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Sprint and Strength Training Modulates Autophagy and Proteostasis in Aging Sprinters

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falsification, or inappropriate data manipulation. The authors also state that results of the present study do not constitute endorsement by ACSM.

ABSTRACT

Purpose: Exercise and aging may modulate muscle protein homeostasis and autophagy, but few studies examine highly-trained middle-aged or older individuals. This study elucidated the effects of a new long-term training stimulus on markers of muscle autophagy and unfolded protein response (UPR) and on sprint running performance in masters sprinters.

Methods: Thirty-two male competitive sprinters (aged 40–76 years) were randomly divided into experimental (EX) and control (CTRL) groups. The EX training program was a combination of heavy and explosive strength and sprint exercises aimed at improving sprint performance. Fifteen and thirteen participants completed the 20-week intervention period in EX and CTRL, respectively. The latter were told to continue their routine exercises. Key protein markers were analyzed by western blotting from vastus lateralis (VL) muscle biopsies. Muscle thickness of VL was analyzed by ultrasonography and sprint performance by a 60-meter running test.

Results: EX induced improvement in 60-meter sprint performance when compared to controls (time x group, $P = 0.003$) without changes in VL muscle thickness. Content of lipidated microtubule-associated protein 1A/1B-light chain 3 (LC3-II) increased in EX ($P = 0.022$) suggesting increased autophagosome content. Additionally, an autophagosome clearance marker sequestosome 1 (p62) decreased in EX ($P = 0.006$). Markers of UPR selectively modulated with decreases (e.g. ATF4, $P = 0.003$) and increases (e.g. EIF2α, $P = 0.019$) observed in EX.

Conclusions: These findings suggest that a new intensive training stimulus that combines strength training with sprint training may increase muscle autophagosome content in a basal state without any evidence of impaired autophagosome clearance in masters sprinters. Simultaneously, the combined training may have a selective effect on the content of UPR signaling components.

Keywords: masters athlete, skeletal muscle, unfolded protein response, mTOR, ATF4

INTRODUCTION

Aging is associated with progressive loss of muscle mass and function (sarcopenia) that may ultimately result in functional limitations and dependent lifestyle (1, 2). Resistance exercise (RE) is known to increase muscle strength and size and to counteract sarcopenia (3) but it is unclear to what extent the age-related reductions in physical activity levels and aging *per se* contribute to decreased muscle function caused by aging. Masters athletes are a population with lifelong participation in systematic training and good health habits, but they also inevitably lose muscle mass and strength despite high levels of physical activity. Therefore, they represent an ideal model to investigate the effects of biological aging on muscle properties *per se* (4, 5). Moreover, aging athletes who can tolerate and are accustomed to vigorous exercise can serve as specialized group to examine the plasticity of an already trained neuromuscular system to intensified or altered exercise stimulus.

To maintain protein homeostasis, for example during and after strenuous exercise, cells have developed a network of several integrated biological processes that contribute to the control of functional proteome (6). Of these quality control processes, autophagy-lysosome and ubiquitin proteasome pathways are crucial in the degradation of dysfunctional and misfolded proteins (7). Previous studies suggest an age-related reduction in the control of proteostasis, including autophagy, that may lead to a cytotoxic accumulation of misfolded protein aggregates (8). We previously showed that an acute bout of RE and 21-weeks of resistance training (RT) increased autophagosome content marked by increased content of lipidated LC3 protein in previously untrained young men around the age of 27. However, the increased autophagosome content was not observed in previously untrained older men around the age of 61 (9). Contrary to our previous

results, a bout of RE increased acutely with a two day latency autophagy markers in previously untrained older (~70 years) men (10) and long-term RT increased muscle autophagy in aged rats (11). Thus, the discrepancy between previous studies warrant further studies to elucidate the effects of strength training on autophagy in aged muscle. Furthermore, decreased muscle autophagosome content has been observed in sedentary older (~71 years) men, but not among similarly aged recreational exercisers with a lifelong background mainly in endurance exercise (12).

Protein homeostasis may also disrupt in endoplasmic reticulum (ER), in which premature proteins fold by the assistance of chaperones to their functional three-dimensional structure. When protein homeostasis disrupts in the ER, unfolded protein response (UPR_{ER})activates, aiming to balance the homeostasis in ER (13). UPR may mediate some exercise-induced adaptations in rodents (14), and an unaccustomed RE bout increased UPR markers in humans regardless of age (9, 10). UPR-induced transcription factor ATF4 may be involved in age-related muscle atrophy (15), but overall findings regarding basal levels of UPR markers in aged muscle are unclear (16). Moreover, how a different type of vigorous exercise, especially in the context of training intervention, could modulate muscle ER stress/UPR markers in the basal state in already highly trained aging athletes is not known.

The purpose of the present study was to investigate the effect of a new long-term training stimulus, which consisted of the integration of strength and explosive strength training into a conventional sprint training regimen (EX), on muscle autophagy and unfolded protein response (UPR) markers in basal state as well as sport performance in middle-aged and older (40–76 years) male sprinters. We hypothesized that as an adaptation to the altered combined training stimulus the muscle autophagy and UPR markers are modulated in the basal state.

MATERIALS AND METHODS

Study design and participants

The present study is part of a larger research project investigating the association of age and training with sprint performance and musculoskeletal characteristics among male athletes (17–21). A total of 111 athletes aged 17–84 years with a very long-term training background and a successful competition history on the national or international level in sprinting events (100–400 m) were contacted with a personal letter as previously described (17, 18). The letters contained detailed questionnaires regarding: (1) current and previous training status, (2) competition results as well as (3) possible injuries and diseases that could preclude from maximal physical performance tests and training. Based on the received questionnaires, 83 athletes received an invitation (nationwide) to participate in the cross-sectional study of running biomechanics and musculoskeletal characteristics (17).

The participants for the original randomized controlled training intervention were recruited from the masters athletes aged ≥40 years who took part in the cross-sectional study that served as baseline measurements (17). Participants were excluded from the study if they had uncontrolled medical conditions or musculoskeletal disorders that prohibited participants from conducting the planned training program. The health status was confirmed in a short interview with a physician and by a clinical examination that included, for example, a resting ECG for participants ≥55 years old. In total, 40 participants were originally randomly divided to an experimental group (EX) and 32 to a control group (CTRL) after the baseline measurements. These participants did not have previous experience of consistent heavy strength training that was conducted in the EX group in this study. The participants in the EX group completed a periodized 20-week training program in

which maximal and explosive strength training was integrated to running-based sprint training routines (Figure 1). The participants in the CTRL group were told to continue their accustomed training regimen that mainly consisted of sprint training. All the participants from the original EX group of whom muscle samples were available were included in this study (n = 16). Consequently, 16 participants from the original CTRL group were chosen for the present study based on muscle sample availability and age in order to match the age spectrum of the groups. Based on the per protocol analysis three participants in the CTRL group were excluded, because their reported weekly strength training volume during the experimental period was above the average volume of the EX group. In addition, one participant was excluded from the EX group due to poor adherence to the experimental training program. Thus, 15 participants in the EX group and 13 in the CTRL group were included in this study (Table 1). Three participants in the EX group were not able to conduct the post-performance tests due to illness or injury but were included in the study because their total training adherence was adequate to be included to the study.

The ethical committee of the University of Jyväskylä approved the study and it was conducted according to the declaration of Helsinki. All the participants were carefully informed about the study design, possible risks and discomfort related to the study and every participant signed a written informed consent.

Measurements

The main laboratory-based measurements were conducted before the 20-week intervention period and immediately after the training period on two consecutive days. Participants were told to prepare for the measurements as they would for an important competition and to restrain from vigorous exercise two days before the measurements. On the first measurement day, performance

tests and anthropometric measurements were conducted. On the morning of the following day, muscle biopsies were obtained after an overnight fast (12 h) (Figure 1).

Anthropometric measurements. Height was measured with a height gauge, body mass with a balance beam scale and the percentage of body fat and lean body mass were analyzed with a bioelectrical impedance device (Spectrum II; RJL Systems, Detroit, MI) (17). Vastus lateralis (VL) muscle thickness at the mid-region was measured by a B-mode ultrasound instrument (SSD-1400, Aloka Japan) (17). In brief, a 5-cm linear-array probe (7.5 MHz) was positioned perpendicular to the surface of the VL muscle and thickness was determined as the distance between the adipose tissue-muscle interface and intermuscular interface.

Performance tests. For the assessment of sprinting performance, the subjects ran two 60-meter trials on a synthetic indoor running track while wearing track spikes. Running times were recorded by double-beam photocell-gates connected to an electronic timer (20). Squat jump on a contact mat was carried out to examine the explosive strength production (20). Participants squatted, hands on their hips, to an approximately 90° knee angle, stopped and rapidly jumped as high as possible and landed on the contact mat with their legs extended. Jump height was calculated from the flight time.

