Large increases in resistance training volume do not impair muscle hypertrophy or anabolic–catabolic molecular signaling in trained individuals

Running title: Resistance training volume and skeletal muscle adaptation

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Abstract

Skeletal muscle hypertrophy results from the integrated regulation of anabolic and proteolytic processes in response to mechanical loading. Although increases in resistance training (RT) volume are used to increase mechanical stress, it remains uncertain whether large and abrupt volume progressions could exceed muscle adaptive capacity by disrupting the balance between anabolic and catabolic signaling. The present study investigated whether a large increase in weekly RT volume (+120%) leads to impaired hypertrophic outcomes and intracellular regulatory responses compared with a modest increase (+20%). Twenty-five resistance-trained men and women (18–35 years old) completed an 8-week randomized, single-blind, within-subject unilateral intervention. Each participant trained both legs twice weekly, with one leg assigned to the large (VOL120) and the contralateral leg to the modest (VOL20) weekly volume progressions relative to habitual training volume. Vastus lateralis muscle cross-sectional area (mCSA) was assessed by ultrasonography before and after training. Muscle biopsies were obtained at baseline, post-intervention, and 24 h after the last session to quantify muscle fiber cross-sectional area (fCSA), satellite cell myonuclear content, and anabolic/catabolic signaling markers. Both protocols induced increases in mCSA over time (p<0.001), with no protocol vs. time interaction. No significant effects were observed for fCSA nor satellite cell number or myonuclear content. Additionally, molecular responses related to translational regulation and protein degradation were largely similar between protocols. A large abrupt increase in weekly resistance training volume (+120%) did not attenuate muscle hypertrophy relative to a modest increase (+20%) in resistance-trained individuals, and most acute and chronic molecular markers assessed likewise did not differ significantly between protocols.

NEW & NOTEWORTHY

A large, abrupt increase in weekly resistance training volume (+120% relative to habitual training) did not enhance muscle hypertrophy compared with a modest increase (+20%) in resistance-trained individuals. We found no evidence that the larger volume progression attenuated adaptation under the present experimental conditions. Acute and chronic molecular responses likewise did not differ significantly between protocols.

Keywords: exercise; muscle adaptation; protein synthesis, skeletal muscle fiber, training.

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Introduction

Skeletal muscle hypertrophy represents a complex adaptive process that emerges from the coordinated regulation of protein synthesis, protein degradation, and structural remodeling in response to repeated mechanical loading [1]. While the intrinsic regulatory capacity underlying such processes ultimately constrains the magnitude and sustainability of hypertrophic adaptations [1], progressive increases in loading during resistance training (RT) are commonly implemented to maintain adaptive responses across prolonged training periods [2, 3]. Among the variables used to manipulate loading, RT volume represents a practical means of experimentally increasing cumulative mechanical stress, particularly in resistance-trained individuals [4]. Consistent with this rationale, experimental and meta-analytical evidence has frequently reported greater hypertrophy following higher-volume RT protocols, supporting a dose-dependent relationship between volume and muscle growth [5-8]. More recently, meta-regression analyses suggest diminishing returns with increasing weekly set volume, yet whether an upper boundary exists beyond which additional volume attenuates further hypertrophic adaptation remains unresolved, particularly in resistance-trained individuals [9]. Notably, when examining studies conducted exclusively in resistance-trained participants, several investigations have reported comparable hypertrophic adaptations despite substantial differences in prescribed RT volume [10-13]. These heterogeneous findings raise the possibility that abrupt, high magnitude increases in training volume (i.e., implemented all at once rather than progressively) may challenge the muscle's adaptive capacity and attenuate hypertrophic responses rather than enhance them [14].

Understanding these heterogeneous hypertrophic outcomes requires integrating whole-muscle responses with the intracellular pathways that regulate translational efficiency, translational capacity, and protein breakdown. Activation of the mammalian target of rapamycin complex 1 (mTORC1) regulates translational efficiency and myofibrillar protein synthesis through downstream targets such as p70S6K and 4E-BP1 [1]. In parallel, ribosome biogenesis also contributes to translational capacity [15], while satellite cell–mediated myonuclear accretion supports sustained fiber growth by expanding the cellular machinery required for elevated protein synthesis [16, 17]. Indeed, training volume has been shown to modulate several of these processes, including satellite cell responses and ribosome biogenesis [18]. In contrast, the effects of chronic, progressive increases in RT volume on canonical anabolic signaling pathways remain insufficiently characterized in resistance-trained humans, as most available evidence

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derives from acute studies employing relatively modest exercise volumes [7, 19-21]. Hypertrophic adaptations are also influenced by the regulation of muscle protein breakdown, which contributes to net protein balance and structural remodeling. Calpains and the ubiquitin–proteasome system play complementary roles in the degradation of damaged or misfolded proteins and cytoskeletal turnover in skeletal muscle [22, 23]. Experimental evidence from animal models indicates that excessive mechanical loading or insufficient recovery between resistance exercise bouts can sustain proteolytic signaling without proportional increases in protein synthesis, thereby impairing hypertrophic adaptations [24, 25]. In this sense, it is possible that large and abrupt increases in RT volume shift the balance between anabolic and catabolic processes toward a proteolytic-dominant state, effectively uncoupling intracellular signaling responses from morphological adaptation.

Collectively, these observations suggest a non-linear relationship between RT volume and muscle hypertrophy, whereby volume-dependent gains occur up to a threshold beyond which further increases in mechanical stress exceed the adaptive capacity of skeletal muscle. Under this framework, moderate increases in volume may optimize adaptive signaling, whereas excessive volumes may compromise net protein balance and tissue remodeling, consistent with an inverted "U"-shaped volume–hypertrophy relationship [14, 26]. Evidence consistent with this model has emerged from individual-level analyses in resistance-trained humans. Inspection of the individual responses reported by Scarpelli et al. [27] suggests that limbs exposed to large volume progressions (83–120% above baseline) showed smaller increases in vastus lateralis muscle cross-sectional area (mCSA) than contralateral limbs subjected to a more modest increase (~20%). Accordingly, Haun et al. [28] reported that, in resistance-trained men (n = 31), large weekly increases in back-squat volume over 6 weeks resulted in no increases in vastus lateralis muscle thickness or type I and type II fiber cross-sectional area (fCSA). However, findings from both studies were not derived from a randomized controlled design specifically intended to test whether large, abrupt increases in RT volume directly attenuate hypertrophy or to determine whether such effects are accompanied by coordinated alterations in anabolic and proteolytic signaling pathways.

Therefore, the primary aim of the present study was to determine whether a large and abrupt increase in weekly RT volume (+120%; VOL120) attenuates hypertrophic adaptations at both the whole-muscle and muscle fiber levels compared with a protocol involving a modest volume increase (+20%; VOL20) in resistance-trained individuals.

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Additionally, we examined acute and chronic responses of key anabolic and proteolytic biomarkers under these distinct volume-progression protocols. We hypothesized that the larger volume progression would exceed muscle adaptive capacity, leading to attenuated hypertrophy and molecular responses consistent with a shift toward catabolic regulation relative to the modest progression.

Methods

Ethical approval and participants

Healthy young men and women aged 18–35 years were recruited. Eligibility criteria included a minimum of 1 year and a maximum of 5 years of consistent lower-limb RT experience, with a habitual quadriceps training volume ranging from 12 to 20 sets per week, including leg press and leg extension exercises. Baseline weekly volume was determined via structured interviews in which participants reported the number of quadriceps-focused exercises routinely performed, sets per exercise, and weekly training frequency. These interviews were conducted by an experienced researcher, and participants were eligible only if they reported consistent lower-limb resistance training experience and regular inclusion of leg press and leg extension exercises within the predefined habitual volume range. Participants were excluded if they reported chronic use of anabolic steroids, vitamin or ergogenic supplements, or anti-inflammatory medications, or if they had any musculoskeletal injury or neuromuscular disorder that could interfere with participation in the prescribed training protocols. All participants provided written informed consent after receiving a detailed explanation of the study procedures, risks, and benefits. The study was approved by the Institutional Review Board (approval no. 6.642.786), conducted in accordance with the Declaration of Helsinki, and registered in the Brazilian Registry of Clinical Trials (RBR-2f836zh).

Experimental design

This study employed a randomized, single-blind, within-subject unilateral design with repeated measures. This approach was selected to minimize inter-individual variability, allow each participant to serve as their own control, and increase statistical sensitivity for detecting training-induced adaptations while reducing the required sample size (MacInnis et al., 2017). Each lower limb represented an independent experimental unit and was randomly allocated to one of two resistance training (RT) volume-progression protocols: a modest increase in weekly volume (+20%; VOL20) or a large,

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abrupt increase (+120%; VOL120) both relative to each participant's habitual pre-study weekly set volume, and maintained throughout the 8-week training period (16 sessions). Vastus lateralis mCSA was the pre-specified primary outcome of the trial. Secondary outcomes included muscle fibre morphology, satellite cell and myonuclear content, as well as molecular markers associated with anabolic and proteolytic pathways, which were included to provide a broader mechanistic characterization of the two volume-progression protocols. Accordingly, the molecular analyses should be interpreted cautiously, particularly given the number of molecular outcomes assessed.

Figure 1 provides an overview of the experimental timeline. Briefly, vastus lateralis mCSA was assessed by ultrasonography at baseline and then reassessed 72 h later (pre-biopsy) to determine the typical error and coefficient of variation of the ultrasound method. After completion of the training intervention, mCSA was reassessed 96 h after the final training session, followed by bilateral muscle biopsies to evaluate chronic adaptations (Pre vs. Post). Participants then completed an additional training session (session 17), after which muscle biopsies were obtained 24 h later to assess acute molecular responses (0 h vs. 24 h). During this final session, each limb performed the same RT protocol to which it had been allocated throughout the intervention.

INSERT FIGURE 1 HERE

Sample size estimation

Sample size estimation was performed using a simulation-based power analysis (10,000 simulations) for a two-way repeated-measures design, implemented with the Superpower package in R [29]. Statistical power was set at 80% with a two-sided α level of 0.05. Based on previous unilateral training studies, a correlation coefficient of $r = 0.78$ was assumed for changes in vastus lateralis muscle mCSA between limbs. Although prior work investigating training volume reported effect sizes of approximately $d = 0.75$, a more conservative effect size ($d = 0.50$) was adopted to account for differences in experimental design. Accordingly, a Cohen's f value of 0.20 was assumed for the interaction effect. Under these assumptions, the estimated minimum sample size was 24 participants. To account for potential attrition, recruitment exceeded the minimum number required by the power analysis.

Resistance training protocols

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Participants completed two supervised RT sessions per week for 8 weeks, with all sets performed to concentric muscle failure within a target repetition range of 9–12 repetitions and a fixed 2-min inter-set rest interval. Training loads were adjusted on a set-by-set basis: loads were increased if more than 12 repetitions were completed and reduced if fewer than 9 repetitions were achieved. Load reductions between sets were permitted when necessary to allow participants to remain within the prescribed 9–12 repetition range while continuing to perform sets to momentary concentric failure. These adjustments were not intended to avoid failure, but rather to maintain participants within the target repetition range while still reaching momentary concentric failure across the session. Both RT protocols were individualized based on each participant's habitual quadriceps training volume prior to study entry. Baseline weekly volume was determined via structured interviews in which participants reported the number of exercises, sets per exercise, and weekly training frequency for the quadriceps muscles. Weekly volume was calculated as the product of exercises per session, sets per exercise, and weekly frequency. Based on this value, each limb was assigned to either a modest volume increase (+20%; VOL20) or a large, abrupt volume increase (+120%; VOL120), which was maintained throughout the intervention period. The exercises performed in each session were the 45° leg press and the leg extension (Effort NKR; Nakagym, São Paulo, Brazil). Both protocols were performed concurrently within the same participant, with each leg assigned to one training protocol. Limb allocation was randomized using block randomization stratified by limb dominance (right/left). The exercise order was kept constant across all sessions (45° leg press followed by leg extension). To minimize potential order effects between protocols, the limb corresponding to each protocol alternated as the first limb trained across sessions. Volume load (load × sets × repetitions) was recorded for all sessions.

