Clusterin is upregulated in serum and muscle tissue in idiopathic inflammatory myopathies and associates with clinical disease activity and cytokine profile

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Abstract

Objective

The aim of this cross-sectional study was to explore the circulating and skeletal muscle expression of clusterin (CLU) in inflammatory myopathies (IIM) and its potential implication in pathogenetic mechanisms of the disease.

Methods

A total of 85 IIM patients and 86 healthy controls (HC) were recruited. In addition, 20 IIM patients and 21 HC underwent a muscle biopsy. Circulating CLU was measured by ELISA. Serum cytokine profile of patients and HC was assessed by Cytokine 27-plex Assay. Immunohistochemical localisation of CLU was assessed in 10 IIM and 4 control muscle tissue specimens. The expression of CLU and myositis related cytokines in muscle was determined by qPCR.

Results

Serum levels of CLU were significantly increased in IIM patients compared to controls (86.2 (71.6–99.0) vs. 59.6 (52.6–68.4) μg/mL, p<0.0001) and positively correlated with myositis disease activity assessment (MYOACT) (r=0.337, p=0.008), myositis intention-to-treat activity index (MITAX) (r=0.357, p=0.004) and global disease assessment evaluated by physician (r=0.309, p=0.015). Moreover, serum CLU correlated with cytokines and chemokines involved in IIM and their combined effect on disease activity was revealed by multivariate redundancy analysis. In muscle tissue, CLU mRNA was increased in IIM patients compared to controls (p=0.032) and CLU accumulated in the cytoplasm of regenerating myofibres.

Conclusion

We suggest that the up-regulation of clusterin in circulation and skeletal muscle of IIM patients may be an inflammation and atrophy induced response of the organism intended to limit the environment, favouring further muscle damage.

Key words

clusterin, myositis, muscle, inflammation, cytokines

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Introduction

Idiopathic inflammatory myopathies (IIMs) are a heterogeneous group of autoimmune muscle disorders characterised by skeletal muscle weakness and damage, inflammation and extramuscular manifestations affecting skin, lungs and other organs. Laboratory findings include elevated levels of muscle enzymes in circulation and the presence of myositis-specific auto-antibodies (1). Based on clinical, immunological and histopathological features, the IIMs can be divided into several subtypes including dermatomyositis (DM), polymyositis (PM), sporadic inclusion body myositis (IBM) and immune-mediated necrotising myopathy (IMNM) (2-4). Recent findings suggest that immunological as well as non-immunological processes, such as endoplasmic reticulum stress (5), hypoxia (6, 7), mitochondrial (8) and metabolic dysfunction (8-10), are involved in the pathogenesis of IIMs. However, the differences between disease subtypes indicate an unequal contribution of individual pathophysiologic mechanisms and pathways (11).

Clusterin (CLU), also known as apolipoprotein J, is a secreted heterodimeric glycoprotein involved in a variety of physiological and pathophysiological processes. The presence of CLU has been demonstrated in a broad spectrum of body fluids and tissues (12). Its expression is dysregulated in numerous disease states including cancer (13), neurodegenerative (14) and inflammatory disorders (15, 16). CLU acts as a small heat shock protein-like (sHSP) molecular chaperone (17, 18) and exerts cytoprotective and anti-apoptotic properties (19, 20). In addition, CLU has been reported to play a protective function in the development of tissue injury, inflammation, and autoimmunity and is involved in the maintenance of immune homeostasis (15, 21, 22). In a model of apoptotic-cell induced autoimmunity, CLU-deficient mice develop signs of autoimmunity, such as immunoglobulin and complement component deposition or production of autoantibodies (22).

Recent findings suggest a possible role of CLU in myoblast differentiation and proliferation as well as in muscle tissue damage repair and inflammatory response (23). However, very little is known about a potential pathophysiological function of CLU in inflammatory myopathies. A significantly higher skeletal muscle expression of CLU was found in girls with longer duration of juvenile DM compared to those with short disease duration (24). Moreover, CLU immunoreactivity in association with abnormal protein deposits in myofibrillar myopathies and IBM, and in target structures in denervation atrophy was reported (25). The aim of our study was to explore whether there is a dysregulation in the circulating or local expression of CLU in myositis and its potential implication in the pathogenic mechanism of the disease.

Methods

Patients

A total of 85 IIM patients and 86 healthy controls were recruited into this cross-sectional study. For serological analysis, sixty-five patients with IIM (27 with DM, 28 with PM and 10 with IMNM), and 65 age- and sex-matched healthy individuals were included (Table I). Apart from the IIM patients, serum levels of CLU were measured in patients with systemic lupus erythematosus (SLE), representing a cohort with autoimmune disease without muscle involvement (n=56; 5 males, mean age ± SD: 42.8±15.6 years), and in respective age-/sex-matched control subjects HC^{SLE} (n=56; 5 males, mean age ± SD: 43.5±14.3 years). In another group of 20 IIM patients (6 with DM, 10 with PM and 4 with IMNM) and 21 healthy controls, a needle muscle biopsy has been performed and samples were used for gene expression studies (Table II). Additionally, muscle samples taken during the diagnostic open muscle biopsy from a distinct group of 14 patients were investigated by immunohistochemistry (Table III). Patients with PM and DM fulfilled the definite or probable criteria of Bohan and Peter (26), patients with necrotising myopathy were diagnosed using the ENMC criteria (27). All patients were recruited from the inpatient and outpatient departments of the Institute of Rheumatology in Prague during the period of July 2007 to January

2017, therefore the 2017 ACR/EULAR IIM criteria (28) have not been applied retrospectively. All study participants were ≥18 years of age, and each of them signed an informed consent form. The study design and informed consent form were approved by the local ethics committee at the Institute of Rheumatology in Prague. No individual personal data are included.

Clinical disease activity and physical functioning were evaluated by the disease activity core set measures proposed by International Myositis Assessment & Clinical Studies Group (IMACS): myositis disease activity assessment (MYOACT), patient and physician global activity using visual analogue scales (VAS), myositis intention-to-treat activity index (MITAX), manual muscle testing (MMT) and the health assessment questionnaire (HAQ) (29).

Laboratory measurements

Peripheral blood samples were collected and immediately processed. Serum aliquots were stored at -80°C until analysis. Serum CLU levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (BioVendor, Brno, Czech Republic). The detection limit was 5 ng/mL and the detection range of the assay was 5–160 ng/mL. The absorbance was determined by a Sunrise ELISA reader (Tecan, Salzburg, Austria) with 450 nm as the primary wavelength.

Cytokine profile of patients and healthy controls was assessed by Bio-Plex Pro Human Cytokine 27-plex Assay kit (Bio-Rad, Hercules, CA, USA). Data were collected and analysed using a Bio-Plex 200 system and Bio-Plex Manager software v. 6.1 (Bio-Rad, Hercules, CA, USA).

Serum levels of the muscle-associated enzymes [creatine kinase (CK), lactate dehydrogenase (LDH)], C-reactive protein (CRP), as well as glycaemia and serum lipid profile, were measured by routine laboratory techniques from fresh sera after at least 8 hours of overnight fasting in an accredited laboratory of the Institute of Rheumatology in Prague.

