Rheumatology, 2025, **00**, 1–9
https://doi.org/10.1093/rheumatology/keaf436
Advance access publication 18 August 2025
Original Article

Basic science

Aquaporin-4 downregulation in dysphagic patients with idiopathic inflammatory myopathies: myofiber vulnerability and inflammation drivers

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Abstract

Objectives: To evaluate whether the impairment of aquaporin-4 (AQP4), a water channel protein important for muscle function, may be associated with dysphagia in idiopathic inflammatory myopathies (IIMs). Swallowing muscle inflammation in IIM has been documented in case reports, but direct correlations with limb muscles impairment are lacking.

Methods: AQP4 was immunolocalized in limb muscle biopsies. The percentage of AQP4 expressing myofibers was compared in IIM patients with and without dysphagia, and healthy controls, and correlated with muscular strength, creatine kinase level and myositis damage index. Additionally, myosin heavy chain (fast and neonatal), cluster of differentiation 68 (CD68) and matrix metalloproteinase-2 (MMP2) were immunolocalized with sarcolemma AQP4 to evaluate whether an increase in innate immunity and extracellular matrix degradation were reflected in the modified AQP4 expression.

Results: AQP4 immunostaining was significantly decreased in myofibers of dysphagic patients compared with normophagic patients and controls. Moreover, although both IIM groups had CD68-positive cells surrounding AQP4-negative myofibers, only in dysphagic patients AQP4-negative myofibers surrounded by CD68-positive and MMP2-positive cells were significantly increased, suggesting that inflammation from blood vessels and inflammatory cells affects AQP4 immunolocalization.

Conclusion: Myofiber hypotrophy appears to be due to inflammation from CD68-positive and MMP2-positive cells, whose activity disrupts the extracellular matrix and may detach AQP4 from the sarcolemma.

Keywords: myositis, AQP4, fast-twitch myofibers, dysphagia, MMP2.

Rheumatology key messages

- Reduced AQP4 immunolocalization in limb muscle is novel in IIM, particularly in dysphagic patients.
- Reduced AQP4 immunolocalization is associated with both muscle damage and dysphagia.
- Chronic inflammation from $CD68^{POS}$ cells and $MMP2^{POS}$ vessels likely contributes to AQP4 loss.

Introduction

Idiopathic inflammatory myopathies (IIMs) are acquired immune-mediated diseases with muscle inflammatory infiltrates, increased levels of creatine kinase (CK) and variable involvement of other organs, such as joints, lungs, skin, gastrointestinal tract and heart. Inflammation of the swallowing muscles can impair pharynx/oesophagus contractility, laryngeal elevation and cricopharyngeal function, determining a rapidly progressive course and leading to serious complications such as nutritional deficits and aspiration pneumonia,

Received: 17 March 2025. **Accepted:** 28 July 2025

posing significant mortality risks [1, 2]. Dysphagia occurs in 32–84% of patients with IIM [3]. Given that moderate to severe oropharyngeal dysphagia strongly suggests cancer according to international IIM-associated Cancer Screening guidelines [4], it is crucial to identify biomarkers for this complication. In addition, the prevalence of dysphagia was shown to be significantly higher in drug-resistant IIM patients than patients responding to therapy [5].

Biopsies in several IIM case reports have shown inflammatory involvement of swallowing muscles similar to that in limb skeletal muscles [6, 7]. However, most IIM patients do not undergo a muscle biopsy of the upper digestive tract and correlation studies between the myopathology of the two sites are lacking. Therefore, specific causal factors, molecular drivers and specific biomarkers have not been identified for patients with IIM and dysphagia. Suprahyoid muscles, mostly impaired in dysphagia related to IIM [8], are mainly composed by type 2 (fast-twitch, glycolytic) myofibers with a lower proportion of type I (slow-twitch, oxidative) [9]. Recently, the cell type mapping of IIM quadriceps femoris muscle highlights selective type 2A myofiber vulnerability in inclusion body myositis (IBM) [10] and also that dermatomyositis (DM) patients with dysphagia confirmed at fiberoptic endoscopic evaluation of swallowing, showed a significantly higher atrophy coefficient of type 2 myofibers than age-matched DM patients without dysphagia [3].

Aquaporin-4 (AQP4) is a water channel involved in transcellular water transport and maintenance of osmotic gradients. It is most abundantly localized in the muscular system and central nervous system, especially on astrocyte endfeet at the blood–brain barrier [11]. AQP4 expression, firstly reported in skeletal myofibers [12], allows rapid water flows across the sarcolemma balancing those changes in osmotic pressure and hydrostatic forces during myofiber contraction and subsequent relaxation, maintains morphological homeostasis, and is involved in the metabolic and fatigue resistance processes of skeletal muscles [11, 13]. AQP4 impaired expression, reducing rapid volume changes during contraction, is associated with premature fatigue during forced and voluntary muscle contractions as has been observed in different pathological conditions in both animal models of myopathies [14] and human myopathies [15, 16].

In the present study, AQP4 immunolocalization has been investigated by high-resolution confocal microscopy in muscle biopsies of IIM patients with or without dysphagia to foster a deeper understanding of the pathomechanisms underlying the progression of tissue damage and clinical outcome. The results suggest that myofiber hypotrophy observed in normophagic IIM patients is worse in dysphagic patients, where it appears to be supported by a combined intervention of CD68^{pos} inflammatory cells and MMP2^{pos} perivascular cells.

