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# Aged gastrocnemius muscle of mice positively responds to a late onset adapted physical training

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**Introduction:** A regular physical training is known to contribute to preserve muscle mass and strength, maintaining structure and function of neural and vascular compartments and preventing muscle insulin resistance and inflammation. However, physical activity is progressively reduced during aging causing mobility limitations and poor quality of life. Although physical exercise for rehabilitation purposes (e.g., after fractures or cardiovascular events) or simply aiming to counteract the development of sarcopenia is frequently advised by physicians, nevertheless few data are available on the targets and the global effects on the muscle organ of adapted exercise especially if started at old age.

**Methods:** To contribute answering this question for medical translational purposes, the proteomic profile of the gastrocnemius muscle was analyzed in 24-month-old mice undergoing adapted physical training on a treadmill for 12 weeks or kept under a sedentary lifestyle condition. Proteomic data were implemented by morphological and morphometrical ultrastructural evaluations.

**Results and Discussion:** Data demonstrate that muscles can respond to adapted physical training started at old age, positively modulating their morphology and the proteomic profile fostering protective and saving mechanisms either involving the extracellular compartment as well as muscle cell components and pathways (i.e., mitochondrial processes, cytoplasmic translation pathways, chaperone-dependent protein refolding, regulation of skeletal muscle contraction). Therefore, this study provides important insights on the targets of adapted physical training, which can be regarded as suitable benchmarks for future *in vivo* studies further exploring the effects of this type of physical activity by functional/metabolic approaches.

## KEYWORDS

ageing, skeletal muscle, physical training, matrix, proteomics, electron microscopy

## 1 Introduction

Physical activity is progressively reduced and/or impaired during aging causing mobility limitations and consequently a poor quality of life. In later stages, this decline may be associated to sarcopenia (Cruz-Jentoft et al., 2019; Chen et al., 2020), a muscle disease characterized by loss of skeletal muscle mass and strength associated with increased risk of frailty, falling and mortality (Cruz-Jentoft and Sayer, 2019). Many factors contribute to the progressive loss of muscle efficiency such as age, gender, comorbidities, body mass index, and

malnutrition (Landi et al., 2013; Batsis et al., 2014; Beaudart et al., 2017; Robinson et al., 2018). Therefore, preventive issues and/or treatment of elderly require multidisciplinary approaches and a broad spectrum of competences.

A regular physical activity and/or exercise, performed throughout life, can slow down the age-dependent decline of muscle mass and strength, maintaining structure and function of neural and vascular compartments as well as muscles (Gao et al., 2020), and can prevent both age-associated muscle insulin resistance and inflammation (Novelli et al., 2004; Mikkelsen et al., 2013). However, lifelong physical training is a relatively rare situation in the general population, while physical exercise in adulthood or in the elderly for rehabilitation purposes (e.g., after fractures or cardiovascular events) or simply to delay the effects of ageing is a rather frequent event. Therefore, it is important to understand if and how the muscle organ can respond to physical training started in old age. Furthermore, it is also important to take into consideration the intensity of exercise to avoid cardiovascular system overload opposing the widely described benefits of physical activity (La Gerche et al., 2017; Graziano et al., 2022). Within this context, adapted exercise, i.e., any modalities of physical training tailored on the residual physical capacity of the elderly in terms of frequency, intensity, and volume, was proposed as a way to optimize functional outcomes of physical exercise in both fit and frail older adults (Imagita et al., 2020; Izquierdo et al., 2021).

Investigations focusing on the role of physical activity in aged subjects indicate that it preserves fiber's morphology, mitochondrial function (i.e., ATP production) and antioxidant capacity (Proctor et al., 1995; Lanza et al., 2008; Safdar et al., 2010; Zampieri et al., 2015). However, these studies were mostly performed through reductionist-based approaches that, being restricted to specific molecular targets, can only improve the understanding of single biological mechanisms, without a global evaluation of the whole muscular microenvironment in response to physical activity and/or exercise.

In recent years, omic's-based techniques (e.g., transcriptomics, and proteomics) have been applied to explore the skeletal muscle behavior in different experimental conditions (Vissing and Schjerling, 2014; Lang et al., 2017; Starnes et al., 2017). The proteome profile of skeletal muscles has been investigated in aging (Doran et al., 2009; Gueugneau et al., 2014; Gonzalez-Freire et al., 2017; Murgia et al., 2017; Lofaro et al., 2021), and in metabolic diseases (Kleinert et al., 2018), as well as in response to high altitude hypoxia (Levett et al., 2015) and after interval, endurance or strength training (Holloway et al., 2009; Son et al., 2011; Schild et al., 2015; Petriz et al., 2017). Results clearly indicate that the response of skeletal muscles varies depending on the type and duration of the exercise (e.g., few days vs. few weeks) (Egan and Zierath, 2013). However, it is still unclear whether the effects of physical training on the proteomic profile of skeletal muscle also occur when training is undertaken at old age.

To contribute answering this question, we analyzed the proteomics of the gastrocnemius muscle (GAST) in 24-month-old mice undergoing adapted physical training (treadmill running 3 days a week for 12 weeks, O-T) or kept in cages under a sedentary lifestyle condition (O-S). Proteomic data were further implemented by morphological and morphometrical ultrastructural evaluations.

Our findings demonstrate that: i) old muscles respond to adapted physical training started at old age, ii) adapted physical training positively modulates the morphology and the proteomic profile of GAST either involving the extracellular compartment as well as muscle cell components and pathways (i.e., mitochondrial processes, cytoplasmic translation pathways, chaperone-dependent protein refolding, regulation of skeletal muscle contraction).

All these data emphasize that adapted physical training started at old age activates protective and saving mechanisms that act in synergy to counteract the age-dependent phenotype of GAST muscle.

## 2 Materials and methods

### 2.1 Mice

A total of 18 male BALB/c mice aged 24 months were used in this study. Male mice were chosen to avoid sex and hormonal status influence. The mice, housed in groups of 3–4, were maintained under standard conditions ( $24^{\circ} \pm 1^{\circ}\text{C}$  ambient temperature,  $60\% \pm 15\%$  relative humidity, and 12 h light/dark cycle) and fed *ad libitum* with standard commercial chow. The mice were allocated to the sedentary old (O-S,  $n = 8$ ) and training old (O-T,  $n = 10$ ) group with the “= Rand()” function in Microsoft Excel. O-S mice were only allowed only spontaneous free-moving activity in the cage. O-T mice underwent training on a treadmill (Harvard Instruments, Crisiel, Rome, Italy) for 30 min at 8 m/min belt speed (0% incline), 3 days a week for 12 weeks. Running on a treadmill is a widely used training modality in laboratory animals because it allows for accurate control and extensive modulation of exercise intensity, duration, and frequency, making it suitable even for older individuals (Massett et al., 2021). Current treadmill protocols for adult individuals consistently use 1 h running a day at belt speed  $>10$  m/min. In this work, physical training was adapted to optimize old mice compliance to training (Fabene et al., 2008). To minimize stress several provisions were adopted. Running was continuously supervised by an experimenter. Mice familiarized for 1 week to the treadmill with a single 30-min daily session on the stopped treadmill. During the experiment, mice were transported to the running room 1 h before each daily session of exercise. According to the standard protocol of our laboratory to prevent injury to the hind limbs provoked by the posterior wall of the treadmill a metal-beaded curtain, as non-noxious stimulus, was used as an incentive; no shock-plate incentive was used. To avoid possible interference of acute with chronic effects of physical exercise, the mice were sacrificed 3 days after completion of the experimental training protocol. Animals were deeply anesthetized with tribromoethanol drug and sacrificed by cervical dislocation or perfused via the ascending aorta with 0.1 M PBS followed by 4% paraformaldehyde in PBS depending on the analyses to be performed.

Evaluation of the results and data analysis were carried out blind to the mouse group.

Mice were handled according to the regulations of the Italian Ministry of Health (DL 4 March 2014, n. 26) and to the European Communities Council (Directive 63/2010/EU of the European Parliament and the Council) directives. The experimental

protocol was approved by the Italian Ministry of Health (Ref.: 538/2015-PR).

The gastrocnemius muscle was selected for this study since it is one of the primary movers of the hindlimb, being involved in gait; moreover, it is prevalently composed of fast-twitch fibers (Zancanaro et al., 2007), which are especially affected by atrophy during aging (Lexell, 1995).

## 2.2 In tube-gel digestion

For proteomic analysis, whole gastrocnemius muscles were quickly removed from three O-S and three O-T (sacrificed by cervical dislocation) and frozen.

Tissue protein digestion was performed as previously described (Lofaro et al., 2021). Briefly, three subsequent extraction protocols were used to isolate proteins with different solubility grade. Therefore, frozen muscles were homogenized in phosphate buffer (PBS) on ice using a glass homogenizer to solubilize cellular and hydrophilic proteins. The resulting homogenates were then centrifuged at 8,000× g for 30 min at 4°C. The supernatants (PBS extract) were collected, whereas pellets were resuspended in urea-thiourea buffer and incubated at 4°C for 24 h with continuous shaking to favor the solubilization of hydrophobic proteins. The samples were centrifuged at 150,00× g for 20 min at 4°C to obtain the supernatant (U/T extract), while the pellet was homogenized in guanidinium-HCl buffer (pH = 8.5, 1:5 w/v), heated at 100°C for 10 min, and collected (GuHCl extract). This last extraction is considered the most appropriate for insoluble ECM proteins. The protein concentration of each fraction was determined using the Bradford method (Bradford, 1976).

