# High-intensity interval training blunts exercise-induced changes in markers of mitochondrial biogenesis in human skeletal muscle

Cesare Granata[1,2,*], Rodrigo S.F. Oliveira[1], Jonathan P. Little[3] and David J. Bishop[1,4,]

[1]Institute for Health and Sport, Victoria University, Melbourne, VIC, Australia

[2]Department of Diabetes, Central Clinical School, Monash University, Melbourne, VIC, Australia

[3]School of Health and Exercise Sciences, University of British Columbia Okanagan, Kelowna, BC, Canada

[4]School of Medical and Health Sciences, Edith Cowan University, Joondalup, WA, Australia.

***Corresponding author:** Cesare Granata, Department of Diabetes, Central Clinical School, Faculty of Medicine, Nursing and Health Sciences, Monash University, Alfred Centre, 99 Commercial Rd, Melbourne, 3004, VIC, Australia. Tel: +61 3 99030531; Email: cesare.granata@monash.edu

**Short title:** Blunted mitochondrial adaptations post training

## Abbreviations

20k-TT, 20-km cycling time trial; ACC, acetyl-CoA carboxylase; ACTB, β-actin; B2M, beta-2-microglobulin; BM, body mass; cyt *c*, cytochrome *c*; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GXT, graded exercise test; HIIE, high-intensity interval exercise; HIIT, high-intensity interval training; HVT, high-volume training; LDHA, lactate dehydrogenase A; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; NVT, normal-volume training; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PHF20, plant homeodomain finger-containing protein 20; TBP, TATA-binging protein; TFAM, mitochondrial transcription factor A; $\dot{V}O_{2Peak}$, peak oxygen uptake; $\dot{W}_{LT}$, power at the lactate threshold; $\dot{W}_{peak}$, peak power output.

## ABSTRACT

Exercise-induced increases in peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and p53 protein content in the nucleus mediate the initial phase of exercise-induced mitochondrial biogenesis. Here we investigated if these exercise-induced increases were maintained when repeating a high-intensity interval exercise (HIIE) session performed at the same absolute exercise intensity before and after 40 sessions of twice-daily high-volume high-intensity interval training (HVT) (Pre-HVT and Post-HVT, respectively). Vastus lateralis muscle biopsies were collected from 10 healthy participants before, immediately post, and 3h after the HIIE sessions. Protein contents of select markers of exercise-induced mitochondrial biogenesis were assessed in nuclear- and cytosolic-enriched fractions by immunoblotting; mRNA contents of key transcription factors and mitochondrial genes were assessed by qPCR. Despite exercise-induced increases in PGC-1α, p53, and plant homeodomain finger-containing protein 20 (PHF20) protein content, the phosphorylation of p53 and acetyl-CoA carboxylase (p-p53$^{Ser15}$ and p-ACC$^{Ser79}$, respectively), and PGC-1α mRNA Pre-HVT, no significant changes were observed Post-HVT. Forty sessions of twice-daily high-intensity interval training blunts exercise-induced molecular changes regulating the early events of mitochondrial biogenesis observed Pre-HVT. Future studies should determine if this loss relates to the decrease in relative exercise intensity, habituation to the same exercise stimulus, or a combination of both.


**Keywords**: endurance exercise, HIIT, mitochondrial adaptations, p53, PGC-1α

## Introduction

Mitochondria are responsible for the production of the majority of the energy required to sustain daily activities and are key regulator of energy homeostasis (1). The importance of mitochondria is underlined by the links between a healthy mitochondrial pool and enhanced endurance performance (2), improved health (3) and a reduced risk of several lifestyle-related chronic diseases (4, 5). Exercise has long been known to induce mitochondrial biogenesis (6) - the making of new components of the mitochondrial reticulum (7). However, despite the current knowledge on this topic (8-10), more research is required to better understand the molecular mechanisms mediating exercise-induced mitochondrial adaptations.

A key regulator of exercise-induced mitochondrial biogenesis is peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (11). In both rat (12) and human (13-16) skeletal muscle it has been observed that there is a post-exercise increase of PGC-1α protein content in the nucleus, where PGC-1α performs its transcriptional activity (17). In addition, the PGC-1α protein itself (18), as well as other proteins (e.g., p53 (19)), contribute to the exercise-induced upregulation of PGC-1α mRNA (20). However, this exercise-induced increase in PGC-1α mRNA appears to be reduced as a training intervention progresses (21-23). While this suggests the exercise-induced increase of PGC-1α protein in the nucleus may also be reduced after a short training intervention, no study has investigated exercise-induced changes in the content of nuclear proteins before and after a training intervention. Given that increased PGC-1α protein content in the nucleus represents an important process that contributes to the initial phase of exercise-induced mitochondrial biogenesis (12), it is important to better understand how the molecular response to exercise changes with training.

p53 is another important regulator of exercise-induced mitochondrial biogenesis in human skeletal muscle (24). Nuclear accumulation of p53 protein has been reported immediately (13), or 3 hours (25), after a single session of exercise. While the mechanisms underlying the nuclear accumulation of p53 are complex (26, 27), they have partly been attributed to phosphorylation of p53 at serine 15 (p-p53$^{Ser15}$) (27) - a posttranslational modification that enhances p53 protein stability (28) and prevents its nuclear export and cytosolic degradation (27, 29). However, once again, these molecular events have only been investigated following a single exercise session, and it is not known if they are altered by training. Given that the majority of the p53 activity takes place in the nucleus (27), it is important to determine if the early events of the p53-mediated exercise-induced mitochondrial biogenesis are differentially regulated in this subcellular compartment as the training intervention progresses.

To maintain the effectiveness of exercise to stimulate mitochondrial biogenesis, it is important to understand the molecular signals that are altered when an exercise stimulus is repeated. Therefore, the aim of our study was to investigate if a session of high-intensity interval exercise (HIIE), performed at the same absolute workload before and after a period of high-volume training (HVT; 40 sessions of high-intensity interval training [HIIT] performed twice-daily for 20 consecutive days), induces similar increases in the protein content of PGC-1α, p53, and p-p53$^{Ser15}$ in the nucleus. Upstream signaling, as well as mRNA content of several genes involved in exercise-induced mitochondrial biogenesis, were also investigated before and after HVT. We hypothesized that 40 sessions of HIIT would result in significantly reduced exercise-induced increases in these events mediating exercise-induced mitochondrial biogenesis.

## Materials and methods

### *Participants*

Ten healthy men (20 ± 2 y; 180 ± 12 cm; 80 ± 15 kg; 46.2 ± 7.6 mL · min$^{-1}$ · kg$^{-1}$), who were not regularly engaged in cycling-based sports, were moderately-trained (i.e., undertaking moderate, unstructured aerobic activity for less than 3 to 4 hours per week for at least 6 months prior to the study), and were non-smokers and free of medications, volunteered to participate in this study. Upon passing an initial medical screening participants were informed of the study requirements, risks, and benefits, before giving written informed consent. All experimental protocols and study's procedures were approved by the Victoria University Human Research Ethics Committee and conformed to the standards set by the latest revision of the Declaration of Helsinki. All participants completed the study; however, due to the limited amount of muscle tissue harvested during the second biopsy trial, data from one participant were excluded (including physiological and performance data).

### *Study design and testing*

This research was part of a larger, previously-published study investigating the effect of different training volumes on mitochondrial adaptations (30). The experimental protocol specific to the portion of the study described in this manuscript consisted of three tests, each separated by 48 to 72 hours, repeated before and after the HVT: a 20-km cycling time trial (20k-TT), a graded exercise test (GXT) and a HIIE biopsy trial (Pre-HVT and Post-HVT). During the 20 days of HVT participants performed HIIT twice a day (Figure 1). Prior to beginning this phase of the larger study, participants were familiarized with the 20k-TT, the GXT and the HIIE, and completed the normal volume training (NVT) phase, (12 HIIT sessions in 4 weeks; Figure 1). It has

been reported that the transcriptional response to the first session of exercise is reflective of muscle damage (31) and can differ significantly from the response to subsequent exercise sessions (22). Thus, the NVT phase served to habituate participants to the exercise stimulus. Participants were required to refrain from vigorous exercise for the 72 h preceding each test, from alcohol and any exercise for 24 h before testing, and from food and caffeine consumption for 3 h before each test.

*20k-TT.* Cycling time trials were performed on an electronically-braked cycle ergometer (Velotron, RacerMate, USA) after a 6-min warm-up were participants cycled for 4 min at 66% of the power attained at the lactate threshold ($\dot{W}_{LT}$), followed by 2 min at $\dot{W}_{LT}$, and 2 min of rest. During these tests, participants were only allowed access to cadence and completed distance. Heart rate was monitored (Polar-Electro, Finland) during all exercise trials and training sessions.

*GXT.* A discontinuous graded exercise test was performed on an electronically-braked cycle ergometer (Lode Excalibur, v2.0, The Netherlands) to determine peak oxygen uptake ($\dot{V}O_{2peak}$), peak power ($\dot{W}_{peak}$), and $\dot{W}_{LT}$ (using the modified $D_{max}$ method (32)), and the exercise intensity for both the biopsy trial and the HVT training sessions, as previously described (33). Briefly, the test began at 60, 90, or 120 W, depending on participants' fitness levels, and was increased by 30 W every 4 min. Stages were interspersed with 30-s breaks for measurement of fingertip capillary blood lactate concentration using a pre-calibrated blood-lactate analyzer (YSI 2300 STAT Plus, YSI, USA). Participants were instructed to keep a cadence above 60 rpm and were only allowed access to cadence and elapsed time; the GXT was terminated when participants reached volitional exhaustion or cadence dropped below 60 rpm. The $\dot{W}_{peak}$ was determined as the power of the last completed stage plus 7.5 W for every additional minute completed. $O_2$ and $CO_2$ concentrations were analyzed from expired air using a pre-calibrated gas analyzer (Moxus 2010, AEI technologies, USA), and $\dot{V}O_2$ values were recorded every 15 s. The average of the two highest consecutive 15-s values was recorded as a participant's $\dot{V}O_{2peak}$. The same GXT was performed after 20 days of training to determine the relative exercise intensity of the Post-HVT biopsy trial.

