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Title: Monitoring progression of Amyotrophic Lateral Sclerosis using ultrasound morpho-textural muscle biomarkers: A pilot study.

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Abstract: There is an increasing need for progression biomarkers, which allow the loss of motor neurons in amyotrophic lateral sclerosis (ALS) to be monitored in clinical trials. In this prospective longitudinal study, muscle thickness, echointensity, echovariation and grey level co-occurrence matrix textural features are examined as possible progression ultrasound biomarkers in ALS patients during a five months' follow-up period. Thirteen patients, subjected to three measurements for twenty weeks, showed a significant loss of muscle, an evident tendency to loss of thickness, and increased echointensity and echovariation. As regards textural parameters, muscle heterogeneity tended to increase as a result of the neoformation of non-contractile tissue through denervation. Taking into account some limitations of the study, the quantitative muscle ultrasound biomarkers evaluated showed a promising ability to monitor patients affected by ALS.

Suggested Reviewers:

Opposed Reviewers:

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Murcia, Spain, September 9th, 2017

Dr. Christy K. Holland
***Ultrasound in Medicine and Biology*,**

Dear Editor-in-chief:

Enclosed is the manuscript **"Monitoring progression of amyotrophic lateral sclerosis using ultrasound morpho-textural muscle biomarkers: A pilot study"**, to your consideration.

The manuscript is a resubmission Ref. Ms. No. UMB-D-17-00412 which includes reviewer's recommendations. Thanks you very much because your comments have improved our manuscript. According editorial recommendation we have revised our manuscript extensively after giving careful consideration to the points that the referees' have made.

We have made a point-by-point response letter and we have added a copy of the rewritten manuscript.

Please address all correspondence concerning this manuscript to me at jmartinez@ucam.edu

Thank you for your consideration of this manuscript.

Sincerely,
Jacinto J. Martínez-Payá

We have responded point-by-point to all the comments made by the reviewers pointing out where reviewers will be able to find those changes in the text. We have added a copy of the rewritten manuscript Ref. Ms. No. UMB-D-17-00412. The title of the revised version is, as editor in chief suggested, "Monitoring progression of amyotrophic lateral sclerosis using ultrasound morpho-textural muscle biomarkers: A pilot study".

General Comments

Reviewers #1 and #2 agreed that there are two important improvements that we need to consider: the small size of the cohort (n=13) and the short interval between measurements (e.g. 4 weeks vs. 10 weeks).

- We understand and appreciate the methodological suggestions of both reviewers and these improvements will be undoubtedly included in future studies.
- The aim of this pilot study was to develop a proof of concept of the potential value of several ultrasound biomarkers, obtained with a new methodology, in the evaluation of ALS progression. Previous studies concluded that ultrasonography was not suitable to monitor disease progression. However, in our study, several ultrasound biomarkers emerge as candidates for progression monitoring. Of course, these results must be validated in large-scale and long follow-up studies, but we think that our results are a good starting point for future research.

Introduction

Reviewer #1 wrote: "The introduction should include mention of electrical impedance myography in ALS."

- We have included it (page 3; line 8-13) and we have added the following reference:
  - Rutkove SB, Caress JB, Cartwright MS, Burns TM, Warder J, David WS, Goyal N, Maragakis NJ, Clawson L, Benatar M, Usher S, Sharma KR, Gautam S, Narayanaswami P, Raynor EM, Watson ML, Shefner JM. Electrical impedance myography as a biomarker to assess ALS progression. Amyotroph Lateral Scler 2012;13:439–445.

Reviewer #2 suggested these two minor changes:

- Line 3: Please change "upper (UMN) and lower (LMN) motor neurons" to "upper motor neurons (UMN) and lower motor neurons (LMN)".
- Line 8: Please change "incorporatedin" to "incorporated in".

- We have made these changes in the main document on page 1, lines 3 and 8 respectively.

Methods

Reviewer #1 asked: If the patients were recruited September 2013-April 2014 and follow-up was only 20 weeks, why the 3-year delay to analysis/publication?

- After the follow-up, the image analysis was performed, the database was constructed and we completed the statistical analysis. We spent almost one year

in all these actions because the members of this research team do not dedicate themselves exclusively to research, but combine this work with university teaching and / or clinical practice.

- This paper is the third and last in a series of papers in which we present and discuss the results of the "Longitudinal ultrasound study of muscular degeneration in patients with amyotrophic lateral sclerosis". To avoid overlapping the information and analysis we have had to wait for the journals to publish the two previous ones, which explains why the delay between data analysis period and publication is almost 2 years.

Reviewer #1 also asked: Similarly, why were the patients not followed for more than 20 weeks?

- A longer period of follow-up would have been desirable but our initial sample was small and patients were in a moderate to advanced stage ALS, limiting the ability of long follow-up.
- Moreover, progression biomarkers are of especial interest to shorten the duration of clinical trials and make them more efficient. Therefore, a good progression biomarker should be able to find changes within 20 weeks, which is approximately the duration of many phase I and II trials in ALS. Consequently, we thought that, for a pilot study, this duration should be enough to find differences.
- Finally, previous studies had similar periods of follow-up:
  - Arts IMP, Overeem S, Pillen S, Schelhaas HJ, Zwarts MJ. Muscle changes in amyotrophic lateral sclerosis: a longitudinal ultrasonography study. Clinical neurophysiology 2011;122:623–8.
  - Lee CD, Song Y, Peltier AC, Jarquin-Valdivia AA, Donofrio PD. Muscle ultrasound quantifies the rate of reduction of muscle thickness in amyotrophic lateral sclerosis. Muscle & nerve 2010;42:814–9.

Reviewer #1 wrote: The Cartwright et al. reference is not the appropriate citation for the ALSFRS-r. Please cite the source reference (J Neurol Sci. 1999 Oct 31;169(1-2):13-21.) Also check references for the MRC global score.

- We agree with the reviewer that the cited reference was erroneous. We have corrected it and we have added these appropriate references in the text (page 5, lines 16 and 18):
  - Cedarbaum JM, Stambler N, Malta E, Fuller C, Hilt D, Thurmond B, Nakanishi A. The ALSFRS-R: a revised ALS functional rating scale that incorporates assessments of respiratory function. BDNF ALS Study Group (Phase III). J Neurol Sci 1999;169:13–21.
  - Florence JM, Pandya S, King WM, Robison JD, Baty J, Miller JP, Schierbecker J, Signore LC. Intrarater reliability of manual muscle test (Medical Research Council scale) grades in Duchenne's muscular dystrophy. Physical therapy 1992;72:115-22–6.

Reviewer #1 asked: Was there any blinding of image acquisition performed in the current study? This is a potential source of bias.

- Researchers who acquired and analyzed the images were blinded for diagnosis. We added this information in the document (page 5; lines 22).

Reviewer #1 wrote: Please provide more detail on how the ROI was selected. Were more superficial or deep areas favoured? Was there a bias toward including the most affected areas?

