Elevated serum creatine kinase in the early stage of sporadic amyotrophic lateral sclerosis

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ABSTRACT

Objective: To assess the changes of muscle-related biomarkers at the early stage of amyotrophic lateral sclerosis, and to confirm these findings in an experimental animal model.

Methods: Thirty-nine subjects with sporadic amyotrophic lateral sclerosis and 20 healthy controls were enrolled and longitudinally evaluated. We evaluated serum creatine kinase and creatinine levels and appendicular lean soft tissue mass using dual X-ray absorptiometry. The levels of biomarkers at early ALS stages were estimated using linear mixed models with unstructured correlation and random intercepts. We also analyzed the longitudinal changes of serum creatine kinase and creatinine, together with the mRNA levels of acetylcholine receptor subunit γ (*Chrng*) and muscle-associated receptor tyrosine kinase, markers of denervation, in the gastrocnemius muscle of superoxide dismutase 1 (SOD1)^{G93A} transgenic mice, an animal model of amyotrophic lateral sclerosis.

Results: The estimated levels of creatine kinase were higher in subjects with amyotrophic lateral sclerosis at the early stage than in healthy controls, although the estimated appendicular lean soft tissue mass and creatinine levels were equivalent between both groups, suggesting that the elevation of creatine kinase precedes both muscular atrophy and subjective motor symptoms in sporadic amyotrophic lateral sclerosis. In SOD1^{G93A} mice, the serum levels of creatine kinase were elevated at 9 weeks of age (peri-onset) when *Chrng* started to be up-regulated, and were then down-regulated at 15 weeks of age, consistent with the clinical data from patients with sporadic amyotrophic lateral sclerosis.

Interpretation: Creatine kinase elevation precedes muscular atrophy and reflects muscle denervation at the early stage.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by the selective loss of upper and lower motor neurons. ALS causes rapidly progressive muscle weakness and atrophy, leading to death in approximately 3–5 years from onset, chiefly due to respiratory failure [1, 2]. Mutations in genes such as chromosome 9 open reading frame 72 (*C9orf72*), TAR DNA binding protein, fused in sarcoma, and superoxide dismutase 1 (*SOD1*) have been identified in 50–70% of familial cases, as well as in a smaller population of sporadic cases, suggesting that several biological mechanisms are involved in the pathophysiology of ALS, e.g., RNA metabolism, protein homeostasis, nucleocytoplasmic trafficking, and neuroinflammation [3–5]. These insights have inspired a myriad of attempts to develop disease-modifying therapies for this devastating disease; nevertheless, most agents that have shown promise in animal studies have failed to demonstrate clear efficacy in clinical trials [6, 7]. This failure in translation may be attributable to various clinical and biological factors, among which disease progression before the onset and early stages of neurological symptoms is a key issue for the successful development of a cure for ALS.

It is now widely accepted that pathological changes begin long before the clinical symptoms manifest, indicating the preclinical progression of neurodegenerative processes [8–10]. This hypothesis is explicitly emphasized in Alzheimer's disease, in which amyloid β deposition, a component of senile plaques, precedes brain volume loss and cognitive decline by a couple of decades [8]. Similar changes of biomarkers have been reported in most neurodegenerative diseases, including Huntington's disease, Parkinson's disease, and spinal and bulbar muscular atrophy, another adult-onset motor neuron disease [11, 12]. These findings provide a theoretical basis for the development of preventive therapies that target the preclinical phase of neurodegenerative disorders [13, 14].

As for ALS, a few markers have been identified that show changes in gene mutation carriers at the preclinical stage. For instance, dipeptide repeat proteins are detectable in the cerebrospinal fluid of asymptomatic *C9orf72* mutation carriers [15]. In addition, alterations of microRNA expression profiles in cerebrospinal fluid have also been reported in *C9orf72* mutation carriers [16]. It is also reported that short-interval intracortical inhibition, an index of cortical hyperexcitability in electrophysiological studies, is affected in asymptomatic *SOD1* mutation carriers [17]. Furthermore, a decrease in the estimated number of motor units, another electrophysiological marker of motor neuron degeneration, is detectable in asymptomatic *SOD1*

mutation carriers [18]. However, there is no report on the preclinical changes of biomarkers in sporadic ALS, except for scattered studies on altered body fat and lipid metabolism as risk factors for ALS [19, 20].

Here, we conducted a longitudinal study to estimate the changes of biomarkers at the very early stage of ALS by analyzing clinical data from sporadic patients at the early stage. Our results indicate the elevation of the serum levels of creatine kinase (CK) in the early stage of ALS, which was reproduced in G93A mutant SOD1 ($SOD1^{G93A}$) mice, an animal model of ALS.

Materials and Methods

Ethics and consent

This study was conducted according to the Declaration of Helsinki, the Ethics Guidelines for Human Genome/Gene Analysis Research, and the Ethical Guidelines for Medical and Health Research Involving Human Subjects endorsed by the Japanese government. This study was approved by the Ethics Review Committee of Nagoya University Graduate School of Medicine (Nos. 2013-0035 and 2015-0041), and all participants gave written informed consent before participation.

Participants

Subjects who were clinically diagnosed with the revised El Escorial Criteria of definite, probable, or possible ALS were recruited consecutively. The principal inclusion criteria were no family history and disease duration of ≤2 years at the time of enrollment. Subjects who had severe complications such as malignancy, heart failure, or renal failure were excluded from this study. Age- and sex-matched healthy controls were also recruited during the same period as the ALS patients. Subjects with sporadic ALS were assessed during hospitalization at the initial evaluation and followed up every 6 months at our outpatient clinic as long as they were able to continue to attend out-patient clinic. Healthy controls were followed up every 6 or 12 months at the outpatient clinic.

Subjects with ALS who were evaluated twice or more were analyzed longitudinally. Results of DXA which were performed within 1 week after a radiological examination with contrast agents or radioisotopes were excluded as they influence the results of dual-energy X-ray absorptiometry (DXA).

We also retrospectively analyzed serum CK before the onset, which had been measured for the purpose of medical practice, in patients with or without follow-up data. All study subjects were

Japanese and observed at the Nagoya University Hospital between May 2013 and March 2018.

Definition of disease onset and onset site

Disease onset was defined as the time point when the subject felt weakness of any body part. Subjects with ALS were classified into two groups, limb-onset type and bulbar-onset type, according to the site where they first felt weakness. The limb-onset type included subjects who felt weakness in either of the four extremities first, while the bulbar-onset type included subjects who felt dysarthria or dysphagia first. There were no patients with respiratory-onset type ALS.