Muscle biopsies. Muscle biopsies were obtained from the mid-part of the vastus lateralis (VL) defined as a midpoint between the greater trochanter and the lateral joint line of the knee of the dominant leg by using a needle biopsy technique. The skin surface area around the biopsy obtainment site was cleaned with an antiseptic solution and then anesthetized with 1% lidocaine containing epinephrine, before the biopsy obtainment. A needle (5 mm) was inserted into the muscle belly at a depth of ~1.5–2.5 cm below the surface of the skin, and, with the aid of suction, ~100–150 mg of muscle tissue was removed. The muscle sample was cleaned of any visible

connective and adipose tissue and was frozen immediately in liquid nitrogen and stored at −80°C until homogenization and protein extraction. The scar of the muscle biopsy of the baseline measurement was used to localize the same biopsy site at POST time-point (17).

Experimental training program

The 20-week training program in the EX group was a combination of heavy and explosive strength training that was integrated to the their normal running-based sprint training as previously described (18, 19). The training program was carefully designed and periodized to fit participants' indoor and outdoor competition schedule. The general aim of the training program was to enhance overall sprint performance by improving the explosive force production capacity of the muscle groups that are crucial for acceleration and maximum speed sprinting (i.e. quadriceps, hamstrings, gluteus, calves, hips, core muscles). There were two similar 9- and 11-week periods in the training program that were further divided into three 3–4-week phases that differed in intensity, volume and type of training. The strength training in the first phase consisted of strength endurance and muscle hypertrophy exercises (3–4 sets x 8–12 repetitions, 50–70% of 1RM). The strength training in the latter two phases involved mainly maximal strength training exercises (2–3 sets x 4–6 repetitions, 70%–85% 1RM), explosive-type weight lifting (2–3 sets x 4–6 repetitions, 35%–60% of 1RM) and plyometric exercises (2–3 sets of 3–10 repetitions). The first training period was repeated by a rather similar three-phase training period with a progressive increase in overall training intensity and a reduction in volume to peak the performance at the end of the training period. There were two strength and plyometric training sessions on non-consecutive days per week and plyometric exercises were conducted before the sprinting sessions. The main strength exercises were leg press and/or half squat on machines, clean pull (from knee height) and/or stiff

leg deadlift using free weights. In addition, dynamic hip extension, hip flexion, knee flexion, knee extension, and ankle plantar flexion conducted on machines were included in the training program as supplementary exercises. Furthermore, exercises to increase core and upper body strength were also included in the training program. The plyometric training started with lower-intensity vertical jumps and progressed to higher intensity horizontal jumps. Furthermore, the sprint training progressed from speed endurance training to maximal speed and sprint acceleration exercises, and was rather similar in the both training periods. In the EX group, approximately one-half of the total training volume was strength and plyometric training while the other half composed of sprint training. In order to avoid injuries, the strength training intensity of the older participants (≥65 years; n = 6) was planned to be slightly lower (lower load, more repetitions) than in younger participants.

Training instructions and monitoring

Training programs with detailed written, pictorial and videotaped instructions were mailed to the EX group (18). The participants of the both groups completed detailed training logs to monitor their participation in the training program and the logs were collected every fifth week during field tests (organized in athletes' home cities). Based on the logs, training mode specific volume was calculated as training hours per week as well as training sessions per week. Based on the training logs, the only significant difference in the training mode specific volume between the groups was in the strength training (Table 1, $P = 2.16 \cdot 10^{-5}$).

Homogenization and protein extraction

The frozen muscle samples were put in Eppendorf tubes and were homogenized manually with plastic rods (VWR Disposable Pestle, 1.5 ml, Cat. No. 47747-358) in an ice-cold Hans lysis buffer (pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol) at a dilution of 15 $\mu l \cdot mg^{-1}$ of wet weight muscle. Before homogenization, Hans buffer was supplemented with protease and phosphatase inhibitors including 1mM pepstatin and 0.5 mM EDTA-containing HALT Protease and phosphatase inhibitor coctail (Pierce, 78440). The homogenate was centrifuged at 1000 rpm for 1 minute at 4 °C and the supernatant containing the cytoplasmic fraction of the proteins was collected. The remaining pellet was re-suspended to 360 µl of inhibitor-supplemented Hans lysis buffer. At this point, the NaCl concentration of Hans buffer was increased by adding 40 µl of 3 M NaCl. Suspension was subsequently rotated for 1 h in 4° C and centrifuged at 14000 rpm for 15 minutes in 4° C. The supernatant containing nuclear fraction of the proteins was then collected. The total protein concentration of the nuclear and cytosolic fractions was measured using the Bicinchoninic acid protein assay (BCA Protein Assay-kit, Pierce, #23227).

Western blotting

We pooled cytosolic and nuclear fractions for the western blot analysis because (i) we were interested in the changes in the total protein content and not in their subcellular localization and (ii) the nuclear fractions contained cytosolic proteins (e.g., GAPDH and EIF2α, not shown), indicating that the fractionation protocol was not thoroughly reliable. Samples were mixed together with Laemmli sample buffer + β-mercaptoethanol followed by 10 minutes of boiling at 95 °C to denature proteins. The proteins were separated by SDS-PAGE using Criterion™ TGX Stain-

Free™ Precast Gels (4%–20 %) (Bio-Rad Laboratories) and were transferred to a PVDF membrane with a Trans-Blot® Turbo™ Transfer system (Bio-Rad Laboratories). The membrane was blocked (5% nonfat dry milk in TBS-T) for 2 hours in room temperature and incubated with specific primary antibodies at 4 °C overnight. Next day, the membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h in room temperature. The membranes were again washed with TBS-T and proteins were visualized by enhanced chemiluminescence (SuperSignal west femto maximum sensitivity substrate, Pierce Biotechnology) using a ChemiDoc MP device (Bio-Rad Laboratories). The signal was quantified by Image Lab software and normalized to the whole-lane signal of the stain-free blot, because the stain-free signal is suggested to be more stable and accurate loading control than singular housekeeping genes, especially when analyzing muscle samples from older people or from exercise intervention studies (22). The protocol to measure ubiquitinated proteins differed from the protocol described above slightly. The membrane was blocked over-night at 4° C instead of 2 hours at room temperature and because the primary antibody contained the horse-radish conjugate, secondary antibodies were not used. The other steps were similar as in the above-described protocol.

Antibodies

The following were purchased from Cell Signaling Technology: primary antibodies that recognized activating transcription factor 4 (ATF4; #11815), phosphorylated acetyl-CoA carboxylase at ser79 (p-ACC^{s79}; #3661), acetyl-CoA carboxylase (ACC; #3676), apoptosis regulator bcl-2 (BCL-2; #3498), Beclin-1 (Beclin-1; #3738), eukaryotic translation initiation factor

2A (EIF2α; #5324), and its phosphorylation at ser51 (p-EIF2α^{s51}; #3398), Filamin C (FLNC; #86972), 78 kDa glucose-regulated protein (GRP78; #3177), Inositol requiring protein 1 (IRE1α; #3294), PRKR-like endoplasmic reticulum kinase (PERK; #3192), phosphorylation of ribosomal protein S6 kinase at thr389 (p-P70^{t389}; #9234), Sequestome-1 (SQSTM1/P62; #5114), Ribosomal protein S6 (S6; #2217) and its phosphorylation at ser240 and 244 (p-S6$^{s240/244}$; #2215), transcription factor EB (TFEB; #37785), Serine/threonine-protein kinase ULK1 (ULK1; #8054) and its phosphorylation at ser757 (p-ULK1^{s757}; #6888). An antibody that recognized two modifications of microtubule associated protein 1 light chain 3 (LC3-I and LC3-II; L7543) was purchased from Sigma Aldrich. Antibodies that recognized ubiquitinated proteins (sc-8017) and cytochrome C (CYCS; sc-8385) were purchased from Santa Cruz Biotechnology. The nontruncated full-length splice variant of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α1) was analysed with an antibody against the C-terminus of the protein (amino acids 777-797) (Calbiochem, Merck KGaA, Darmstadt, Germany). BAG family molecular chaperone regulator 3 (BAG3; #10599-1-AP) was measured with an antibody from Proteintech®. Cathepsin L (ab103574) and GAPDH (ab9485) were analysed with antibodies purchased from Abcam. As secondary antibodies, horseradish peroxidase conjugated IgG produced in rabbit, mouse or goat were used (Jackson ImmunoResearch Laboratories, PA).

Enzymatic assays

The enzymatic activity of the phosphofructokinase (PFK) and citrate synthase (CS) were analyzed with commercial kits (Activity Colorimetric Assay Kit, Sigma-Aldrich, St. Louis, USA) by an automated spectrophotometer (Konelab 20XTi – Thermo Scientific, Vantaa, Finland) according to the manufacturer's instructions for muscle homogenates.

