

# Features of repeated muscle biopsies and phenotypes of monocytes in paired blood samples and clinical long-term response to treatment in patients with idiopathic inflammatory myopathy: a pilot study

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## Abstract

### Objective

*In a pilot study we aimed to identify biomarkers in repeated muscle biopsies and paired blood samples, taken before and after conventional immunosuppressive therapy, in order to predict long-term therapeutic response in patients with idiopathic inflammatory myopathies (IIM).*

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### Methods

*Muscle biopsies were selected from 13 new onset patients, six responders and seven non-responders. Repeated muscle biopsies after a median of 11 months follow-up were available from 9 patients and paired peripheral blood mononuclear cells (PBMCs) from 5 patients. Treatment response after 3 years was defined by MMT-8 measuring muscle strength and the ACR/EULAR 2016 improvement criteria. Frozen biopsy sections were immunohistochemically stained for expression of CD3, CD66b, IL-15, CD68, CD163 and myosin heavy chain neonatal (MHCn). PBMCs were analysed by flow cytometry for monocyte phenotypes (CD14, CD16, CD68, CX3CR1, and CCR2).*

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### Results

*Before treatment there were no significant differences in any clinical or muscle biopsy variables or monocyte subsets between responders and non-responders. MMT-8 was significantly higher compared to baseline in the responders at 3-year follow-up. In responders the expression of CD68 in the repeated biopsies was significantly lower compared to non-responders ( $p < 0.05$ ).*

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### Conclusion

*Baseline biopsy, monocyte profile or clinical data did not predict long-term treatment response, but in the repeated biopsy within 1 year of immunosuppressive treatment, the lower number of macrophages (CD68<sup>+</sup>) seemed to predict a more favourable long-term clinical response with regard to improved muscle strength.*

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### Key words

muscle, blood, phenotypes, prognosis, myositis

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## Introduction

The idiopathic inflammatory myopathies (IIM) collectively termed myositis, is a group of systemic autoimmune diseases primarily affecting skeletal muscles and leading to muscle weakness. Other organs are often affected including the skin, joints, heart, and lung (1). Based on different clinical and histopathological features IIM can be divided into dermatomyositis (DM), polymyositis (PM), sporadic inclusion body myositis (sIBM), and more recently also immune-mediated necrotising myopathy (IMNM) (2). Treatment of IIM is based on immunosuppressive agents but response varies among the clinical subsets (3). Patients with sIBM do not usually show improved muscle strength with immunosuppressive treatment and for adult patients with PM, DM or IMNM we do not have any confirmed biomarkers to predict treatment response. There is thus a high unmet need to identify features that could predict clinical improvement following immunosuppressive treatment.

The molecular mechanisms leading to muscle weakness have not been clarified but most patients with IIM have inflammatory infiltrates and cytokines detectable in muscle biopsies, suggesting the important contributions of immune response to the pathogenesis of myositis (4, 5). T cells of both CD4 and CD8 phenotypes as well as macrophages, B cells, and neutrophils may be present (6-8). However, limited information is available on their usefulness in prediction of responsiveness to immunosuppressive treatment. Some data suggest that an apoptosis-resistant subset of T cells, so-called CD28<sup>null</sup> T cells of both CD4 and CD8 phenotype, may predominate and accumulate in the tissue after immunosuppressive treatment in some IIM individuals and thereby contribute to the persistence of inflammation (9). In this context interleukin-15 (IL-15), which is important for T cell activation and differentiation, is of interest as its expression in pretreatment biopsies correlated negatively to improvement of muscle function in patients with DM or PM (10, 11).

Another cell type that may be of importance in relation to treatment re-

sponse is circulating monocytes. Human monocytes can be classified into three subgroups according to the expression of surface markers CD14 and CD16: classical monocytes (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate monocytes (CD14<sup>++</sup>CD16<sup>+</sup>), and non-classical monocytes (CD14<sup>+</sup>CD16<sup>+</sup>) (12). The intermediate subset with varying functions in different conditions is considered a promising predictor of disease activity and treatment response, like rheumatoid arthritis (RA). Intermediate monocytes from healthy donors were reported as the main source of both pro-inflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$  and TNF- $\alpha$ ) and the anti-inflammatory cytokine IL-10 following LPS stimulation in two studies (13, 14). The frequency of the intermediate subset was increased in the peripheral blood of RA patients compared to healthy controls and it also induced Th17 cell expansion *in vitro* (14, 15). The frequency of intermediate monocytes in IIM patients has not yet been investigated.

In this pilot study we aimed to identify biomarkers in repeated muscle biopsies to predict effects of immunosuppressive treatment on muscle performance and disease activity, and to investigate the effect of immunosuppressive treatment on monocyte subsets in paired blood samples and in relation to treatment response.

