Received: 28 July 2023 | Revised: 7 August 2024 | Accepted: 9 August 2024

DOI: 10.1002/mus.28237

BASIC SCIENCE RESEARCH ARTICLE

MUSCLE&NERVE WILEY

Amyotrophic Lateral Sclerosis and swim training affect copper metabolism in skeletal muscle in a mouse model of disease

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Funding information
Narodowe Centrum Nauki; National Science Centre, Grant/Award Numbers: DEC-2013/09/NZ7/02538, 2020/39/B/NZ7/03366

Abstract

Introduction/Aims: Swim training and regulation of copper metabolism result in clinical benefits in amyotrophic lateral sclerosis (ALS) mice. Therefore, the study aimed to determine whether swim training improves copper metabolism by modifying copper metabolism in the skeletal muscles of ALS mice.

Methods: *SOD1G93A* mice ($n = 6$ per group) were used as the ALS model, and wild-type B6SJL (WT) mice as controls ($n = 6$). Mice with ALS were analyzed before the onset of ALS (ALS BEFORE), at baseline ALS (first disease symptoms, trained and untrained, ALS ONSET), and at the end of ALS (last stage disease, trained and untrained, ALS TERMINAL). Copper concentrations and the level of copper metabolism proteins in the skeletal muscles of the lower leg were determined.

Results: ALS disease caused a reduction in the copper concentration in ALS TERMINAL untrained mice compared with the ALS BEFORE (10.43 ± 1.81 and 38.67 ± 11.50 μg/mg, respectively, $p = .0213$). The copper chaperon for SOD1 protein, which supplies copper to SOD1, and ATPase7a protein (copper exporter), increased

Abbreviations: ALS, amyotrophic lateral sclerosis; AOC3, membrane primary amine oxidase; ATOX1, copper transport protein; CCS, copper chaperon for SOD1; C5orf63, glutaredoxin-like protein homolog; COX11, cytochrome c oxidase assembly protein COX11; COX17, cytochrome c oxidase copper chaperone; CTR1, copper transporter-1; Cu, copper; CuATSM, diacetyl-bis (4-methylthiosemicarbazonato)copper(II); DMT1, divalent metal transporter-1; ELISA, enzyme-linked immunosorbent assay; LOXL1, lysyl oxidase homolog 1; mutSOD1, mutant SOD1; MT, metallothionein; PRIO, major prion protein; SCO1, SCO1 cytochrome c oxidase assembly protein; SOD1, superoxide dismutase 1; STEAP3, metalloreductase; TBST, tris-buffered saline with 0.1% Tween® 20 detergent; WT, wild type.

at the terminal stage of disease by 57% ($p = .0021$) and 34% ($p = .0372$), while the CTR1 protein (copper importer) decreased by 45% ($p = .002$). Swim training moderately affected the copper concentration and the concentrations of proteins responsible for copper metabolism in skeletal muscles.

Discussion: The results show disturbances in skeletal muscle copper metabolism associated with ALS progression, which is moderately affected by swim training. From a clinical point of view, exercise in water for ALS patients should be an essential element of rehabilitation for maintaining quality of life.

KEYWORDS
ALS, copper metabolism, copper transport, exercise, neurodegeneration

1 | INTRODUCTION

Pathologic variants in the superoxide dismutase 1 (*SOD1*) gene occur in 15% of individuals with familial amyotrophic lateral sclerosis and 1%–2% of those with sporadic ALS.[1] The discovery of the role of this pathogenic variantin the etiology of ALS contributed to constructing animal test models of this disease—*SOD1 G93A* mice. Many studies have shown that ALS disease is strongly associated with disturbed copper metabolism in patients with ALS[2–4] and animal models of the disease.[5–8] Disrupted copper availability is not only observed in mice ALS models but is also a feature of sporadic human ALS. Therefore, the benefits of copper metabolism stabilization can be expected in sporadic ALS cases, not just those involving mutant SOD1.[9] The disturbed metabolism of copper in cells manifested by an insufficient supply of this metal to SOD1 results in modification of the SOD1 protein and the formation of ZnSOD1 aggregates that become toxic to cells and contribute to developing disease and death.[10]

Cells maintain copper homeostasis with copper uptake proteins (copper transporter 1 [CTR1], divalent metal-ion transporter 1 [DMT1]), copper-storing proteins (metallothionein [MT]), proteins supplying copper to other proteins/enzymes (e.g., chaperone for SOD1 [CCS]), and copper-exporting proteins (ATPase7a and ATPase7B).