- We select the ROI in the most reflective region avoiding anisotropic zones and minimizing the risk of loss of the most affected areas.
- In a previous published study, we demonstrated that the method of ROI selection was reproducible and valid. Reference of this reliability study has been included in the text (page 6; lines 20-21). .

Results/Discussion

Reviewer #1 wrote: The number of patients lost to follow-up is of concern. Analysis show that these were the most affected patients, which is not surprising. However, the muscle will be more affected in these patients and less likely to show change (e.g. "End-stage muscle"). We have seen this in some older Duchenne muscular dystrophy patients, making longitudinal assessments challenging.

- We absolutely agree. The advanced state of patients entering the study is a limitation of the present investigation and future studies will be undoubtedly focused in early stage patients. However, as we have explained above, the aim of this pilot study was to develop and test a new ultrasound monitoring method. As we emphasize in the strengths and limitation section "larger studies assessing longitudinal changes in muscle EV from diagnosis to an advanced stage are warranted to confirm our results and hypotheses and to establish the role of EI, EV and GLMC as progression and prognostic biomarkers" (page 13, lines 20-23).

Reviewer #1 wrote: Upon reading the results section, it is unclear if EI and GLCM changed prior to onset of clinical change or if the change was delayed as with MTh. Table 4 makes this clearer, but please clarify in the text.

- We agree with this concern and we have included a paragraph in the text to clarify the information provided in tables 4 and 5 (now tables 2 and 3) (page 9; lines 9-15 and page 11; lines 15-18).

Reviewer #1 wrote: A primary weakness of this approach, similar to all studies on muscle ultrasound, is that these are derived measures and the images are often highly anisotropic. How does the current approach offer advantages or measuring echointensity alone? How might this approach hold up to intra-observer and inter-observer agreement analysis - not only for obtaining the image, but selecting the ROI?

- The echovariation and textural parameters add information about the granularity of the muscle compared to the analysis of the echointensity. We have shown the advantages of these measurements in ALS patients in previous articles: Ultrasound Med Biol 2017;43:1153–1162 / Ultrason Imaging 2017; In press. DOI: 10.1177/0161734617711370. However, since each parameter measures different properties of the muscle and each muscle display different echographic characteristics depending on its function or the stage of the disease, it is possible that different biomarkers show the best performance according to the studied muscle and the stage of the disease. Larger studies are warranted to address this issue.
- In previous published studies we have studied the reliability and reproducibility of the ROI selection method, obtaining very good ICC values. Ultrasound Med Biol 2017;43:1153–1162 / Ultrason Imaging 2017; In press. DOI: 10.1177/0161734617711370.

Reviewer #1 wrote: More information is needed on the conflicting results presented on page 12, paragraph 2. The current results are in opposition with the authors' earlier publication on this topic. The explanation provided is very short and hypothetical.

Thank you for the recommendation. Our apparently contradictory results suggest that changes in textural parameters (unlike EI) vary with disease progression, with an initial decrease of tissue heterogeneity and granularity, followed by a gradual increase. Increases in EI found in ALS patients have been previously interpreted as a result of fibrotic and fatty tissue formation after muscle denervation, since these tissues are more echointense than muscle (Caresio et al. 2015). However, the first muscle change during the course of denervation, is an edemalike phase. This phase is characterized by an increase of both T1 and T2 relaxation times in muscle MRI (Bryan et al. 1998). With advanced denervation, muscle is replaced by fat and fibrotic tissue, which is characterized by a decrease in T1 relaxation time, and a further increase in the T2 relaxation time. Thus, during the progressive stages of muscle denervation, the T2 relaxation time increases continuously, whereas the T1 relaxation time increases and then decreases (Bryan et al. 1998). We think that something similar happens with muscle biomarkers. The initial increase in EI and decrease in homogeneity and granularity are due to this edemalike phase, which explains that ALS patients show less homogeneity and granularity than controls. However, as the disease progresses and muscle tissue is progressively replaced by fat and fibrotic tissue, the formation both non-contractile tissues would decrease the homogeneity, while the EI continues to increase. Moreover, the fatty tissue is more echointense than the fibrotic one (Walker and Cartwright 2011) which means that the degree of irregular distribution of both non-contractile tissues would affect muscle texture in a different degree in each muscle group. These phenomena would explain the diversity of changes in EV, granularity and contrast found in different muscle groups, since each might be in a different denervation stage and with a variable proportion of fatty and fibrotic tissue infiltration. However, given the limitations of our study, more studies on muscle textural biomarkers are needed to figure out in more detail the dynamics of change of muscle biomarkers during the disease progression.

- For further explanation of this point, we have included a paragraph in the main document (page 12, lines 8-25 and page 13, lines 1-13).

Figures and Tables

Reviewer #1 suggested that tables 2 and 3 not additive to the text and might be deleted or provided as supplementary material.

- As suggested, we have removed tables 2 and 3 from the main document by enclosing them as supplementary material.
- In the *Results* section we have changed the number of the remaining tables and we have pasted the description of the tables to the end of the section (page 9, line 13-22).

TITLE: Monitoring progression of Amyotrophic Lateral Sclerosis using ultrasound morpho-textural muscle biomarkers: A pilot study.

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ABSTRACT.

There is an increasing need for progression biomarkers, which allow the loss of motor neurons in amyotrophic lateral sclerosis (ALS) to be monitored in clinical trials. In this prospective longitudinal study, muscle thickness, echointensity, echovariation and grey level co-occurrence matrix textural features are examined as possible progression ultrasound biomarkers in ALS patients during a five months' follow-up period. Thirteen patients, subjected to three measurements for twenty weeks, showed a significant loss of muscle, an evident tendency to loss of thickness, and increased echointensity and echovariation. As regards textural parameters, muscle heterogeneity tended to increase as a result of the neoformation of non-contractile tissue through denervation. Taking into account some limitations of the study, the quantitative muscle ultrasound biomarkers evaluated showed a promising ability to monitor patients affected by ALS.

Keywords: Amyotrophic lateral sclerosis; motor neuron disease; neuromuscular Diseases; biomarkers; ultrasonography; disease progression; Image Processing Computer-Assisted.

INTRODUCTION.

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder affecting both upper motor neurons (UMN) and lower motor neurons (LMN), leading to gradual muscle weakness and wasting (Wijesekera and Leigh 2009). The variable degree of impairment of UMN and LMN results in pathological and clinical heterogeneity, which hinders diagnosis, prognosis and the monitoring of progression (Kinsley and Siddique 1993). Electromyography allows LMN impairment to be detected before the onset of overt symptoms and consequently has been incorporated in successive diagnostic criteria (Brooks et al. 2000). However, despite some promising new neurophysiological techniques such as MUNIX (Neuwirth et al. 2015) and electrical impedance myography (Rutkove et al. 2012), clinical tools such as Medical Research Council (MRC) (Florence et al. 1992) and the revised ALS functional rating scale, (ALSFRS-r) (Simon et al. 2014) remain the gold standard biomarkers for progression monitoring in clinical trials or clinical practice.