## Patients and methods

### Patients

We selected 13 newly diagnosed IIM patients (8 women and 5 men) from our myositis registry, SweMyoNet, between 2007 and 2014 from which we had one pre-treatment biopsy, one post-treatment biopsy, and data of clinical disease activity. Of these, six were responders and seven non-responders after 3 years of treatment using the definition of MMT-8 reaching  $\geq 78/80$  together with minimal improvement according to the ACR/EULAR 2016 improvement criteria (Supplementary Table I). Their diagnoses at the time of the first biopsy were: DM (definite n=4), PM (definite n=4, probable n=3), IBM (definite n=2) based on the 2017 EULAR/ACR classification criteria (16). The median disease duration, defined as

**Table I.** Clinical information of each patient.

Pat. no.*	Sex/age <sup>+</sup>	Diagnosis <sup>#</sup>	Auto-antibody	Duration between 2 biopsies**	Treatment between 2 biopsies	Extra- muscular disease	Clinical outcome
1	M/34	DM (definite)	Neg	9	GC, MTX	A, SR	responder
2	F/60	PM (definite)	Jo-1	13	GC, CyA, MMF	ILD	responder
3	F/67	PM (probable)	Neg	8	GC, MTX	Dys	responder
4	M/56	PM (definite)	Jo-1	NA	GC, Cycloph, MMF	ILD	responder
5	F/57	PM (definite)	Jo-1	13	GC, Cycloph	ILD, Dys, R	responder
6 <sup>++</sup>	M/59	DM (definite)	PM/Scl	NA	GC, Cycloph	ILD, SR	responder
7	F/73	PM (definite)	Neg	NA	GC, MTX	Dys	non-responder
8	M/40	DM (definite)	Ro52, MDA5	11	GC, Cycloph, AZA	ILD, SR	non-responder
9	F/57	PM (probable)	U1RNP	8	GC, MMF	A, Dys, R	non-responder
10	M/46	DM (definite)	Neg	13	GC, MTX, AZA, MMF	A	non-responder
11	F/69	IBM (definite)	SSA	8	GC, MTX, Anakinra	Dys	non-responder
12	F/69	PM (probable)	Neg	11	GC, AZA, MTX	A	non-responder
13	F/63	IBM (definite)	Neg	11	GC, MTX	Dys	non-responder

\*Patient number; <sup>+</sup>M: male, F: female. Age at the baseline (diagnosis) point presented by years.

<sup>#</sup>Diagnosis based on the baseline muscle biopsy. PM: polymyositis, DM: dermatomyositis, IBM: inclusion body myositis.

\*\*Duration between two muscle biopsies presented by months; <sup>++</sup>Patient was given treatment before the baseline muscle biopsy.

Jo-1: histidyl transfer RNA (tRNA) synthetase; PM/Scl: a nuclear/nucleolar particle composed of several polypeptides of which two have been identified as autoantigens; Ro52: 52 kDa ribonucleoprotein autoantigen Ro; MDA5: anti-melanoma differentiation-associated gene 5; U1RNP: U1 small nuclear ribonucleoprotein 70 kD; SSA: Sjögren's syndrome A, also known as Ro, ribonucleoprotein autoantigen; GC: glucocorticoid; MTX, methotrexate; Cycloph: Cyclophosphamide; CyA: cyclosporine A; MMF: Mycophenolate Mofetil; AZA: azathioprine. NA: not available. A: arthritis; SR: skin rash; ILD: interstitial lung disease; Dys: dysphagia; R: Raynaud.

time from onset of symptoms relating to IIM and diagnosis was 13 months, and median age at time of diagnosis was 65 years (range 45–84). Demographic data and clinical characteristics are summarised in Table I. Clinical information was collected from time of diagnosis, at 1- and 3-year follow-up. Treatment was based on a combination of glucocorticoids and immunosuppressive agents according to the decision of the treating physician (Table I).

Autoantibody profile was determined by RNA and protein immunoprecipitation including Jo1, PL12, PL7, OJ, EJ, KS, Mi2, MDA5, TIF1 $\gamma$ , SRP, PmScl, Ro/SSA, U1RNP, and Ku in collaboration with Prof. T. Mimori, Kyoto, Japan. Sera collected after 2013 were screened by line-blot assay (Euroline myositis panel 4 by Euroimmun, Lübeck, Germany) in collaboration with Prof. J Rönnelid, Uppsala University, Uppsala, Sweden.

#### ACR/EULAR 2016 improvement criteria

The improvement score (TIS) (from 0–100) is calculated by adding the values of each core set measure of the International Myositis Assessment and Clinical Studies (IMACS) disease activity score (17, 18). The scores  $\geq 20$ ,  $\geq 40$ , and  $\geq 60$  represent minimal, mod-

erate, and major improvement, respectively. Each component of IMACS core set measures at 3-year follow-up and the clinical outcome are presented in Supplementary Table I.