Materials and methods

This is a single-centre retrospective study. We selected patients with IIMs who had undergone open muscle biopsy and fiberoptic endoscopic evaluation of swallowing (FEES) for diagnostic purposes between 2010 and 2018.

The presence of myositis-specific autoantibodies (MSAs) and myositis-associated autoantibodies (MAA) was determined by commercially available immunoassays (MYO12D-24, D-Tek, Belgium and Euroline Autoimmune Inflammatory Myopathies, Euroimmun, Germany). Patients with DM and polymyositis (PM) were classified according to the 2017 EULAR/ACR classification criteria for idiopathic inflammatory myopathies [17]. Sporadic inclusion body myositis (sIBM) was diagnosed based on the presence of characteristic histopathological features, including rimmed vacuoles. Patients with antisynthetase syndrome (ASyS) were defined by the presence of antisynthetase antibodies and at least one clinical manifestation among arthritis, interstitial lung disease (ILD), or myositis. Immune-mediated necrotizing myopathy (IMNM) was defined by the presence of anti-SRP or anti-HMGCR antibodies, or by histological features consistent with necrotizing myopathy in the absence of MSAs [18]. Patients with overlap myositis (OM) were defined by the presence of clinical or serological features of myositis in association with features of another connective tissue disease.

Single open biopsies of muscle tissue obtained from patients with clinically and pathologically confirmed IIMs were retrospectively reviewed. Suitable biopsy was available for 54 patients, and these samples were included in the analysis. Among them, representative sections were selected for further immunohistochemical analysis based on tissue quality and availability. The cohort selected before the initiation of any new treatment, included four patients with anti-HMGCR^{pos} IMNM (one patient exposed to a statin, three patients not exposed), two patients with anti-SRP^{pos} IMNM, two patients with isolated anti-SSA/Ro52^{pos} IMNM, two patients with seronegative IMNM (absence of any MSA and MAA), eight patients with adult anti-Mi2a/b^{pos} DM, one patient with adult anti-TIF1γ^{pos} DM, one patient with adult anti-MDA5^{pos} DM, two patients with isolated anti-SSA/Ro52^{pos} DM, eight patients with seronegative DM, 10 patients with PM, one patient with isolated anti-SSA/Ro52^{pos} PM, three patients with systemic sclerosis PM/Scl^{pos}-overlap myositis (SSc-OM), four patients with anti-Jo1^{pos} ASyS and six patients with sIBM. Non-inflammatory muscle samples from six additional age-matched apparently healthy subjects—with mildly elevated serum levels of CK but without any histological abnormality, including inflammatory infiltrates or MHC class I up-regulation—were used as the control group. Clinical characteristics were collected at the time of the muscle biopsy or, when not available at that time, from the closest clinical visit before or after the biopsy. These included age at the time of biopsy, disease duration, gender, muscle strength assessed by manual muscle testing (MMT-8) (range: 0–80), serum CK levels at the time of biopsy and muscle damage evaluated using the visual analogue scale (VAS) according to the myositis damage index (MDI) [19]. The presence of dysphagia was assessed using FEES (performed by M.L.F.), when feasible, as a routine procedure at the time of diagnosis to screen for organ involvement. Dysphagia was defined according to the findings of the FEES, based on the presence of impaired bolus transit, penetration or aspiration during the ingestion of solids, semi-solids or liquids.

All procedures in this study were performed in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments. The study was reviewed and approved by the Medical Ethics Committee of the Regional Policlinico University Hospital of Bari (study n. 6229, approval n. 84762, 2020/11/06; comitatoetico@policlinico.ba.it). All patients gave signed informed consent to diagnostic and research analyses and specimens inclusion in a muscle biobank.

The muscle biopsy specimens underwent histochemical and immunohistochemical (IHC) stainings and quantitative evaluations, as detailed in Supplementary Data S1. Briefly, the muscle specimens were rapidly frozen, sectioned and subjected to standard immunofluorescence protocol using the following primary antibodies used to recognize: AQP4 (1:1000; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany; code: A5971, rabbit IgG), myosin heavy chain (fast) (MHCfast, 1:40; Monosan, Uden, The Netherlands; code: MONX10807; mouse IgG1), CD68 (1:50; Dako, code: M0876, mouse IgG3), MMP-2 (1:50; Chemicon, Temecula, USA; code: MAB13431, mouse IgG1), myosin heavy chain (neonatal) (1:30; Monosan, Uden, The Netherlands; code: MONX10808; mouse IgG1). The sections were incubated with appropriate secondary antibodies and then counterstained with TO-PRO-3.

Categorical variables were expressed as number and percentage; continuous variables as mean ± standard deviation. Comparisons between groups were performed using *t*-test, and one-way ANOVA followed by Bonferroni's multiple comparison test was used to compare morphology data. Differences were considered statistically significant at a *P*-value of <0.05. Pearson's correlation coefficient test was used to estimate correlations between AQP4 myofibers and clinical data; one-tailed Spearman's coefficient was used for correlating the percentage of $AQP4^{neg}$ myofibers and the semi-quantitative scores of CD8, C5b9, MHC expression levels as described in the diagnostic reports. All statistical analyses were performed using GraphPad Prism (release 6.0, GraphPad Software, La Jolla, USA).