*Pre- and Post-HVT HIIE biopsy trials.* Each participant performed the two biopsy trials in the morning and at the same time, to avoid variations caused by circadian rhythms. Participants were provided with a standardized dinner (55 kJ·kg$^{-1}$ body mass (BM), providing 2.1 g carbohydrate·kg$^{-1}$ BM, 0.3 g fat·kg$^{-1}$ BM, and 0.6 g protein·kg$^{-1}$ BM) and breakfast (41 kJ·kg$^{-1}$ BM, providing 1.8 g carbohydrate·kg$^{-1}$ BM, 0.2 g fat·kg$^{-1}$ BM, and 0.3 g protein·kg$^{-1}$ BM) to minimize variability in muscle gene and protein expression attributable to diet, as previously described (30). While participants rested in the supine position, and after injection of

local anesthetic (1% xylocaine) into the skin and fascia of the vastus lateralis muscle, three small incisions were made about 2-3 cm apart. A resting muscle biopsy was taken (Rest) using a biopsy needle with suction. Approximately ten minutes after participants were helped to an electronically-braked cycle ergometer (Velotron, RacerMate, USA) and began a warm up consisting of cycling for four minutes at 66% of $\dot{W}_{LT}$, followed by 2 min at $\dot{W}_{LT}$, and 2 min of rest, after which the Pre-HVT HIIE session began. HIIE consisted of five 4-min intervals at an exercise intensity equal to $\dot{W}_{LT} + 0.2 (\dot{W}_{peak} - \dot{W}_{LT})$, interspersed with two minutes of recovery at 60 W. Immediately after termination of HIIE (~5 to 10 s) a second skeletal muscle biopsy was taken (+0 h), while a third one was obtained after three hours of recovery (+3 h), during which time participants were allowed access to water *ab libitum* and had no access to food. Skeletal muscle samples were rapidly cleaned of excess blood, fat, and connective tissue, were snap frozen in liquid nitrogen, and later stored at -80°C for subsequent analyses. By design, the Post-HVT HIIE biopsy trial was performed at the same absolute exercise intensity used during the Pre-HVT trial, and followed an identical format.

*HVT.* The day following the Pre-HVT HIIE biopsy trial participants began HIIT twice a day for 20 consecutive days. Training sessions were performed in the morning and afternoon and consisted of either five to twelve 4-min intervals at relative exercise intensities ranging from $\dot{W}_{LT} + 0.3 (\dot{W}_{peak} - \dot{W}_{LT})$ to $\dot{W}_{LT} + 0.8 (\dot{W}_{peak} - \dot{W}_{LT})$, interspersed with a 2-min recovery at 60 W, or eight to twenty-two 2-min intervals at relative exercise intensities ranging from $\dot{W}_{LT} + 0.5 (\dot{W}_{peak} - \dot{W}_{LT})$ to $\dot{W}_{LT} + 0.8 (\dot{W}_{peak} - \dot{W}_{LT})$, interspersed with a 1-min recovery at 60 W (30). Single-session duration increased from 30–35 min to 70–80 min. A 10 km cycling time trial was performed before, and at regular weekly intervals during, the HVT to monitor participants for signs of overreaching, as previously described (30). If performance would have decreased by more than 10%, the training load would have been reduced to prevent overreaching (34). No participants experienced a performance loss throughout the entire study, and the training protocol was completed as planned. All participants completed a minimum of 36 (equivalent to 90%) training sessions; average compliance was 96.5% of the prescribed number of sessions.

*Skeletal muscle analyses*

*Subcellular fractionation.* Nuclear and cytosolic fractions were prepared from 35 to 50 mg of skeletal muscle using a commercially-available nuclear extraction kit (NE-PER, Pierce, USA). Briefly, muscle samples were washed in phosphate-buffered saline (PBS), homogenized in CER-I buffer containing a protease/phosphatase

inhibitor cocktail (Cell Signaling Technology [CST], 5872) and centrifuged at ~16,000 g. The supernatant was taken as the crude cytosolic fraction. The pellets containing nuclei was washed six times in PBS to minimize cytosolic contamination and nuclear protein were extracted by centrifugation (~16,000 g) in a high-salt NER buffer supplemented with the same inhibitors cocktail and following the manufacturers' instructions. Protein concentration was determined in triplicate using a commercial colorimetric assay (Bio-Rad Protein Assay kit-II; Bio-Rad, Gladesville, NSW, Australia). Nuclear and cytosolic fraction enrichment was confirmed by blotting the separated fractions against a nuclear (histone H3) and a cytosolic (lactate dehydrogenase A [LDHA]) protein; histone H3 was mainly detected in nuclear fractions, whereas LDHA was mainly detected in cytosolic fractions (Figure 2A), indicating the subcellular fractionation enrichment was successful.

*Immunoblotting.* Muscle lysates (10 to 50 µg) were separated by electrophoresis using SDS-PAGE gels (8-15%) as previously described (13). An internal standard was loaded in each gel, and each lane was normalized to this value to reduce gel-to-gel variability. Whole-lane Coomassie blue staining (35) was performed to verify correct loading and equal transfer between lanes (Figure 2B). The following primary antibodies were used (supplier, catalogue number): histone H3 (CST, 9715), LDHA (CST, 2012), p53 (CST, 2527), p-acetyl-CoA carboxylase (p-ACC$^{Ser79}$; CST, 3661), PGC-1α (Calbiochem, st-1202), plant homeodomain finger-containing protein 20 (PHF20; CST, 3934), and p-p53$^{Ser15}$ (CST, 9284). Representative images for all target proteins are presented in Figure 2C.

*Total RNA isolation.* Total RNA was isolated from ~15 mg of muscle tissue as previously described (36). Briefly, samples were homogenized (FastPrep FP120 Homogenizer; Thermo Savant) in the presence of 1 g of zirconia/silica beads (1.0 mm; Daintree Scientific, St. Helens, TAS, Australia) and 800 µL of TRIzol® Reagent (Invitrogen, Melbourne, Australia). Lysates were centrifuged at 13,000 rpm for 15 min at 4°C; the supernatant was collected, combined with chloroform (Sigma-Aldrich, St Louis, USA), and total RNA was extracted using the TRIzol® protocol as per manufacturer's instructions. RNA precipitation was performed for at least 2 h at -20°C in the presence of 400 µL of isopropanol and 10 µL of 5 M NaCl (both Sigma-Aldrich, St Louis, USA). RNA concentration was determined spectrophotometrically (Nanodrop ND1000, Thermo Fisher Scientific, USA) by measuring the absorbance at 260 (A260) and 280 (A280) nm, with A260/A280 ratios > 1.8 indicating high-quality RNA (37). To ensure RNA was free of DNA contamination samples were DNase treated using an RQ1 RNase-free DNase kit (Promega Corporations, Madison, USA).

*Real-time PCR (qPCR)*. First-strand cDNA synthesis was performed on 300 ng of total RNA using a thermal cycler (S1000 Thermal Cycler; Bio-Rad; Bio-Rad, Gladesville, NSW, Australia) and the commercially available iScript™ cDNA synthesis kit (Bio-Rad, Gladesville, NSW, Australia) in the presence of random hexamers and oligo(dT)s, according to the manufacturer's directions. Forward and reverse primers for all genes investigated (Table 1) were designed based on NCBI RefSeq using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/BLAST/), and specificity of the amplified product was confirmed by melting point dissociation curves. The mRNA expression of cytochrome *c* (cyt *c*), nuclear respiratory factor 1 (NRF-1) and 2 (NRF-2), p53, PGC-1α, PHF20, and mitochondrial transcription factor A (TFAM) were quantified by quantitative real-time PCR (Mastercycler® RealPlex2, Eppendorf, Germany), using SYBR Green chemistry (iTaqTM Universal SYBR® Green Supermix; Bio-Rad, Gladesville, NSW, Australia) (10 µL PCR reaction volume). All samples were run in duplicate simultaneously with template free controls, using an automated pipetting system (epMotion 5070, Eppendorf, Germany) to reduce technical variation (37). The following PCR cycling patterns were used: initial denaturation at 95°C (3 min), 40 cycles of 95°C (15 s) and 60°C (60 s). Relative changes in mRNA content were calculated using the $2^{-\Delta\Delta Ct}$ method. To account for the efficiency of RT and initial RNA concentration, the mRNA expression of four housekeeping genes was quantified, and their stability was determined using the BestKeeper software (38). Cyclophilin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and beta-2-microglobulin (B2M) were classified as stable, whereas TATA-binging protein (TBP) was reported as unstable and was therefore excluded. These results were confirmed by the Normfinder algorithm (39).

*Statistical analysis*

All values are reported as mean ± SD unless otherwise specified. To investigate the influence of exercise (Rest, +0 h, and +3 h) and training (Pre-HVT and Post-HVT), and the interaction between these two variables, two-way ANOVA with repeated measures were performed on absolute values, followed by Tukey's honestly significant difference post-hoc tests to assess differences between time points. Specifically, significant interactions and main effects were further analyzed with pre-planned contrasts comparing the Pre- and Post-HVT trials separately. Resting protein and mRNA content values in the Pre- and Post-HVT trials were also compared with a pre-planned paired t-test. Sigma Stat software (Jandel Scientific, USA) was used for all statistical analyses. The level of statistical significance was set a priori at $P < 0.05$.