Muscle ultrasonography (MUS) is an accessible, painless and easy to perform method to detect structural muscle changes in ALS (Mayans et al. 2012). More specifically, MUS reveals marked diminished muscle thickness (MTh), increased echointensity (EI) and fasciculations (Arts et al. 2012; Martínez-Payá et al. 2017a). However, in a longitudinal study, Arts et al. (2010) observed that these ultrasound changes found in ALS patients were highly variable and did not show evident correlation with functional measures like muscle strength or disability during 6 months monitoring (Arts et al. 2011a).

We have previously described a new first-order ultrasound biomarker, echovariation (EV), which distinguished the muscles of ALS patients from healthy controls, with higher effect sizes than MTh or EI and correlating better with other clinical variables (Martínez-Payá et al. 2017a).

While EV is a fast and easy method to obtain information on tissue homogeneity (Aggarwal and Agrawal 2012), it does not provide information concerning the relative positions of the various grey levels within the image. This issue can be resolved by a second-order statistics features based on the grey level co-occurrence matrix (GLCM), where the pixels are considered in pairs. GLCM detects the relationship between neighbouring pixel intensities and provides information about grey level patterns (Haralick et al. 1973). Moreover, GLCM parameters showed reduced granularity in the muscles of ALS patients compared with controls and a similar discrimination capacity to EV (Martínez-Payá et al. 2017b). However, the usefulness of the textural features of EV and GLCM as progression biomarkers has not been analysed. Hence, we designed a prospective longitudinal study in patients with ALS to evaluate these new biomarkers and compare them with MTh and EI, during a follow-up period of 20 weeks.

MATERIAL AND METHODS.

Subjects.

Patients were recruited from the ALS Association (ADELA) of Valencia (Spain) between September 2013 and April 2014. Twenty-six patients diagnosed with ALS according to the revised El Escorial Criteria (Brooks et al. 2000) by an experienced neurologist (JFVC) were included. The same cohort had been used previously to assess changes in EV and GLMC between patients (at recruitment) and controls (Martínez-Payá et al. 2017a; Martínez-Payá et al. 2017b). For this longitudinal study, each patient was evaluated twice more with an interval of 10 weeks ± 7 days.

Standard protocol approval, recruitment, and patient consent.

This study was approved by the Ethics Committee of the Universidad Católica de Murcia (Spain). All participants provided written informed consent.

Recorded variables.

Demographical and clinical characteristics (sex, age, weight, height, body mass index, time from diagnosis) were recorded. Muscle strength was measured using the MRC global score with a maximum value of 100 (Florence et al. 1992), as described and segmented by upper (max. 50) and lower limbs (max. 30) and neck muscles (max. 20). The ALSFRS-r rating scale (Cedarbaum et al. 1999), was assessed by the same investigator (JM-P) on the same day as the MUS was performed.

Ultrasonography.

MUS was performed in four muscle groups of each side by the same experienced examiner (JM-P) blinded for the diagnosis, with a phased array real-time scanner LOGIQe BT12 (General Electric Healthcare, China) and a 5–13 MHz linear array transducer (12L–RS) as previously published (Martínez-Payá et al. 2017a; Martínez-Payá et al. 2017b).

Applying the standardized protocol described (Arts et al. 2011a; Martínez-Payá et al. 2017a), bilateral transverse ultrasound images of the biceps/brachialis, forearm flexors group, quadriceps femoris and tibialis anterior were obtained and measured. Three images were taken of every muscle in order to minimize variation in measurement parameters (Arts et al. 2008).

The resulting images had a resolution of 820 x 614 pixels (with a scale of 99.5px/cm for tibialis anterior muscle and 83.5px/cm for other muscles) involving 256 grey levels, and were stored as TIFF files without compression or loss (Wiggins et al. 2001).

Image analysis.

MTh was measured with electronic calipers as previously described (Arts et al. 2010; Martínez-Payá et al. 2017a; Martínez-Payá JJ et al. 2017b). This parameter was measured in all three images of each muscle group by an expert ultrasonographer (JM-P) and the mean of the three values was used for the corresponding analysis.

The image processing and analysis was performed by one researcher (JR-D), blind to diagnosis, using the ImageJ (v.1.48, National Institute of Health, Bethesda, Maryland, USA, 2015) software. The region of interest (ROI) was selected with the ROI Manager application for ImageJ, with a size of 71 x 40 pixels for tibialis anterior and 73 x 73 pixels for other muscle groups on an 8-bit grey scale. The ROI was defined as the muscle region without bone and fascia with the best reflection (Martínez-Payá et al. 2017a; Martínez-Payá et al. 2017b) (Figure 1). This ROI selection method has been found reproducible and valid in a previous study. (Martínez-Payá et al. 2017a). From the ROI we obtained EI, EV (Martínez-Payá et al. 2017a) and GLCM (Martínez-Payá JJ et al. 2017b) textural features.

The texture analysis based on a GLCM is derived from the angular relationship between neighbouring pixels (Nanni et al. 2013), among which five parameters were selected: Energy or Angular Second Moment (ASM); Textural correlation (TC); Homogeneity or Inverse

Difference Moment (IDM); Contrast (CON) and Entropy (ENT). A homogeneous image would be the result of a greater ASM/TC/IDM and a smaller CON/ENT.

Statistical analysis.

Data were analysed using IBM SPSS Statistics for Windows 19.0 (IBM Corp. Released 2010, Armonk, NY, USA). Data were summarized by mean, standard deviations, range and 95% confidence intervals for continuous variables and absolute and relative frequencies for categorical variables. Significance was fixed at 0.05 in all the statistical tests.

The follow-up group with 3 measurements (initial, 10^{th} week and 20^{th} week) was compared with the lost to follow-up group at baseline with a one-way analysis of variance (ANOVA) for continuous variables and chi-squared test for categorical variables.

All continuous variables were distributed normally and thus the ANOVA assumptions were not violated. A repeated measures ANOVA was used to compare the effect of time (three levels) on MRC, ALSFRS-r, EI, EV, and the five GLCM textural features. The Mauchly test was used to evaluate the assumption of sphericity, in case it was violated; a Greenhouse-Geisser corrected test for degrees of freedom was performed. Univariate paired post-hoc t-test (with Bonferroni correction) analyses for each dependent measure with respect to baseline were performed.

Cohen's d statistic (taking the SD at baseline as reference) was used to determine the effect size in pairwise comparisons (< 0.1 small, 0.25 medium and > 0.4 large effect size) (Kelley and Preacher 2012). To compare ultrasound parameters of various muscles, besides ALSFRS-R and MRC scores for the 20 weeks, the percentage change from baseline was calculated for each parameter.

RESULTS.

Study subject's characteristics.