Participants were instructed to refrain from any unsupervised quadriceps resistance training or high-intensity lower-limb exercise throughout the study period. Unsupervised training targeting the upper body, gluteal, hamstring, and calf muscles was permitted, with participants explicitly instructed to avoid exercises likely to substantially load the quadriceps (e.g., squat variations, lunges, deadlifts).

Dietary adherence

To minimize dietary confounding, participants were instructed to maintain their habitual dietary intake throughout the study and to refrain from using nutritional supplements other than that provided by the researchers. Immediately after each RT

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session throughout the intervention, participants ingested 30 g of whey protein (Adaptogen®, São Paulo, Brazil) to standardize post-exercise protein intake and reduce between-participant variability in the acute muscle protein synthetic response to exercise [30-32]. Dietary adherence was assessed by a self-reported 24-h dietary records using MyFitnessPal software (http://www.myfitnesspal.com), which presents good relative validity for tracking energy and macronutrient consumption [33]. Dietary recall was collected before each of the 3 muscle biopsy samples. Participants were instructed on how to properly report all food items and their respective portion sizes consumed for each meal. An experienced researcher inserted each food item into the software, which provided information regarding total energy consumption, and total grams for all macronutrients (i.e., proteins, fats, and carbohydrates).

Ultrasound assessment

Vastus lateralis mCSA was obtained through B-mode ultrasound (US) images using a 7.5 MHz linear probe (MySono U6, Samsung), following the procedures validated by Lixandrão et al. [34]. Participants were instructed to refrain from vigorous physical activity for at least 72 h before each assessment. Before the assessments, participants laid down for 15 minutes to ensure fluid redistribution. Then, the assessor measured the distance between the greater trochanter and the lateral epicondyle of the femur to determine the length of the right and left femur. The point corresponding to 50% of the length was marked with a dermographic pen as a reference point for image acquisition. From the 50% point, the skin was marked in the medial and lateral directions every 2 cm to guide the displacement of the probe in the sagittal plane, parallel to the long axis of the femur. Water-soluble transmission gel was applied to ensure acoustic coupling of the US probe without compressing the epidermis. To obtain the mCSA, the US probe was displaced in the sagittal plane, starting at the alignment point of the upper edge of the probe with the most medial skin mark (over the rectus femoris muscle) and ending on the lateral side of the thigh. Then, the images were opened in the software Power Point (Microsoft, USA) and manually rotated to reconstruct the entire fascia of the vastus lateralis muscle to allow panoramic visualization of the entire mCSA [35]. The reconstructed image was opened in ImageJ software, and the “polygonal” function was used to circle and measure the CSA of the vastus lateralis, excluding the underlying muscle fascia and bone tissue as much as possible. The typical error and the coefficient of variation were 0.68 cm^2 and 2.31%, respectively. Fascicle length and pennation angle

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of vastus lateralis were measured at the same time and site of the mCSA acquisition, with the probe placed longitudinally to the muscle belly. The PA was defined as the angle between the intersection of a fascicle and deep aponeurosis. Fascicle length was defined as the distance from fascicle origin in the deep aponeurosis to insertion in the superficial aponeurosis. The mean value of three images was used to determine pennation angle and fascicle length using the "Angle" tool [36] and "Straight" tool [37], respectively, of the ImageJ software (1.50b). The typical error and the coefficient of variation for pennation angle and fascicle length were 1.95° (8.74%) and 0.35 cm (4.87%) respectively. Finaly, to reduce assessment bias, ultrasound assessors were blinded to limb protocol during image acquisition and subsequent analyses (muscle cross-sectional area, pennation angle, and fascicle length). Participants were instructed and repeatedly reminded not to disclose limb allocation.

Muscle biopsies

Muscle tissue samples were obtained from the vastus lateralis by percutaneous needle biopsy performed by an experienced medical professional. The skin over the biopsy site was cleaned with an antiseptic solution, and local anesthesia was administered subcutaneously (2–3 mL of 1% lidocaine). A small incision was made with a sterile scalpel, and a Bergström needle with manual suction was inserted to a depth of approximately 4 cm to obtain ~100 mg of muscle tissue. Immediately after collection, samples were carefully cleaned of visible blood and connective tissue and subdivided for downstream analyses. Approximately 70–80 mg of tissue was placed into pre-labeled cryotubes for protein and enzymatic activity analyses. An additional 20–30 mg was embedded in optimal cutting temperature (OCT) compound with fibers oriented perpendicular to the cutting surface and rapidly frozen in isopentane cooled with liquid nitrogen for immunohistochemical analyses. All samples were snap-frozen in liquid nitrogen and stored at −80°C for further processing.

Immunohistochemical analyses

The OCT-embedded samples were sectioned at 7 µm using a criostato (Leica Biosystems, Buffalo Grove, IL, USA), mounted on slides, and stored at -80°C until batch analysis muscle fiber cross-sectional area (fCSA), myonuclei, and satellite cell (SC) number. All samples from a given participant were processed simultaneously to minimize inter-assay variability. Sections were air-dryed (2 h) and fixed with ice-cold acetone at -

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20°C (5 min). Sections were incubated with 3% H2O2 (10 min), followed by an autofluorescence-quenching reagent (TrueBlack®, cat. no. 23007; Biotium, Fremont, CA, USA) (1 min) and then blocked with a solution of 5% goat serum, 2.5% horse serum, and 0.1% Triton X-100 (1 h). Subsequently, endogenous biotin and streptavidin binding sites were blocked using a Streptavidin/Biotin Blocking Kit (15 min each) before overnight incubation at 4°C with a primary antibody cocktail containing: mouse anti-Pax7 IgG1 (1:20; Supernatant; Developmental Studies Hybridoma Bank; Iowa City, IA, USA) + rabbit anti-dystrophin (1:100; cat. no. GTX57970; GeneTex, Irvine, CA, USA) + mouse anti-MHC I (1:100; BA-D5; RRID: AB_2235587; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) in 2.5% horse serum/PBS. Notably, the same staining protocol has been used prior by our laboratory and is available in an open access journal article elsewhere [38]. The following day, sections were rinsed and incubated with biotin-SP-conjugated goat anti-mouse IgG1 (1:1000; Jackson ImmunoResearch; West Grove, PA, USA) (90 min), followed by incubation in a secondary cocktail containing streptavidin-HRP (SA-HRP) (1:500; cat. no. S-911; Thermo Fisher Scientific) + goat anti-rabbit Alexa Fluor 488 (1:250; Vector Laboratories; Burlingame, CA, USA) + goat anti-rabbit Alexa Fluor 647 (1:250; cat. no. A-21242; Thermo Fisher Scientific) (1 h). Tyramide Signal Amplification (TSA) Alexa Fluor 594 (1:200; cat. no. B-40957; Thermo Fisher Scientific) was applied (20 min), followed by DAPI (1:10,000) (10 min). Finally, slides were mounted in PBS/glycerol (1:1) and digital images (10× and 20× objectives) were captured (Zeiss Axio Imager.M2). SC analysis (Pax7+/DAPI+), fiber typing, fCSA, and myonuclei were quantified manually and via MyoVision software [39]. Only regions free of freezing artifacts were analyzed, and an average of ~540 fibers per sample were included for quantification. A list of antibodies utilized for experiments are provided in Supplementary Material.

Muscle tissue homogenization and protein extraction

Approximately 20 mg of whole muscle tissue was placed in 1.7 mL microcentrifuge tubes with 300 µL of a general cell lysis buffer (cat. no. 9803; Cell Signaling Technology, Danvers, MA, USA). The samples were homogenized using hard plastic pestles and centrifuged at 500 × g at 4°C (5 min). The supernatants were transferred to a new set of microtubes. Approximately 20 µL of the resulting cell lysates were used to determine total protein concentrations using a commercially available bicinchoninic acid (BCA) protein assay kit (cat. no. 23227; Thermo Fisher Scientific) and

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a spectrophotometer (BioTek Synergy H1, Winooski, VT, USA), following the manufacturer's instructions.

Western blotting

The homogenized lysates were added to 4× Laemmli buffer and deionized water (diH2O) at a concentration of 1 µg/µL. The solutions were then denatured at 100°C (5 min) before being stored at -80°C until analysis. Next, the prepared samples (15 µL) were loaded onto 4–15% SDS-polyacrylamide gels (cat. no. 5671085, Criterion TGX; Bio-Rad Laboratories, Hercules, CA, USA) and subjected to electrophoresis at 180 V (50 min) with pre-made 1× SDS-PAGE running buffer (VWR-Avantor, Radnor, PA, USA). The proteins were transferred to pre-activated polyvinylidene difluoride membranes (cat. no. 1620177; Bio-Rad Laboratories) at 200 mA (2 h). The membranes were stained with Ponceau S (10 min), rinsed with distilled water for 1 minute, air-dried for 30 minutes, and digitally photographed using a gel documentation system (ChemiDoc Touch; Bio-Rad Laboratories). This total protein staining was used for normalization of band density. For proteins with widely separated molecular weights, membranes were cut horizontally after transfer and Ponceau visualization, allowing different membrane sections to be probed sequentially with distinct antibodies. For proteins in which phosphorylated and total forms were assessed, quantification was obtained from the same membrane using stripping and reprobing with the respective antibodies. The membranes were then reactivated in methanol and blocked with 5% skimmed milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature (1 h). The membranes were incubated overnight at 4°C with the following antibodies at a dilution of 1:1000 in TBST with 5% bovine serum albumin (BSA): rabbit anti-MyHC (cat. no: 64038, Cell Signaling Technology); rabbit anti-polyubiquitin (cat. no: 3933, Cell Signaling Technology); rabbit 20S antibody cocktail (cat. no: PW8155, Enzo Life Sciences); rabbit anti-calpain-1 (cat. no: 2556, Cell Signaling Technology); rabbit anti-calpain-2 (cat. no: 70655, Cell Signaling Technology); rabbit anti-LC3 (cat. no: 2775, Cell Signaling Technology); rabbit anti-FOXO1 (cat. no: 9454, Cell Signaling Technology); rabbit anti-FOXO3 (cat. no: 24975, Cell Signaling Technology); rabbit anti-RPS6 (cat. no: 2217, Cell Signaling Technology); rabbit anti-4EBP1 (cat. no: 9644, Cell Signaling Technology); rabbit anti-phospho-4EBP1 (cat. no: 2855, Cell Signaling Technology); rabbit anti-p62 (cat. no: 5114, Cell Signaling Technology); mouse anti-SKIV2L2 (cat. no: sc-515828, Santa Cruz Technology); mouse anti-G3BP1 (cat. no: sc-365338, Santa Cruz Technology); rabbit

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anti-p70S6K (cat. no: 9234, Cell Signaling Technology); rabbit anti-phospho-p70S6K (cat. no: 2983, Cell Signaling Technology); rabbit anti-mTOR (cat. no: 5536, Cell Signaling Technology); rabbit anti-phospho-mTOR (cat. no: 2971, Cell Signaling Technology). The following day, the membranes were incubated with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (cat. nos.: 7076 and 7074; Cell Signaling Technology) (1 h). The membranes were then developed with a chemiluminescent substrate (Immobilon Forte, cat. no. WBLUF0500; MilliporeSigma, Burlington, MA, USA), and protein bands were visualized using a digital imaging system (ChemiDoc Touch, Bio-Rad Laboratories). Band densities were quantified using Image Lab software (Bio-Rad), and none of the bands presented unquantifiable overexposure values as detected using software parameters. Target protein signals were normalized to total protein staining (Ponceau). Samples from each participant, including both limb conditions and all relevant time points, were loaded on the same membrane to minimize the influence of inter-gel variability on within-subject comparisons. Gel-to-gel correction coefficients derived from the average Ponceau signal of each membrane were applied to account for differences in transfer intensity across membranes. Representative immunoblots are provided in the main manuscript (Figure 5).

Calpain and proteasome activity assays

Calpain and proteasome enzymatic activities were quantified using commercially available luminescence-based assay kits (cat. nos. G8502 and G8622; Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Briefly, aliquots of muscle lysate were diluted 1:10 with diH_2O and 25 µL were added to white 96-well plates containing the kit-specific reagent mixture. After a 10-min incubation at room temperature, luminescence was measured using a microplate luminometer (BioTek Synergy H1, Winooski, VT, USA). Signals were normalized to total soluble protein concentration determined by BCA assay and expressed as relative luminescence units (RLU) per µg of protein. All samples were analyzed in duplicate. The coefficients of variation between duplicates averaged 2.4% for calpain activity and 3.1% for proteasome activity.