Data analysis

The between groups effect was analysed by a repeated measures ANOVA. If the data was not normally distributed, the data was log-transformed before the repeated measures ANOVA-analysis. In addition, within-group effects in the CTRL and EX groups were analysed by a paired *t* test or a related samples Wilcoxon signed rank-test based on the distribution of the data (Shapiro-Wilk). To analyze possible differences in the baseline between the EX and CTRL groups, independent *t* test or Mann-Whitney U-test were used. Pearson's product moment correlation was used to analyze the correlations between the variables. All the statistical tests were conducted with PASW statistics version 24.0 (SPSS, Chicago, USA) or Excel software. The level of statistical significance was set at $P \leq 0.05$. Data in the text is expressed as means ± SD. In the figures, dashed lines depict single values whereas bolded lines depict the average of the groups.

RESULTS

Sprint performance improved by combined training in the EX group

As a result of 20-week periodized experimental training program (EX), 60-meter sprint-running time improved in EX ($P = 0.029$), whereas it declined in CTRL ($P = 0.042$, time x group-effect: $P = 0.003$) (Figure 2A). In the baseline, there was no significant difference in sprint performance between the groups ($P = 0.524$). Squat jump height ($P = 0.076$) tended to increase in the EX group (Figure 2B). VL muscle thickness did not change in either of the groups (Figure 2C). In the baseline, 60-meter sprint-running time and squat jump height correlated significantly with age ($r = 0.825$, $P = 3.86 \cdot 10^{-7}$; $r = -0.782$, $P = 6.43 \cdot 10^{-6}$, respectively; Figures 2D & E). In addition, there was a non-significant trend in the correlation ($r = -0.352$, $P = 0.066$) between VL thickness and

age (Figure 2F). Furthermore, there was a negative correlation (r = -0.487, *P* = 0.009) between age and body lean mass, while body fat percentage did not correlate significantly with age (Figures 2G & H).

Muscle autophagosome content increased in the EX group

At the baseline, of all the variables analysed, the only (*P* < 0.05) difference between the groups was in BCL-2 (*P* = 0.014). As a marker of autophagosome content, lipidated LC3 (LC3-II) increased only in the EX group (*P* = 0.022) whereas the unlipidated LC3-I was unchanged in both EX and CTRL (Figures 3A & B). An adaptor protein acting as a link between recyclable cellular material and autophagosomes, p62/SQSTM1 decreased only in the EX group (*P* = 0.006; Figure 3C). The rest of the analysed markers of autophagy (p-ULK1 at ser757, total ULK1, Beclin-1 and BCL-2) were unchanged in both groups (Figures 3D–F). Furthermore, TFEB, which among other functions regulates lysosome biogenesis, quantified at 53 kDa (calculated molecular mass) decreased by EX [time x group-effect: *P* = 0.002; see Supplemental Figure 1A, Supplemental Digital Content 1, Content of transcription factor EB (TFEB) and lysosomal protease (Cathpesin L) before (PRE) and after (POST) the 20-week intervention period in the experimental (EX) and control (CTRL) groups, http://links.lww.com/MSS/B954] and quantified at 65–70 kDa (predicted observed band size by the antibody manufacturer) increased only in the EX group (*P* = 0.040; see Supplemental Figure 1B, Supplemental Digital Content 1, http://links.lww.com/MSS/B954). In addition, the content of lysosome-resident Cathepsin L remained unchanged (see Supplemental Figure 1D, Supplemental Digital Content 1, http://links.lww.com/MSS/B954).

LC3-II content analysed from the baseline biopsies and the change in LC3-II did not correlate with age (r = 0.178, *P* = 0.364; r = -0.162, *P* = 0.410, respectively; Figures 3G & H). The indicators of

mTOR signaling, phosphorylated S6 at ser240/244 and total S6 did not change in either of the groups (Figures 4A & B). In addition, p-S6 at ser240/244 and another indicator of mTOR signaling, p-p70S6K at thr389 analysed from the baseline (PRE) biopsies did not correlate with age (r = -0.248, *P* = 0.203; r = -0.200, *P* = 0.308, respectively; Figures 4C & D). While mTOR can inactivate autophagy, AMP kinase (AMPK) is able to activate autophagy and we observed that downstream of AMPK, phosphorylated ACC at ser79 increased in the EX group (*P* = 0.014) and tended to increase in CTRL [*P* = 0.064; see Supplementary figure 2A, Supplemental Digital Content 2, Phosphorylated ACC at ser79 and total ACC (A & B) as well as indicators of protein ubiquitination (C) and mechanosensitive protein markers, http://links.lww.com/MSS/B955], while total ACC remained unchanged (see Supplementary figure 2B, Supplemental Digital Content 2, http://links.lww.com/MSS/B955).

To achieve a more comprehensive view of the protein degradation pathways, we measured the total content of ubiquitin-tagged proteins. Contrary to increased autophagosome content, marked by LC3-II, in the EX group, the content of ubiquitinated proteins did not change (see Supplementary figure 2C, Supplemental Digital Content 2, http://links.lww.com/MSS/B955). In addition, BAG3 that in response to mechanical strain regulates mTOR activity and is responsible for chaperone assisted selective autophagy (CASA) of actin and filamin (23) did not change in either of the groups (see Supplementary figure 2D, Supplemental Digital Content 2, http://links.lww.com/MSS/B955). Furthermore, the mechanosensitive filamin C (FLNC) did not change (see Supplementary figure 2E, Supplemental Digital Content 2, http://links.lww.com/MSS/B955).

Combined training in the EX group selectively modulated UPR components

To understand muscle protein homeostasis more thoroughly, we analysed several markers that are typically induced by endoplasmic reticulum (ER) stress that consequently activates unfolded protein response (UPR_{ER}). Of those markers, ATF4 content decreased only in the EX group (P = 0.003; Figure 5A). Upstream of the canonical signaling pathway that regulates ATF4 gene expression, phosphorylation of EIF2α at ser51 tended (P = 0.067; Figure 5B) to increase and total EIF2α increased (P = 0.019; Figure 5C) in the EX group while PERK did not change (Figure 5D). Additionally, the content of IRE1α and GRP78, which act as an upstream regulator of UPR and as an ER-resident chaperone, respectively, did not change in either of the groups (Figures 5E & F). However, IRE1α increased when compared to controls (time x group, P = 0.050, Figure 5E). At baseline, there was no significant correlation (r = -0.173, P = 0.380) between age and ATF4 protein content (Figure 5G).

Markers of glycolytic metabolism increased in the EX group

To get an overview of the experimental training induced changes in the energy metabolism pathways and to put autophagy and UPR results into context, we analysed a few key markers of oxidative and glycolytic energy metabolism. The content of cytochrome c (CYT C), which plays a key role in the electron transport chain in the mitochondria, increased in EX [P = 0.008; see Supplemental Figure 3A, Supplemental Digital Content 3, Indicators of oxidative (A-C) and glycolytic energy metabolism (D & F) before (PRE) and after (POST) the 20-week intervention period in experimental (EX) and control (CTRL) groups, http://links.lww.com/MSS/B956], while the master regulator of oxidative metabolism, PGC1-α1, did not change in either of the groups (see Supplemental Figure 3B, Supplemental Digital Content 3, http://links.lww.com/MSS/B956).

Additionally, the enzyme activity of the citrate synthase (CS) did not change (see Supplemental figure 3C, Supplemental Digital Content 3, http://links.lww.com/MSS/B956). GAPDH content increased only in EX suggesting an improvement in glycolytic capacity (P = 0.022; see Supplemental Figure 3D, Supplemental Digital Content 3, http://links.lww.com/MSS/B956). In addition, phosphofructokinase (PFK) activity non-significantly increased (P = 0.097) in EX (see Supplemental figure 3E, Supplemental Digital Content 3, http://links.lww.com/MSS/B956).

DISCUSSION

We found that a new training stimulus combining maximal and explosive strength exercises with sprint exercises improved sprinting performance without changes in vastus lateralis muscle thickness in competitive middle-aged and older male masters sprinters (40–76 years) with a long training history. The training program in the EX group was accompanied by an increased muscle LC3-II content suggesting increased muscle autophagosome content without any evidence of impaired autophagosome clearance (p62 decreased in EX) in basal state. In addition, we observed that UPR was selectively modulated in the EX group.