#### Muscle biopsies

The first muscle biopsy was taken for diagnostic purposes before treatment (pre-treatment biopsies) except for one patient who was given intravenous glucocorticoids for three days before the biopsy due to medical need. A repeat muscle biopsy after a median of 11 months (8–13 months) of treatment was available for 9/13 patients (post-treatment biopsies). The post-treatment biopsies of the other four patients were excluded due to poor quality in one, and more than 16 months between the two biopsies for the other three. The paired muscle biopsies included are presented in Supplementary Table II.

Muscle biopsies were taken from vastus lateralis or tibialis anterior muscles using a semi-open approach under local anesthesia and frozen in isopentane pre-chilled by liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until sectioned (19). Frozen muscle biopsies were cut into  $7\mu\text{m}$  sections using a cryostat (Cryostar NX70, Thermo Scientific, US), placed on chrome-gelatine slides, then air dried for 30 minutes. Some sections were fixed with

2% formaldehyde before freezing. All sections were stored at  $-80^{\circ}\text{C}$  until use. Muscle biopsies were evaluated by an experienced neuropathologist (I.N.) and variables were scored from 0 to 3+ for level for lesion severity. A summary of the histopathological features is summarised in Supplementary Table III. All samples were taken with the patients' written informed content. The study was approved by the regional ethics committee in Stockholm, Sweden.

#### Immunohistochemistry staining

CD3 (T cells), CD66b (neutrophils), CD68 and CD163 (macrophages), IL-15, and myosin heavy chain neonatal (MHCn) were investigated by immunohistochemical staining. For expression of CD3, CD66b, and IL-15 sections were fixed in 2% formaldehyde and for expression of CD68, CD163, and MHCn acetone-fixed slides were used (50% acetone followed by 100% acetone). The staining protocol applied was the same as previously reported (8, 11, 20). Tonsil sections served as positive controls. Information concerning the antibodies used is presented in Supplementary Table IV.

#### Quantification of immunohistochemistry

Stained sections were evaluated on

coded slides using a Reichert Polyvar II microscope (Reichert-Jung, Vienna, Austria). Quantification was performed using computerised image analysis with a Leica DMRXA2 (Leica, Cambridge, UK) and a Leica digital camera DFC450 C (Leica, Cambridge, UK). CD3, CD66b, and IL-15 expression was evaluated as the number of positively stained mononuclear cells per mm<sup>2</sup> in the whole section. The expression of CD68 and CD163 was evaluated as the percentage of positively stained area of total tissue area (mm<sup>2</sup>). MHCn expression was estimated as number of positively stained muscle fibres divided by the total number of fibres. The number of positively stained cells of CD3, CD66b, and IL-15 was counted by two independent persons (QT and CW or IEL) and the mean value was used for analysis.

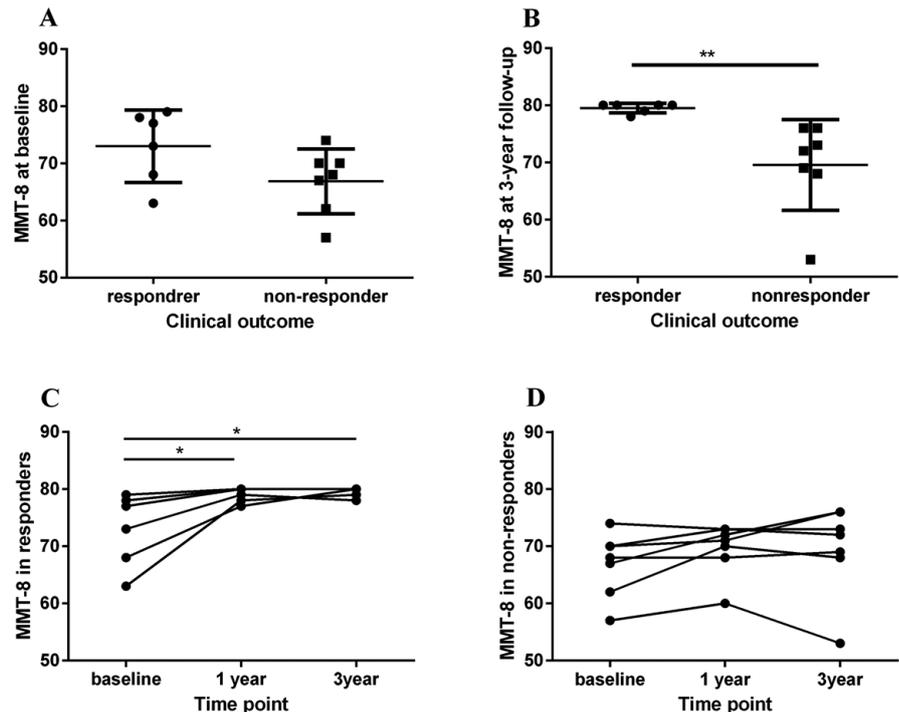
#### Flow cytometry acquisition of peripheral blood mononuclear cells

Paired frozen peripheral blood mononuclear cells (PBMCs) were available from 5/9 patients taken at the similar time as the muscle biopsies (Suppl. Table II) and 3 healthy controls.