Twenty-six patients with ALS (8 women) were included in this study and 13 patients were lost to follow-up (6 deaths and 7 due to high disability that prevented the ultrasound study). Therefore, 13 patients were followed for 20 weeks (2 women, mean age 56.3 years, SD 10.41) (Table 1).

Follow-up vs lost to follow-up cohort.

The clinical and ultrasound characteristics of the first measurement were compared between patients who finished all three measurements and the patients lost to follow-up. Differences in demographical and clinical characteristics between both cohorts can be found in Table 1. As expected, patients lost to follow-up were more disabled, had less muscle strength and a longer disease duration since diagnosis.

Quantitative muscle ultrasound biomarkers and progression monitoring in ALS.

A significant decrease in muscle strength and ALSFRS-r rating scale was evident at the first and second follow-up (Table 2). The mean of the MRC global values and ALSFRS-r score had fallen by about 20% at week 20 (approx. 1%/week) with a rate of change of -0.55 points/week (max. 100) and -0.33 points/week (max 48), respectively. The decrease in MRC in lower limbs was similar in both follow-up measurements. Conversely, in upper limbs, the decrease in MRC occurred predominantly between the 10th and 20th week (Table 2).

We observed consistent changes with time in the first-order MUS biomarkers (Table 2). MTh showed a trend to decrease in all muscle groups but with a low effect size ($\leq$0.43) and rate of change (-0.4%/week). Moreover, this decrease was not linear and occurred late (between the second and third measurements). Conversely, EI increased and showed a greater rate of change (1.1%-1.3%/week) and effect sizes (1.22-1.49) than clinical variables, except for tibialis anterior. Moreover, changes in EI were similar in both follow-up measurements,

suggesting a linear increase. EV tended to increase in all muscle groups, except biceps brachialis. However, greater heterogeneity in effect sizes and rates of change was found depending on muscle groups and the follow-up time.

Regarding GLCM textural parameters, most of them showed significant changes with time, ASM/TC/IDM decreasing and CON/ ENT increasing (Table 3). Although effect sizes and rate of change of each parameter varied as per muscle group, overall CON was the parameter showing the greatest effect sizes (0.35-1.08) and rates of change (between 1.4 and 3.3%/week).

MTh of all muscles showed similar or worse size effects in both measurements than the respective clinical variables. However, compared with ALSFRS-r, all biomarkers except IDM showed greater effect sizes in at least one muscle of the lower and upper limbs. Compared with the MRC in upper limbs, all biomarkers except EV and CON, showed better effect sizes in both upper limbs muscles. Finally, in lower limbs, all biomarkers except IDM showed better effect sizes than MRC in quadriceps, but not in tibialis anterior, suggesting that muscle changes are more evident in those more preserved ones.

Differences in first-order MUS variables between cohorts were also analysed (Supplementary Table 1). We observed a lower MTh and a greater EI in most evaluated muscles in the lost to follow-up cohort. Quadriceps femoris was the muscle showing greatest differences, whereas no relevant differences were found in forearm flexor. Greater EV in the quadriceps femoris was found in the lost to follow-up group, but no differences in EV in other muscle groups. Analyses of GLCM textural features showed that, again, quadriceps femoris presented the greatest differences (lower ASM, TC and IDM and a greater CON and ENT in the lost to follow-up group), whereas no differences were found in the upper limb muscle groups (Supplementary Table 2).

DISCUSSION.

We have previously described an increased EI and reduced MTh, EV and granularity (i.e. reduced muscle heterogeneity) in ALS patients compared with healthy controls (Martínez-Payá et al. 2017a; Martínez-Payá et al. 2017b). MTh is a measure of muscle atrophy, which is a well-known feature of ALS. EI measures the mean pixel intensity of an ROI and has been shown to increase in ALS patients (Arts et al. 2008; Martínez-Payá et al. 2017a) probably due to the infiltration of fatty and connective tissue after neurogenic denervation (Pillen et al. 2009). EV is a parameter that quantifies the deviation of the level of grey from the average, and is the result of dividing the standard deviation by the mean pixel intensity (EI), thus providing information on tissue homogeneity (greater EV reflects greater tissue heterogeneity). GLMC variables investigate the relationship between neighbouring pixel intensities (Haralick et al. 1973) and provide information about grey level patterns (granularity) (Gdynia et al. 2009).

Study design.

In this longitudinal study, the evolution of all these quantitative MUS parameters as the disease progressed was analysed in the same cohort of ALS patients (Martínez-Payá et al. 2017a; Martínez-Payá et al. 2017b).

Half of the patients were lost to follow-up due to poor short-term prognosis (death or increasing disability that prevented continuing in the study). As expected, lost to follow-up patients were more disabled at recruitment. Interestingly, they showed lower MTh and greater EI than the follow-up cohort. Although our study was not designed to evaluate MUS as a prognosis biomarker, these results reinforce previous findings by Arts et al. (2011) who found that EI predicts prognosis (Arts et al. 2011b). Greater differences between groups were found in the lower limb muscles, which could be because more patients in the lost to follow-up

group had a lower limb onset. No significant differences between groups were found in EV and GLMC, except as regards quadriceps femoris, where the follow-up cohort showed reduced heterogeneity. However, the effect sizes of each biomarker varied considerably according to the muscle group, suggesting that differences both in the muscle characteristics and in the degree of impairment influence MUS biomarkers.

Quantitative MUS parameters as progressions biomarkers.

ALS is characterized by a great heterogeneity in the disease onset and spread (Ravits and La Spada 2009), which results in a variable degree of muscle impairment, depending on the region of onset, the spread of the disease and the muscle group studied in each single region. This hinders an accurate measurement of disease progression in clinical trials. The patients followed in this longitudinal study are representative of those usually included in clinical trials, namely patients in a moderate stage of the disease with at least two body regions affected. Despite this disease-related heterogeneity and the low number of studied subjects, we were able to find consistent changes as the disease progressed in all the studied MUS biomarkers. Moreover, although decreases in MTh were mild and late, a linear variation with greater effect sizes than that of clinical variables (ALSFRS-r and MRC) was observed in most first and second order parameters. Among all, EI showed the greater effect sizes in most muscles. This suggests that EI (a measure of fatty and connective tissue infiltration) is more sensitive to changes with disease progression than clinical variables. Surprisingly, Arts et al. (2011) previously failed to find changes in EI and MTh with disease progression (Arts et al. 2011a), whereas Lee et al. (2010) found changes in MTh but not in EI (Lee et al. 2010). Both studies included a small number of patients and any divergences from our results are probably attributable to methodological differences.

Regarding EV, a gradual increase in forearm flexor and quadriceps femoris muscle groups but not in biceps/brachialis and tibialis anterior was found for this parameter, reflecting an

increase in tissue heterogeneity with disease progression and agreeing with the increased muscle heterogeneity reported in qualitative visual assessment following neurogenic denervation (Pillen et al. 2008).