Outcome measures

Disease severity was assessed with the Japanese version of the revised ALS Functional Rating Scale (ALSFRS-R), a validated questionnaire-based functional rating scale for ALS [21, 22]. Venous blood samples were collected in the supine position after more than 12 h of fasting and just after waking up during hospitalization. At the outpatient clinic, they were collected while the subjects were in the sitting or supine position after more than 12 h of fasting. We measured the following serological indices: CK, creatinine, cystatin C, albumin, blood glucose, hemoglobin A1c, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase (ALP), total bilirubin (T-bil), lactate dehydrogenase, uric acid, uric nitrate, white blood cell count, hemoglobin, and platelet count. The levels of serum CK were log transformed before analysis as they had a non-normal distribution.

Body composition was assessed with DXA using fan-beam technology (Discovery A; Hologic, Inc., Bedford, MA). DXA has been utilized frequently to evaluate body composition including bone mineral content, fat mass, and lean body mass [23]. We calculated the sum of appendicular lean soft tissue (ALST) mass as an index of skeletal muscle mass [24].

ALS model animal

Transgenic mice overexpressing the human *SOD1* gene carrying the G93A mutation, $SOD1^{G93A}$ mice, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained as hemizygotes by mating transgenic males with B6/SJLF1 females [25]. Mutant male mice and their male littermate were sacrificed, and their venous blood was collected by puncturing the right ventricle using a 26G needle under pentobarbital anesthesia at the age of 5, 9, and 15 weeks: the pre-onset, peri-onset, and symptomatic stage, respectively [26]. Serum CK and creatinine levels were measured with L-Type CK (Wako, Japan) and an enzymatic method, respectively, at Oriental Yeast Co., Ltd. (Tokyo, Japan). Hemolyzed samples were excluded

from the analysis. All of the animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the Nagoya University Animal Experiment Committee (No. 29170).

Quantitative real-time PCR of murine muscle

At the age of 5, 9, and 15 weeks, gastrocnemius muscles from mutant SOD1^{G93A} mice and their littermates were dissected and snap-frozen with powdered CO_2 in acetone. Total RNA was extracted from mouse skeletal muscles using TRIzol and a PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA). The extracted RNA was reverse-transcribed into first-strand cDNA with ReverTra Ace reverse transcriptase (TOYOBO Co., Osaka, Japan). Quantitative real-time PCR was performed using KOD SYBR® qPCR Mix (TOYOBO Co., Osaka, Japan), and the product was detected using the CFX96™ real-time system (Bio-Rad Laboratories, Hercules, CA). The reaction conditions were 98°C for 2 min, followed by 40 cycles of 10 s at 98°C, 10 s at 60°C, and 30 s at 68°C. The expression levels of acetylcholine receptor subunit γ (*Chrng*) and muscle-associated receptor tyrosine kinase (*Musk*) in gastrocnemius muscles were measured as markers of muscle denervation [27]. The expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was also quantified and used as an internal standard control. The primers used were 5′-AGCCTCCCCAGCCATCCAGG-3′ and 5′-AGCCTCCCCAGCCATCCAGG-3′ for *Chrng*, 5′-ATCACCACGCCTCTTGAAAC-3′ and 5′-TGTCTTCCACGCTCAGAATG-3′ for *Musk* [27], and 5′-GAATTTGCCGTGAGTGGAGT-3′ and 5′-CGTCCCGTAGACAAAATGGT-3′ for *Gapdh*.

Statistical analysis

Linear mixed models with unstructured correlation and random intercepts were applied to estimate the average trajectories of each biomarker [28]. Polynomial basis functions were included to incorporate quadratic smoothing. Estimated values and 95% confidence intervals are shown from 0 to +50 months relative to symptom onset at 5-months intervals in subjects with ALS.

The chi-square test, unpaired t-test, and Mann-Whitney U test were used for the comparison of variables between two groups, and Pearson's correlation coefficient was used for analyzing correlations among parameters. A partial correlation was performed to determine the relationship between ALSFRS-R and log CK while controlling for age, disease duration, and ALST mass. $P < 0.05$ was considered to be significant, and correlation coefficients (r) were interpreted as follows; greater than 0.8 was very strong, 0.6-0.8 was moderately strong and 0.3-

0.5 was fair [29]. All data are presented as the mean ± standard deviation unless stated otherwise.

Statistical Package for the Social Sciences 25.0J software (IBM Japan, Tokyo, Japan) and SAS version 9.4 (SAS Institute, Inc., Cary, NC) were used to perform all statistical analyses.

Results

Participants

A total of 81 subjects with ALS and 20 age- and sex-matched healthy controls were recruited. As a result, we analyzed the longitudinal data from 39 patients with sporadic ALS and 20 healthy controls. Four subjects with ALS could not undergo longitudinal evaluation of DXA although they underwent other longitudinal evaluation including blood test. The data prior to the initial evaluation from 8 subjects with ALS were also analyzed retrospectively (Fig 1).

Baseline characteristics

Mean age at the initial evaluation, sex ratio, and body mass index were equivalent between both groups (Table 1). The mean disease duration was 12.6 ± 5.3 months, indicating that the subjects with ALS in our study were early cases. The clinical backgrounds of the limb-onset and bulbar-onset groups were equivalent.

Blood tests and body composition

The baseline levels of serum creatinine and ALST mass, which reportedly correlate with whole muscle mass [24, 30], were lower in subjects with ALS than in healthy controls. In contrast, the baseline levels of serum CK were higher in subjects with ALS (Table 2). Both male and female subjects had similar results (Supplemental table 1). As for the onset sites of ALS, the baseline levels of CK tended to be higher and those of creatinine were lower in limb-onset type ALS compared to the bulbar-onset group (CK, 262.5 ± 257.2 vs. 135.6 ± 79.7 U/L, respectively, $p = 0.105$; creatinine, 0.62 ± 0.15 vs. 0.80 ± 0.27 mg/dL, respectively, $p = 0.013$).

We followed up the subjects with ALS and healthy controls for 8.2 ± 4.2 months and 11.4 ± 3.8 months, respectively. The annualized differences of those parameters between the initial and final evaluation are shown in Table 2. The levels of CK, creatinine, and ALST mass decreased longitudinally in subjects with ALS, although they rarely changed in healthy controls. These changes were also common in both male and female subjects (Supplemental table 1). These results suggest that ALST mass in DXA could be a biomarker reflecting muscular atrophy in ALS patients. In addition, the levels of LDL-C, AST, ALP, and T-bil increased longitudinally in subjects

with ALS, although they rarely changed in healthy controls (Table 2).

