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Moderate-Intensity Interval Training Is Sufficient to Increase Muscle Mitochondrial Respiratory Capacity and Exercise Tolerance in Young Untrained Men

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Running Title: MUSCLE ADAPTATION TO ENDURANCE TRAINING

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ABSTRACT

Introduction: There is considerable evidence that physical activity can stimulate mitochondrial biogenesis in muscles, enhance maximal mitochondrial oxygen consumption rate, improve physical fitness, and help maintain overall health in humans. On the other hand, there is growing evidence that excessive training load may have a detrimental effect on mitochondria and glucose homeostasis. **Hypothesis:** We hypothesized that moderate-intensity interval training (MIIT) would be sufficient to increase muscle mitochondrial oxidative capacity and exercise tolerance in untrained healthy people. **Methods:** Eleven young, healthy, untrained males took part in this study. They performed a 7-week endurance training program (MIIT) on a cycle ergometer, composed of 4 sessions per week, each lasting 40 min. Before and after the MIIT training program, a pulmonary maximal oxygen consumption ($\dot{V}O_{2max}$) as well as lactate threshold (LT) were determined and a muscle biopsy from the *m. vastus lateralis* was taken to evaluate the level of muscle proteins related to energy metabolism and mitochondrial biogenesis, and to measure the muscle's maximal mitochondrial respiration rate. **Results:** The training resulted in an increase of skeletal muscle homogenate $\dot{V}O_{2max}$ (by ~50%, P=0.001), due to increased mitochondrial mass and the enrichment of mitochondria with the individual respiratory chain complexes. The MIIT increased the power output at the lactate threshold (PO at LT) by ~43 W (P=0.001) and the pulmonary $\dot{V}O_{2max}$ by 7% (P=0.01) and had no adverse effect on blood glucose homeostasis. **Conclusions:** MIIT, which is a less intense form of training than the commonly used types of interval training, effectively induces a range of beneficial muscular and systemic adaptations that contribute to improvement of physical fitness and maintenances good health in humans. **Key Words:** BIOENERGETICS, EXERCISE, LACTATE THRESHOLD,

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MAXIMAL OXYGEN UPTAKE, MITOCHONDRIAL BIOGENESIS, RESPIRATORY EXCHANGE RATIO, INSULIN SENSITIVITY

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INTRODUCTION

Physical exercise is a widely recognized non-pharmacological intervention, effective in the prevention and treatment of many pathological conditions such as obesity, hypertension, and insulin resistance in humans (1,2). Indeed, there is a growing body of evidence that the beneficial effect of physical training can be observed in varied organs, especially in muscle tissue, improving insulin sensitivity (2,3). Since Holloszy's seminal work (4) demonstrating in rats that endurance training increases mitochondrial protein content and enzyme activity in hindlimb muscles, considerable attention has been given to the effects of physical training on mitochondrial adaptations in skeletal muscle (5,6). Beyond exercise capacity, it is now widely recognized that optimal mitochondrial function plays a pivotal role in maintaining overall human health (7-9). In the context of physical performance, an increase in muscle oxidative phosphorylation (OXPHOS) capacity is considered central to these training-induced adaptations (10-13). Specifically, enhanced muscle OXPHOS activity attenuates metabolic perturbations during exercise of a given intensity (10-14).

Despite these well-established benefits, a critical question remains: what is the specific exercise dose or training workload required to elicit the mitochondrial adaptations that optimally translate into both improved physical performance and human health? As early as 30–40 years ago, it was found that relatively low-intensity training (< 70% of HR_{max}) is sufficient to improve cardiovascular fitness in humans (15-17). More recently, high-intensity interval training (HIIT), involving repeated bouts of near-maximal or maximal effort interspersed with recovery periods, has been widely promoted as an effective alternative to traditional endurance training, capable of inducing comparable or even superior physiological adaptations (18). Notably, HIIT performed at intensities >90% of maximal power output (i.e., power output at $\dot{V}O_{2max}$) has been shown to

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elicit the greatest absolute increases in mass-specific mitochondrial respiration, whereas sprint interval training (SIT) appears to be the most time-efficient strategy for improving mitochondrial respiratory function (13). Consequently, both HIIT and SIT have been widely recommended for athletes, untrained individuals, and patients as effective training strategies to improve mitochondrial function and exercise performance (19-23). Nevertheless, most evidence supporting these beneficial effects of HIIT and SIT on mitochondrial biogenesis and oxidative capacity is derived from short-term training interventions, and the effects of prolonged training remain unclear (24). Interestingly, Flockhart et al. (25) recently found that even a short period of HIIT training in its extreme form, lasting just 3 weeks, can impair mitochondrial function and disrupt glucose homeostasis in recreationally trained individuals. Similarly, DesOrmeaux et al. (26) have recently reported that, in the case of healthy, highly trained endurance athletes competing in various sports, adding three HIIT sessions per week to their usual training program over a three-week period led to a decline in their performance. Nevertheless, this training overload, in contrast to the findings of Flockhart et al. (25), did not adversely affect mitochondrial bioenergetics, mitochondrial function or insulin signaling in the skeletal muscles of the athletes studied. These findings highlight the need for further research to determine the impact of varied training loads on metabolic health in individuals with different training histories. Consistently, HIIT and SIT interventions require greater caution, particularly in cardiac patients (27-30). Taken together, these findings underscore the need to better define the optimal exercise dose to ensure long-term efficacy and safety.