Results

Population

Demographic and clinical features of IIM patients and age-matched controls are reported in Table 1. None of the IIM patients referred a family history of muscle disease. None of the 54 patients had cancer-associated myositis (3 years before/after the symptoms onset). The average age at disease onset and the gender distribution were similar among the different groups (Table 1). At disease onset, no differences were found in physician assessment of the MMT-8. CK levels were higher in normophagic than dysphagic patients without reaching statistical significance ($P > 0.05$) (Table 1). The cohort of dysphagic patients had a longer interval, on average twice as long, between the onset and the time of biopsy than the normophagic patient cohort (Table 1). Dysphagic patients exhibited a significantly higher MDI compared with normophagic patients ($P < 0.01$).

Table 1. Demographic and clinical characteristics

Variables	Normophagic (29 pts.)	Control (6 pts.)	Dysphagic (25 pts.)
Female, *n* (%)	19 (65.5)	4 (44.7)	21 (84)
Age at disease diagnosis, median (IQR)	55 (43–64)	53 (35–61)	60 (53–67)
Disease duration at biopsy time (no. of months), mean (S.D.)	24 (32)	/	45 (63)
IIMs subset, *n* (%)			
DM	9 (31)		11 (44)
ASyS	4 (13.8)		0 0
PM	4 (13.8)		7 (28)
IBM	4 (13.8)		2 (8)
IMNM	8 (27.6)		2 (8)
OM	0 0		3 (12)
MMT-8 at disease diagnosis, mean (S.D.)	74 (6)	/	68 (11)
CK at biopsy time, mean (S.D.)	1628 (2025)	/	919 (1272)
Myositis damage index	1.5 (1.8)		3.4 (2.1)*

* $P < 0.01$.
Abbreviations: CK = creatine kinase; IQR = inter quartile range; MMT = manual muscle test.

AQP4 expression in dysphagic, normophagic patients and control group

As already described in non-inflammatory muscle samples [20], AQP4 immunolocalization formed a continuous rim along the sarcolemma of type 1^{like} (97.46–100%) and type 2 (99.05–100%) myofibers (Fig. 1B). The staining was more pronounced in type 2 (Corrected Total AQP4 Fluorescence (CTAF): 356 645 ± 121 488 AU) compared with type 1^{like} (CTAF: 281 625 ± 60 590 AU) myofibers (Fig. 1B).

Given the variability in myofiber response to inflammatory stimuli, we analysed a range of myofiber parameters using AQP4/MHCfast immunofluorescence by means of high-resolution confocal microscopy computer-aided morphometry in muscle biopsies from IIM patients with and without dysphagia, as well as non-inflammatory skeletal muscle. Firstly, we quantified the number of $AQP4^{pos}$ and $AQP4^{neg}$ myofiber types and observed a significant reduction in $AQP4^{pos}$ myofiber percentages in dysphagic compared with both normophagic patients and control muscles. The most pronounced changes were observed in anti-$HMGCR^{pos}$ IMNM patients (Fig. 1A). On the contrary, in normophagic patients, there was no change in $AQP4^{pos}$ myofiber percentages compared with control muscles (Fig. 1A). Type 1^{like} myofiber density was significantly increased in normophagic and especially in dysphagic patients compared with the controls, and a consistent proportion of $AQP4^{neg}$ myofibers was found only in dysphagic patients (Fig. 1A; cyan lines indicate mean and SD of $AQP4^{neg}$ myofibers). Conversely, type 2 density was only increased in normophagic patients (Fig. 1A). In dysphagic patients, significant alterations compared with both normophagic and control muscles were also found in myofiber diameter, area, variability coefficient and atrophy factor, in which there was a major contribution of $AQP4^{neg}$ myofibers to determining these alterations (Fig. 1A; cyan). The most pronounced changes in dysphagic patients were detected in type 2 myofiber parameters, mainly due to the smaller size of $AQP4^{neg}$ type 2 myofibers, as evidenced by lower values of diameter and area and consistently higher values of variability and atrophy factors, which were both largely above the normal values (Fig. 1A; gray axes; cyan lines, 1B).

In normophagic patients, beyond the significantly increased type 2 myofiber density, there were other type 2 myofiber parameters that were significantly different from control muscles, namely the mean diameter and related area, which were both significantly reduced, and the variability coefficient, which was significantly increased (Fig. 1A; cyan lines, 1B). As already mentioned for dysphagic patients, also in the normophagic patient cohort, the presence of hypotrophic, $AQP4^{neg}$ type 2 myofibers was the main cause of the significant changes of these parameters when compared with the controls (Fig. 1A; cyan lines, 1B).