The primary aim of the experimental training program was to increase maximal and explosive strength capabilities of leg (especially knee and hip extensors) and core muscles. The increase in maximal and explosive strength capabilities would consequently improve 60-meter sprint running performance in the middle-aged and older masters athletes who showed a clear age-related decline in sprint running performance and explosiveness (Figures 2D & E). Indeed, we showed that the combined training was able to improve sprint performance, which occurred without muscle hypertrophy at least in knee extensor muscles. Moreover, the combined training increased the markers of anaerobic glycolytic metabolism (GAPDH and a trend in PFK activity) in VL muscle,

which might partially explain the improved performance. Previously, fixed plane strength training (squats, deadlifts etc.) and sprint-specific exercises (plyometrics, resisted sprinting etc.) as well as their combination have been shown to improve acceleration, maximum speed and overall sprint performance among high-level young sprinters <35 years (24). Evidence from a very few available studies on aging athletes also indicates that strength exercises combined with sprint run-based interval training can promote additional benefits in sports performance in comparison to sprint training alone (4).

We report that the incorporation of maximal and explosive strength exercises into a conventional run-based sprint-regimen led to an increase in lipidated form of LC3 that is bound to the autophagosome membrane and is thus a correlate of autophagosome content. (25). Thus, as we hypothesized, the results suggest that new training stimuli may be able to increase muscle autophagosome content, which results from increased autophagosome formation or decreased autophagosome clearance. Typically, when autophagosome clearance is decreased or impaired, the p62/SQSTM1 content increases in cells (26). Since p62 was decreased in EX, our results suggest that the increase in autophagosome content occurred without any evidence of impaired autophagosome clearance. However, the decreased content of p62 may also be due to a decrease in its protein synthesis. Therefore, to fully confirm our results, more kinetic methods to assess autophagy flux, in other words autophagosome formation and turnover, are warranted. The increase in the basal content of autophagosomes may be interpreted as an increased capacity to recycle damaged cellular material that is induced by a new exercise stimulus i.e. strength training. To indirectly support this claim, a previous study in young participants (27) reported that an eccentric RE bout disrupted Z-disc orientation, which was accompanied by a co-localization of LC3-II with filamin C that is a Z-disc resident protein and unfolds by mechanical loading.

Additionally, the study reported increased content of chaperone assisted selective autophagy (CASA) components including BAG3 (27). We did not, however, observe a change in BAG3 and filamin C content in EX.

A previous study (12) showed that autophagy markers (LC3-II and ATG7) are decreased in the skeletal muscle of older sedentary men compared with young sedentary men. Interestingly, regular physical activity (mainly endurance exercise) prevented the age-related decline in autophagy in that study. In the current study, we did not have muscle samples from young men (<40 years) nor from the sedentary older men, but autophagosome content analysed from the PRE biopsies did not correlate with age in the overall study population (40–76 years) (Figure 3G) suggesting that there may not be age-related differences in the basal muscle autophagosome content in the current study population of highly trained masters athletes. Moreover, our previous study with previously untrained men suggested that aging may blunt the strength training induced increase in muscle autophagosome content (9). However, in the current study, the change in the autophagosome content did not correlate with age in the EX and CTRL groups (data not shown) nor in the whole population with relatively wide age spectrum (40–76 years) (Figure 3H).

Because EX had an effect on the autophagy-lysosome degradation pathway, we assessed whether EX also had an effect on ubiquitin-proteasome pathway that is another major protein degradation pathway (7). We observed that EX had no effect on the total content of ubiquitinated proteins, suggesting that EX modulated mainly the autophagy-lysosome pathway of the protein degradation pathways. However, our study does not exclude the possible acute effects of exercise on these pathways in these participants.

mTOR signaling is activated acutely by an anabolic stimulus, for example RE or a meal rich in protein (28) and the temporary activation of mTOR is thought to be needed to counteract age-

related muscle atrophy and to induce muscle hypertrophy (29). However, previously it has been shown that muscle mTOR signaling is chronically increased in aged humans (30) and rodents (31) while chronically inhibiting mTOR has counteracted sarcopenia in aging mice (32). Increase in baseline mTOR signaling was suggested to inhibit autophagy and consequently induce muscle atrophy through the accumulation of non-functional cytotoxic cellular material (33). The increased mTOR signaling has also been suggested to contribute to the insulin and anabolic resistance, which are typical phenotypic characteristics of aging tissue (34). Interestingly, in the current study population (40–76 years), there were no associations between age and the indicators of mTOR signaling (Figures 4C & D) even though lean mass and muscle function negatively correlated and muscle thickness tended to correlate negatively with aging (Figures 2D–G). Our results suggest that in the current study population of highly fit and physically active masters athletes, there may not be no age-related increment in basal mTOR activity.

To further explore protein homeostasis, we analysed unfolded protein response (UPR) markers that are induced by protein misfolding in the lumen of endoplasmic reticulum (ER) (13). The current findings related to ER stress/UPR markers in aging muscle are somewhat contradictory (16). However, the upregulation of UPR-induced transcription factor ATF4 has been suggested to be one of the mediators of age-related muscle atrophy in mice (35). Interestingly, we did not find a correlation between ATF4 content and age suggesting that there is no age-related increase in ATF4 content in the current study population of middle-aged and older masters athletes (Figure 5G). Furthermore, ATF4 content in the baseline (PRE) did not correlate with VL muscle thickness (not shown).

Even though ATF4 content was not associated with age or muscle thickness in the baseline in the whole study population, its content was decreased in EX. The observed decrease in ATF4 content

may be considered as a beneficial adaptation because it is was reported to be associated with muscle atrophy (36, 37) and because it may depict an overall decrease in the basal level of ER stress. However, the other results of the present study (EIF2α increased, p-EIF2α tended to increase in EX, PERK and GRP78 unchanged in EX as well as IRE1α increased in EX compared with CTRL) suggest that EX had a selective effect on the content of UPR components. In contrast to the effects of long-term RT on basal muscle UPR components, acutely after single bout of RE in humans (9, 10) the majority of the UPR markers have been reported to consistently increase. Thus, to summarize, a selective and context-dependent pattern of UPR signaling may be expected after acute and long-term exercise.

The strength of the present study trial was that we had a highly specialized and homogeneous group of competitive masters athletes with an extensive experience in sprint training but no background in systematic strength training. Thus, this special population of masters athletes enabled us to study the effects of a new structured training stimulus with increased strength training volume on autophagy and UPR markers in trained population with a relatively wide spectrum of age. In addition, the intervention period was relatively long and thus capable of inducing long-term adaptations to the basal content of autophagy and UPR markers as well as performance. On the other hand, the limitation of our study was that we lacked acute samples after different exercise sessions. Furthermore, the present study was not strictly controlled and supervised because the athletes were living all over Finland. To counteract the lack of training supervision, participants were carefully informed about the training program and regularly completed training logs that were collected during the field tests in their hometowns. The variance in training adherence and the fact that also the competitive participants in the CTRL group pursued to improve their performance and trained throughout the training period, both of which might have increased

variance in the results. These above mentioned reasons might explain the lack of between group differences in the variables that changed within the EX group. Finally, there was no group of younger than 40-year-old athletes or sedentary older people in the present study, which would have enabled us to draw stronger conclusions regarding the effects of aging and life-long exercise training on muscle autophagy and protein homeostasis regulation. Moreover, future studies should include more people over 70 years of age because it is the age when the decline in physical performance begins, along with a rapid decline in muscle mass (4).

As a conclusion, the present study shows that even in middle-aged and older male masters sprinters with a with a long training history, new prolonged training stimulus combining maximal and explosive strength training with sprint training is able improve sprint running performance. Furthermore, possibly as a homeostatic response to the new prolonged training stimulus, muscle autophagosome content appears to increase in a basal state without any evidence of impaired autophagosome clearance that may suggest an increased recycling capacity of damaged cellular material induced by altered long-term training stimulus. Finally, muscle UPR signaling components seem to selectively respond to a new prolonged training stimulus and the physiological significance of these specific results need to be determined in the future studies.

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CONFLICTS OF INTEREST

The authors of this article declare no conflict of interest and that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The authors also state that results of the present study do not constitute endorsement by ACSM.

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FIGURE LEGENDS

Figure 1. Graphic illustration of the study design. The participants for the original randomized controlled training intervention were recruited from the masters athletes aged ≥40 years who took part in the cross-sectional study that served as baseline measurements (17). After the baseline measurements, 40 participants were originally randomly divided to an experimental group (EX) and 32 to a control group (CTRL). A subsample for the present study was selected based on muscle sample availability and to match the EX and CTRL groups with age. The participants in the EX group completed a periodized 20-week training program. The participants in the CTRL group were told to continue their accustomed training regimen. The PRE and POST measurements included anthropometry and performance tests on the first day and on the following morning, muscle biopsy was obtained from vastus lateralis (VL) muscle after an overnight fast (12 h).