Therefore, to address the limited knowledge regarding the effects of less demanding exercise interventions, we investigated the impact of a 7-week endurance training program, primarily composed of moderate-intensity interval cycling (MIIT), on: (i) quadriceps muscle homogenate

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$\dot{V}O_{2max}$ and mitochondrial electron transport chain (ETC) subunits content; (ii) muscle nuclear factor erythroid 2-related factor 2 (NRF2); (iii) blood glucose homeostasis; (iv) power output (PO) at the lactate threshold (LT) and (v) pulmonary maximal oxygen uptake ($\dot{V}O_{2max}$) in young healthy untrained men. We hypothesized that this much less demanding training program (MIIT) would be sufficient to enhance muscle mitochondrial biogenesis, increase mitochondrial OXPHOS activity in skeletal muscles, and improve exercise capacity in young, healthy men.

MATERIAL AND METHODS

Ethics

The study protocol has been approved by the Ethics Committee of Jagiellonian University (no. 1072.6120.104.2025) and conducted according to the Declaration of Helsinki. A schematic illustration of the study outline is presented in Figure 1.

Subjects

Eleven untrained, but physically active young men (mean ± SD: age 21 ± 1 yrs, height 181.0 ± 4.2 cm, and body mass, BM 76.1 ± 17.7 kg) took part in this study. Initially, 12 individuals volunteered for the study, but after initial medical qualification, one participant was excluded due to a medical condition (based on blood test data). The basal anthropometric characteristics of the studied group are presented in Table 1. Participants were informed about the planned course of the experiment and the potential risks associated with taking part in the study. After giving their written consent, participants underwent medical examinations, including a physical examination, electrocardiogram (ECG), cardiac ultrasound (USG) and blood tests. Those qualified for the study were asked to avoid intense physical exercise on the day preceding the laboratory tests and on the day of the tests, as well as to refrain from additional physical activity during the 7-week training period. Moreover, the participants were asked to follow the

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normal mixed diet over the three days prior to the maximal incremental tests (composed of ~55% of carbohydrates, ~25% of fat, and ~20% of protein and to avoid caffeinated and alcoholic beverages 24 h before exercise).

Exercise protocols

Maximal incremental exercise test. A maximal incremental exercise test was performed using the cycloergometer Ergoselect100K (Ergoline GmbH, Bitz, Germany), following a 6-min resting period that allow for the establishing of basal cardio-respiratory parameters at rest. The exercise test was performed at pedaling rates of 60 rev · min^{-1}, starting from a power output (PO) of 30 W and followed by a gradual increase of 30 W every minute until exhaustion. The maximum power output achieved in the final phase of this incremental exercise test is referred to as: PO at $\dot{V}O_{2max}$, which corresponds to the traditionally used term W_{max}. The following criteria of reaching pulmonary $\dot{V}O_{2max}$ during an incremental exercise test were applied: a) evident plateauing in $\dot{V}O_2$ in relation to the increase in power output; b) heart rate (HR) at the end of exercise being close to age-predicted HR_{max}; c) end-exercise respiratory exchange ratio (RER) > 1.0.

Lactate threshold test. The lactate threshold (LT) was studied during another incremental exercise test but with longer exercise bouts, *i.e.*, lasting 6-min each, performed on the cycloergometer Ergomedic 874E (Monark, Vansbro, Sweden), following a 6-min resting period. The exercise was performed at a pedaling rate of 60 rev · min^{-1}, starting from a power output of 30 W and followed by a gradual increase of 30 W every six minutes up to one bout over the LT. During the last minute of rest and the last minute of each exercise bout, a drop of blood from the finger was taken to measure lactate concentration using the analyzer Lactate Scout 4 (SensLab GmbH, Leipzig, Germany). During rest and exercise, the heart rate was recorded continuously

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by POLAR M430 heart rate monitor with H10 sensor (Polar Electro Oy, Kempele, Finland). The LT in this study was defined as the highest power output above which the plasma lactate concentration shows a sustained increase of ≥ 0.5 mmol $\cdot$ L^{-1} per bout (31).