For each fraction (200 µg of proteins), a gel-tube digestion method was performed, as already described (Boraldi et al., 2019). The proteins embedded in the gel-tubes were firstly reduced by incubation with 10 mM dithioerythritol and 100 mM ammonium bicarbonate at 56°C for 45 min. Subsequently, they were alkylated with 55 mM iodoacetamide and 100 mM ammonium bicarbonate for 30 min at room temperature in the dark. Protein digestion was performed by trypsin with an enzyme-to-protein ratio of 1:100 (Promega, Madison, MI, United States). Peptides were extracted from the gel-tubes using 100% acetonitrile and subsequently dried in a SpeedVac (Eppendorf AG, Hamburg, Germany).

## 2.3 LC–MS/MS analysis and MS/MS data processing

Mass spectrometric analysis was performed using an LC coupled to a QExactive mass spectrometer (Thermo Fisher); MS/MS data (.raw) were examined using BatMass (v. 0.3.0), as previously described (Lofaro et al., 2021). Briefly, replicates were aligned using FreeStyle (v.1.5) to assess the run quality. The raw files were then converted to .mzXML format using msConvert ProteoWizard (v.3.0.19239) with default settings and uploaded to the MSFragger (v.3.4.0) for MS/MS Ion Search. The search was conducted using the Uniprot database (2018\_05) limited to *Mus musculus* (Taxonomy ID: 10090). Enzyme was specified to trypsin

with a maximum of 1 missed cleavage. Fix modification was carbamidomethylation of cysteine, whereas variable modifications were deamidation of asparagine and glutamine, oxidation of methionine, and cysteine propionamide. Data searches were performed with a mass error tolerance of 10 ppm for precursor ions and of 0.02 Da for fragment ions with peptide charge states of 2+, 3+, and 4+.

Only confidently identified peptides with a false discovery rate (FDR) ≤ 1 and proteins with at least one unique peptide were exported.

The MSFragger results (interact.pep) obtained for each extraction were imported into Skyline-daily (v.21.0.9.139) to generate spectral libraries using the following parameters: a spectra cut-off score of 0.95, peptide length ranging from 8 to 25 amino acids, and precursor ion charges of 2+, 3+, and 4+. MS1 filters were set to “use high selectivity extraction” with a resolving power of 60,000 at 300 m/z, and repeated and duplicate peptides were removed. Following the Skyline “DDA peptide search” workflow, the raw files (.raw) were imported and matched to the spectral libraries to recover precursor ion intensity. Precursor ion intensity represents the sum of areas under the curve of extracted ion chromatograms (XICs) containing precursor ion isotope peaks (M, M+1, M+2) (Singh and Thongboonkerd, 2018). Fasta files containing proteins with a 1% FDR were imported into Skyline to maintain and fix the FDR.

The quantitative analysis was only conducted on proteins identified with at least two peptides because the quantification of a single peptide across LC-MS/MS runs could be not sufficiently accurate (Tsai et al., 2020). Statistical analysis of proteomic data was performed with the Skyline group comparison tool. Runs were normalized using the “Equalize Medians” method with a confidence level of 0.95, and the Tukey’s Median Polish was used as the summary method (Lofaro et al., 2021).

## 2.4 Protein-protein interaction (PPI) analyses

PPIs were built by STRING database (v. 11.5) to understand functions and interactions of DEPs (Szklarczyk et al., 2021). Furthermore, MCL clustering with a minimum of 3 inflation parameters was employed to identify the top 5 protein clusters, which were imported and edited into Cytoscape (v.3.9.1) to visualize networks.

## 2.5 Immunofluorescence

For identification of diffuse and tissue-abundant antigens, we adopted an immunohistochemical approach, briefly described below.

### 2.5.1 Laminin

GAST muscles from at least three O-S and three O-T mice were frozen in liquid nitrogen-precooled isopentane, transversally sectioned into 5-µm thick cryosections and incubated with 1% bovine serum albumin, 2% normal goat serum, 0.3% Triton® X-100 in PBS for 1 h and immunolabelled with a rabbit polyclonal

antibody direct against laminin, diluted 1:800 (Abcam, Cambridge, United Kingdom). After washing with PBS, cryosections were stained with the secondary antibody Alexafluor 594-anti-rabbit diluted 1:200. The cryosections were finally counterstained for DNA with 0.1 mg/mL Hoechst 33,258 and mounted in PBS: glycerol (1:1). An Olympus BX51 microscope equipped with a 100 W mercury lamp (Olympus Italia, Milan, Italy) was used under the following conditions: 540 nm excitation filter (excf), 580 nm dichroic mirror (dm), and 620 nm barrier filter (bf) for Alexa 594; 330–385 nm excf, 400 nm dm, and 420 nm bf for Hoechst 33,258. Images were recorded with an Olympus Magnifire digital camera system (Olympus Italia). In anti-laminin immunolabeled samples, the minimum Feret's diameter (the minimum distance of parallel tangents at opposing borders of the muscle fibers) was measured on 200 myofibers per mice group. The minimum Feret's diameter is very insensitive against deviations from the "optimal" cross-sectioning profile, therefore, reliably detecting differences between muscles (Briguet et al., 2004).

### 2.5.2 Heavy chain of skeletal fast fiber myosin

GAST samples (about 1 mm<sup>3</sup>) from perfused animals (three O-S and three O-T mice) were placed for 2 h at 4°C in 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M PBS, washed in PBS, treated with 0.5 M NH<sub>4</sub>Cl solution in PBS for 45 min at 4°C to block free aldehyde groups, dehydrated in graded concentrations of ethanol at room temperature and embedded in LRWhite resin. For fiber typing, 2 µm-thick cross sections were submitted to immunohistochemical procedures to distinguish fast and slow fibers (Egan and Zierath, 2013). Briefly, sections were incubated for 2 h at room temperature with a mouse monoclonal antibody recognizing the heavy chain of skeletal fast fiber myosin (clone MY-32, Sigma-Aldrich, Buchs, Switzerland) diluted 1:200 in PBS; the antigen-antibody complex was revealed with an Alexa 488 conjugated antibody against mouse IgG (Molecular Probes, Invitrogen, Milan, Italy). The sections were finally counterstained for DNA with 0.1 µg/mL Hoechst 33,258. Micrographs were taken with an Olympus BX51 microscope equipped with a 100 W mercury lamp under the following conditions: 450- to 480-nm excitation filter (excf), 500-nm dichroic mirror (dm), and 515-nm barrier filter (bf) for Alexa 488; 330- to 385-nm excf, 400-nm dm, and 420-nm bf for Hoechst 33,258. Images were recorded with an Olympus Camedia C-5050 digital camera. In immunolabeled samples, the percentage of fast and slow muscle fibers was calculated on 100 myofibers per animal, with 300 myofibers measured per group (O-S and O-T). Micrographs were taken at magnification of ×20 and processed with the ImageJ software (NIH).

## 2.6 Ultrastructure and morphometric evaluations

GAST samples (about 1 mm<sup>3</sup>) from perfused animals (three O-S and three O-T mice) were placed for 2 h at 4°C in 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, United States) plus 2% paraformaldehyde. After fixation, samples for ultrastructural morphology were rinsed with PBS, postfixed with 1% OsO<sub>4</sub> (Electron Microscopy Sciences) and 1.5% potassium

ferrocyanide for 2 h at 4°C, dehydrated with acetone, and embedded in Epon 812 resin (Electron Microscopy Sciences).

For morphometrical evaluation of ultrastructural variables, ultrathin sections (70–90 nm thick) from three O-S and three O-T mice were stained with lead citrate for 1 min and observed in a Philips Morgagni transmission electron microscope operating at 80 kV and equipped with a Megaview III camera for digital image acquisition.

The morphometric evaluation of the endomysium thickness was performed on 20 randomly selected electron micrographs (×5,600) of longitudinally sectioned muscle. The distance between the sarcolemma of two adjacent muscle cells was measured every 1 µm of sarcolemma length, for a total of 50 measurements per animal with a total of 150 measurements for mice group (O-S and O-T). The thickness of the basement membrane covering the myofiber was measured on randomly selected electron micrographs (×36,000) of longitudinally sectioned muscles. A minimum of 30 measurements per animal were performed for a total of 100 measurements for mice group (O-S and O-T).