## Results

*Total work during the biopsy trials*

By design, the Pre- and Post-HVT HIIE sessions were performed at the same absolute exercise intensity (231.1 ± 33.1 W, Figure 3) and resulted in the same total work (277.3 ± 39.8 kJ). There was an increase (9.0 ± 6.1%, $P = 0.002$) in the power attained at the lactate threshold ($\dot{W}_{LT}$) following training (215.5 ± 32.2 *vs.* 234.7 ± 36.8 W, Pre- and Post-HVT, respectively; Figure 3), which resulted in the relative exercise intensity of the Pre-HVT biopsy trial (107.4 ± 1.2% of $\dot{W}_{LT}$) being greater than the Post-HVT biopsy trial (98.8 ± 5.2% of $\dot{W}_{LT}$). Following training, there was also an increase in peak power ($\dot{W}_{peak}$) (7.8 ± 4.4%, $P = 0.001$; 292.5 ± 37.9 *vs.* 315.2 ± 42.3 W, Pre- and Post-HVT, respectively; Figure 3); consequently, the exercise intensity expressed relative to $\dot{W}_{peak}$ was also greater in the Pre-HVT biopsy trial (78.9 ± 2.4% of $\dot{W}_{peak}$) than the Post-HVT biopsy trial (73.3 ± 3.7% of $\dot{W}_{peak}$). Post-HVT, there was an increase in peak oxygen uptake ($\dot{V}O_{2peak}$) (11.7 ± 7.6%, $P = 0.001$; 46.2 ± 7.6 *vs.* 51.4 ± 7.8 mL · min$^{-1}$ · kg$^{-1}$, Pre- and Post-HVT, respectively), whereas 20-km cycling time trial (20k-TT) time was decreased (5.2 ± 2.3%, $P < 0.001$; 2140.8 ± 99.9 *vs.* 2028.1 ± 87.5 s, Pre- and Post-HVT, respectively). The participants' BM did not change post training (0.2 ± 1.6 %, $P = 0.720$; 80.4 ± 14.8 *vs.* 80.6 ± 14.5 kg, Pre- and Post-HVT, respectively).

*Muscle analyses (representative immunoblots are presented in Figure 2C)*

*PGC-1α protein content.* There was an interaction effect in both subcellular compartments (nucleus: $P = 0.044$, cytosol: $P = 0.004$). In the nucleus (Figure 4A), PGC-1α was increased at +3 h compared with Rest during the Pre-HVT (3.1-fold, $P = 0.002$), but not during the Post-HVT (1.0-fold, $P = 0.869$) biopsy trial. During the Pre-HVT, nuclear PGC-1α was also greater at +3 h compared with Post-HVT (3.1-fold, $P = 0.015$). There was no significant difference in nuclear PGC-1α protein content at Rest between the two biopsy trials (1.8-fold, $P = 0.178$).

In the cytosol (Figure 4B), PGC-1α increased compared with Rest both at +0 h (1.8-fold, $P = 0.036$) and +3 h (2.2-fold, $P < 0.001$) during the Pre-HVT, but not during the Post-HVT (1.1-fold, $P = 1.000$ at +0 h; 0.8-fold, $P = 0.070$ at +3 h) biopsy trial. During the Pre-HVT biopsy trial, cytosolic PGC-1α was also greater at +3 h (1.5-fold, $P = 0.017$) compared with the same time point of the Post-HVT biopsy trial. At Rest, cytosolic PGC-1α was greater Post-HVT compared with Pre-HVT (2.0-fold, $P = 0.002$).

*Gene expression*. There was an interaction effect for PGC-1α mRNA content ($P = 0.020$; Figure 5A), which was increased at +3 h compared with Rest during the Pre-HVT (3.6-fold, $P < 0.001$), but not during the Post-HVT (2.0-fold, $P = 0.129$) biopsy trial. During the Pre-HVT biopsy trial, the mRNA content of PGC-1α at +3 h was also greater (1.9-fold, $P < 0.001$) compared with that recorded at the same time point during the Post-HVT biopsy trial. There was no change in p53 mRNA content throughout (interaction: $P = 0.425$; main effect of exercise: $P = 0.379$; Figure 5B). Results for the mRNA content of cyt *c*, NRF-1 and NRF-2, PHF20, and TFAM are reported in Table 2.

*Phosphorylation of acetyl-CoA carboxylase (ACC) at serine 79 (p-ACC$^{Ser79}$) protein content.* p-ACC$^{Ser79}$ was not detected in nuclear fractions (Figure 2C). In the cytosol (Figure 6), no interaction effect was reported ($P = 0.774$); however, there was a main effect of exercise ($P < 0.001$), whereby p-ACC$^{Ser79}$ was greater compared with Rest at +0 h (1.7-fold, $P < 0.001$). Pre-planned comparisons within biopsy trials indicated that at +0 h cytosolic p-ACC$^{Ser79}$ was greater compared with Rest during the Pre-HVT (2.0-fold, $P = 0.013$), but not during the Post-HVT (1.4-fold, $P = 0.114$) biopsy trial.

*p53 protein content.* In the nucleus (Figure 7A), there was an interaction effect ($P = 0.016$); nuclear p53 was increased at +3 h compared with Rest during the Pre-HVT (2.8-fold; $P = 0.004$), but not during the Post-HVT (1.2-fold, $P = 0.328$) biopsy trial. At Rest, nuclear p53 was greater Post-HVT compared with Pre-HVT (1.6-fold, $P = 0.042$)

In the cytosol (Figure 7B), the interaction effect was not statistically significant ($P = 0.051$); however, there was a main effect of exercise ($P = 0.003$). Cytosolic p53 increased compared with Rest both at +0 h (1.9-fold, $P = 0.019$) and +3 h (2.2-fold, $P = 0.004$). Pre-planned comparisons within trials revealed that during the Pre-HVT biopsy trial cytosolic p53 was greater compared with Rest at both +0 h (2.6-fold, $P = 0.020$) and +3 h (3.2-fold, $P < 0.001$); however, during the Post-HVT biopsy trial no differences compared with Rest were reported at +0h (1.3-fold, $P = 0.440$) and +3 h (1.2-fold, $P = 0.835$). There was no difference between the two biopsy trials at Rest (1.9-fold, $P = 0.108$).

*PHF20 protein content.* No interaction effects were observed in the nuclear ($P = 0.151$) and cytosolic ($P = 0.132$) fractions. In the nucleus (Figure 7C), there was a main effect of exercise ($P = 0.049$), and PHF20 was greater compared with Rest at +3 h (2.0-fold, $P = 0.040$). Pre-planned comparisons within biopsy trials indicated that at +3 h nuclear PHF20 was greater compared with Rest during the Pre-HVT (2.8-fold, $P =$

0.025), but not during the Post-HVT (1.3-fold, $P = 0.991$) biopsy trial. There was no difference between the two biopsy trials at Rest (1.9-fold, $P = 0.339$).

In the cytosol (Figure 7D), a main effect of exercise ($P = 0.014$) was also reported; cytosolic PHF20 was increased compared with Rest at +3 h (1.6-fold, $P = 0.012$). Pre-planned comparisons within biopsy trials indicated that at +3 h cytosolic PHF20 was greater compared with Rest during the Pre-HVT (2.2-fold, $P = 0.016$), but not the Post-HVT (1.1-fold, $P = 0.988$) biopsy trial. There was no difference between the two biopsy trials at Rest (1.5-fold, $P = 0.618$).

*p-p53$^{Ser15}$ protein content.* In the nucleus (Figure 7E), there was an interaction effect ($P = 0.021$); nuclear p-p53$^{Ser15}$ was increased compared with Rest at +3 h during the Pre-HVT (2.2-fold; $P = 0.001$), but not during the Post-HVT (1.3-fold, $P = 0.970$) biopsy trial.

In the cytosol (Figure 7F), there was no interaction effect ($P = 0.136$); however, there was a main effect of exercise ($P = 0.017$). Cytosolic p-p53$^{Ser15}$ was increased compared with Rest at +3 h (2.1-fold, $P = 0.014$). Pre-planned comparisons within biopsy trials indicated that cytosolic p-p53$^{Ser15}$ was greater compared with Rest at +3 h during the Pre-HVT (3.2-fold, $P = 0.003$), but not during the Post-HVT (1.1-fold, $P = 0.963$) biopsy trial.

## Discussion

We report for the first time that 40 sessions of HIIT resulted in the loss of all measured exercise-induced molecular changes recorded Pre-HVT. Despite exercise-induced increases in the protein content of PGC-1α, p-ACC$^{Ser79}$, p53, PHF20, and p-p53$^{Ser15}$, and the mRNA content of PGC-1α prior to HVT, there were no significant changes in any of these parameters when a session of HIIE was repeated at the same absolute exercise intensity post training. In most instances, this did not seem to be explained by increases in resting protein values Post-HVT. It remains to be determined if this loss stems from the decrease in the relative exercise intensity post-training (98.8 *vs.* 107.4% of $\dot{W}_{LT}$ for Post- and Pre-HVT, respectively), habituation to the HIIE stimulus, or a combination of both.

We observed a significant exercise-induced increase in PGC-1α protein content in both the nuclear and cytosolic fractions Pre-HVT, consistent with most previous research (13-16). However, for the first time we report that these exercise-induced increases were absent post training in both subcellular fractions. Although no direct comparison with previous research is possible, our findings are consistent with the lack of exercise-

induced increase in PGC-1α protein content in whole-muscle lysates reported after a 12-day, 10-session, training intervention (23). A possible explanation for our findings is that Post-HVT the relative exercise intensity elicited during the session was lower compared with Pre-HVT (98.8 *vs.* 107.4% of $\dot{W}_{LT}$ for Post- and Pre-HVT, respectively), suggesting that metabolic perturbations may have been reduced post-training. It has been proposed that metabolic perturbations (e.g., increases in intracellular calcium [$Ca^{2+}$], adenosine monophosphate [AMP] to adenosine triphosphate [ATP] ratio, oxidized nicotinamide adenine dinucleotide [$NAD^+$] to NADH ratio, reactive oxygen species [ROS] production) provide an important stimulus for exercise-induced mitochondrial biogenesis (8), and promote an increase in the nuclear content of PGC-1α protein (12). Indeed, as described more in depth later in the discussion, we also observed no significant changes in cytosolic p-$ACC^{Ser79}$ Post-HVT, which supports this hypothesis.

