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Review

Statin-associated autoimmune myopathy: A distinct new IFL pattern can increase the rate of HMGCR antibody detection by clinical laboratories

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ABSTRACT

Background and objective: Statin-associated autoimmune myopathy (SAAM) with anti-HMGCR antibodies has recently been described. Several specific immunoassays are in use to detect HMGCR antibodies. In the course of systematic autoantibody screening we recognized a new distinct IFL staining pattern on rat liver sections that regularly coincided with anti-HMGCR antibodies. In this study we investigated whether this new IFL pattern is specifically associated to statin-associated autoimmune myopathy and corresponds to anti-HMGCR antibodies.
Patients and methods: Twenty-three patients positive for anti-HMGCR antibodies (14 diagnosed with SAAM) were investigated for anti-HMGCR antibodies by two ELISA assays and confirmed by immmunoblot. HMGCR associated liver IFL pattern (HALIP) was detected by indirect IFL and the reactivity against HMGCR was confirmed by immunoabsorption using purified human HMGCR antigen. 90 patients with other autoimmune diseases and 45 non-autoimmune statin treated patients were studied as controls.
Results: 21 out of 23 (91%) anti-HMGCR positive patients were HALIP positive. The staining was completely and specifically removed by immunoabsorption with human purified HMGCR. None of the control sera from autoimmune patients or non-autoimmune statin treated subjects was positive for HALIP. Statistical concordance between HALIP and anti-HMGCR antibody specific tests was 98.7%, kappa 0.95.
Conclusions: A new and distinct IFL staining pattern (HALIP) is associated to HMGCR associated myopathy. Absorption and concordance studies indicate that the antigen recognized in the liver by HALIP is HMGCR or a closely related protein. Awareness of this new pattern can help to detect HMGCR autoantibodies in statin treated patients tested for autoimmune serology.

Contents

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1. Introduction

Inflammatory myopathies comprise a group of systemic autoimmune diseases affecting proximal muscles that are characterized by an inflammatory infiltrate [1]. It is generally accepted that dermatomyositis (DM), polymyositis (PM) and sporadic inclusion body myositis are members of the group. A fourth subtype with few scattered inflammatory cells and high degree of necrosis designated immune-mediated necrotizing myopathy (IMNM) is also included by most authors in this group [1,2].

IMNM has been associated with malignancy, connective tissue diseases and recently, with exposure to statins [2–4]. Statins seem to act as a trigger for a specific type of IMNM characterized by autoantibodies directed against 3-hydroxy-3-methyl glutaryl-coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol synthesis [5]. HMGCR-associated myopathy has shown a strong association with HLA-DR11 allele and can benefit from immunosuppressive therapy [4,6]. Although the immunoprecipitation assay is still considered the gold standard for the detection of these anti-HMGCR antibodies, ELISAs, addressable laser bead immunoassay and immunoblot tests have been recently developed and made commercially available [7].

Indirect immunofluorescence (IFL) is the most widely used test for autoantibody screening [8]. A combination of rat stomach, liver and kidney is the usual substrates (rat triple tissue). Antinuclear, antimitochondrial, anti-liver-kidney microsome (LKM), anti-smooth muscle and anti-gastric parietal cell autoantibodies are the main autoantibodies detected. In the course of routine autoantibody screening on rat triple tissue sections we have recognized an IFL staining pattern on rat liver that we could not ascribe to known patterns, regularly coexisted with HMGCR antibodies and all corresponded to patients with statin associated autoimmune myopathy. We report here that this IFL staining pattern on rat liver correlates very closely with HMGCR antibodies and may provide a new tool for the screening for HMGCR antibodies.

2. Patients and methods

2.1. Patients

The study included 23 patients positive for anti-HMGCR antibodies, 14 of them diagnosed with statin associated autoimmune myopathy (1 PM, 1 DM, 12 IMNM), 3 diagnosed with autoimmune myopathy (1 PM, 2 IMNM) without known exposure to statins, and 6 patients whose sera were referred the immunology laboratory but lacking clinical and anatomopathological information. A total of 90 patients diagnosed of other autoimmune diseases (systemic lupus erythematous, autoimmune hepatitis, systemic sclerosis, Sjøgren syndrome, rheumatoid arthritis and dermatomyositis), and 45 patients treated with statins and not suffering from autoimmune medical conditions were also included in the study as controls (patient flow chart is shown in Fig. 1).