The morphometric evaluation of the perimysium collagen bundle size was performed on 25 longitudinally sectioned collagen bundles per animal, for a total of 75 measurement for each mice group (O-S and O-T). The index of collagen bundle linearity (X/Y, expressed as the ratio between the real length of the bundle profile and the corresponding linear length) was assessed on 10 longitudinally sectioned collagen bundles per animal, for a total of 30 measurements per mice group (O-S and O-T). For morphometric evaluation of collagen fibrils, measurement of fibril size, as well as the distance between single collagen fibrils, was performed on a minimum of 30 longitudinally sectioned collagen fibrils per animal with a total of 100 measurements per mice group (O-S and O-T).

The density of the perinuclear and intermyofibrillar mitochondria was assessed in 10 randomly selected micrographs (×11,000) of longitudinally sectioned muscle per animal for a total of 30 measurements per mice group (O-S and O-T). For the assessment of perinuclear mitochondria density, the cytoplasmic area surrounding myonuclei and devoid of myofibrils bundles was considered. For the intermyofibrillar mitochondria density, cytoplasmic areas far from the myofiber periphery were considered. The number of mitochondria was counted, and the mitochondrial density was expressed as number of mitochondria/area (µm<sup>2</sup>).

The sectional area of both intermyofibrillar and perinuclear mitochondria as well as the length of outer and inner mitochondrial membrane was measured in 20 mitochondria (×36,000) per animal for a total of 60 mitochondria per mice group (O-S and O-T). The inner/outer membrane ratio, an assessment of cristae extension independent of mitochondrial size, was calculated as a reliable morphological parameter of mitochondrial function (*i.e.*, respiratory activity). In fact, the cristae density is considered a predictor of maximum oxygen uptake in relation to mitochondrial volume (Nielsen et al., 2017).

Morphometrical analysis of the nucleolus and the nucleolar components, *i.e.*, fibrillar centers (FCs, circular in shape, contain ribosomal genes and enzymes necessary for transcription (Recher et al., 1969; Goessens, 1984), dense fibrillar component (DFC, usually edges the FCs, is the site of transcription and processing

of rRNA (Schwarzacher and Wachtler, 1991) and granular component (GC, site of maturation and storage of ribosomal subunit (Schwarzacher and Wachtler, 1991), was performed on 10 randomly selected nucleoli of longitudinally sectioned muscles ( $\times 18,000$ ) per animal for a total of 30 measurements per mice group (O-S and O-T). Area of the nucleolus as well as each nucleolar component were measured, and the percentage of the nucleolar areas occupied by FCs, DFC, and GC was calculated.

All measurements were made by using the Radius software for image acquisition and elaboration implemented in the Philips Morgagni transmission electron microscope.

## 2.7 Ultrastructural immunocytochemical analyses

For identification of sub-cellular and less abundant antigens, we adopted an ultrastructural, immune-electron microscopy approach allowing for more accurate and sensitive antigen localization in spite of the less intense signal compared to light microscopy immunohistochemistry.

GAST samples (about  $1 \text{ mm}^3$ ) from perfused animals (three O-S and three O-T mice) were placed for 2 h at  $4^\circ\text{C}$  in 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M PBS, washed in PBS, treated with 0.5 M  $\text{NH}_4\text{Cl}$  solution in PBS for 45 min at  $4^\circ\text{C}$  to block free aldehyde groups, dehydrated in graded concentrations of ethanol at room temperature and embedded in LRWhite resin. This procedure, while providing poorer morphology vs. standard embedding (e.g., eposidic resin), makes samples much more suitable for antigen detection.

Longitudinally-cut sections (70–90 nm thick), collected on Formvar-carbon-coated nickel grids, were floated on normal goat serum (NGS) diluted 1:100 in PBS for 3 min and then incubated at  $4^\circ\text{C}$  for 17 h with a polyclonal antibody directed against telethonin (sc-8725, Santa Cruz biotechnology, Dallas, United States) diluted 1:25 or a monoclonal antibody directed against mitochondrial hsp70 (mtHsp70; Enzo Life Sciences, Farmingdale, NY, United States) diluted 1:100, both diluted in PBS containing 0.05% Tween and 0.1% BSA. After rinsing with PBS, Tween and PBS, the grids were incubated with NGS as above, and then incubated for 30 min at room temperature with the appropriate secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA, United States) coupled with colloidal gold, diluted 1:20 in PBS. The grids were rinsed with PBS and distilled water. Sections were stained for 35 min at room temperature with Uranyl Less EM stain (Electron Microscopy Sciences, Hatfield, PA, United States), followed by Reynolds' lead citrate for 1 min (Lacavalla and Cisterna, 2023). Sections were then observed with a Philips Morgagni transmission electron microscope operating at 80 kV.

As controls, some grids were floated on the incubation mixture without the primary antibody, and then treated as above. Immunolabelling was absolutely negligible.

A semiquantitative assessment of the anti-telethonin immunolabeling was carried out by estimating the gold grain density on 10 myofiber area for a total of 30 myofiber area per each mice group. The gold grains were counted, and the labelling density was expressed as number of gold grains/myofiber area ( $\mu\text{m}^2$ ).

A semiquantitative measurement of anti-mithsp70 immunolabelling was carried out on a minimum of 10 intermyofibrillar mitochondria per animal for a total of 40 measurements per mice group. The gold grains were counted, and the labelling density was expressed as number of gold grains/mitochondrial area ( $\mu\text{m}^2$ ).

## 2.8 Statistical analysis

Quantitative and semiquantitative values for individual variables were pooled according to the experimental group (O-S and O-T) and are presented as mean  $\pm$  standard error of the mean (SEM). Between-group comparison of body mass was carried out with the *t*-test for independent samples (O-T vs. O-T at baseline; GAST mass at endpoint; nucleolar area at endpoint) or the *t*-test for paired samples (O-S and O-T before and after training). For all other variables, the Shapiro-Wilk test showed non normal distribution of the residuals of the regression of the variable on mouse ordinal number (intended as a categorical variable); therefore, the non-parametric Mann-Whitney test was used throughout. The statistical significance was set at *p*-value  $< 0.05$ . The IBM-SPSS (v.25, Armonk, NY, United States) statistical package was used for all analyses.

## 3 Results and discussion

### 3.1 Effects of adapted physical training on mice

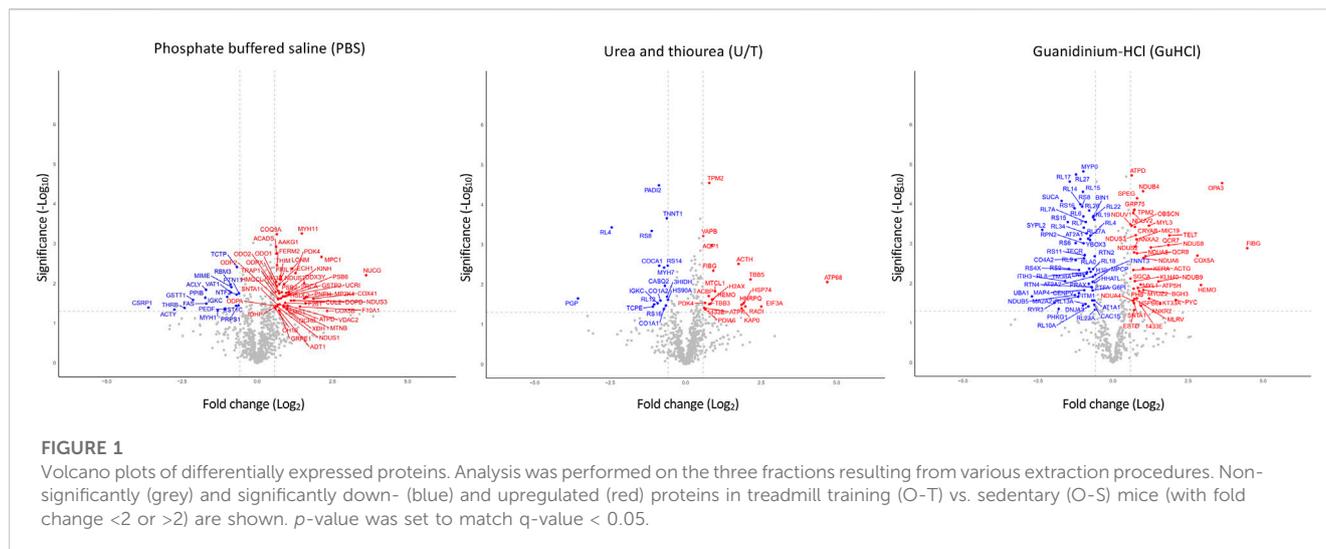
All mice in the O-T group were able to correctly carry out the physical training protocol (see *materials and methods*).

At baseline, body mass was similar in the two groups of mice (O-S,  $39.7 \pm 1.92 \text{ g}$ ; O-T,  $39.7 \pm 0.76 \text{ g}$ ; *p* = N.S.). Body mass showed statistically significant decrease in O-S at the end of the training period ( $34.8 \pm 1.37 \text{ g}$ , *t* = 3.954, *p* = 0.011), whereas it was not statistically significant different in O-T ( $37.8 \pm 1.58 \text{ g}$ ; *t* = 1.031, *p* = 0.350). These findings indicate that adapted physical training contributes preventing age-related weight loss. At the end of the training period the average mass of GAST was slightly (*p* = 0.21) higher in O-T ( $170 \pm 20 \text{ mg}$ ) than O-S ( $160 \pm 17 \text{ mg}$ ).