Correlation between muscle-related biomarkers and clinical indices

ALSFRS-R was weakly correlated with log serum CK, but not with ALST mass or serum creatinine, at the initial evaluation (Fig 2A–C, Supplementary Table 2). The correlation between log CK and ALSFRS-R at baseline was still significant after being adjusted for age, disease duration, and ALST mass (partial correlation coefficient = 0.453, p = 0.006). Furthermore, the annualized differences (shown as delta) of all of these muscle-related markers were correlated with those of ALSFRS-R (Fig 2D–F). However, log serum CK, ALST mass, and serum creatinine levels at the initial evaluation were not correlated with the longitudinal change of motor function (data not shown). These results suggest that serum CK levels reflect the motor function of each individual, although they do not indicate the prognosis of ALS.

Estimation of muscle-related biomarkers at the early stage of ALS

Muscle-related biomarkers at early ALS stages and average trajectories were estimated using a linear mixed model with quadratic smoothing (Fig 3, Table 3). The estimated values of serum CK at early ALS stages were higher in ALS subjects than in controls, though estimated serum creatinine and ALST mass were equivalent between both groups in this statistical model. These results suggest that, at the early stage of ALS, serum CK levels are elevated, while there appears to be little muscular atrophy.

Retrospective analysis of serum CK before disease onset

For 8 male subjects with sporadic ALS, their serum CK levels before clinical onset had been recorded as a part of medical practice independent of ALS. In these subjects, the levels of serum CK started to be elevated before onset, reached their maximum approximately around onset, and then decreased after onset (Fig 4).

Muscle-related biomarkers in an animal model of ALS

The results of the clinical part of the present study led us to conduct biomarker analysis in an animal model of ALS. We examined the changes of muscle-related serum markers, CK and creatinine, in SOD1^{G93A} transgenic mice. We also measured the mRNA levels of *Chrng* and *Musk* in the gastrocnemius muscles of the mice, as they are reportedly elevated with denervation [27]. At week 5, the levels of all indices were equivalent between mutant SOD1 mice and their wild-type littermates (Fig 5A–D). At week 9, the timing of onset, serum CK levels were elevated and *Chrng* mRNA expression was up-regulated significantly in mutant SOD1 mice compared with wild-type mice (Fig 5A and C). Although not statistically significant, *Musk*

was also up-regulated in SOD1^{G93A} transgenic mice at week 9 (Fig 5D). At the symptomatic stage, week 15, serum CK levels were decreased in mutant SOD1 mice, despite the continued up-regulation of *Chrng* mRNA (Fig 5A and C). These results suggest that serum CK starts to be elevated at a very early stage when muscle denervation emerges, and declines with disease progression, consistent with the results of our clinical study.

Discussion

In the present study, we found that the estimated level of serum CK was elevated at the onset of motor symptoms in patients with sporadic ALS. Our study also indicated that other muscle-related markers, i.e., serum creatinine and ALST mass in DXA, were not substantially altered at the early stage, although they declined with disease progression during the symptomatic phase of the disease. These results indicate that serum CK is a potential biomarker that reflects the progression of ALS at the early stage.

Several studies have documented the elevation of serum CK in ALS [31–37]. As for the pathophysiology underlying this phenomenon, an association between active denervation and the elevation of serum CK was demonstrated in a previous electromyographic study [38]. Increased serum CK levels are associated with muscle cramp in patients with ALS [33] and Guillain-Barre syndrome [39]. Furthermore, denervation reportedly induces membrane instability in muscle tissue and leakage of CK into blood *in vivo* [40]. Taken together, the elevation of serum CK at the early stage in ALS appears to be caused by membrane instability or the destruction of muscle tissue due to the denervation and hyperexcitability of motor neurons.

We also revealed that mutant SOD1 mice had increased serum CK levels, together with up-regulation of *Chrng*, at the onset of motor dysfunction. *Chrng* encodes the gamma subunit of the acetylcholine receptor protein, which is up-regulated upon muscle denervation, together with *Musk*. Given a previous report on denervation and compensative reinnervation in skeletal muscle of mutant SOD1 mice at their preclinical stage [41], our findings suggest that the elevated levels of CK likely reflect denervation at the preclinical stage of ALS.

However, it is also possible that other factors are involved in the elevation of serum CK. Several reports have suggested that serum CK can be elevated by energetic compensatory changes of muscle metabolism, as CK is an enzyme that phosphorylates creatine for the contraction of muscle fibers [30, 36]. The level of serum CK is also influenced by physical activity [42]. Although ALS is classified as a motor neuron disease, a primary change of muscle

is also suggested [43]. For example, microRNA-206, which regulates histone deacetylase 4 (HDAC4), is up-regulated in skeletal muscle and has a protective effect on disease progression in a mouse model of ALS [44]. The relative mRNA expression level of HDAC4 in skeletal muscle of ALS patients reportedly correlates with disease progression [45]. Alternatively, the elevation of CK might reflect the difference in the proportion of type 1 and 2 muscle fibers affected. In mutant SOD1 mice, the selective vulnerability of fast-fatigable motor neurons, which innervate type 2b muscle fibers, is observed in the pre-symptomatic stage [41]. Given that the enzyme activity of CK is higher in type 2 fibers [46], the proportion of affected myofiber types might be another determinant of CK elevation and motor function at the early stage of ALS.

The decrease of serum CK levels with disease progression both in patients and animals in the present study appears to be associated with the loss of muscle volume, given the progressive decline in serum creatinine levels and ALST mass in DXA of sporadic ALS patients. Serum creatinine levels and ALST in DXA are both regarded as clinical biomarkers for the skeletal muscle mass of patients with neuromuscular disorders including ALS [24]. However, it is of note that the estimated values of these indices were not decreased at the onset of sporadic ALS, suggesting that muscle volume is not an appropriate biomarker at the early stage, although they can be used to monitor disease progression at the symptomatic stage of ALS. This view is in accordance with a previous observation that there is little muscle atrophy at disease onset when motor neuron loss is supposed to have already begun [43].