All the patients diagnosed with systemic autoimmune diseases were followed at the Vall d'Hebron General Hospital in Barcelona (Spain) between 1983 and 2016. All patients and controls included in the study gave informed consent for the use of their sera for research purposes.

Statin associated myopathy included patients with exposure to statins, positive anti-HMGCR antibodies and IMNM, PM or DM. Diagnostic criteria for IMNM were based on the diagnosis of IMNM diagnostic and classification criteria proposed by the Muscle Study Group/European Neuro Muscular Centre (MSG/ENMC) [9]. The diagnosis of dermatomyositis/polymyositis was based on the criteria of Bohan and Peter [10,11] and only patients with definite or probable disease were included in this study.

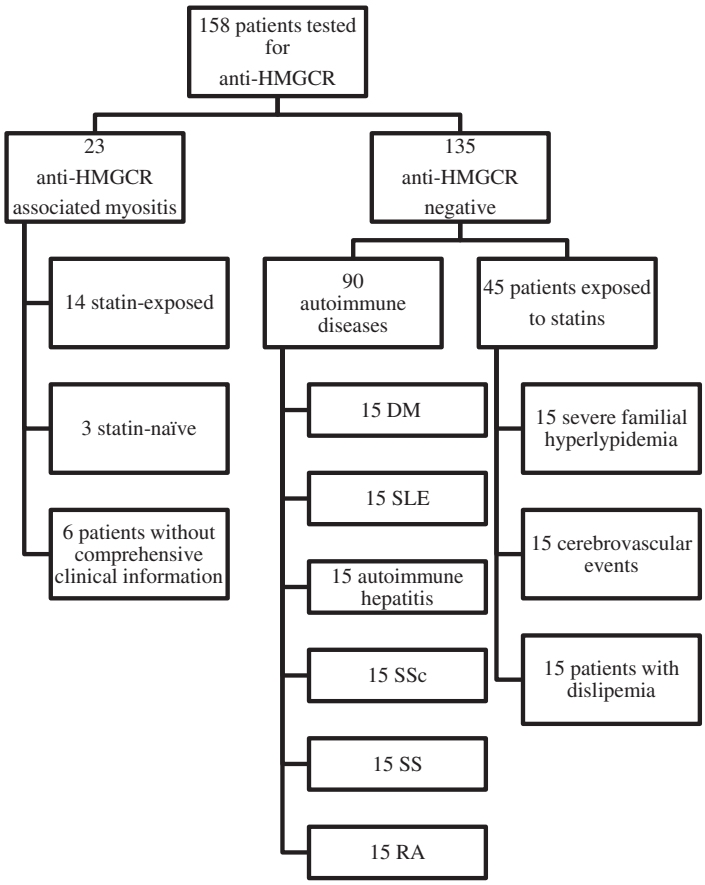
Clinical data was obtained using a standardized questionnaire, comprehensive physical examination, and a systematic chart review. Demographic data, signs and symptoms of the disease, clinical course, number and type of treatments were also recorded. Treatment response was defined as complete if the patient recovered totally without evidence of active disease (including normal CK level), no response if there was no evidence of clinical improvement and persistently high CK values and partial if partial, in the rest of the cases. Subjects' written consent was obtained in accordance to the Declaration of Helsinki [12].

2.2. Methods

2.2.1. Anti-HMGCR antibody detection

Anti-HMGCR antibodies were measured by two ELISA tests (one home-made, one commercial) and confirmed by immunoblot.