Training procedure. A 7-week endurance training program was performed on a cycle ergometer Ergomedic 874E (Monark, Vansbro, Sweden) at a pedaling rate of 60 rev $\cdot$ min^{-1}, according to two different protocols, each lasting 40 min. In order to objectively assess the training load for each participant, in this study we adopted the classical four exercise-intensity domains concept (see: 32,33). First endurance cycling session (protocol "A") was carried out at the power output corresponding to the LT and at the power output corresponding to 25% difference between PO at $\dot{V}O_{2max}$ and PO at LT (25%Δ) in the following order: 15 min at the PO at LT, 10 min at 25%Δ, 15 min at the PO at LT (40 min in total). After 2nd week of the training program, this order of exercise session was changed to: 10 min at the PO at LT, 8 min at 25%Δ, 10 min at the PO at LT, 8 min at 25%Δ, 4 min at the PO at LT; and after 4th week to: 8 min at the PO at LT, 10 min at 25%Δ, 8 min at the PO at LT, 10 min at 25%Δ, 4 min at the PO at LT. The second endurance cycling session (protocol "B") was carried out at the PO at LT and at the power output corresponding to 35% difference between the PO at $\dot{V}O_{2max}$ and the PO at LT (35%Δ) in the following order: 10 min at the PO at LT, 6 min at 35%Δ, 10 min at the PO at LT, 6 min at 35%Δ, 8 min at the PO at LT (40 min in total). After 2nd week of the training program, the exercise segments with 35%Δ was replaced by 60%Δ and after 4th week of training, the time frame of the exercise segments was changed to: 8 min at the PO at LT, 8 min at 60%Δ, 8 min at the PO at LT, 8 min at 60%Δ, 8 min at the PO at LT (40 min in total). The power output corresponding to 25/35/60%Δ was calculated for each subject according to the formula: 25/35/60% Δ = PO at LT + 0.25/0.35/0.60 (PO at $\dot{V}O_{2max}$ - PO at LT). The heart rate of each participant was monitored

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during each training session by the Polar H10 sensor (Polar Electro Oy, Kempele, Finland) connected with the Polar Flow app. Each training session was also supervised by one member of the research team. After each training session, participants rated their perceived exhaustion on a 20-point Borg scale (34). Endurance training protocols "A" were performed on Mondays and Thursdays, and the training protocols "B" on Tuesdays and Fridays of each training week (see Figure 1).

Therefore, according to the above-mentioned exercise-intensity domains (see 32,33), the subjects performed their training mainly in the moderate-intensity domain with some dose of cycling in the heavy-intensity domain. It is worth noting that in accordance with the recently proposed terminology regarding physical activity and exercise intensity (the statement by experts from the ACSM and ESSA, see (35)), the moderate-intensity domain as described by Whipp (32) and Poole and Jones (33) corresponds to very low and low exercise intensity, while the high-intensity domain in the convention proposed by Whipp (32) (for overview see also Poole and Jones (33)), corresponds to moderate-exercise intensity (see Figure 3 in the Bishop et al. (35)). Accordingly, when using the proposed terminology from the experts' statement of the ACSM and ESSA, our participants performed their training almost exclusively at low and moderate exercise intensities (see Bishop et al. (35)).

Blood sampling

Blood samples were collected from the antecubital vein at rest after overnight fasting between 7:00 and 8:30 a.m., both before and after performing 7-week cycling endurance training. Blood samples for determination of glycohemoglobin (HbA_{1c}) were collected into EDTA tubes (S-Monovette tubes, Sarstedt, Nümbrecht, Germany) whereas blood for serum

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glucose and insulin measurements was collected into tubes with coagulation activator and then centrifuged at 4 000 *g* for 5 min at 20 °C.

Muscle biopsy

Muscle biopsies were obtained under local anesthesia (Lignocainum Hydrochloricum WZF 2%, Polfa, Warszawa, Poland) from the right *vastus lateralis m. quadricipitis femoris*, approximately 15 cm above the upper margin of the *patella.* Before taking muscle biopsies, the planned region of the needle puncture was visualized in each subject using the ultrasound system Logiq E10s (GE Ultrasound Korea, Seongnam-si, Republic of Korea) in order to minimize potential harm to the blood vessels. The biopsies were taken using a φ = 2 mm biopsy needle (14GA x 10 cm; Argon Medical Devices, Inc., Athens, TX, USA). The depth of the biopsy was set automatically by ProMag^{TM} system (ProMag^{TM} Ultra 2.2; Argon Medical Devices, Inc., Athens, TX, USA). The muscle specimens were immediately frozen in liquid nitrogen (LN_2). Muscle biopsies were taken before and after 7 weeks of endurance training. The second muscle biopsy procedure (after completing the training program) in the studied group was conducted 46.4 ± 3.2 hours after the last training session.

Measurements

Biochemical analyses in blood. The HbA_{1c} concentration was assayed by the HPLC method on Bio-Rad D-10 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) autoanalyzer. The concentration of glucose was determined using an enzymatic method (Cobas c503 analyzer), and insulin was determined by the electrochemiluminescence method ECLIA (Cobas e801 analyzer). The measurement range for the glucose was 0.11-41.6 mmol $\cdot L^{-1}$, and the limit of detection was 0.11 mmol $\cdot L^{-1}$. Intra- and inter-assay coefficients of variation (CVs) for glucose concentrations within the clinical range (> 3.3 mmol $\cdot L^{-1}$) were < 0.7% and < 0.8%, respectively.

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The measurement range for the insulin was 0.4-1000 µU · $mL^{-1,}$ and the limit of detection was 0.4 µU · mL^{-1}. Intra- and inter-assay CVs for insulin concentrations were < 4.4% and < 5.4%, respectively.

Homeostasis Model Assessment of Insulin Resistance (HOMA IR) was calculated using fasting serum glucose and insulin levels, based on the formula: (fasting glucose [mmol · L^{-1}] × fasting insulin [µU · mL^{-1}]) / 22.5.