Immunohistochemistry

Skeletal muscle tissue specimens were obtained from 5 patients with DM (4 females, mean age ± SD: 39.8±19.7 years), 5 patients with PM (4 females, mean age ± SD: 58.6±13.3 years) and 4 control individuals with no proven inflammatory muscle disease (3 females, mean age ± SD: 50.5±9.0 years). Diagnostic muscle biopsies were performed in all individuals from *m. vastus lateralis* (or *medialis*) using open biopsy technique. The sites of biopsies were selected using magnetic resonance imaging (MRI) (30).

Serial cryostat sections of muscle tissue were fixed in cold acetone. Immunohistochemical labelling of CLU was performed after washing in phosphate buffered saline (PBS), blocking endogenous peroxidase activity by Dual Endogenous Enzyme Block (Dako, Glostrup, Denmark), washing in PBS and blocking in PBS supplemented with 2% bovine serum albumin. Sections were incubated with rabbit monoclonal anti-CLU antibody (ab92548) diluted 1:100 (Abcam, Cambridge, UK) overnight at 4°C, washed in PBS and incubated with peroxidase-conjugate mouse anti-rabbit secondary antibody diluted 1:200 (Dako, Glostrup, Denmark). Isotype-specific antibodies were used as negative controls. Liquid DAB+ Substrate Chromogen System (Dako, Glostrup, Denmark) was used for visualisation. The slides were counterstained with Mayer's Haematoxylin, dehydrated and fixed. The samples were analysed by a BX53 microscope using a DP80 Digital Camera and CellSens Standard Software (Olympus, Center Valley, PA, USA).

Gene expression analysis in samples of skeletal muscle

Samples of skeletal muscle (*m. vastus lateralis*) were taken by Bergström needle biopsy under local anesthesia in the fasted state (31). Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation. Total RNA was isolated from muscle tissue (~15 mg) using TRIzol Reagent (ThermoFisher Scientific, Waltham, MA, USA). Concentration and purity of RNA were tested using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was obtained by reverse transcription with a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA, USA) in a thermal cycler MyCycler (Bio-Rad, Hercules, CA, USA). Quantitative PCR (qPCR) was carried out using TaqMan Gene Expression Assays (ThermoFisher Scientific, Waltham, MA, USA) in the QuantStudio 7 Flex RT-PCR system (ThermoFisher Scientific, Waltham, MA, USA). Data were analysed using the dCt method for relative quantification and expressed as $2^{-dCT}*1000$. The combination of *ACTB* and *RPL13* was used as an endogenous control after evaluation of 4 housekeeping genes (*GAPDH*, *ACTB*, *RPL13,* and *B2M*) by NormFinder software (MOMA, Aarhus University Hospital, Denmark).

Apart from the mRNA expression of CLU, gene expression of a set of cytokines possibly related to myositis pathology was analysed in skeletal muscle samples: cytokines interleukin (IL)-1β, IL-6, IL-8, IL-18, interferon (IFN)-γ, tumour necrosis factor (TNF), transforming growth factor (TGF)-β1; chemokine monocyte chemoattractant protein (MCP)-1; interferon-induced genes Mx-1 and interferon-stimulated gene (ISG)-15; vascular endothelial growth factor (VEGF); atrophy markers Atrogin-1 and muscle RING-finger protein (MuRF)-1; mitochondrial biogenesis inductor peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α; and myogenesis factors paired box (PAX)7 and myogenic factor (MYF)6. Their expression was compared between IIM patients and controls and associated to the CLU gene expression.

Statistical analysis

Data are expressed as median (interquartile range, IQR). The normal distribution was determined by Shapiro-Wilk normality test. According to normality status, Student's t-test or Mann-Whitney test were used for comparison of two groups of subjects. One-way ANOVA or the Kruskal-Wallis test and corresponding *post-hoc* analyses were

Table I. Characteristics of patients with idiopathic inflammatory myopathies and healthy controls with serum samples.

	HC	IIM	DM	PM	IMNM
Number	65	65	27	28	10
Gender, male/female	21/44	19/46	9/18	8/20	2/8
Age, years	44 (37-59)	58 (46-65)*	58 (49-67)*	51 (39-61)	63 (56-72)*
BMI, kg/m^2	25.5 (22.3-28.0)	26.0 (22.3-28.7)	27.4 (23.7-29.9)	25.2 (22.0-27.8)	27.7 (23.6-32.8)
CRP, mg/L	1.5 (0.5-2.5)	2.3 (1.3-4.0)*	2.5 (1.9-4.6)**	2.1 (0.9-3.0)	1.8 (1.3-4.1)
CK, ukat/L	1.7 (1.2-2.1)	3.1 (0.8-11.8)	1.0 (0.6-4.3)	4.4 (1.1-13.5)*	11.8 (6.3-47.9)**
LDH, ukat/L	2.7 (2.4-3.0)	4.5 (3.1-7.5)***	4.1 (3.1-5.1)***	4.5 (2.9-6.6)***	7.5 (4.7-12.0)***
Disease duration from the first symptoms, years	NA	1.2 (0.6-5.6)	1.2 (0.5-10.7)	1.3 (0.6-4.3)	0.8 (0.6-3.9)
Disease duration from diagnosis, years	NA	0.15 (0.03-1.9)	0.18 (0.02-7.5)	0.10 (0.03-0.9)	0.42 (0.05-1.2)
Treatment, GC/MTX/AZA/CPA/CSA	NA	49/16/8/2/1	18/6/3/1/-	23/6/3/1/1	8/4/2/-/-
Prednisone dose equivalent, mg/day	NA	20.0 (8.8-40)	17.5 (4.7-41.3)	20.0 (5.0-40.0)	50.0 (26.3-60)
Patient global disease activity, mm	NA	50.0 (38.5-67.3)	50.0 (36.0-72.0)	50.0 (39.0-67.0)	50.0 (39.3-64.0)
Physician global disease activity, mm	NA	24.0 (11.5-47.0)	15.0 (6.0-40.5)	28.0 (13.8-48.5)	39,5 (10.0-55.8)
MITAX	NA	0.14 (0.08-0.25)	0.13 (0.08-0.21)	0.19 (0.09-0.29)	0.11 (0.06-0.16)
MYOACT	NA	0.06 (0.01-0.15)	0.05 (0.01-0.15)	0.06 (0.01-0.15)	0.01 (0.00-0.05)
MMT8	NA	66.0 (55.0-74.0)	65.0 (55.5-76.0)	71.0 (58.0-74.0)	55.0 (49.5-68.5)
HAQ	NA	0.88 (0.25-1.59)	0.63 (0.25-1.63)	0.88 (0.13-1.53)	1.38 (0.38-1.75)
Autoantibodies:					
ANA nuclear/ANA cytoplasmic	NA	33/4	18/1	12/3	3/0
Jo-1/PL-7/Mi-2/TIF1g/ MDA5/HMGCR/SRP/ MSA negative	NA	12/2/5/4/2/8/2/30	2/1/5/4/2/1/0/12	10/1/0/0/0/1/2/14	0/0/0/0/0/6/0/4
Ro52/Ro60/La/ PM-Scl/U1RNP/Ku72/86/ MAA Negative	NA	16/3/1/7/1/2/40	7/2/1/4/0/1/16	9/1/0/3/1/1/15	0/0/0/0/0/0/9