2.2.1.1. In house ELISA test. Briefly, wells of a 96-well polystyrene high binding microtiter plate (Costar, Cambridge, MA, USA) were coated with 50 μl of PBS containing 100 ng of purified HMGCR (Sigma, St. Lois, MO, USA) or with PBS alone, to measure non-specific binding for each sample. Plates were wrapped in aluminium foil and incubated overnight at 4 °C. After washing twice with PBS/0.05% Tween 20 (washing solution), blocking was performed with 200 μl of blocking buffer [PBS/0.05% Tween 20/2% bovine serum albumin (Sigma)]. Plates were incubated 1 h at room temperature; afterwards, two washes with the washing solution were performed. Sera (50 μl diluted at 1:400 in blocking buffer) were added in duplicate (as well as to the well which did not contain the antigen) and were incubated for 1 h at 37 °C. After six washes, plates were incubated for 30 min. at 37 °C with 50 μl of horseradish peroxidase-labelled goat anti-human IgG (γ-chain specific) antibody (Sigma) (1:10,000 in blocking solution). After washing six times, colour was developed with 50 μl of TMB substrate reagent (BD Biosciences, San Diego, CA, USA). Plates were kept for 30 min at 37 °C and then 50 μl of a solution of 2 N H_2SO_4 were added to stop the reaction. The reaction was read as Optical Density (OD) at 450 nm in a spectrophotometer (Lab systems iEMS reader MF, Barcelona, Spain). Non-specific background OD (wells without the antigen) was subtracted from the corresponding tested sample. A positive reference sample (kindly provided by Dr. Andrew Mammen) was included in each plate. The cut-off point for positivity on ELISA was established at two SD above the mean value obtained in a sample of 50 healthy control serums (blood donors).



SAAM: statin-associated autoimmune myopathy; DM: dermatomyositis; PM: polymyositis; SLE: systemic lupus erithematosus; SSc: systemic sclerosis; SS: Sjögren syndrome: RA: rheumatoid arthritis

Fig. 1. Patient flow chart.

2.2.1.2. Commercial ELISA. HMCGR antibodies were measured in the same samples using the QUANTA Lite HMGCR kit (INOVA Diagnostics, San Diego, CA) according to the manufacturer's instructions. A sample was considered positive when more than 20 units were detected.

2.2.1.3. Confirmation by immunoblotting. Briefly, 5 μg of HMGCR catalytic domain human, recombinant GST fusion protein expressed in *Escherichia coli* (Sigma-Aldrich, St Louis, MO) were run on 4% to 12% polyacrylamide-SDS minigels with MOPS running buffer, and western blot was performed on a nitrocellulose membrane using the Invitrogen NuPAGE (Carlsbad, CA) electrophoresis system, as previously described [13,14]. Nitrocellulose was cut into several strips and incubated for 1 h at room temperature (RT) in PBS containing 3% non fat dry milk (blocking buffer). Each strip was then incubated with the corresponding human serum sample diluted 1:100 in blocking buffer for 1 h at RT. After washing, phosphatase alkaline-labelled goat anti-human immunoglobulin antibody (Invitrogen, Frederick, MD) was added to each strip which were incubated for 1 h at RT. Colour development was performed in phosphatase reagent (BCIP/NBT, PR omega, Madison, WI). Based on signal intensity, the results were classified into negative or positive.

2.2.2. IFL on kidney/stomach/liver and on HEp-2 substrates

For the IFL on tissue or cells we used ready-made multispot slides from commercial sources i.e., cryostat triple tissue sections (rat liver, kidney and stomach) and fixed HEp-2 cells (Nova Lite range of reagents, Inova Diagnostics). We also tested substrates from other suppliers (Euroimmune, BioSystems, and Aeskulides) in order to rule out artefacts generated during the preparation of the substrates. To develop the reaction we used a goat anti-human IgG FITC labelled serum (INOVA). Slides were stained using an automatic processor (QUANTA-Lyres® INOVA) according to the manufacturer's instructions.

Briefly, sera from patients and controls were diluted in PBS at 1/40 for the rat triple tissue staining and at 1/80 for the HEp2-human cells staining. Multispot slides were incubated with 40 μl of prediluted sera for 30 min at room temperature. After 3 washes with PBS, 40 μl of the FITC conjugated serum was added, incubated for another 30 min and after several washing steps slides were mounted in buffered glycerol with anti-fade and examined under a UV-light microscope (Nikon eclipse 50i).

All the sera (both positive and negative for anti-HMGCR autoantibodies) were tested blindly using this technique at two different laboratories, at the Vall d'Hebron Immunology Department, and at the Immunology Department of the Sant Pau Hospital.

