the 117 proteins included was added to one specific bead ID. In addition to the biotinylated proteins or PrESTs, internal controls were included and immobilized to beads. As positive controls, a rabbit anti-human IgG antibody (Jackson ImmunoResearch) and Epstein-Barr virus nuclear antigen 1 (Abcam) were used, and as negative controls, the antigen tags were used; one bead ID with His_6ABP (PrEST) and one ID with biotin added to immobilized neutravidin (biotinylated proteins). The following day all beads were pooled, and the volume was adjusted to enable the addition of 500 beads per ID to each sample well in a 384 well plate.

Plasma or serum was diluted (1:250) in assay buffer (phosphate buffered saline (PBS), 0.05% (v/v) Tween-20, 3% (w/v) bovine serum albumin (BSA), 0.01 mg/ml neutravidin and 0.16 mg/ml hexahistidine and albumin binding protein tag (His_6ABP)) and incubated for 1 h, to block potential antibodies binding the tag of the recombinant proteins. Beads and diluted plasma or serum were added to each well, and the plate was incubated for 2 h before washing three times with PBS-T (0.05% (v/v) Tween-20). Captured antibodies were fixated to the beads in 0.2% paraformaldehyde [37] for 10 min before washing three times with PBS-T. Secondary R-Phycoerythrin conjugated Goat F (ab')2 Fragment anti-Human IgG (γ) (H10104, Invitrogen) was added, the plate was incubated for 30 min before washing three times with PBS-T and final addition of PBS-T to each well. The samples were analyzed on FLEXMAP3D (Luminex Corp.), using xPONENT software (Luminex Corp.), recording median fluorescence intensity (MFI).

2.6. ELISA

An ELISA was developed to validate the new anti-aaRS findings. Streptavidin-coated 384 well plates were incubated overnight at 4 °C. The next day, plates were washed four times in PBS-T (0.05% (v/v) Tween-20) and blocked for 2 h at room temperature with blocking buffer (PBS with 0.05% (v/v) Tween-20 and 5% (w/v) BSA). Biotinylated recombinant protein was added at 1 μg/ml diluted in PBT (PBS with 0.05% (v/v) Tween-20 and 0.5% (w/v) BSA), before incubation for 1 h. Plates were washed four times with PBS-T and plasma diluted (in a five-fold dilution series from 25× to 16500× dilution) and incubated for 1 h in dilution buffer (PBS with 0.05% (v/v) Tween-20 and 1% (w/v) BSA) were added in duplicates and let to incubate for 1.5 h. Subsequently, plates were washed four times in PBS-T and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG antibody (Dako #P0214), diluted 1:4000, in PBT for 1 h at RT. After washing four times, TMB substrate (Thermo Scientific) was added to the plates, and the reaction was stopped after 3 min by addition of 1 M H_2SO_4. The optical density (OD) was read at 450 nm with 620 nm background removal

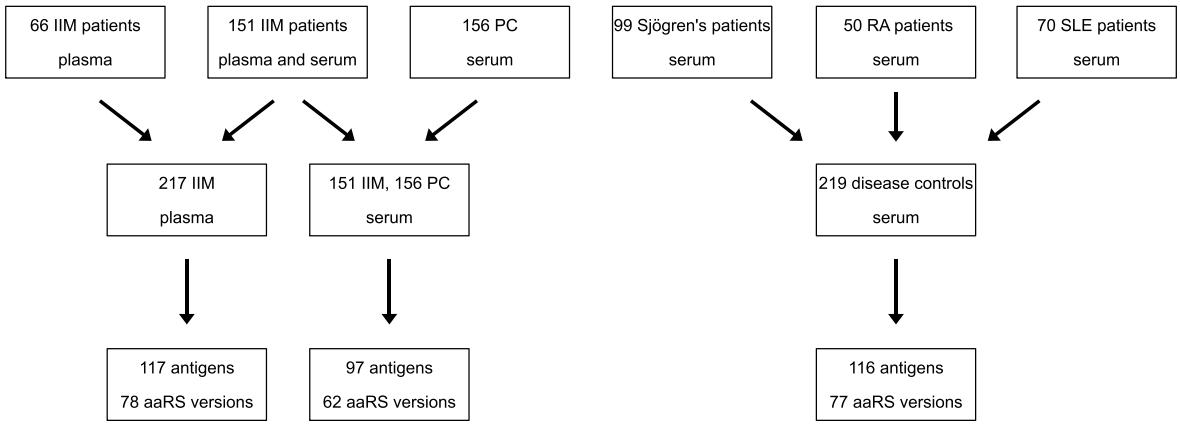


Fig. 1. Experimental design of the study. 217 IIM plasma samples were analyzed using the multiplex bead array assay against 117 antigens. For 151 of the 217 IIM patients, serum was available and analyzed together with serum from 156 PC using the multiplex bead array assay against 97 antigens. As disease controls, sera from 50 patients with RA, 99 with Sjögren's syndrome, and 70 with SLE were included and analyzed using the multiplex bead array assay against 116 antigens. aaRS, aminoacyl tRNA synthetase; IIM, idiopathic inflammatory myopathies, PC, population control; RA, rheumatoid arthritis; Sjögren's, Sjögren's syndrome; SLE, systemic lupus erythematosus.

using a microplate reader (SpectraMaxPlus384) and SoftMax Pro 6.5 software (Molecular Devices). All samples were tested against MDA5 as a reference antigen, and streptavidin (SA) as background. In addition, sample from a patient with anti-MDA5 autoantibodies was used as control plasma for background signals of the specific autoantigen.

2.7. Western blot

Western blot was performed as previously described [38]. Briefly, 1 μg of each aaRS was run under denatured conditions in a SDS-PAGE for 1 h and 20 min at 100 V. Thereafter, proteins were transferred onto a nitrocellulose membrane at 25 V for 7 min using iBlot dry blotting system (Invitrogen). The membranes were blocked for 1 h in blocking

Table 1
Demographic data of the 217 patients with IIM included in the study, 50 with ASSD, 165 without ASSD, and two with unknown ASSD status.

	IIM (n = 217)	ASSD (n = 50)	Non-ASSD (n = 165)	p-value*
Age at diagnosis, mean years (SD)	56.6 (15.2)	50.6 (15.6)	58.3 (14.6)	**0.001**
Sex, n (%) women	137 (63.1)	34 (68.0)	101 (61.2)	NS
IIM subgroup, n (%)				**0.0001**
No myositis, n (%)	1 (0.5)	1 (2.0)	0 (0.0)	
PM, n (%)	99 (45.6)	37 (74.0)	62 (37.6)	
DM, n (%)	75 (34.6)	9 (18.0)	64 (38.8)	
ADM, n (%)	5 (2.3)	1 (2.0)	4 (2.4)	
sIBM, n (%)	31 (14.3)	0 (0.0)	31 (18.8)	
IMNM**, n (%)	4 (1.8)	0 (0.0)	4 (2.4)	
Ethnicity, n (%) White	206 (94.9)	49 (98.0)	155 (93.9)	NS
Disease duration, median years (IQR)	0 (3)	0 (3)	0 (3)	NS
Follow-up duration, mean years (SD)	11.2 (7.8)	12.4 (8.6)	11 (7.5)	NS
Dead during follow-up, n (%)	92 (42.4)	17 (34.0)	73 (44.2)	NS
Age at death, mean (SD)	75 (10.9)	73.1 (12.5)	75.7 (10.6)	NS
Autoantibodies				
anti-Jo1, n (%)	45 (20.7)	45 (90)	0 (0.0)	**0.0001**
anti-PL7, n (%)	2 (0.9)	2 (4.0)	0 (0.0)	0.055
anti-PL12, n (%)	1 (0.5)	1 (2.0)	0 (0.0)	NS
anti-EJ, n (%)	1 (0.5)	1 (2.0)	0 (0.0)	NS
anti-OJ, n (%)	1 (0.5)	1 (2.0)	0 (0.0)	NS
anti-Mi-2, n (%)	5 (2.3)	0 (0.0)	5 (3.1)	NS
anti-SRP, n (%)	7 (3.2)	0 (0.0)	7 (4.3)	NS
anti-MDA5 n (%)	14 (6.5)	0 (0.0)	14 (8.6)	**0.02**
anti-TIF1γ, n (%)	23 (10.6)	0 (0.0)	22 (13.6)	**0.003**
anti-SSA*, n (%)**	63 (29)	25 (50.0)	38 (23.2)	**0.0001**
anti-SSB, n (%)	9 (4.1)	1 (2.0)	8 (4.9)	NS
anti-U1RNP, n (%)	19 (8.8)	6 (12.0)	13 (7.9)	NS
anti-Ku, n (%)	2 (0.9)	0 (0.0)	2 (1.2)	NS
anti-Pm-Scl*, n (%)**	20 (9.2)	3 (6.5)	17 (10.5)	NS
Seronegative (no MSAs), n (%)	115 (53)	0 (0.0)	114 (69.1)	**0.0001**
Clinical manifestations				
Other autoimmune disease, n (%)	45 (20.7)	10 (20.0)	35 (21.0)	NS
Cancer, n (%)	59 (27.2)	9 (18.0)	48 (29.1)	NS
Muscle involvement, n (%)	216 (99.5)	49 (98.0)	165 (100)	NS
Myopathic weakness, n (%)	201 (92.6)	44 (88.0)	155 (95.7)	NS
Muscle enzyme elevation, n (%)	198 (91.2)	45 (91.8)	151 (94.4)	NS
Myopathic EMG, n (%)	137 (63.1)	29 (72.5)	106 (74.6)	NS
Pathological muscle biopsy, n (%)	169 (77.9)	38 (80.9)	129 (82.7)	NS
Skin involvement, n (%)	106 (48.8)	24 (48.0)	80 (48.5)	NS
Raynaud's phenomenon, n (%)	56 (25.8)	21 (42.0)	35 (21.2)	**0.04**
Arthritis, n (%)	56 (25.8)	29 (58.0)	27 (17.0)	**0.0001**
ILD, n (%)	69 (31.8)	39 (79.6)	30 (18.9)	**0.0001**
Cardiac involvement, n (%)	19 (8.8)	10 (21.3)	9 (6.0)	**0.004**
Dysphagia, n (%)	108 (49.8)	13 (26.5)	93 (57.4)	**0.0001**
Smoking, n (%)	110 (50.7)	24 (57.1)	85 (64.4)	NS
Treatment at time of sample, n (%)	99 (45.6)	25 (51)	74 (45.4)	NS

