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Published in:
BioMed Research International

DOI:
10.1155/2016/5930621

Publication date:
2016

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):

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Research Article

Skeletal Muscle Remodelling as a Function of Disease Progression in Amyotrophic Lateral Sclerosis

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Received 22 December 2015; Revised 7 March 2016; Accepted 24 March 2016

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Muscle weakness is considered the pivotal sign of amyotrophic lateral sclerosis (ALS). Knowledge about the skeletal muscle degeneration/regeneration process and the myogenic potential is limited in ALS patients. Therefore, we investigate these processes in a time course perspective by analysing skeletal muscle biopsies from ALS patients collected before and after a 12-week period of normal daily activities and compare these with healthy age-matched control tissue. We do this by evaluating mRNA and protein (immunohistochemical) markers of regeneration, neurodegeneration, myogenesis, cell cycle regulation, and inflammation. Our results show morphological changes indicative of active denervation and reinnervation and an increase in small atrophic fibres. We demonstrate differences between ALS and controls in pathways controlling skeletal muscle homeostasis, cytoskeletal and regenerative markers, neurodegenerative factors, myogenic factors, cell cycle determinants, and inflammatory markers. Our results on Pax7 and MyoD protein expression suggest that proliferation and differentiation of skeletal muscle stem cells are affected in ALS patients, and the myogenic processes cannot overcome the denervation-induced wasting.

1. Introduction
Amyotrophic lateral sclerosis (ALS), also known as motor neuron (MN) disease, is the most common motor neuron disorder among adults [1]. ALS is characterized by degeneration of upper motor neurons leading to spasticity and lower motor neurons resulting in interruption of the nerve connection at the neuromuscular junction (NMJ) and myofibre loss [2, 3]. The affected neuromuscular system attempts to restore function by nerve sprouting for reinnervation and by myogenesis to regenerate the muscle fibres [4]. Eventually, persistent muscle wasting exceeds the ability to repair and the consequence is progressive muscle atrophy, respiratory insufficiency, and usually death within 3–5 years of diagnosis [5]. Knowledge about the skeletal muscle degeneration/regeneration process and the myogenic potential is limited in ALS patients and has, to our knowledge, never been investigated in a time course perspective.

Muscle weakness is considered the cardinal sign of ALS and debate still exists as to whether denervation originates from the neuron or the muscle [4]. Much of our current knowledge about ALS disease progression is established within the superoxide dismutase (SOD1) G93A mouse model [6]. Previous studies show that muscle specific expression of mutant SOD1 in mice leads to an ALS phenotype and degradation of MNs [7, 8], demonstrating that abnormalities in skeletal muscle can induce degradation of MNs and cause ALS symptoms.

Human ALS samples are rare, but autopsy of an ALS patient demonstrated skeletal muscle changes with clear signs of denervation and reinnervation. However, the patient had normal appearing motor neurons; thus pathological changes...
in skeletal muscle appear to be present before the motor neurons are affected [2], thus providing human evidence for the “dying-back” phenomenon.

Healthy skeletal muscle has a remarkable ability to regenerate after injury and tissue damage. The skeletal muscle stem cells, the satellite cells (SCs), that reside under the basal lamina of muscle fibres are responsible for this repair [9]. Upon activation, the SCs become myoblasts and proliferate and differentiate to restore and replace damaged muscle fibres [10]. In vitro cultures of SCs from ALS patients demonstrate a senescent-like morphology, disturbed differentiation, and an apparent inability to proceed through the myogenic program [11, 12] resulting in a decreased ability to regenerate and mature to functional myofibres.

Together, these findings suggest an important role of skeletal muscle homeostasis, metabolic condition, and regenerative ability in the development of ALS. To slow down disease progression, maintenance of skeletal muscle function may be the most promising therapeutic target as of today. Thus, improved knowledge about the skeletal muscle degenerative/regeneration process is warranted, particularly in a time course perspective.

Therefore, in the present study we describe the degenerative and regenerative processes of skeletal muscle of human ALS patients and how different markers change during 12 weeks of disease progression. The hypothesis is that the myogenic program is ineffective in patients with ALS compared with healthy age-matched subjects.