A strength of our study design is that participants were habituated to HIIE (12 sessions) during the NVT phase (30). This reduces the possibility that the different molecular responses to the Pre- and Post-HVT trials were due to muscle damage (31) or being unfamiliar with this type of exercise during the Pre-HVT trial (40). Future studies should investigate the effects of a single session of exercise performed at the same relative exercise intensity before, at different time points during, and after, a high volume of training (e.g., >25-30 sessions), to determine if the complete loss of the exercise-induced increases in nuclear and cytosolic PGC-1α protein content also occurs if the relative exercise intensity is maintained pre- and post-training.

The reported increase in PGC-1α protein content in both the nucleus and the cytosol may be attributable, at least in part, to increased protein stability (41). Both p38 mitogen-activated protein kinase (MAPK) (42) and AMP-activated protein kinase (AMPK) (43) act as signaling proteins that increase PGC-1α stability via phosphorylation. Due to the limited amount of enriched lysates obtained during subcellular fractionation, we could not measure phosphorylation of p38 MAPK and/or AMPK directly. However, due to its molecular weight (~280 kDa), when blotting for lower molecular weight proteins we were also able to measure p-$ACC^{Ser79}$, a downstream target and commonly-used marker of AMPK activation (44-46). As expected, cytosolic p-$ACC^{Ser79}$ (p-$ACC^{Ser79}$ was not detected in nuclear fractions as previously reported (13, 16)) increased immediately post exercise Pre-HVT, but not Post-HVT, suggesting that abrogation of AMPK signaling may explain, at least in part, the abrogation of exercise-induced increases in PGC-1α protein content post-training. Subcellular translocation is another factor that has been associated with increased PGC-1α protein content in the nucleus (12). While our data do not seem to indicate cytosolic/nuclear shuttling of PGC-

1α, protein translocation is a complex series of cellular processes that cannot be assessed by subcellular fractionation coupled with the immunoblotting technique (47).

The PGC-1α protein itself has been reported to increase PGC-1α transcriptional activity following exercise (19, 20) via an autoregulatory loop (18). The exercise-induced increase in PGC-1α mRNA content observed Pre-HVT is consistent with previous findings investigating HIIE (21, 22, 48-52) and with the notion that increased nuclear PGC-1α protein content and stability is associated with greater PGC-1α transcriptional activity (53). No exercise-induced increase in PGC-1α mRNA content was reported Post-HVT, suggesting that 20 days of HVT also blunted the exercise-induced increase in PGC-1α transcription. However, previous studies have reported a reduction (rather than complete loss) of the exercise-induced upregulation of PGC-1α mRNA content post-training compared to pre-training when the exercise session was repeated at the same relative (22) or absolute (21, 23) exercise intensity. This discrepancy may relate to the much greater number of sessions performed between exercise biopsy trials in our study compared with these three previous studies (40 *vs.* 7 to 12, respectively), and a likely greater reduction in the relative exercise intensity between the Pre- and Post-HVT trials. Moreover, in contrast to the three previous studies, our participants were habituated to HIIE; this raises the possibility that the greater molecular response recorded pre-training in the previous studies may be partly attributable to the "first bout" effect (31, 40). Finally, findings from Perry, et al. (22) seem to suggest that it is the repetition of the same exercise session (even at the same relative exercise intensity) rather than exercise intensity per se that may be driving this decrease/elimination in exercise-induced increases in PGC-1α mRNA (and protein) content post-training.

To better characterize the effect of 40 sessions of HIIT on exercise-induced mitochondrial adaptations to HIIE, we also measured the mRNA content of nuclear (NRF-1 and NRF-2 (54)) and mitochondrial (TFAM (55)) transcription factors regulating mitochondrial biogenesis that are transcriptionally controlled by PGC-1α (11). The mRNA content of cyt *c* (a gene under the regulation of PGC-1α and NRF1 (11)), p53 (a transcriptional regulator of PGC-1α gene expression (19)) and PHF20 (a transcription factor that activates p53 gene expression (56)), were also measured. Apart from a decrease in cyt *c* mRNA content at +0 h in both HIIE trials, we observed no exercise-induced increases in any other gene either Pre- or Post-HVT. This is consistent with the majority of previous findings, as discussed in more detail in a previously published review article (9). A possible explanation for our findings may relate to biopsy timing, as there is evidence that the exercise-induced upregulation of some of these genes peaks more than 3 hours post-exercise (9, 48, 56-59).

353 Similar to our results for PGC-1α protein we observed an exercise-induced increase in p53 protein content
354 pre-training in the nuclear and cytosolic fractions, as previously demonstrated (13, 25). However, this
355 exercise-induced increase in both subcellular fractions was blunted following 40 training sessions. No study
356 has previously investigated exercise-induced changes in p53 protein content pre- and post-training in
357 subcellular fractions. Nonetheless, our results are consistent with findings showing reduced/blunted exercise-
358 induced mitochondrial adaptations (e.g., PGC-1α mRNA, PGC-1α protein in whole-muscle lysates) when the
359 same exercise session is repeated post-training both at the same absolute (21, 23) or relative (22) exercise
360 intensity.

361 A possible factor contributing to the lack of exercise-induced changes in nuclear p53 protein content Post-
362 HVT is that 40 sessions of HIIT increased the resting values of p53 in the nucleus. This does not apply to the
363 cytosolic fraction, however, given that resting values of p53 protein remained unchanged. A second factor
364 relates to a possible decrease in subcellular shuttling (60); however, simply immunoblotting subcellular
365 enriched fractions for p53 (or PGC-1α) protein is not a valid technique to demonstrate p53 (similar to PGC-
366 1α) nuclear/cytosolic shuttling - a process requiring an intricate and tightly synchronized series of events (26,
367 60). Nonetheless, we observed a concomitant increase in p53 and PHF20 protein content in the nucleus pre-
368 HVT (as previously demonstrated (13)), but not Post-HVT. In this regard, PHF20 has been reported to
369 increase p53 protein stability (27) by disrupting the murine double minute-2 (MDM2)-p53 interaction (61)
370 responsible for p53 protein degradation (27, 29). Although we were not able to measure the interaction
371 between these two proteins due to limited lysate availability, it is plausible that our findings may indicate
372 greater p53-PHF20, and reduced p53-MDM2, interaction Pre- vs Post-HVT. In the cytosolic enriched
373 fractions, findings are similar; however, the increase in p53 protein content at +0 h was not matched by an
374 increase in PHF20 protein content. This could suggest that cellular events other than the p53 interaction with
375 PHF20 may also induce p53 protein stability.

376 A second important event disrupting the p53-MDM2 interaction and promoting p53 stability is
377 phosphorylation of p53 at serine 15 (28). Pre-HVT, and consistent with this notion, nuclear p-p53$^{Ser15}$
378 increased in parallel with the increase in p53 protein content, as previously reported (13). In the cytosol,
379 however, p53 protein content increased earlier than p-p53$^{Ser15}$ (+0 h *vs.* +3 h, respectively), indicating once
380 again that other cellular mechanisms may be involved in the regulation of the p53 protein stability. In
381 contrast, no exercise-induced changes in p-p53$^{Ser15}$ in either the nuclear or cytosolic fractions were reported
382 Post-HVT.

Phosphorylation of p53 at serine 15 is regulated by signaling kinases such as p38 MAPK (62) and AMPK (63). For the same reasons explained above, we were only able to measure p-ACC$^{Ser79}$ as a readout of AMPK activation (46). In the cytosol, p-ACC$^{Ser79}$ was increased at +0 h during the Pre-HVT, but not during the Post-HVT biopsy trial, consistent with the exercise-induced increase reported in p-p53$^{Ser15}$. This change may have contributed to the abrogation of the p53-mediated exercise-induced changes Post-HVT.

This research adds novel information regarding the early events regulating the exercise-induced mitochondrial response to an HIIE session repeated at the same absolute exercise intensity before and after a training intervention. We provide evidence that 40 sessions of HIIT blunted the exercise-induced increases recorded pre-training in all of the molecular events measured. Future studies should investigate if the loss (or reduction) of the exercise-induced increases in markers of mitochondrial adaptations post-training relates solely to the decrease in relative exercise intensity, and/or if this is exacerbated by the continuous repetition of the same exercise stimulus during the training intervention. Well-designed experiments comparing exercise sessions repeated pre- and post-training at the same relative exercise intensity and at different time points during the training intervention (even only after 1 or 2 training sessions to determine the role, if any, of the "first bout effect") should provide valuable insight into the mechanisms driving this phenomenon. Where possible, these experiments should also employ more novel and sophisticated techniques for the assessment of a multitude of exercise-induced mitochondrial changes (e.g., transcriptomics, proteomics), as well as measuring metabolic perturbations post-exercise (e.g. lactate and other metabolites).

## Acknowledgements

We thank the participants for their time, effort and commitment to this study. The authors would like to acknowledge Ms. Elise Brentnall and Mr. Maarten Missinne for their valuable help in data collection and biochemical analyses, respectively.

## Author contributions

D. J. Bishop and C. Granata designed the research; C. Granata and R. S. F. Oliveira conducted the research; C. Granata, R. S. F. Oliveira, J. P. Little, and D. J. Bishop analyzed and interpreted the data; C. Granata wrote the manuscript; C. Granata, R. S. F. Oliveira, J. P. Little, and D. J. Bishop critically revised and contributed to the manuscript; C. Granata and D. J. Bishop have primary responsibility for final content. Data collection took place at Victoria University. Muscle analysis took place at Victoria University and the University of British Columbia Okanagan. All persons designated as authors qualify for authorship, and all those qualifying for authorship are listed. All authors have read and approved the final manuscript.

## Conflict of interest

The authors declare no conflict of interest.

## Funding

This study was funded by a grant from the ANZ-MASON Foundation provided to DJB and a Natural Sciences and Engineering Research Council of Canada Discovery Grant to JPL.