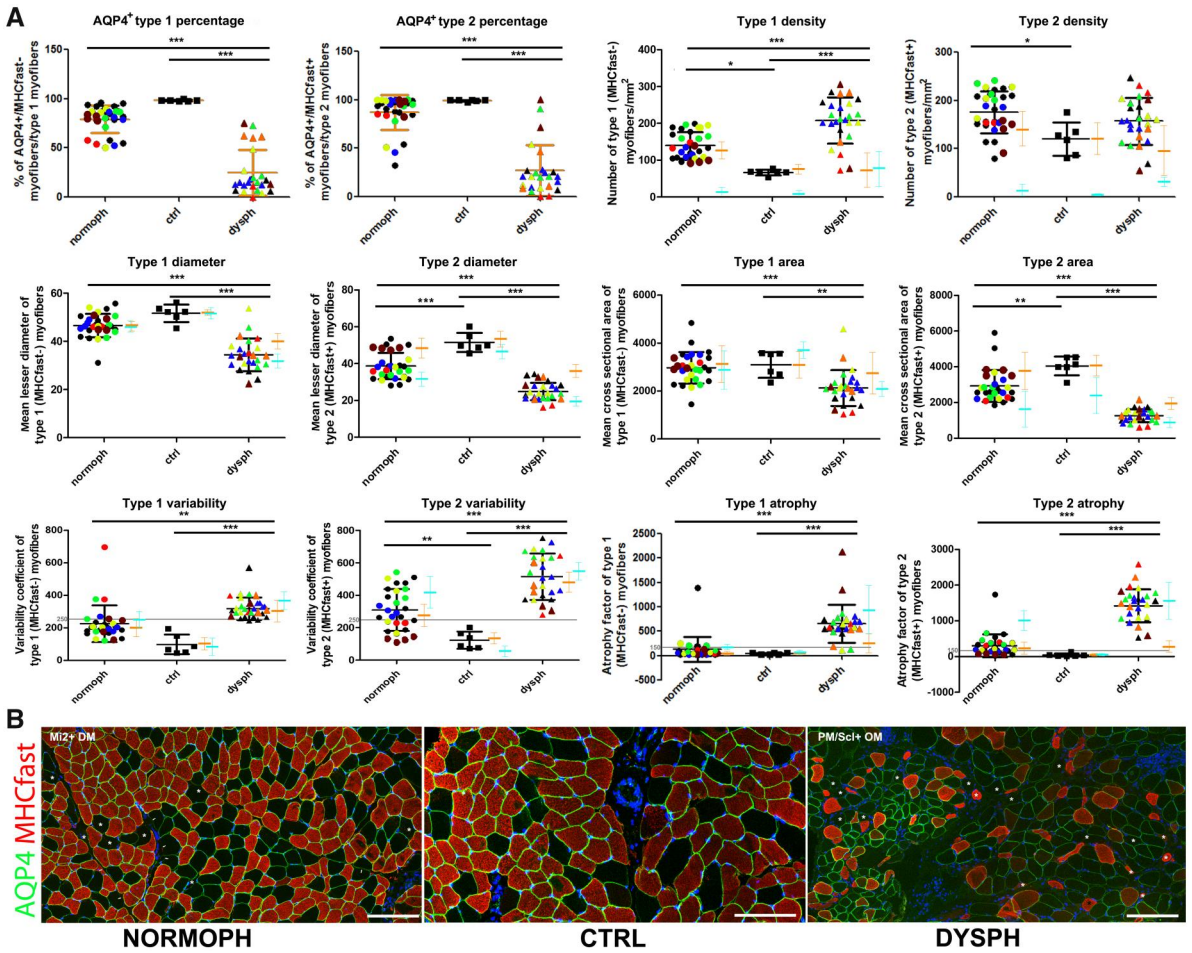

Figure 1. Comparison of myofiber type parameters between normophagic ($n = 29$) and dysphagic IIM-affected patients ($n = 25$), and controls ($n = 6$). (**A**) Quantification of percentage of acquaporin-4^{pos} (AQP4^{pos}; orange bars) and AQP4^{neg} (cyan bars) myofiber types, mean density, lesser diameter, cross-sectional area, variability coefficient and atrophy factor. Normophagic idiopathic inflammatory myositis (IIM) patients are indicated as: blue circles (n. 4 seronegative polymyositis = PM), green circles (n. 4 seronegative dermatomyositis = DM), yellow circles (n. 4 Mi2^{pos} DM), brown circles (n. 4 sporadic inclusion body myositis—sIBM), red circles (n. 2 HMGCR^{pos} immune-mediated necrotizing myositis = IMNM) and black circles (n. 1 MDA5^{pos} DM, 2 SRP^{pos} IMNM, 2 ro52^{pos} IMNM, 2 seronegative IMNM, 4 Jo1^{pos} antisynthetase syndrome (ASyS)). Dysphagic IIM patients are indicated as: blue triangles (n. 6 seronegative PM), green triangles (n. 5 seronegative DM), yellow triangles (n. 4 Mi2^{pos} DM), brown triangles (n. 2 sIBM), red triangles (n. 2 HMGCR^{pos} IMNM), orange triangles (n. 3 Pm/Scl^{pos}-overlap myositis (OM)) and black triangles (n. 1 ro52^{pos} PM, 1 TIF1γ^{pos} DM, 1 ro52^{pos} DM). The controls are indicated as squares. In each group, means and standard deviation ranges are shown as solid black lines for grouped data without discriminating between AQP4^{pos} (shown as orange bars on the right) and AQP4^{neg} (shown as cyan bars on the right) myofibers. Statistically significant adjusted *P*-values after Bonferroni correction are shown (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The horizontal grey lines indicate the upper normal limits of the examined parameters. (**B**) Representative confocal microscope images of muscle biopsy immunolabeled with anti-AQP4 and anti-myosin heavy chain fast (MHCfast) myofibers derived from a normophagic patient affected by anti Mi2^{pos} DM (quadriceps, 75 years, F), from a normophagic patient affected by transient hyperCKemia used as control (quadriceps, 39 years, F), and a dysphagic patient affected by anti PM/Scl^{pos} OM (deltoid, 49 years, F). AQP4^{neg} (asterisks) type 1 or type 2 (MHCfast^{pos}) myofibers preferentially cluster at the perimysial border in the anti Mi2^{pos} DM patient where a thinned rim of AQP4 channels prospects the perimysial vascular compartment (arrows). AQP4^{neg} (asterisks) type 1 or type 2 (MHCfast^{pos}) myofibers are also seen in the centre of fascicles in the dysphagic PM/Scl^{pos} OM patient. Scale bars: 200 μm

In summary, we found type 2 myofiber hypotrophy in IIM patients, that were particularly evident in dysphagic but also present in normophagic IIM patients, characterized by a selective absence of AQP4 channel in the smaller myofibers and were particularly evident in anti-HMGCR-positive immune-mediated necrotizing myopathy.