Data are presented as numbers or median (IQR). *(p<0.05), **(p<0.01),***(p<0.001) - statistically significant difference compared to HC. ANA: antinuclear autoantibodies; AZA: azathioprine; BMI: body mass index; CK: creatine kinase; CPA: cyclophosphamide; CRP: C-reactive protein; CSA: cyclosporine A; DM: dermatomyositis; GC: glucocorticoids; HAQ: health assessment questionnaire; HC: healthy controls; HMGCR: anti-3-hydroxy -3-methylglutaryl-coenzym A reducase antibody; IIM: idiopathic inflammatory myopathy; IMNM: immune mediated necrotising myopathy; Jo-1: anti-histidyl-tRNA synthetase antibody; Ku72/86: anti-72/86 kDa DNA- dependent protein kinase catalytic subunit complex; LDH: lactate dehydrogenase; MAA: myositis associated autoantibodies; MITAX: myositis intention to treat index; Mi-2: anti-nuclear helicase antibody; MMT: manual muscle testing; MTX: methotrexate; MSA: myositis specific autoantibodies; MYOACT: myositis disease activity assessment; NA: not available; PM: polymyositis; PM-Scl: anti-polymyositis-scleroderma antibody; Ro52: anti-52kDa Ro (TRIM21) antibody; Ro60: anti-60kDa Ro antibody; SRP: anti-signal recognition particle antibody; TIF1-γ: anti-transcriptional intermediary factor 1γ antibody; U1RNP: anti-U1 ribonucleoprotein antibody.

Table II. Characteristics of patients and healthy controls with skeletal muscle samples.

	HC	IIM
Number (PM/DM/IMNM)	21	20 (10/6/4)
Gender, male/female	4/17	4/16
Age, years	53 (42-59)	55 (48-67)
BMI, kg/m^2	27.7 (26.6-29.1)	25.7 (23.0-29.3)
CRP, mg/L	1.8 (1.0-4.3)	2.4 (1.0-4.0)
CK, ukat/L	1.5 (1.1-2.4)	1.8 (1.3-8.9)
LDH, ukat/L	3.3 (2.9-3.6)	3.9 (3.6-4.8)
Disease duration from the first symptoms, years	NA	2.6 (0.9-6,9)
Treatment:		
GC/MTX/AZA/CPA/LEF/MMF	NA	16/13/7/2/1/1
Prednisone dose equivalent, mg/day	NA	10.0 (5.0-35.0)
MMT8	NA	63.5 (55.3-70.0)
Autoantibodies	NA	ANA nuclear: 12, ANA cytoplasmic: 4, Jo-1: 4, Mi-2: 2, TIF1γ: 1, HMGCR: 2, SRP: 1, MSA negative: 10, Ro52: 6, Ro60: 3, PM-Scl: 1, U1RNP: 2, Ku72/86: 1, MAA negative: 8

Data are presented as numbers or median (IQR). ANA: antinuclear autoantibodies; AZA: azathioprine; BMI: body mass index; CK: creatine kinase; CPA: cyclophosphamide; CRP: C-reactive protein; CSA: cyclosporine A; DM: dermatomyositis; GC: glucocorticoids; HC: healthy controls; HMGCR: anti-3-hydroxy -3-methylglutaryl-coenzym A reducase antibody;IIM: idiopathic inflammatory myopathy; IMNM: immune mediated necrotising myopathy; Jo-1: anti-histidyl-tRNA synthetase antibody; Ku72/86: anti-72/86 kDa DNA- dependent protein kinase catalytic subunit complex; LDH: lactate dehydrogenase; MAA: myositis associated autoantibodies; Mi-2: anti-nuclear helicase antibody; MMT: manual muscle testing; MTX: methotrexate; MSA: myositis specific autoantibodies; NA: not available; PM: polymyositis; PM-Scl: anti-polymyositis-scleroderma antibody; Ro52: anti-52kDa Ro (TRIM21) antibody; Ro60: anti-60kDa Ro antibody; SRP: anti-signal recognition particle antibody; TIF1-γ: anti-transcriptional intermediary factor 1γ antibody; U1RNP: anti-U1 ribonucleoprotein antibody.

Table III. Characteristics of patients and controls with immunohistochemistry analysis.

Biopsy #	Age, years	Sex, male/female	Diagnosis	Treatment*	Histopathological Features - ENMC criteria
1	56	F	DM	none	c, d
2	8	M	DM	NA	c, d, e
3	48	F	DM	none	c, d, e
4	21	F	DM	none	c, d, e
5	42	F	DM	prednisone 30 mg	c, d, e
6	61	F	PM	none	a
7	54	F	PM	methylprednisolone 4 mg	a
8	57	M	PM	prednisone 40 mg	b
9	33	F	PM	prednisone 60 mg	a
10	67	F	PM	NA	a
11	43	M	C	none	--
12	54	F	C	none	--
13	41	F	C	NA	--
14	60	F	C	none	--

*at the time of biopsy. DM: dermatomyositis, PM: polymyositis, C: control, NA: not available.
ENMC criteria (27):
a: endomysial inflammatory cell infiltrate (T-cells) surrounding and invading non-necrotic muscle fibres.
b: endomysial CD8 þ T-cells surrounding, but not definitely invading non-necrotic muscle fibres, or ubiquitous MHC-1 expression.
c: perifascicular atrophy.
d: MAC depositions on small blood vessels, or reduced capillary density, or tubuloreticular inclusions in endothelial cells on EM, or MHC-1 expression of perifascicular fibres.
e: perivascular, perimysial inflammatory cell infiltrate.