Figure 2. Performance and muscle thickness before (PRE) and after (POST) the 20-week intervention period in the experimental (EX) and control (CTRL) groups (A–C) as well as correlations between performance and body composition (PRE) with age (D–H). A) 60-meter sprint-time; n = 12 in EX and n = 13 in CTRL. B) Squat jump height; n = 11 in EX and n = 13 in CTRL. C) Vastus lateralis (VL) muscle thickness; n = 15 in EX and n = 13 in CTRL. D) Correlation between 60-meter sprint time (PRE) and age. E) Correlation between squat jump height (PRE) and age. F) Correlation between vastus lateralis (VL) muscle thickness (PRE) and age. G) Correlation between body lean mass (PRE) and age, n = 15 in EX and n = 13 in CTRL. H) Correlation between body fat percentage (PRE) and age, n = 15 in EX and n = 13 in CTRL.

In A–C, dashed lines depict individual values, whereas bolded lines represent the average of the groups. In A–C, *P* values above the dashed lines depict the statistical significance within a group (PRE vs POST) whereas *P* values in the middle depict time x group effect.

Figure 3. Autophagy markers before (PRE) and after (POST) the 20-week intervention period in the experimental (EX) and control (CTRL) groups analysed from muscle biopsies obtained from vastus lateralis muscle and their representative immunoblots (A–F) as well as correlations between LC3-II and age (G & H). Protein contents of A) lipidated form of LC3 (LC3-II), B) cytosolic free form of LC3 (LC3-I), C) sequestome1/P62, D) the ratio of phosphorylated ULK1 at ser757 and total ULK1, E) Beclin-1 and F) BCL-2. G) Correlation between LC3-II (PRE) and age. I) Correlation between the fold-change in LC3-II (PRE vs. POST) and age. n = 15 in EX and n = 13 in CTRL. In A–F, dashed lines depict individual values, whereas bolded lines represent the average of the groups. In A–F, *P* values above the dashed lines depict the statistical significance within a group (PRE vs POST) whereas *P* values in the middle depict time x group effect. Stain-free blots are cropped at 25–42 kDa to save space but the whole lane was quantified.

Figure 4. Indicators of mTOR signaling, before (PRE) and after (POST) the 20-week intervention period in the experimental (EX) and control (CTRL) groups analysed from muscle biopsies obtained from vastus lateralis muscle and their representative immunoblots (A & B) as well as correlation between age and indicators of mTOR signaling (PRE). A) Content of phosphorylated S6 at ser240/244, B) total protein content of S6 and C) correlation between phosphorylated S6 at ser240/244 (PRE) and age. D) Correlation between phosphorylated p70S6K (PRE) at thr389 and age. n = 15 in EX and n = 13 in CTRL. In A & B, dashed lines

depict individual values, whereas bolded lines represent the average of the groups. In A & B, *P* values above the dashed lines depict the statistical significance within a group (PRE vs POST) whereas *P* values in the middle depict time x group effect. Stain-free blots are cropped at 25–42 kDa to save space, but the whole lane was quantified.

Figure 5. Endoplasmic reticulum stress induced unfolded protein response (UPR) markers before (PRE) and after (POST) the 20-week intervention period in experimental (EX) and control (CTRL) groups analysed from muscle biopsies obtained from vastus lateralis muscle and their representative immunoblots (A–F) as well as correlation between ATF4 (PRE) and age (G). Protein content of A) ATF4, B) phosphorylated EIF2α at ser51, C) total EIF2α, D) PERK, E) IRE1α, F) GRP78 and H) correlation between ATF4 content (PRE) and age. *n* = 15 in EX and *n* = 13 in CTRL. In A–F, dashed lines depict individual values, whereas bolded lines represent the average of the groups. In A–F, *P* values above the dashed lines depict the statistical significance within a group (PRE vs POST) whereas *P* values in the middle depict time x group effect. Stain-free blots are cropped at 25–42 kDa to save space, but the whole lane was quantified. Note that in B, C & D the representative blots were cropped from the same IDs analysed in the same run and thus share the same stain-free blot.

LIST OF SUPPLEMENTAL DIGITAL CONTENT

Supplemental digital content 1 (pdf.) Content of transcription factor EB (TFEB) and lysosomal protease (Cathpesin L) before (PRE) and after (POST) the 20-week intervention period in the experimental (EX) and control (CTRL) groups analysed from muscle biopsies obtained from vastus lateralis muscle and the representative immunoblots. Protein content of A) TFEB (observed band at 53 kDa which is its calculated molecular mass), B) TFEB (observed band at 65–70 kDa, predicted band size provided by the antibody manufacturer) and C) the quantified sum signal of the 53 kDa and 65–70 kDa bands of TFEB. D) Protein content of Cathepsin L. *n* = 15 in EX and *n* = 13 in CTRL. Dashed lines depict individual values, whereas bolded lines represent the average of the groups. *P* values above the dashed lines depict the statistical significance within a group (PRE vs POST) whereas *P* values in the middle depict time x group effect. Stain-free blots are cropped at 25–42 kDa to save space, but the whole lane was quantified.

Supplemental digital content 2 (pdf.). Phosphorylated ACC at ser79 and total ACC (A & B) as well as indicators of protein ubiquitination (C) and mechanosensitive protein markers (D & E) before (PRE) and after (POST) the 20-week intervention period in the experimental (EX) and control (CTRL) groups analysed from muscle biopsies obtained from vastus lateralis muscle and their representative immunoblots. A) Phosphorylated ACC at ser79, B) total ACC, C) ubiquitinated proteins, D) BAG3 and E) filamin C (FLNC). *n* = 15 in EX and *n* = 13 in CTRL except in C (*n* = 14 in EX). Dashed lines depict individual values, whereas bolded lines represent the average of the groups. *P* values above the dashed lines depict the statistical significance

within a group (PRE vs POST) whereas *P* values in the middle depict time x group effect. In figures A, B, D and E, stain-free blots are cropped at 25–42 kDa but the whole lane was quantified.

Supplemental digital content 3 (pdf.). Indicators of oxidative (A-C) and glycolytic energy metabolism (D & F) before (PRE) and after (POST) the 20-week intervention period in experimental (EX) and control (CTRL) groups analysed from muscle biopsies obtained from vastus lateralis muscle and the representative immunoblots. A) Cytochrome c (CYT C) and B) PGC1-α1. C) Enzymatic activity of citrate synthase (CS) and D) protein content of GAPDH. F) Enzymatic activity of phosphofructokinase (PFK). *n* = 15 in EX and *n* = 13 in CTRL in all variables except in PFK (*n* = 15 in EX and *n* = 12 in CTRL) and CS (*n* = 13 in EX and *n* = 12 in CTRL). Dashed lines depict individual values, whereas bolded lines represent the average of the groups. *P* values above the dashed lines depict the statistical significance within a group (PRE vs POST) whereas *P* values in the middle depict time x group effect. Stain-free blots are cropped at 25–42 kDa to save space, but the whole lane was quantified. Note that in A & B the representative blots from CTRL were cropped from the same IDs analysed in the same run and thus share the same stain-free blot.