Similar to EV, GLMC parameters showed a progressive increase in tissue granularity and contrast with disease progression, The textural variable most sensitive to changes was CON, although as with EV, differences were not statistically significant in biceps brachialis and tibialis anterior.

These results appear to conflict with our previous findings of reduced EV, granularity and contrast in ALS patients compared with healthy controls (Martínez-Payá et al. 2017a; Martínez-Payá et al. 2017b). These apparently contradictory results suggest that changes in these biomarkers (unlike EI) vary with disease progression, with an initial decrease of tissue heterogeneity and granularity, followed by a gradual increase. Increases in EI found in ALS patients have been previously interpreted as a result of fibrotic and fatty tissue formation after muscle denervation, since these tissues are more echointense than muscle (Caresio et al. 2015). However, the first muscle change during the course of denervation, is an edemalike phase. This phase is characterized by an increase of both T1 and T2 relaxation times in muscle MRI (Bryan et al. 1998). With advanced denervation, muscle is replaced by fat and fibrotic tissue, which is characterized by a decrease in T1 relaxation time, and a further increase in the T2 relaxation time. Thus, during the progressive stages of muscle denervation, the T2 relaxation time increases continuously, whereas the T1 relaxation time increases and then decreases (Bryan et al. 1998). We think that something similar happens with muscle biomarkers. The initial increase in EI and decrease in homogeneity and granularity are due to this edemalike phase, which explains that ALS patients show less homogeneity and granularity than controls. However, as the disease progresses and muscle tissue is progressively replaced by fat and fibrotic tissue, the formation both non-contractile tissues

would decrease the homogeneity, while the EI continues to increase. Moreover, the fatty tissue is more echointense than the fibrotic one (Walker and Cartwright 2011) which means that the degree of irregular distribution of both non-contractile tissues would affect muscle texture in a different degree in each muscle group. These phenomena would explain the diversity of changes in EV, granularity and contrast found in different muscle groups, since each might be in a different denervation stage and with a variable proportion of fatty and fibrotic tissue infiltration.

Our results, together with those previously published (Martínez-Payá et al. 2017a; Martínez-Payá et al. 2017b) suggest that EI would be the best muscle biomarker to monitor disease progression changes (at least in late disease stages), since it continuously increases throughout the disease course. However, textural parameters would discriminate better early muscle changes and would therefore perform better as diagnostic biomarkers (Martínez-Payá et al. 2017a; Martínez-Payá et al. 2017b).

Strengths and limitations.

To the best of our knowledge, our study represents the most thorough analysis to date of muscle biomarkers to assess disease progression in ALS patients. Moreover, the highly reliable methodology identified consistent changes in muscle biomarkers with disease progression, where others failed (Arts et al. 2011a; Lee et al. 2010). However, the studied sample was small, patients were in a moderated to advanced stage of the disease and follow-up was limited to 20 weeks. Therefore, larger studies assessing longitudinal changes in muscle EV from diagnosis to an advanced stage are warranted to confirm our results and hypotheses and to establish the role of EI, EV and GLMC as progression and prognostic biomarkers.

CONCLUSIONS.

Our pilot study suggests that, unlike previously found, quantitative MUS parameters are feasible progression biomarkers in ALS and could be more sensitive than clinical variables for monitoring progression. In moderately disabled ALS patients, disease progression results in a decrease in MTh, and an increase in EI and in muscle heterogeneity (measured with both EV and GLMC monitoring parameters). Overall, EI appears to be the most sensitive and reliable biomarker for progression in a moderate to advanced disease stage. Changes in muscle homogeneity and granularity differ according to muscle group and disease stage, which could reflect distinct denervation phases.

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FIGURE CAPTIONS

Figure 1. Analysis of thickness and echotexture in the biceps/brachialis muscle group in ALS patients after a follow-up of 20 weeks. In this patient, we observed a loss of 25% of muscle thickness and an increase of 26% of the muscle echointensity (ROI A. 74 points vs ROI B. 98 points).

TABLES.

Table 1. Baseline differences between patients subjected to 3 measurements and lost patients.

Baseline characteristics	Follow-up cohort (n=13)	Lost to follow-up cohort (n=13)	p-value
Women (n) (%)	2 (15.4 %)	6 (46.2 %)	0.016
Age (yr)	56.3 (10.41); 52.1 to 60.5	60.9 (11.46); 56.3 to 65.6	0.137
Weight (kg)	66.9 (14); 61.3 to 72.6	68.6 (17.22); 61.7 to 75.6	0.699
Height (m)	1.7 (0.075); 1.67 to 1.73	1.62 (0.074); 1.59 to 1.65	0.001
BMI (kg/m^2)	23 (3.6); 21.6 to 24.5	25.8 (4.98); 23.7 to 27.8	0.027
Disease onset-diagnosis (months)	14.1 (9.01); 10.5 to 17.8	18.5 (10.43); 14.3 to 22.7	0.115
Region of onset (n) (%)			
Right Lower Limb	3 (23.1 %)	6 (46.2 %)	
Left Lower Limb	3 (23.1 %)	2 (15.4 %)	
Right Upper Limb	1 (7.7 %)	0 (0 %)	0.321
Left Upper Limb	2 (15.4 %)	2 (15.4 %)	
Bulbar	4 (30.8 %)	3 (23.1 %)	
ALFSFR-r (max. 48)	29.3 (11.92); 24.5 to 34.1	23 (10.72); 18.7 to 27.3	0.050
MRC (max. 100)	62.9 (19.72); 54.9 to 70.8	54.2 (28.66); 42.6 to 65.8	0.210
MRC upper limbs (max. 50)	29.6 (13.79); 24.1 to 35.2	27.5 (18.53); 20 to 35	0.636
MRC lower limbs (max 30)	17.9 (5.42); 15.7 to 20	13 (11.03); 8.5 to 17.4	0.048

Data are presented as mean (SD); C.I. 95%. P-value for Chi-Square (Sex), and T-Student for independent samples. BMI: Body Mass Index. ALFSFR-r: Amyotrophic lateral sclerosis functional rating scale. MRC: Medical Research Council Scale for muscular Strength. P- value for Chi-square test for sex differences and T-Student test for independent samples for age, weight, height and body mass index differences

Table 2. Evolution in clinical and first order statistic ultrasound variables.