### 3.2 Effects of adapted physical training on the protein profile of GAST

By using three different extraction buffers (Lofaro et al., 2021) (see *materials and methods*) followed by LC-MS/MS analyses, 1,493 different proteins were identified in aged mice (Supplementary Table S1), of which 369 were present in all 3 extracts, while 416, 182, and 51 proteins were detected in phosphate buffered saline (PBS), urea and thiourea (U/T) and guanidinium-HCl (GuHCl) extracts, respectively. A label free quantification was applied to the proteins identified with at least two peptides (Supplementary Table S2).

Figure 1; Supplementary Table S3 show the differentially expressed proteins (DEPs) between O-T and O-S mice in each extract: 50 and 18 proteins were up- or downregulated in PBS



fraction; 21 and 17 were up- or downregulated proteins in U/T fraction and 43 and 60 proteins were up- or downregulated in GuHCl fraction. The use of subsequent different extraction procedures allowed to markedly increase the number of identified proteins compared, for instance, to similar proteomic studies (Li et al., 2019).

Taken together, these results show that a total of 197 proteins were significantly modified by adapted physical training.

### 3.3 Pathway enrichment analysis

To understand functions and interactions of DEPs, we used the STRING database, version 11.5 (Szklarczyk et al., 2021). Figure 2 shows the five different subnetworks that were identified by STRING according to the protein-protein interaction (PPI) network of DEPs. These clusters were enriched in proteins associated with mitochondrial processes (cluster 1; GO:0061732); cytoplasmic translation pathways (cluster 2; GO:0002181); regulation of skeletal muscle contraction (cluster 3; GO:0014724); chaperone-dependent protein refolding (cluster 4; GO:0051085); collagen-activated signaling pathways (cluster 5; GO:0038063). These data clearly indicate that DEPs are not scattered in the secretome but are mostly connected within specific pathways/functions. Disclosing protein-protein interactions through the interactome may contribute to understand the cell process-regulating machinery and to pave the way for the identification of potentially new biomarkers (Feng et al., 2015).

### 3.4 Effects of adapted physical training on mitochondria processes and cytoplasmic translation pathways

Skeletal muscle contractile activity affects mitochondrial and ribosome biogenesis (Mesquita et al., 2021), however studies revealed apparently opposing results depending on the training program (e.g., endurance vs. resistance; endurance followed by resistance or vice versa; frequency and/or duration of exercise). Exercise, in fact, can simultaneously influence ribosome and mitochondrial biogenesis, or can increase ribosome biogenesis

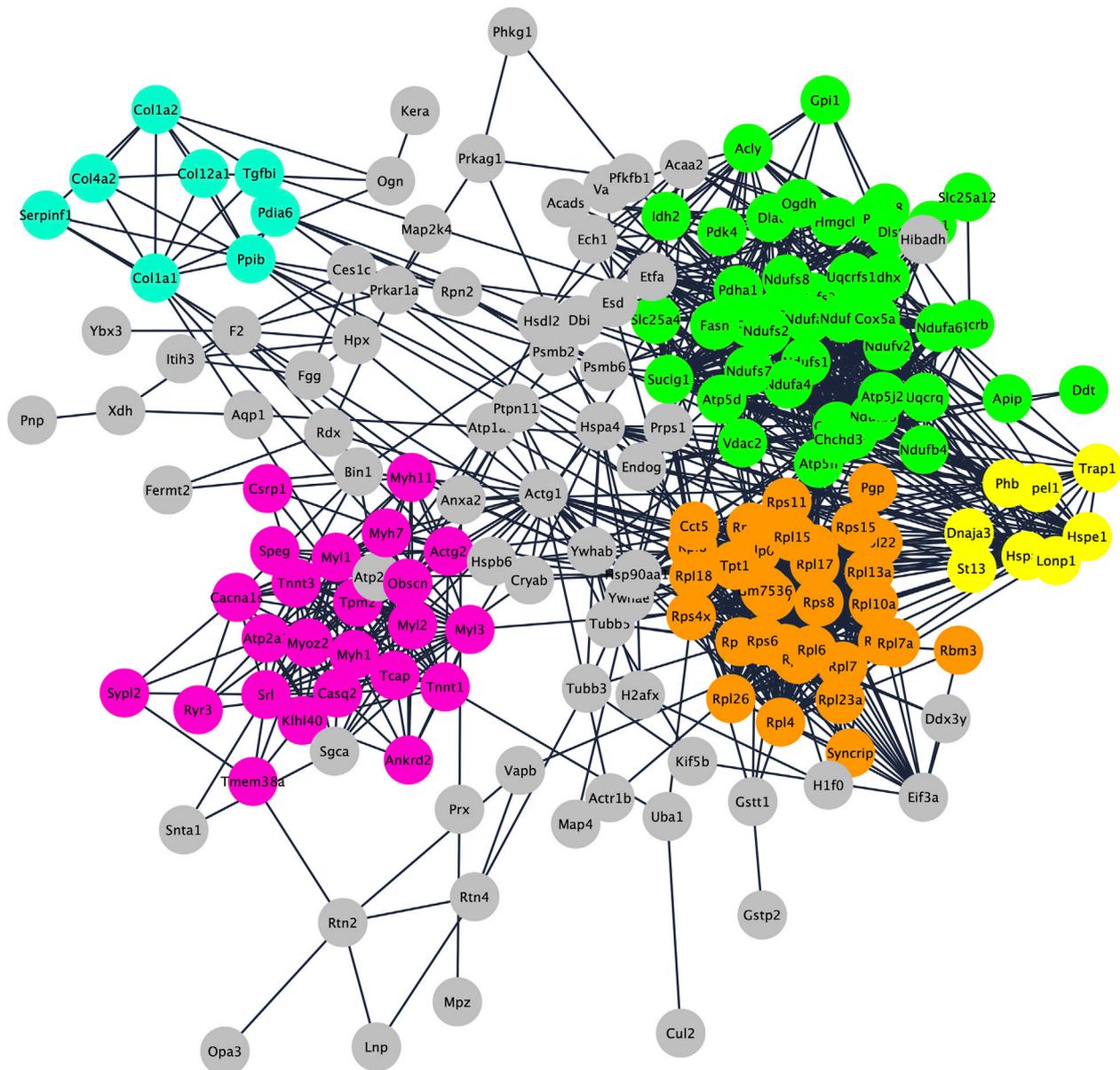
over mitochondrial biogenesis or vice versa as a consequence of the competition between the two processes (Morrison et al., 1989; Wilkinson et al., 2008; Gibala et al., 2009; Hansson et al., 2019; Figueiredo et al., 2021).

In our experimental conditions we found that: i) 39 and 6 polypeptides related to mitochondrial processes were up- or downregulated by physical training, respectively (Figure 3A; Supplementary Table S3), whereas ii) 33/34 DEPs related to ribosomes were downregulated after the training period (Figure 4A; Supplementary Table S3).

Therefore, adapted long-term treadmill running increased the level of mitochondrial proteins, many of which belong to the mitochondrial complex I, a multimeric enzyme that modulates the transfer of electrons from NADH to ubiquinone, facilitating ATP synthesis (Galkin et al., 2006). These data are in agreement with those observed in the rat model (Li et al., 2019), thus further highlighting the role of mitochondria metabolism/activity as a preferential target of physical training, although the extent of these changes may differ according to the age and/or to the type of exercise. Present data demonstrate that mitochondria are still responsive in aged animals.

Since it has been demonstrated that structure and function are tightly connected and that mitochondrial morphology is strictly linked with changes in energy production (Buffa et al., 1970; Muscatello and Pasquali-Ronchetti, 1972; Glancy et al., 2020; Lofaro et al., 2020), the ultrastructure of myofibers and of mitochondria was investigated.

The typical organization of the myofibers was maintained in both O-S and O-T. Many myonuclei were in subsarcolemmal position and the longitudinally aligned myofibrils occupied almost the entire cytoplasm. Cisternae of sarcoplasmic reticulum, often surrounded by abundant glycogen deposits, were distributed between the myofibrils. Few lipid droplets were found in the myofibers. Well preserved ovoid mitochondria were located in the subsarcolemmal region. The great majority of mitochondria were lined between the myofibrils (Figure 3B), consistently with the role of intermyofibrillar mitochondria that are the site of biochemical pathways related to muscle contraction (Müller, 1976). The density of both intermyofibrillar and perinuclear mitochondria was significantly



**FIGURE 2**

Protein–protein interaction and enrichment analysis of 192 DEPs obtained by STRING. Networks (clusters) with 8 or more proteins (densely connected nodes) were colored in green (cluster 1; mitochondrial processes), orange (cluster 2; cytoplasmic translation pathways), pink (cluster 3; regulation of skeletal muscle contraction), light blue (cluster 4; chaperone-dependent protein refolding) and yellow (cluster 5; collagen-activated signaling pathways).