In the spinal cord, the concentration of CTR1 and MT increases, while ATP7a decreases,[6,11] accumulating copper ions inside the cell.[11] Therefore, disturbance in the homeostatic control of intracellular copper ions is considered a pathological hallmark in rodent models of SOD1-ALS. CCS, another protein related to copper metabolism, facilitates the maturation of apo-SOD1 to active holo-SOD1 by promoting disulfide bond formation and the insertion of copper.[12] Interestingly, *CCS* overexpression in SOD1G93A mice has been shown to produce severe mitochondrial pathology and accelerate the disease course.[7] Moreover, the survival of motor neurons was significantly reduced in $CCS^{-/-}$ mice, which retained only 20% of their SOD1 activity.[13]

Regulation of copper concentration in cells by, among other things, MT overexpression[5] or oral treatment with the therapeutic agent diacetyl-bis(4-methylthiosemicarbazonato)copper(II) (CuATSM)[10,14] results in extending the lives of ALS mice similarly to swim training.[15,16] Swim training improves bioenergetics, iron, and glucose metabolism in skeletal muscle, reduces skeletal muscle fibers and weight loss,[15–18] and attenuates the reduction in muscle strength in ALS mice.[19] However, no data have documented how ALS and swim training affect copper concentrations and the levels of copper metabolism proteins in the skeletal muscle of ALS mice, a change that may underlie muscle degeneration in ALS.

We hypothesized that the beneficial effects of swim training observed earlier in the ALS SOD1 model also resulted from improved impaired copper metabolism in the skeletal muscles of diseased mice. Therefore, the study aimed to determine whether swim training improves copper metabolism by modifying copper metabolism in the skeletal muscles of ALS mice.

2 | METHODS

2.1 | Animals

Transgenic mice (male) with the human pathogenic variant *G93A SOD1* B6SJL-Tg (SOD1G93A)1Gur/J (ALS mice) (five groups, $n = 6$ in each group) and wild-type (WT) B6SJL mice ($n = 6$) (male) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed in a controlled environment room (23 ± 1°C with a 12-h light–dark cycle), were provided standard mouse chow, and had ad libitum access to water. After the acclimation period, the mice were randomized according to disease progression and training status as previously described[20]: ALS BEFORE (untrained ALS mice with no apparent disease symptoms), ALS ONSET (ALS mice with first disease symptoms), and untrained and trained ALS TERMINAL (untrained and trained ALS mice in the last stage of the disease).

The mice were euthanized with cervical dislocation. The animals in the ALS BEFORE group were euthanized at the 10th week of age. The ALS ONSET mice were euthanized when the first disease symptoms were observed in untrained animals (16th week of age). The ALS TERMINAL groups were euthanized when the last stage of disease was observed in untrained animals (18th to 19th week of life), as described previously.[15] After cervical dislocation, the posterior group

of the lower leg muscle and the tibialis anterior muscle were separated quickly, and the visible connective tissue was trimmed, weighed, and frozen in liquid nitrogen.

2.2 | Swim training protocol

ALS mice (ALS ONSET trained and TERMINAL ALS trained) started the training procedure at 10 weeks of age, as described,[16] and modified previously.[15] A unique pool with regulated water flow was used to conduct the swim training. The mice swam five times a week for 30 min. The water temperature was 30°C, with a maximum flow speed of 5 L/min. The training frequency was reduced to three times a week at 105 days of age. The exercise time and water flow were adapted to individual mouse capabilities. The training ended at 115 days of life.

2.2.1 | Tissue homogenization and preparation of the posterior group of lower leg muscle lysates for western blotting and enzyme-linked immunosorbent assay

After the tissue was thawed, the material was rinsed, drained, cut, and transferred to a 2 mL Eppendorf tube. Then 200 µL of RIPA lysis buffer (Sigma Aldrich, St. Louis, MO, USA) containing a protease cocktail (Sigma Aldrich, St. Louis, MO, USA) and phosphatase inhibitors (PhosSTOP™, PHOSS-RO Sigma Aldrich, St. Louis, MO, USA) were added to the material.

The pieces of tissue were homogenized using a glass Teflon homogenizer (10% w/v). Homogenization was performed manually 20 times. Then, each lysate was frozen at −70°C, thawed at 30°C three times, and again manually homogenized 10 times. Finally, using the Sigma 3 K30 centrifuge, the samples were centrifuged at 15,000 × *g* at 4°C for 10 min. The supernatant was decanted and frozen at −80°C for further analysis.