Real-time quantitative PCR

Total RNA was isolated from frozen vastus lateralis muscle samples (~20 mg) using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Samples were manually

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homogenized with plastic pestles, and RNA was precipitated using an isopropanol-glycogen mixture. The resulting RNA pellets were washed twice with 75% ethanol, dried, and reconstituted in 30 μL of RNase-free water. RNA concentration and purity were determined in duplicate by spectrophotometry (NanoDrop Lite, Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using a commercial reverse transcription kit (qScript cDNA SuperMix; Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's protocol. Quantitative PCR was performed using SYBR Green chemistry (Quanta Biosciences) on a real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Reactions were carried out in a final volume of 20 μL containing gene-specific forward and reverse primers (final concentration: 2 μM each) and 25 ng of cDNA. All samples were analyzed in duplicate. Primer sequences are provided in Supplementary Material. Relative gene expression was calculated using the ΔΔCt [40], with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the reference gene. Results are expressed as fold change using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All statistical analyses were performed using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). Visual inspection of residual plots was also performed to verify model assumptions. Primary and secondary outcomes were analyzed using linear mixed-effects models to account for the within-subject unilateral design. Training protocol (VOL20 vs. VOL120), time (pre-intervention, post-intervention [chronic], 0 h and 24 h post-exercise [acute], when applicable), and their interaction were included as fixed effects, and participant was included as a random effect. Model fit was evaluated by inspection of residual distributions and influence diagnostics. Separate models were constructed for each dependent variable, including mCSA, fCSA (mixed, type I and II), myonuclear number, satellite cell content, protein expression/phosphorylation markers, proteolytic activity measures, and gene expression outcomes. Additionally, whenever different between-protocols baseline values were detected, the absolute changes for the dependent variables were compared using an analysis of covariance (ANCOVA). When a significant main effect or interaction was detected, pairwise comparisons were performed using Tukey-adjusted post hoc tests. An unpaired t test was used to compare the number of weekly sets and volume load (sets × repetitions × load [kg]) accumulated

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throughout the intervention between the experimental protocols. Descriptive data are presented as mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$.

Results

Participant flow and adherence

Participant characteristics by protocol are presented in Table 1. Of 138 volunteers screened for eligibility, 29 met the inclusion criteria and consented to participate, and their limbs were randomized to the two training protocols. Of these, 28 participants initiated the training intervention after baseline biopsies, and three withdrew during follow-up due to persistent muscle or joint pain. As the study used a within-subject unilateral design, these withdrawals occurred at the participant level and therefore both protocol assignments (VOL20 and VOL120) from each withdrawn participant were excluded simultaneously. Thus, 25 participants (18 men and 7 women) completed the intervention and were included in the final analyses. Across the 8-week intervention, a total of 400 supervised training sessions were scheduled, of which 394 were completed, corresponding to a mean adherence rate of 98.5%. A CONSORT flow diagram is shown in figure 2.

INSERT TABLE 1 HERE

INSERT FIGURE 2 HERE

Number of sets and volume load

The unpaired t-test revealed a significantly higher number of weekly sets for protocol VOL120 (32.8 ± 5.9) compared to protocol VOL20 (18.0 ± 3.3 sets; $p < 0.001$). The accumulated volume load throughout the intervention was significantly greater in VOL120 (237,125 ± 91,078 kg, $p < 0.0001$) than in VOL20 (138,416 ± 52,6931 kg).

Dietary adherence

Macronutrient distribution did not significantly change throughout the study period. Mean dietary intake corresponded to 21.8 ± 5.7% of total energy from protein, 49.8 ± 9.5% from carbohydrates, and 28.4 ± 18.1% from fat (detailed data not shown).

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Muscle cross-sectional area, fascicle length and pennation angle

Mixed model analysis indicated a significant main effect of time was observed for vastus lateralis mCSA ($F_{[1,72]}$ = 61.02, p < 0.001). Both protocols showed significant increases from pre- to post-training (Figure 3A). Additionally, a significant main effect of protocol was observed ($F_{[1,72]}$ = 9.71, p = 0.002). The VOL120 protocol showed higher mCSA value compared to the VOL20 protocol. No protocol vs. time interaction was observed for mCSA ($F_{[1,72]}$ = 1.70, p = 0.197). Additionally, no significant main effects or interactions were observed for fascicle length or pennation angle (all p > 0.05) (Figure 3B and 3C).

Muscle fiber cross-sectional area

Mixed model analysis indicated no significant effects of time, protocol, or protocol vs. time interaction were detected for mixed, type I and type II fCSA (all p > 0.05) (Figure 3D, 3E and 3F).

INSERT FIGURE 3 HERE

Satellite cells and myonuclei

Mixed model analysis showed no significant effects of time, protocol, or protocol vs. time interaction were observed for myonuclear content, or satellite cell content for either protocol in chronic or acute analyses (all p > 0.05) (Figure 4A-F).

INSERT FIGURE 4 HERE

Chronic responses of molecular markers

The mixed-model analysis revealed a significant main effect of time only for CALP-1 ($F_{[1,69]}$ = 9.17, p = 0.003), CALP-2 ($F_{[1,72]}$ = 17.10, p < 0.0001), LC3-II ($F_{[1,63]}$ = 8.95, p = 0.003), and SKIVL2 ($F_{[1,72]}$ = 4.46, p = 0.038) protein expression, with higher values at post compared with pre-intervention. In contrast, FOXO3 protein expression ($F_{[1,66]}$ = 5.00, p = 0.028) was higher at pre- compared with post-intervention. A significant main effect of protocol was observed only for CALP-2 ($F_{[1,72]}$ = 4.82, p = 0.031), with higher protein expression in VOL120 compared with VOL20. Additionally, ANCOVA adjusted for baseline values also indicated a significant between-protocol difference for G3BP1, with higher adjusted values in VOL20 (p = 0.005). No significant

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main effects of protocols and time, or interactions protocol vs. time were detected for any other proteins (all $p > 0.05$). Mean ± SD values for chronic protein expression are presented in Table 2. The mixed-model analysis revealed a significant main effect of time for 45S pre-rRNA gene expression ($F_{[1,69]} = 4.41$, $p = 0.039$), with higher values at post compared with pre-intervention. MSTN mRNA expression also showed a significant main effect of time ($F_{[1,23]} = 6.98$, $p = 0.014$), with lower values at post compared with pre-intervention. A significant main effect of protocol was observed only for 45S pre-rRNA gene expression ($F_{[1,69]} = 6.82$, $p = 0.011$), with higher values in VOL120 compared with VOL20. No significant main effects of protocol and time, or protocol vs. time interaction were observed for the remaining gene targets analyzed (all $p > 0.05$). Mean ± SD values for chronic gene expression data are presented in Table 3. The mixed model analysis revealed no significant main effects of protocol or time, and no protocol vs. time interaction for calpain and proteasome chronic activity.

INSERT TABLE 2 HERE

INSERT TABLE 3 HERE

Acute responses of molecular markers

The mixed-model analysis revealed significant main effects of time for MyHCfrag ($F_{[1,71]} = 4.16$, $p = 0.045$) and p-p70S6k ($F_{[1,66]} = 14.36$, $p = 0.0003$) protein expression with higher values at 24 h post-exercise compared with 0 h. Significant main effects of time were also observed for p70S6K ($F_{[1,29]} = 12.61$, $p = 0.001$), CALP-1 ($F_{[1,71]} = 17.06$, $p < 0.0001$), CALP-2 ($F_{[1,71]} = 4.16$, $p = 0.045$), SKIV2L2 ($F_{[1,71]} = 10.07$, $p = 0.002$), 4EBP1 ($F_{[1,74]} = 4.65$, $p = 0.035$), p-4EBP1($F_{[1,73]} = 15.14$, $p = 0.0002$), and ($F_{[1,29]} = 12.67$, $p = 0.001$), but with lower protein expression at 24 h post-exercise compared with 0 h. Significant main effects of protocol were only observed for CALP-1 ($F_{[1,70]} = 5.03$, $p = 0.028$), and RPS6 ($F_{[1,24]} = 5.89$, $p = 0.023$), with higher protein expression in VOL120 compared with VOL20. No significant main effects of protocol and time, or interaction protocol vs. time were detected for any other proteins (all $p > 0.05$). Mean ± SD values for acute protein expression are presented in Table 4. The mixed-model analysis revealed a significant main effect of time only for 45S pre-rRNA gene expression ($F_{[1,22]} = 9.10$, $p = 0.006$), 28S rRNA gene expression ($F_{[1,31]} = 4.82$, $p = 0.035$), and FST mRNA expression ($F_{[1,22]} = 4.54$, $p = 0.044$), with higher values at 24 h post-exercise compared

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with 0 h. No significant main effects of time or protocol, and no protocol vs. time interactions, were observed for the remaining gene targets analyzed. Mean ± SD values for acute gene expression are presented in Table 5. The mixed model analysis revealed a significant main effect of time for calpain ($F_{[1,22]} = 15.98$; $p = 0.001$), and for proteasome activity ($F_{[1,22]} = 6.57$; $p = 0.017$), both with higher values at 24h-post exercise compared with 0h. A significant main effect of protocol was observed only for proteasome activity ($F_{[1,23]} = 7.76$; $p = 0.010$), with higher values for VOL20 compared to VOL120. No protocol vs. time interaction was observed for the acute calpain and proteasome activities.

INSERT FIGURE 5 HERE

INSERT TABLE 4 HERE

INSERT TABLE 5 HERE

Discussion

We sought to determine whether a large abrupt increase in weekly RT volume would attenuate hypertrophic adaptation and alter molecular markers related to anabolic and proteolytic regulation in resistance-trained individuals. Contrary to our initial hypothesis, the present data did not show that the larger weekly volume progression attenuated hypertrophy relative to the modest progression. Both protocols increased vastus lateralis mCSA over time, and no statistically significant protocol × time interaction was detected. Most acute and chronic molecular markers assessed also did not show statistically significant between-protocol differences. Although no statistically significant between-protocol difference was detected, these findings should not be interpreted as formal evidence of equivalence.

Evidence in resistance-trained individuals does not consistently indicate a hypertrophic advantage with higher volumes. While some independent studies report greater gains with higher volumes [5, 6, 8], others show comparable adaptations despite substantial differences in prescribed volume [10-13]. In line with the latter, Aube et al. [10] observed similar vastus lateralis muscle thickness increases despite markedly different relative increases in weekly sets (~30% vs. ~98% above prior training volume), although progression magnitude was not experimentally imposed, which limits causal inference. Similarly, Barsuhn et al. [41] and Enes et al. [11] also reported comparable

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hypertrophic responses across different volume progressions in trained individuals. Collectively, these findings indicate that the magnitude and direction of any volume-related effect in trained muscle is contingent on experimental design and biological context rather than uniformly expressed across studies [14].

Several methodological factors likely contribute to such discrepant findings. First, in many prior investigations, training volume was prescribed in absolute terms without reference to participants' habitual volume, such that the effective relative overload likely varied substantially between individuals [5, 6]. In the present study, both interventions were anchored to each participant's prior weekly set volume, allowing the comparison to more directly examine the magnitude of relative progression (+20% vs +120%). While this feature does not by itself explain divergent literature outcomes, it reduces an important source of prescription heterogeneity that is often unaccounted for in volume-comparison studies [42]. Second, most prior investigations have employed between-subject designs, which are inherently more susceptible to inter-individual variability in hypertrophic responsiveness driven by genetic, molecular, and training-history factors [5, 6, 11, 43]. The unilateral within-subject design adopted here reduces biological heterogeneity and increases sensitivity for detecting true protocol-dependent effects [44]. Under these more controlled conditions, the absence of a protocol × time interaction suggests that the magnitude of mCSA increase from pre- to post-intervention did not differ detectably between progression magnitudes. Importantly, although a main effect protocol was detected for mCSA, the between-protocol difference in mCSA increases (2.7%) was small and close to the measurement coefficient of variation based on the typical error of the ultrasound method (2.3%). This was accompanied by a small standardized effect favoring VOL120, but with a wide 95% CI crossing zero (ES = −0.36, 95% CI −1.35 to 0.64; based on VOL120 − VOL20 pre-to-post percent changes in mCSA), reinforcing a cautious interpretation of protocol superiority. Moreover, hypertrophy was not accompanied by detectable changes in fascicle length or pennation angle, in agreement with prior findings in resistance-trained cohorts undergoing moderate-duration RT interventions [45, 46]. At the muscle fiber level, neither protocol produced significant increases in fCSA. While this may appear counterintuitive given the mCSA increases, similar dissociations between macroscopic and microscopic hypertrophy measures have been previously reported in trained individuals [47-49]. Fiber-level adaptations assessed via immunohistochemistry typically show greater sampling variability and lower sensitivity to detect change than imaging-based whole-

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muscle measures, particularly in previously trained muscle [47]. An alternative interpretation for the dissociation between mCSA and the absence of detectable changes in fCSA is that early increases in whole-muscle size in the absence of detectable fiber hypertrophy may reflect edema-induced swelling, likely of extracellular origin, particularly when muscle damage is pronounced [50-52]. However, this explanation appears less likely in the present study, given that our participants were resistance-trained, post-training assessments were obtained 96 h after the final session, and the acute molecular responses at the end of the intervention did not indicate a disproportionate elevation in proteolytic markers under the higher-volume condition. This interpretation is also consistent with recent acute data in trained men showing that, despite different session volumes, muscle thickness and echo-intensity returned to baseline within 24 h, with no evidence of sustained edema up to 72 h post-exercise [53].