US parameters	Time	Mean (SD)	95% C.I.	Minimum	Maximum	p-value*	Effect size**	Mean % change
			Clinical variables					
MRC total (max. 100)	Initial	62.9 (19.72)	54.9 to 70.8	31.4	94.6			
	10th week	58.3 (23.13)	48.9 to 67.6	12.0	94.6	0.006	0.22	-8.2
	20th week	51.8 (25.46)	41.5 to 62.0	12.0	94.6	0.001	0.56	-20.8
MRC upper limbs (max. 50)	Initial	29.6 (13.79)	24.1 to 35.2	2.0	46.7			
	10th week	27.5 (15.11)	21.4 to 33.6	0.0	46.7	0.009	0.16	-9.4
	20th week	22.9 (16.68)	16.2 to 29.7	0.0	46.7	0.007	0.48	-34.7
MRC lower limbs (max. 30)	Initial	17.9 (5.42)	15.7 to 20	6.0	28.0			
	10th week	15.9 (7.51)	12.9 to 19	0.0	28.0	0.005	0.35	-15.7
	20th week	14.5 (8.02)	11.2 to 17.7	0.0	28.0	<0.001	0.62	-26.5
ALSFRS-r (max. 48)	Initial	29.3 (11.92)	24.5 to 34.1	5.0	48.0			
	10th week	26.5 (12.53)	21.5 to 31.6	5.0	48.0	0.012	0.23	-11.2
	20th week	22.7 (11.34)	18.1 to 27.3	5.0	46.0	<0.001	0.55	-21.3
			Muscle thickness(MTh; mm)					
Biceps/brachialis	Initial	31.1 (6.13)	28.5 to 33.7	21.4	41.1			
	10th week	30.1 (7.24)	27.0 to 33.1	10.0	42.5	0.847	0.20	-6.3
	20th week	28.9 (5.38)	26.6 to 31.1	18.0	39.1	<0.001	0.40	-7.6
Forearm flexor	Initial	31.1 (7.89)	27.8 to 34.4	16.4	44.2			
	10th week	31.0 (6.63)	28.2 to 33.8	15.6	44.2	1.000	0.05	0.4
	20th week	27.8 (6.55)	25.0 to 30.6	19.2	39.8	0.177	0.43	-7.2
Quadriceps femoris	Initial	26.5 (7.84)	23.2 to 29.8	15.6	40.7			
	10th week	26.4 (7.08)	23.4 to 29.4	10.4	41.5	1.000	0.04	-0.9
	20th week	24.1 (7.67)	20.8 to 27.3	10.8	35.6	0.095	0.32	-8.5
Tibialis anterior	Initial	21.8 (5.73)	19.4 to 24.2	11.3	32.4			
	10th week	19.8 (3.83)	18.2 to 21.4	12.4	25.4	0.318	0.32	-1.5
	20th week	19.5 (4.04)	17.7 to 21.2	12.1	27.4	0.034	0.39	-7.1
			Echointensity (EI; 0 – 255 levels)					
Biceps/brachialis	Initial	87.7 (13.72)	81.9 to 93.5	63.3	120.3			
	10th week	102.4 (16.67)	95.4 to 109.5	76.4	143.1	0.001	0.99	17.5
	20th week	108.0 (14.98)	101.7 to 114.4	88.1	151.4	<0.001	1.49	25.8
Forearm flexor	Initial	99.3 (16.49)	92.4 to 106.3	66.5	130.1			
	10th week	109.2 (14.68)	103.0 to 115.4	82.0	142.1	0.115	0.61	12.0
	20th week	119.0 (13.26)	113.4 to 124.6	94.8	140.9	<0.001	1.22	23.3
Quadriceps femoris	Initial	95.6 (15.38)	89.1 to 102.1	74.4	123.9			
	10th week	103.0 (13.22)	97.4 to 108.6	79.6	123.2	0.069	0.53	10.3
	20th week	114.3 (15.55)	107.7 to 120.9	81.5	143.8	<0.001	1.22	22.3
Tibialis anterior	Initial	110.1 (14.28)	104.1 to 116.1	75.1	129.8			
	10th week	114.3 (13.25)	108.7 to 119.8	94.0	137.4	0.618	0.29	4.2
	20th week	116.3 (11.36)	111.5 to 121.1	100.0	134.8	0.096	0.48	7.6
			Echovariation (EV; 0 - 100 points)					
Biceps/brachialis	Initial	24.5 (7.65)	21.3 to 27.8	9.5	38.3			
	10th week	21.1 (4.89)	19.0 to 23.2	11.0	30.5	0.089	0.42	-7.4
	20th week	22.4 (5.47)	20.1 to 24.7	12.6	31.3	0.577	0.27	-0.2
Forearm flexor	Initial	19.0 (4.42)	17.1 to 20.9	10.6	26.5			
	10th week	21.3 (4.11)	19.6 to 23.1	14.3	29.0	0.173	0.50	9.8
	20th week	22.7 (4.02)	21.0 to 24.4	14.3	31.2	<0.001	0.77	21.6
Quadriceps femoris	Initial	16.9 (3.42)	15.5 to 18.4	11.0	24.2			
	10th week	21.5 (5.35)	19.3 to 23.8	12.8	33.2	0.005	1.24	22.5
	20th week	22.7 (4.18)	20.9 to 24.4	14.8	29.8	<0.001	1.61	37.7
Tibialis anterior	Initial	17.2 (3.92)	15.6 to 18.9	11.5	25.2			
	10th week	19.1 (4.45)	17.2 to 21.0	12.3	28.1	0.181	0.54	17.1
	20th week	18.4 (4.35)	16.5 to 20.2	12.8	29.1	0.447	0.40	13.9

S.D: standard deviation. 95% C.I.: 95% confidence interval. * The reference is the initial explorationEffect size was estimated with Cohen's d Statistic (<0.1 small, 0.25 medium and > 0.4 large effect size).**

Table 3. Evolution of second order statistical ultrasound variables through Grey Level Co-occurrence Matrix (GLCM) textural features.