2.2.2 | Tissue homogenization and preparation of the posterior group of lower leg muscle lysates for the measurement of the copper concentration

After the tissue was thawed, a 10% homogenate was made using the buffer containing 5 mM 2-{[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino}ethane-1-sulfonic acid, 0.3 M sucrose, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, and bovine serum albumin 0.1%. The homogenate was then diluted 1:4 with 4× distilled water and stored until further analysis.

2.2.3 | Determination of copper concentration

Copper levels in skeletal muscle homogenates were analyzed with ICP-OES (Ciros Vision, SPECTRO Analytical Instruments Kleve, Germany) after wet-washing samples with 65% nitric acid as previously described.[21] The copper concentration in the skeletal muscle homogenates was expressed as micrograms per milligram of protein.

2.2.4 | Immunoblotting

To assess protein levels in lysates, equal amounts of total protein (50 µg per sample) were separated on 4%–20% gradient gels and transferred to polyvinylidene difluoride membranes. Then, blocking buffer (5% [w/vol] skim milk powder in 1× tris-buffered saline with 0.1% Tween® 20 detergent [1× TBST: 150 mM NaCl, 20 mM tris(hydroxymethyl)aminomethane pH 7.5, and 0.1% polysorbate 20]) was used. For 1 h, the membranes were blocked in the blocking buffer at room temperature. Next, the membranes were rinsed three times in 1× TBST for 5 min, incubated with primary antibodies dissolved in blocking buffer with gentle shaking, and left overnight at 4°C.

The following rabbit polyclonal antibody was used: CTR1/SLC31A1 (Cat. No. 13086, Cell Signaling, Danvers, MA, USA, 1:1000), DMT1/anti-SLC11A1 (Cat. No. FNab07905, Fine Test, Wuhan, China, 1:500), anti-ATP7a (Cat. No. ab 125,137, Abcam, Cambridge, UK, 1:1000), and rabbit monoclonal antibody; CCS/CCS antibody (Cat. No. ab137131, Abcam, Cambridge, UK, 1:1000).

Following the washing procedure (3 × 5 min in 1× TBST), the membranes were incubated for 1 h at room temperature and gently shaken with secondary antibodies: anti-rabbit IgG-peroxidase (Cat No. A9169, 1:20,000, Sigma Aldrich, St. Louis, MO, USA). After blocking, immunoblots were detected and visualized using improved chemiluminescent reagents (Pierce; Thermo Fisher Scientific, Inc., MA, USA). Changes in protein levels were assessed with immunoreactive band densitometry. Then, it was normalized to the total amount of protein in the samples measured on the membranes after the transfer using stain-free technology, which allows for avoiding mistakes related to housekeeping proteins (which may change during devastating diseases like ALS; for more information, please see Supplementary Methods). In this technique, following a brief UV light activation, Stain-Free fluorochromes are covalently bound to protein molecules in the gel, allowing them to be imaged repeatedly on the gel or a membrane post-transfer. The ChemiDoc image analysis system (Bio-Rad Laboratories, Inc., CA, USA) analyzed and quantified the relative levels of the protein (original blots were added into Figures S1–S4). Next, each result for the ALS groups was normalized to the mean of the WT groups.

2.2.5 | Metallothionein measurement in the posterior group of lower leg muscles

The MT protein level was determined with enzyme-linked immunosorbent assay (ELISA) according to the method described in the Datasheet for ELK2455 Mouse MT1 (Metallothionein 1) ELISA Kit (ELK Biotechnology, Wuhan, China). A 10% dilution of 1:10 with phosphate buffered saline was used for the experiment. The results were read at

a 450 nm wavelength using a Thermo Scientific™ Multiskan™ GO Microplate reader.

2.2.6 | Chemicals

All chemicals, except those for which the manufacturer is described in the materials and methods section, were purchased from Merck (Darmstadt, Germany).