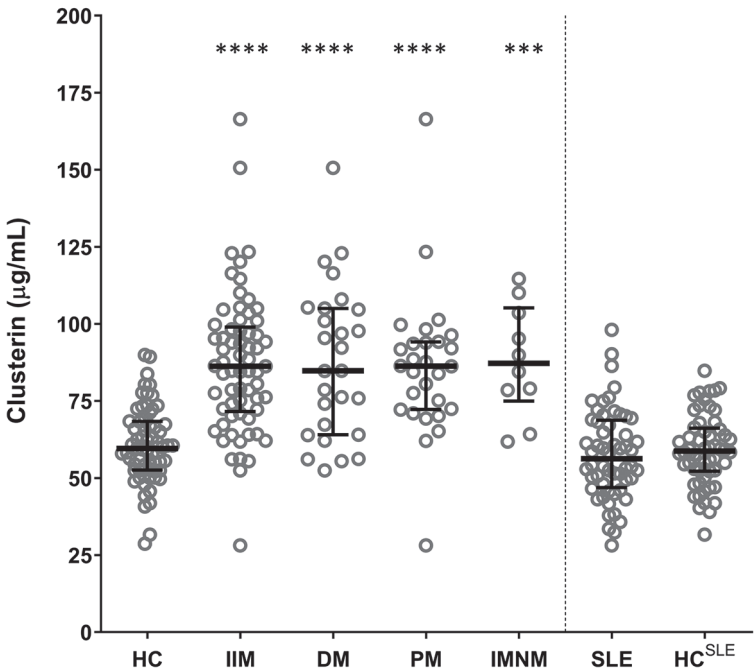

Fig. 1. Serum levels of clusterin are increased in all patients with idiopathic inflammatory myopathies (IIM) as well as in individual IIM subtypes (DM, dermatomyositis; PM, polymyositis; IMNM, immune mediated necrotising myopathy) in comparison to healthy controls (HC) and systemic lupus erythematosus patients (SLE). HC (n=65), IIM (n=65), DM (n=27), PM (n=28), IMNM (n=10), SLE (n=56), HC^{SLE} (n=56). Horizontal lines represent the median and interquartile range. (***p<0.001, ****p<0.0001).

conducted for comparison of data obtained from three and more groups of subjects. The associations of serum and gene expression levels with clinical and laboratory parameters were assessed by Pearson's or Spearman's correlation coefficients, as applicable. A correlation coefficient of 0.1 to 0.3 was considered weak, 0.3 to 0.5 moderate and 0.5 to 1.0 a strong correlation. p-values less than 0.05 were considered statistically significant. The analysis and the graphs were performed using GraphPad Prism 6 software, version 6.05 (GraphPad Software, San Diego, CA, USA).

Principal component analysis (PCA) is a mathematical algorithm that reduces the dimensionality of the data while retaining most of the variation in the data set. It accomplishes this reduction by identifying directions, called principal components, along which the variation in the data is maximal (32). Most multivariate methods of data analysis require that variables be independent or at least uncorrelated. Since cytokines function in an intricate network, they are highly dependent upon one another and do not meet this requirement. Therefore, a redundancy analysis (RDA) was selected to obtain an overview of the combined effects of circulating CLU levels and cytokine profile on disease activity at the whole patients' group level. PCA and RDA were performed in R 3.6.0 software (33) within R Studio 1.1.423 and with libraries ggplot2 (34), ggbiplot (35) and vegan (36). All variables were Z-normalised. For RDA, analysis of variance like permutation test with 999 permutations was applied to evaluate the significance of the individual independent variables as well as the model as a whole. This approach helped us to avoid a problem with possible correlations among numerous independent variables (*e.g.* cytokines). The significantly fitting variables were chosen for the final model. Terms above the level of significance (p≥0.05) were dropped from the final model.

Results

Circulating clusterin is increased in myositis patients and associates with disease activity and circulating cytokine profile

Serum levels of CLU were significantly increased in all IIM patients compared to healthy subjects (86.2 (71.6–99.0) *vs*. 59.6 (52.6–68.4) µg/mL, p<0.0001) and also in individual subsets of patients in comparison with the control group (PM: 86.3 (72.3–94.1) µg/mL, p<0.0001; DM: 84.8 (64.0–105.0) µg/

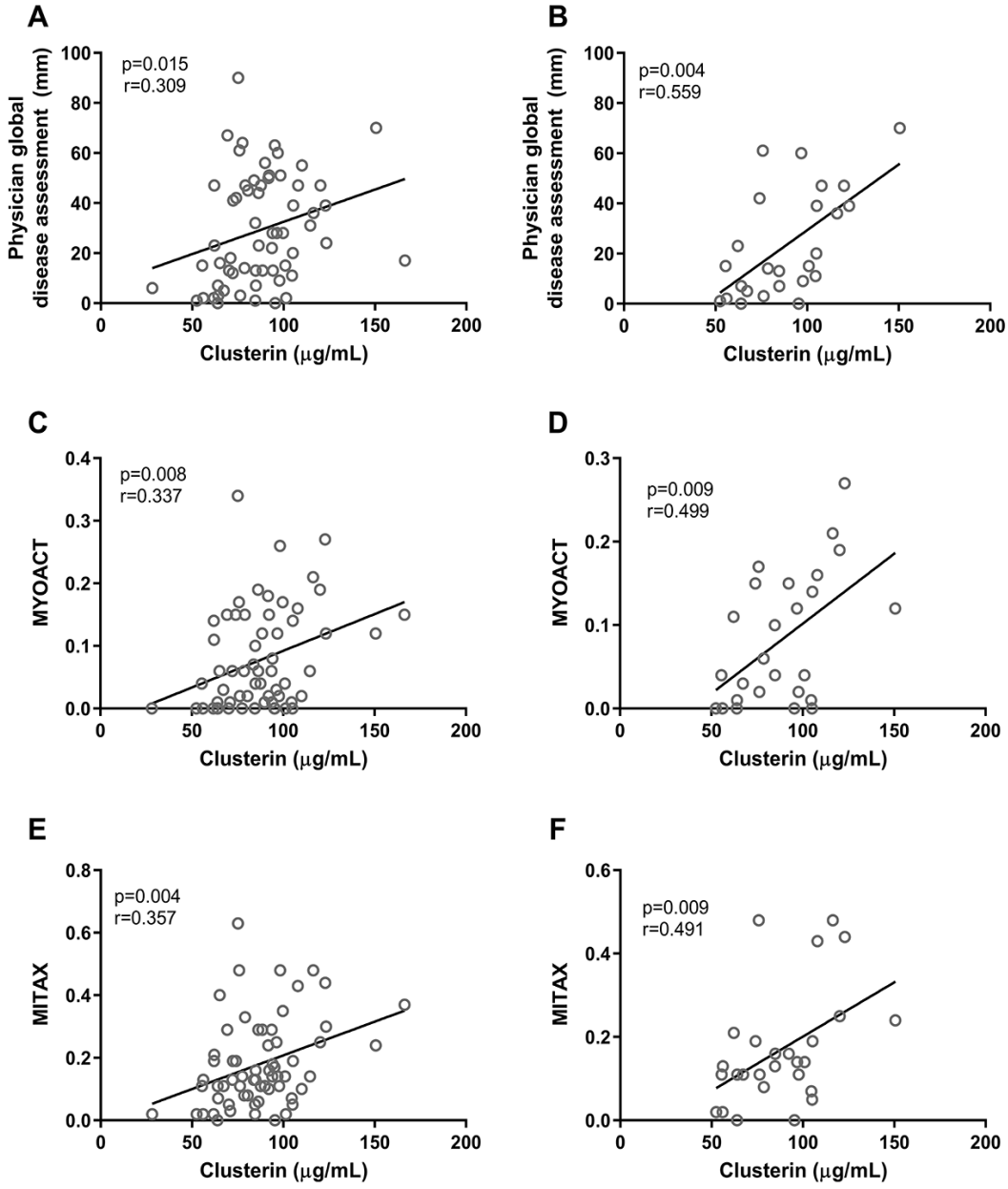

Fig. 2. Clusterin serum levels positively correlate with global disease assessment evaluated by physician, MYOACT and MITAX in all patients (**A**-**C**-**E**) and in patients with dermatomyositis (**B**-**D**-**E**). **A** (n=61), **B** (n=25), **C** (n=61), **D** (n=26), **E** (n=63), **F** (n=27).
MITAX, myositis intention to treat index; MYOACT, myositis disease activity assessment visual analogue scales.

mL, $p<0.0001$; IMNM: 87.2 (75.0–105.2) μg/mL, $p<0.001$). There were no differences in the CLU levels among particular IIM subtypes. CLU levels in patients with SLE were comparable with those in healthy controls (HC^{SLE}) (56.3 (46.9–68.8) *vs*. 58.8 (52.2–66.2) μg/mL, $p=0.538$) (Fig. 1).