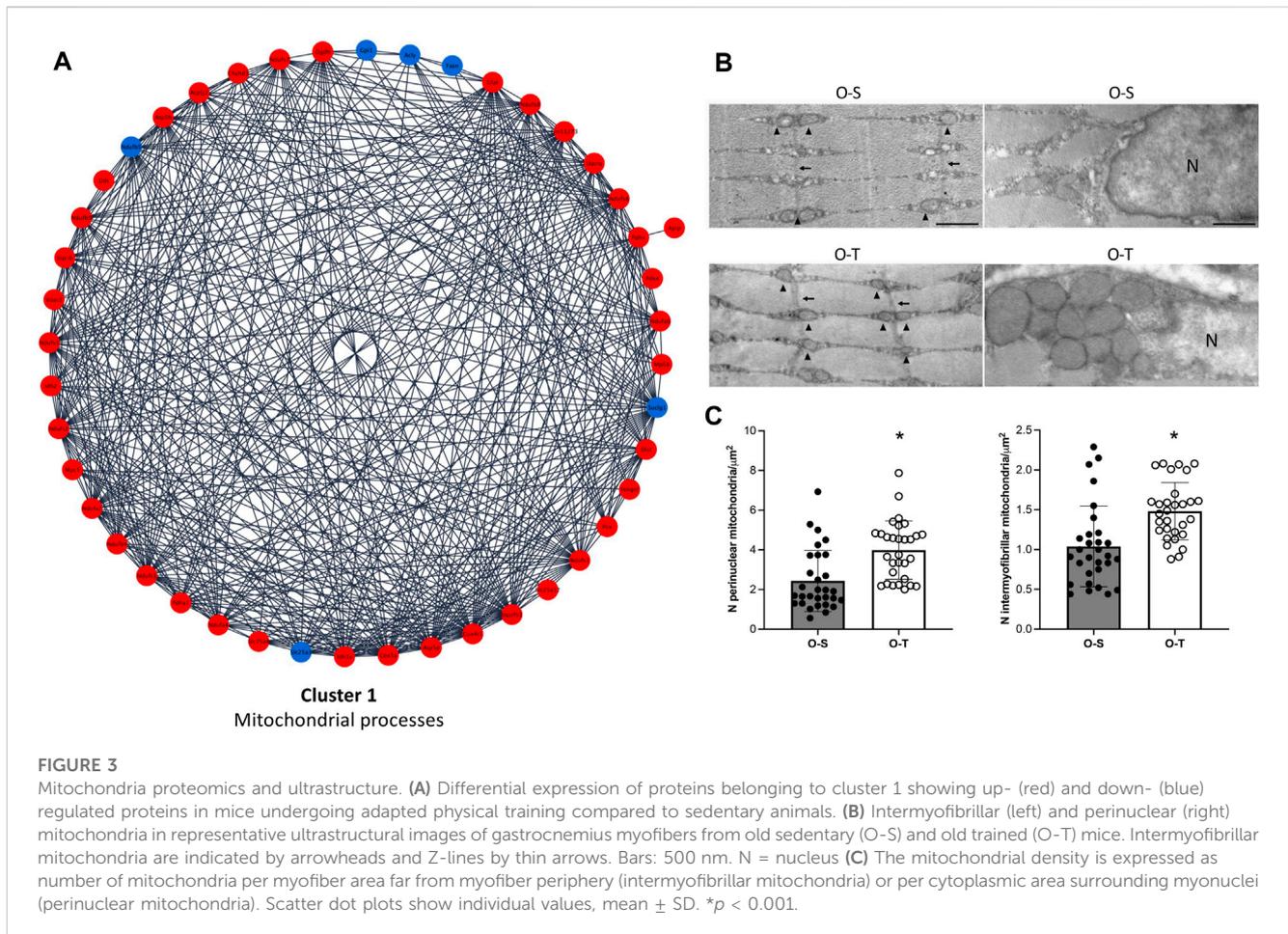
higher in O-T vs. O-S (Figure 3C). No significant difference was found in the size of the intermyofibrillar mitochondria in O-S vs. O-T ( $0.084 \pm 0.009 \mu\text{m}^2$  vs.  $0.076 \pm 0.007 \mu\text{m}^2$ ;  $p = 0.68$ ) as well as in the mitochondrial cristae extension, a reliable morphological parameter of mitochondrial function (Nielsen et al., 2017) ( $1.206 \pm 0.055$  vs.  $1.233 \pm 0.082$ ;  $p = 0.44$ ). The mitochondrial size and the cristae extension were instead significantly higher in perinuclear mitochondria of O-T vs. O-S ( $0.137 \pm 0.017$  vs.  $0.055 \pm 0.004 \mu\text{m}^2$ ,  $p < 0.001$  and  $1.615 \pm 0.111$  vs.  $1.286 \pm 0.069$ ,  $p = 0.01$ , respectively).

The significant increase of mitochondrial density in trained mice is in agreement with data indicating that in aged skeletal muscle, the decreased mitochondrial density as well as the reduced respiratory

activity are mostly related to decreased physical activity (Rasmussen et al., 2003; Russ and Kent-Braun, 2004; Distefano et al., 2018). Therefore, it can be hypothesized that the higher density of both intermyofibrillar and perinuclear mitochondria as well as the increased size and cristae extension of the latter may lead to increased ATP availability (Glancy et al., 2015; Glancy and Balaban, 2021; Willingham et al., 2021).

These findings also suggest that the perinuclear mitochondrial population is more sensitive to the stimulating effect of physical training in the skeletal muscle of old mice.

In our model, ribosome proteins were significantly downregulated after training (Figure 4A; Supplementary Table



S3), suggesting a general reduction/delay of protein synthesis, although some proteins/pathways may be specifically upregulated as shown by analyses of DEPs. Indeed, these data are consistent with the observation that reduced protein synthesis allows to increase lifespan (Tavernarakis, 2007; 2008), since it may represent a saving mechanism reducing the risk of accumulating damaged proteins (Hipkiss, 2007), thus avoiding the overloading of the protein quality control system, and the development of a pro-aging environment (López-Otín et al., 2013).

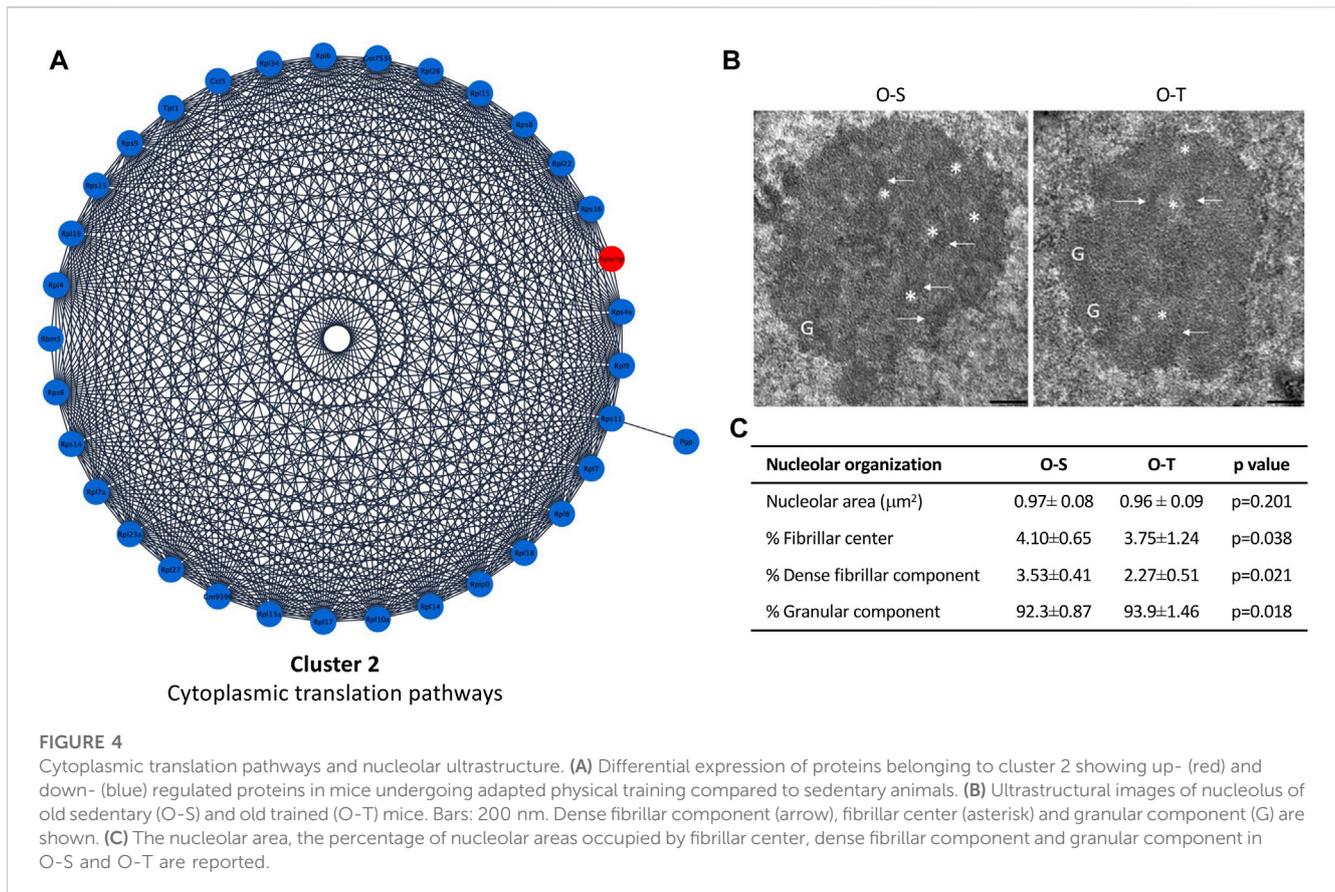
Moreover, protein synthesis is one of the most energy-consuming cellular processes (Lane and Martin, 2010), therefore depletion of ribosomal proteins and of translational factors can increase stress resistance allowing the switch to the selective production of proteins required not only to sustain muscle contraction, but also to foster cellular maintenance, repair, and turnover pathways (Gonskikh and Polacek, 2017).