2.2.7 | Proteomic analysis

To evaluate the effect of swim training on the levels of proteins involved in copper metabolism, we performed a comparative proteomic analysis of skeletal muscle in mice. Principal component analysis (PCA) enabled the linear transformation of the 15 variables into 2D or 3D space, simultaneously retaining the maximum information about the analyzed individual variables. The levels of 15 investigated proteins (membrane primary amine oxidase, AOC3; metalloreductase, STEAP3; glutaredoxin-like protein, C5orf63 homolog; ceruloplasmin, copper transport protein, ATOX1; superoxide dismutase 1, SOD1; cytochrome c oxidase assembly protein, SCO1; cytochrome c oxidase subunit 1; cytochrome c oxidase subunit 2; copper homeostasis protein cut C homolog; lysyl oxidase homolog 1, LOXL1; cytochrome c oxidase assembly protein, COX11; cytochrome c oxidase copper chaperone, COX17; copper chaperone for superoxide dismutase, CCS; and major prion protein, PRIO) were determined in the BEFORE ALS and TERMINAL ALS untrained and trained groups using liquid chromatography MS3 spectrometry (LC–MS/MS) at the Thermo Fisher Center for Multiplexed Proteomics (Department of Cell Biology, Harvard Medical School, Cambridge, MA, USA). Preparation of the samples (the tibialis anterior muscle) was described previously in reference 22. An Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and an LC-MS3 data collection strategy were used to analyze the peptide fractions. A free software environment for statistical computing and graphics was chosen to visualize the changes between all studied groups. To perform PCA, R statistical software (Foundation for Statistical Computing, Vienna, Austria) was used.

2.2.8 | Statistical analysis

The data were statistically analyzed using the software package Statistica v. 13.0 (StatSoft. Inc., Tulsa, OK, USA). The results are shown as the mean ± standard error of the mean. The distribution's normality and the variance's similarity were examined to determine which statistical test should be used. One-way analysis of variance or the Kruskal–Wallis test was used to identify the differences associated with disease progression and the changes between the ALS and WT groups. Tukey's post hoc test determined the significance level between the previously mentioned groups. The significance of swim training-associated changes (ALS ONSET untrained vs. trained and ALS TERMINAL untrained vs. trained) was verified with the Student's *t*-test. The data were considered statistically significant at $p < .05$.

3 | RESULTS

3.1 | Effects of ALS disease progression and swim training on skeletal muscle copper concentration

Copper concentrations in the ALS TERMINAL untrained mice decreased compared with those in the ALS BEFORE and untrained ALS ONSET groups of mice. No statistically significant differences were found between the trained and untrained groups at each stage of the disease (Figure 1A).

3.2 | Effects of ALS disease progression and swim training on copper metabolism proteins profiles in skeletal muscle

The content of the CCS protein was significantly higher in the ALS groups at the onset and terminal stages of the disease than in the WT group. In addition, the progression of the disease resulted in higher CCS protein content in the untrained groups at the onset and terminal stages compared with the ALS BEFORE group of mice (Figure 1B).

The concentration of the MT protein was higher in the untrained ALS ONSET and trained TERMINAL groups than in the WT group. In addition, the concentration of the MT protein was higher in the untrained ALS ONSET group than in the BEFORE ALS group and the untrained TERMINAL ALS group. At the terminal stage of the disease, the MT concentration decreased significantly in the untrained mice compared with the trained group (Figure 1C).

The ATP7a protein content increased significantly between the WT group and all ALS groups. At the terminal stage of the disease, the ATP7a concentration increased significantly in the untrained mice compared with the ALS BEFORE and ALS ONSET untrained groups of mice. Interestingly, swim training at the onset stage of the disease induced an increase in the ATP7a concentration (Figure 2A).

The content of the CTR1 protein was significantly lower in the ALS groups at the onset and terminal stages of the disease than in the WT group. Similarly, the progression of the disease resulted in a lower content of the CTR1 protein in the untrained groups at the onset and terminal stages than in the ALS BEFORE group of mice. At the terminal stage of the disease, the CTR1 content significantly decreased in the trained group compared with the untrained group (Figure 2B).

The content of the DMT1 protein was significantly higher in the untrained and trained ALS ONSET groups than in the WT group. There was also an increase in the content of the DMT1 protein in the untrained ALS ONSET group compared with the ALS BEFORE group and the untrained ALS TERMINAL group of mice (Figure 2C).