Inflammation and AQP4 expression

We sought to evaluate whether the observed myofiber hypotrophy was accompanied by a significantly higher proportion of AQP4^{neg}/neonatal myosin^{pos} regenerating myofibers and found a significantly increased percentage of AQP4^{neg}/neonatal myosin^{pos} myofibers only in dysphagic patients compared with both normophagic and control patients (Fig. 2A and B). Thus, in line with pathological criteria for IMNM [18], the highest proportion of AQP4^{neg}/neonatal myosin^{pos} regenerating myofibers was found in dysphagic anti-HMGCR^{pos} IMNM patients (Fig. 2A and B). Conversely, neonatal myosin^{pos} regenerating myofibers rarely co-expressed the AQP4 water channel and their low percentages were similar among the three groups (Fig. 2A and B).

Additionally, we sought to evaluate whether an increase in innate immunity was reflected in the AQP4 expression by measuring the percentage of myofibers surrounded by at least three CD68^{pos} cells. Both normophagic and dysphagic IIM patients showed a significant increase in CD68^{pos} surrounded AQP4^{neg} myofibers compared with controls (Fig. 2C and D). Dysphagic IIM patients also had a closer mean distance of

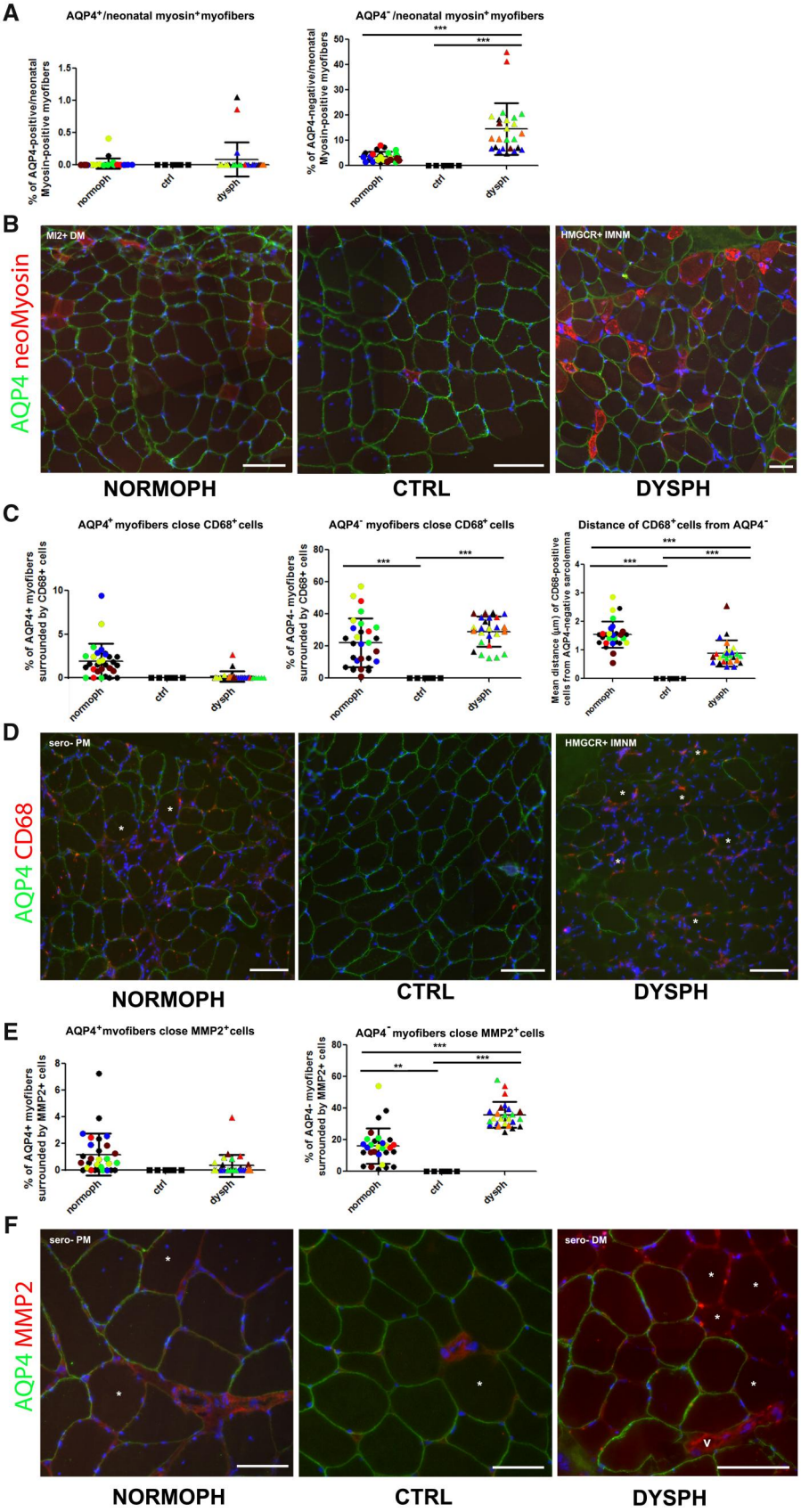

Figure 2. Comparison of AQP4 sarcolemma immunolocalization between normophagic ($n = 29$) and dysphagic IIM-affected patients ($n = 25$), and controls ($n = 6$). (**A**, **C**, **E**) Quantification of the percentage of acquaporin-4^{pos} (AQP4^{pos}) (on total number of AQP4^{pos}) and AQP4^{neg} myofibers (on the total number of AQP4) coexpressing neonatal myosin (**A**), surrounded by CD68^{pos} cells (**C**) and by MMP2^{pos} vessels (**E**) are shown together with the mean distance of CD68^{pos} cells from AQP4^{neg} sarcolemmas (**C**). Normophagic idiopathic inflammatory myositis (IIM) patients and dysphagic IIM patients are indicated as in Fig. 1. The controls are indicated as squares. Means and standard deviation ranges are shown as solid lines in each group. Statistically significant

CD68^{pos} cells to the AQP4^{neg} sarcolemma compared with normophagic patients (Fig. 2C and D). The proportion of CD68^{pos}-surrounded AQP4^{pos} was significantly lower than the CD68^{pos}-surrounded AQP4^{neg} sarcolemmas ($P < 0.0001$ in both IIM groups), and the former did not differ between the three groups (Fig. 2C and D). As expected, CD68^{pos} cells accumulated preferentially around affected AQP4^{neg} myofibers in anti-HMGCR^{pos} IMNM, anti-Mi2^{pos} DM and seronegative PM samples of both normophagic and dysphagic IIM patients (Fig. 2C and D). In addition, there were statistically significant positive correlations between the percentage of AQP4^{neg} type 2 myofibers and semi-quantitative scores of CD8^{+} lymphocyte infiltration and sarcolemmal expression of C5b9, class I MHC and class II MHC in both normophagic and dysphagic IIM patients (Supplementary Fig. S1). Considering the interactions between AQP4 and the dystrophin–glycoprotein complex (DGC) and the proteolytic activity of metalloproteinase 2 (MMP2) on different extracellular matrix (ECM) molecules and β-dystroglycan [21, 22], we investigated the relationship between reduced AQP4 and MMP2 immunolocalization. Notably, the proportions of AQP4^{neg} myofibers surrounded by MMP2^{pos} vessels were significantly higher than of AQP4^{pos} myofibers ($P < 0.0001$ in both IIM groups) and also significantly higher in dysphagic compared with both normophagic patients and non-inflammatory controls (Fig. 2E and F). These MMP2^{pos} structures resembled transversely cut endomysial microvessels and their thicker parental vessels (Fig. 2F). Critically, AQP4 immunolocalization was almost entirely absent (<2% of total AQP4^{pos} myofibers) from sarcolemmas adjacent to these MMP2^{pos} vessels across all the groups (dysphagic, normophagic and controls), indicating that inflammatory stimuli derived from blood vessels and inflammatory cells impair AQP4 sarcolemmal immunolocalization. These findings suggested a more severe impairment of myofibers in dysphagic patients due to sustained inflammation and ECM degradation.

AQP4 and clinical characteristics

Considering the overall cohort of IIM patients, the percentage of AQP4^{pos} type 1^{like} myofibers was correlated with the MMT-8 value of muscular strength at the baseline visit (r_S: 0.383; P: 0.007) and accordingly, the percentage of both AQP4^{neg} type 1^{like} (r_S: −0.389; P: 0.006) and AQP4^{neg} type 2 (r_S: −0.288; P: 0.047) myofibers was inversely correlated with MMT8. Neither the overall cohort of IIM patients, nor the normophagic and dysphagic subgroups showed any correlation with CK level near the disease diagnosis. Since MMT-8 can serve as an indicator of muscle damage, we evaluated the muscle MDI [19] and correlated it with the percentage of AQP4^{pos} and AQP4^{neg} type 1 and type 2 myofibers. In the overall cohort, we observed that the muscle MDI was negatively correlated with the percentage of AQP4^{pos} type 1 (r_S: −0.433; P: 0.001) and type 2 (r_S: −0.265; P: 0.05) myofibers and positively correlated with the percentage of AQP4^{neg} type 1 (r_S: 0.442; P: 0.001) and type 2 (r_S: 0.265; P: 0.05) myofibers.

Discussion

A deeper understanding of the mechanisms underlying the pathogenesis and progression of inflammatory and degenerative IIMs is essential for the development of reliable biomarkers and targeted therapies. This study, using immunofluorescence and confocal microscopy, reveals a novel finding: a significantly reduced AQP4 sarcolemma immunolocalization in limb skeletal muscle of IIM patients, with a more pronounced impairment of the water channel in dysphagic compared with normophagic patients. To our knowledge, a reduced limb muscle AQP4 immunolocalization has been previously observed only in a subset of patients with neuromyelitis optica CNS spectrum disorders associated with anti-AQP4 antibodies [23] and has not been described in IIM before. The more pronounced morphological alterations—such as mean lesser diameter, cross sectional area, variability coefficient and atrophy factor—observed in dysphagic IIM patients (including those with impaired swallowing of solids, semi-solids and liquids) compared with normophagic patients suggest an accelerated hypotrophy of type 2 myofiber. This highlights the greater vulnerability of type 2 fibres, whereas type 1 fibres become affected as the inflammatory process progresses [10, 24]. Type 1 myofibers exhibit a greater resilience to inflammation due to their higher oxidative capacity, capillary density, regenerative potential, endurance and larger myotendinous junctions [24–26]. However, they can still be affected by severe or prolonged inflammatory conditions.