There are some reports describing a change of lipid metabolism in ALS [20, 47, 48]. A preclinical study of Swedish ALS patients demonstrated elevation of LDL-C and HDL-C 10 years before the disease onset, being more conspicuous in female [20]. However, our cohort study did not show a change of HDL-C or LDL-C in ALS patients at the baseline, although prospective evaluation showed a longitudinal increase in LDL-C levels. The disagreement between our study and previous report may be explained by several factors. Our study population was relatively male dominant. The level of LDL-C in our study tended to be higher in 14 female patients than in the male patients (data not shown), and this might have influenced the results. Furthermore, a difference in genetic background might be another explanation for the discrepancy of cholesterol, considering that the proportion of sporadic ALS patients who carry the hexanucleotide repeat expansion of C9orf72 is 0.4% in Japan, which is rather small compared to patients in Western countries (3–21%) [49]. As for the longitudinal elevation of LDL-C, it was accompanied by longitudinal elevations of AST, ALP, and T-bil. This might reflect

dyslipidemia and steatosis of liver, both of which have been observed during progression of ALS [47, 48] . The present study has several limitations. First, as the number of subjects with ALS in the present study was small, selection bias could not be excluded. Secondly, the estimation of values was based on a statistical model, even though we confirmed that estimation using retrospective data from patients was in agreement with the animal experimental data. Moreover, we did not analyze comprehensive changes of metabolism in ALS patients. More detailed analysis should be performed focused on creatine and its related metabolism to discuss the changes of creatinine and CK. A further large-scale study is required to assess the preclinical changes in subjects with ALS.

References

1. Rowland L, Shneider N (2001) Amyotrophic lateral sclerosis. N Engl J Med 344:1688–1700.
2. Wijesekera L, Leigh P (2009) Amyotrophic lateral sclerosis. Orphanet J Rare Dis 4:3
3. Renton AE, Chiò A, Traynor BJ (2014) State of play in amyotrophic lateral sclerosis genetics. Nat Neurosci 17:17–23.
4. Nakamura R, Sone J, Atsuta N, et al (2016) Next-generation sequencing of 28 ALS-related genes in a Japanese ALS cohort. Neurobiol Aging 39:219.e1-219.e8.
5. Taylor JP, Brown RH, Cleveland DW (2016) Decoding ALS: From genes to mechanism. Nature 539:197–206.
6. Bensimon G, Lacomblez L, Meininger V (1994) A Controlled Trial of Riluzole in Amyotrophic Lateral Sclerosis. N Engl J Med 330:585–591.
7. Abe K, Itoyama Y, Sobue G, et al (2014) Confirmatory double-blind, parallel-group, placebo-controlled study of efficacy and safety of edaravone (MCI-186) in amyotrophic lateral sclerosis patients. Amyotroph Lateral Scler Front Degener 15:610–617.
8. Villemagne VL, Burnham S, Bourgeat P, et al (2013) Amyloid β deposition, neurodegeneration, and cognitive decline in sporadic Alzheimer's disease: A prospective cohort study. Lancet Neurol 12:357–367.
9. Cooper CA, Chahine LM (2016) Biomarkers in Prodromal Parkinson Disease: A Qualitative Review. J Int Neuropsychol Soc 22:956–967.

10. Vos SJB, Xiong C, Visser PJ, et al (2013) Preclinical Alzheimer's disease and its outcome: A longitudinal cohort study. Lancet Neurol 12:957–965.

11. Katsuno M, Tanaka F, Sobue G (2012) Perspectives on molecular targeted therapies and clinical trials for neurodegenerative diseases. J Neurol Neurosurg Psychiatry 83:329–335.

12. Hijikata Y, Hashizume A, Yamada S, et al (2018) Biomarker-based analysis of preclinical progression in spinal and bulbar muscular atrophy. Neurology. https://doi.org/10.1212/WNL.0000000000005360

13. Solomon A, Mangialasche F, Richard E, et al (2014) Advances in the prevention of Alzheimer's disease and dementia. J Intern Med 275:229–250.

14. Schapira AHV, Olanow CW, Greenamyre JT, Bezard E (2014) Slowing of neurodegeneration in Parkinson's disease and Huntington's disease: Future therapeutic perspectives. Lancet 384:545–555.

15. Lehmer C, Oeckl P, Weishaupt JH, et al (2017) Poly-GP in cerebrospinal fluid links *C9orf72* -associated dipeptide repeat expression to the asymptomatic phase of ALS/FTD. EMBO Mol Med 9:859–868.

16. Freischmidt A, Müller K, Zondler L, et al (2014) Serum microRNAs in patients with genetic amyotrophic lateral sclerosis and pre-manifest mutation carriers. Brain 137:2938–2950.

17. Vucic S, Nicholson GA, Kiernan MC (2008) Cortical hyperexcitability may precede the onset of familial amyotrophic lateral sclerosis. Brain 131:1540–1550.

18. Aggarwal A, Nicholson G (2002) Detection of preclinical motor neurone loss in SOD1 mutation carriers using motor unit number estimation. J Neurol Neurosurg Psychiatry 73:199–201.

19. Gallo V, Wark PA, Jenab M, et al (2013) Prediagnostic body fat and risk of death from amyotrophic lateral sclerosis: The EPIC cohort. Neurology 80:829–838.

20. Mariosa D, Hammar N, Malmström H, et al (2017) Blood biomarkers of carbohydrate, lipid, and apolipoprotein metabolisms and risk of amyotrophic lateral sclerosis: A more than 20-year follow-up of the Swedish AMORIS cohort. Ann Neurol 81:718–728.

21. Ohashi Y, Tashiro K, Itoyama Y, et al (2001) Study of functional rating scale for amyotrophic lateral sclerosis: revised ALSFRS(ALSFRS-R) Japanese version. No To Shinkei 53:346–355.

22. Hashizume A, Katsuno M, Suzuki K, et al (2015) A functional scale for spinal and bulbar muscular atrophy: Cross-sectional and longitudinal study. Neuromuscul Disord 25:554-562.

23. Haarbo J, Gotfredsen A, Hassager C, Christiansen C (1991) Validation of body composition by dual energy X-ray absorptiometry (DEXA). Clin Physiol 11:331–341.

24. Hijikata Y, Katsuno M, Suzuki K, et al (2016) Impaired muscle uptake of creatine in spinal and bulbar muscular atrophy. Ann Clin Transl Neurol 537–546.

25. Gurney ME, Pu H, Chiu AY, et al (1994) Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 264:1772–1775.

26. Leitner M, Menzies S, Lutz C (2009) Working with ALS Mice. Jax 1–28

27. Rocchi A, Milioto C, Parodi S, et al (2016) Glycolytic-to-oxidative fiber-type switch and mTOR signaling activation are early-onset features of SBMA muscle modified by high-fat diet. Acta Neuropathol 132:127–144.