IIM, idiopathic inflammatory myopathies; ASSD, anti-synthetase syndrome; PM, polymyositis; DM, dermatomyositis; ADM, amyopathic dermatomyositis; sIBM, sporadic inclusion body myositis; IMNM, immune-mediated necrotizing myopathy; Jo1, HisRS; PL7, ThrRS; PL12, AlaRS; EJ, GlyRS; OJ. IleRS; Mi-2, chromatin organization modifier helicase (CHD) 3 and 4; SRP, signal recognition particle; MDA5, interferon-induced helicase C domain-containing protein 1; TIF1γ, E3 ubiquitin-protein ligase TRIM33; SSA, Ro52 (tripartite motif containing 21 (TRIM21)) and Ro60 (TROVE domain family member 2); SSB, Sjogren syndrome antigen B; U1RNP, small nuclear ribonucleoprotein U1 subunit 70; Ku, X-ray repair cross complementing (XRCC) 6; Pm-Scl, polymyositis-scleroderma overlap syndrome-associated antigen 75 (exosome component 9) and 100 (exosome component 10); MSA, myositis-specific autoantibodies; EMG, electromyography; ILD, interstitial lung disease. Disease duration = interval between the time of diagnosis and the time of sampling; follow-up duration = interval between the time of diagnosis and the time of the last recorded visit at the Rheumatology Unit, Karolinska University Hospital, Sweden. Patients with unknown data were not included in the table nor the comparison for each information. * the reported p-value is for comparisons between the ASSD and the non-ASSD group of patients (information on ASSD status was not available for two patients, excluded from the two groups). ** all patients with IMNM tested positive for anti-HMGCR antibodies. *** with regard to anti-SSA antibodies, information on reactivity to the individual Ro52 (TRIM21) or Ro60 (TROVE2) was not available for all patients and therefore not reported. The same applied to Pm-Scl where the commercial test included both Pm-Scl 75 and Pm-Scl 100 but information regarding the separate antigens was not available.

buffer. After incubation, plasma diluted in dilution buffer was added to the membrane and incubated for 1 h. After washing with PBS-T (3 × 5 min), the membranes were incubated with HRP-conjugated rabbit anti-human IgG antibody (Dako #P0214) for 1 h. The membranes were washed 3 × 5 min with PBS-T, and TMB substrate (Thermo Scientific) was added. The substrate was removed, and membranes were washed in ultra-pure water after a visible signal appeared.

2.8. Statistical analysis

The bead array data were processed in R using RStudio. Based on the quality control analysis, the MFI signals were normalized by antigen in the analysis of population control and IIM sera, scaling the 25th percentile of each antigen to a common value. All samples were normalized per sample by transforming the median fluorescence intensity (MFI) values per sample into number of median absolute deviations (MADs) around the sample median for both sample types [37]. The multiplex bead array assay was run twice for plasma for reproducibility purposes. Samples that yielded a higher value than the cut-off in both runs, for any of the included versions of the specific protein, were assigned positive. Four different cut-offs were tested before selecting 100xMAD (Supplementary Table 2, Supplementary Figs. 1–2). The cut-off 100xMAD was also used for the control cohorts.

Statistical analyses were performed with Statistical Package for the Social Sciences (SPSS, version 22.0, IBM software, USA). Continuous variables with normal distribution were presented as means with standard deviations (SD), while variables that violated normality were presented as medians with interquartile range (IQR). Groups were compared using the independent sample *t*-test and Mann-Whitney U tests. Differences in distributions of categorical variables between groups were tested using the chi-square test and Fisher's exact test when appropriate. Agreement between the results obtained by different tests was calculated using Cohen's Kappa coefficient.

Principal component analysis (PCA) for binary data was performed in R using Rstudio (prcomp) to dimensionally reduce the binary data of clinical manifestations. Variables were centered but not scaled. If a patient was positive for the specific manifestation or phenotype, 1 was assigned and 0 was assigned if negative. 140/2449 (5.7%) of the data points were not available (NA). The PCA analysis was done in three different ways assigning NA to either; 0, 1 or randomly 0 or 1, to evaluate that the NA did not affect the analysis (data not shown), and randomly selected 0 or 1 was used. After analysis, the patients were grouped according to ASSD status.

3. Results

3.1. IIM cohort: comparison between ASSD and non-ASSD patients

Demographics, laboratory, and clinical data of the IIM cohort (95% Caucasian), comparing 50 patients with ASSD and 165 without ASSD (ASSD status not available for 2/217 patients), is presented in Table 1. Raynaud's phenomenon, arthritis, ILD, and cardiac disease were statistically more frequent in the ASSD group, while dysphagia was more prevalent among the non-ASSD patients (Table 1). Among myositis-specific autoantibodies (MSAs), anti-Jo1 reactivity was most frequent in the ASSD group, while anti-TIF1γ was most common in the non-ASSD group. 69% of patients without ASSD were seronegative for any MSAs.

3.2. Autoantibodies detected in the multiplex bead array assay - IIM cohort

In the IIM cohort, autoantibodies against all cytoplasmic aaRS proteins except three (IleRS (OJ), LeuRS, and AspRS) were detected (Fig. 2, Fig. 3, and Table 2). Autoantibodies against any of the aaRS were present in one-third (n = 72, 33%), and of these, seven patients were positive for two and one patient for three anti-aaRS antibodies (Supplementary Table 3). Nine patients from the non-ASSD group were positive for anti-Jo1, -PL7, -PL12, or -EJ (Supplementary Table 4). In addition, we detected reactivities to other MSA antigens (MDA5, Mi-2, and TIF1γ), myositis-associated autoantibody (MAA) antigens (SSA (Ro52 (TRIM21)), SSB, U1RNP, and Pm-Scl), and to AIMP-1 and AIMP-2, two of the MSC scaffold proteins (Figs. 2 and 3D, Supplementary Table 5).