2. Materials and Methods

Five subjects clinically diagnosed with ALS (aged 65 ± 9 yrs) agreed to participate in this study. The patients were diagnosed at the Department of Neurology, Odense University Hospital according to the El Escorial criteria [13]. Four of them were diagnosed within one year from inclusion, whereas the last one was diagnosed 15 years earlier (subject 4, Figure 1). Inclusion criteria were definite or probable ALS, being ambulatory at onset of study, and willingness to comply with the study protocol. Patients were excluded for other neurological or serious medical problems including cardiovascular disease and diabetes mellitus. See Table 1 for additional clinical details. All subjects were evaluated using the revised ALS functional rating scale (ALSFRS-R) [14], which showed that all patients presented with a similar functional score at the time of inclusion and their disease symptoms showed a comparable decline over the 12-week period (Table 1), thus allowing us to group the patients for all analyses. The participants were informed about the purpose of the study and gave written informed consent before their participation. The study was conducted according to the Declaration of Helsinki and was approved by the Local Ethics Committee of the Region of Southern Denmark, Denmark.

2.1. Sample Collection. Muscle biopsies were obtained at baseline (BL) and after 12 weeks (12 wk.). They were collected from m. vastus lateralis under local anaesthetic (1% lidocaine; Amgros, Copenhagen, Denmark) using a 5 mm Bergstrøm needle. Biopsies were taken in the same region and depth of the weakest leg and care was taken to avoid any damaging effect of multiple biopsies [15] by separating incisions by about 5 cm. Fifty to hundred mg tissue was excised and divided into multiple pieces. These were either snap-frozen in liquid nitrogen or embedded in Tissue-Tek (Sakura Finetek, Alphen aan den Rijn, Netherlands) and frozen in precooled isopentane. The samples were stored at −80°C for further analysis. Furthermore, muscle biopsies were collected from healthy aged-matched control subjects (♂ aged 65.1 ± 0.8 yrs) using the above protocol.

2.2. RNA Extraction and cDNA Synthesis. Tissue samples were homogenized and total RNA was extracted according to the manufacturers directions including a salt wash [16] and RNA concentration was measured using the Nanodrop (ND1000, Thermo Scientific, Copenhagen, Denmark) returning 260/280 ratios above 1.8 for all samples, and intact RNA was confirmed by denaturing agarose gel electrophoresis (Bioanalyzer; Agilent Technologies, Glostrup, Denmark). 500 ng of RNA was converted into complementary DNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Copenhagen, Denmark).

2.3. Real-Time Reverse Transcriptase Polymerase Chain Reaction. Real-time RT-PCR for ACTA1, CHRNA1, GDNF, CDK5, MYOG, MYOD1, NCAM, CD68, and PTPRC/CD45 was performed using TaqMan Low Density Arrays. Each port on the card was loaded with cDNA equivalent to 125 ng total RNA and run at 50 cycles in triplicate on the 7900 Sequence Detection System (all reagents from Applied Biosystems, Copenhagen, Denmark). For MYF5, PAX7, P21, P27, SMAD3, and IGF1 real-time RT-PCR was performed in reactions of 25 µL by mixing 6.25 ng cDNA with appropriate TaqMan primers/probes and run in triplicate as described above. Specific primer details can be found in
Table S1 “(see Supplementary Material available online at http://dx.doi.org/10.1155/2016/5930621)” Data was collected and analysed using SDS 2.4 or QuantStudio 12K software analyses: $\Delta C_t = 5$ for ALS and $\Delta C_t = 7$ for controls. Scale bar = 100 m.

2.4. MicroRNA Analysis. Thirty g total RNA was reverse transcribed using a TaqMan MicroRNA Reverse Transcription Kit (4366596) and RT primers specific for miR-1,
miR-27b, mir-133a, miR-26a, miR-29b, miR-23a, miR-455, and controls RNU24 and RNU6b according to manufacturers instructions. The RT products were then amplified in triplicate using sequence complementary TaqMan Small RNA assays. All reagents were from Applied Biosystems. Data was collected and analysed using SDS 2.4 and normalized to RNU24 using the qBase+ software (Biogazelle, Zwijnaarde, Belgium): □ = 5 for ALS and □ = 7 for controls.