## References


1. Handschin, C., and Spiegelman, B. M. (2006) Peroxisome proliferator-activated receptor γ coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocr. Rev.* **27**, 728-735
2. Jacobs, R. A., and Lundby, C. (2013) Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes. *J. Appl. Physiol.* **114**, 344-350
3. Nunnari, J., and Suomalainen, A. (2012) Mitochondria: in sickness and in health. *Cell* **148**, 1145-1159
4. Booth, F. W., Gordon, S. E., Carlson, C. J., and Hamilton, M. T. (2000) Waging war on modern chronic diseases: primary prevention through exercise biology. *J. Appl. Physiol.* **88**, 774-787
5. Luft, R. (1994) The development of mitochondrial medicine. *Proc Natl Acad Sci USA* **91**, 8731-8738
6. Holloszy, J. O. (1967) Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J. Biol. Chem.* **242**, 2278-2282
7. Granata, C., Jamnick, N. A., and Bishop, D. J. (2018) Training-induced changes in mitochondrial content and respiratory function in human skeletal muscle. *Sports Med.* **48**, 1809–1828
8. Egan, B., and Zierath, J. R. (2013) Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab.* **17**, 162-184
9. Granata, C., Jamnick, N. A., and Bishop, D. J. (2018) Principles of exercise prescription, and how they influence exercise-induced changes of transcription factors and other regulators of mitochondrial biogenesis. *Sports Med.* **48**, 1541–1559
10. Hood, D. A. (2009) Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle. *Appl. Physiol. Nutr. Metab.* **34**, 465-472
11. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C., and Spiegelman, B. M. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98**, 115-124
12. Wright, D. C., Han, D. H., Garcia-Roves, P. M., Geiger, P. C., Jones, T. E., and Holloszy, J. O. (2007) Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1α expression. *J. Biol. Chem.* **282**, 194-199
13. Granata, C., Oliveira, R. S. F., Little, J. P., Renner, K., and Bishop, D. J. (2017) Sprint-interval but not continuous exercise increases PGC-1α protein content and p53 phosphorylation in nuclear fractions of human skeletal muscle. *Sci. Rep.* **7**, 44227

14. Heesch, M. W., Shute, R. J., Kreiling, J. L., and Slivka, D. R. (2016) Transcriptional control, but not subcellular location, of PGC-1α is altered following exercise in a hot environment. *J. Appl. Physiol.* **121**, 741-749

15. Little, J. P., Safdar, A., Bishop, D., Tarnopolsky, M. A., and Gibala, M. J. (2011) An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1alpha and activates mitochondrial biogenesis in human skeletal muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R1303-1310

16. Little, J. P., Safdar, A., Cermak, N., Tarnopolsky, M. A., and Gibala, M. J. (2010) Acute endurance exercise increases the nuclear abundance of PGC-1α in trained human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **298**, R912-R917

17. Puigserver, P., and Spiegelman, B. M. (2003) Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α): Transcriptional coactivator and metabolic regulator. *Endocr. Rev.* **24**, 78-90

18. Handschin, C., Rhee, J., Lin, J., Tarr, P. T., and Spiegelman, B. M. (2003) An autoregulatory loop controls peroxisome proliferator-activated receptor γ coactivator 1α expression in muscle. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 7111-7116

19. Irrcher, I., Ljubicic, V., Kirwan, A. F., and Hood, D. A. (2008) AMP-activated protein kinase-regulated activation of the PGC-1α promoter in skeletal muscle cells. *PLoS One* **3**

20. Pilegaard, H., Saltin, B., and Neufer, D. P. (2003) Exercise induces transient transcriptional activation of the PGC-1α gene in human skeletal muscle. *J. Physiol.* **546**, 851-858

21. Morrison, D., Hughes, J., Della Gatta, P. A., Mason, S., Lamon, S., Russell, A. P., and Wadley, G. D. (2015) Vitamin C and E supplementation prevents some of the cellular adaptations to endurance-training in humans. *Free Radic. Biol. Med.* **89**, 852-862

22. Perry, C. G. R., Lally, J., Holloway, G. P., Heigenhauser, G. J. F., Bonen, A., and Spriet, L. L. (2010) Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J. Physiol.* **588**, 4795-4810

23. Stepto, N. K., Benziane, B., Wadley, G. D., Chibalin, A. V., Canny, B. J., Eynon, N., and McConell, G. K. (2012) Short-term intensified cycle training alters acute and chronic responses of PGC1α and cytochrome c oxidase IV to exercise in human skeletal muscle. *PLoS One* **7**

24. Saleem, A., Carter, H. N., Iqbal, S., and Hood, D. A. (2011) Role of p53 within the regulatory network controlling muscle mitochondrial biogenesis. *Exerc. Sport Sci. Rev.* **39**, 199-205

25. Tachtsis, B., Smiles, W., Lane, S., Hawley, J., and Camera, D. M. (2016) Acute endurance exercises induces nuclear p53 abundance in human skeletal muscle. *Front. Physiol.* **7**

26. Marchenko, N. D., Hanel, W., Li, D., Becker, K., Reich, N., and Moll, U. M. (2010) Stress-mediated nuclear stabilization of p53 is regulated by ubiquitination and importin-α3 binding. *Cell Death Differ.* **17**, 255-267

27. Oren, M. (1999) Regulation of the p53 tumor suppressor protein. *J. Biol. Chem.* **274**, 36031-36034

28. Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91**, 325-334

29. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387**, 296-299

30. Granata, C., Oliveira, R. S. F., Little, J. P., Renner, K., and Bishop, D. J. (2016) Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle. *FASEB J.* **30**, 3413-3423

31. Murton, A. J., Billeter, R., Stephens, F. B., Des Etages, S. G., Graber, F., Hill, R. J., Marimuthu, K., and Greenhaff, P. L. (2013) Transient transcriptional events in human skeletal muscle at the outset of concentric resistance exercise training. *J. Appl. Physiol.* **116**, 113-125

32. Bishop, D. J., Jenkins, D. G., McEniery, M., and Carey, M. F. (2000) Relationship between plasma lactate parameters and muscle characteristics in female cyclists. *Med. Sci. Sports Exerc.* **32**, 1088-1093

33. Granata, C., Oliveira, R. S. F., Little, J. P., Renner, K., and Bishop, D. J. (2016) Training intensity modulates changes in PGC-1α and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle. *FASEB J.* **30**, 959-970

34. Halson, S. L., Bridge, M. W., Meeusen, R., Busschaert, B., Gleeson, M., Jones, D. A., and Jeukendrup, A. E. (2002) Time course of performance changes and fatigue markers during intensified training in trained cyclists. *J. Appl. Physiol.* **93**, 947-956

35. Welinder, C., and Ekblad, L. (2011) Coomassie staining as loading control in Western blot analysis. *J. Proteome Res.* **10**, 1416-1419

36. Eaton, M., Granata, C., Barry, J., Safdar, A., Bishop, D., and Little, J. P. (2018) Impact of a single bout of high-intensity interval exercise and short-term interval training on interleukin-6, FNDC5, and METRNL mRNA expression in human skeletal muscle. *Journal of Sport and Health Science* **7**, 191-196

37. Kuang, J., Yan, X., Genders, A. J., Granata, C., and Bishop, D. J. (2018) An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PLoS One* **13**, e0196438

509 38. Pfaffl, M. W., Tichopad, A., Prgomet, C., and Neuvians, T. P. (2004) Determination of stable
510 housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool
511 using pair-wise correlations. *Biotechnol. Lett.* **26**, 509-515
512 39. Andersen, C. L., Jensen, J. L., and Ørntoft, T. F. (2004) Normalization of Real-Time Quantitative
513 Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for
514 Normalization, Applied to Bladder and Colon Cancer Data Sets. *Cancer Res.* **64**, 5245-5250
515 40. Bishop, D. J., Botella, J., Genders, A. J., Lee, M. J.-C., Saner, N. J., Kuang, J., Yan, X., and Granata,
516 C. (2019) High-Intensity Exercise and Mitochondrial Biogenesis: Current Controversies and Future Research
517 Directions. *Physiology* **34**, 56-70
518 41. Rodgers, J. T., Lerin, C., Gerhart-Hines, Z., and Puigserver, P. (2008) Metabolic adaptations through
519 the PGC-1α and SIRT1 pathways. *FEBS Lett.* **582**, 46-53
520 42. Puigserver, P., Rhee, J., Lin, J., Wu, Z., Yoon, J. C., Zhang, C. Y., Krauss, S., Mootha, V. K., Lowell,
521 B. B., and Spiegelman, B. M. (2001) Cytokine Stimulation of Energy Expenditure through p38 MAP Kinase
522 Activation of PPARγ Coactivator-1. *Mol. Cell* **8**, 971-982
523 43. Canto, C., and Auwerx, J. (2009) PGC-1α, SIRT1 and AMPK, an energy sensing network that
524 controls energy expenditure. *Curr. Opin. Lipidol.* **20**, 98-105
525 44. Chen, Z. P., Stephens, T. J., Murthy, S., Canny, B. J., Hargreaves, M., Witters, L. A., Kemp, B. E.,
526 and McConell, G. K. (2003) Effect of exercise intensity on skeletal muscle AMPK signaling in humans.
527 *Diabetes* **52**, 2205-2212
528 45. Chen, Z.-P., McConell, G. K., Michell, B. J., Snow, R. J., Canny, B. J., and Kemp, B. E. (2000)
529 AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase
530 phosphorylation. *American Journal of Physiology-Endocrinology And Metabolism* **279**, E1202-E1206
531 46. Jäger, S., Handschin, C., St-Pierre, J., and Spiegelman, B. M. (2007) AMP-activated protein kinase
532 (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proc Natl Acad Sci USA* **104**,
533 12017-12022
534 47. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2007) *Molecular Biology of*
535 *the Cell. 5th edition*, Garland Science, New York, NY:
536 48. De Filippis, E., Alvarez, G., Berria, R., Cusi, K., Everman, S., Meyer, C., and Mandarino, L. J. (2008)
537 Insulin-resistant muscle is exercise resistant: Evidence for reduced response of nuclear-encoded mitochondrial
538 genes to exercise. *Am. J. Physiol. Endocrinol. Metab.* **294**, E607-E614