CLU levels in all patients with IIM positively correlated with MYOACT (r=0.337, $p=0.008$), MITAX (r=0.357, $p=0.004$) and global disease assessment evaluated by physician (r=0.309, $p=0.015$). After dividing the IIM patients into disease subtypes, only patients with DM showed moderate to strong correlations between the CLU levels and MYOACT (r=0.499, $p=0.009$), MITAX (r=0.491, $p=0.009$) and physician's disease assessment (r=0.559, $p=0.004$) (Fig. 2). Moreover, a significant positive correlation between the CLU levels and HAQ in patients with DM was found (r=0.470, $p=0.014$). No such associations were observed in PM and IMNM subsets of patients.

There was no significant association between the CLU levels and sex, age (r = -0.019, $p=0.883$), body mass index (BMI) (r=-0.121, $p=0.365$), disease duration from the first symptoms (r=0.008, $p=0.951$) or glucocorticoid dose equivalent to prednisone (r=0.091, $p=0.534$). Furthermore, CLU levels did not correlate with the levels of CRP, CK, and LDH in any of the studied groups.

Serum levels of most cytokines/chemokines as detected by Cytokine 27-plex Assay kit were elevated in patients with IIM compared with healthy subjects (Table IV). Serum CLU levels correlated weakly with IL-7, IL-8, IL-12, IL-13, fibroblast growth factor (FGF), interferon gamma-induced protein (IP)-10 and IL-1 receptor antagonist (IL-1Ra) in the whole group of IIM patients (Table IV). Much stronger cor-

Table IV. Summary of correlations between clusterin and cytokines in sera of healthy controls and myositis patients.

	HC [a]		IIM [b]		DM [c]		PM [d]		IMNM [e]	
	r	p	r	p	r	p	r	p	r	p
IL-1b***	0.211	0.097	0.289	**0.027***	0.453	**0.018***	0.166	0.439	0.071	0.868
IL-2	0.219	0.082	0.251	0.089	0.399	**0.044***	-0.020	0.936	0.557	0.624
IL-4***	0.124	0.331	0.198	0.129	0.353	0.077	0.020	0.924	0.222	0.597
IL-5***	-0.161	0.203	0.230	0.085	0.208	0.309	0.368	0.070	0.237	0.651
IL-6	0.276	**0.027***	0.220	0.103	0.359	0.072	0.017	0.938	0.689	0.130
IL-7*	0.274	0.063	0.329	**0.047***	0.451	**0.027***	0.136	0.690		
IL-10*	0.295	**0.018***	0.011	0.942	0.238	0.262	-0.266	0.271	0.245	0.842
IL-12	0.221	0.136	0.374	**0.021***	0.297	0.159	0.485	0.110		
GM-CSF***	0.084	0.507	0.154	0.290	0.368	0.084	-0.137	0.553	0.809	0.098
IFN-g**	-0.057	0.658	0.188	0.153	0.127	0.529	0.322	0.116	0.011	0.981
IL-8***	0.154	0.225	0.327	**0.010***	0.447	**0.019***	0.218	0.284	0.172	0.685
IL-13***	-0.072	0.572	0.267	**0.041***	0.503	**0.010***	0.198	0.332	-0.408	0.316
IL-17*	0.241	0.055	0.156	0.242	0.024	0.911	0.223	0.273	0.267	0.523
MIP-1a***	0.140	0.269	0.122	0.351	0.036	0.858	0.258	0.203	-0.140	0.742
FGF basic***	0.120	0.344	0.273	**0.042***	0.242	0.224	0.257	0.236	0.746	0.089
TNF*	0.051	0.686	0.170	0.191	0.071	0.724	0.225	0.270	0.494	0.213
IL-9*	0.182	0.149	0.094	0.471	0.265	0.182	-0.126	0.539	0.335	0.417
IL-15***	-0.227	0.071	0.177	0.234	0.142	0.518	0.226	0.352	-0.243	0.694
Eotaxin**	0.222	0.078	0.101	0.437	0.019	0.927	0.093	0.653	0.495	0.212
G-CSF***	0.114	0.370	0.214	0.132	0.305	0.147	0.161	0.485	-0.140	0.792
MCP-1***	0.012	0.922	0.119	0.361	-0.007	0.971	0.294	0.144	0.401	0.325
MIP-1b	0.175	0.167	0.115	0.379	0.074	0.715	0.124	0.547	0.322	0.436
IP-10***	0.162	0.202	0.269	**0.036***	0.038	0.851	0.466	**0.016***	0.644	0.085
RANTES	0.095	0.456	-0.143	0.272	-0.314	0.110	-0.111	0.591	0.416	0.306
PDGF-bb***	-0.004	0.975	0.030	0.817	-0.052	0.797	0.033	0.872	0.616	0.104
VEGF***	-0.201	0.111	0.252	0.069	0.436	**0.042***	0.168	0.433	-0.019	0.967
IL-1ra***	-0.055	0.665	0.266	**0.038***	0.408	**0.035***	0.190	0.353	-0.168	0.691

*, **,***IIM values significantly increased compared to HC (*p<0.5, **p<0.01, ***p<0.001).
[a] n=64, [b] n=61, [c] n=27, [d] n=26, [e] n=8.

relation of CLU with IL-1β, IL-2, IL-7, IL-8, IL-13, VEGF, and IL-1Ra levels was seen in DM and with IP-10 in PM patients (Table IV). A weak correlation of CLU serum levels with circulating IL-6 and IL-10 was detected in control individuals.

Clusterin gene expression is elevated in skeletal muscle of myositis patients and localised mainly to regenerating myofibres

CLU mRNA expression in skeletal muscle tissues was significantly increased in patients with IIM compared to healthy donors (p=0.032) (Fig. 3A). There was no difference in the mRNA expression levels between the IIM subgroups and no associations between the CLU expression and clinical or laboratory parameters of the disease were found. Moreover, we did not observe a significant association of serum CLU with CLU gene expression in muscle tissue neither in IIM patients (r= -0.035, p=0.885), nor in HC (r= -0.274, p=0.242).