2.5. Immunohistochemistry and Quantifications. Ten m sections of frozen tissue were fixed, permeabilised, and blocked before they were incubated with primary antibodies (1 h, at room temperature). Primary antibodies include MHC-slow 1: 2000 (M8421, Sigma-Aldrich, Copenhagen, Denmark), MHC-fast 1: 2000 (M4276, Sigma-Aldrich), MyoD1 1: 25 (863R-16, Cell Marque/Sigma-Aldrich), Pax7 1: 100 (BD Biosystems). Data was collected and analysed using SDS Vision by Zeiss (Brock & Michelsen, Copenhagen, Denmark), Envision+/DAB+ (K4001, K4003, and K3468, Dako), NCAM 1: 100 (Leu19/MY31, BD Biosciences, Albertslund, Denmark), and CD68 1: 100 (M0814, Dako). Then, ImmPRESS AP Ig polymer detection kit and Vector Blue or Vector Red AP Substrate Kit (Vector Laboratories, Herlev, Denmark), Envision+/DAB+ (K4001, K4003, and K3468, Dako, Copenhagen, Denmark), or Alexa Fluor 488 or 555 anti-mouse or anti-rabbit, accordingly 1: 500 (Thermo Fisher Scientific, Denmark), were applied according to manufacturers protocols. Immunofluorescence stainings were mounted with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Digital image processing and analyses were performed using Axio imager M1 and AxioVision by Zeiss (Brock & Michelsen, Copenhagen, Denmark).

In all quantifications the investigator was blinded to the identity of the patients. Cross-sectional area (CSA) of mixed fibres, which is reduced at 12 wk.

<table>
<thead>
<tr>
<th>Group</th>
<th>BL</th>
<th>12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fibres (mean)</td>
<td>300.8 ± 83.5</td>
<td>207 ± 38</td>
</tr>
<tr>
<td>Type I (%)</td>
<td>52.5 ± 6.3</td>
<td>54.9 ± 9.3</td>
</tr>
<tr>
<td>Type II (%)</td>
<td>47.5 ± 4.5</td>
<td>40.3 ± 7.5</td>
</tr>
<tr>
<td>Mixed (%)</td>
<td>4.9 ± 2.0</td>
<td>4.8 ± 1.5</td>
</tr>
</tbody>
</table>

Table 2: Fibre type distribution in ALS muscle.

3. Results

3.1. Visible Morphological Changes in ALS. Figure 1 shows HE stainings from ALS subjects at BL and 12 wk. and from two control samples and reveals morphological changes with increasingly disorganised muscle fibres including increased number of atrophic fibres and compensatory hypertrophy in all ALS biopsies. Subjects 2 and 5 are particularly affected by disease progression demonstrating a high degree of atrophy, compensatory hypertrophy, and areas of macrophage infiltration after 12 wk., while the remaining subjects present with milder morphological changes including the same features. Furthermore, increased amounts of inflammatory cells as well as nuclear clumps are present between the muscle fibres at 12 weeks compared to BL.

The morphological changes are supported by an increase in actin (ACTA1), since -actin is known to be induced during active remodelling, for example, exercise and regeneration [18, 19]. Furthermore, denervation is evident by increases in the nerve and NMJ promoting factors acetylcholine receptor (CHRNA1), glial derived neurotropic factor (GDNF), and cyclin-dependent kinase 5 (CDK5), when compared to controls (Figure 2).

3.2. Changes in Fibre Type Distribution and Fibre Cross-Sectional Area. Baseline and 12 wk. biopsies demonstrate individual disease-induced changes in fibre type distribution and mean CSA, but none of the changes are significant at a group level (Table 2).

When comparing the frequency distributions of CSA at BL 12 wk. in both type I and type II fibres (□ < 0.05); thus ALS muscle contains a larger number of atrophic fibres at 12 wk.

No significant differences are observed for any parameters except for the cross-sectional area (CSA) of mixed fibres, which is reduced at 12 wk.