539 49. Edgett, B. A., Foster, W. S., Hankinson, P. B., Simpson, C. A., Little, J. P., Graham, R. B., and Gurd,
540 B. J. (2013) Dissociation of increases in PGC-1α and its regulators from exercise intensity and muscle
541 activation following acute exercise. *PLoS One* **8**
542 50. Nordsborg, N. B., Lundby, C., Leick, L., and Pilegaard, H. (2010) Relative workload determines
543 exercise-induced increases in PGC-1α mRNA. *Med. Sci. Sports Exerc.* **42**, 1477-1484
544 51. Popov, D., Zinovkin, R., Karger, E., Tarasova, O., and Vinogradova, O. (2014) Effects of continuous
545 and intermittent aerobic exercise upon mRNA expression of metabolic genes in human skeletal muscle. *J.*
546 *Sports Med. Phys. Fitness* **54**, 362-369
547 52. Popov, D. V., Zinovkin, R. A., Karger, E. M., Tarasova, O. S., and Vinogradova, O. L. (2013) The
548 effect of aerobic exercise on the expression of genes in skeletal muscles of trained and untrained men. *Hum.*
549 *Physiol.* **39**, 190-195
550 53. Anderson, R. M., Barger, J. L., Edwards, M. G., Braun, K. H., O'Connor, C. E., Prolla, T. A., and
551 Weindruch, R. (2008) Dynamic regulation of PGC-1α localization and turnover implicates mitochondrial
552 adaptation in calorie restriction and the stress response. *Aging Cell* **7**, 101-111
553 54. Scarpulla, R. C. (2002) Nuclear activators and coactivators in mammalian mitochondrial biogenesis.
554 *Biochim. Biophys. Acta, Gene Struct. Expression* **1576**, 1-14
555 55. Scarpulla, R. C. (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and
556 function. *Physiol. Rev.* **88**, 611-638
557 56. Park, S., Kim, D., Dan, H. C., Chen, H., Testa, J. R., and Cheng, J. Q. (2012) Identification of Akt
558 interaction protein PHF20/TZP that transcriptionally regulates p53. *J. Biol. Chem.* **287**, 11151-11163
559 57. Cartoni, R., Léger, B., Hock, M. B., Praz, M., Crettenand, A., Pich, S., Ziltener, J. L., Luthi, F.,
560 Dériaz, O., Zorzano, A., Gobelet, C., Kralli, A., and Russell, A. P. (2005) Mitofusins 1/2 and ERRα
561 expression are increased in human skeletal muscle after physical exercise. *J. Physiol.* **567**, 349-358
562 58. Egan, B., O'Connor, P. L., Zierath, J. R., and O'Gorman, D. J. (2013) Time course analysis reveals
563 gene-specific transcript and protein kinetics of adaptation to short-term aerobic exercise training in human
564 skeletal muscle. *PLoS One* **8**
565 59. Hammond, K. M., Impey, S. G., Currell, K., Mitchell, N., Shepherd, S. O., Jeromson, S., Hawley, J.
566 A., Close, G. L., Hamilton, L. D., Sharples, A. P., and Morton, J. P. (2016) Postexercise high-fat feeding
567 suppresses p70S6K1 activity in human skeletal muscle. *Med. Sci. Sports Exerc.* **48**, 2108-2117
568 60. Gottifredi, V., and Prives, C. (2001) Getting p53 out of the nucleus. *Science* **292**, 1851-1852

569 61. Cui, G., Park, S., Badeaux, A. I., Kim, D., Lee, J., Thompson, J. R., Yan, F., Kaneko, S., Yuan, Z.,
570 Botuyan, M. V., Bedford, M. T., Cheng, J. Q., and Mer, G. (2012) PHF20 is an effector protein of p53 double
571 lysine methylation that stabilizes and activates p53. *Nature Structural and Molecular Biology* **19**, 916-924
572 62. She, Q. B., Bode, A. M., Ma, W. Y., Chen, N. Y., and Dong, Z. (2001) Resveratrol-induced activation
573 of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer*
574 *Res.* **61**, 1604-1610
575 63. Jones, R. G., Plas, D. R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M. J., and Thompson, C.
576 B. (2005) AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol. Cell* **18**, 283-
577 293

578

579 Tables

580 Table 1. Primers used for real-time PCR analyses of mRNA expression.

| **Gene** | **Primer efficiency** | **Forward primer (5'→3')** | **Reverse primer (5'→3')** |
|---|---|---|---|
| ACTB | 107% | GAGCACAGAGCCTCGCCTTT | TCATCATCCATGGTGAGCTGGC |
| B2M | 98% | TGCTGTCTCCATGTTTGATGTATCT | TCTCTGCTCCCCACCTCTAAGT |
| cyt *c* | 98.8% | GGGCCAAATCTCCATGGTCT | TCTCCCCAGATGATGCCTTT |
| GAPDH | 106% | AATCCCATCACCATCTTCCA | TGGACTCCACGACGTACTCA |
| NRF-1 | 80.7% | CTACTCGTGTGGGACAGCAA | AGCAGACTCCAGGTCTTCCA |
| NRF-2 | 92% | AAGTGACAAGATGGGCTGCT | TGGACCACTGTATGGGATCA |
| p53 | 101.8% | GTTCCGAGAGCTGAATGAGG | TTATGGCGGGAGGTAGACTG |
| PGC-1α | 103.6% | GGCAGAAGGCAATTGAAGAG | TCAAAACGGTCCCTCAGTTC |
| PHF20 | 117.5% | GTGGGGCCGTGAGGAGAATA | AACTGGGCTCCCACTTCAAA |
| TBP | 99% | CAGTGACCCAGCAGCATCACT | AGGCCAAGCCCTGAGCGTAA |
| TFAM | 109.3% | CCGAGGTGGTTTTCATCTGT | GCATCTGGGTTCTGAGCTTT |

581 ACTB, β-actin; B2M, beta-2-microglobulin; cyt *c*, cytochrome *c*; GAPDH, glyceraldehyde 3-phosphate dehydrogenase;
582 NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; PGC-1α, peroxisome proliferator-activated
583 receptor γ coactivator-1α; PHF20, plant homeodomain finger-containing protein 20; TBP, TATA-binging protein;
584 TFAM, mitochondrial transcription factor A.

Table 2. mRNA content of cytochrome *c* (cyt *c*), nuclear respiratory factor 1 (NRF-1) and 2 (NRF-2), plant homeodomain finger-containing protein 20 (PHF20), and mitochondrial transcription factor A (TFAM) measured immediately post (+0 h) and 3 h (+3 h) after a single session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training (HVT), in the vastus lateralis muscle of young healthy men. Values are expressed relative to TATA-binging protein (TBP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) housekeeping genes.

| Gene | Time Point | Pre-HVT | Post-HVT |
|---|---|---|---|
| cyt *c* | Rest | 2.06 ± 0.89 | 2.79 ± 1.55 |
| | +0 h [#] | 1.63 ± 0.44 | 1.46 ± 0.67 |
| | +3 h | 1.70 ± 0.64 | 2.48 ± 1.37 |
| NRF-1 | Rest | 0.14 ± 0.08 | 0.13 ± 0.06 |
| | +0 h | 0.19 ± 0.09 | 0.14 ± 0.04 |
| | +3 h | 0.15 ± 0.05 | 0.16 ± 0.05 |
| NRF-2 | Rest | 0.23 ± 0.07 | 0.21 ± 0.06 |
| | +0 h | 0.23 ± 0.09 | 0.19 ± 0.05 |
| | +3 h | 0.28 ± 0.13 | 0.26 ± 0.15 |
| PHF20 | Rest | 0.33 ± 0.08 | 0.27 ± 0.04 |
| | +0 h | 0.31 ± 0.07 | 0.24 ± 0.05 |
| | +3 h | 0.31 ± 0.08 | 0.46 ± 0.50 |
| TFAM | Rest | 0.48 ± 0.14 | 0.44 ± 0.09 |
| | +0 h | 0.40 ± 0.11 | 0.37 ± 0.08 |
| | +3 h | 0.47 ± 0.13 | 0.44 ± 0.12 |

[#] Main effect of exercise ($P < 0.05$) *vs.* Rest. All values are mean ± SD. n = 9 for NRF1; n = 8 for all other genes.

## Figures

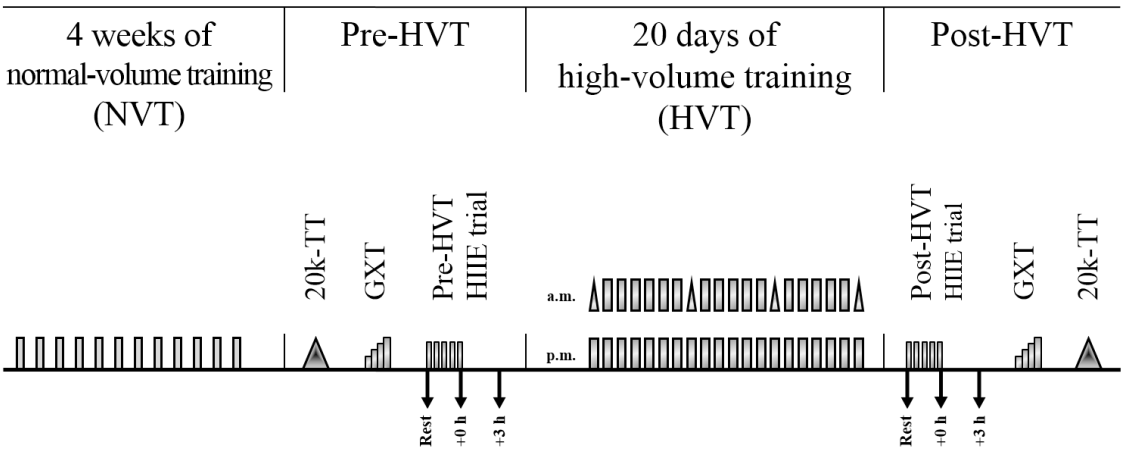



Figure 1. **Study design.** Grey rectangles indicate a HIIE session; grey triangles within HVT indicate a 10-km cycling time trial; each rectangle and/or vertical pair of rectangles and/or vertical pair of rectangles and triangles represents a training day; arrows indicate a skeletal muscle biopsy. 20k-TT: 20-km cycling time trial; GXT: graded exercise test; HIIE: high-intensity interval exercise; Rest: skeletal muscle biopsy at rest; +0 h: skeletal muscle biopsy taken at the end of the HIIE session; +3 h: skeletal muscle biopsy taken three hours after the completion of the HIIE session.