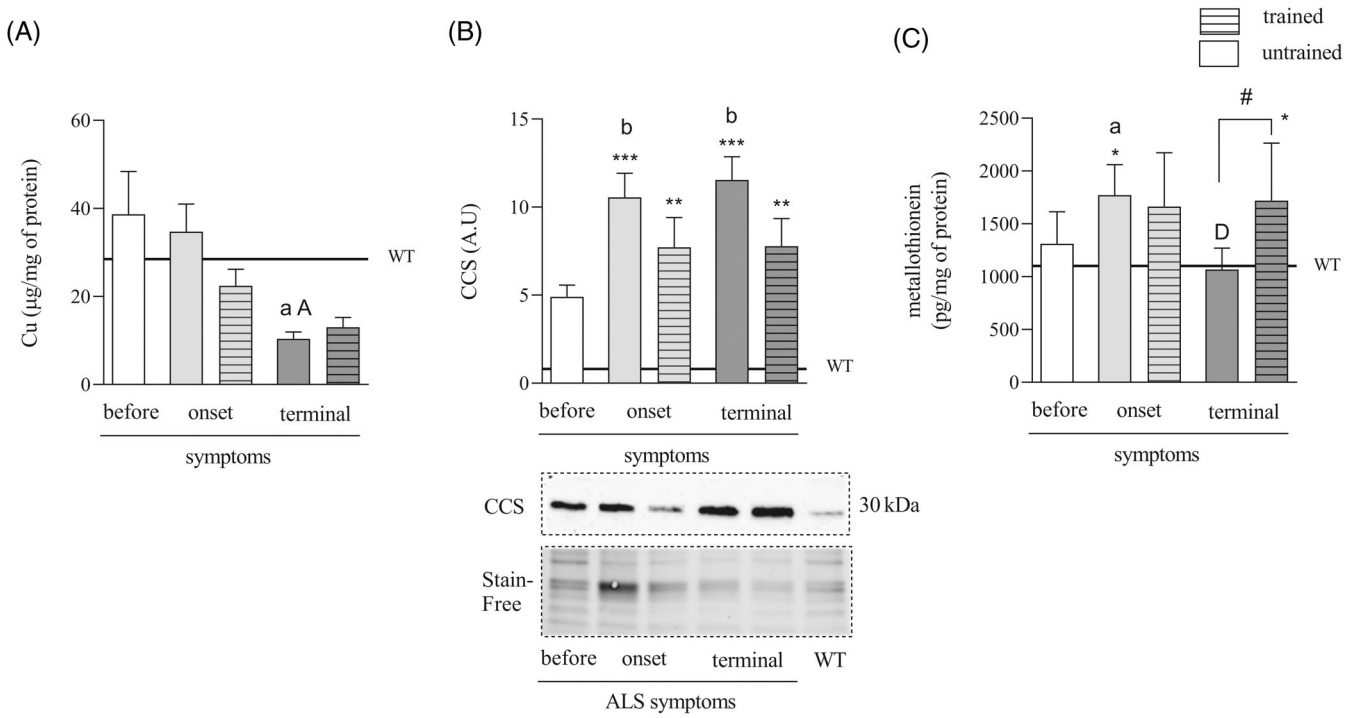

FIGURE 1 Effect of amyotrophic lateral sclerosis (ALS) disease progression and swim training on the copper concentration, copper chaperon for SOD1 (CCS), and metallothionein (MT) protein level in skeletal muscle. Copper concentration (A), CCS (B) and MT (C) levels were measured in mice skeletal muscle. The cropped blot was used in the figure. Full-length blot was shown in Figure S1. There were significant differences between the groups: $^{a}p < .05$, $^{b}p < .01$ versus ALS BEFORE group, $^{A}p < .05$, $^{D}p < .001$ versus ALS ONSET untrained group of mice,$^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus wild type (WT) group of mice, (Tukey's post hoc test), $^{\#}p < .05$ between the indicated groups (Student *t*-test). The data are presented as the means ± SD ($n = 6$ in each group).