Blood lactate concentration [La^-] was analyzed using a portable Lactate Scout 4 analyzer (SensLab GmbH, Leipzig, Germany) in 2 µL of fresh capillary whole blood taken from the fingertip. The concentration of lactate was determined using an enzymatic amperometric detection method. The measurement range for the lactate was 0.5-25 mmol · L^{-1}. For lower lactate concentrations (up to 7.5 mmol · L^{-1}), the precision of lactate measurement is < 0.3 mmol · $L^{-1,}$ and for higher lactate concentrations (above 7.5 mmol · L^{-1}), the coefficients of variation is < 4%.

Anthropometric Measurements. Anthropometric measurements were performed in the morning hours. Body mass and height of each subject were measured using standard procedures and equipment (Radwag WPT 150, Radwag, Radom, Poland). Body fat percentage was determined using the method based on skinfold thickness measurement as described by Jackson and Pollock (36) using three-site skinfold measurements (triceps, chest, and thigh) with a Harpenden caliper (Baty International, Burgess Hill, UK).

Gas exchange and cardio-vascular variables. Gas exchange variables were recorded continuously using the Quark CPET with Mixing Chamber system (COSMED srl, Rome, Italy), starting from 6 min rest before exercise until the end of the exercise test. The gas analyzers before each test were calibrated with certified calibration gases containing 16.00% O_2 and 5.00%

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CO_2 – balanced with NO_2, according to the manufacturer's manual. At rest and during exercise test, the ECG was monitored using integrated 12-lead Stress Testing ECGs (COSMED srl, Rome, Italy). Blood pressure was measured using Tango M2 (SunTech Medical, Morrisville, NC, USA) and blood saturation (SpO_2) using the pulse oximeter ONYX Vantage 9590 (Nonin Medical Inc., Plymouth, MN, USA).

Mitochondrial respiration. Mitochondrial oxygen consumption in the muscle biopsies was measured using a Clark electrode (5300A Biological Oxygen Monitor; YSI, Rye Brook, NY, USA) in a thermostated chamber maintained at 37 °C. All the biopsies collected from the volunteers before and after training were frozen in liquid nitrogen. Muscle biopsies (vastus lateralis) were homogenized in the KCl-based buffer (180 mM KCl, 50 mM Tris-HCl, 0.1 mM EGTA, pH 7.4). Depending on the biopsy size, 0.4-1.0 mg of protein suspended in 0.5 mL of the homogenization buffer was used for the measurements. Due to the freezing and homogenization procedures mitochondria could be damaged (e.g., the outer mitochondrial membrane could be broken and endogenous cytochrome c could be released). Using such a material a full spectrum of bioenergetics parameters (e.g., ADP-stimulated respiration) of mitochondria was limited only to evaluation of the maximal capacity of the mitochondrial respiratory chain. To counteract the limitation of the maximal rate of the electron flow along the ETC as a result of an endogenous cytochrome *c* release caused by biopsy freezing and/or homogenization-related outer mitochondrial membrane rupture, an exogenous cytochrome c (20 μM final concentration) was added to the measurement chamber. This allowed for the measurement of the maximal rate of oxygen consumption in frozen biopsies in the presence of the substrate, ensuring that the oxygen consumption rate was not limited by a deficiency of endogenous cytochrome c. Moreover, it is known that the freezing process itself and subsequent tissue storage can also affect the

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permeability of the inner mitochondrial membrane, potentially resulting in uncoupling the mitochondria. Therefore, to ensure a unique degree of uncoupling across all mitochondria in the preparation (specifically, to reach the maximal uncoupling), maximal oxygen consumption was measured in the presence of succinate as a substrate for ETC and 2 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as an uncoupler. Subsequent additions of small doses of CCCP resulted in the gradual increase in the rate of O_2 consumption and allowed achievement of the maximal rate of the muscle homogenate oxygen consumption (skeletal muscle homogenate $\dot{V}O_{2max}$) in the presence of the optimal CCCP concentration without its inhibitory effect on ETC. Addition of 2 μM antimycin A (AA) resulted in the inhibition of ETC-related respiration and enabled evaluation of the "zero" baseline of mitochondrial O_2 consumption. Additionally, the efficiency of tissue homogenization was assessed using digitonin (0.02% final concentration) added to the homogenate (in the presence of succinate, cytochrome c, and CCCP) to permeabilize the cell membranes of any cells that remained intact after homogenization. The lack of an increase in the oxygen consumption rate following its addition confirmed that mitochondrial substrate was already accessible, indicating effective skeletal myocyte cell membrane disruption during the homogenization procedure. According to a recent publication (37) succinate – substrate for complex II of ETC – seems to be the best option to investigate the activity of the mitochondrial respiratory chain in frozen samples.