28. Yau W-YW, Tudorascu DL, McDade EM, et al (2015) Longitudinal assessment of neuroimaging and clinical markers in autosomal dominant Alzheimer's disease: a prospective cohort study. Lancet Neurol 14:804–813.

29. Chan YH (2003) Biostatistics 104: Correlation Analysis. Singapore Med J 44:614–619

30. Wyss M, Kaddurah-Daouk R (2000) Creatine and Creatinine Metabolism. Physiol Rev 80:1107–1213.

31. Panitch HS, Franklin GM (1972) Elevation of serum creatine phosphokinase in amyotrophic lateral sclerosis. Neurology 22:964–966.

32. Harrington TM, Cohen MD, Bartleson JD, Ginsburg WW (1983) Elevation of creatine kinase in amyotrophic lateral sclerosis. Potential confusion with polymyositis. Arthritis Rheum 26:201–205.

33. Gibson SB, Kasarskis EJ, Hu N, et al (2015) Relationship of creatine kinase to body composition, disease state, and longevity in ALS. Amyotroph Lateral Scler Front

399 Degener 16:473–477.

400 34. Felice KJ, North WA (1998) Creatine kinase values in amyotrophic lateral sclerosis. J
401 Neurol Sci 160:3–5.

402 35. Iłzecka J, Stelmasiak Z (2003) Creatine kinase activity in amyotrophic lateral sclerosis
403 patients. Neurol Sci 24:286–287.

404 36. Rafiq MK, Lee E, Bradburn M, et al (2016) Creatine kinase enzyme level correlates
405 positively with serum creatinine and lean body mass, and is a prognostic factor for
406 survival in amyotrophic lateral sclerosis. Eur J Neurol 23:1071–1078.

407 37. Tai H, Cui L, Guan Y, et al (2017) Correlation of creatine kinase levels with clinical
408 features and survival in amyotrophic lateral sclerosis. Front Neurol 8:1–5.

409 38. Tai H, Cui L, Liu M, et al (2018) Creatine kinase level and its relationship with
410 quantitative electromyographic characteristics in amyotrophic lateral sclerosis. Clin
411 Neurophysiol 129:926–930.

412 39. Satoh J, Okada K, Kishi T, et al (2000) Cramping pain and prolonged elevation of serum
413 creatine kinase levels in a patient with Guillain-Barré syndrome following Campylobacter
414 jejuni enteritis. Eur J Neurol 7:107–109.

415 40. Linkhart TA, Wilson BW (1975) Appearance of acetylcholinesterase and creatine kinase
416 in plasma of normal chickens after denervation. J Neurol Sci 26:193–201.

417 41. Pun S, Santos AF, Saxena S, et al (2006) Selective vulnerability and pruning of phasic
418 motoneuron axons in motoneuron disease alleviated by CNTF. Nat Neurosci 9:408–419.

419 42. Kim J, So W-Y (2018) High body mass index is associated with the extent of muscle
420 damage after eccentric exercise. Int J Environ Res Public Health.
421 https://doi.org/10.3390/ijerph15071378

422 43. Eisen A, Kiernan M, Mitsumoto H, Swash M (2014) Amyotrophic lateral sclerosis: A long
423 preclinical period? J Neurol Neurosurg Psychiatry 85:1232–1238.

424 44. Williams AH, Valdez G, Moresi V, et al (2009) MicroRNA-206 delays ALS progression
425 and promotes regeneration of neuromuscular synapses in mice. Science 326:1549–
426 1554.

427 45. Bruneteau G, Simonet T, Bauché S, et al (2013) Muscle histone deacetylase 4

upregulation in amyotrophic lateral sclerosis: Potential role in reinnervation ability and disease progression. Brain 136:2359–2368.

46. Andrés V, Cussó R, Carreras J (1990) Effect of denervation on the distribution and developmental transition of phosphoglycerate mutase and creatine phosphokinase isozymes in rat muscles of different fiber-type composition. Differentiation 43:98–103.

47. Dupuis L, Corcia P, Fergani A, et al (2008) Dyslipidemia is a protective factor in amyotrophic lateral sclerosis. Neurology 70:1004–1009.

48. Ikeda K, Hirayama T, Takazawa T, et al (2012) Relationships between disease progression and serum levels of lipid, urate, creatinine and ferritin in Japanese patients with amyotrophic lateral sclerosis: a cross-sectional study. Intern Med 51:1501–8.

49. Ogaki K, Li Y, Atsuta N, et al (2012) Analysis of C9orf72 repeat expansion in 563 Japanese patients with amyotrophic lateral sclerosis. Neurobiol Aging 33:11–16.

Figure legends

Figure 1. Flowchart of study population enrollment

Flowchart describing the enrollment and exclusion of the study population. Data before the onset were retrospectively analyzed in 4 subjects without follow-up data[a] and 4 subjects on whom longitudinally analysis was performed[b]. Longitudinal data of body compositions was not available in 4 subjects with ALS.

ALS, amyotrophic lateral sclerosis; DXA, dual X-ray absorptiometry.

Figure 2. Correlation between ALSFRS-R and muscle-related biomarkers

A–C. Scatter plots of ALSFRS-R and muscle related biomarkers [A, logCK (n=39); B, ALST mass (n=35); C, Cr (n=39)] at the initial evaluation. D–E. Scatter plots of the annualized differences of ALSFRS-R and muscle related biomarkers [D, ΔlogCK (n=39); E, ΔALST mass (n=35); F, ΔCr (n=39)]. Significant correlation coefficients and p-values are annotated.

ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised ALS Functional Rating Scale; ALST, appendicular lean soft tissue; CK, creatine kinase; Cr; creatinine; Δ(delta) is the annualized difference of each parameter between the initial and final evaluation.

Figure 3. Raw data and estimated average trajectories of muscle-related biomarkers

A–C. Raw data of muscle-related biomarker in subjects with ALS [A, LogCK (n=39); B, ALST mass (n=35); C, Cr (n=39)]. D–F. Estimated average trajectories and their confidence intervals in subjects with ALS. G–L. Raw data and average trajectories in HCs (n=20). The date of the initial evaluation of HCs was set at the mean disease duration (12.6 months) of subjects with ALS. M–O. Merged average trajectories of both groups. Blue lines indicate the data of the subjects for whom retrospective data of serum CK before onset was also analyzed (Patients ALS1, 2, 3, and 4 in Fig 4). Red lines and red dotted lines demonstrate average trajectories and their 95% confidence intervals in subjects with ALS. Black trajectories and black dotted lines demonstrate average trajectories and their 95% confidence intervals of HCs.