Autoantibodies towards nine aaRS (LysRS, GlnRS, TrpRS, SerRS, EPRS, ArgRS, MetRS, ValRS, and CysRS), not previously associated with IIM/ASSD were detected in 12 patients (Fig. 3C, Table 2). Of these, four were in the seronegative group, *i.e.,* not presenting any other MSAs, while eight had previously tested positive for MSAs (anti-Jo1 (n = 3), -MDA5 (n = 2), -Mi2 in combination with -TIF1γ (n = 1) -TIF1γ (n = 1) and -SRP (n = 1), Table 3). Of these eight, we could confirm anti-Jo1 autoantibodies in two of three patients but not the other previously reported MSAs (Table 3). Reactivities to known aaRS autoantigens in ASSD, not previously tested in this cohort, were found in 10 individuals: AsnRS (KS, n = 2), PheRS (Zo, n = 5), and TyrRS (HA, n = 3) (Fig. 3B,

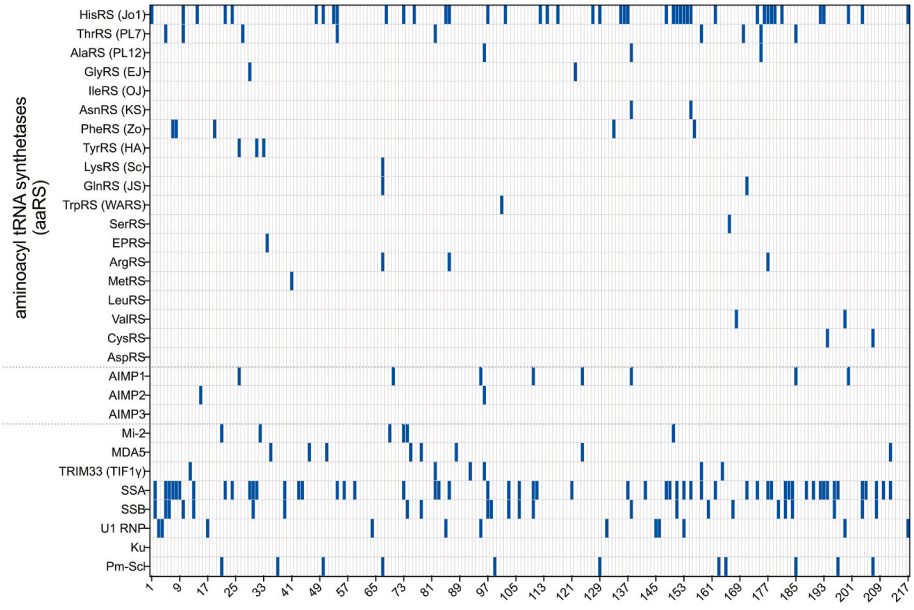


Fig. 2. Autoantibody reactivities for IIM patients. Reactivity against a panel of 30 antigens for 217 IIM patients as assessed by the multiplex bead array assay. Each column represents one patient, (patient 1–217), and each row represents one potential autoantigen. Reactivity was assigned positive (blue) if the criteria as defined in the method section were met for at least one of the included versions of a particular protein antigen. All cytoplasmic aaRS proteins are displayed above the dotted gray lines, the AIMPs are in between dotted lines, and below are the additional myositis-related proteins included in the study. With regard to anti-SSA reactivities, all 56 positive patients were reactive against Ro52 (TRIM21) and none against the included versions of Ro60.

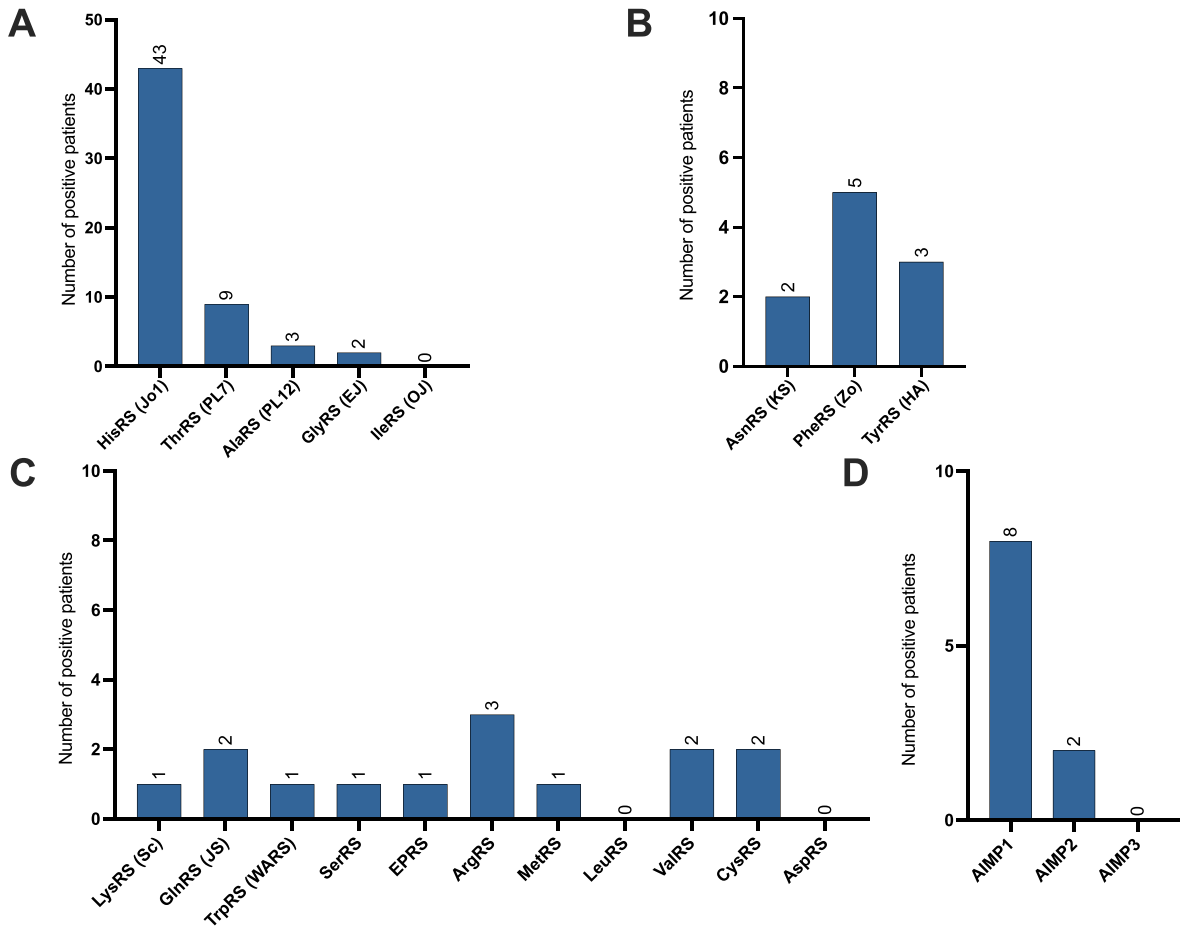


Fig. 3. IIM patients positive for autoantibodies against aaRS and AIMPs using the multiplex bead array assay. Patients with autoantibodies targeting; **(A)** the five aaRS autoantigens usually tested for in the clinic, **(B)** known ASSD-associated aaRS autoantigens usually not tested for in clinical settings, and **(C)** the remaining eleven human cytoplasmic aaRS not previously associated to IIM/ASSD as autoantigens**. (D)** Patients positive for autoantibodies targeting AIMP (1–3), the three scaffold proteins that are part of the multi-synthetase complex (MSC).

Table 2). In addition, we identified patients with multiple reactivities, both with known and potential novel anti-aaRS as well as other MSAs (Supplementary Table 3).

3.3. Autoantibodies detected in the multiplex bead array assay – control cohorts

In the population control (PC) cohort, autoantibodies against nine of the nineteen aaRS were detected (Fig. 4A, Table 2). Autoantibodies against any of the aaRS were present in 15/156 (9.6%) individuals with the highest frequency to HA (n = 4), ArgRS (n = 3), CysRS (n = 2), and LeuRS (n = 2) (Fig. 4, Table 2, Supplementary Figs. 3–5). Of the nine novel anti-aaRS reactivities found in the IIM cohort, we detected reactivity in PC against five aaRS antigens: LysRS (n = 1), SerRS (n = 1), EPRS (n = 1), ArgRS (n = 3), CysRS (n = 2). Reactivities against Mi-2 (n = 9), MDA5 (n = 2), SSA (n = 5), SSB (n = 5), and Pm-Scl (n = 2) were also detected and in total did 32/156 (20.5%) displayed reactivity against any of the included antigens (Fig. 4A).