Western Blot. Muscle homogenates were diluted in RIPA (R0278, Sigma-Aldrich) buffer 1:1 and kept on ice for 30 min for protein extraction. Subsequently, lysates were centrifuged at 16 000 × g for 20 min. Supernatants were transferred into fresh tubes, and the pellets were discarded. Protein concentration was measured using Bio-Rad Protein Assay Dye Reagent Concentrate, 450 mL (Bio-Rad #5000006), according to the manufacturer's instructions. Lysates

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were then mixed with LDS Sample Buffer (4×) (Invitrogen, #B0008) and Sample Reducing Agent (10×) (Invitrogen, #B0004) to a final 1× concentration, mixed and resolved in Bolt 4%–12% Bis-Tris Plus Mini Protein Gels (Invitrogen) 15 µg per lane under reducing conditions and transferred onto 0.45 µm Immun-Blot® Low Fluorescence PVDF Membrane (Bio-Rad, #1620264) using a wet transfer system (Mini Blot Module, Invitrogen) at 10 V for 60 min. After the transfer membranes were washed with milli-Q water and the total protein staining was performed in order to verify uniform protein loading and transfer efficiency with the use of Ponceau S (Sigma-Aldrich #P3504) or Revert Total protein Revert™ 700 Total Protein Stain for Western Blot Normalization (LICORbio - U.S., #P/N 926-11010) according to the manufacturer's protocol. Membranes were blocked in EveryBlot Blocking Buffer (Bio-Rad, #12010020) for 15 min at room temperature, then incubated overnight at 4 °C with primary antibodies: Total OXPHOS Rodent WB Antibody Cocktail (Abcam #AB110413), and anti-TOM20 antibody - Mitochondrial Marker (Abcam #ab186735) and PPARGC1A (PGC1α) Polyclonal Antibody (Invitrogen # PA5-72948), pAMPK (Cell Signaling #2535), AMPK (Cell Signaling #5831), TFAM (Cell Signaling #7495), NRF2 (NFE2L2) (Proteintech #16396-1-AP), catalase (Santa Cruz Biotechnology #sc-271803), SOD1 (Santa Cruz Biotechnology #sc-101523), SOD-2 (Santa Cruz Biotechnology #sc-133134), pAKT (Cell Signaling #2965), AKT (Cell Signaling #4691), pFOXO1 (Cell Signaling #9464), FOXO1 (#2880), pPKD (Cell Signaling #2051), PKD (Cell Signaling #90039), SCD1 (Cell Signaling #2794). After washing with TBS-Tween 0,5%, membranes were incubated for 1 h at room temperature with a peroxidase (HRP)-conjugated goat anti-rabbit (Cytiva #RPN4301; 1:10000) or StarBright Blue 700 goat anti-rabbit (Bio-Rad, #12004161; 1:5000) secondary antibody, and rhodamine-conjugated anti-β-actin (Bio-Rad, #12004263; 1:5000) as a loading control. Fluorescence signals

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were detected using a ChemiDoc MP Imaging System (Bio-Rad) and quantified using Image Lab software (v6.1.0, Bio-Rad, 2020). When a secondary antibody conjugated to horseradish peroxidase was used, signals were detected on autoradiography film using an enhanced chemiluminescence substrate (Bio-Rad) and quantified using ImageJ software.

Statistics

The results are presented as median with interquartile range (IQR: Q1-Q3), and minimum-maximum value (details of data presentation are given in each table and figure label). The significance of the difference of the variables measured before and after the training was tested with the matched-pairs Wilcoxon signed-rank test, and the exact (*i.e.*, non-asymptotic) two-sided *P*-values were computed. We have also performed an a priori sample size calculation (G*Power version 3.1.9.4, Düsseldorf, Germany), estimating the effect size for main experimental outcomes. In case of performance variables (PO at LT and PO at $\dot{V}O_{2max}$) the effect size based on our previous results (38,39) amounted to about 1.1 and in the case of maximal mitochondrial oxidative capacity (citrate synthase and cytochrome c oxidase activity) the effect size amounted to about 1.4 (11,40). With an estimated effect size of 1.1-1.4, a level of significance of 0.05 and statistical power of 0.8, a minimum of 7-10 participants was requested. Considering potential dropout, 12 men were originally recruited to the study, one of whom was excluded for medical reasons (see the section Subjects). Statistical analysis was carried out using Origin2026 software (OriginLab Corporation, Northampton, MA, USA) and Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA).

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RESULTS

Training workload: heart rate and Borg's scale score during the training program

As shown in Figure 2A, the HR increased only moderately during the applied MIIT. Namely, most of the applied training workloads (95% of the total training time) were performed with HR not exceeding 150 beats · min^{-1} *i.e.*, < 83% of HR_{max}. As shown in Figure 2B, 92% of the total training time was performed at a HR not exceeding 80% of HR_{max}, and 98% at a HR below 85% of HR_{max}. The subjective assessment of effort during training, made by participants on a 20-point Borg's scale (34), was 11.4 ± 1.7 points.

Body composition and cardiorespiratory parameters before and after training

The 7-weeks of moderate-intensity endurance training had no significant effect on body mass, fat mass, however slightly decreased body mass index (BMI) **(Table 1).** Basic cardiorespiratory parameters (HR_{max}, $\dot{V}_{Emax}$, $\dot{V}CO_{2max,}$ and RER_{max}) measured at the end of the maximal incremental exercise test were also not significantly different after the training program, although the maximal pulmonary minute carbon dioxide production ($\dot{V}CO_{2max}$), which tended to be higher after the training (see Table 1).