These samples were thawed, filtered through a Falcon 40 µm cell strainer and counted using a Scepter™ automated cell counter (Millipore, Germany). Single cell suspensions (1x10<sup>6</sup> cells per sample) were plated in 96-well V-bottom plate and stained with the antibodies specific either for surface markers CD14, CD16, CD68, CX-3CR1, and CCR2 (used to define the subsets of monocytes) (all from Biolegend, Germany), or for surface markers CD3, CD4, CD8, CD25 and CD127 (to define subsets of T cells), all from BD Biosciences, Germany. Dead cells were identified and removed from analysis using LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen, Germany). Cell samples were run in a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and analysed using Kaluza v. 1.1 software (Beckman Coulter).

#### Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 (San Diego, California, USA). Mann-Whitney U-test was used for the comparison between



**Fig. 1.** MMT-8 of the patients.

A: Comparison of MMT-8 between responders and non-responders at baseline point.

B: Comparison of MMT-8 between responders and non-responders at 1-year follow-up point.

C: Comparison of MMT-8 at baseline point, 1-year follow-up, and 3-year follow-up in responders.

D: Comparison of MMT-8 at baseline, 1-year follow-up, and 3-year follow-up in non-responders.

\* means significantly different. \* $p < 0.05$ , \*\* $p < 0.01$ .

two independent groups. Fisher's exact test was used to calculate the differences of basic clinical information between responders and non-responders. Wilcoxon matched-pairs signed rank test was applied to compare two paired groups. Friedman test was used to calculate the differences of MMT-8 at three time points with paired samples, followed by a Dunn's post-test to correct for multiple comparisons. Spearman's Rank correlation was applied to calculate the correlation between molecular expression in muscle tissue and clinical outcome measures. The coefficient value of 0.10–0.69 represented a weak correlation, 0.40–0.69 a moderate correlation, 0.70–0.89 a strong correlation, and 0.90–1.00 a very strong correlation (21).  $p < 0.05$  was considered to be significantly different.

## Results

### Clinical results

Before treatment no significant difference in MMT-8 was evident between responders and non-responders (Fig. 1A), but the MMT-8 value for respond-

ers was already significantly higher compared to non-responders at 1-year follow-up (median of responders: 79.50, non-responders: 71.00,  $p < 0.05$ , data not shown) and this difference was maintained at the 3-year follow up (median of responders: 80.00, non-responders: 72.00) ( $p < 0.01$ , Fig. 1B-C-D).

Conventional histopathology assessment is summarised in Supplementary Table III. The number of patients with regenerating/degenerating fibres, endomyrial infiltrates, perymysial infiltrates, necrotic fibres, and perifascicular atrophy was similar in the post-treatment biopsies compared to the pre-treatment muscle biopsy. In pre-treatment muscle biopsies, two patients (non-responders) had rimmed vacuoles and were both diagnosed with IBM. One additional patient (responder) had rimmed vacuoles in the second biopsy (diagnosis PM).

### Expression pattern of different markers in pre-treatment muscle biopsies

Using immunohistochemistry we observed inflammatory cells in infiltrates or as scattered cells in the pre-treatment

**Table II.** Immunohistochemistry data of muscle biopsies before and after treatment for T cells (CD3), neutrophils (CD66b), macrophages (CD68, CD163), IL-15, and neonatal myosin heavy chain (MHCn).