ALS, amyotrophic lateral sclerosis; ALST, appendicular lean soft tissue; CK, creatine kinase; Cr, creatinine; HC, healthy control; mo, months.

Figure 4. Retrospective data of serum CK levels in 8 subjects with ALS

Serum CK levels in 8 subjects with sporadic ALS, for whom serum CK levels had been measured for the purpose of medical practice other than ALS. Age, sex, and the site of onset were described above each graph. Vertical dotted lines demonstrate the timing of the first symptom of weakness and arrow heads demonstrate the timing of the initial evaluation.
ALS, amyotrophic lateral sclerosis; CK, creatine kinase.

Figure 5. Serum CK, creatinine, and mRNA expression of denervation markers in SOD1^{G93A} transgenic mice and their littermates

Serum CK (A), serum creatinine (B), and *Chrng* (C) and *Musk* (D) mRNA levels in the gastrocnemius muscles (wild-type, male, n = 5; SOD1^{G93A}, male, n = 5). *$p < 0.05$. Data are presented as the mean ± standard error.
CK, creatine kinase; Cr, creatinine; *Chrng*, acetylcholine receptor subunit γ; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; mSOD1, SOD1^{G93A} transgenic mice; *Musk*, muscle-associated receptor tyrosine kinase; N.S., not significant; WT, wild-type mice.

Table 1. Baseline characteristics

	Total ALS (n = 39)	HC (n = 20)	p-value	Bulbar (n = 12)	Limb (n = 27)	p-value
Sex (male%)	64.1	65	0.946	66.7	63.0	0.824
Age at the first evaluation (years)	66.4 ± 7.3	64.4 ± 6.9	0.314	69.6 ± 5.8	65.0 ± 7.6	0.071
Disease duration (months)	12.6 ± 5.3	N.A.	N.A.	12.0 ± 5.8	12.9 ± 5.2	0.788
Height (cm)	160.4 ± 7.9	162.9 ± 8.9	0.293	158.5 ± 7.3	161.3 ± 8.2	0.320
Body weight (kg)	56.7 ± 10.9	62.2 ± 11.0	0.071	54.6 ± 9.3	57.6 ± 11.6	0.428
Body mass index	21.9 ± 3.0	23.3 ± 2.8	0.081	21.6 ± 2.6	22.0 ± 3.1	0.685
ALSFRS-R	40.9 ± 4.7	47.8 ± 0.3	<0.001	40.5 ± 4.8	41.1 ± 4.8	0.730

ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised Amyotrophic Lateral Sclerosis Functional Rating Scale; Bulbar, bulbar-onset ALS; HC, healthy control; Limb, limb-onset ALS; N.A., not available. Data represent mean ± standard deviation.

Table 2. Baseline and changes of blood tests and body composition

	Baseline			Change at 48 weeks		
	ALS	**HC**	**p-value**	**ALS**	**HC**	**p-value**
Blood test	**ALS (n = 39)**	**HC (n = 20)**		**ALS (n = 39)**	**HC (n = 20)**	
CK (U/L)	223.5 ± 225.0	107.4 ± 53.5	0.004	-77.0 ± 136.9	2.2 ± 49.7	0.002
log CK	2.21 ± 0.34	1.99 ± 0.20	0.008	-0.19 ± 0.31	0.004 ± 0.14	0.001
Cr (mg/dL)	0.68 ± 0.21	0.77 ± 0.16	0.100	-0.09 ± 0.14	0.02 ± 0.08	<0.001
CysC (mg/L)	0.96 ± 0.20	0.90 ± 0.14	0.221	0.09 ± 0.16	0.01 ± 0.08	0.017
UN (mg/dL)	15.8 ± 4.6	13.4 ± 2.2	0.010	0.36 ± 8.31	0.26 ± 2.60	0.941
UA (mg/dL)	5.1 ± 1.3	5.5 ± 1.0	0.328	-0.69 ± 1.53	0.28 ± 1.28	0.012
AST (U/L)	24.3 ± 7.3	25.6 ± 6.3	0.502	7.4 ± 24.7	-1.0 ± 4.3	0.044
ALT (U/L)	22.1 ± 10.8	21.1 ± 7.8	0.695	5.4 ± 27.7	-1.0 ± 6.4	0.178
T-Bil (mg/dL)	0.87 ± 0.36	0.92 ± 0.42	0.688	0.25 ± 0.49	-0.31 ± 0.22	0.001
ALP (U/L)	209.2 ± 61.6	242.1 ± 59.8	0.055	29.6 ± 80.9	-30.8 ± 45.9	0.003
γ-GTP (U/L)	34.2 ± 26.6	56.2 ± 57.8	0.120	17.7 ± -10.6	-10.6 ± 71.0	0.159
TP (g/dL)	7.0 ± 0.4	7.2 ± 0.3	0.065	0.17 ± 0.96	-0.09 ± 0.76	0.291
Alb (g/dL)	4.2 ± 0.3	4.4 ± 0.3	0.077	0.10 ± 0.81	-0.09 ± 0.43	0.354
LDH (U/L)	210.7 ± 42.8	185.4 ± 34.7	0.026	-19.8 ± 49.5	-15.0 ± 33.9	0.699
LDL-C (mg/dL)	119.4 ± 36.6	117.3 ± 27.9	0.822	23.0 ± 57.9	1.2 ± 19.7	0.043
HDL-C (mg/dL)	54.6 ± 15.9	59.7 ± 18.2	0.282	4.0 ± 18.3	0.85 ± 7.9	0.365
HbA1c (%)	5.9 ± 0.8	5.9 ± 0.4	0.935	-1.4 ± 1.9	0.10 ± 0.25	<0.001
WBC (10^3/μL)	5.7 ± 2.1	5.8 ± 1.7	0.910	0.9 ± 3.0	-0.18 ± 1.36	0.072
Hb (g/dL)	14.6 ± 4.9	14.6 ± 1.4	0.970	-1.6 ± 10.1	-0.3 ± 1.1	0.571
Plt (10^3/μL)	232.8 ± 69.5	222.3 ± 57.0	0.561	8.0 ± 75.6	-5.7 ± 26.1	0.313
Body composition	**ALS (n = 39)[a]**	**HC (n = 20)**		**ALS (n = 35)**	**HC (n = 20)**	
ALST mass (kg)	16.65 ± 4.01	19.20 ± 4.5	0.030	-2.56 ± 2.27	-0.42 ± 1.22	<0.001
BMC (kg)	1.90 ± 0.39	1.94 ± 0.42	0.716	-0.09 ± 0.12	0.02 ± 0.12	0.002
Total fat mass(kg)	14.25 ± 4.12	15.21 ± 3.79	0.385	-0.97 ± 4.49	0.46 ± 1.65	0.094

Change at 48 weeks shows the annualized difference of each parameter between the initial and final evaluation.