Figure 1

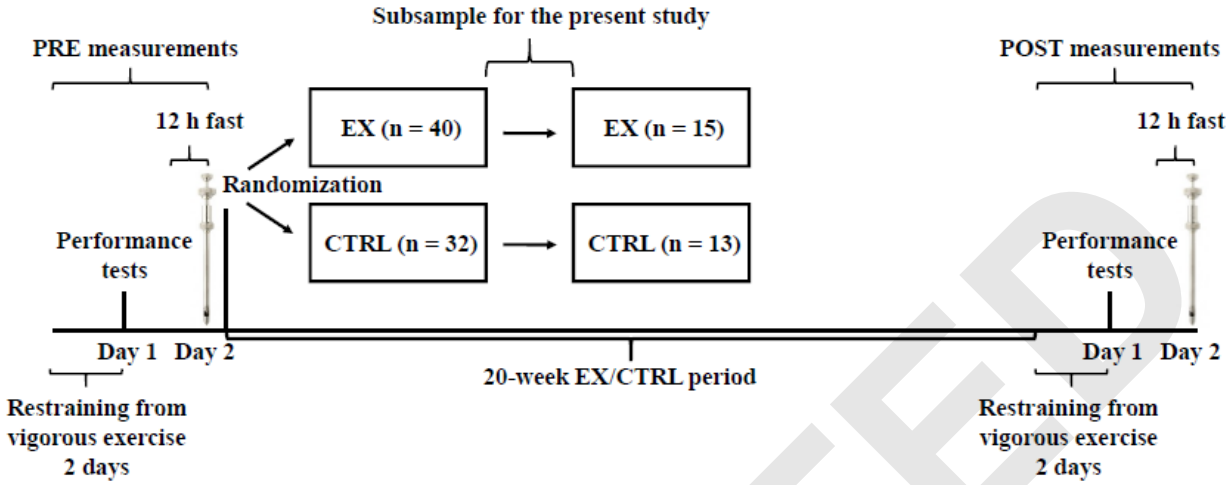


Figure 2

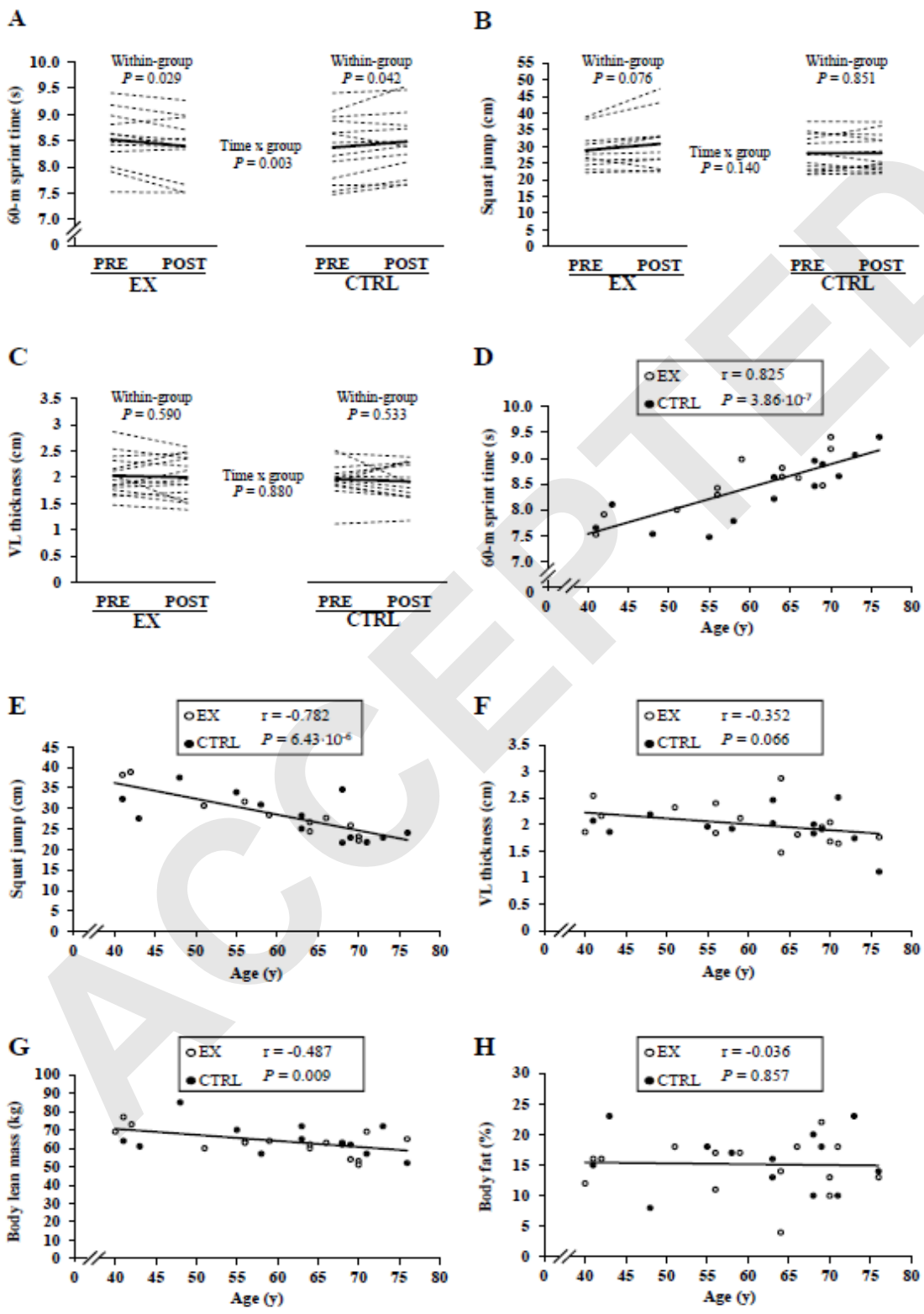


Figure 3

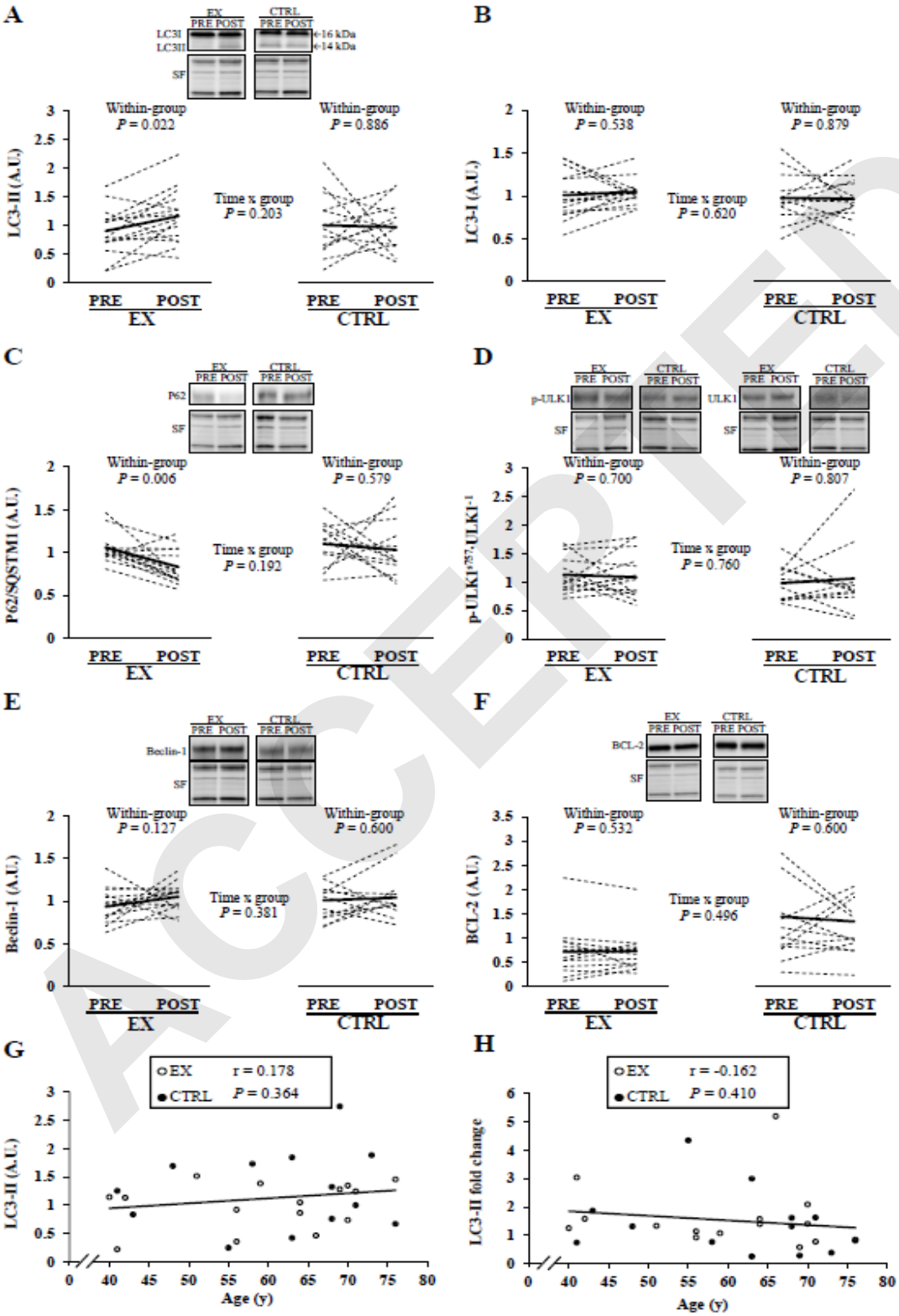