To assess whether an adapted physical exercise affects protein synthesis by influencing production and/or export of ribosomal subunits, morphometrical evaluation of the nucleolar area as well as the percentage of nucleolar areas occupied by fibrillar center (FC, where rDNA is located), dense fibrillar component (DFC, site of transcription and processing of rRNA, and assembly of the ribosomal subunits) and granular component (GC, site of maturation and storage of ribosomal subunits) was carried out (Figure 4B). No significant difference in nucleolar area was found

in O-T vs. O-S. On the contrary, the percentage of the nucleolar areas occupied by FC and DFC was significantly lower in O-T vs. O-S (Figure 4C), thus suggesting a decreased production of pre-ribosomes and, hence, decreased ribosomal protein requirement. Moreover, the observed downregulation of ribosomal proteins, concomitant with the enlargement of GC (Figure 4C), suggests a delay in the processing and release of the pre-ribosomes (Jamison et al., 2010). Evidence demonstrated that high rates of rDNA transcription may lead to genome instability and to accumulation of rDNA damage, which are typical features of cellular aging (Tsekrekou et al., 2017). Therefore, our results are consistent with the occurrence of protective mechanisms in trained old mice.

### 3.5 Effects of adapted physical training on the regulation of skeletal muscle contraction

In the present study, adapted physical training in old mice was associated to remarkable modifications in the contractile muscle system (Figure 5A) characterized by both a reduction of Myh7 ( $\log_2$ Fold change O-T/O-S = -0.7131) and Tnnt1 ( $\log_2$ Fold change O-T/O-S = -0.623) and an increase of Myoz2 ( $\log_2$ Fold change O-T/O-S = 0.8695), Myl1 ( $\log_2$ Fold change O-T/O-S = 0.9223), Myl2 ( $\log_2$ Fold change O-T/O-S = 0.7101) and Myl3 ( $\log_2$ Fold change



O-T/O-S = 1.1973) (Supplementary Table S3). These findings indicate a reorganization of the skeletal muscle contraction system in O-T compared with O-S mice.

According to data in the literature, in response to physiological and/or pathological signals, muscle fibers can reforge their phenotype to the environmental demand (*i.e.*, switch from fast to slow fibers and vice versa) (Bassel-Duby and Olson, 2006; Talbot and Maves, 2016). During aging a fast to slow fiber transition can be observed (Ohlendieck, 2011) and exercise may cause changes in the composition of muscle fiber types depending on the type/frequency of exercise and on the type of muscle (Lee et al., 2022).

Interestingly, in our experimental model, immunolabeling for the heavy chain of skeletal fast fiber myosin (Supplementary Figure S1) revealed no statistically significant difference in the percentage of fast myofibers in O-S vs. O-T (98.28  $\pm$  1.21 vs. 99.75  $\pm$  0.25,  $p = 0.47$ ), with a negligible percentage of slow fibers in both mice groups.

Moreover, it is well known that aging is characterized by a progressive reduction in both muscle mass and myofiber size and that physical training can prevent muscle functional deficiency (Zancanaro et al., 2007). In the present study, to determine the size of myofibers, the sections of GAST muscle were immunolabelled with laminin, a basal lamina protein that individually outlines myofibers, and whose expression was not affected by adapted physical training (Supplementary Table S2). We observed an increase of the size of myofibers in O-T vs. O-S (Figure 5B), confirmed by the minimum Feret's diameter distributions (Figure 5C) showing that more myofibers were in

higher size classes in O-T vs. O-S with mean values of 26.74  $\pm$  0.47  $\mu\text{m}$  vs. 24.23  $\pm$  0.41  $\mu\text{m}$  ( $p < 0.001$ ).

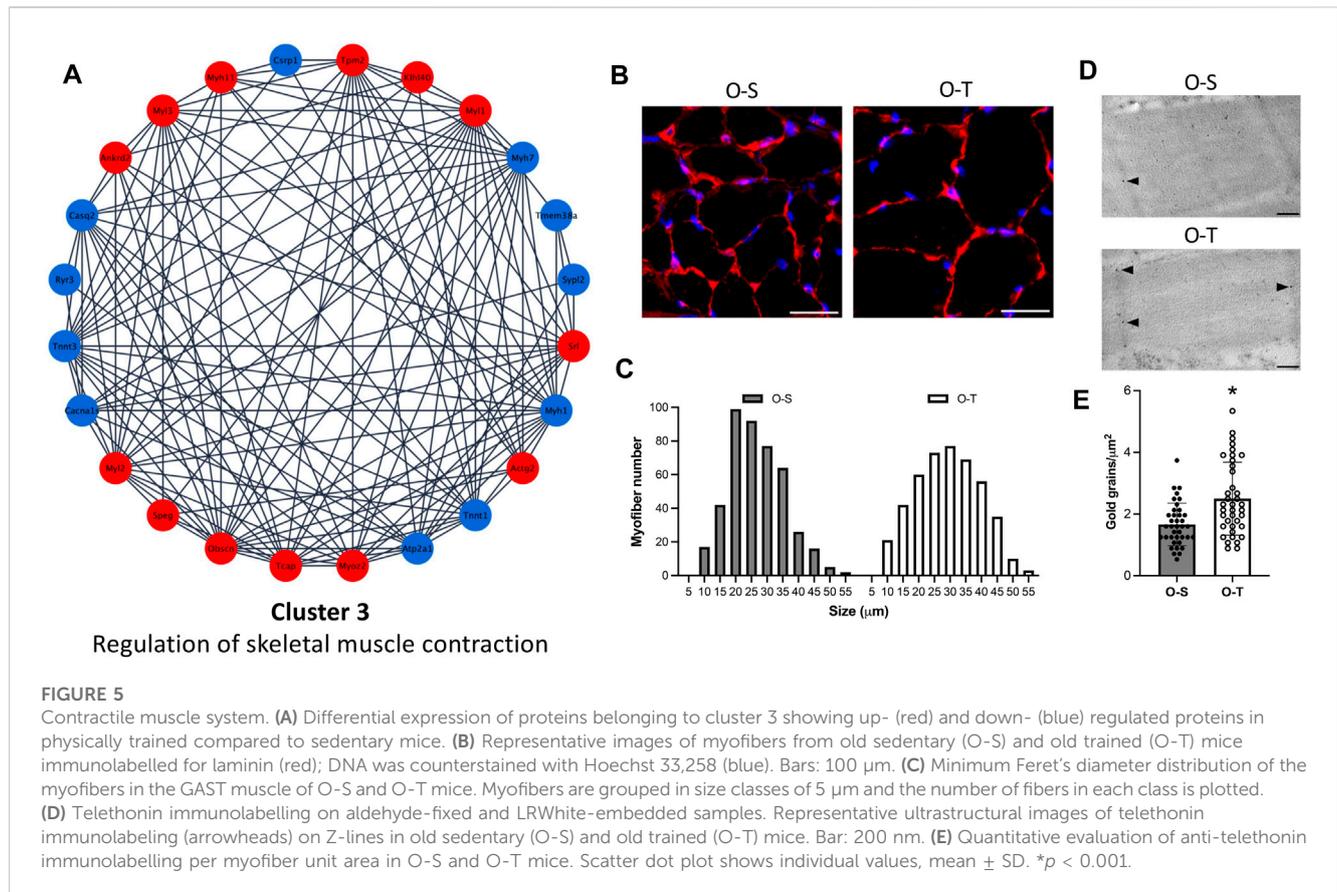
It is well known that increase of fiber cross-sectional area is accompanied by a proportional increase of myofibrillar proteins that are essential for the generation and propagation of mechanical forces and are under the control of regulatory components (*e.g.*, titin, obscurin and telethonin).

Interestingly, regulatory proteins (*i.e.*, ankyrin, obscurin, telethonin) were all upregulated in O-T mice (log<sub>2</sub>Fold change O-T/O-S = 1.5712, 0.6121, and 1.8802, respectively) (Figure 5A; Supplementary Table S3) and immunolabelling confirmed the upregulation of telethonin in O-T compared to O-S mice (Figures 5D, E).