3.3. Disease Induced Changes in Fibre CSA. The distribution of individual fibre CSA displays large variability in fibre size with fibres ranging from below 3000 µm to more than 10,000 µm (Figures 3(i) and 3(j)). Furthermore, fibres with MHC-fast (type II fibres) demonstrate atrophy and compensatory hypertrophy of both fibre types (Figures 3(a) and 3(b)), as well as areas with fibre type grouping dominated by either type I fibres or type II fibres (Figures 3(c)–3(h)). The distribution of individual fibre CSA displays large variability in fibre size with fibres ranging from below

When comparing the frequency distributions of CSA at BL 12 wk. in both type I and type II fibres (□ < 0.05); thus ALS muscle contains a larger number of atrophic fibres at 12 wk.
12 wk. in ALS patients. () indicates a tendency for a significant difference (\( \Delta < 0.1 \)). and 12 wk. there is a shift towards a smaller fibre size compared to BL.
3.3. Myogenic Changes in ALS. Skeletal muscle of ALS patients shows a reduction in mRNA levels of satellite cell markers, whereas the mRNA levels of the late myogenic markers PAX7 and MYF5 compared to age-matched controls (p < 0.1 compared to BL). Asterisks indicate significant differences from controls; *p < 0.05 and **p < 0.001.

The mRNA results suggest that the satellite cells might be affected in the ALS patients. We therefore analysed the activation status of the satellite cells using immunohistochemistry. Pax7* cells were found next to both hypertrophied fibres (Figure 4(e) and 4(f), arrows) and in smaller fibres (Figure 4(f), arrowhead). The overall number of Pax7 cells/fibre did not change significantly, whereas the number of MyoD+ cells/fibre decreased in ALS subjects, which trends to reach statistical significance (p = 0.078), but no changes in NCAM+ cells were observed (Figures 5(e) and 5(f)).

4.4. Alterations in miRNAs, Cell Cycle Regulators, and...
not change between BL and 12 wk. (Table 3). To investigate
performed double immunofluorescence staining for Pax7 and
MyoD. We find that that very few Pax7+ cells express MyoD
are negative for MyoD (Table 3 and Figures 4(g)–4(i)). On
an interesting note, we observed a large amount of MyoD
positive nuclei in the tissue in general for all patients (Figures
Inflammation in ALS. Skeletal muscle specific microRNAs
(myomiRs) 1, 26a, and 133a along with miR-455 are reduced
in ALS compared to controls (Figure 6(a)); however, disease
progression does not induce further change between BL and 12 wk. mRNA expression of cyclin-dependent kinase inhibitor 1A (p21) is induced, while cyclin-dependent kinase inhibitor 1B (p27) and mothers against decapentaplegic homolog 3 (SMAD3) mRNA transcripts are reduced compared with controls (Figures 6(b)–6(d)). Insulin-like growth factor 1 (IGF1), an inducer of muscle tissue growth, is not affected in ALS compared to controls (Figure 6(e)). Both mRNA levels of CD68 and CD45 along with protein expression of CD68 demonstrated higher levels of inflammatory cells in skeletal muscle of ALS patients compared to controls (Figure 7). The CD68 cells were localized mainly in the connective tissue between muscle fibres.

4. Discussion

In this paper we describe the degenerative and regenerative processes in skeletal muscle of ALS patients over a short-term period to observe disease development and progression. Moreover, we compare the skeletal muscle of ALS patients to that of healthy, age-matched muscle. We find morphological changes in ALS muscle indicative of active denervation and
reinnervation along with a reduction in CSA, yet none of the examined mRNA markers change significantly within the 12-week time period. However, we demonstrate differences between ALS and controls in pathways controlling skeletal muscle homeostasis, cytoskeletal and regenerative markers, neurodegenerative factors, myogenic factors, cell cycle determinants, and inflammatory markers.