Figure 4

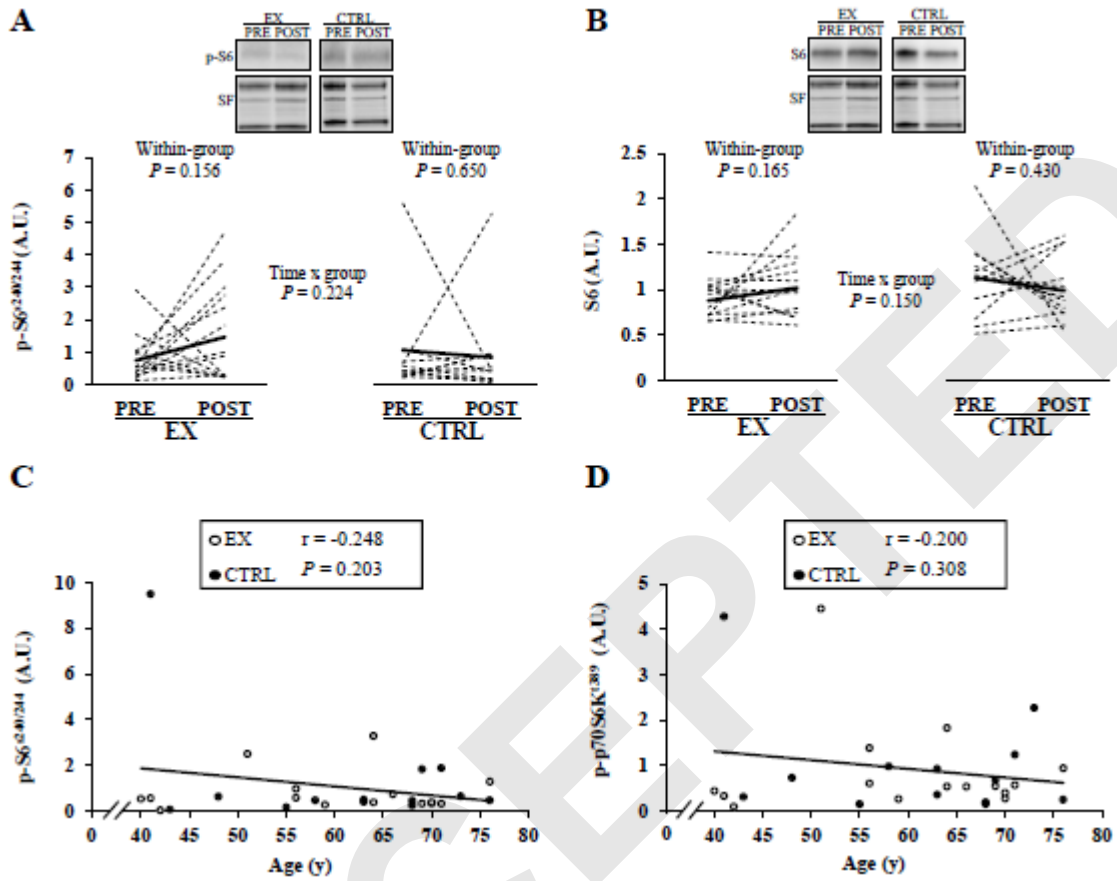
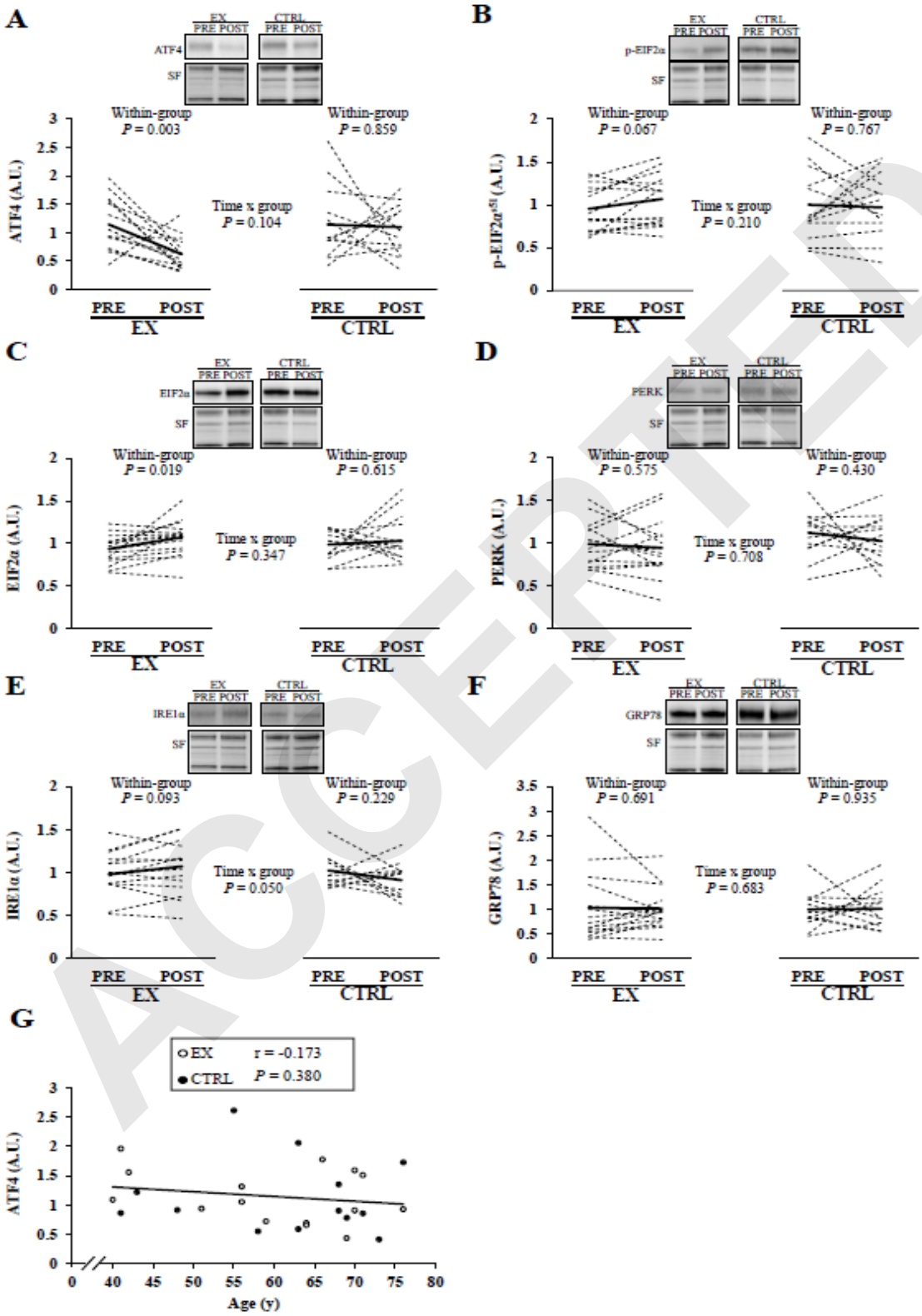
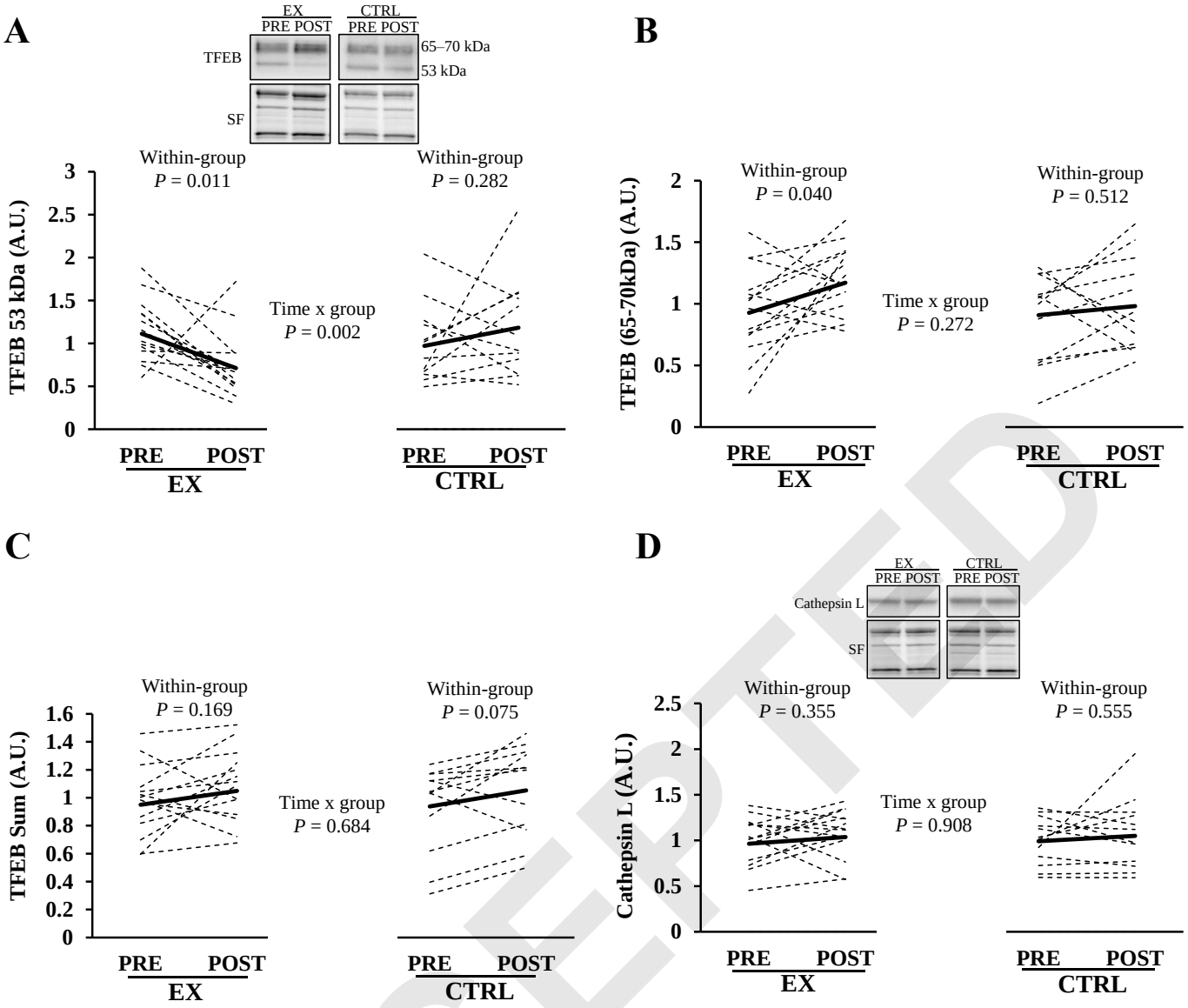