Glucose homeostasis before and after training

As shown in Table 1, MIIT did not significantly affect fasting blood HbA_{1c} level, glucose and insulin concentrations, and HOMA IR.

Exercise capacity before and after training

The training program resulted in an increase in the PO measured at the lactate threshold (PO at LT; P = 0.001; Figure 3A) and at the final stage of the maximal incremental exercise test (PO at $\dot{V}O_{2max}$; P = 0.01; Figure 3B) by about 80% and 8%, respectively. Before the training, the PO at LT amounted to 54.6 ± 26.2 W and increased to 98.2 ± 33.1 W after the 7-week training

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program. PO at $\dot{V}O_{2max}$ amounted to 244.1 ± 41.8 W before the training and was increased to 263.6 ± 42.0 W after the training. Furthermore, $\dot{V}O_{2max}$ expressed in absolute terms, increased from 2.60 ± 0.48 L $O_2 \cdot min^{-1}$ before training to 2.80 ± 0.44 L $O_2 \cdot min^{-1}$ after training (P = 0.01) (Figure 4A). Similarly, the relative value of $\dot{V}O_{2max}$ increased from 34.7 ± 3.5 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$ before training to 37.0 ± 4.3 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$ after training (P = 0.01) (Figure 4B).

Mitochondrial maximal respiration rate before and after training

As presented in Supplemental Figure 1 (Supplemental Digital Content, https://links.lww.com/MSS/D518), the addition of exogenous cyt. c increases the rate of oxygen consumption, indicating outer mitochondrial membrane rupture caused by biopsy freezing and/or homogenization procedure. For this reason, all further measurements of O_2 consumption in homogenates of frozen vastus lateralis muscle samples were performed in the presence of 20 μM exogenous cyt. c, so the electron flow along the ETC is not limited by the level of cyt. c. The skeletal muscle homogenate $\dot{V}O_{2max}$ has been achieved in the presence of the optimal (2 μM) CCCP concentration (evaluated as described in the Materials and Methods section).

Measurements of the oxygen consumption in the biopsies of vastus lateralis muscle collected before and after endurance training revealed, on average, a 50% increase of muscle homogenate $\dot{V}O_{2max}$, indicating higher ETC capacity after training (Figure 4C). In the case of one participant, the effect of training on the ETC capacity and the skeletal muscle homogenate $\dot{V}O_{2max}$ was negligible (Figure 4C). It is important to note that the ~50% increase in maximal uncoupled mitochondrial respiration observed in the homogenate reflects the intrinsic capacity of the respiratory chain rather than typical *in vivo* physiological rates. *In vivo*, whole-body oxygen consumption is primarily limited by systemic oxygen delivery and cellular ATP demand. This explains why a substantial expansion in mitochondrial capacity resulted in a more modest (~7%)

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increase in whole-body $\dot{V}O_{2max}$, highlighting the role of systemic constraints in translating cellular adaptations to the whole-organism level (see also Discussion section).

Mitochondria protein content/activity before and after the training

Mitochondrial content in the homogenates prepared from muscle biopsy samples was assessed by Western blot based on the level of a housekeeping mitochondrial protein, mitochondrial outer membrane translocase (TOM20), routinely used as a mitochondrial marker and normalized to total protein assessed by whole PVDF membrane staining after transfer (Figure 5A). The level of TOM20 protein was approximately 20% higher (Figure 5C), on average, in the biopsies of vastus lateralis muscle taken after training. This indicates increased mitochondrial mass after training in the muscle of participants.

Next, we investigated whether the increase in mitochondrial mass was also accompanied by an enrichment of the inner mitochondrial membrane in the ETC complexes and ATP synthase. Figure 5A shows the levels of representative core subunits of the mitochondrial oxidative phosphorylation (OXPHOS) system: NDUFB8 (complex I), SDHB (complex II), UQCRC2 (complex III), MTCO1 (complex IV), and ATP5A (ATP synthase) (for reference see: 41-43). When normalized to total cellular protein (Figure 5B), MTCO1, UQCRC2, and NDUFB8 levels were elevated in post-training biopsies, whereas ATP5A levels were numerically higher, although this difference did not reach statistical significance (Figure 5E).

Similarly, when the levels of representative OXPHOS subunits were normalized to the mitochondrial marker protein TOM20, they were found to be elevated in the post-training biopsies. Specifically, the mitochondrial enrichment in complexes I, III, IV, and ATP synthase reached statistical significance. Taken together, these observations indicate that the enhanced maximal mitochondrial respiration observed after training reflects the combined effects of

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increased mitochondrial mass and enrichment of mitochondria with OXPHOS complexes in skeletal muscle.

We assessed total PGC-1α and TFAM protein abundance, two well-established regulators of mitochondrial biogenesis (44). PGC-1α is a key transcriptional coactivator that promotes mitochondrial biogenesis by coordinating the expression of nuclear and mitochondrial genes, including TFAM, and its expression and activity in skeletal muscle increase in response to exercise, contributing to exercise-induced mitochondrial adaptations (45). Neither PGC-1α nor TFAM differed significantly between baseline and post-training (Figure 5D and Figure 6), suggesting that sustained activation of the PGC-1α-TFAM regulatory axis was not evident at the time of sampling. Moreover, we observed a trend toward an increase ($P = 0.06$) in NRF2 protein content following the exercise intervention (Figure 6). To further assess antioxidant defense, we measured the protein content of key antioxidant enzymes, including SOD1, SOD2, and catalase (Figure 6), none of which showed significant changes following training. Thus, the observed NRF2 response was not accompanied by detectable changes in the abundance of the antioxidant enzymes assessed.