Patients with IIM showed significantly higher muscle gene expression of IL-6 (p=0.024), TGF-β1 (p=0.007), MCP-1 (p=0.009) and ISG-15 (p=0.015) (Fig. 3B). Moreover, gene expression of CLU correlated significantly with IL-1β (r=0.489, p=0.034), IL-6 (r=0.581, p=0.009), TNF (r =0.485, p=0.035), MYF6 (r=0.548, p=0.012) and PGC-1α (r=0.709, p=0.001) mRNA (data not shown).

Immunohistochemistry performed on skeletal muscle sections from 5 PM and 5 DM patients and 4 controls demonstrated the presence of CLU protein in all muscle specimens represented by weak extracellular staining. In PM and DM samples, CLU accumulation in the cytoplasm of regenerating myofibres was revealed (Fig. 4).

Clusterin is associated with disease activity and pro-inflammatory profile regardless of myositis subtype

First, PCA was performed to see whether there is an association of serum cytokine profile with a particular IIM subtype (Fig. 5A). The first two components of PCA explained together 54.8% of the variability in data. DM patients were characterised by higher values of VEGF, IL-7, IL-12, RANTES, IL-2, IL-15, FGF, IL-17 and IL-10. On the other hand, the lowest levels of all cytokines were observed in IMNM patients. Second, RDA (Df =7, Variance=1.42, F=2.73, p=0.003) was used to obtain an overview of the combined effects of cytokine profile with circulating CLU on disease activity in particular patient groups (Fig. 5B). The first two axes explained together 81.1% of the variance in data. High levels of CLU were associated with high values of MITAX and MYOACT. Moreover, the association of CLU with chemokines MCP-1, MIP-1, and IFN-γ was revealed in the studied group. Patients with the highest muscle disease activity (MDA) had the highest levels of IL-4 and lowest MMT8 values. Patients were classified according to the PM/DM/IMNM subtype to see whether they cluster based on the cytokine+CLU profile. No clustering of subjects after the addition of CLU to the analysis was observed, suggesting that the associations of cytokines+CLU

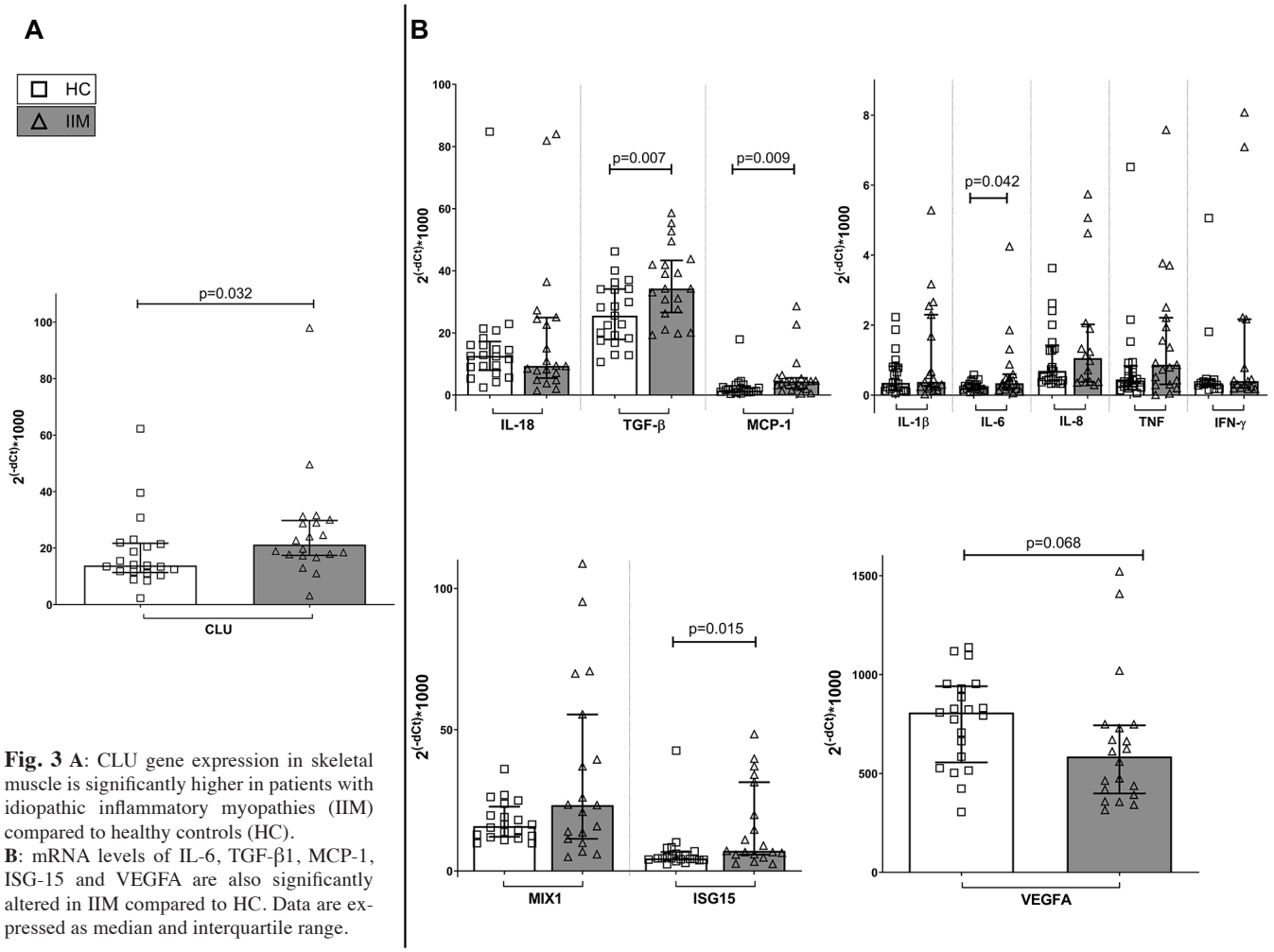

Fig. 3 A: CLU gene expression in skeletal muscle is significantly higher in patients with idiopathic inflammatory myopathies (IIM) compared to healthy controls (HC).
B: mRNA levels of IL-6, TGF-β1, MCP-1, ISG-15 and VEGFA are also significantly altered in IIM compared to HC. Data are expressed as median and interquartile range.

with disease activity measures are not specific for the particular IIM subtype.

Discussion

The present study focuses on clusterin (CLU) involvement in autoimmune inflammatory myopathies. Even though CLU is a well-studied protein with a significant contribution to the pathogenesis of various diseases, there is little evidence on its role in inflammatory myopathies and skeletal muscle biology in general. Herein, we report increased circulating levels as well as skeletal muscle expression of CLU in patients with IIM compared to healthy individuals. Serum CLU was significantly associated with myositis disease activity, particularly in DM patients. In addition to that, circulating CLU was correlated with circulating levels of cytokines and chemokines involved in IIM and their combined effect on disease activity was observed. The expression of CLU in the skeletal muscle of myositis patients was histologically localised to regenerating muscle fibres and CLU mRNA levels correlated with the gene expression of pro-inflammatory cytokines in muscle tissue.