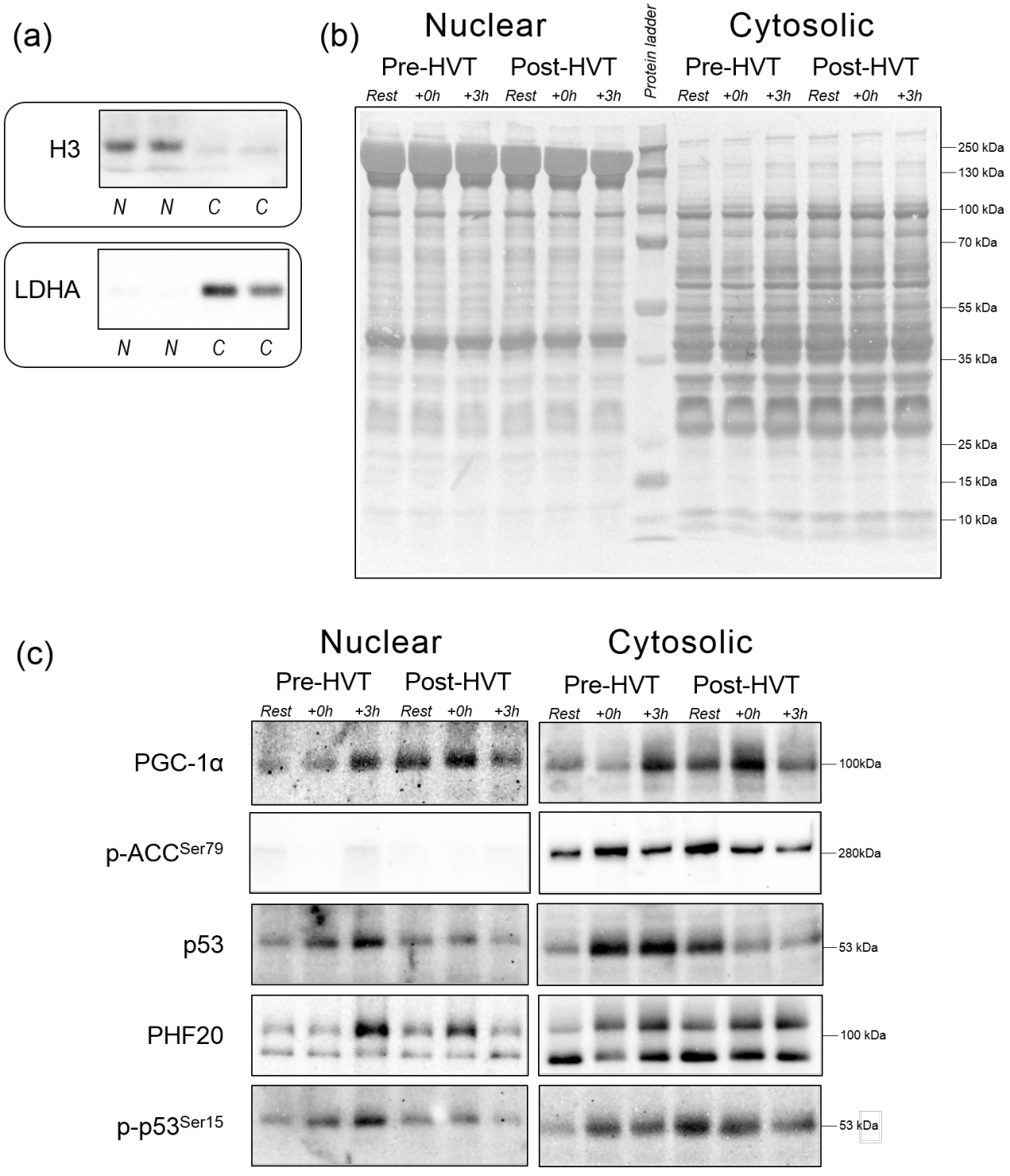



Figure 2. **Representative immunoblots, subcellular enrichment and protein loading controls.** (a) Representative immunoblots of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), acetyl-CoA carboxylase phosphorylated at serine 79 (p-ACC$^{Ser79}$), p53, plant homeodomain finger-containing protein 20 (PHF20), and p53

phosphorylated at serine 15 (p-p53$^{Ser15}$) measured in the nuclear and cytosolic fractions obtained from human vastus lateralis muscle biopsies, before (Rest), immediately post (+0 h), and 3 h (+3 h) after a single session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training (HVT). PHF20: top band at ~105 kDa. No band was detected in the nuclear fractions for p-ACC$^{Ser79}$. (b) Histone H3 and lactate dehydrogenase A (LDHA) were used as indicators of cytosolic and nuclear enrichment, respectively. N: nuclear fractions; C: cytosolic fractions. (c) Whole-lane Coomassie blue staining for both nuclear and cytosolic fractions was used to verify equal loading between lanes. The immunoblot and whole-lane Coomassie images in this figure were cropped to improve the conciseness and clarity of the presentation.

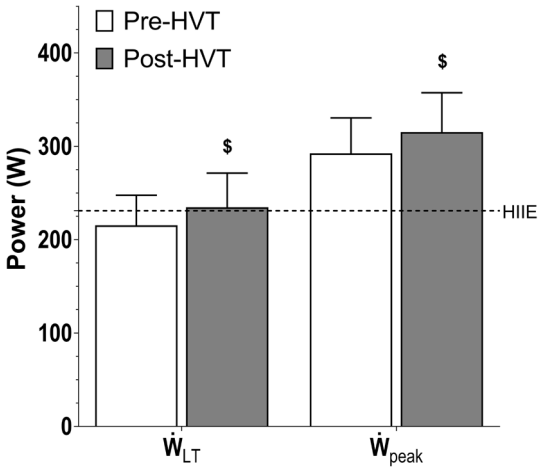



Figure 3. **Power attained at the lactate threshold ($\dot{W}_{LT}$), peak power achieved during the graded exercise test ($\dot{W}_{peak}$), and mean power of the Pre- and Post-HVT high-intensity interval exercise (HIIE) biopsy trials.** $\dot{W}_{LT}$ and $\dot{W}_{peak}$ were assessed before (Pre-HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training (HVT). The dotted line represents the mean power during the Pre- and Post-HVT HIIE biopsy trials. n = 9. $^{\$}$ $P < 0.05$ *vs.* Pre-HVT.

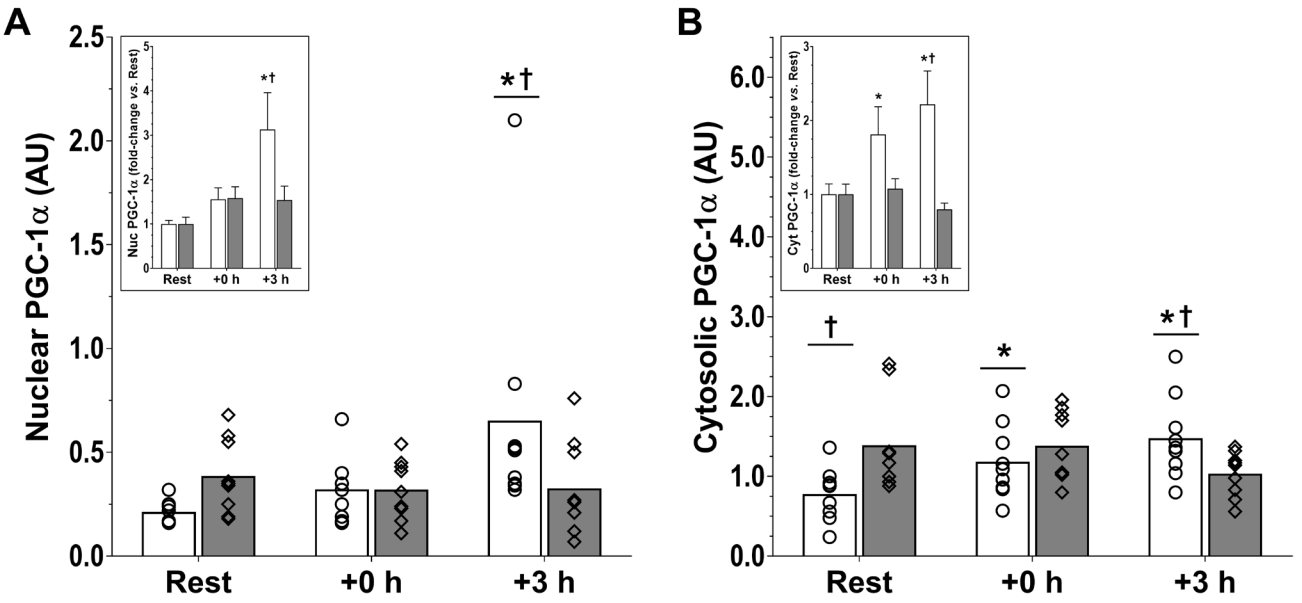



Figure 4. **Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) protein.** Protein content of PGC-1α in nuclear (a), and cytosolic (b) sub fractions before (Rest), immediately post (+0 h), and 3 h (+3 h) after a single session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-HVT) and (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training (HVT), in the vastus lateralis muscle of young healthy men (n = 9). Open circles (Pre-HVT) and open diamonds (Post-HVT) represent individual values; white (Pre-HVT) and grey (Post-HVT) bars represent mean values. * $P < 0.05$ *vs.* Rest of the same group; † $P < 0.05$ *vs.* same time point of Post-HVT trial. To more clearly depict fold-changes in post-exercise values from potentially different Rest values in the untrained and trained state, an inset has been added to each main figure (note, significant differences between trained and untrained values at Rest are not reported in insets as these values are both normalized to 1).