Metabolic markers of skeletal muscle metabolism before and after training

To assess the effect of the training intervention on skeletal muscle metabolism, we next examined AMPK protein levels, a key cellular energy sensor (46). We observed no significant effect of the MIIT on the phosphorylation ratio of pAMPK (Thr172) to total AMPK in vastus lateralis muscle biopsies collected after the training intervention compared with pre-training samples from the same individuals (Figure 7), suggesting no changes in AMPK activity following training. Moreover, no changes were observed in the phosphorylation ratio of pAKT (Thr308) to total AKT, nor in its downstream target pFOXO1 (Thr24) relative to total FOXO1

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(Figure 7), indicating no detectable alterations in insulin signaling under these conditions (46). Additionally, we did not observe any changes in the phosphorylation ratio of pPKD (Ser910) to total PKD or in SCD1 protein levels, both of which have been associated with lipid-induced insulin resistance in skeletal muscle (47,48). Taken together, these data suggest a neutral effect of the training intervention on skeletal muscle metabolism and insulin sensitivity.

DISCUSSION

The main finding of our study was that the applied training program (MIIT), consisting of 40-min cycling sessions performed 4 times a week, mainly at a HR not exceeding 150 bt. per min, i.e., < 83% of HR_{max} and lasting 7 weeks resulted in an increase of the PO at LT and the pulmonary $\dot{V}O_{2max}$ as well as in an enrichment of the skeletal muscle mitochondrial with OXPHOS complexes, increase in skeletal muscle homogenate $\dot{V}O_{2max}$ and clear tendency towards increase in muscle NRF2 protein level in young, healthy, untrained men.

According to the recently proposed classification of training intensity (49), the training protocol used in our study (taking into account the recorded heart rate, see Figure 2A-B) can be classified as moderate-intensity interval training (MIIT). This form of training is far less demanding in terms of fatigue than the other popular forms of interval training, *i.e.*, the high-intensity interval training (HIIT) or the sprint interval training (SIT) (see 24, 49). It is worth mentioning that the training workload applied in our study was well tolerated by the young, healthy, untrained men. Namely, they reported that their "level of exhaustion" after each training session amounted to 11.4 ± 1.7 points in the 20-point Borg's scale (*fairly light*) (34). Furthermore, opposite to the recently reported harmful effects of a HIIT on the glucose homeostasis in young, healthy people (25), in the present study we have found no detrimental effect of the MIIT on the blood glucose balance in our participants, as judged by the levels of the

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plasma glucose, HbA_{1c} levels and the HOMA IR, measured before and after training (see Table 1). Given that skeletal muscle is the primary site of postprandial glucose disposal, we also assessed AMPK signaling, together with selected components of insulin and metabolic signaling pathways. We found no significant training-induced changes in AMPK phosphorylation, the AKT-FOXO1 pathway, PKD phosphorylation, or SCD1 protein content (Figure 7). As exercise-induced AMPK activation is generally transient and has been reported to return toward basal levels within several hours after exercise (50), the biopsies obtained approximately 46 h after the final exercise session primarily reflect the post-training resting state rather than the acute exercise response. At the same time, we cannot exclude the possibility that AMPK activity in the muscles of our participants during the preformed training sessions increased significantly above resting levels (50-52), which could potentially influence a range of muscular adaptive responses (50). It is worth mentioning, that in the light of the recent studies, the role of AMPK in muscle metabolism during physical exercise does not appear to be as significant as previously thought (52,53). Our finding of no significant change in resting muscle AMPK signaling following training is consistent with the earlier study by McConell et al. (51), who showed that 10 days of moderate-to-heavy endurance cycling training had no effect on resting muscle AMPK activity in humans. In contrast, Frøsig et al. (54) reported that three weeks of one-legged knee-extensor endurance training resulted in an increase in basal muscle AMPK activity that persisted for 55 h after the final exercise bout. The authors suggested that sustained changes in AMPK activity may contribute to training-induced adaptations in metabolism and gene expression. This apparent discrepancy may reflect differences in training modality and load, as well as participants' training status and metabolic health. Moreover, the lack of changes in SCD1 protein content and PKD1 phosphorylation status, both of which have been linked to lipid-induced insulin resistance

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(48,55) together with unchanged AKT–FOXO1 signaling pathway, a key downstream component of insulin receptor signaling, suggests that insulin signaling was not adversely affected by the conducted MIIT intervention.