Pat. no.	CD3*		CD66b*		CD68 <sup>+</sup>		CD163 <sup>+</sup>		IL-15*		MHCn <sup>#</sup>	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
<b>Responders</b>												
1	13.0	NA	1.1	NA	0.3	NA	0.2	NA	0	NA	0.005	NA
2	6.5	2.1	63.4	4.3	0.2	0.04	0.2	0.04	17.7	0.5	0.06	0.03
3	266.0	7.4	8.7	21.2	3.7	0.1	5.2	0.2	1.2	0.3	0.4	0.08
4	2.3	NA	5.1	NA	0.02	NA	0.06	NA	0.9	NA	0.1	NA
5	17.2	17.4	5.5	21.5	0.9	0.1	0.9	0.4	1.4	2.6	0.02	0.03
6	21.2	NA	35.7	NA	0.7	NA	0.8	NA	11.6	NA	0.1	NA
Median	15.1	7.4	7.1	21.2	0.5	0.1	0.5	0.2	1.3	0.5	0.07	0.03
<b>Non-responders</b>												
7	0	NA	2.2	NA	0.06	NA	0.03	NA	0.9	NA	0.04	NA
8	5.8	2.0	12.4	1.4	0.5	0.4	0.4	0.3	3.5	2.6	0.001	0.3
9	217.4	3.2	1.5	4.2	1.7	0.3	1.4	0.2	1.6	1.5	0.4	0.02
10	0	10.0	3.0	1.7	0.02	0.3	0.04	0.1	0.8	0.05	0.006	0.008
11	74.9	18.6	2.2	3.3	0.4	0.2	0.4	0.1	1.9	0.6	0.4	0.1
12	6.1	6.0	17.2	20.0	0.7	0.2	0.07	0.2	5.6	14.7	0.009	0.08
13	0	39.6	2.1	1.6	0.05	0.4	0.04	0.1	0	1.4	0	0.06
Median	5.8	8.0	2.2	2.5	0.4	0.3	0.07	0.2	1.6	1.4	0.009	0.07

\*Numbers of CD3, CD66b, and IL-15 indicate the number of positively stained mononuclear cells per mm<sup>2</sup> in the whole section.

\*Numbers of CD68 and CD163 mean the percentage of positively stained area of total tissue area (mm<sup>2</sup>).

#Numbers of neonatal myosin heavy chain indicate the ratio of positively stained fibres divided by the total number of fibres.

NA: Not available. Responders: patients with MMT-8 ≥78 and at least minimal improvement according to ACR/EULAR 2016 improvement after three years of treatment. Non-responders: patients with MMT-8 <78 and no improvement according to ACR/EULAR 2016 improvement criteria after three years of treatment.

biopsies from all the patients. CD3<sup>+</sup> T cells were present in 10/13 patients and the location of the infiltrates was predominantly surrounding the muscle fibres (Table II) (Fig. 2A). Occasional neutrophils were observed in biopsies from all 13 patients, mainly in perimysial areas (Table II).

CD68<sup>+</sup> and CD163<sup>+</sup> macrophages were detectable in all 13 patients, mainly surrounding and occasionally invading necrotic muscle fibres (Table II; Fig. 2B-C). IL-15<sup>+</sup> mononuclear cells were observed in biopsies of 11 patients (Table II; Fig. 2D). MHCn was expressed in scattered muscle fibres in 12 patients (Table II; Fig. 2E).

*Expression pattern of markers in post-treatment muscle biopsies*

Repeated muscle biopsies from nine patients were included in the analysis (Suppl. Table II).

Using immunohistochemistry we detected inflammatory cells in all post-treatment biopsies either as infiltrates or as scattered cells. CD3<sup>+</sup> T cells, neutrophils, CD68<sup>+</sup> and CD163<sup>+</sup> macrophages, IL-15<sup>+</sup> cells, and fibres expressing MHCn were present in all nine biopsies. The localisation and compo-

sition of the stained markers were similar to the baseline biopsies. The median expression of investigated markers in responders and non-responders after treatment is presented in Table II.

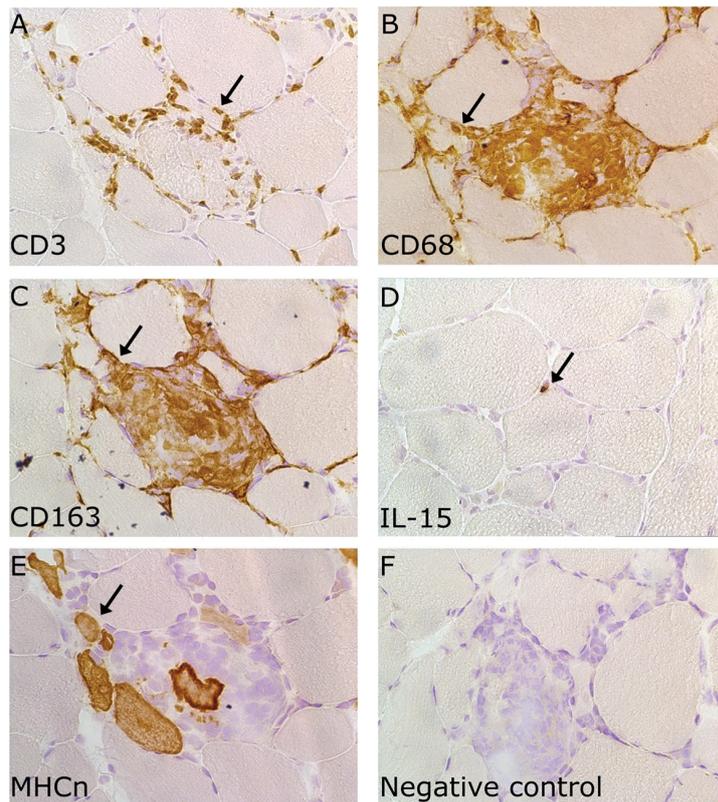
*Molecular expression in muscle tissue in responders and non-responders before and after treatment in relation to clinical response*