In the disease control cohort (n = 219 (Sjögren's syndrome n = 99, RA n = 50, and SLE n = 70)), autoantibodies against fifteen of the nineteen aaRS were detected (Fig. 4B, Table 2). Autoantibodies against any of the aaRS were present in 29/219 (13.2%) of the disease controls, with the highest frequency to Zo (n = 5), HA (n = 4), ValRS (n = 4), PL12 (n = 3), OJ (n = 3) and SerRS (n = 3). Of the nine novel anti-aaRS reactivities found in the IIM cohort, we detected reactivity in the disease controls against six: ValRS (n = 4), SerRS (n = 3), CysRS (n = 1), MetRS (n = 1), GlnRS (n = 1), and TrpRS (n = 1). In addition, we detected reactivities against the two remaining aaRS not found in the IIM cohort: AspRS (n = 1) and LeuRS (n = 1) (Fig. 4, Table 2, Supplementary Figs. 3–5). In total, 91/219 (41.6%) disease controls displayed reactivity against any of the included antigens (Fig. 4B). The majority, 58/91 (63.7%), of the disease control individuals testing positive were reactive to SSA, SSB, or Pm-Scl, or a combination of these. Reactivities against MSA-antigens Mi-2 (n = 4), and MDA5 (n = 1), were also detected.

Since plasma was analyzed in the IIM cohort and sera for the controls, 151/217 matched sera and plasma samples from patients with IIM were re-analyzed to check for possible sample discrepancies, finding result agreement in 134/151 (89%) (Supplementary Fig. 6). The reactivity discrepancies between serum and plasma of 17 patients showed three different explanations; a) one is a technical explanation for four individuals where the reactivity could not be confirmed due to that the reactive antigen was missing in one of the experiments (Supplementary Data), b) for eleven individuals, in one of the experiments the signal did not reach the high cut-off, even though we could observe an increased signal, and c) in two individuals (2/151, 1.3%) reactivities did not correlate between the samples, both individuals had reactivities against one of the ArgRS versions).

3.4. Clinical manifestations of IIM individuals with novel anti-aaRS autoantibodies

Clinical manifestations of the 22 patients with autoantibodies against novel aaRS and previously not tested aaRS are summarized in Table 3. Myositis was diagnosed in all patients with anti-HA, -Zo, or -KS

Table 2
Number of individuals with reactivity against aaRS in 217 IIM, 156 PC, and 219 disease controls. The autoantigen for the specific autoantibody is stated in the table.

Antigen	217 IIM n (%)	156 PC n (%)	99 Sjögren's syndrome n (%)	50 RA n (%)	70 SLE n (%)
HisRS (Jo1)	43 (19.8)	1 (0.6)	1 (1.0)	0 (0.0)	1 (1.4)
ThrRS (PL7)	9 (4.1)	0 (0.0)	0 (0.0)	1 (2.0)	1 (1.4)
AlaRS (PL12)	3 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	3 (4.3)
GlyRS (EJ)	2 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.4)
IleRS (OJ)	0 (0.0)	0 (0.0)	2 (2.0)	0 (0.0)	1 (1.4)
AsnRS (KS)	2 (0.9)	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)
PheRS (Zo)	5 (2.3)	0 (0.0)	2 (2.0)	3 (6.0)	0 (0.0)
TyrRS (HA)	3 (1.4)	4 (2.6)	2 (2.0)	0 (0.0)	2 (2.9)
LysRS (Sc)	1 (0.5)	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)
GlnRS (JS)	2 (0.9)	0 (0.0)	1 (1.0)	0 (0.0)	0 (0.0)
TrpRS (WARS)	1 (0.5)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)
SerRS	1 (0.5)	1 (0.6)	2 (2.0)	1 (2.0)	0 (0.0)
EPRS	1 (0.5)	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)
ArgRS	3 (1.4)	3 (1.9)	0 (0.0)	0 (0.0)	0 (0.0)
MetRS	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.4)
LeuRS	0 (0.0)	2 (1.3)	0 (0.0)	0 (0.0)	1 (1.4)
ValRS	2 (0.9)	0 (0.0)	0 (0.0)	1 (2.0)	3 (4.3)
CysRS	2 (0.9)	2 (1.3)	0 (0.0)	0 (0.0)	1 (1.4)
AspRS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.4)

aaRS, aminoacyl tRNA synthetase; IIM, Idiopathic inflammatory myopathies; PC, population control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

(n = 10), while ILD affected three. Arthritis was reported by one patient with anti-HA antibodies. The three anti-HA and one anti-Zo positive patients had Raynaud's phenomenon. None presented with mechanic's hands. All patients with novel aaRS (n = 12) had either muscle weakness and/or muscle enzyme elevation. Electromyography showed myopathic changes in 7/9 patients and muscle biopsy was consistent with myositis in 8/12 patients. Out of eight patients with pathological muscle biopsy, all presented widespread up-regulation of major histocompatibility complex class I (MHC-I), five with perivascular and/or endomysial inflammatory infiltrates even invading non-necrotic muscle fibers and two with perifascicular atrophy. The patient with anti-SerRS antibody reactivity had perifascicular necrosis, which has been proposed to be specific for ASSD [39–41] (missing information in 5/8 pathological muscle biopsies). None of the patients suffered from Raynaud's phenomenon. Two of the three patients with ILD, of which one also displayed arthritis and mechanic's hands, had previously tested positive for anti-Jo1 autoantibodies. Of the 12 patients with novel anti-aaRS, only three had negative anti-nuclear antibodies (ANA) by indirect immunofluorescence (IIF), while six presented with ANA positivity and homogeneous, nucleolar, or granular pattern (information not available for three patients).

According to Connors criteria [24], the 22 IIM patients described above could be re-classified as having ASSD. After including these in the previous classified ASSD group, we ended up with 68 patients with ASSD and 147 with non-ASSD. The frequencies of clinical manifestations in the two groups were the same as in the analysis reported in Table 1. Principal component analysis of the clinical manifestations did not show any clear differentiation between the two groups, and the 22 patients with newly detected anti-aaRS reactivities were closer to the non-ASSD group (Fig. 5).

3.5. Measurement of agreement - IIM cohort

The autoantibody results obtained from this study were compared with previously documented autoantibodies in these cases (Supplementary Figs. 1–2) by calculating the kappa coefficient (Table 4). The previously known autoantibodies had been detected in the clinical setting using line blot, IP, or ELISA. 45/50 previously known anti-synthetase autoantibodies could be detected in this study, all except for four anti-Jo1 and one anti-OJ (Supplementary Fig. 1), whose reactivities were all close to zero for all included versions of the specific antigen in the multiplex bead array assay. The samples in this study were not always taken at the same time point as the samples previously used for the detection of autoantibodies used in the clinical setting.

3.6. Validation of new autoantibody reactivities - IIM cohort

To validate the findings of new anti-aaRS autoantibody reactivities in IIM, one patient representing each new autoantigen was selected, and an ELISA method was developed. In addition, the same sample was analyzed using western blot. We could confirm all new autoantibody reactivities in at least one of the methods (Fig. 6 and Supplementary Figs. 7–9). We further validated one patient with reactivity against AIMP-1 and AIMP-2, respectively, as well as individuals with more than one anti-aaRS reactivity using ELISA and western blot. Also for these proteins, the results from the multiplex bead array assay could be confirmed (Supplementary Fig. 7 and Supplementary Fig. 8).

4. Discussion

In this study, a well-characterized IIM cohort and control cohorts were screened for autoantibody reactivities against the entire family of cytoplasmic aminoacyl-tRNA synthetases (aaRS). Our results indicate that all cytoplasmic aaRS but two display autoantigenic properties in patients with IIM.

Myositis-specific autoantibodies (MSAs) represent a fundamental diagnostic tool, helping to identify different IIM subgroups characterized by distinct clinical manifestations and histopathological features as well as to predict disease prognosis [42]. However, more than 40% of IIM patients test negative for the commonly tested, generally described MSAs [11], indicating a possibility to identify yet unknown autoantigens. The discovery of new myositis-specific antibodies could help, especially in the seronegative group, to diagnose IIM in the early stage of the disease and start immune-modulating treatment before irreversible damage occurs.