In addition, our morphometric analysis revealed abnormally small type 2 myofibers in both normophagic and dysphagic IIM patients, with a significantly increased type 2 variability coefficient compared with the controls. This vulnerability of type 2 myofibers to contraction-induced damage and myonecrosis from inflammation, trauma and genomic stress is well-established, paralleling findings in conditions such as excessive eccentric contraction, fasting, cachexia, sepsis, critical conditions, sIBM, Duchenne and Becker, facioscapulohomeral muscular dystrophies, Pompe and McArdle diseases [24]. While swallowing muscles are predominantly type 2 fast myofibers [9], their invasive biopsy and the comparable diagnostic accuracy of alternative methods in inflammatory muscle disorders explain the scarcity of IIM studies specifically analysing these muscles.

Figure 2. Continued
adjusted P-values after Bonferroni correction are shown (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (**B**) Representative fluorescence microscope images of muscle biopsy derived from a normophagic patient affected by anti Mi2^{pos} DM (quadriceps, 81 years, F), from a normophagic patient affected by transient hyperCKemia used as control (quadriceps, 61 years, M) and a dysphagic patient affected by anti-HMGCR^{pos} IMNM (deltoid, 67 years, F), immunolabeled with anti-AQP4 and anti-neonatal myosin antibodies. Neonatal myosin^{pos} myofibers preferentially appear AQP4^{neg}. Scale bars: 100 μm. (**D**) Representative fluorescence microscope images of muscle biopsy derived from a normophagic patient affected by seronegative PM (deltoid, 72 years, M), from a normophagic patient affected by transient hyperCKemia used as control (quadriceps, 72 years, F) and a dysphagic patient affected by anti-HMGCR^{pos} IMNM (quadriceps, 36 years, F), immunolabeled with anti-AQP4 and anti-CD68 antibodies. AQP4^{neg} (asterisks) myofibers appear preferentially surrounded by CD68^{pos} cells in all patients. Scale bars: 100 μm. (**F**) Representative fluorescence microscope images of muscle biopsy derived from a normophagic patient affected by seronegative PM (quadriceps, 56 years, F), from a normophagic patient affected by transient hyperCKemia used as control (quadriceps, 61 years, M) and a dysphagic patient affected by seronegative DM (quadriceps, 44 years, M), immunolabeled with anti-AQP4 and anti-MMP2 antibodies. AQP4^{neg} (asterisks) myofibers appear preferentially surrounded by MMP2^{pos} perimysial (v) and endomysial vessels especially in the dysphagic patient. Scale bars: 100 μm

The IIM pathogenesis involves a complex interplay of inflammatory signals driving chronic immune cell accumulation such as CD8^{pos} (cluster of differentiation 8) T cells, CD 68^{pos} macrophages and neutrophils, along with the release of pro-inflammatory molecules and microRNAs, with an impact on disease severity and the efficacy of current anti-inflammatory-based treatments [27–29].

Myofiber hypotrophy in IIMs may result from: (1) regeneration attempts triggered by inflammatory-induced satellite cells activation and proliferation [30]; (2) concomitant neuromuscular junction injury and functional denervation [10] and (3) ECM detachment of damaged myofibers [31]. An increased proportion of CD56^{pos} regenerating myofibers was previously observed in our cohort of IIM patients [29, 32, 33], consistent with the elevated expression of Pax7, MyoD, myogenin and neonatal myosin heavy chain during the healing phase of inflammation [34]. However, these hypotrophic regenerating myofibers unfortunately upregulate autoantigens, perpetuating autoimmune attacks [35, 36]. This likely explains the observed absence of AQP4 immunolocalization in neonatal myosin^{pos} regenerating myofibers across all study groups.

While AQP4 expression decreases in denervated atrophied skeletal muscles but remains unchanged during unloading (e.g. tail suspension) [13, 14], its recovery after injury is slow, requiring at least 4 weeks post-reversible denervation [13, 37]. Reduced AQP4 expression may also result from disuse [38] due to muscle weakness and pain or from corticosteroid administration [39], although in our study most biopsies were obtained prior to diagnosis and corticosteroid treatment.

Additionally, we observed a significantly higher number of AQP4^{neg} myofibers surrounded by CD68^{pos} cells compared with AQP4^{pos} myofibers, suggesting that CD68 cells contribute to an inflammatory attack linked to the suppression of AQP4 sarcolemma immunolocalization. This downregulation may occur through mechanisms similar to those observed in LRRK2-associated Parkinson's disease and in transgenic mouse models, where interferon-γ (IFNγ) induces AQP4 delocalization from astrocytic endfeet, leading to glymphatic dysfunction [40]. Hyperactivation of IFNGR-2, by phosphorylating mutant LRRK2, indirectly abolishes AQP4 expression in astrocyte endfeet probably by promoting its phosphorylation and delocalization [40].