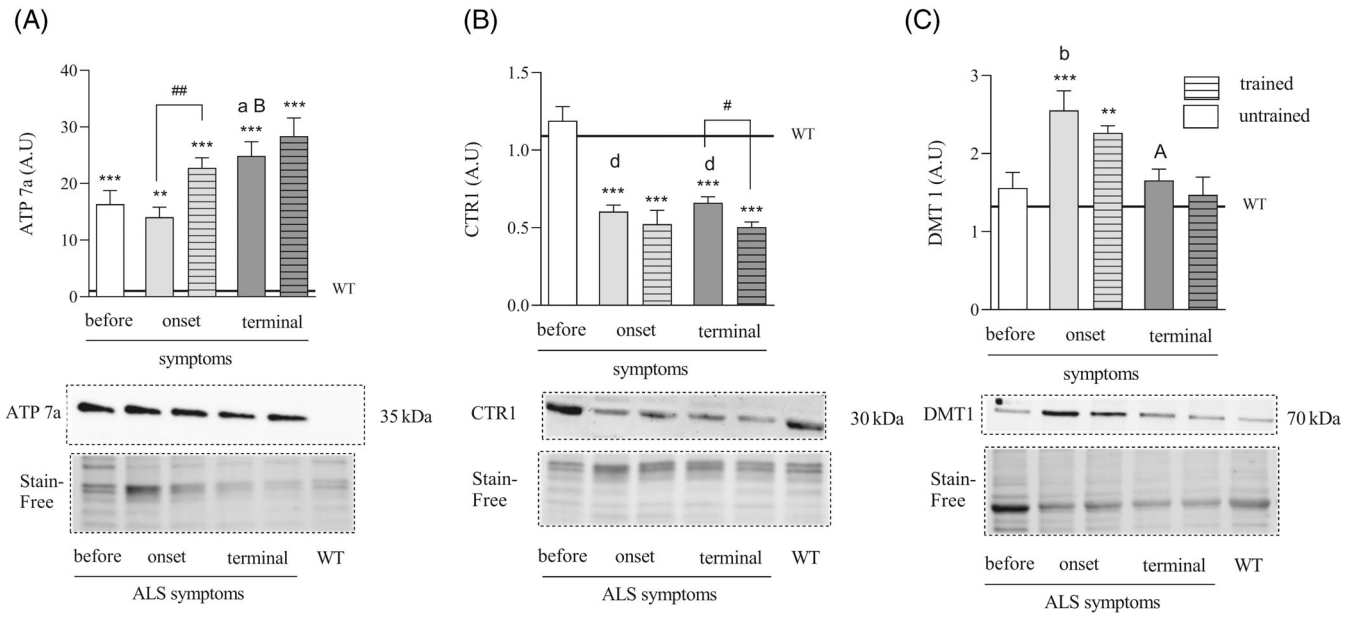

FIGURE 2 Effects of amyotrophic lateral sclerosis (ALS) disease progression and swim training on ATP7a, CTR1, and DMT1 protein level in skeletal muscle. ATP7a (A), CTR1 (B), and DMT1 (C) levels were measured in mice skeletal muscle. The cropped blots were used in the figure. Full-length blots are shown in Figures S2–S4. There were significant differences between the groups: $^{a}p < .05$, $^{b}p < .01$, $^{d}p < .001$ versus ALS BEFORE group, $^{A}p < .05$, $^{B}p < .01$ versus ALS ONSET untrained group of mice, $^{**}p < .01$, $^{***}p < .001$ versus wild type (WT) group of mice (Tukey's post hoc test), $^{\#}p < .05$, $^{\#\#}p < .01$ between the indicated groups (Student *t*-test). The data are presented as the means ± SD ($n = 6$ in each group).