[a]Longitudinal data of body compositions was not available in 4 subjects with ALS.

Alb, albumin; ALP, alkaline phosphatase; ALS, amyotrophic lateral sclerosis; ALST, appendicular lean soft tissue; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMC, bone mineral content; CK, creatine kinase; Cr, creatinine; CysC, cystatin C; γ-GTP, gamma glutamyl transpeptidase; Hb, hemoglobin; HbA1c, hemoglobin A1c; HC, healthy control; HDL-C, high-density lipoprotein cholesterol; LDH, lactate dehydrogenase; LDL, low-density lipoprotein cholesterol; Plt, platelet count; T-Bil, total bilirubin; TP, total protein; UA, uric acid; UN, uric nitrate; WBC, white blood cell count. Data represent mean ± standard deviation.

505 **Table 3. Estimated values of muscle-related biomarkers at onset**

	ALS		HC (n = 20)	
	Estimation	95% CI	Estimation	95% CI
Log CK	2.34 (n =39)	1.68–3.00	1.97	1.57–2.37
ALST mass (kg)	19.91 (n = 35)[a]	11.73–28.09	19.32	9.76–28.89
Cr (mg/dL)	0.80 (n = 39)	0.38–1.22	0.75	0.41–1.09

506

507 [a]Longitudinal data of body compositions was not available in 4 subjects with ALS.

508 The values were calculated using a linear mixed model with unstructured correlation and

509 random intercepts.

510 ALS, amyotrophic lateral sclerosis; ALST, appendicular lean soft tissue; CI, confidence interval;

511 CK, creatine kinase; Cr, creatinine; HC, healthy controls

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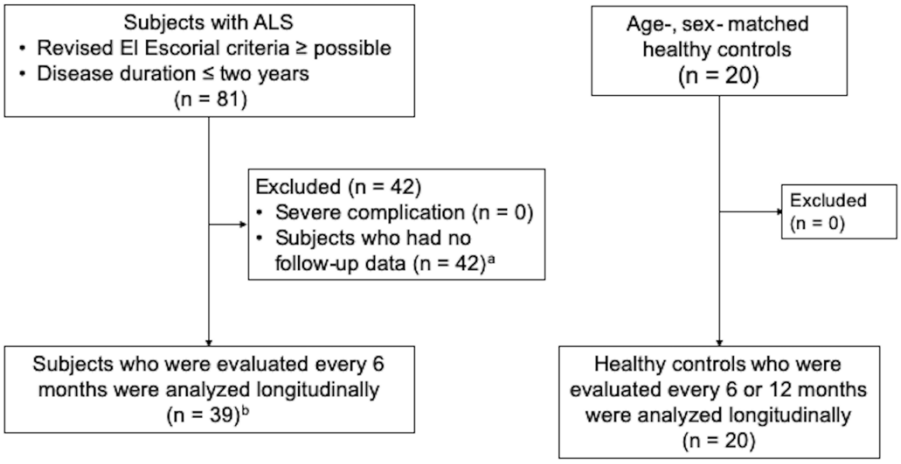
514 Figure 1



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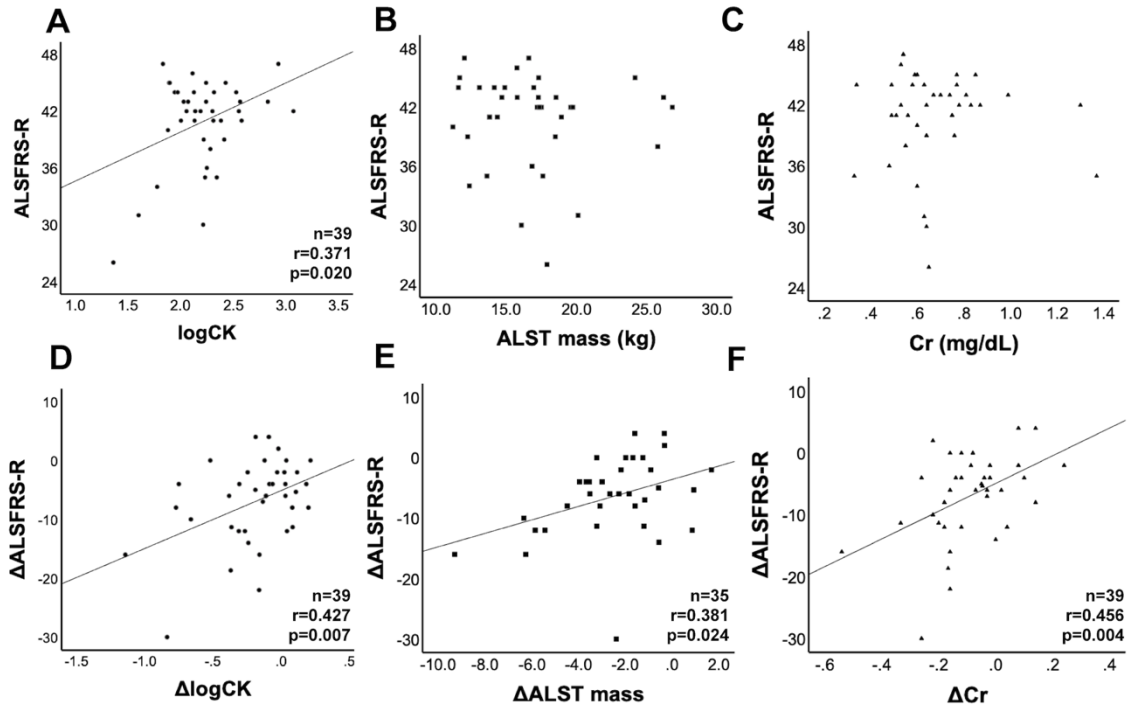
517 Figure 2