Here, we explored if patients with IIM test positive for autoantibodies against any of the nineteen cytoplasmic aaRS, using a multiplex bead array assay. To increase the possibility of detecting new autoantigens, we included different versions of the same aaRS, either full-length or truncated versions, to allow for detection of autoantibodies targeting both conformational dependent and -independent epitopes. We found that more than one-third of the IIM cohort tested positive for any anti-aaRS antibody, independently of previous autoantibody status. In the IIM cohort, we could detect autoantibodies against 16/19 cytoplasmic aaRS, including nine aaRS proteins that, to our knowledge, have never been described as autoantigens in IIM before or have only been

Table 3
Brief characteristics of the patients with IIM who were positive for the new aaRS autoantibody specificities not previously tested in this cohort.
Upper part: IIM patients (n = 12) testing positive for anti-aaRS autoantibodies other than the eight usually described.
Lower part: IIM patients (n = 10) testing positive for autoantibodies anti-KS, -HA, and -Zo in this study. Previously known autoantibody status, smoking status, and clinical manifestations are included. The autoantigen for the specific autoantibody is stated in the table.

Patients with IIM (n = 12) positive for novel anti-aaRS autoantibodies							
Patient	**Clinical subgroup**	**Known antibody positivity**	**aaRS detected in this study**	**Smoking status**	**HLA-DRB1 Allele 1/ Allele 2**	**Clinical manifestations**	**Validated by ELISA and WB**
34	non-ASSD	seroneg	EPRS	yes	*03/*15	PM	Fig. 6
41	non-ASSD	seroneg	MetRS	yes	*03/*07	PM	Fig. 6
67	non-ASSD	seroneg	LysRS, GlnRS and ArgRS	yes	*03/*04	DM, mechanic's hands	Suppl. Fig. 7
86	ASSD	HisRS (Jo1)	HisRS (Jo1) and ArgRS	na	*03/*09	DM, mechanic's hands, ILD, arthritis, cancer	
101	non-ASSD	MDA5	TrpRS (WARS)	na	*03/*04	PM, arthritis	Fig. 6
166	non-ASSD	Mi-2 and TIF1g	SerRS	na	*04/*15	DM, ILD, DM skin features, dysphagia, cancer	Fig. 6
168	ASSD	HisRS (Jo1)	ValRS	yes	*08/*14	PM	Fig. 6
171	non-ASSD	MDA5	GlnRS	yes	*01/*03	PM	
177	ASSD	HisRS (Jo1)	HisRS (Jo1) and ArgRS	yes	na	Muscle weakness^{+}, ILD	
194	non-ASSD	TIF1g	CysRS	yes	*01/*03	DM, cancer	Suppl. Fig. 7
199	non-ASSD	SRP^{++}	ValRS	yes	*04/*15	PM	
207	non-ASSD	seroneg	CysRS	no	*03/*13	PM	
Patients with IIM (n = 10) positive for anti-aaRS autoantibodies previously not tested for							
Patient	**Clinical subgroup**	**Known antibody positivity**	**aaRS detected in this study**	**Smoking status**		**Clinical manifestations**	**Validated by ELISA and WB**
7	non-ASSD	seroneg	PheRS (Zo)	yes		PM	
8	non-ASSD	seroneg	PheRS (Zo)	yes		PM, ILD	Suppl. Fig. 9
19	non-ASSD	seroneg	PheRS (Zo)	yes		PM, Raynaud's, dysphagia	
26	non-ASSD	seroneg	TyrRS (HA)	na		sIBM, Raynaud's	
31	non-ASSD	seroneg	TyrRS (HA)	yes		DM, DM skin features, arthritis, Raynaud's, dysphagia	
33	non-ASSD	TIF1g and HGMCR	TyrRS (HA)	no		DM, DM skin features, ILD, Raynaud's, cancer, dysphagia	Suppl. Fig. 9
133	non-ASSD	TIF1g	PheRS (Zo)	yes		DM, DM skin features, cardiac involvement, dysphagia, cancer	
138	non-ASSD	seroneg	AlaRS (PL12) and AsnRS (KS)	yes		PM, calcinosis, dysphagia	Suppl. Fig. 8
155	ASSD	HisRS (Jo1)	HisRS (Jo1) and AsnRS (KS)	yes		DM, DM skin features, ILD, cancer, dysphagia	
156	non-ASSD	SRP^{++}	PheRS (Zo)	yes		DM, DM skin features, calcinosis, dysphagia	

Upper part: Seven of these twelve patients were selected for validation using ELISA and the result are shown in the figure stated in the last column. Lower part: Three of the ten patients were selected for validation using ELISA and the results are shown in the figure stated in the last column. HLA-DRB1 data was only included for the 12 IIM patients in the upper part of the table. aaRS, aminoacyl tRNA synthetase; ASSD, anti-synthetase syndrome; PM, polymyositis; DM, dermatomyositis; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; ILD, interstitial lung disease; MDA5, interferon-induced helicase C domain-containing protein 1; Mi-2, chromatin organization modifier helicase (CHD) 3 and 4; sIBM, sporadic inclusion body myositis; na, not available; seroneg, previously no myositis specific autoantibodies detected; Suppl., supplementary; SRP, signal recognition particle; TIF1γ, E3 ubiquitin-protein ligase TRIM33; WB, western blot. Smoking status = yes (ever smoker) no (never smoker). DM skin features = any of periungual erythema, Gottron's sign, Gottron's papules, V-sign, shawl sign, erythroderma, periorbital edema, heliotrope rash. +muscle weakness based on manual muscle test-8 (MMT-8) below 80 and/or impaired muscle endurance by myositis functional index-2; patient 177 did not fulfill European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) criteria for the classification of idiopathic inflammatory myopathies. ++SRP was not included in the multiplex bead array assay.

reported in occasional individuals [13–16]. Importantly, reactivities against these novel proteins were identified in patients previously classified as seronegative for MSAs.

For anti-Jo1, -PL12, -PL7, -EJ, and -OJ, we could confirm previously known anti-aaRS autoantibodies in 45/50 patients, missing only four anti-Jo1 and one anti-OJ reactivities. The low kappa coefficient for anti-PL12, -PL7, and -EJ could be explained by new reactivities found in this study, not previously detected, or tested for. In addition, some of the samples used in this study were not from the same time point as the previously tested sample in the clinic, and the autoantibody reactivities were reported as ever present. As levels of anti-Jo1 autoantibodies may change over time [38], this could be one explanation for the observed discrepancy in a few individuals and could also explain the low kappa coefficient. As explained above, the inclusion of several antigens from the same protein might increase the possibility of detecting autoantibodies. Moreover, limitations with conventional methods used in the clinic have been noted. For example, anti-aaRS antibodies may be negative in line blot [43], but can show a cytoplasmic ANA pattern by IIF as aaRS are located mainly in the cytoplasm [6,44].

Thirteen patients had co-existence of anti-aaRS autoantibodies, or anti-aaRS autoantibodies together with other MSAs. This is of particular interest as anti-aaRS autoantibodies are usually described as mutually exclusive [9–12]. Since the sequence similarities between the aaRS proteins are low (Supplementary Table 3), it is unlikely that the multiple reactions are due to cross-reactivity [45,46]. Nevertheless, studies have suggested that autoantibodies from the same individual could target several members of the multi-synthetase complex (MSC) [17,18,47]. Here, we found one patient, P67, with autoantibodies targeting three MSC members (ArgRS, GlnRS, and LysRS) corroborating this hypothesis, and we also found individuals with autoantibodies targeting the AIMPs that are a part of the MSC.