US parameters	Time	Mean (SD)	95% C.I.	Minimum	Maximum	p-value*	Effect size**	Mean % change
			Angular Second Moment (ASM)					
	Initial	19.4 (8.71)	15.8 to 23.1	8.2	44.0			
Biceps/brachialis	10th week	14.7 (6.84)	11.8 to 17.6	6.1	41.0	0.083	0.55	-11.8
	20th week	12.8 (4.04)	11.1 to 14.5	6.3	21.0	0.001	0.76	-25.6
	Initial	15.4 (6.57)	12.6 to 18.1	7.7	31.0			
Forearm flexor	10th week	11.1 (4.00)	9.4 to 12.8	5.2	21.0	0.040	0.65	-7.5
	20th week	9.8 (2.54)	8.8 to 10.9	6.1	16.0	0.005	0.84	-25.6
	Initial	18.3 (8.56)	14.7 to 21.9	7.2	48.0			
Quadriceps femoris	10th week	14.0 (5.77)	11.5 to 16.4	6.4	29.0	0.154	0.50	-5.5
	20th week	11.4 (3.65)	9.8 to 12.9	5.7	18.0	0.007	0.81	-25.9
	Initial	19.4 (8.71)	15.8 to 23.1	8.2	44.0			
Tibialis anterior	10th week	14.7 (6.84)	11.8 to 17.6	6.1	41.0	0.061	0.65	-14.4
	20th week	12.8 (4.04)	11.1 to 14.5	6.3	21.0	0.018	0.65	-18.1
			Contrast (CON)					
	Initial	194.0 (99.06)	152.2 to 235.8	84.1	517.6			
Biceps/brachialis	10th week	229.3 (71.16)	199.3 to 259.4	75.3	365.9	0.087	0.36	39.9
	20th week	228.4 (91.28)	189.9 to 267.0	127.0	535.9	0.540	0.35	32.8
	Initial	210.3 (73.38)	179.4 to 241.3	87.3	335.0			
Forearm flexor	10th week	247.1 (77.88)	214.2 to 280.0	105.8	447.4	0.388	0.50	24.4
	20th week	289.5 (92.40)	250.4 to 328.5	145.9	486.6	0.010	1.08	58.6
	Initial	203.5 (84.39)	167.9 to 239.2	107.5	394.6			
Quadriceps femoris	10th week	227.9 (94.05)	188.2 to 267.6	89.4	508.3	1.000	0.29	23.2
	20th week	288.0 (150.67)	224.4 to 351.6	151.9	852.8	0.076	1.00	65.6
	Initial	263.2 (100.28)	220.9 to 305.6	78.3	476.2			
Tibialis anterior	10th week	304.6 (111.42)	257.5 to 351.6	142.3	554.0	0.100	0.41	21.8
	20th week	312.2 (139.01)	253.5 to 370.9	140.8	601.4	0.192	0.49	28.3
			Textural Correlation (TC)					
	Initial	18.7 (9.1)	14.9 to 22.6	7.3	47.0			
Biceps/brachialis	10th week	16.0 (7.65)	12.8 to 19.2	6.1	38.0	0.639	0.30	1.0
	20th week	14.2 (5.43)	11.9 to 16.5	5.3	26.0	0.085	0.50	-10
	Initial	24.4 (10.21)	20.1 to 28.7	10.3	46.5			
Forearm flexor	10th week	16.0 (6.25)	13.3 to 18.6	6.3	28.0	0.056	0.59	-7.3
	20th week	12.1 (5.54)	9.8 to 14.5	4.4	30.5	<0.001	0.94	-38
	Initial	20.2 (9.31)	16.2 to 24.1	9.3	44.0			
Quadriceps femoris	10th week	14.2 (5.13)	12 to 16.3	6.5	27.5	0.005	0.90	-13.9
	20th week	10.6 (3.28)	9.2 to 12	6.0	20.0	<0.001	1.31	-40.2
	Initial	17.2 (8.16)	13.7 to 20.6	8.0	49.0			
Tibialis anterior	10th week	13.8 (5.21)	11.6 to 16.0	5.8	32.0	0.350	0.41	-9.1
	20th week	14.0 (5.05)	11.8 to 16.1	6.6	24.0	0.268	0.40	-10.2
			Inverse Difference Moment (IDM)					
	Initial	25.7 (5.28)	23.5 to 28.0	17.7	34.5			
Biceps/brachialis	10th week	22.6 (4.50)	20.7 to 24.5	16.2	37.5	0.005	0.60	-9.6
	20th week	22.0 (3.72)	20.4 to 23.6	15.4	28.9	<0.001	0.71	-13.0
	Initial	21.5 (3.97)	19.8 to 23.1	15.7	29.0			
Forearm flexor	10th week	20.3 (4.76)	18.3 to 22.3	15.2	32.6	1.000	0.29	2.7
	20th week	19.5 (3.35)	18.0 to 20.9	14.5	25.4	0.333	0.51	-5.7
	Initial	22.5 (4.12)	20.8 to 24.3	15.5	30.7			
Quadriceps femoris	10th week	22.3 (4.65)	20.3 to 24.2	14.0	32.3	1.000	0.06	5.6
	20th week	21.0 (3.84)	19.3 to 22.6	15.7	27.0	0.567	0.38	-4.7
	Initial	22.3 (4.54)	20.4 to 24.3	14.6	31.4			
Tibialis anterior	10th week	20.0 (4.33)	18.2 to 21.9	14.3	28.6	0.014	0.51	-7.5
	20th week	20.0 (3.57)	18.5 to 21.5	14.6	25.7	0.015	0.52	-10.0
			Entropy (ENT)					
	Initial	7.0 (0.41)	6.8 to 7.1	5.9	7.7			
Biceps/brachialis	10th week	7.1 (0.38)	7.0 to 7.3	6.0	7.8	0.148	0.44	2.5
	20th week	7.2 (0.33)	7.1 to 7.4	6.6	7.9	0.004	0.63	3.7

US parameters	Time	Mean (SD)	95% C.I.	Minimum	Maximum	p-value*	Effect size**	Mean % change
	Initial	7.1 (0.38)	6.9 to 7.2	6.3	7.6			
Forearm flexor	10th week	7.3 (0.34)	7.2 to 7.5	6.6	8.0	0.097	0.63	2.1
	20th week	7.4 (0.24)	7.3 to 7.5	6.9	7.9	0.005	0.96	5.3
	Initial	6.9 (0.40)	6.7 to 7.1	5.9	7.7			
Quadriceps femoris	10th week	7.2 (0.38)	7.0 to 7.3	6.4	7.8	0.139	0.59	2.5
	20th week	7.3 (0.34)	7.2 to 7.5	6.8	7.9	0.004	1.01	6.1
	Initial	6.9 (0.27)	6.8 to 7	6.2	7.5			
Tibialis anterior	10th week	7.1 (0.29)	7.0 to 7.2	6.3	7.5	0.026	0.78	3.0
	20th week	7.1 (0.24)	7.0 to 7.2	6.7	7.5	0.016	0.71	3.4

S.D: standard deviation. 95% C.I.: 95% confidence interval. * The reference is the initial explorationEffect size was estimated with Cohen's d Statistic (<0.1 small, 0.25 medium and > 0.4 large effect size).**

Supplementary Table 1. First order statistics ultrasound variables between the follow-up group and the lost to follow-up group.