2.2.3. Immunoabsorption experiment

Immunoabsorption experiments were performed in 5 patients with a positive IFL pattern by using HMGCR human antigen (Sigma, St. Lois, MO, USA) diluted in PBS with 10% foetal bovine serum at the following increasing antigen concentrations (0.33, 0.84, 1.64, 3.37, 6.74 and 10.37 μg/ml). We used as controls an anti-HMGCR negative healthy human sera, and two sera positive for anti-mitochondrial antibodies and anti-gastric parietal cell antibodies respectively.

2.2.4. Statistical analysis

Dichotomous variables were expressed as percentages and absolute frequencies, and quantitative features were reported as means and standard deviations (SD). Creatine kinase (CK) level, a highly positively

skewed variable, was expressed as median, first, and third quartile (Q1–Q3).

The Kappa statistic was used to assess agreement both between ELISA methods for detecting anti-HMGCR antibodies and also between HALIP and the anti-HMGCR antibodies measured by ELISA. Analyses were performed with Stata, version 14.0 (StataCorp).

3. Results

3.1. Clinical features of patients with anti-HMGCR associated myopathy

Detailed clinical data and follow-up were available for 17 patients with HMGCR-associated myopathy. Among these 17 patients (41% women), 14 were exposed to statins at the onset of the disease (82%) and 88% (n = 15) showed an immune mediated necrotizing myositis in the muscle biopsy (with the other two patients showing features compatible with an unspecified PM and DM). Mean age was 63 years-old (SD 18 years) and median CK was 6941 IU/l (Q1-Q3 6428–9596 IU/l). Data from patients exposed to statins whose clinical characteristics were available is summarized in Table 1. Concordance between our in house ELISA and the commercial ELISA (QUANTA Lite assays, INOVA Diagnostics) showed a very high reproducibility (overall agreement 97%, Kappa 0.9).

3.2. IFL on kidney/stomach/liver and on HEp-2 substrates

Anti-HMGCR positive sera stained scattered hepatocytes with a centro-lobular distribution. The staining was confined to the cytoplasm and spared clearly the nuclei (Fig. 2). No more than 10% of the hepatocytes were stained in a given liver lobule (zone 3 of the lobule around/near the centrilobular hepatic veins). The bile duct, the endothelium and the cells in the sinusoids were not stained. This pattern was clearly different from the patterns produced by anti-mitochondrial, anti-liver–kidney-microsomal or LC1 antibodies usually seen using this technique. Stomach and kidney did not show any characteristic pattern (see Fig. 3, a to d). Most (21 out of 23 [91%]) sera from patients with anti-HMGCR (14 out of 14 [100%] diagnosed with SAAM) were positive for this IFL pattern and thus we named it anti-HMGR Antibody Associated Liver IFL Pattern (HALIP). The sera from the 90 patients from the autoimmune systemic disease group or from the 45 non-autoimmune patients treated with statins did not produce this distinct IFL pattern. The overall agreement between HALIP and ELISA results confirmed by blot was 99% (Kappa 0.95). HALIP pattern was recognized independently by experienced technical staff at two immunology laboratories (Vall d'Hebron Hospital and at the Sant Pau Hospital) showing perfect agreement. Immunoabsorption study showed that HALIP was specifically removed by incubation with the human purified antigen, confirming the reactivity of the autoantibodies against anti-HMGCR antigen (see Fig. 4). This highly specific IFL pattern was observed irrespective of the commercial source of the triple tissue multispot slides (see Fig. 3). On human HEp-2 cells a granular cytoplasmic fluorescence pattern was observed in only 6 out of 23 (35%) patients, which gives a rather poor concordance with ELISA results confirmed by blot and with HALIP (overall agreement less than 35% for both).