In the pre-treatment biopsies there were no significant differences in the expression of CD68 between responders and non-responders (Fig. 3A). In the post-treatment biopsies the expression of CD68 in responders was significantly lower than that in non-responders after 1-year treatment, although post-treatment biopsies were only available from three responders ( $p < 0.05$ , Fig. 3B). The expression of CD68 showed a within-group trend towards decrease after treatment in responders (median before: 0.87, after: 0.13), but there was no trend in non-responders (Fig. 3C-D). The same pattern was observed for the expression of CD163 and IL-15. For other markers (CD3, CD66b, and MHCn), there were no changes observed after treatment in either responders or non-responders.

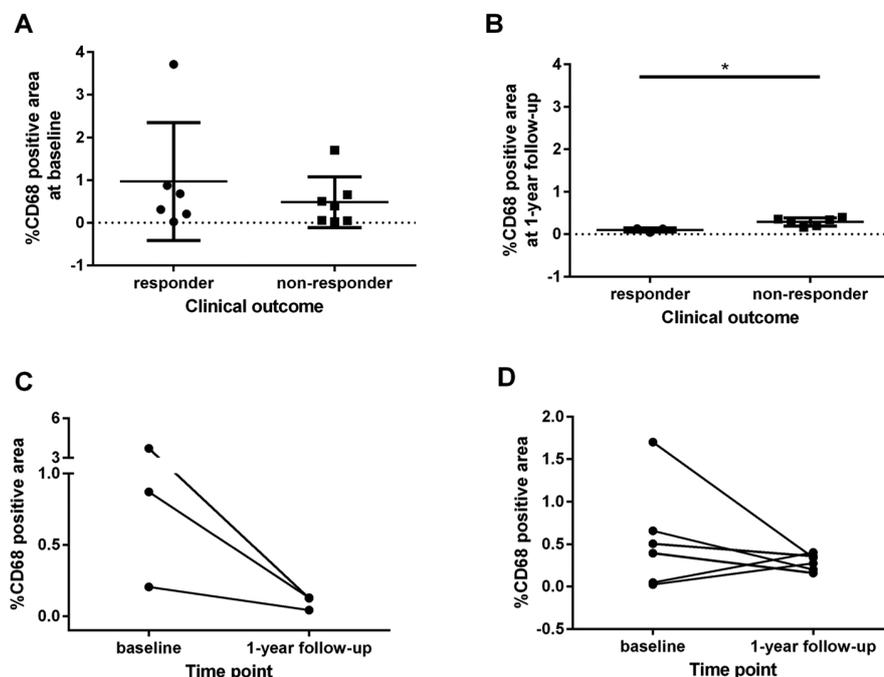
*Correlations between muscle biopsy variables and clinical data*

In the pre-treatment biopsies there was a strong correlation between CD68 expression and pre-treatment serum levels of LD ( $r_s = 0.7671, p = 0.005$ ) and a moderately negative correlation with MMT-8 ( $r_s = -0.5641, p = 0.04$ ). In the post-treatment biopsy the muscle biopsy expression of CD3 correlated positively to: aspartate aminotransferase (ASAT) ( $r_s = 0.8333, p = 0.0083$ ), and creatine kinase (CK) levels at the 1-year follow-up ( $r_s = 0.8619, p = 0.004$ ).

We did not find any markers expressed in muscle biopsies in the pre-treatment biopsies that correlated with the respective clinical variables at 1- or 3-year follow-ups, and there were no significant correlations between expression of molecular markers in post-treatment muscle biopsies and clinical variables at 3 years follow-up. The clinical variables include muscle enzymes (ASAT, ALAT, LD, CK), global response, and each domain of the response criteria (health assessment questionnaire (HAQ), physician’s global disease activity assessment, patient’s global disease activity assessment, extramuscular disease activity, and MMT-8).



**Fig. 2.** Immunohistochemistry staining of (A) CD3, (B) CD68, (C) CD163, (D) IL-15, (E) myosin heavy chain neonatal (MHCn), and (F) negative control of mouse IgG1 in the same area of muscle sections from one PM patient. Brown is positive staining. The black arrows indicate the positive cells or muscle fibres. Blue is stained for nucleus by haematoxylin. The magnification is 250x.