Figure 5





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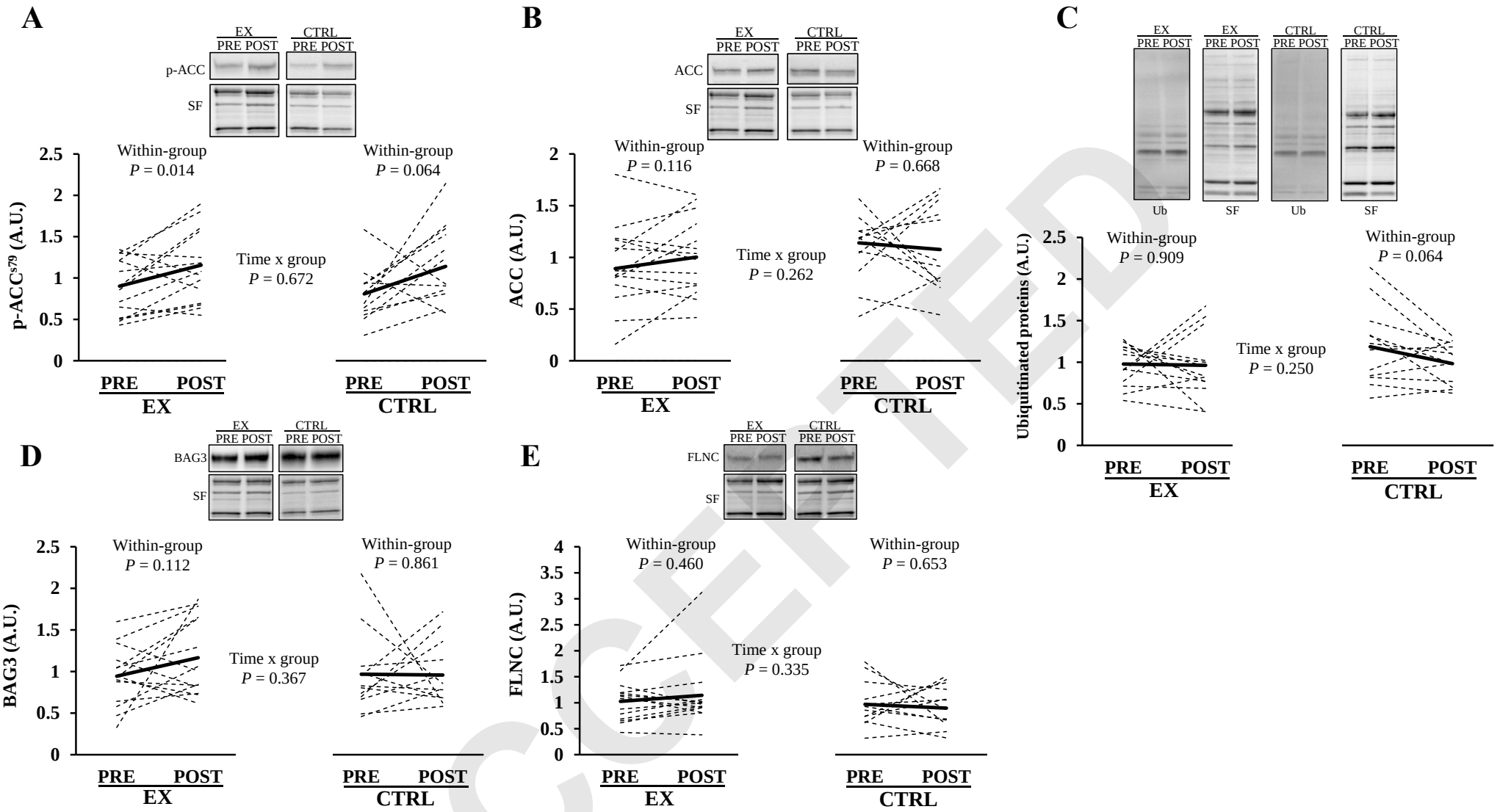

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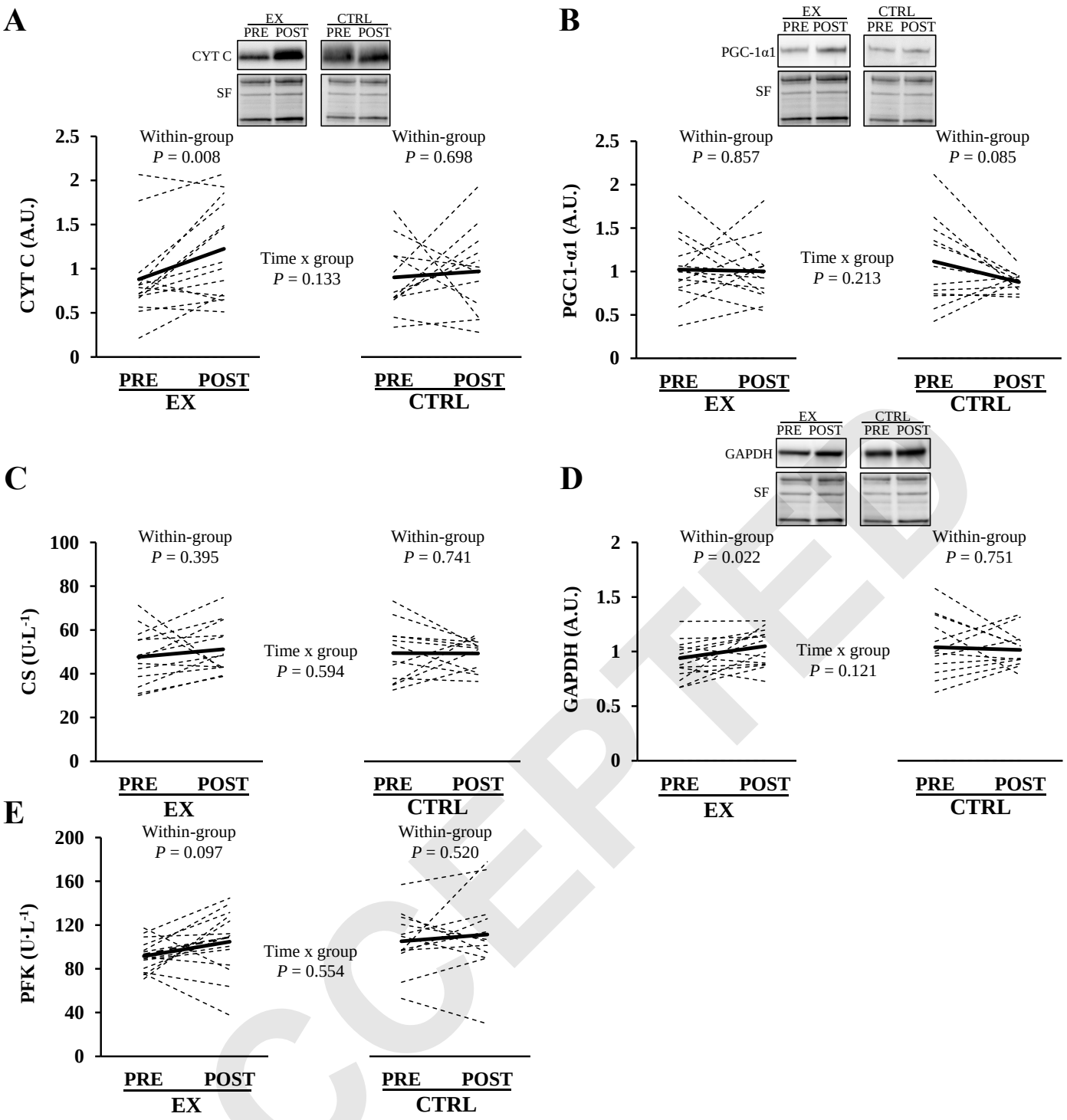

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