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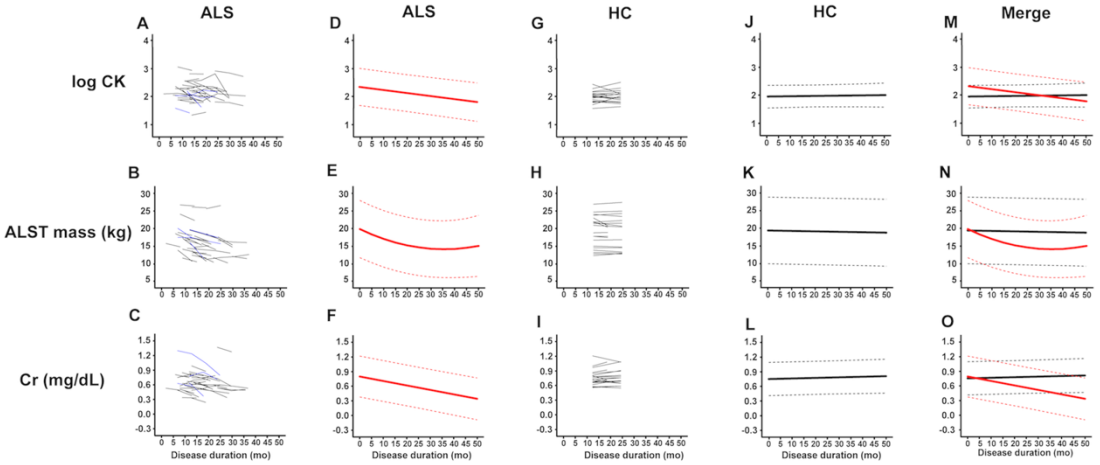
520 Figure 3



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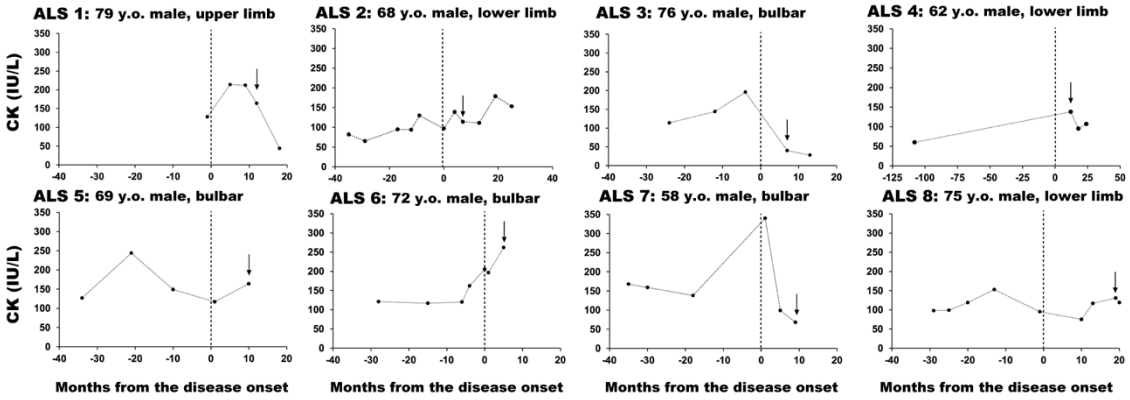
524 Figure 4



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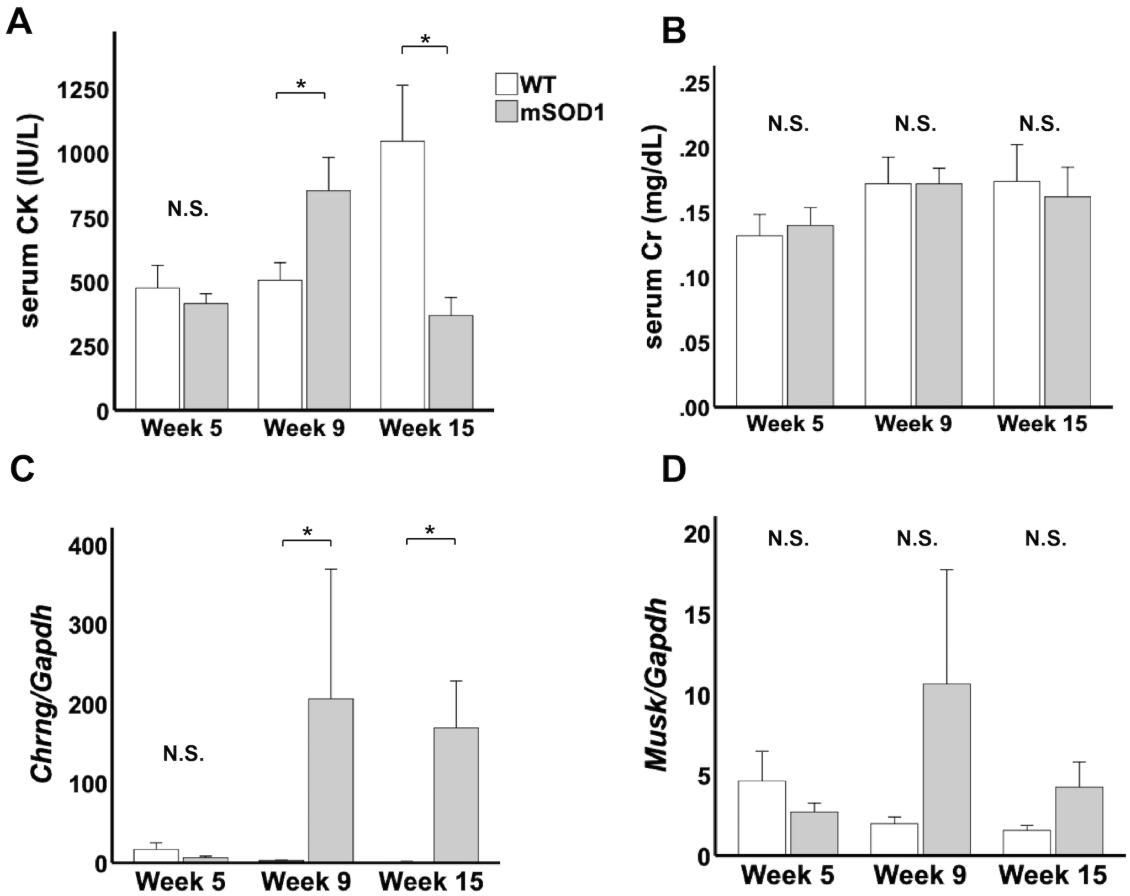
529 Figure 5



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