There are, to our knowledge, only a limited number of studies

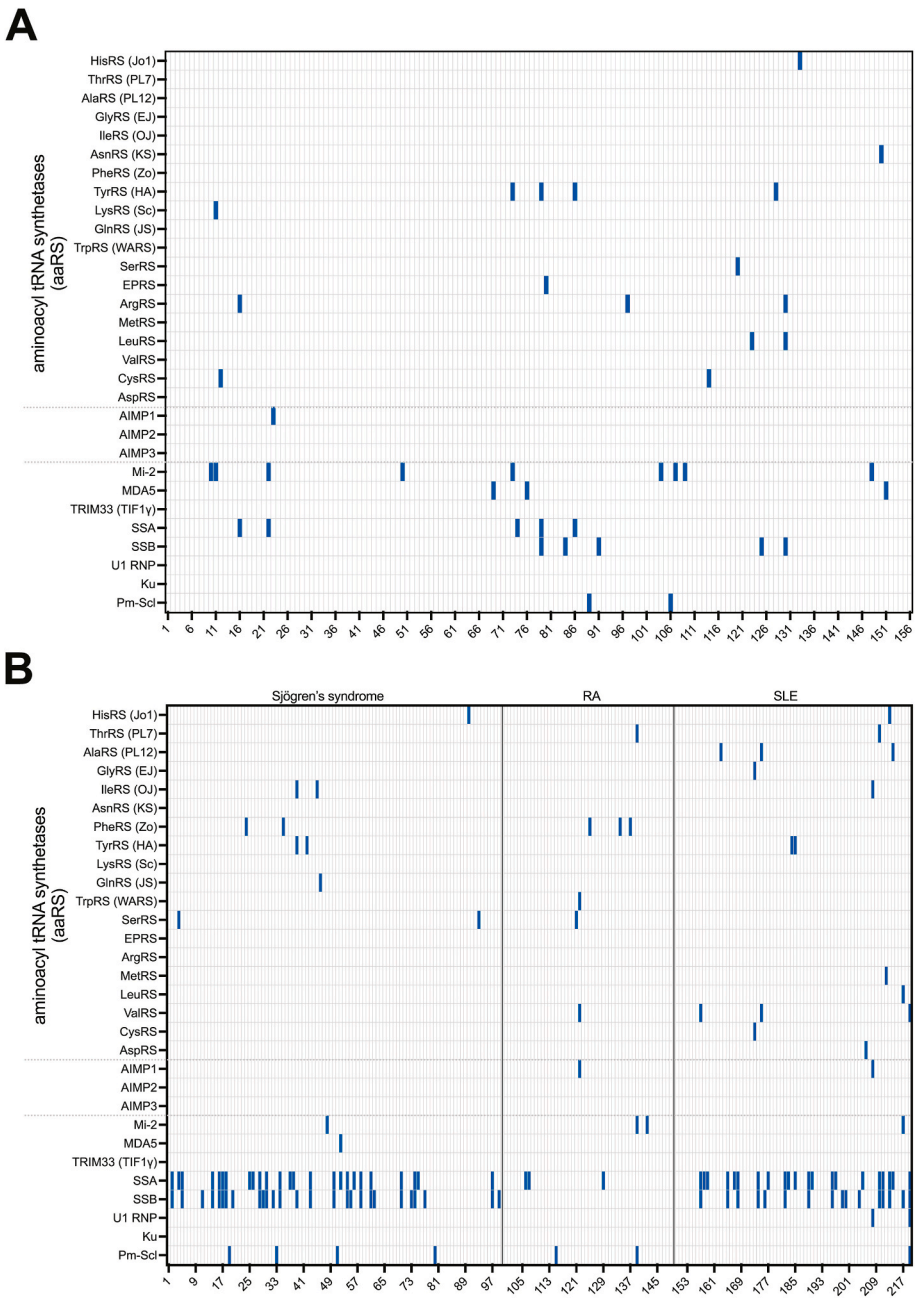


Fig. 4. Autoantibody reactivities for controls. Reactivity against a panel of 30 antigens for (**A**) 156 population controls and (**B**) 219 disease controls with Sjögren's syndrome (n = 99), RA (n = 50), or SLE (n = 70), as assessed by the multiplex bead array assay. Each column represents one individual, and each row represents one potential autoantigen. Reactivity was assigned positive (blue) if the criteria defined in the method section were met for at least one of the included versions of a particular protein antigen. All cytoplasmic aaRS proteins are displayed above the dotted gray lines, the AIMPs are in between dotted lines, and below are the additional myositis-related proteins included in the study. The black vertical lines in (**B**) separate the three disease control groups in the cohort. PC, population control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

published investigating the presence of anti-aaRS autoantibodies in population controls, particularly regarding the rarer anti-aaRS autoantibodies [48–50]. Our study gives additional insight into this. Autoantibodies targeting aaRS and other autoantigens were observed at low frequencies, as expected in control cohorts [51]. However, the relatively high frequency of reactive subjects in the PC with the rarer anti-aaRS was a surprise. The fact that we used population controls that might have other autoimmune diseases could explain some of the reactivities. For other autoantibodies, such as ACPA, the detection of ACPA in healthy individuals is associated with an increased risk of developing RA [52]. In addition, the autoantibody specificity and isotype distribution of autoantibodies have been suggested to be an important part of the disease pathogenesis [53]. Previous studies have also shown that autoantibody levels might correlate with disease activity [38,54]. The multiplex bead array assay, as used in this study, is not for absolute quantification, and could not investigate the difference in autoantibody levels between individuals with IIM and controls. Thus, further in-depth studies of anti-aaRS autoantibody levels, specificity, and isotype distribution in IIM and population controls are needed. We could also detect aaRS reactivities in the disease control cohorts, both to previously known aaRS autoantigens as well as the newly discovered aaRS autoantigens in this study. Recent studies reported a relatively high prevalence of anti-Zo, -KS, and -HA in a broad spectrum of ILD patterns [55], and ILD has been reported as the primary clinical feature of anti-KS patients [56]. In our cohort, ten patients with IIM were identified with these autoantibodies, and ILD was reported only in three. Patient selection, in our study from a rheumatology clinic, may explain these differences. In addition, nine individuals from the disease control cohort did also test positive for anti-Zo (n = 5) or anti-HA (n = 4). Furthermore, both TrpRS and SerRS have previously been suggested as autoantigens in other diseases [14,15]. Indeed, we did detect autoantibody reactivities against both TrpRS and SerRS in disease controls (n = 1 and n = 3, respectively). Notably, anti-HA autoantibodies were found at a higher frequency in PC than in the IIM cohort (2.6 vs. 1.4%). The exact meaning