To our knowledge, there are only two studies on CLU with the involvement of myositis patients. Ferrer *et al*. demonstrated an association between the skeletal muscle protein expression of CLU and abnormal protein deposits in myofibrillar myopathies and IBM (24). In the second study, Chen *et al*. (25) found significantly upregulated skeletal muscle gene expression of CLU in children with juvenile DM with long disease duration compared to those with short disease duration (25). The present study shows increased circulating and skeletal muscle gene expression levels of CLU in adult patients with IIM compared to healthy individuals. However, no association of CLU expression with disease duration was observed. The serum CLU in patients with IIM was also increased compared to patients with SLE used as an autoimmune control group. It may be speculated that elevated serum CLU is, at least in this experimental setting, more specific for myositis. CLU upregulation in inflammatory diseases has been reported previously, namely in acute pancreatitis and autoimmune myocarditis (15, 21). In these studies, CLU-deficient mice showed more severe inflammation and tissue destruction, suggesting a protective function of CLU against further damage. Decreased circulating and local tissue CLU has been reported in RA and OA (37, 38). Similarly, a significant decrease of CLU in patients with SLE was observed and CLU correlated inversely with disease activity, arthritis and other disease specific features (39). The lack of CLU and of its protective effect could contribute to the patho-

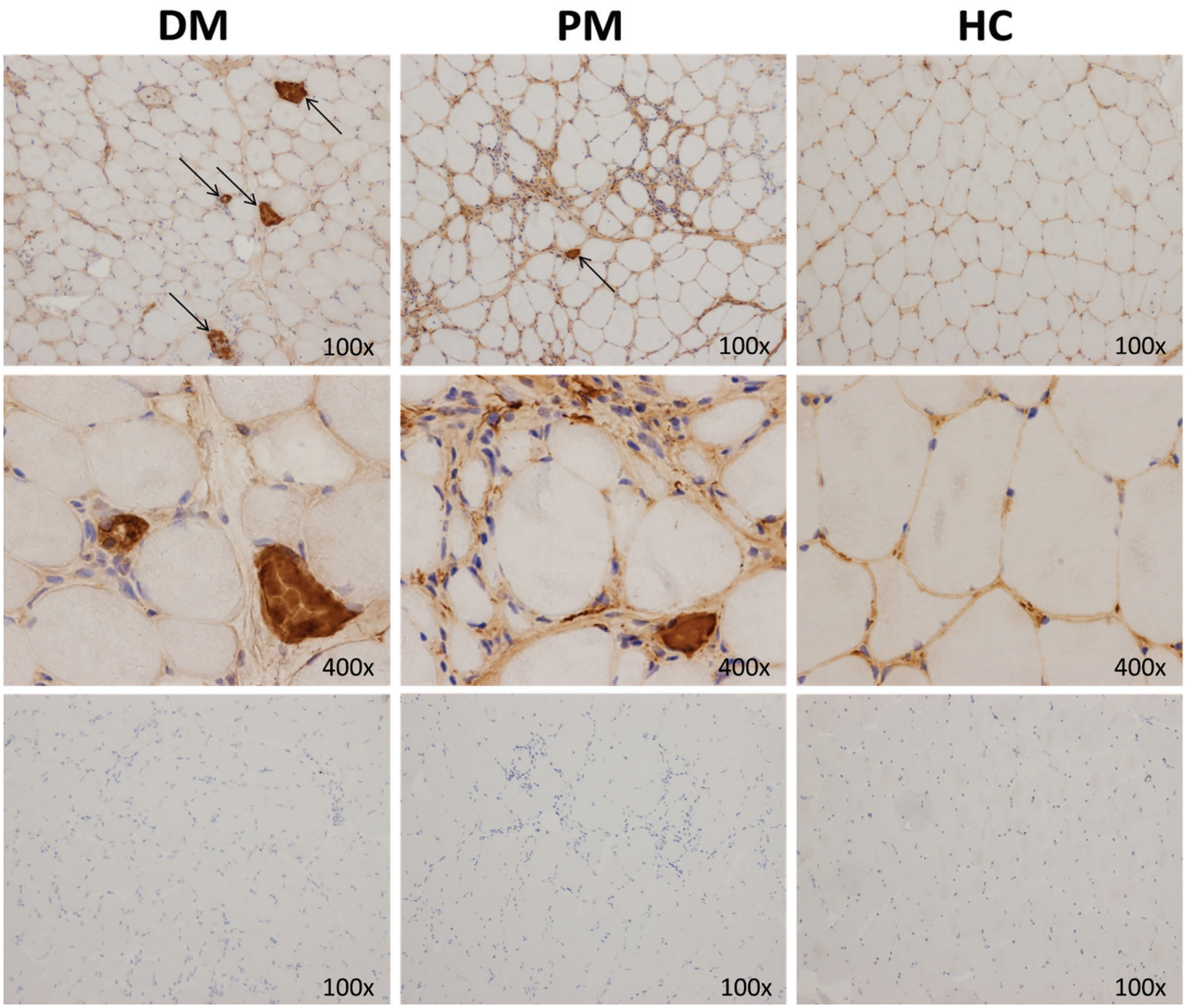

Fig. 4. Immunohistochemistry of clusterin in skeletal muscle tissues revealing weak extracellular staining in all muscle samples. In polymyositis (PM) / dermatomyositis (DM), intense cytoplasmic positivity is present in regenerating myofibres (indicated by arrows). Isotype-specific antibody was used as a negative control. HC, healthy controls.

genesis of those diseases, which is in line with the study of Cunin *et al*. (22) demonstrating signs of autoimmunity in CLU-deficient mice in a model of apoptotic cell-induced autoimmunity.

This study reports a positive relationship between systemic CLU levels and myositis disease activity measures, particularly in patients with DM. The strongest association of CLU with disease activity in DM patients may suggest an involvement of CLU in DM-specific pathogenic pathways, such as vascular damage. CLU is an inhibitor of complement activation and protects cells or tissues from membrane attack complex (MAC) (40, 41) which may have a strong role in autoimmune diseases. Complement activation and subsequent damage of the small vessels in muscle tissue resulting in ischaemic muscle fibre damage is considered one of the crucial processes in the immunopathology of DM (42). Based on several studies (43, 44) showing CLU at sites of vascular damage caused by ischaemia, we can hypothesise that its accumulation in DM may represent an attempt to protect the tissue from damage. Furthermore, CLU can play a role in new vessel formation as it was shown to be implicated in angiogenesis (45-47) and positively correlates with VEGF in our cohort of DM patients (VEGF upregulation in IIM was observed in this study similarly to (6, 48)).