The regulation of skeletal muscle hypertrophy in response to different resistance training volumes depends on the coordinated interaction between anabolic and catabolic molecular pathways that collectively determine net protein balance and tissue remodeling [1]. In the present study, some molecular targets were examined under both acute and chronic conditions because these time points address distinct biological questions. Acute measurements reflect the short-term post-exercise signaling milieu, whereas chronic measurements inform whether repeated exposure to the training protocols is accompanied by persistent alterations in basal protein abundance or signaling status. Experimental evidence indicates that increases in exercise volume can augment anabolic signaling, including p70S6K phosphorylation [19], satellite cell activation [18], and ribosome biogenesis [54]. Yet other studies suggest that the muscle protein synthetic response reaches a ceiling beyond which further increases in volume do not proportionally enhance anabolic signaling [21]. In parallel, very high training volumes have been proposed to increase activation of proteolytic systems, including markers of the ubiquitin–proteasome and autophagy-related pathways, potentially shifting protein balance away from net accretion [24, 25]. In this context, the present study was designed to integrate molecular and morphological assessments to examine whether large abrupt increases in weekly training volume disrupt regulatory signaling in trained muscle. Importantly, the acute molecular trial was performed only after completion of the training intervention to minimize the confounding influence of early-phase exercise-induced muscle damage and remodeling, which are known to dissociate acute molecular signaling from hypertrophic outcomes during the initial weeks of RT [52, 55, 56]. Overall, our data indicate that most

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anabolic and catabolic signaling markers responded similarly under modest and large volume progressions, consistent with the comparable hypertrophic adaptations observed between protocols.

With respect to chronic anabolic signaling, most protein and gene targets associated with mTORC1 signaling and translational regulation were not significantly altered by the intervention, nor did they differ between protocols. Although much of the literature on chronic molecular responses derives from untrained samples, our findings align with prior reports in resistance-trained cohorts showing limited sustained changes in canonical anabolic signaling proteins following high-volume training phases [57]. Satellite cell and myonuclear content were likewise unchanged, in agreement with previous studies in trained individuals undergoing RT interventions [56, 57]. One plausible interpretation is that previously trained muscle may already possess an expanded myonuclear domain from prior training exposure, reducing the necessity for additional myonuclear accretion over moderate-duration interventions [16, 17]. Among the markers related to ribosome biogenesis and RNA homeostasis, 45S pre-rRNA and SKIV2L2 protein levels were the only targets upregulated following the 8-week intervention period. The first and rate-limiting step of ribosome biogenesis is the transcription of rDNA into the 47S pre-rRNA transcript, which is subsequently processed to 45S pre-rRNA and eventually into the mature 28S, 18S, and 5.8S rRNAs [58]. The increase in basal (i.e., 72-hour post bout) 45S pre-rRNA expression observed here is consistent with previous rodent mechanical overload [59] and human training [58] studies, suggesting that mechanical overload can chronically stimulate increases in translational capacity. In parallel, SKIV2L2 is an RNA helicase and a core cofactor of the nuclear RNA exosome involved in RNA surveillance and rRNA processing/turnover [60]. Its upregulation may therefore reflect modulation of RNA quality-control dynamics rather than increased ribosome biogenesis per se. Along similar lines, G3BP1, an RNA-binding protein central to stress granule assembly and RNA homeostasis [61], displayed a protocol effect, although this was not accompanied by divergent hypertrophic outcomes. Collectively, these results suggest subtle modulation of RNA regulatory pathways with sustained loading, even when morphological and canonical anabolic signaling outcomes are comparable between volume progressions. Acute anabolic signaling was not enhanced under the higher-volume protocol. At 24 h post-exercise, most anabolic signaling targets were similar between conditions, and p70S6K protein abundance was reduced despite a time-dependent increase in p-p70S6K. This contrasts with studies

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reporting volume-dependent increases in anabolic signaling, after higher per-session set numbers [7, 19-21, 54]. However, much of this literature involves untrained participants and assessments conducted within the early post-exercise window. Notwithstanding, our findings extend prior work by indicating that, in trained individuals, large abrupt increases in weekly volume do not necessarily amplify chronic and acute anabolic signaling responses, which is consistent with the similar hypertrophic outcomes observed between protocols.

For the chronic proteolytic and remodeling-related signaling, the molecular profile did not indicate a maladaptive catabolic shift under the higher-volume progression. MSTN gene expression decreased after the intervention, independent of protocol, in agreement with evidence linking reduced myostatin-related markers with RT–induced hypertrophy [62, 63], although this response in trained individuals have been inconsistent [64]. Calpain-1 and calpain-2 protein content increased chronically, whereas enzymatic calpain activity did not, a dissociation that is physiologically plausible given the tight regulation of calpain activity by Ca^{2+} availability, autolysis, subcellular localization, and calpastatin inhibition [65, 66]. With respect to autophagy-related markers, LC3-I was unchanged, whereas LC3-II protein levels were chronically increased. This apparent divergence between isoforms has been reported previously [67] and may reflect increased LC3-I production with subsequent conversion to LC3-II, rather than a simple accumulation pattern. Members of the FOXO family are well-established transcription factors involved in whole-body energy metabolism, skeletal muscle mass regulation, and substrate utilization [68]. In the present study, FOXO3 protein levels were chronically reduced, whereas FOXO1 remained unchanged. This differential behavior is mechanistically plausible, as FOXO3 is highly responsive to cellular stress and is strongly associated with autophagy-related gene regulation, whereas FOXO1 has been more closely linked to overt catabolic conditions such as prolonged disuse or severe energy deprivation [68]. Accordingly, the absence of change in FOXO1 supports the interpretation that the training intervention did not induce a chronic catabolic state, while the reduction in FOXO3 likely reflects adaptive remodeling rather than disproportionate proteolytic activation. This interpretation is further supported by the largely unchanged levels of polyubiquitinated proteins and proteasome subunits after the intervention, in agreement with prior studies in similar trained cohorts [46, 57]. Consistent with the chronic findings, acute markers associated with ubiquitin–proteasome and autophagy-related pathways were largely unchanged across protocols and time points. This agrees

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with several prior studies reporting minimal acute modulation of these pathways following resistance exercise in humans [65, 66]. Notably, the only prior experimental study directly comparing proteolytic signaling across distinct RT volumes was conducted in rodents, and no volume effect on ubiquitin or LC3 responses were reported [69]. Taken together, the present acute and chronic proteolytic signaling data indicate that a large weekly volume progression does not disproportionally alter markers associated with protein breakdown pathways, supporting the interpretation that hypertrophic adaptations were not constrained by a volume-induced catabolic imbalance under the protocols studied.

Experimental considerations

The present study is not without limitations. Although dietary habits were recorded prior to each muscle biopsy session, daily macronutrient intake was not strictly controlled throughout the intervention. Nutritional variability can influence hypertrophic outcomes. However, available evidence suggests that inter-individual biological factors and training-related variables contribute more strongly to RT–induced hypertrophy than self-reported macronutrient intakes [56, 70, 71]. Importantly, the within-subject design reduces the likelihood that nutritional variability differentially biased the comparison between volume protocols, as both protocols were implemented within the same individuals. In addition, participants were instructed to maintain their habitual dietary patterns, and a standardized 30 g whey protein supplement was provided after each training session to support post-exercise anabolic responses [32]. A second limitation relates to participant characteristics. The sample consisted of recreationally resistance-trained individuals, and therefore extrapolation to highly trained strength athletes, clinical populations, or untrained individuals should be made with caution. Training history and baseline adaptive status are known to modulate both hypertrophic and molecular signaling responses to resistance exercise, which may constrain generalizability across populations. A third limitation relates to the sex distribution of the sample. Although both men and women were included, the sample was unbalanced (18 men and 7 women), and the study was not designed or powered to detect sex-specific differences in hypertrophic or molecular responses. Therefore, the present findings are likely more representative of resistance-trained men than of a sex-balanced trained sample. A fourth limitation relates to the ascertainment of habitual pre-study quadriceps training volume. Although baseline weekly volume was obtained through structured interviews conducted by an experienced

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744 researcher, it relied on participant self-report and was not objectively verified through
745 training logs or other external records. This may have introduced some imprecision in the
746 estimation of each participant's habitual baseline volume. However, participants met
747 strict inclusion criteria regarding lower-limb training experience and habitual quadriceps
748 training volume, and the within-subject design likely reduced part of the variability
749 associated with this limitation. A fifth limitation relates to dietary assessment. Although
750 participants were instructed to maintain their habitual dietary intake throughout the
751 intervention, dietary intake was assessed only at three timepoints surrounding the biopsy
752 sessions and therefore does not fully capture day-to-day nutritional variability across the
753 entire 8-week study period. However, given the within-subject design of the present
754 study, dietary variability would be expected to affect both training conditions within the
755 same participant, similarly, thereby reducing its potential to confound the primary
756 between-protocol comparison. In addition, participants received standardized whey
757 protein supplementation after every supervised training session. Finally, although a broad
758 panel of anabolic and proteolytic signaling markers was assessed, dynamic measurements
759 of muscle protein synthesis and breakdown rates were not performed. Such measures
760 would provide a more direct index of net protein balance and could further clarify how
761 different volume progression strategies influence muscle remodeling. This is particularly
762 relevant given the known temporal dissociation between acute signaling protein
763 activation and integrated myofibrillar protein synthesis responses [72]. An additional
764 consideration is the large number of molecular outcomes assessed. Although these markers were
765 included to provide mechanistic insight into anabolic and proteolytic regulation, the number of
766 statistical tests performed increases the possibility of type I error across these secondary
767 outcomes. Therefore, the molecular findings should be interpreted cautiously and primarily as
768 exploratory rather than definitive at the level of individual markers.
769
770 **Conclusion**
771 A large abrupt increase in weekly resistance training volume (+120%) did not
772 attenuate muscle hypertrophy relative to a modest increase (+20%) in resistance-trained
773 individuals, and most acute and chronic molecular markers assessed likewise did not
774 differ significantly between protocols.
775
776

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Supplemental material

The supplemental material can be found at https://doi.org/10.6084/m9.figshare.32408943

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Conflicts of interest

The authors declare no conflict of interest related to the content of this article.

Data availability statement

Raw data will be made available by the corresponding author upon reasonable request.

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Figure legends

Figure 1. Experimental design. VOL20: modest increase in weekly resistance training volume (+20%); VOL120: large increase in weekly resistance training volume (+120%).

Figure 2. CONSORT flow diagram of participants through the study.

Figure 3. Muscle cross-sectional area (mCSA), fascicle length, pennation angle, and fiber-type-specific morphology. Values for the VOL20 (white bars) and VOL120 (gray bars) protocols before (Pre) and after (Post) 8 weeks of resistance training. (A) mCSA; (B) fascicle length; (C) pennation angle; (D) mixed, (E) type I, and (f) type II fiber cross-sectional areas (fCSA). The lower panels show representative images of the techniques utilized: (G) ultrasound scan of the vastus lateralis used for mCSA measurement; (H) B-mode ultrasound image demonstrating the assessment of pennation angle and fascicle length; and (I) representative immunohistochemical micrograph of type I (green) and type II (black) fibers delineated by dystrophin (white). Scale bar = 100 μm. Values are presented as means (standard deviation).