Early studies suggested that AQP4 phosphorylation by various kinases could contribute to its downregulation, though this has not been conclusively demonstrated. For instance, stress-induced casein kinase II was proposed to enhance AQP4 clathrin-mediated endocytosis, targeting it for lysosomal degradation [41]. Similarly, protein kinase (PK) A and C were reported to promote AQP4 endosomal internalization and degradation [42]. However, these mechanisms remain unconfirmed, and further studies are needed to clarify their role in AQP4 regulation.

Several kinases can be activated by the IFNGR-2 signalling pathway [43], though their potential impact on AQP4 regulation in skeletal muscle of IIM patients and models remains unexplored. However, increasing evidence underscores the key role of IFN in sustaining inflammation through the activation of transmembrane receptors coupled to the janus kinase-signal transducer and activator of transcription pathway. IFNs appear to drive IIM pathogenesis through multiple mechanisms, including the stimulation of *atrogin* and *MuRF1* genes, which downregulate myosin heavy chain IIa expression and drive type 2 muscle atrophy [44]. Notably, atrogin also causes AQP4 ubiquitination and degradation [45].

Another key effect of IFNγ signalling is the disruption of endothelial barrier integrity observed both *in vivo* and *in vitro* [46]. Our study reveals a strong association between the absence of AQP4 in myofibers and the presence of adjacent MMP2^{pos} endomysial and perimysial vascular cells. MMP2, an endopeptidase that degrades ECM molecules, plays a crucial role in tissue remodelling during angiogenesis, embryogenesis, tumorigenesis, lipid metabolism and inflammation [47]. Its activity in inflammatory and remodelling processes promotes cell transmigration through leaky blood microvessels [48]. Among the numerous ECM substrates of the MMP2 activity, laminin and dystroglycans (DG) [21, 22] are particularly relevant due to their potential role in anchoring AQP4 to the sarcolemma [49]. Laminin has been shown to induce clustering of β-DG, syntrophin and AQP4 via PKCδ phosphorylating activity in astrocyte culture [50], suggesting that MMP2-mediated laminin degradation could disrupt AQP4 anchoring, leading to its delocalization and degradation. This effect may be particularly pronounced in type 2 myofibers, where both laminin and AQP4 are highly expressed [51].

Finally, we observed a negative correlation between the percentage of AQP4^{pos} fibres and muscle damage, and a positive correlation between the percentage of AQP4^{neg} myofibers and muscle damage. This suggests that a decrease in AQP4^{pos} fibres and an increase in AQP4^{neg} fibres could serve as potential markers of muscle damage. However, this correlation was not observed when analysing dysphagic and non-dysphagic patients separately, likely due to the small sample size. Larger cohorts are needed to confirm these findings.

This study has additional limitations, including its retrospective design and diagnostic heterogeneity between dysphagic and normophagic IIM groups. Furthermore, in the three PM-SCL patients, we cannot exclude the possibility that the dysphagia resulted from concurrent oesophageal smooth muscle dysfunction. However, given the rarity of IIMs, all samples were obtained from a local biobank limiting broader population-based analysis.

Despite these limitations, our findings offer insights into two poorly understood aspects of IIM etiopathogenesis: myofiber-type-specific AQP4 impairment is more evident in dysphagic than normophagic patients, potentially reflecting different disease stages and/prognosis. Further investigation into the differential responses of slow and fast myofiber in IIMs is crucial, as these may influence disease manifestation and progression. While our study provides a cross-sectional view of skeletal muscle pathology at the time of biopsy, future research is needed to determine whether the observed changes could serve as therapeutic targets or biomarkers for clinical subtype stratification and prognosis.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

The data underlying this article are available in the article and its online supplementary material. The deidentified, patient-level data supporting the findings of this study are available upon reasonable request from the corresponding

author [F.G.]. The data are not publicly available due to privacy or ethical restrictions, as they contain information that could compromise the privacy of research participants. Researchers with a pertinent research question may submit a formal request for access to the data. This request should include a detailed research proposal outlining the study's objectives, methods and a plan for data security and management. All data access requests will be reviewed by the Institutional Data Access Committee of Ospedale Consorziale Policlinico di Bari to ensure the proposed research aligns with the original consent provided by the study participants and complies with all relevant ethical and legal regulations. Data will be shared under a formal Data Sharing Agreement that outlines the terms of use, including restrictions on data redistribution and requirements for data security and destruction upon completion of the approved research project. For inquiries and to initiate a data access request, please contact the corresponding author at francesco.girolamo@uniba.

Author contributions

Investigation/Formal analyses: M.F., D.D'A., M.L.F., M.E., O.V., F.G. Writing—original draft preparation: M.F., M.G., F.G. Writing—review & editing: M.F., D.V., A.F., F.G. Resources: D.D'A. Conceptualization/Supervision/Project administration/Funding acquisition: D.V., A.F., F.I., F.G.

Funding

This work was supported by Intramural Funding (to F.G. and F.I.) and the Italian Ministry of Education, University and Research (MIUR, Finanziamento delle attività base di ricerca 2017).

Disclosure statement: The authors have declared no conflicts of interest.

Acknowledgements

The authors thank M.V.C. Pragnell, BA, for linguistic help and M. de Giorgis, Tech., for technical help in IHC.

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Rheumatology, 2025, 00, 1–9
https://doi.org/10.1093/rheumatology/keaf436
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