The role of CLU in IIM-associated mechanisms was investigated in a context of inflammatory milieu characteristic for systemic as well as muscle tissue level in myositis. The infiltration of muscle tissue by a variety of activated immune cells that is heavily dependent on the presence of multiple cytokines is one of the shared pathogenic mechanisms between DM, PM, IMNM and IBM (49, 50). It has been reported that CLU is differentially regulated by cytokines, growth factors and stress-inducing agents (51). In our cohort, circulating CLU significantly correlated with

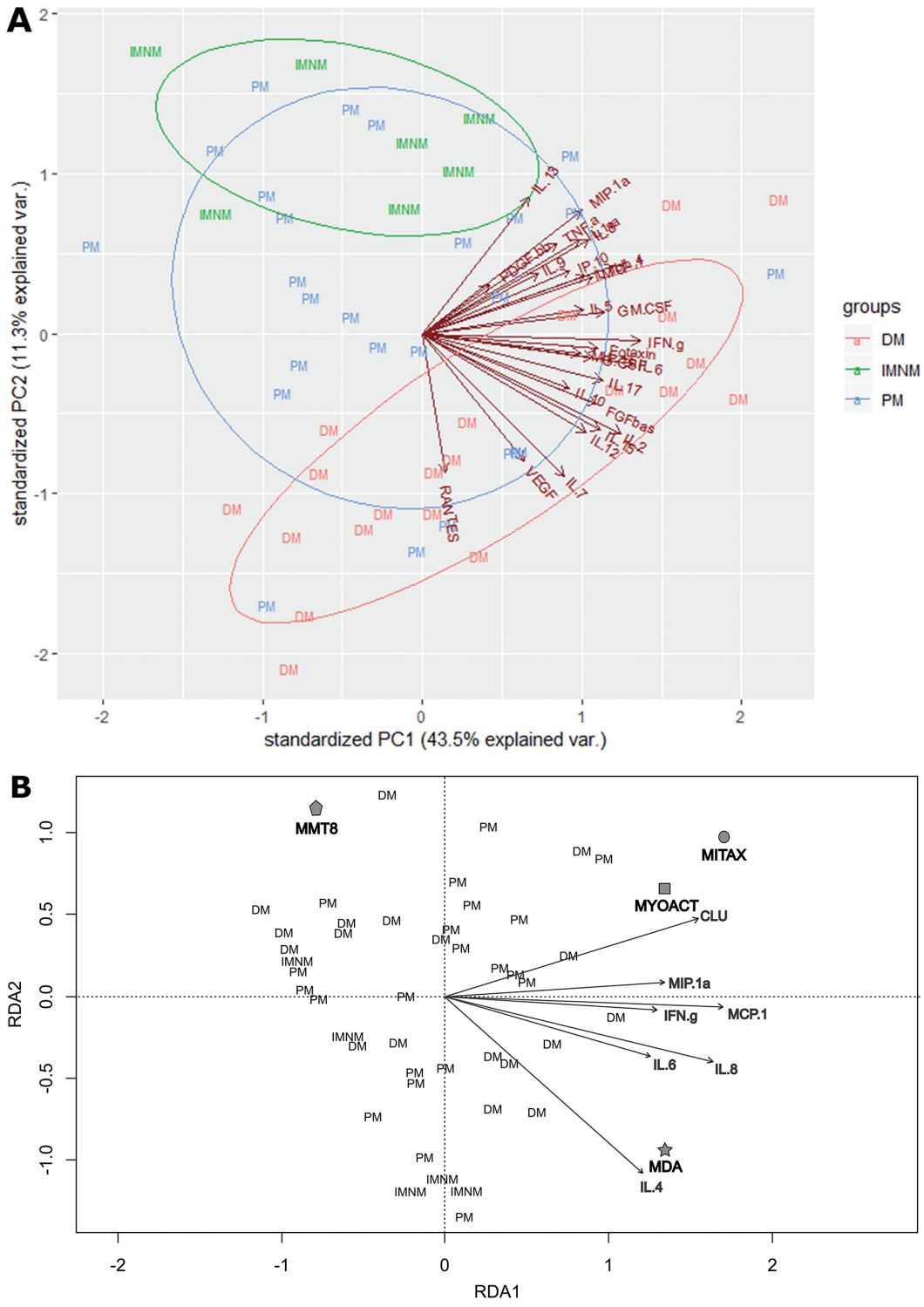

Fig. 5. PCA and RDA plots showing the associations of cytokines with particular IIM subgroups (**A**) and the association of clusterin+cytokine profile with disease activity measures in all IIM patients (**B**).

A: The diagram represents a PCA biplot of a serum cytokines data set. "PM/DM/IMNM" symbols represent particular patient samples classified according to their IIM subtype and plotted in two dimensions using their projections onto the first two principal components PC1 and PC2 represented by the axes.

Reading clue: The lengths of the vectors indicate the strengths of the relationships. The size of the angle between vectors indicates the direction of the correlation: a small angle between two vectors (or a vector and an object) represents a positive correlation; a 90° angle indicates no correlation and opposed vectors indicate a negative correlation.

B: The diagram represents the RDA triplot of CLU and serum cytokines significantly associated with the model. Patients are displayed as "PM/DM/IMNM" symbols. Explanatory variables (CLU, cytokines) are plotted as vectors and response variables (parameters of disease activity) are plotted as geometrical objects. Reading clue is the same as for plot A.

MMT8: manual muscle testing of 8 muscle groups; MITAX: myositis intention to treat index; MYOACT: myositis disease activity assessment visual analogue scales; MDA: muscle disease activity.

cytokine profile in sera of DM patients but not in PM and IMNM. The association of high cytokine levels with DM was also demonstrated by PCA with clear clustering of particular IIM subgroups according to cytokine profile. Moreover, the muscle tissue expression of CLU positively correlated with the expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF, known to stimulate CLU secretion (52). Apart from the cytokine milieu, cell death induced by conditions such as hypoxia can also result in the release of signals that activate CLU gene expression and secretion (53). This is in line with an association of CLU with chemokines MCP-1 and MIP-1 in serum revealed by multivariate RDA analysis in this study. CC chemokines like MCP-1 (CCL2), MIP-1α (CCL3) and MIP-1 (CCL4) are known to be majorly involved in macrophage recruitment and activation (54) and are upregulated in response to muscle injury (55) and muscle ischaemia followed by angiogenesis and regeneration (56).

In the present study, we show a strong expression of CLU in regenerating muscle fibres in patients with IIM. Based on the above-mentioned CLU functions, it could be involved in several stages of muscle regeneration: i) in an early phase following necrosis when the affected site is invaded by leukocytes and macrophages actively secreting cytokines and growth factors and creating a microenvironment promoting CLU upregulation (57); ii) in subsequent activation and differentiation of muscle stem cells followed by maturation of the myofibres and paralleled by formation of new vessels by angiogenesis to revascularise the newly formed myofibres (57). Hypothetically, a strong expression of CLU in regenerating muscle fibres could serve as a protection against pro-inflammatory and hypoxic environment. The presented data suggest that the upregulation of clusterin in circulation and skeletal muscle of IIM patients may be an inflammation and atrophy induced response of the organism intended to limit the development of an environment favouring further muscle damage. However, further studies are needed to establish the exact effect of this multifunctional protein on muscle metabolism and regeneration in myositis.

The study has some limitations. First, the design was cross-sectional. Second, circulating, skeletal muscle gene expression and protein localisation data have been analysed in different cohorts of patients based on the biological material availability. Despite these limitations, this study clearly shows abnormalities in systemic levels and local expression of clusterin and as such it provides new insights into the potential implication of clusterin in myositis pathogenesis.

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