US parameters	Group	Mean (SD)	95% C.I.	Minimum	Maximum	p-value	Effect size*
Muscle thickness (MTh; mm)							
Biceps/brachialis	Follow-up	30.6 (6.21)	28.0 to 33.1	21.4	41.1	0.021	0.63
	Lost	26.6 (5.93)	24.2 to 28.9	16.6	39.9		
Forearm flexor	Follow-up	30.4 (7.92)	27.2 to 33.6	16.4	44.2	0.729	0.10
	Lost	29.5 (11.33)	24.9 to 34.1	18.5	72.0		
Quadriceps femoris	Follow-up	25.6 (8.1)	22.4 to 28.9	15.1	40.7	0.027	0.61
	Lost	20.2 (9.13)	16.5 to 23.9	8.1	47.0		
Tibialis anterior	Follow-up	21.1 (5.95)	18.7 to 23.5	11.3	32.4	0.007	0.73
	Lost	17.0 (4.43)	15.3 to 18.8	10.1	26.7		
Echointensity (EI; 0 - 255 levels)							
Biceps/brachialis	Follow-up	88.7 (14.11)	83 to 94.4	63.3	120.3	0.074	0.49
	Lost	95.8 (14.07)	90.1 to 101.5	66.6	124.2		
Forearm flexor	Follow-up	99.1 (16.39)	92.5 to 105.8	66.5	130.1	0.283	0.30
	Lost	103.7 (13.96)	98.1 to 109.4	81.5	131.0		
Quadriceps femoris	Follow-up	95.3 (15.05)	89.2 to 101.4	74.4	123.9	0.033	0.59
	Lost	105.8 (19.46)	98.0 to 113.7	78.2	146.1		
Tibialis anterior	Follow-up	110.1 (13.75)	104.6 to 115.7	75.1	129.8	0.008	0.72
	Lost	121.9 (16.86)	115.1 to 128.7	92.1	152.1		
Echovariation (EV; 0 - 100 points)							
Biceps/brachialis	Follow-up	24.3 (7.4)	21.3 to 27.3	9.5	38.3	0.213	0.35
	Lost	21.7 (7.18)	18.8 to 24.6	10.4	36.8		
Forearm flexor	Follow-up	19.2 (4.29)	17.4 to 20.9	10.6	26.5	0.786	0.08
	Lost	19.5 (4.87)	17.5 to 21.5	11.5	30.0		
Quadriceps femoris	Follow-up	17 (3.29)	15.6 to 18.3	11.0	24.2	0.001	0.85
	Lost	20.8 (4.73)	18.8 to 22.7	11.2	29.5		
Tibialis anterior	Follow-up	16.8 (4)	15.2 to 18.5	10.8	25.2	0.623	0.14
	Lost	16.2 (4.66)	14.4 to 18.1	7.5	26.4		

S.D: standard deviation. 95% C.I.: 95% confidence interval. *Effect size was estimated with Cohen's d Statistic (<0.1 small, 0.25 medium and > 0.4 large effect size).

Supplementary Table 2. Second order statistical ultrasound variables measured from grey level co-occurrence matrix (GLCM) between patients with 3 measurements and lost patients group.

US parameters	Group	Mean (SD)	95% C.I.	Minimum	Maximum	p-value	Effect size*
			Angular Second Moment (ASM)				
Biceps/brachialis	Follow-up	18.8 (8.63)	15.3 to 22.3	8.2	44.0	0.823	0.06
	Lost	18.3 (9.09)	14.6 to 21.9	8.5	44.0		
Forearm flexor	Follow-up	15.3 (6.48)	12.7 to 17.9	7.7	31.0	0.269	0.31
	Lost	13.4 (5.51)	11.2 to 15.7	6.3	26.5		
Quadriceps femoris	Follow-up	18.4 (8.27)	15.1 to 21.8	7.2	48.0	0.008	0.72
	Lost	12.9 (6.12)	10.4 to 15.4	4.8	28.0		
Tibialis anterior	Follow-up	16.9 (5.50)	14.6 to 19.1	8.2	33.0	0.557	0.17
	Lost	15.8 (6.99)	13.0 to 18.6	7.6	36.0		
			Contrast (CON)				
Biceps/brachialis	Follow-up	198.6 (96.97)	159.5 to 237.8	84.1	517.6	0.706	0.11
	Lost	209.6 (111.27)	164.7 to 254.6	74.3	556.6		
Forearm flexor	Follow-up	207.7 (72.11)	178.6 to 236.8	87.3	335.0	0.143	0.41
	Lost	240.1 (84.43)	206.0 to 274.2	114.0	451.0		
Quadriceps femoris	Follow-up	200.9 (82.51)	167.6 to 234.3	107.5	394.6	0.011	0.69
	Lost	287.0 (143.97)	228.9 to 345.2	103.4	667.8		
Tibialis anterior	Follow-up	261.0 (97.49)	221.6 to 300.3	78.3	476.2	0.028	0.60
	Lost	340.1 (149.27)	279.8 to 400.4	173.1	701.4		
			Textural Correlation (TC)				
Biceps/brachialis	Follow-up	18.3 (8.85)	14.8 to 21.9	7.3	47.0	0.825	0.06
	Lost	17.8 (8.38)	14.4 to 21.2	10.0	46.0		
Forearm flexor	Follow-up	20.0 (9.05)	16.3 to 23.6	9.3	44.0	0.267	0.31
	Lost	17.5 (7.06)	14.6 to 20.3	7.1	31.0		
Quadriceps femoris	Follow-up	24.4 (9.94)	20.4 to 28.4	10.3	46.5	0.004	0.79
	Lost	16.4 (8.78)	12.9 to 20.0	4.8	35.5		
Tibialis anterior	Follow-up	17.4 (7.87)	14.2 to 20.6	8.0	49.0	0.091	0.47
	Lost	14.2 (5.26)	12.1 to 16.3	6.6	26.0		
			Inverse Different Moment (IDM)				
Biceps/brachialis	Follow-up	25.5 (5.17)	23.4 to 27.6	17.7	34.5	0.954	0.02
	Lost	25.4 (5.92)	23.0 to 27.8	17.3	36.9		
Forearm flexor	Follow-up	21.5 (3.96)	19.9 to 23.1	15.7	29.0	0.161	0.39
	Lost	20.0 (3.61)	18.6 to 21.5	15.0	28.3		
Quadriceps femoris	Follow-up	22.8 (4.09)	21.2 to 24.5	15.5	30.7	0.064	0.51
	Lost	20.7 (3.82)	19.2 to 22.3	15.1	29.4		
Tibialis anterior	Follow-up	22.6 (4.48)	20.8 to 24.4	14.6	31.4	0.204	0.35
	Lost	21.1 (4.28)	19.3 to 22.8	15.1	31.0		
			Entropy (ENT)				
Biceps/brachialis	Follow-up	7.0 (0.41)	6.8 to 7.1	5.9	7.7	0.925	0.03
	Lost	7.0 (0.46)	6.8 to 7.2	5.9	7.6		
Forearm flexor	Follow-up	7.1 (0.38)	6.9 to 7.2	6.3	7.6	0.315	0.28
	Lost	7.2 (0.39)	7.0 to 7.3	6.4	7.8		
Quadriceps femoris	Follow-up	6.9 (0.39)	6.8 to 7.1	5.9	7.7	0.004	0.78
	Lost	7.3 (0.44)	7.1 to 7.4	6.4	8.1		
Tibialis anterior	Follow-up	6.9 (0.28)	6.8 to 7.0	6.2	7.5	0.391	0.24
	Lost	6.9 (0.36)	6.8 to 7.1	6.0	7.5		

S.D: standard deviation. 95% C.I.: 95% confidence interval. *Effect size was estimated with Cohen's d Statistic (<0.1 small, 0.25 medium and > 0.4 large size effect).

Figure 1

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