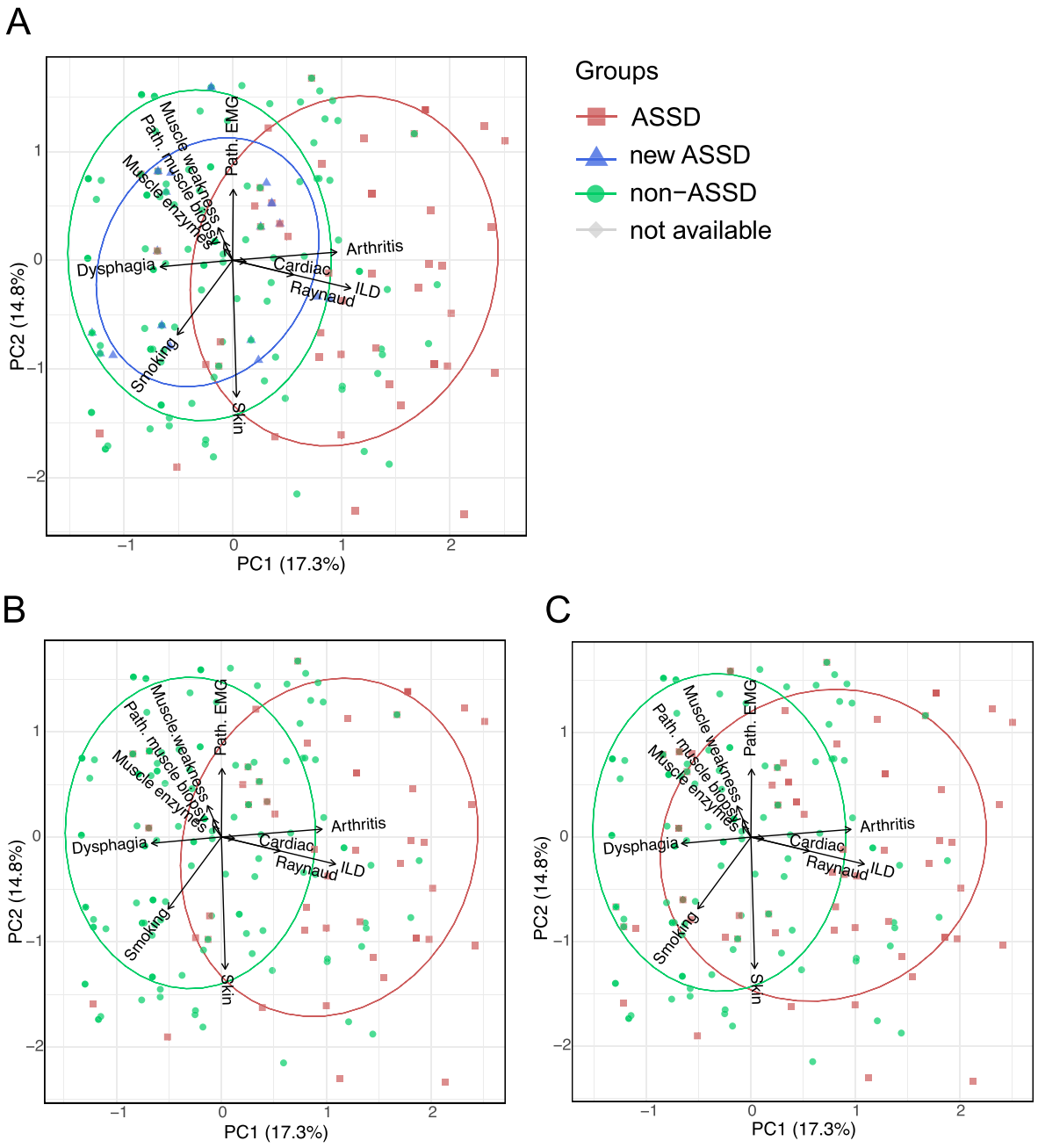
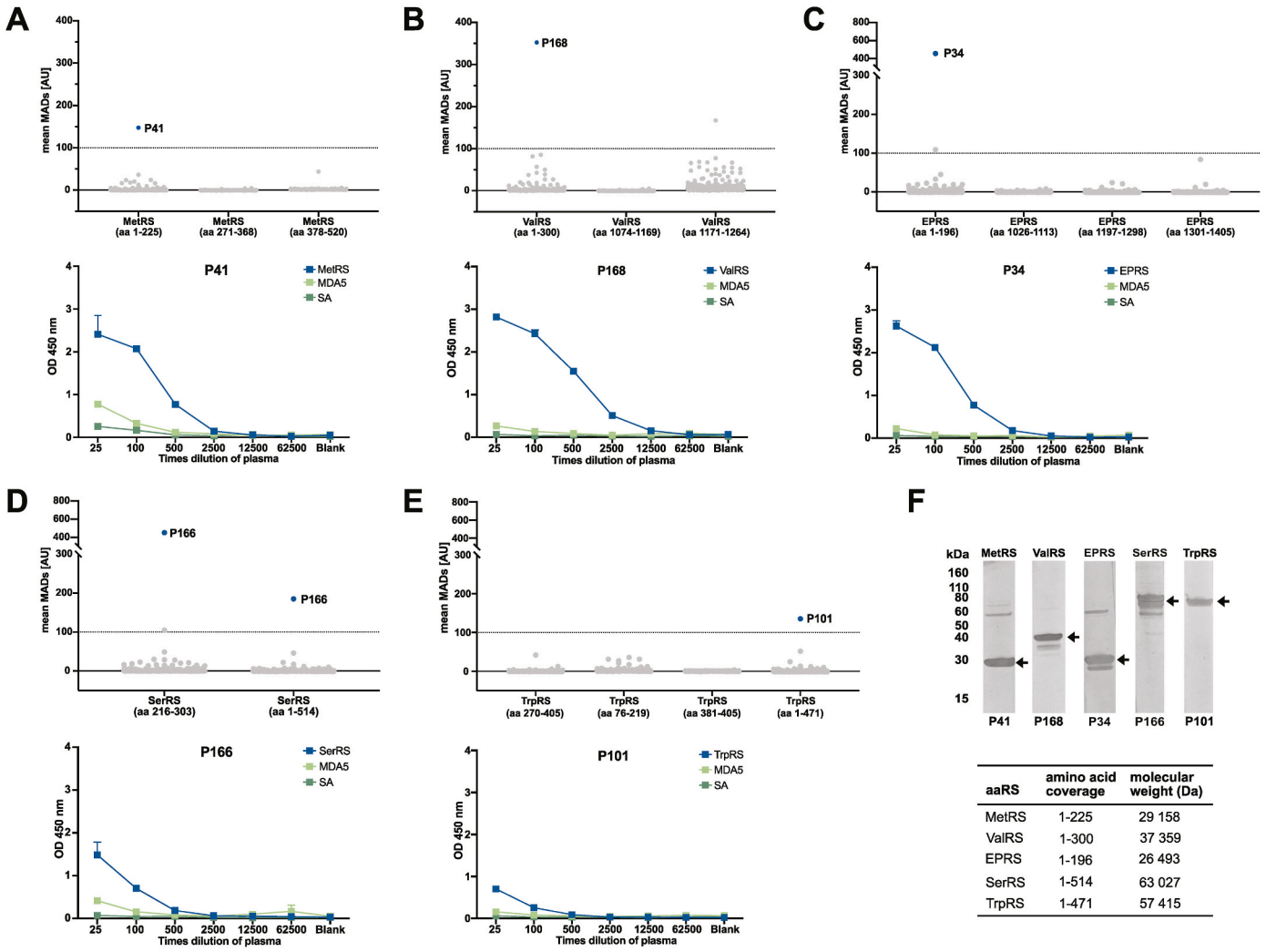


Fig. 5. Principal component analysis (PCA) of clinical manifestations and phenotypes for the IIM cohort. Analysis based on the binary data of the variables; muscle involvements (pathological muscle biopsy, muscle enzymes elevation, pathological EMG, and muscle weakness), skin involvement, Raynaud's phenomenon, Arthritis, interstitial lung disease (ILD), cardiac involvement, dysphagia, and smoking. Scores plots PC1 vs PC2 are shown, each dot represents one patient and the contribution of each variable to PC1 and PC2 are included. Some dots are overlapping represented by the change of color intensity. Grouping is based on **(A)** ASSD classification, ASSD (n = 50, red), non-ASSD (n = 147, green), not available ASSD status (n = 2, gray), patients with a new ASSD classification after this study (n = 18, blue). **(B)** Patients are grouped based on ASSD status from clinical information (before this study) ASSD (n = 50, red), non-ASSD (n = 165, green), and not available ASSD status (n = 2, gray). **(C)** Patents grouped based on ASSD status after reclassifying 22 patients into the ASSD group ASSD (n = 68, red), non-ASSD (n = 147, green), and not available ASSD status (n = 2, gray). Demographic data is according to Table 1. The analysis indicates no clear differentiation between groups in scores plot of PC1 vs PC2.

of this result needs further investigation, and the low frequencies of the rare anti-aaRS autoantibodies found in both IIM, PC, and disease controls should be further validated in larger cohorts. Even though a low frequency of all anti-aaRS autoantibodies were found in control cohorts, we do not believe that this is undermining their clinical value, as only the presence of anti-aaRS autoantibodies is not sufficient for the classification or diagnosis of ASSD, and as discussed above, also other autoantibodies are known to exist in healthy individuals.