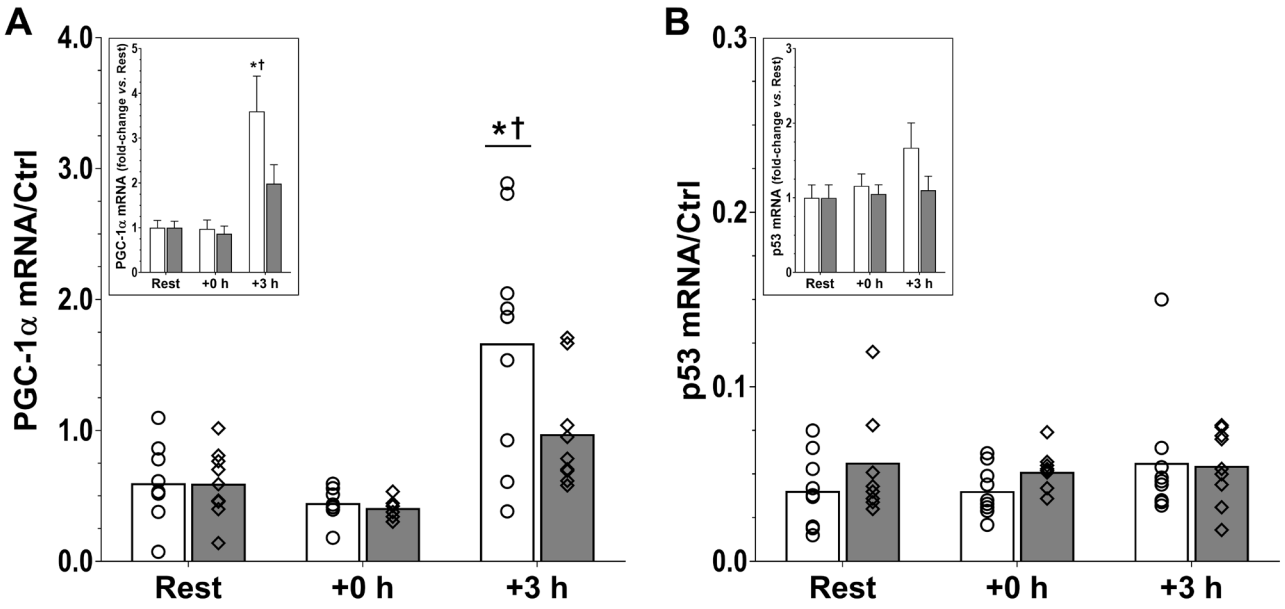



Figure 5. **Gene expression.** mRNA content of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (a), and p53 (b) before (Rest), immediately post (+0 h), and 3 h (+3 h) after a single session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training (HVT), in the vastus lateralis muscle of young healthy men (n = 9). Values are expressed relative to TATA-binging protein (TBP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) housekeeping genes (Ctrl in the figure). Open circles (Pre-HVT) and open diamonds (Post-HVT) represent individual values; white (Pre-HVT) and grey (Post-HVT) bars represent mean values. * $P < 0.05$ *vs.* Rest of the same group; † $P < 0.05$ *vs.* same time point of Post-HVT trial. To more clearly depict fold-changes in post-exercise values from potentially different Rest values in the untrained and trained state, an inset has been added to each main figure.

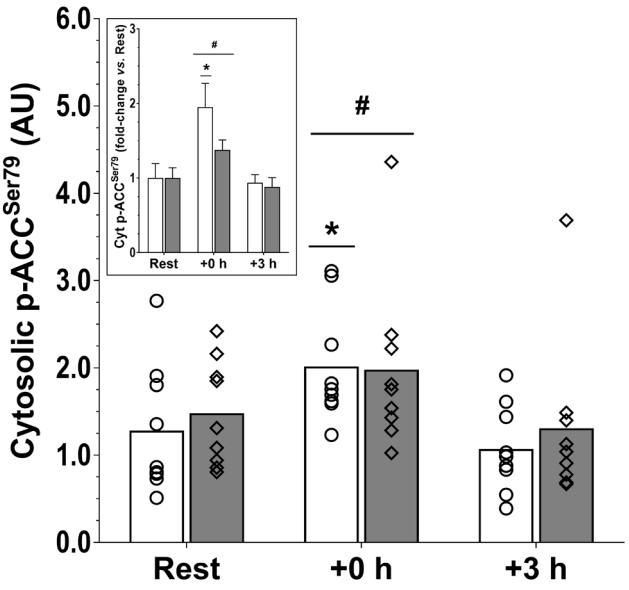
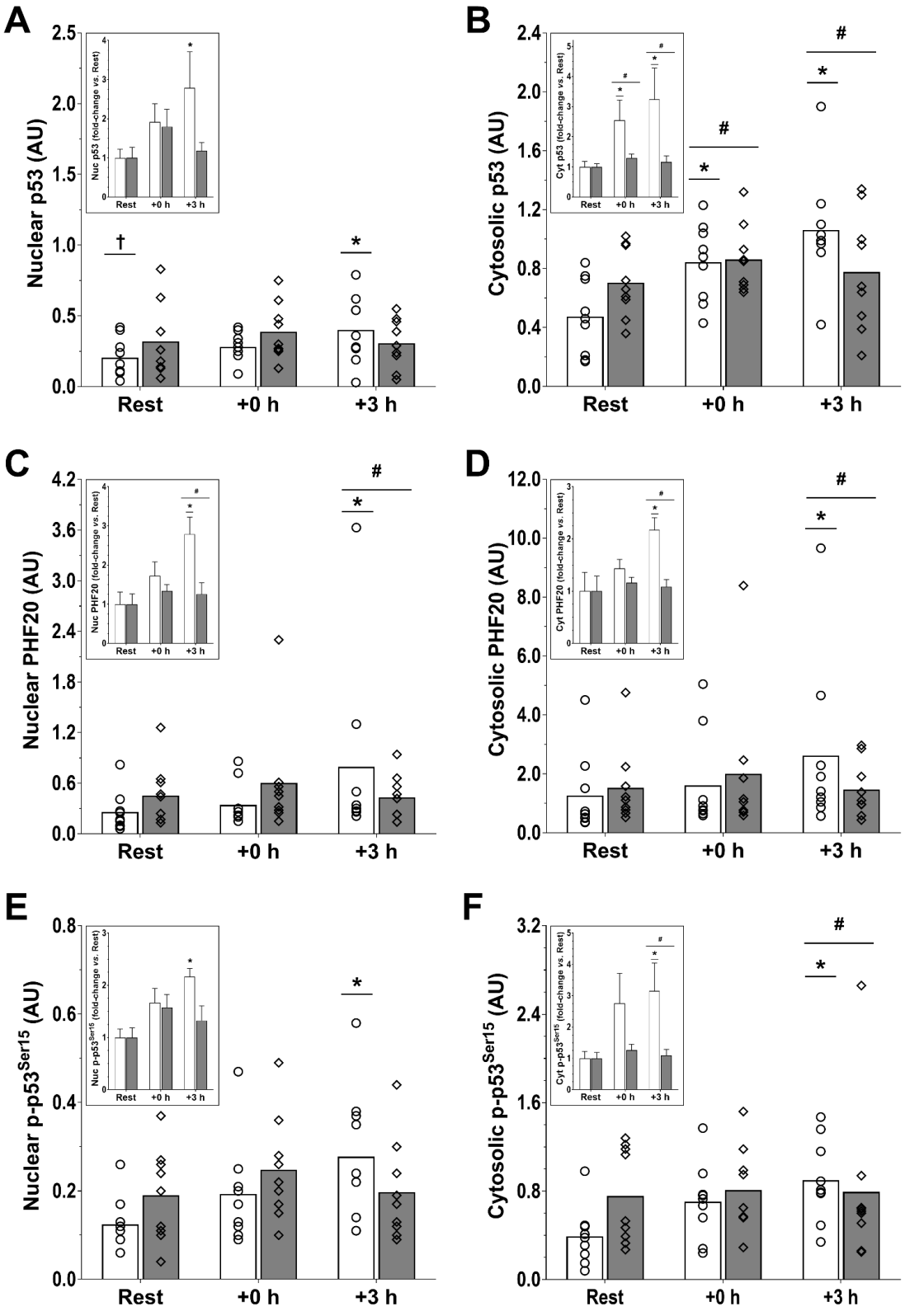



Figure 6. **Phosphorylation of acetyl-CoA carboxylase (ACC) at serine 79 (p-ACC$^{Ser79}$).** Protein content of cytosolic p-ACC$^{Ser79}$ before (Rest), immediately post (+0 h), and 3 h (+3 h) after a single session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training (HVT), in the vastus lateralis muscle of young healthy men (n = 9). Open circles (Pre-HVT) and open diamonds (Post-HVT) represent individual values; white (Pre-HVT) and grey (Post-HVT) bars represent mean values. # main effect of exercise ($P < 0.05$) *vs.* Rest; * $P < 0.05$ vs. Rest of the same group. To more clearly depict fold-changes in post-exercise values from potentially different Rest values in the untrained and trained state, an inset has been added to each main figure.




650

Figure 7. **p53 and plant homeodomain finger-containing protein 20 (PHF20) protein.** Protein content of nuclear (a) and cytosolic (b) p53, of nuclear (c) and cytosolic (d) PHF20, and of nuclear (e) and cytosolic (f) p-p53$^{Ser15}$ assessed before (Rest), immediately post (+0 h), and 3 h (+3 h) after a single session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training (HVT), in the vastus lateralis muscle of young healthy men (n = 9). Open circles (Pre-HVT) and open diamonds (Post-HVT) represent individual values; white (Pre-HVT) and grey (Post-HVT) bars represent mean values. $^{\#}$ main effect of exercise ($P < 0.05$) *vs.* Rest; * $P < 0.05$ *vs.* Rest of the same group; $^{\dagger}$ $P < 0.05$ *vs.* same time point of Post-HVT trial. To more clearly depict fold-changes in post-exercise values from potentially different Rest values in the untrained and trained state, an inset has been added to each main figure (note, significant differences between trained and untrained values at Rest are not reported in insets as these values are both normalized to 1).