Data from the present study indicate that adapted physical training started at old age is able to counteract the age-associated decrease of myofibers' size, a typical feature of skeletal muscle aging (Zancanaro et al., 2007), and to upregulate the expression of regulatory proteins supporting sarcolemmal integrity (Subramaniam et al., 2022). Instead, physical training did not affect the percentage of slow fibers in GAST.

### 3.6 Effects of adapted physical training on chaperone-dependent protein refolding

Among chaperon proteins, heat shock proteins (HSPs), in stress conditions, are rapidly induced to stabilize misfolded or denatured



polypeptides (Stetler et al., 2010), but also in normal conditions are involved, for instance, in folding and maturation of synthesized proteins. Hspe1 and Hspa9 were upregulated ( $\log_2$ Fold change O-T/O-S = 0.5995 and 0.7155, respectively) after training (Figure 6A; Supplementary Table S3).

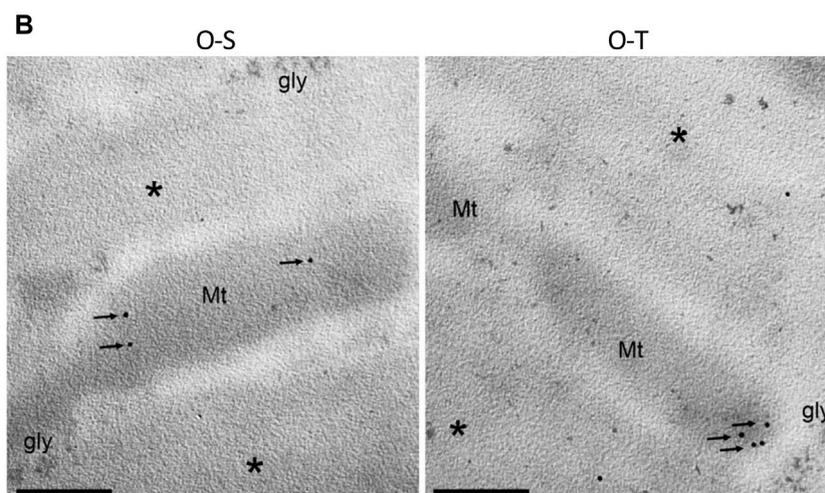
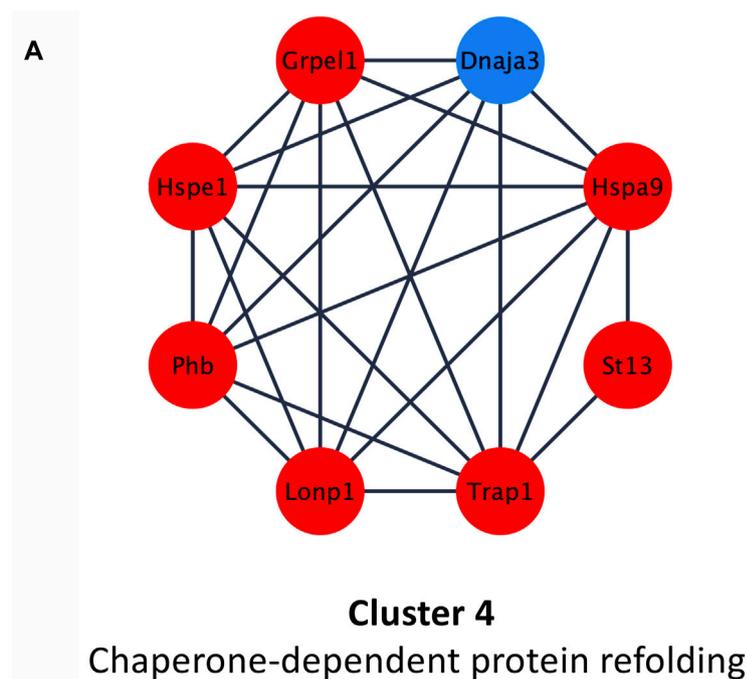
It is known that during aging there is an increased oxidative stress which determines damages to biological macromolecules (e.g., protein carbonylation). Kayani and collaborators, for instance, demonstrated that muscles of adult and old mice overexpressing Hspe1/Hsp10 showed a lower susceptibility to contraction-induced damages, because this molecule, providing a protection against ROS, reduces the mitochondrial protein carbonyl content (Kayani et al., 2010).

In agreement with these observations, the upregulation of Hspe1 and of several mitochondrial components (e.g., proteins belonging to cluster 1), demonstrated in our experimental model, may contribute to preserve an efficient mitochondrial function in O-T.

In addition, adapted physical training determined an upregulation of Hspa9 (also named Grp75 or mortalin or mtHsp70). This chaperone is mainly localized into mitochondria, and its expression correlates with increased mitochondrial activity (Ornatsky et al., 1995). Moreover, it is involved in several functions such as: i) protein transfer from nucleus to mitochondria (Dolezal et al., 2006), ii) energy metabolism (Xu et al., 2009), iii) proteasomal degradation, iv) immune response (Pilzer and Fishelson, 2005) and

v) life span extension (Kaula et al., 2000). It has been shown that mortalin is over-expressed under various stress conditions (e.g., caloric restriction, low doses of ionizing radiation) (Ornatsky et al., 1995; Merrick et al., 1997; Sadekova et al., 1997) and also after physical exercise, suggesting that Hspa9/mortalin exerts a cytoprotective effect counteracting ROS accumulation (Liu et al., 2005; Xu et al., 2009). Moreover, the increased expression of Hspa9 favors mitochondrial protein biogenesis in synergy with the upregulation of co-chaperones (e.g., Grpel) and of LONP1 (Wiedemann and Pfanner, 2017; Shin et al., 2021), a serine protease that mediates the selective degradation of misfolded/unfolded/oxidized proteins (De Gaetano et al., 2020; Gibellini et al., 2020), possibly reducing mitochondrial proteotoxicity. These data are in agreement with the upregulation of Grpel ( $\log_2$ Fold change O-T/O-S = 0.722) and Lonp1 ( $\log_2$ Fold change O-T/O-S = 1.1642) in trained mice (Figure 6A; Supplementary Table S3).

MtHsp70 immunolabelling per mitochondrion (Figure 6B) did not reveal significant differences between the two experimental groups ( $20.70 \pm 2.88$  vs.  $16.95 \pm 4.71$   $p = 0.5$ ). This result seems to contradict the proteomic data (i.e., upregulation of this proteins in O-T vs. O-S). This apparent discrepancy is because immunolabelling considers the protein quantity per mitochondrion, while MS-based quantitative proteomics data refer to the whole muscle in which the mitochondrial density is significantly increased in O-T vs. O-S (Figure 3C).



**FIGURE 6**

Chaperone-dependent protein refolding. **(A)** Differential expression of proteins belonging to cluster 4 showing up- (red) and down- (blue) regulated proteins in trained compared to sedentary mice. **(B)** mtHsp70 immunolabeling on aldehyde-fixed and LRWhite-embedded samples. Representative ultrastructural images of mtHsp70 immunolabeling (arrows) on mitochondria (Mt) in old sedentary (O-S) and old trained (O-T) mice. \*myofibrils; gly, glycogen deposit. Bar: 200 nm.