Upper and lower motor neuron injuries induce different morphological changes, revealing a preferential type II fibre atrophy and type I hypertrophy and muscle fibre group

Figure 4: Myogenesis in ALS muscle. mRNA transcripts of (a) MYF5, (b) PAX7, (c) MYOG, and (d) MYOD1 in ALS during disease progression compared to controls. (e and f) Representative immunohistochemical images of Pax7− cells in ALS muscle. Arrows indicate Pax7− cells associated with hypertrophied fibres and arrowheads indicate Pax7− cells associated with atrophic fibres. (g–l) Double immunofluorescence stainings of MyoD (yellow), Pax7 (green), and DAPI (blue). (j–l) Double immunofluorescence stainings of MyoD (yellow), Ki67 (green), and DAPI (blue). (*) $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. In (a–d) for ALS and for controls and in (g–l) $\square = 5$ for ALS. Scale bar = 50 μm for all images.
atrophy and fibre type grouping, respectively [20]. Previous studies have evaluated muscle morphometry in ALS patients [21] and neuromuscular disorders [22] and described characteristic features as a mix of the above with muscle fibre atrophy and hypertrophy and a large degree of fibre type grouping, which is in line with our findings. Our results demonstrate a reduction in CSA from BL to 12 wk. and a large variability in fibre CSA ranging from below 100 \( \mu m^2 \) to over 18,000 \( \mu m^2 \) indicating an active remodelling process with atrophy of some fibres and compensatory hypertrophy in others, the latter in order to overcome the loss of muscle fibres. The fact that we do not find a significant increase in hypertrophied fibres is likely a matter of lack of power, as we do see a tendency for an increase. Higher levels in \( \alpha \)-actin

Table 3: Analysis of activation status of satellite cells in ALS.

<table>
<thead>
<tr>
<th></th>
<th>Pax7/fibre</th>
<th>Pax7/MyoD</th>
<th>Pax7/MyoD</th>
<th>Ki67/MyoD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.071</td>
<td>12</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.066</td>
<td>30</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.074</td>
<td>23</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.092</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.068</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.074 ±0.005</td>
<td>19.6±3.6</td>
<td>1.4±0.6</td>
<td>0.8 ±0.37</td>
</tr>
</tbody>
</table>

Satellite cells/myoblast in BL and 12 wk. patient samples were stained for expression of Pax7, MyoD, and Ki67 using immunofluorescence and evaluated by counting. The number of identified cells in each section is noted.

Figure 5: NCAM in ALS. (a) NCAM mRNA transcript in ALS during disease progression compared to controls. (b and c) Immunohistochemical staining for NCAM (blue) showed positive fibres (arrows) and cells (arrowheads) in ALS, but no staining except for a few positive satellite cells in controls (d). Stereological analysis of (e) NCAM fibres (\(*\) difference from controls; \(\text{SEM} = 0.078\)) and (f) NCAM cells. Asterisk in (d) indicates significant difference from controls.
mRNA levels compared to controls further underline this, as active remodelling leads to an activation of the actin promoter [19]. In line with this, our data show substantially elevated NCAM mRNA levels compared to controls, and high muscle fibre NCAM protein expression also suggests that regeneration is activated [23]. NCAM+ fibres in ALS can represent both denervated and regenerating fibres, and the observed decrease in NCAM- fibres from BL to 12 wk. could be a result...
NCAM mRNA levels compared to controls, and high muscle of either an exhausted or an impaired regeneration. It must
be kept in mind that it is the number of NCAM fibres that have been evaluated, rather than the expression level of each fibre, which might change over time.

Denervated, atrophic myofibres tend to become angular and eventually result in “nuclear clumps” with little or no cytoplasm left [24], while new myogenic fibres are small and round [25]. We find that NCAM fibres in ALS patients are mainly small and angular, which suggest that these are denervated fibres rather than newly formed, regenerating fibres.

As a measure of the myogenic potential we evaluated the number of Pax7+ satellite cells in ALS skeletal muscle. Previous studies have established satellite cell numbers of young and old individuals to be around 0.05 Pax7+ cells/fibre [26] and around the same magnitude in ALS patients [27]. Our data of Pax7+ cells/fibre is in line with previous findings [26], indicating that satellite cells are present for potential activation [28]. We therefore performed double stainings for MyoD and Pax7 to evaluate the actual activation status of the Pax7+ cells present in ALS muscle. We find that very few Pax7+ cells express MyoD, suggesting that the satellite cells are in a nonactive, resting state and are not activated; thus corresponding to the increased expression on mRNA level we observe a high amount of MyoD+ cells in general, cells to regenerate the muscle; however, this process has somehow been halted or distorted due to the ALS pathology.