Twelve patients were identified with new anti-aaRS autoantibodies. Two-thirds of these were HLA-DRB1*03 positive and current or previous smokers, in line with the known association between HLA-DRB1*03 haplotype, smoking, and anti-aaRS antibodies [57–60]. The ANA-positivity, without cytoplasmic pattern, reported in 6/12 patients could be explained by the co-existents of other MSA or MAA. When investigating the clinical and histopathological features of the 12 patients with novel anti-aaRS autoantibodies, we could not verify the typical characteristics of ASSD, neither in clinical nor histopathological features. However, this small group of patients and the fact that five of



aaRS	amino acid coverage	molecular weight (Da)
MetRS	1-225	29 158
ValRS	1-300	37 359
EPRS	1-196	26 493
SerRS	1-514	63 027
TrpRS	1-471	57 415

Fig. 6. Validation of bead array assay results with ELISA and western blot for five of the novel anti-aaRS reactivities. Results from the bead array assay (top) and ELISA (bottom) for IIM patients with reactivity against (**A**) MetRS, (**B**) ValRS, (**C**) EPRS, (**D**) SerRS, and (**E**) TrpRS. The mean MADs are shown for the bead array assay, with the IIM patient with novel anti-aaRS reactivity highlighted in blue. The distribution of the other 216 patients for each antigen is shown in gray. The dotted gray line represents the cut-off at 100xMADs. Antibody reactivities against the aaRS were measured by ELISA and absorbance values (450 nm) were obtained. MDA5 (light green) was used as a control protein, and streptavidin (SA, green) represents the background signal. (**F**) Western blot showing the reactivity of the five IIM patients against their respective new autoantigen (top) and table showing the amino acid coverage and molecular weight of the protein loaded on the gel. The antigens used in ELISA and western blot are listed in the table in (**F**). Validation of the other four novel anti-aaRS autoantibodies is shown in Supplementary Figs. 7–8. As a positive control for ELISA, a plasma sample from an anti-MDA5 positive patient was used as a control for protein-specific background (Supplementary Fig. 10). aa, amino acid; MADs, median absolute deviations; OD, optical density; P, patient; SA, streptavidin.

these autoantibodies were also found in controls, makes it difficult to draw conclusions regarding their potential association with ASSD. Similarly, anti-TrpRS autoantibodies, although previously detected in patients with autoimmune diseases [15], have not been suggested as a serological marker for ASSD since the related clinical phenotype was more similar to rheumatoid arthritis than ASSD [17,61]. Nevertheless, all IIM patients with novel anti-aaRS antibodies presented with muscle involvement.

The novel anti-aaRS autoantibodies were mostly found in the non-ASSD group and in four who were previously known as seronegative. Even though some co-existence of anti-aaRS autoantibodies was found, the majority of anti-aaRS positive individuals only had one detectable anti-aaRS autoantibody. For individuals previously known as seropositive, with novel anti-aaRS autoantibodies detected here (n = 8), the previous autoantibody positivity could only be verified in two individuals. The possible reasons for these discrepancies are discussed later.

It is previously known that individuals with anti-aaRS autoantibodies might be amyopathic and only present with clinical manifestations such as ILD or arthritis [62]. As these manifestations are also present in other autoimmune diseases, there might be many individuals not tested for anti-aaRS autoantibodies diagnosed with, for example, RA. The findings of anti-aaRS autoantibodies in both IIM and disease controls found in this study could open up for the discussion of the definition of ASSD, which has been suggested previously [63]. In addition, our results indicate that anti-aaRS autoantibodies should be tested not only in patients with suspected IIM but also in other rheumatic autoimmune diseases. Also, this study highlights the importance of including population controls in research, but also in clinical routines to define appropriate cut-offs.

The limitations of this study include the following. Firstly, the new reactivities were detected at a low frequency in IIM patients, and also in controls, thus confirmation in larger cohorts is needed. Secondly, some samples were retrieved after the patient started immune-modulating treatment, which could affect the presence and detection of autoantibodies [64,65]. Thirdly, we did not cover the full-length protein of all

Table 4
Measurement of agreement between this study and previously known antibody status. The comparison was made using the total number of positive patients for myositis-specific autoantibodies and Cohen's kappa coefficient.

	Known positivity, n	Positivity detected in current study, n	Kappa coefficient	p-value
Anti-Jo1	45	43	0.91	0.0001
Anti-PL7	2	9	0.39	0.0001
Anti-PL12	1	3	0.49	0.014
Anti-EJ	1	2	0.66	0.0001
Anti-OJ	1	0	/	/
Anti-Mi-2	5	6	−0.027	NS
Anti-MDA5	14	8	0.52	0.0001
Anti-TIF1γ	23	6	0.31	0.0001

Jo1, HisRS; PL7, ThrRS; PL12, AlaRS; EJ, GlyRS; OJ. IleRS; Mi-2, chromatin organization modifier helicase (CHD) 3 and 4; SRP, signal recognition particle; MDA5, interferon-induced helicase C domain-containing protein 1; TIF1γ, E3 ubiquitin-protein ligase TRIM33.

autoantigens, indicating that we may have some false negatives. For example, anti-OJ reactivity in patient P95 could not be confirmed in this study, in which only shorter protein versions of IleRS were included. Fourthly, sample collection did not always match the timepoint for MSA detection in clinic, and for some patients, clinical data were missing. This could explain why some patients presented discordant results. Finally, to minimize the risk of false positives, we decided to use a high cut-off for all antigens, even though this means a higher risk for false negatives.

In conclusion, our results suggest autoantigenic properties for the cytoplasmic aaRS family, as well as the AIMPs, and we hypothesize that in a larger cohort, all aaRS might be found autoantigenic. However, to infer distinct clinical phenotypes, these results need to be tested in another large study. There are still remaining seronegative patients left in our cohort, and we suggest using more multiplex assays including additional proteins to explore and investigate new potential autoantigens. Combining serological, clinical, and histopathological findings makes it possible to define more homogeneous groups in IIM to achieve an improved understanding of the pathophysiology behind the muscular and extra-muscular manifestations and aim at a more personalized treatment. Here, we found low frequencies of the novel and also previously recognized anti-aaRS autoantibodies in control cohorts. For several of the anti-aaRS autoantibodies, frequencies were similar between IIM patients and controls, and this study emphasizes the importance to include population and disease controls in screening for new autoantibodies.

Abbreviations

aaRS, aminoacyl transfer (t) RNA synthetase(s); ADM, amyopathic dermatomyositis; ANA, anti-nuclear antibodies; ASSD, anti-synthetase syndrome; BSA, bovine serum albumin; DM, dermatomyositis; EMG, electromyography; ENMC, European Neuromuscular Centre; EULAR/ACR, European League Against Rheumatism/American College of Rheumatology; His_6ABP, hexahistidine and albumin binding protein tag; HLA, human leukocyte antigen; HRTC, high-resolution computed tomography; IIF, indirect immunofluorescence; IIM, idiopathic inflammatory myopathies; ILD, interstitial lung disease; IQR, inter-quartile range, IMNM, immune-mediated necrotizing myopathy; MAA, myositis-associated autoantibodies; MADs, median absolute deviations; MFI, median fluorescence intensity; MHC, major histocompatibility complex; MSA, myositis-specific autoantibodies; MSC, multi-synthetase complex; PBS, phosphate buffered saline; PC, population control; PCA, principal component analysis; PM, polymyositis; PrEST, protein epitope signature tag; RA, rheumatoid arthritis, sIBM, sporadic inclusion body myositis; SD, standard deviation; SLE, systemic lupus erythematosus.

A list of all proteins used in this study and abbreviations thereof are available in Supplementary Data.

Author contributions

All authors have read and approved on the final version to be published. Charlotta Preger: Conceptualization, Data Curation, Methodology, Investigation, Formal analysis, Visualization, Writing – Original draft, Writing – Review & Editing. Antonella Notarnicola: Conceptualization, Data Curation, Investigation, Formal analysis, Visualization, Writing – Original draft, Writing – Review & Editing. Cecilia Hellström: Data Curation, Methodology, Investigation, Formal analysis, Writing – Review & Editing. Edvard Wigren: Investigation, Formal analysis, Writing - Review & Editing. Cátia Fernandes-Cerqueira: Conceptualization, Writing – Review & Editing. Marika Kvarnström: Investigation, Funding acquisition, Resources, Writing – Review & Editing. Marie-Wahren-Herlenius: Investigation, Funding acquisition, Resources, Writing – Review & Editing. Helena Idborg: Formal analysis, Writing – Review & Editing. Ingrid E. Lundberg: Conceptualization, Investigation, Funding acquisition, Supervision, Resources, Writing – Review & Editing. Helena Persson: Conceptualization, Resources, Supervision, Writing – Review & Editing. Susanne Gräslund: Conceptualization, Funding acquisition, Resources, Supervision, Writing – Review & Editing. Per-Johan Jakobsson: Conceptualization, Supervision, Funding acquisition, Writing – Review & Editing.

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Declaration of competing interests

I.E. Lundberg has received a research grant from Bristol Myers Squibb and from Astra Zeneca and has served on advisory board of Corbus Pharmaceuticals, Inc., Argenx, Kezaar, Octapharma, Orphazyme, EMD Serono Research & Development Institute, and Janssen. I. E Lundberg has stock shares in Roche and Novartis. The other authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2022.102951.

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