Figure 4. Myonuclear and satellite cell (SC) content per fiber type. Values for the VOL20 (white bars) and VOL120 (gray bars) protocols. For chronic measures (A–D), Pre and Post refer to baseline and 8 weeks of training; for acute measures (E–F), 0 h and 24 h refer to before and after a single session. (A) chronic myonuclei changes per type I fiber; (B) chronic myonuclei changes per type II fiber; (C) chronic satellite cells changes per type I fiber; (D) chronic SC per type II fiber; (E) acute SC per type I fiber; and (F) acute SC per type II fiber. The right panels display representative immunohistochemical micrographs: (G) DAPI staining for nuclei (blue); (H) Pax7 staining for SCs (red); (I) merged DAPI and Pax7 channels, where white arrows indicate SCs (Pax7+/DAPI+); and (J) full composite image including dystrophin (white) and type I fiber (green). Scale bar = 50 μm. Values are presented as means (standard deviation).

Figure 5. Representative Western blot images of skeletal muscle protein expression in VOL20 and VOL120 across the experimental time points. The left panels show full-lane images for myosin heavy chain (MyHC) fragmentation and polyubiquitinated proteins, together with their corresponding molecular weight ladder and Ponceau staining. The

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remaining panels show representative cropped immunoblots for the analyzed protein targets, organized by condition and time point (Pre, Post, and 24 h), with the expected molecular weight indicated for each target.

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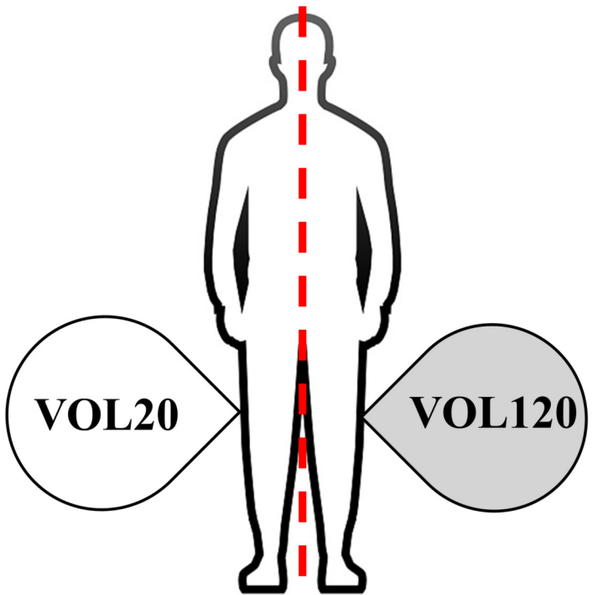

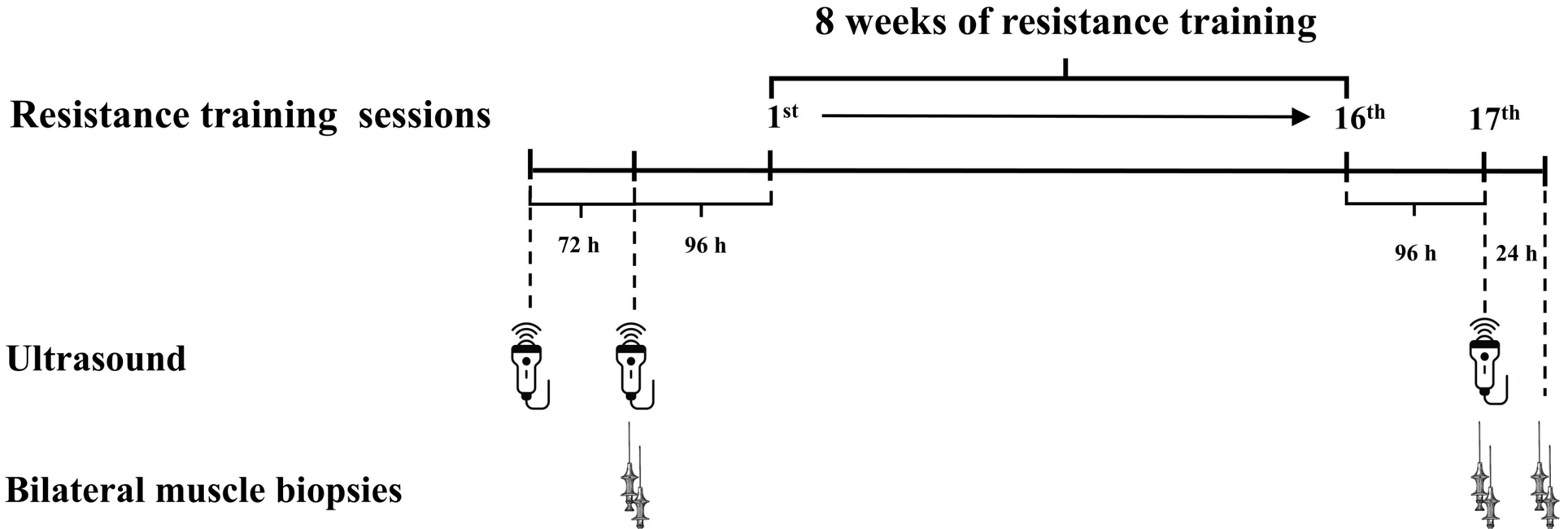

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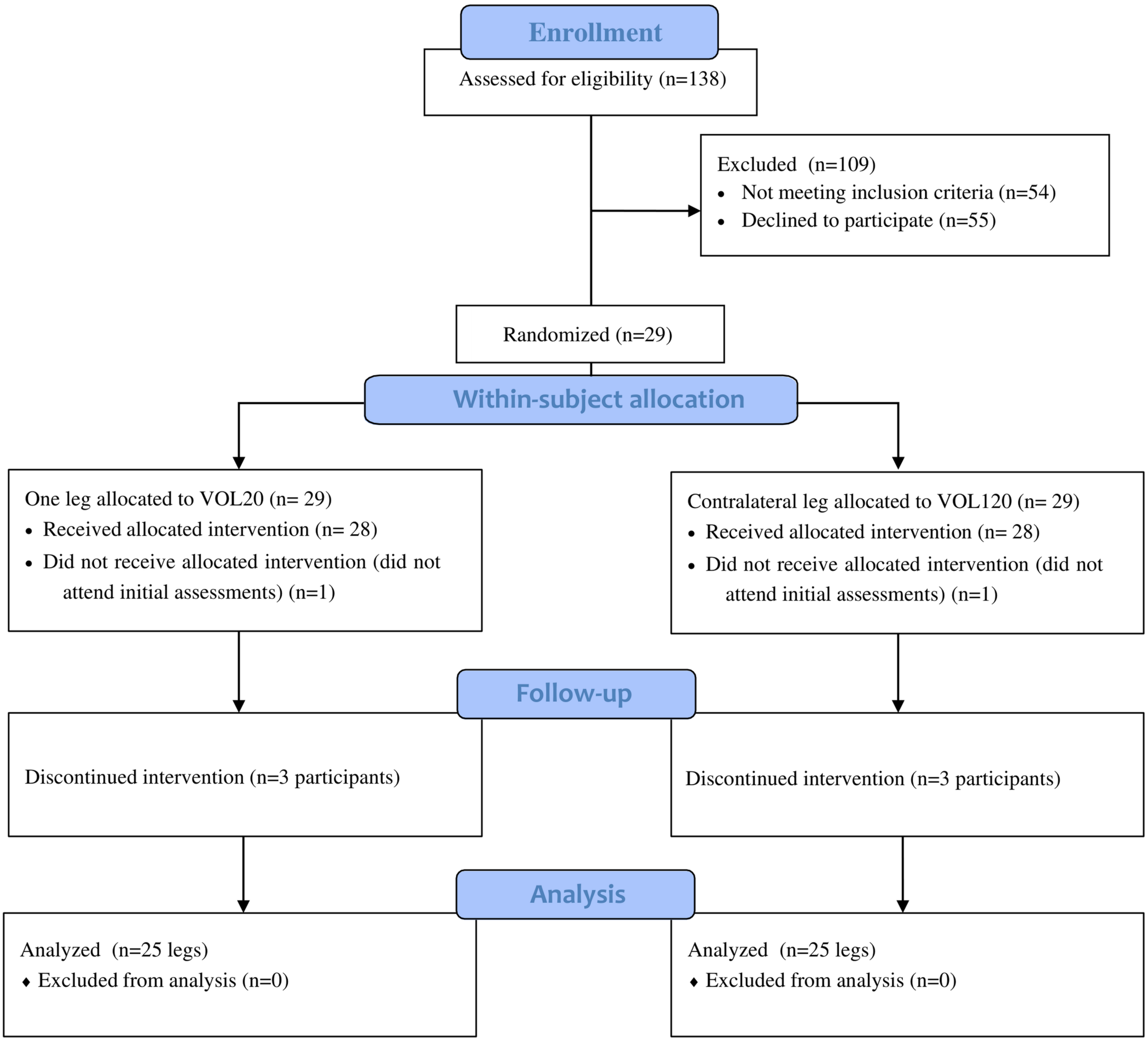

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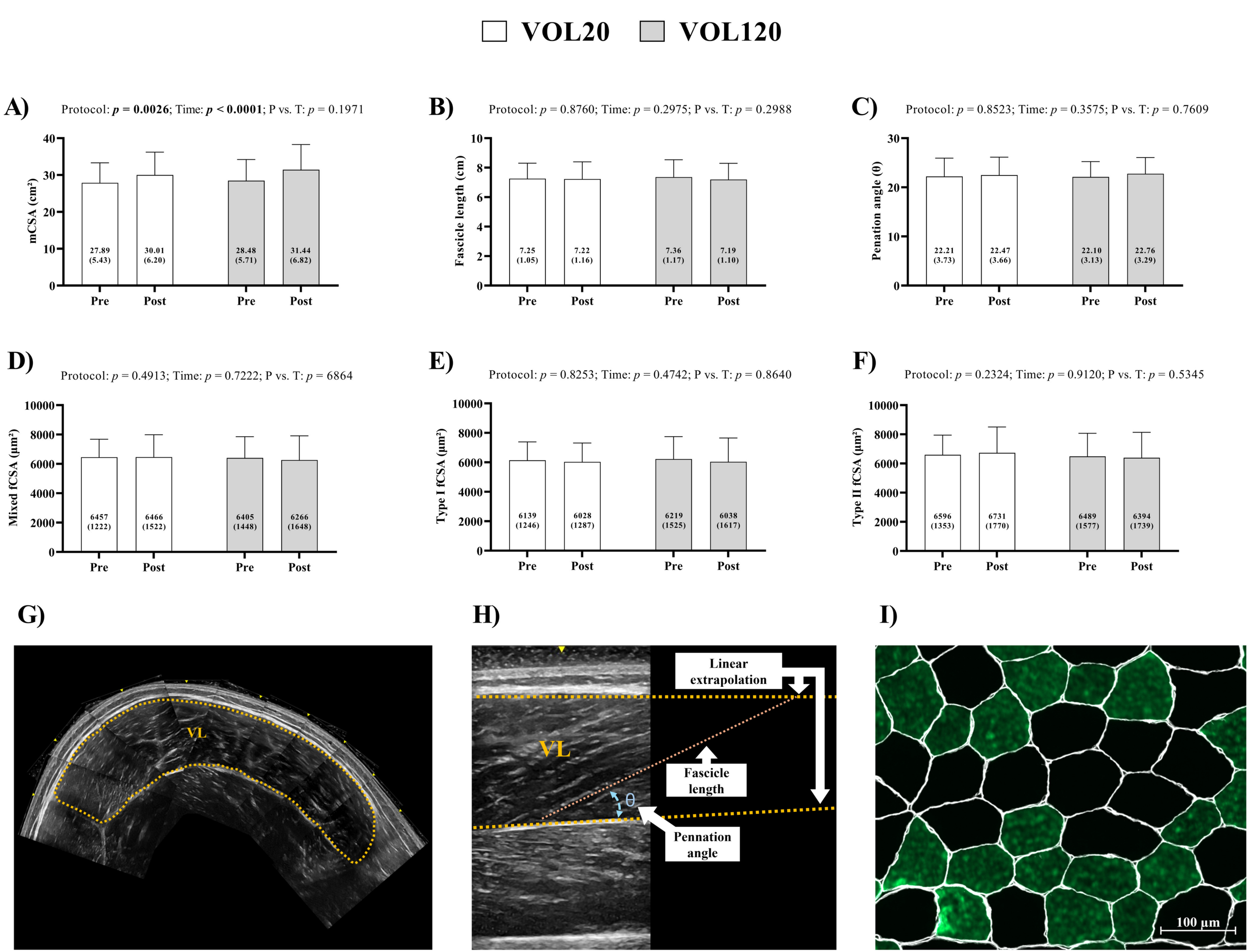