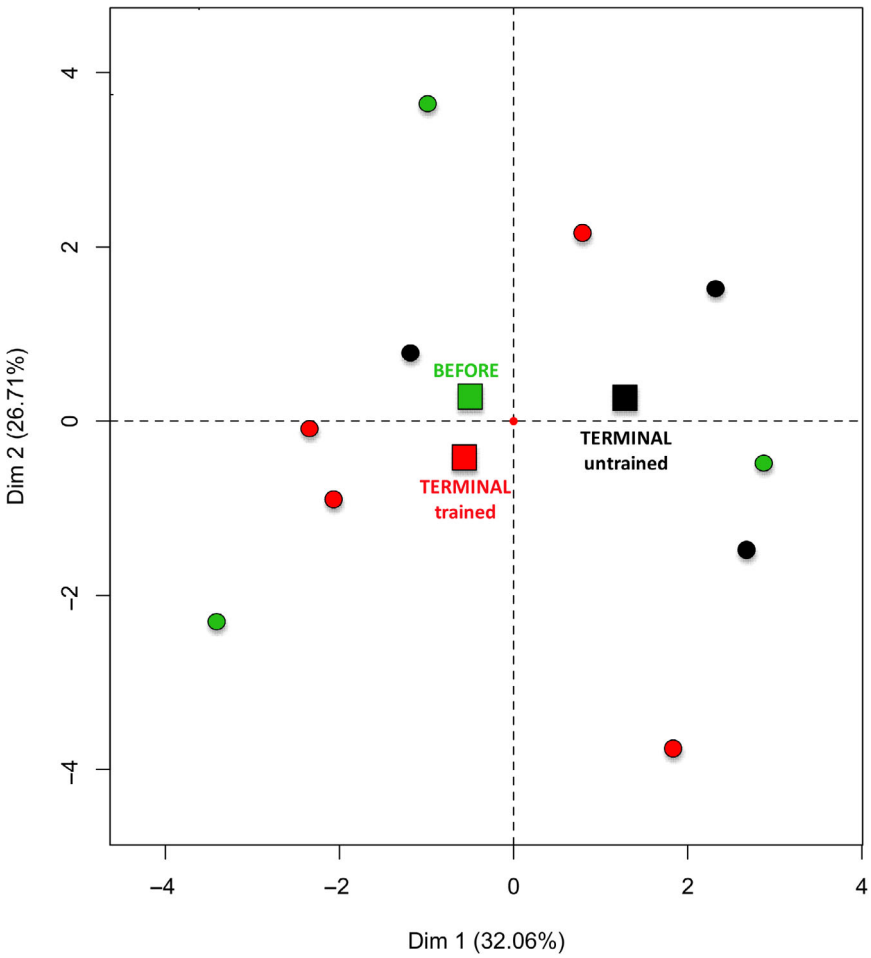

FIGURE 3 Principal component analysis (PCA) presenting the profile of copper metabolism proteins A 2D graph of variables PC1 and PC2 was created using PCA based on the level of 15 identified proteins (individual data, dots; means, squares) (membrane primary amine oxidase, AOC3; metalloreductase; STEAP3; glutaredoxin-like protein, C5orf63 homolog; ceruloplasmin, copper transport protein, ATOX1; superoxide dismutase 1, cytochrome c oxidase assembly protein, SCO1; cytochrome c oxidase subunit 1, cytochrome c oxidase subunit 2, copper homeostasis protein cut C homolog; lysyl oxidase homolog 1, LOXL1; cytochrome c oxidase assembly protein, COX11; cytochrome c oxidase copper chaperone, COX17; copper chaperone for superoxide dismutase, CCS; and major prion protein, PRIO) in the skeletal muscle of the ALS BEFORE (green), ALS TERMINAL untrained (black), and ALS TERMINAL trained mice (red).

3.3 | Effects of swim training on copper metabolism proteomic profiles of skeletal muscle of ALS mice

In the case of 2D space, the newly created PCA variables PC1 and PC2 indicated possible differences in the copper metabolism proteomic profiles between the ALS BEFORE, ALS TERMINAL untrained, and ALS TERMINAL trained groups (Figure 3). Additionally, in the case of 3D space, 3 newly created PCA variables (PC1, PC2, and PC3)—3D graph from the 15 identified proteins listed above (Figure S5). The ALS TERMINAL untrained copper metabolism signature (fingerprint created based on the proteomic profile) seemed to differ from those obtained for the ALS BEFORE and ALS TERMINAL trained groups.

4 | DISCUSSION

4.1 | Effect of ALS disease on copper metabolism

The results of our study show that the copper concentration in skeletal muscle increases insignificantly in mice before they show the first symptoms of the disease compared with WT mice and decreases significantly in ALS TERMINAL mice compared with ALS BEFORE and ALS ONSET mice. A similar trend in the increase in the copper concentration in the quadriceps muscle in mice with the first disease symptoms was shown by Enge et al.,[23] although the decrease in the copper concentration in the terminal group was not as significant as in the present study. The age of the ALS mice and the type of analyzed skeletal muscle might explain these differences, but there is a similarity in the overall trend in the results.

The increasing bioavailability of copper in cells due to the oral supply of CuII (atsm) not only increases the number of motor neurons in the spinal cord but also, similar to swim training, increases the lifespan for ALS G93A mice.[10,15,24] Moreover, potential therapeutics for ALS G93A mice have been proposed by lowering intracellular copper levels by small compounds, including three copper chelators, d-penicillamine,[25] trientine,[26] and tetrathiomolybdate. These compounds can chelate and reduce the accumulated copper ions in the spinal cord.[27]

In this study, it can be seen that the development of the disease is accompanied by a significant decrease in the content of the CTR1 protein, which is responsible for the delivery of copper to cells, as well as a significant increase in the amount of the ATPase7a protein, which excretes copper from muscle cells to the outside. Interestingly, the unchanged content of CTR1 and DMT1, another metal transporter, in the BEFORE ALS group of mice and the increased content of DMT1

in the ALS ONSET group of mice correspond to unchanged copper concentrations in the skeletal muscle at these stages of the disease. Only the reduction in the content of the CTR1 protein, and not DMT1 seen in the terminal stage, was accompanied by a decrease in the copper concentration in skeletal muscle. The copper imported into cells is safely accumulated by the MT protein or transferred to other proteins, for example, SOD1, by CCS. Contrary to our expectations, the level of the MT protein was higher in the ALS ONSET untrained group than in the ALS BEFORE and untrained TERMINAL ALS groups. In contrast to MT, significant changes were observed in the CCS protein levels. The increased protein content and its lack in cells lead to disease progression.[7,13] A higher content of CCS protein in skeletal muscles seems to follow the described changes in SOD1, that is, loss of copper binding capacity with the development of the disease.[28]