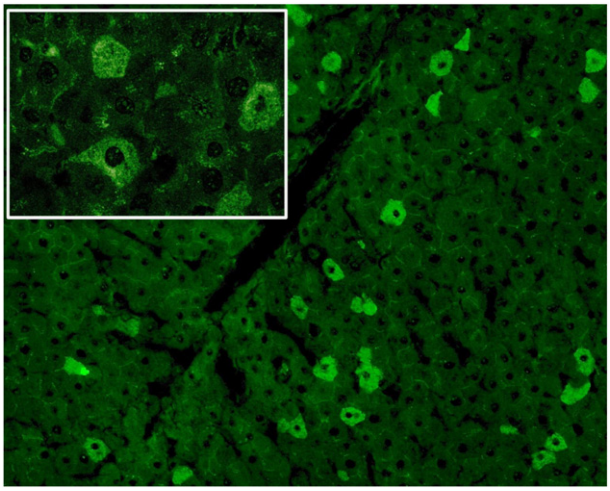


Fig. 2. The HALIP staining. Indirect immunofluorescence on rat liver cryostat section. Patient serum was followed by FITC anti-IgG antisera (for detail see methods). The image was obtained with a spectral confocal microscope FV1000 (Olympus). Notice the clear cytoplasmic staining in distinct scattered hepatocytes, with a granular texture in the inset (original magnification ×200 in the main picture and ×1000 in the inset).

4. Discussion

Autoantibodies to HMGCR are the signature of a specific type of myositis, HMGCR-associated myositis. In this article we report a new and distinctive IFL pattern, HALIP, that is easily detected on the

Table 1
Clinical features of the 14 patients with statin-associated myopathy and anti-HMGCR antibodies.

Case	Age at onset years/sex	Statin use	Anti-HMGCR (HM/Comm)	CK (IU/l)	Muscle biopsy	Proximal weakness	Myalgias	DR11	HALIP	Therapy	Response
1	76/M	Atorvastatin (80 mg/d)	906/>200 U	18,417	DM	(+++)	(++)	(+)	(+)	AZA IVIG	Complete
2	57/F	Atorvastatin (80 mg/d)	918/60 U	2270	IMNM	(+++)	(++)	N/D	(+)	MM IVIG	Complete
3	57/F	Atorvastatin (10 mg/d)	1503/>200 U	3303	IMNM	(+++)	(+)	N/D	(+)	AZA IVIG	Complete
4	52/M	Atorvastatin (20 mg/d)	1312/>200 U	7778	IMNM	(+)	(+++)	(−)	(+)	IVIG	Complete
5	78/F	Atorvastatin (80 mg/d)	997/>200 U	8560	IMNM	(+++)	(+)	(+)	(+)	AZA IVIG	Complete
6[†]	82/M	Atorvastatin (40 mg/d)	896/126 U	8793	IMNM	(+++)	(−)	(+)	(+)	AZA IVIG	Partial
7	73/F	Atorvastatin (80 mg/d)	700/91 U	3304	IMNM	(++)	(−)	(+)	(+)	N/D	Complete
8	67/M	Atorvastatin (20 mg/d)	844/4 U	10,400	IMNM	(+++)	(−)	(+)	(+)	AZA MTX	Partial
9	68/F	Atorvastatin (20 mg/d)	1150/9 U	7200	IMNM	(+++)	(−)	N/D	(+)	AZA MTX	Complete
10	63/F	Sinvastatin (20 mg/d)	884/5 U	11,330	PM	(+++)	(−)	N/D	(+)	AZA MTX IVIG	Complete
11	70/M	Atorvastatin (10 mg/d)	906/90 U	4260	IMNM	(+++)	(−)	N/D	(+)	MTX	Complete
12	69/M	Sinvastatin (20 mg/d)	1184/>200 U	4531	IMNM	(+++)	(++)	N/D	(+)	MTX	Complete
13[&]	55/M	Sinvastatin (20 mg/d)	888/55 U	5187	IMNM	(++)	(−)	N/D	(+)	N/D	Complete
14	76/F	Sinvastatin (40 mg/d)	1217/47 U	12,090	IMNM	(+++)	(+)	N/D	(+)	AZA IVIG	Complete

The cut-off value for a positive result on the home-made (HM) enzyme-linked immunosorbent assay (ELISA) for 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) antibodies was 0.374 absorbance units (Optical Density: OD. 450 nm; 3 SD above the mean in 50 healthy subjects who had never taken statins); a sample was considered positive when it reached more than 20 units in the commercial assay following the indications of the manufacturer.

[†] Deceased not related to IMNM (heart failure); AZA: azathioprine; CK: Creatine kinase, expressed as IU/l Normal value <195); Comm: Commercial ELISA; DM: dermatomyositis; F: female; HALIP: HMGCR antibody associated liver immunofluorescence pattern; IMNM: immune-mediated necrotizing myopathy; IVIG: intravenous immunoglobulin; M: male; MM: Mofetil Mycophenolate; MTX: methotrexate; N/D: not done; all the included patients except number 8, were also treated with glucocorticoids (prednisone 1 mg/kg/d) decreasing the dose 5 mg/per week until withdrawn.