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VOL20 VOL120

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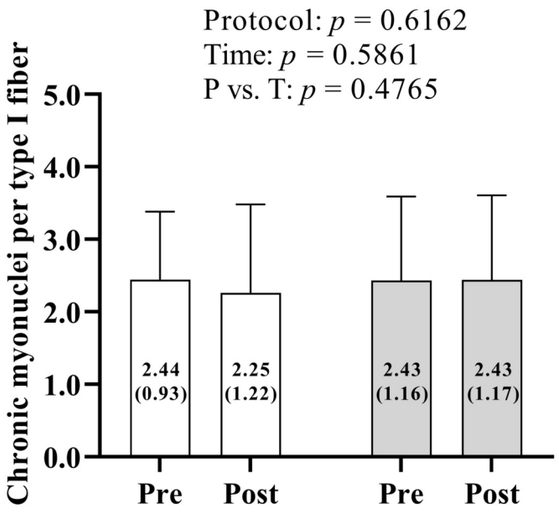

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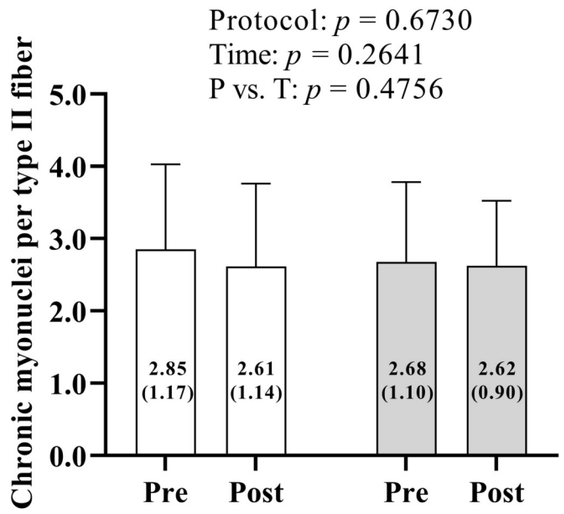

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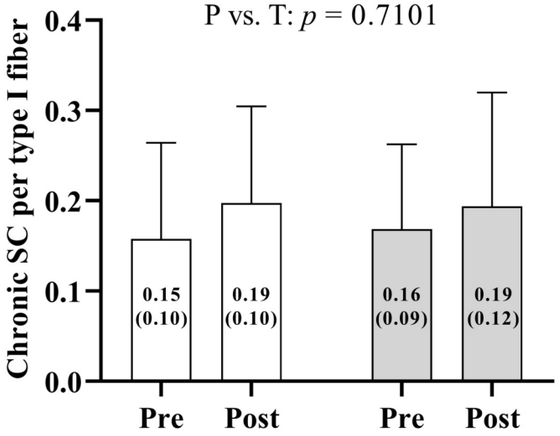

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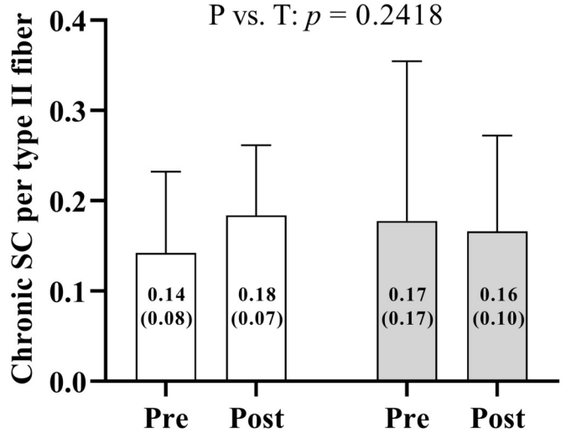

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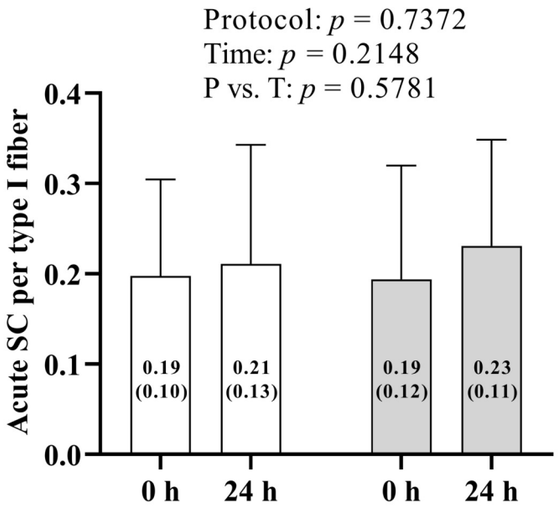

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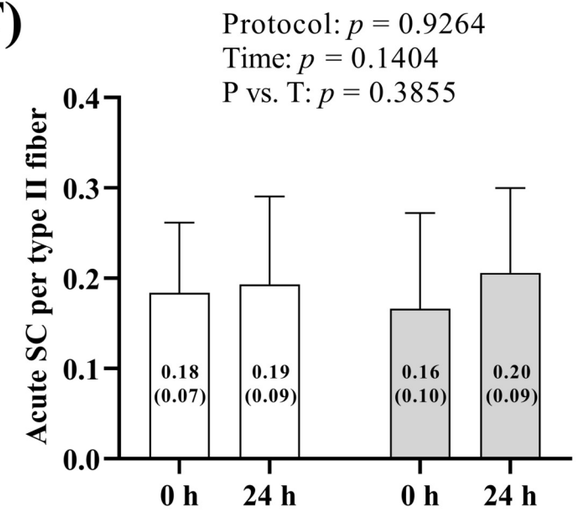

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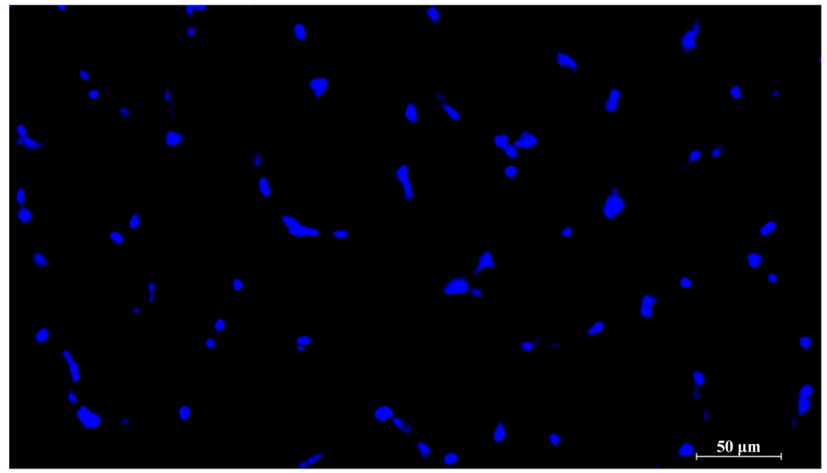

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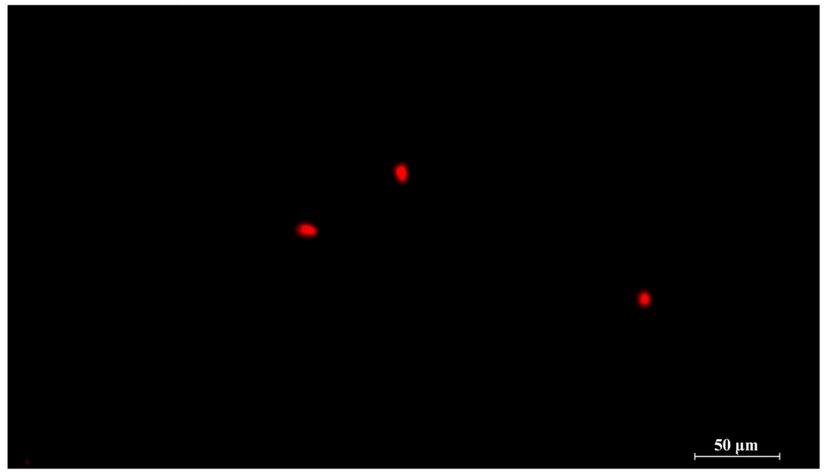

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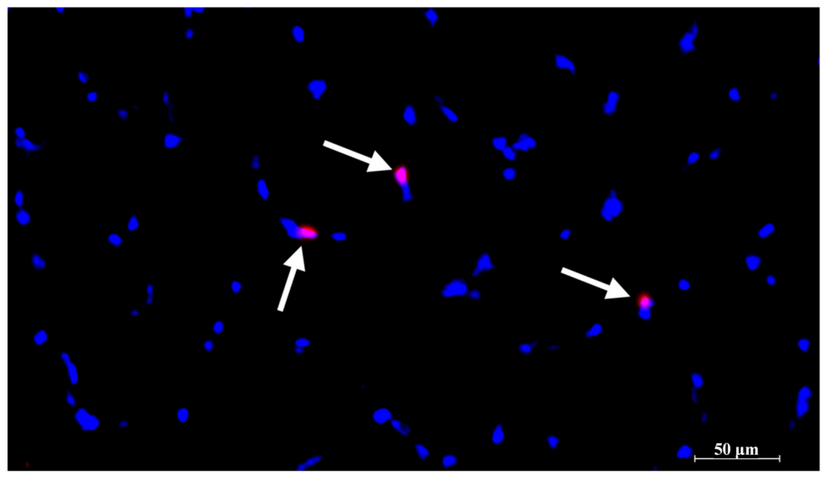

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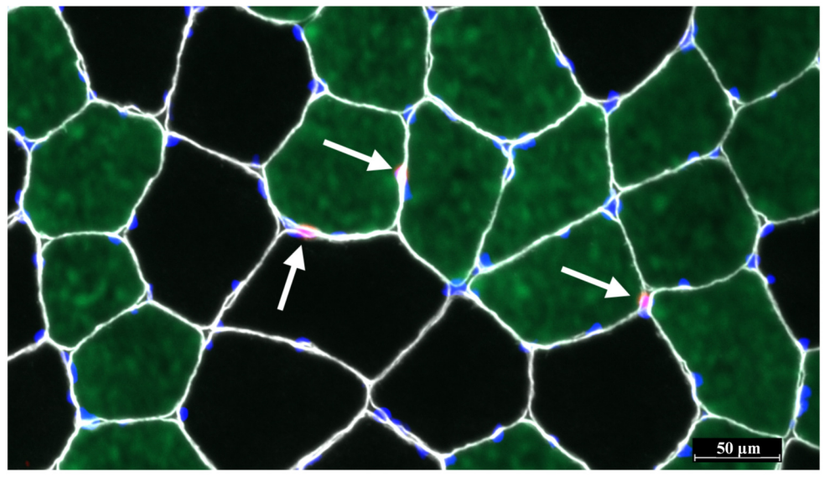