4.2 | Effect of swim training on copper metabolism in ALS mice

We assumed that swim training regulates copper metabolism mainly through the regulation of MT protein levels. It has been shown that overexpressing this protein benefits the quality and life expectancy of ALS mice,[5] similar to those after swim training.[15,16] However, swim training did not cause significant changes in copper concentrations in skeletal muscle. Interestingly, PCA demonstrated that the copper–protein signatures (fingerprint created based on the proteomic profiles) of the ALS BEFORE and ALS TERMINAL trained groups seemed to have a similar proteomic profile. Moreover, their profile differed from those obtained from the untrained ALS TERMINAL group (Figures 3 and S5). This could indicate a positive effect of training on the level of proteins related to copper metabolism. Similar favorable directions were seen in the ALS TERMINAL group for the proteins MT (increase) and CCS (decrease); thus, the opposite of the changes caused by ALS disease. Changes in the content of other proteins were not as visible under the influence of swim training. It follows that the training influenced the proteins regulating the concentration of copper inside the cells and the proteins importing and exporting copper to and outside the muscle cells.

The findings of our study have to be considered in light of some limitations; female mice were not studied and additional studies are needed to confirm the obtained results on other ALS models. Although, in the last decade, the frequency of ALS in women has risen, there is still a higher probability of disease in men.[29,30] Nonetheless, it would be valuable to study the devastating effect of the disease in female ALS mice in whom female sex hormones have significant influences on ALS progression and life duration.[31] Also, research on the cellular model of ALS could help to understand the relationship between cooper metabolism and muscle atrophy.

Moreover, the effects of swim training on the organism are multidimensional. Thus, the influence of swimming might occur through psychological, physiological, and molecular mechanisms that should be considered in future studies.

In summary, the present results show that ALS causes changes in copper metabolism in skeletal muscle. These findings shed new light on the disturbances seen during ALS disease in skeletal muscle and that they are moderately sensitive to a solid protective stimulus for ALS mice, such as swim training. From a clinical point of view, exercise in water for ALS patients might be considered a complement to the program supporting ALS patients. In future studies, it would also be worth verifying how the combination of swim therapy with metal-metabolism-stabilization agents (like CuII (atsm) treatment) influences ALS progression.

AUTHOR CONTRIBUTIONS

Emilia Białobrodzka: Conceptualization; data curation; formal analysis; writing—original draft; investigation. **Damian Jozef Flis:** Conceptualization; methodology; data curation; investigation; formal analysis; writing—original draft. **Banu Akdogan:** Methodology; investigation; writing—review and editing. **Andzelika Borkowska:** Data curation; investigation; writing—review and editing. **Mariusz Roman Wieckowski:** Data curation; investigation; writing—review and editing. **Jedrzej Antosiewicz:** Investigation; writing—review and editing; formal analysis. **Hans Zischka:** Data curation; formal analysis; writing—review and editing. **Katarzyna Patrycja Dzik:** Investigation; data curation; writing—review and editing. **Jan Jacek Kaczor:** Investigation; formal analysis; writing—review and editing. **Wieslaw Ziolkowski:** Conceptualization; methodology; data curation; investigation; supervision; funding acquisition; writing—original draft.

FUNDING INFORMATION

This study was supported by grants from the National Science Centre, Poland (DEC-2013/09/NZ7/02538 and 2020/39/B/NZ7/03366).

CONFLICT OF INTEREST STATEMENT

None of the authors has any conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

All research data are available from the corresponding author upon request.

ETHICS STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. The Local Ethical Committee for Experiments on Animals (Decision No. 11/2013 of April 22, 2013) and the Ministry of the Environment (Decision No. 155/2012) approved the experiment. Regarding housing, use for training and laboratory purposes, and ethical treatment of the research material, the European Union Directive 2010/63/EU guidelines were followed.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Białobrodzka E, Flis DJ, Akdogan B, et al. Amyotrophic Lateral Sclerosis and swim training affect copper metabolism in skeletal muscle in a mouse model of disease. *Muscle & Nerve*. 2024;70(5):1111-1118. doi:10.1002/mus.28237