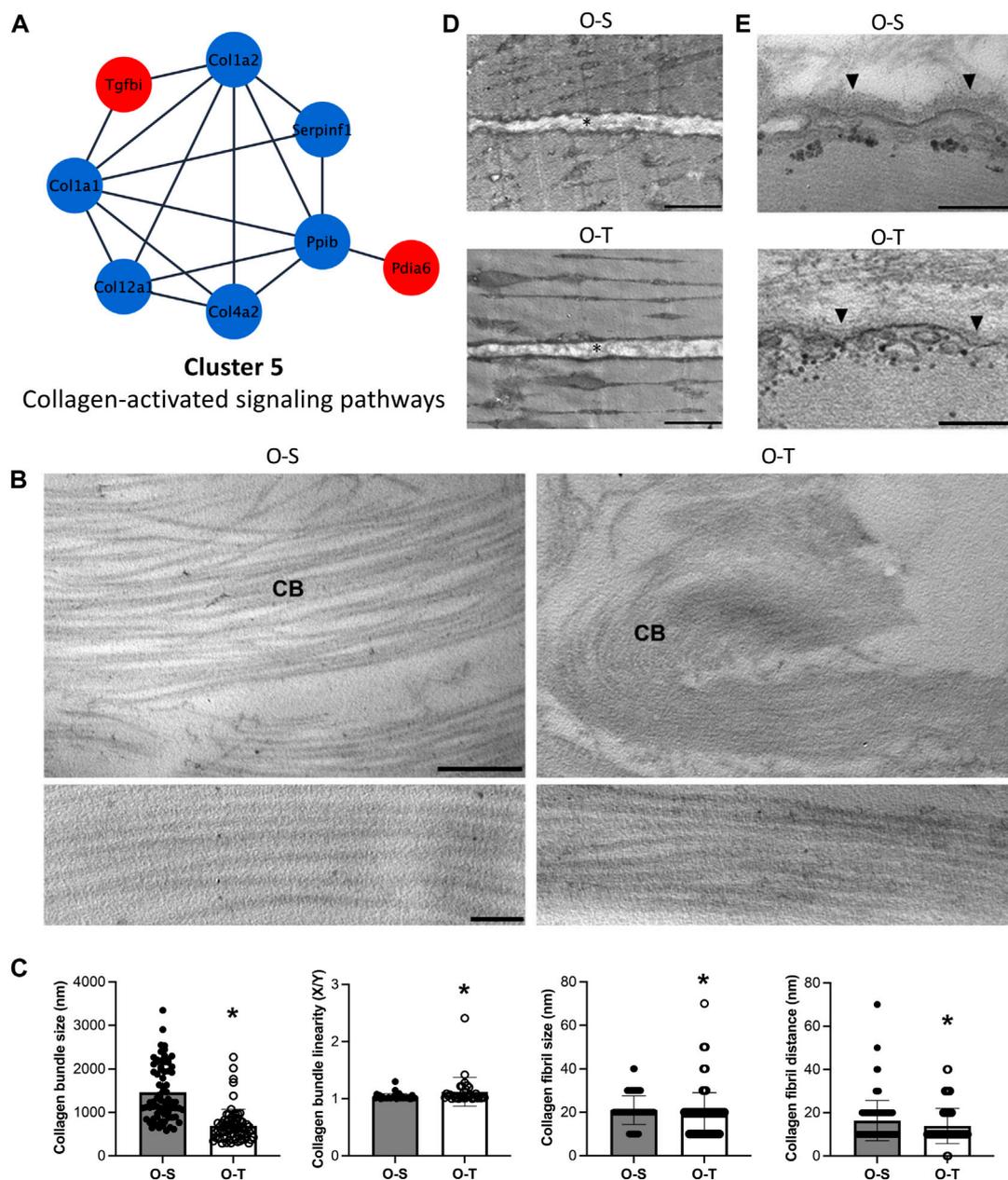
### 3.7 Effects of adapted physical training on collagen-activated signaling pathways

Although intramuscular connective tissue accounts for 1%–10% of skeletal muscle, the amount as well as orientation/organization of ECM is important to regulate the stiffness of the tissue and to transmit forces for optimal contraction (Kjaer, 2004).

It is therefore intriguing to observe that adapted physical training in old mice significantly reduced some collagen types (e.g., Col1a2, Col1a1, Col4a2, and Col12a1) ( $\log_2$ Fold change

O-T/O-S =  $-0.8333$ ,  $-0.7198$ ,  $-0.9642$ , and  $-0.8735$ , respectively) (Figure 7A; Supplementary Table S3).

Collagen type I is the most represented collagen within skeletal muscles, whereas type IV is typically localized in the basement membranes adjacent to the sarcolemma. Interestingly, collagen type XII is highly expressed in muscles where it interacts with the surface of collagen type I fibrils regulating collagen fibril spacing and assembly during fibrillogenesis (Izu and Birk, 2023). The general reduction of all these collagens after exercise could be due to lower synthesis of these molecules and/or to increased susceptibility to



**FIGURE 7**

Collagen proteomics and ultrastructure. **(A)** Differential expression of proteins belonging to cluster 5 showing the up- (red) and down- (blue) regulated proteins in trained compared to sedentary mice. **(B)** Representative ultrastructural images of the perimysium of old sedentary (O-S) and old trained (O-T) GAST muscle. CB, collagen bundle. Bar: 1  $\mu$ m (upper panels), bar: 100 nm (lower panels). **(C)** Morphometric analysis of collagen bundles and collagen fibrils. X/Y, ratio between the real length of the collagen bundle profile and the corresponding linear length. \* $p < 0.05$ . **(D)** Representative ultrastructural images of the endomysium (\*) in old sedentary (O-S) and old trained (O-T) GAST muscle. Scatter dot plots show individual values, mean  $\pm$  SD. **(E)** The basement membrane (arrowhead) covering the myofiber sarcolemma. Bars: 500 nm.

proteolytic activities and turnover (e.g., MMP and cathepsins) as already observed (Rullman et al., 2009; Guzzoni et al., 2018).

The ECM of the perimysium is organized in a network of collagen bundles (Figure 7B) that appeared not only significantly thinner ( $p < 0.001$ ) and less linear ( $p = 0.004$ ) in O-T vs. O-S, but also formed by collagen fibrils significantly smaller ( $p = 0.0018$ ) and closer to each other ( $p = 0.003$ ) in O-T vs. O-S (Figure 7C). These changes may reflect the lower presence of collagen type I and type

XII after training. Therefore, the increase of ECM components, associated with the age-dependent enlargement of interfibrillar spacing (Lofaro et al., 2021), is counteracted/limited by adapted physical training in agreement with the improved turnover and assembly of ECM components (Rullman et al., 2009; Guzzoni et al., 2018). The association of skeletal muscle aging with significant decrease in collagen fiber “tortuosity” (Stearns-Reider et al., 2017) and accumulation of extensively cross-linked collagen as cause of

increased muscle stiffness (Wood et al., 2014; Csapo et al., 2020) has been widely demonstrated. Therefore, the collagen bundle rearrangement in O-T mice, characterized by significantly thinner and more tortuous collagen bundles composed of smaller and more compact fibrils, may be a positive effect counteracting typical hallmarks of muscle aging (Wood et al., 2014).

In both O-S and O-T, the endomysium is a network of collagen fibrils connected to the basement membrane. Morphometrical analyses of the endomysium thickness, performed measuring the distance between two adjacent longitudinally sectioned myofibers, showed no significant difference in O-S vs. O-T (Figure 7D). The basement membrane, which covers the surface of each myofiber as an electrodense sheath, was significantly thinner in O-T vs. O-S ( $30.32 \pm 0.58$  nm vs.  $41.88 \pm 1.07$  nm;  $p < 0.001$ ) (Figure 7E). Within this context, the reduction of type IV collagen in O-T, observed by proteomic analysis and consistent with a thinner basement membrane found by ultrastructural morphometry, indicates that physical training positively influenced the ECM remodeling (Kjaer, 2004; Heinemeier et al., 2009; Hyldahl et al., 2015), as previously observed (Carmeli et al., 2005; Rullman et al., 2009; Ogasawara et al., 2014).

## 4 Conclusion

We are aware that this study shows some limitations: i) the results are related to GAST muscle without comparison with other muscle types (e.g., soleus); ii) proteomic data and morphometric analyses do not provide functional information; iii) we considered male mice, and therefore we cannot exclude the possibility that the effect of exercise on female mice may have a different impact on GAST muscle; iv) data provide a static picture of protein concentration and/or distribution at one point-time and do not capture the dynamic process of changes (i.e., how the protein increase or decrease over time); v) analyses did not focus on protein post-translational modifications which are known to affect protein behavior and characteristics (e.g., protein lifespan, solubility, and assembly).

However, this study has many strengths related to: i) use of different extraction buffer to improve the isolation of both soluble and insoluble proteins; ii) a label free quantification that allows to directly compare the peptide intensities between the samples since data are obtained under identical conditions using high resolution MS/MS; iii) use bioinformatic software to identify the protein-protein interaction of DEPs and the process/pathways in which DEPs are involved; iv) morphological and morphometric analyses which allow to visualize the ultrastructural changes and the localization of proteins in the two experimental conditions (i.e., O-S and O-T).

In summary, data indicate that the GAST muscle positively responds to adapted physical training started at old age, being capable: i) to enhance the size of myofibers and the expression of regulatory proteins supporting sarcolemmal integrity; ii) to increase the density of mitochondria and to modify the expression of mitochondrial components; iii) to reduce the expression of ribosomal proteins and to affect the nucleolar organization establishing protective mechanisms lowering the risk of accumulating damaged proteins; iv) to favor ECM

remodeling. Therefore, this study provides important insights on the targets of adapted physical training, which can be regarded as suitable benchmarks for future *in vivo* studies further exploring the effects of this type of physical activity by functional/metabolic approaches.

Finally, this study reinforces that an adapted physical training initiated in old life can counteract some markers of the aged skeletal muscle, underlining the importance to adopt a physically active behavior at any age. In addition, future studies focused on microvascular characteristics and transcriptome-wide profiling (e.g., mRNA, microRNA, and lncRNA) will allow to advance our current understanding of adapted physical training in old age.

## Data availability statement

The data presented in the study are deposited in the ProteomeXchange (PRIDE) repository, accession number: PXD045483.

## Ethics statement

The animal study was approved by Italian Ministry of Health—Ref.: 538/2015-PR. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

FBor, DQ, and CZ conceived and designed experiments; FDL, BC, and MAL, carried out experiments; FDL, BC, FBos, and FBor performed data curation; FBor and MM supervised the experiments; FBor, CZ, and DQ wrote and reviewed the article. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2023.1273309/full#supplementary-material>

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