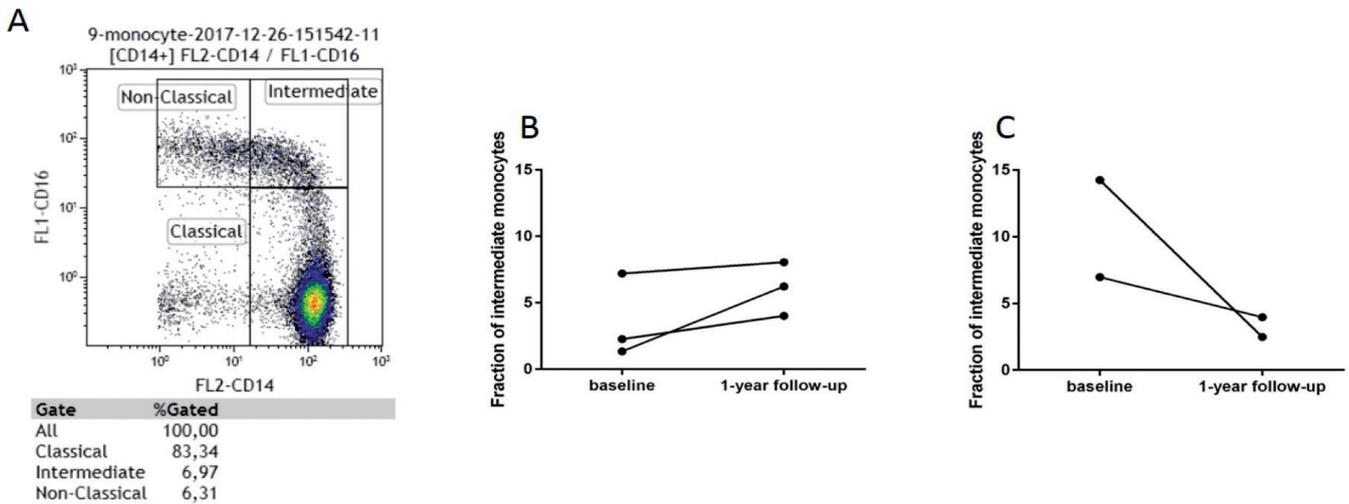
**Fig. 3.** The percentage of CD68 positive area of the whole tissue squares of the patients. **A:** Comparison of percentage of CD68 positive area between responders and non-responders at baseline point. **B:** Comparison of percentage of CD68 positive area between responders and non-responders at 1-year point. **C:** Comparison of percentage of CD68 positive area at baseline point, and 1-year follow-up in responders. **D:** Comparison of percentage of CD68 positive area at baseline point, and 1-year follow-up in non-responders.

*Blood sample results*

Paired blood samples from five patients, 3 responders and 2 non-responders, were available. Three healthy controls were included. Numbers of classical monocytes, intermediate monocytes, and non-classical monocytes were assessed in the blood samples (Fig. 4A). The expression of the intermediate subset of monocytes showed a trend towards an increase in responders, while the opposite was the case in non-responders (Fig. 4B-C). For CD3<sup>+</sup> T cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> T regulatory cells (Treg), we did not find any changes. We did not observe differences between both blood samples from patients and samples from healthy controls.

**Discussion**

In this pilot study we investigated repeated muscle biopsies and blood samples in patients with IIM in order to identify potential biomarkers that could predict response to treatment. In pre-treatment biopsies we did not determine any difference concerning presence of inflammatory cells or signs of muscle regeneration between responders and non-responders. In the post-treatment biopsy there were still inflammatory cells detectable in all biopsies but the expression of CD68 was significantly lower in patients who were classified as responders compared to in non-responders. There was also a trend towards a decreased expression of CD68 and CD163 as well as of IL-15 in the responders in the post-treatment biopsies, but not in the non-responders. We also found correlations between histological markers (CD68, CD3) and muscle enzymes and MMT-8 in different time points, but no correlations with other domains of response criteria. Patients with IIM are usually treated with high doses of glucocorticoids in combination with other immunosuppressive drugs with varying outcomes, and a substantial number of patients develop persisting muscle weakness. To date, no biomarker has been identified to predict treatment response. As skeletal muscle is the major organ targeted by the immune system in patients with IIM and muscle weakness and low muscle endurance are



**Fig. 4.** The fraction of monocytes in blood samples.

**A:** Gating strategy of classical, intermediate, and non-clinical monocytes. **B:** The comparison of fraction of intermediate monocytes in responders between baseline point and 1-year follow-up. **C:** The comparison of fraction of intermediate monocytes in non-responders between baseline point and 1-year follow-up.

the predominating clinical symptoms we aimed to focus on possible biomarkers in muscle biopsies that could predict improved muscle performance and disease activity in patients with IIM with conventional immunosuppressive treatment. With this aim we defined responders as patients that achieved a muscle performance test of  $\geq 78/80$  of the MMT-8 test together with minimal improvement of disease activity according to the ACR/EULAR 2016 response criteria. In this pilot study we included 6/13 patients with IIM that were classified as responders after 3 years of immunosuppressive treatment. The definition of improvement was already fulfilled after 1 year of immunosuppressive treatment for 5/6 responders, and there were no significant changes in the MMT-8 test as a measure of muscle strength between 1-year and 3-year follow-up in neither responders nor non-responders. This suggests that the muscle strength attained at 1 year after diagnosis and treatment is stable even later on and may be indicative of the muscle improvement prognosis at a later time point.