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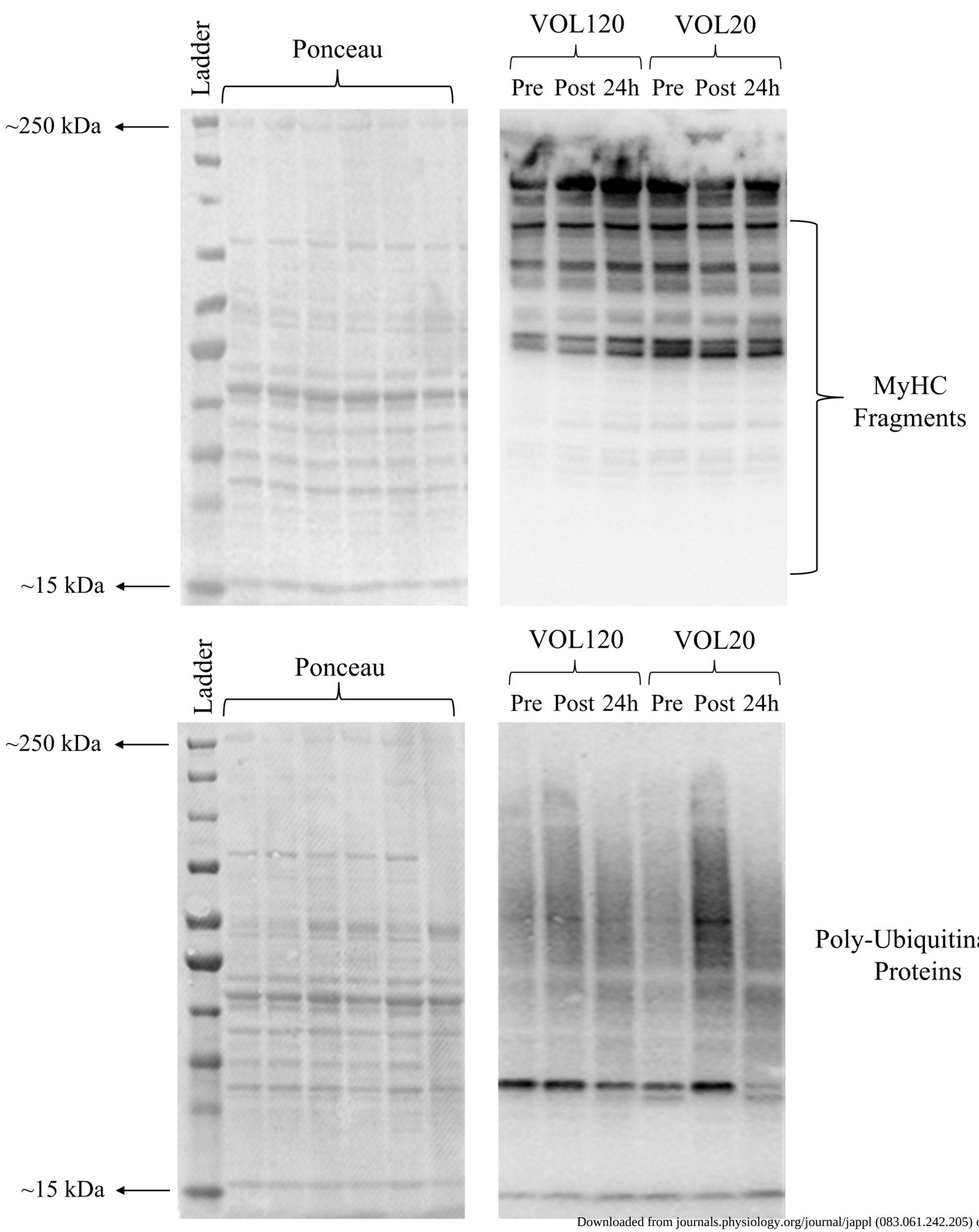

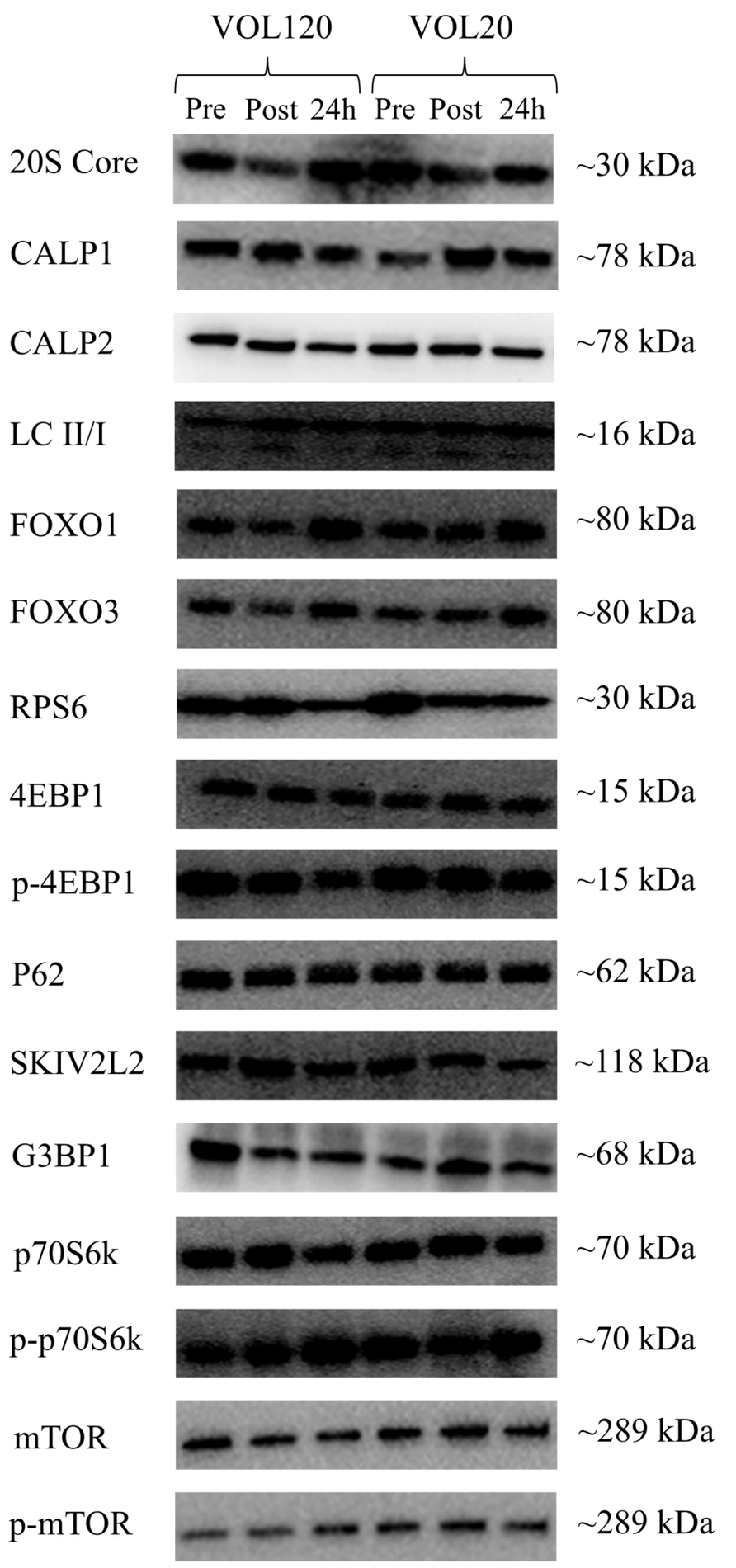

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Table 1. Baseline characteristics of female and male participants.

Variable	Female (n = 7)	Male (n = 18)
Age (years)	25.5 ± 4.8	25.3 ± 5.4
Body mass (kg)	61.0 ± 11.4	86.4 ± 16.8
Height (m)	1.61 ± 0.07	1.79 ± 0.07
Training experience (years)	2.6 ± 1.7	4.8 ± 3.9
Number of exercises for quadriceps per week	4.7 ± 0.4	3.9 ± 0.7
Set volume for quadriceps per week	14.5 ± 1.2	15.0 ± 2.9

kg: kilograms; m: meters. Data presented as mean ± standard deviation.

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Table 2. Chronic changes in skeletal muscle protein abundance following modest (+20%; VOL20) versus large (+120%; VOL120) weekly resistance training volume progression.

Proteins	VOL20		VOL120		Protocol	Time	P vs. T	Direction of significant effect
	Pre	Post	Pre	Post				
MyHCfrag	20.380 ± 8.617	19.517 ± 6.935	19.679 ± 6.068	19.643 ± 7.686	0.799	0.691	0.715	—
PolyUb	7.800 ± 2.738	8.108 ± 2.626	8.025 ± 2.334	8.314 ± 2.838	0.498	0.116	0.962	—
20S_core	0.447 ± 0.093	0.462 ± 0.111	0.483 ± 0.121	0.480 ± 0.117	0.242	0.723	0.642	—
CALP1	0.264 ± 0.046	0.284 ± 0.047	0.281 ± 0.043	0.296 ± 0.045	0.092	**0.003**	0.688	Post > Pre
CALP2	1.363 ± 0.373	1.583 ± 0.291	1.483 ± 0.255	1.690 ± 0.362	**0.031**	**< 0.0001**	0.899	VOL120 > VOL20; Post > Pre
LC3-II	0.095 ± 0.007	0.097 ± 0.007	0.095 ± 0.005	0.098 ± 0.006	0.691	**0.003**	0.510	Post > Pre
LC3-I	0.129 ± 0.015	0.126 ± 0.010	0.126 ± 0.016	0.130 ± 0.013	0.908	0.963	0.239	—
FOXO1	0.162 ± 0.022	0.158 ± 0.018	0.168 ± 0.020	0.162 ± 0.038	0.399	0.270	0.790	—
FOXO3	0.229 ± 0.055	0.210 ± 0.048	0.214 ± 0.047	0.204 ± 0.043	0.263	**0.028**	0.541	Post < Pre
RPS6	0.226 ± 0.098	0.246 ± 0.127	0.220 ± 0.082	0.298 ± 0.201	0.319	0.068	0.064	—
4EBP1	0.200 ± 0.042	0.192 ± 0.032	0.192 ± 0.039	0.191 ± 0.028	0.889	0.893	0.901	—
p-4EBP1	0.155 ± 0.039	0.150 ± 0.030	0.152 ± 0.040	0.143 ± 0.037	0.272	0.136	0.692	—
P62	0.038 ± 0.011	0.039 ± 0.018	0.039 ± 0.011	0.041 ± 0.011	0.452	0.629	0.892	—
SKIV2L2	0.021 ± 0.009	0.024 ± 0.010	0.021 ± 0.008	0.024 ± 0.010	0.891	**0.038**	0.871	Post > Pre
G3BP1	0.209 ± 0.078	0.308 ± 0.113	0.282 ± 0.150	0.226 ± 0.086	**0.005**	-	-	VOL20 > VOL120
p70S6k	0.336 ± 0.056	0.323 ± 0.053	0.288 ± 0.059	0.337 ± 0.050	0.906	-	-	—
p-p70S6k	0.311 ± 0.089	0.313 ± 0.092	0.281 ± 0.058	0.310 ± 0.068	0.332	0.248	0.374	—
mTOR	0.277 ± 0.065	0.295 ± 0.048	0.264 ± 0.045	0.276 ± 0.053	0.125	0.173	0.782	—
p-mTOR	0.394 ± 0.110	0.422 ± 0.123	0.368 ± 0.088	0.389 ± 0.095	0.134	0.199	0.862	—

MyHCfrag: fragments of all myosin heavy chain isoforms; PolyUb: poly-ubiquitin; 20S_core: 20S proteasome core; CALP1: calpain 1; CALP2: calpain 2; LC3: light chain 3; FOXO1: forkhead box O1; FOXO3: forkhead box O3; RPS6: ribosomal protein S6; 4EBP1: eukaryotic translation initiation factor 4E-binding protein 1; p-4EBP1: phosphorylated eukaryotic translation initiation factor 4E-binding

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protein 1; P62: sequestosome 1; SKIV2L2: Superkiller viralicidic activity 2-like 2; G3BP1: Ras GTPase-activating protein-binding protein 1; p70S6K: ribosomal protein S6 kinase beta-1; p-p70S6K: phosphorylated ribosomal protein S6 kinase beta-1; mTOR: mammalian target of rapamycin; p-mTOR: phosphorylated mammalian target of rapamycin. Data are presented as mean ± SD. Protein abundance was determined by Western blotting and expressed in arbitrary units normalized to total protein staining (Ponceau). Pre and Post represent resting muscle samples obtained before and after the 8-week training, respectively. For G3BP1 and p70S6K, *p*-values were derived from ANCOVA using baseline (Pre) values as a covariate. Representative immunoblots are provided in Figure 5.

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Table 3. Chronic changes in skeletal muscle gene expression following modest (+20%; VOL20) versus large (+120%; VOL120) weekly resistance training volume progression.