The higher expression levels of MYOD1 and MYOG mRNA in ALS muscle could indicate an activated differentiation process. When analysing expression of MyoD on protein level as well, a double staining for MyoD and the proliferation marker Ki67 reveals that only few of the MyoD+ cells are in an active proliferative state, suggesting that the activated muscle cells are kept in a MyoD+ postmitotic state. Whether this is due to a long differentiation period or the cells are arrested at this stage needs further investigation. However, it is known that both MYOD1 and MYOG are regulated by electrical stimulation [29]; thus the findings on changes in MyoD on both mRNA and protein level might reflect the changed neural activity in these patients. The observation of reduced early myogenic markers in combination with increased late myogenic markers probably reflects the disease pattern in skeletal muscle of ALS patients.

Denervation is one of the earliest signs of ALS and in line with this, we find the presence of many MyoD+/Ki67 cells, this
points out that there has been activation of satellite

with this the denervation-induced markers CHRNA1, GDNF, and CDK5 are all highly expressed in the ALS patients as we expected. The sensitive nature and large increase of CHRNA1,
GDNF, and CDK5 in ALS muscle allow these to be potential biomarkers of ALS disease development and progression.

In the present study we also investigated the mechanisms of cell cycle progression and skeletal muscle growth to evaluate disturbances in the expression of myomiRs or cell cycle regulators could influence the remodelling and regeneration capacity of ALS skeletal muscle. MicroRNAs are tight regulators of cell fate and tissue homeostasis [30], and our results show that miR-1, miR-133a, and miR-206 are all reduced in ALS muscle suggesting that both myogenic proliferation and differentiation might be altered. Inhibition of miR-1 and miR-206 blocks the downregulation of most targets in differentiating cells, thus indicating that they are required for muscle differentiation [31], while miR-133 expression is diminished in the early stage of muscle regeneration and represses myoblast proliferation [32]. MiR-206 has also been identified as an important player in the repair of the NMJ following nerve injury [33], and one study has identified miR-206 as a potential disease marker in SOD1 mice and ALS patients [34]. Thus miRs appear to be important players in ALS disease progression and muscle restoration.

Multiple studies have identified interactions between miR-1 and IGF-1 showing an inverse relationship between these [35, 36]. We observed a reduction in miR-1 combined with no change in IGF-1 mRNA, which may be due to an insufficient reduction of miR-1, or the interactions between miR-1 and IGF-1 do not play a role in ALS.

We find that p21 mRNA expression is induced in the ALS patients, suggesting that myogenic cells have entered the differentiation and maturation process and thus no longer proliferate [37–39]. This is supported by our findings of MyoD/Ki67 cells. Likewise, our data show a reduction in p27 mRNA in ALS patients compared to controls [40, 41], which is in line with our findings of the presence of primarily nonactivated satellite cells.

The present study has several limitations. The small number of ALS patients studied here and the heterogeneous disease progression and clinical presentation seen in ALS patients in general limit our ability to generalise our findings to larger populations of ALS patients. However, a major strength of the present study is the longitudinal collection of muscle biopsies, which is unique in ALS patients.

Altogether, our results suggest that activation, proliferation, and differentiation of skeletal muscle stem cells are affected and possibly halted in ALS patients and there is therefore a generally insufficient myogenesis that cannot overcome the denervation-induced wasting. It would therefore be necessary to understand which mechanisms are affected in order to impact on the myogenic program if we are to diminish disease progression and skeletal muscle atrophy in ALS patients in the future.

**Competing Interests**
The authors declare that they have no competing interests.

**Acknowledgments**
The authors would like to thank the ALS patients for their participation in the study. This study was supported by grants from the Bevica Foundation and the Hede Nielsen Family Foundation.

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