The molecular mechanisms that cause muscle weakness in patients with IIM have not been clarified in detail. Based on different histopathological features and consistent findings related to the degree of muscle weakness, immune mechanisms have been proposed to contribute (4, 22). Interestingly, in this study we observed a moderate nega-

tive correlation between expression of CD68<sup>+</sup> macrophages in muscle tissue taken before treatment and muscle strength measured by MMT-8 at baseline, and a strong correlation between CD68<sup>+</sup> expression in muscle tissue and serum levels of LD that may reflect leakage of muscle enzymes. These data could indicate that infiltrating macrophages in muscle tissue have an association with muscle weakness, but the underlying molecular mechanisms remain to be clarified.

CD3<sup>+</sup> T cells were detected in 10/13 muscle biopsies taken before treatment and were still detectable after treatment in both responders and non-responders. The expression of CD3<sup>+</sup> T cells in the post-treatment biopsy correlated strongly to serum levels of the muscle enzymes CK and AST at the 1-year follow-up. These data might indicate that T cells or subsets of T cells have a role in muscle weakness but the causality is still unclear. A change in frequency of T cell phenotypes, with a reduced number of FOXP3<sup>+</sup> regulatory T cells following glucocorticoids and immunosuppressive treatment, but a higher frequency of apoptosis-resistant so-called CD28<sup>null</sup> T cells has previously been suggested to have a role in the chronicity of PM and DM (23). However, the phenotype of T cells was not subject to our investigation as the number of biopsies after treatment was low. Some discrepancies were evident in the staining between bi-

opsies used for routine histopathological evaluation and biopsies included for this research project. One explanation could be that some changes are focal, and that different muscle biopsy sample areas were analysed and different staining protocols were used.

The observed reduction of macrophage expression in muscle tissue of patients with IIM after treatment might indicate that these cells were sensitive to given immunosuppressive treatment, in concordance with previous reports (11). We also analysed the fractions of subsets of monocytes in peripheral blood taken at the same time as the muscle biopsies. The frequency of the intermediate monocyte subset increased in responders and decreased in non-responders. To our knowledge, this is the first investigation assessing the expression of intermediate monocytes in IIM patients. The frequency of intermediate monocytes correlated negatively to reduction of disease activity after methotrexate treatment in RA patients, suggesting that the frequency of intermediate monocytes is a potential predictor of the immunosuppressive treatment response (24). Despite the low number of blood samples these results may suggest that inflammatory cells in the periphery and target tissue are differently affected by treatment, and highlights the importance of investigating both blood and tissue compartments in patients with chronic inflammatory disorders.

Interestingly, CD66b-expressing neutrophils were present in the perimysial regions of all of the investigated muscle biopsies, not only at pretreatment but also at post-treatment. Neutrophils have rarely been reported in muscle biopsies of patients with IIM (8), and are usually the first cell type to respond to pathogens. However, why they were still present after almost one year of immunosuppressive treatment is unclear. Likewise, if they have a role in the pathogenesis of IIM requires further investigation (25).

This is a pilot study. Hence, one of its drawbacks is the low number of the patients included, which prevented us from separating the patients into clinical subsets, like subgrouping patients based on autoantibody profile. Secondly, we could not draw any conclusions concerning the effect of specific immunosuppressive treatment, as the treatment strategy for each patient was decided by the treating physician. Thirdly, the missing post-treatment biopsies in 4/13 patients, often due to a switch in treatment as a consequence of lack of improvement, was a drawback in our study. We aimed to evaluate the effects of immunosuppressive agents after a minimum of 6 months, but in some cases the delay was due to organisational issues. The low number of patients and samples may explain the lack of statistically significant changes.

In conclusion, investigations of inflammatory cells using immunohistochemistry analyses in repeated muscle biopsies taken within the first year of treatment, together with evaluation of disease activity and muscle strength may be a way to predict prognosis for recovery of muscle performance. A potential biomarker for treatment response could be the reduction of CD68<sup>+</sup> macrophages in the post-treatment biopsy, but this needs to be confirmed in a larger study.

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