Genes	VOL20		VOL120		Protocol	Time	P vs. T	Direction of significant effect
	Pre	Post	Pre	Post				
18S	1.155 ± 0.753	0.989 ± 0.383	1.220 ± 0.907	1.111 ± 0.640	0.428	0.164	0.699	—
28S	1.178 ± 0.607	1.173 ± 0.556	1.292 ± 0.838	1.739 ± 1.274	0.058	0.223	0.210	—
45S pre-rRNA	1.179 ± 0.666	1.359 ± 0.837	1.448 ± 1.199	2.000 ± 1.472	**0.011**	**0.039**	0.289	VOL120 > VOL20; Post > Pre
MSTN	1.312 ± 1.228	0.770 ± 0.438	1.252 ± 0.894	0.793 ± 0.506	0.879	**0.014**	0.656	Post < Pre
IGF-1	1.292 ± 0.971	1.364 ± 1.162	1.421 ± 1.226	0.815 ± 0.533	0.268	0.160	0.075	—
IL-15	1.164 ± 0.871	1.051 ± 0.520	1.123 ± 0.608	0.941 ± 0.391	0.480	0.208	0.669	—
FST	1.232 ± 0.934	2.675 ± 6.712	1.428 ± 1.621	1.293 ± 1422	0.445	0.370	0.264	—
Total RNA	1297 ± 721	1330 ± 216	1186 ± 362	1346 ± 249	0.579	0.290	0.433	—

18S: 18S ribosomal RNA subunit; 28S: 28S ribosomal RNA subunit; 45S pre-rRNA: 45S pre-ribosomal RNA; MSTN: myostatin; IGF-1: insulin like growth factor 1; IL-15: interleukin 15; FST: follistatin; Total RNA: total RNA concentration. Data are presented as mean ± SD (n = 25). Relative gene expression was determined by real-time quantitative PCR and calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the reference gene. Total RNA was calculated as concentration/weight*volume loaded (ng/mg tissue). Data are presented as mean ± SD. Pre and Post represent resting muscle samples obtained before and after the 8-week training, respectively.

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Table 4. Acute changes in skeletal muscle protein abundance following modest (+20%; VOL20) versus large (+120%; VOL120) weekly resistance training volume progression.

Proteins	VOL20		VOL120		Protocol	Time	P vs. T	Direction of significant effect
	0 h	24 h	0 h	24 h				
MyHCfrag	19.517 ± 6.935	23.303 ± 8.844	19.643 ± 7.686	20.919 ± 7.865	0.372	**0.045**	0.321	24 h > 0 h
PolyUb	8.108 ± 2.626	7.478 ± 1.328	8.314 ± 2.838	7.589 ± 1.155	0.455	0.306	0.821	—
20S_core	0.462 ± 0.111	0.483 ± 0.138	0.480 ± 0.117	0.472 ± 0.109	0.893	0.687	0.508	—
CALP1	0.284 ± 0.047	0.248 ± 0.047	0.296 ± 0.045	0.270 ± 0.055	**0.028**	**< 0.0001**	0.533	VOL120 > VOL20; 24 h < 0 h
CALP2	1.583 ± 0.291	1.457 ± 0.518	1.690 ± 0.362	1.545 ± 0.398	0.154	**0.045**	0.884	24 h < 0 h
LC3-II	0.098 ± 0.006	0.097 ± 0.009	0.097 ± 0.007	0.098 ± 0.007	0.901	0.795	0.365	—
LC3-I	0.126 ± 0.010	0.126 ± 0.019	0.130 ± 0.013	0.135 ± 0.019	0.053	0.226	0.299	—
FOXO1	0.158 ± 0.018	0.162 ± 0.051	0.162 ± 0.038	0.165 ± 0.038	0.630	0.669	0.969	—
FOXO3	0.210 ± 0.048	0.220 ± 0.090	0.204 ± 0.043	0.223 ± 0.038	0.867	0.251	0.570	—
RPS6	0.246 ± 0.127	0.218 ± 0.090	0.298 ± 0.201	0.248 ± 0.109	**0.023**	0.244	0.594	VOL120 > VOL20
4EBP1	0.192 ± 0.032	0.185 ± 0.030	0.191 ± 0.028	0.183 ± 0.032	0.782	**0.035**	0.936	24 h < 0 h
p-4EBP1	0.150 ± 0.030	0.134 ± 0.033	0.143 ± 0.037	0.134 ± 0.030	0.541	**0.0002**	0.314	24 h < 0 h
P62	0.039 ± 0.018	0.043 ± 0.027	0.041 ± 0.011	0.042 ± 0.017	0.928	0.561	0.629	—
SKIV2L2	0.024 ± 0.010	0.017 ± 0.010	0.024 ± 0.010	0.020 ± 0.010	0.258	**0.002**	0.400	24 h < 0 h
G3BP1	0.308 ± 0.113	0.269 ± 0.111	0.226 ± 0.086	0.282 ± 0.107	0.176	-	-	—
p70S6k	0.323 ± 0.053	0.282 ± 0.088	0.337 ± 0.050	0.278 ± 0.058	0.693	**0.001**	0.496	24 h < 0 h
p-p70S6k	0.313 ± 0.092	0.393 ± 0.119	0.310 ± 0.068	0.368 ± 0.111	0.434	**0.0003**	0.545	24 h > 0 h
mTOR	0.295 ± 0.048	0.272 ± 0.074	0.276 ± 0.053	0.284 ± 0.052	0.729	0.570	0.194	—
p-mTOR	0.422 ± 0.123	0.363 ± 0.072	0.389 ± 0.095	0.400 ± 0.091	0.903	0.169	0.056	—

MyHCfrag: fragments of all myosin heavy chain isoforms; PolyUb: poly-ubiquitin; 20S_core: 20S proteasome core; CALP1: calpain 1; CALP2: calpain 2; LC3: light chain 3; FOXO1: forkhead box O1; FOXO3: forkhead box O3; RPS6: ribosomal protein S6; 4EBP1:

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eukaryotic translation initiation factor 4E-binding protein 1; p-4EBP1: phosphorylated eukaryotic translation initiation factor 4E-binding protein 1; P62: sequestosome 1; SKIV2L2: Superkiller viralicidic activity 2-like 2; G3BP1: Ras GTPase-activating protein-binding protein 1; p70S6K: ribosomal protein S6 kinase beta-1; p-p70S6K: phosphorylated ribosomal protein S6 kinase beta-1; mTOR: mammalian target of rapamycin; p-mTOR: phosphorylated mammalian target of rapamycin. Data are presented as mean ± SD. Protein abundance was determined by Western blotting and expressed in arbitrary units normalized to total protein staining (Ponceau). 0 h represents resting muscle samples obtained before the acute exercise session, and 24 h represents samples obtained 24 h after the last session performed under each limb-specific training condition. For G3BP1, *p*-values were derived from ANCOVA using baseline (0 h) values as covariate. Representative immunoblots are provided in Figure 5.

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Table 5. Acute changes in skeletal muscle gene expression following modest (+20%; VOL20) versus large (+120%; VOL120) weekly resistance training volume progression.

Genes	VOL20		VOL120		Protocol	Time	P vs. T	Direction of significant effect
	0 h	24 h	0 h	24 h				
18S	1.075 ± 0.418	1.338 ± 0.791	1.155 ± 0.663	1.201 ± 0.785	0.805	0.215	0.350	—
28S	1.136 ± 0.538	1.700 ± 0.928	1.255 ± 0.919	1.492 ± 0.910	0.798	**0.035**	0.363	24 h > 0 h
45S pre-rRNA	1.252 ± 0.770	2.271 ± 1.811	1.300 ± 0.955	1.911 ± 1.558	0.479	**0.006**	0.416	24 h > 0 h
MSTN	1.168 ± 0.662	0.860 ± 1.037	1.255 ± 0.800	1.021 ± 1.146	0.476	0.112	0.831	—
IGF-1	1.262 ± 1.075	1.622 ± 1.722	1.157 ± 0.756	1.557 ± 1.317	0.749	0.143	0.937	—
IL-15	1.122 ± 0.556	1.300 ± 0.593	1.087 ± 0.451	1.236 ± 0.691	0.623	0.142	0.885	—
FST	2.357 ± 5.913	4.691 ± 7.181	1.381 ± 1.518	3.771 ± 6.485	0.492	**0.044**	0.974	24 h > 0 h
Total RNA	1330 ± 216	1281 ± 269	1346 ± 249	1448 ± 502	0.163	0.783	0.133	—

18S: 18S ribosomal RNA subunit; 28S: 28S ribosomal RNA subunit; 45S pre-rRNA: 45S pre-ribosomal RNA subunit; MSTN: myostatin; IGF-1: insulin like growth factor 1; IL-15: interleukin 15; FST: follistatin; Total RNA: total RNA concentration. Data are presented as mean ± SD (n = 25). Relative gene expression was determined by real-time quantitative PCR and calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the reference gene. Total RNA was calculated as concentration/weight*volume loaded (ng/mg tissue). Data are presented as mean ± SD. 0 h and 24 h represent resting muscle samples obtained before and after the last session performed under each limb-specific training condition.

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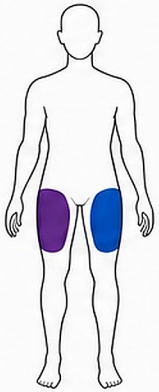
Large increases in resistance training volume do not impair muscle hypertrophy or anabolic–catabolic molecular signaling in trained individuals

PARTICIPANTS & DESIGN

25 resistance-trained
Female and Male
(18–35 years)



VOL120 Large increase (+120%)

VOL20 Modest increase (+20%)

Randomized, single-blind, within-subject unilateral design

Each participant trained both legs twice weekly for 8 weeks

INTERVENTION

8 weeks

2 sessions/week

LEG PRESS

LEG EXTENSION

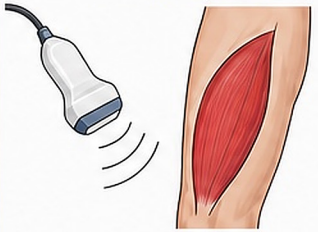
9–12 reps to concentric failure

Volume progression relative to habitual quadriceps volume

- VOL120: +120% weekly set volume
- VOL20: +20% weekly set volume

OUTCOMES ASSESSED

Primary outcome



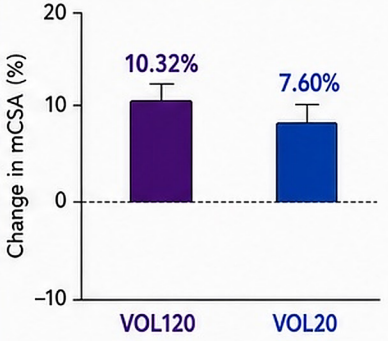
Vastus lateralis muscle cross-sectional area (mCSA)

Secondary outcomes

- Muscle fiber cross-sectional area (fCSA)
- Satellite cells
- Myonuclei content
- Anabolic and catabolic signaling pathways
- Ribosome biogenesis and gene expression

MAIN RESULTS

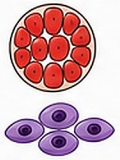
Muscle hypertrophy



Both protocols increased mCSA over time ($p<0.001$)

No protocol × time interaction

Muscle fiber CSA & satellite cells

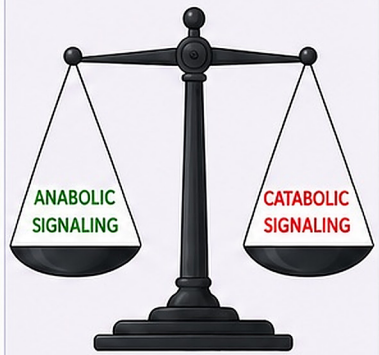


No significant differences between protocols for fCSA, satellite cell number or myonuclear content

Molecular responses

Acute and chronic molecular responses related to anabolic signaling, protein degradation, ribosome biogenesis and gene expression were largely similar between protocols

CONCLUSION



A large, abrupt increase in weekly resistance training volume (+120% relative to habitual training) does not impair muscle hypertrophy relative to a modest increase (+20%) in resistance-trained individuals.

MUSCLE BIOPSIES

- Baseline (Pre)
- Post-intervention (8 weeks)
- 24 h after the last session (Acute response)

ANALYSES PERFORMED

- Histological analysis
- Activity assays
- Gene expression
- Protein expression

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