[&] This patient was rechallenged with rosuvastatin and clinical manifestations resumed.

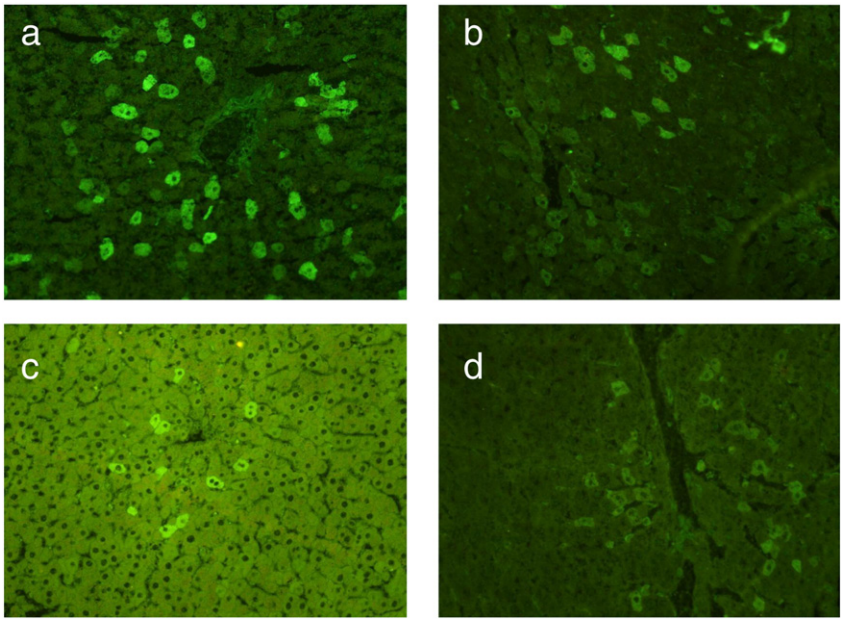


Fig. 3. Anti-HMGCR IFL pattern produced in rat liver by an anti-HMGCR positive serum, using substrate slides from different commercial suppliers. a) Inova Slides. b) Euroimmune. c) BioSystems. d) Aeskulides Diagnostics.

unexpensive standard rat triple tissue auto-immune serology test. The absence of such pattern in patients with other autoimmune diseases or statin-exposed controls indicated that HALIP results identify patients with anti-HMGCR associated myositis reliably compared with the anti-HMGCR auto-antibodies measured by ELISA and confirmed by blot. The immunoabsorption study further confirmed that HALIP recognizes HMGCR in rat tissue.

Taking into account the huge population of patients receiving statins, anti-HMGCR-associated myositis is a rare clinical condition. Its detection is important because early immunosuppressive therapy and/or intravenous immunoglobulin administration may be effective to induce a durable remission [5,15–18]. Accordingly, most of our patients responded to immunosuppression and are now in complete remission.

In our experience, anti-HMGCR autoantibodies are the hallmark of a highly homogeneous disease, the so-called anti-HMGCR associated myopathy, which is triggered by statins, associated to HLA-DR11 and with IMNM biopsy features in elderly patients. But, neither statin exposure, nor the characteristic IMNM biopsy, the HLA or the age of the patient can identify successfully all the patients with the disease. Thus, we consider that the best way to identify this group of patients is based on a thorough assessment of the anti-HMGCR autoantibody status in the presence of enough evidence of an ongoing inflammatory muscle process (using the conventional diagnostic tools suggested by Bohan and Peter [10,11]).

Both statin-exposed and statin-naïve patients were found HALIP positive, suggesting that statin exposure is not required for HALIP

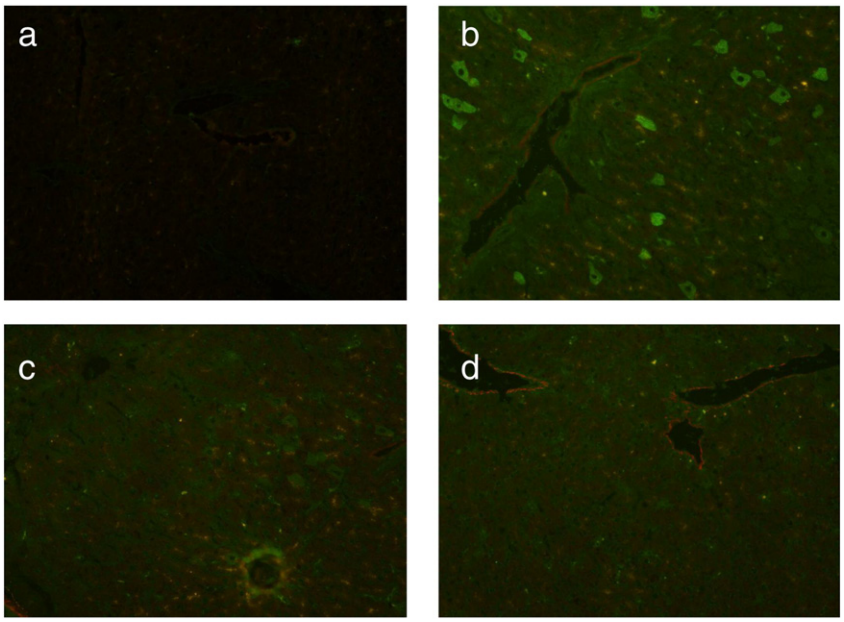


Fig. 4. Immunoabsorption experiment: a) IIF given by an anti-HMGCR negative control sera. b) IIF pattern produced by anti-HMGCR positive serum from patient A. *C. sera* from patient A after immunoabsorption with human antigen HMGCR at concentration of 1.68 μg/m. d) Sera from patient A after immunoabsorption with human antigen HMGCR at concentration of 6.74 μg/ml. (INOVA Diagnostics, Inc., San Diego Lite @ rat kidney/stomach/liver slides).

induction. Besides, classifying anti-HMGCR associated myositis patients on the basis pharmacologic statin exposure may be misleading, since the so-called statin-naïve patients could have been effectively exposed to statins from non-pharmacological sources (like consumption of red yeast extract or some kinds of mushrooms) exposure to which prior to the disease may be difficult to ascertain.

Knowing that human and rat HMGCR share 93% identity (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and HMGCR is highly expressed in liver tissue according with Illumina Body Map (https://www.ebi.ac.uk/gxa/experiments/E-MTAB-513), it is not surprising that human autoantibodies against anti-HMGCR recognize rat HMGCR antigen and thus, can be detected by liver rat IFL. Intriguingly, IFL using HEp-2 cells, in which HMGCR is also strongly expressed, were much less sensitive than rat liver. Most laboratories measure anti-HMGCR antibodies by means of in-house ELISA tests using enzymatic preparations available in the market as antigen. Recently, a commercial ELISA has made this test increasingly available to all laboratories. The IFL pattern that we report here, HALIP, could be of great help in the diagnosis of statin associated autoimmune myopathy because it can be detected by the unexpensive rat triple tissue IFL autoimmune serology which is widely used as a standard technique in most immunology laboratories. Despite this, confirmation of positive results with specific assays will still be required.

In summary, a new distinctive IFL pattern (HALIP) detectable on triple rat IFL autoimmune serology screening is associated with anti-HMGCR autoantibody positivity and statin associated autoimmune myopathy. Awareness of this pattern may help to detect statin associated autoimmune myopathy.

Take-home messages

- A characteristic and specific IFL pattern (HALIP) is herein described. It would help to identify patients with statin-associated autoimmune myopathy in a standard laboratory setting.
- Immunoabsorption and concordance studies indicate that the antigen recognized in the liver by HALIP is HMGCR or a closely related protein.

Contributors

AMS and MAC made the original observation on the HALIP pattern and together with ASOC (guarantor), designed the study. AMS, MAM, LM, and EB, performed the laboratory techniques (ELISA, immunoblot and indirect immunofluorescence). ASOC, JMGJ, PJM, JC, LGM, IPF, and EMA, are responsible for the diagnosis and treatment of the patients with myositis. RP-B contributed to the design of some laboratory tests and to the drafting and revision of the final manuscript. All authors contributed to, and approved, the final manuscript.

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