Impact of the training on the LT and the pulmonary $\dot{V}O_{2max}$

The applied MIIT resulted in a pronounced increase of the PO at the LT (from 55 ± 26 W before to 98 ± 33 W after the training, Figure 3A), and a smaller but significant increase (by about 7%) in the pulmonary $\dot{V}O_{2max}$ and the PO at $\dot{V}O_{2max}$ (see Figures 3 and 4). It is worth noticing that the observed increase of the PO at the LT, PO at $\dot{V}O_{2max,}$ and the $\dot{V}O_{2max}$ (Figure 3A-B and 4A-B) was accompanied by a marked increase of the skeletal muscle homogenate $\dot{V}O_{2max}$ (Figure 4C) (by about 50%) and the enrichment of skeletal muscle mitochondria with OXPHOS complexes (Figure 5). The observed increase in the PO at the LT towards a higher level can be explained by the training-induced increase of the muscle mitochondrial oxidative capacity. Namely, as presented before by others, an increase in muscle's OXPHOS activity attenuates the exercise-induced disturbances in muscle metabolites, particularly attenuates the increase in muscle [ADP], [H^+], [Pi], slows down the decrease in muscle [PCr], glycogen content, and delta G_{ATP} at a given power output during sustained exercise (for an overview see 10,12,14,39). Since these metabolites (Pi, ADP, and H^+), and P_i in particular, play a key role in the development of muscle fatigue (56-58), training-induced attenuation in their levels at a given power output shifts the LT towards higher power output, delays muscle fatigue, and improves endurance performance.

Interestingly, our study also revealed that the substantial increase in the skeletal muscle homogenate $\dot{V}O_{2max}$ (by about 50%, Figure 4C) was accompanied by a much lesser relative increase in the pulmonary $\dot{V}O_{2max}$ (an increase by about 7%, Figure 4A-B). This shows that the

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training-induced increase in muscle OXPHOS activity has a strong impact on the LT, but the training's influence on the pulmonary $\dot{V}O_{2max}$ is far weaker. This finding is consistent with the view that the whole body pulmonary $\dot{V}O_{2max}$ depends mainly on oxygen transport to the mitochondria, in which factors such as cardiac output (or blood flow in the muscles) (59-61) and the red cell-to-mitochondria diffusion rate (62,63) play the key role. Taken together, our study showed that the marked training-induced increase in mitochondrial oxidative capacity in muscle cells following MIIT is accompanied by much greater increases in the LT than by the increase in pulmonary $\dot{V}O_{2max}$ in healthy individuals (Figure 3). This finding may have significant practical implications for the development of training programs for humans, aimed at improving performance during prolonged physical exercise (lasting from several tens of minutes to several hours), which depends to a greater extent on the LT than on the $\dot{V}O_{2max}$.

Impact of the training on the maximal capacity of the mitochondrial respiration chain and the muscle mitochondrial biogenesis

It is worth noticing that in our study, the skeletal muscle homogenate $\dot{V}O_{2max}$ when measured before training amounted to 6.27 nmol O_2 · mg of homogenate's prot.$^{-1}$ · min^{-1} (Figure 4 C), was in the range of values published previously by others (64). This confirms the quality of the studied mitochondria and the correctness of the procedure of the skeletal muscle homogenate $\dot{V}O_{2max}$ measurements. In the present study, we have found that the MIIT resulted in a marked increase (~50%) in skeletal muscle homogenate $\dot{V}O_{2max}$ in the vastus lateralis muscle (Figure 4C). Our data suggest that MIIT promoted mitochondrial remodeling, characterized by increased mitochondrial content, as reflected by increased TOM20 protein abundance, and enrichment of mitochondria with OXPHOS complexes I, III, IV, and ATP synthase (Figure 5). Despite these observations, neither total PGC-1α nor TFAM, two well-established regulators of mitochondrial

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biogenesis, differed in protein abundance between baseline and post-training. This pattern may reflect the transient nature of the molecular regulation of mitochondrial biogenesis, whereby early activation of these regulatory mechanisms precedes persistent mitochondrial adaptations (see for e.g. (65)). Consequently, the lack of detectable changes in PGC-1α and TFAM protein abundance at the time of tissue collection does not preclude mitochondrial biogenesis but may instead reflect the timing of muscle sampling. This interpretation is consistent with previous reports demonstrating that exercise-induced activation of the PGC-1α-TFAM axis is transient and highly dependent on the timing of muscle sampling (66). Accordingly, the enhanced oxygen consumption capacity of the vastus lateralis muscle as observed in post-training muscle biopsy homogenates likely results from both an increased mitochondrial mass and the enrichment of mitochondria in individual respiratory chain complexes.

A clear trend toward increased NRF2 protein content was also detected following training (Figure 6). As a transcription factor, NRF2 (NFE2L2) plays a pivotal role in maintaining mitochondrial function, mitochondrial quality control, and mitochondrial adaptive redox regulation (67). Furthermore, NRF2 (NFE2L2), by regulating redox balance and cellular detoxification, as well as by controlling proteostasis, metabolism, inflammation, and iron homeostasis, can exert a wide range of effects on many of the body's organs (67-69). In our study, muscle biopsies were obtained 46.4 ± 3.2 hours after the final exercise session to assess training-related adaptations beyond the immediate post-exercise period. Thus, the tendency toward increased NRF2 content may represent a training-related adaptation, although a contribution of the response to the final exercise bout cannot be excluded. However, because only total NRF2 protein content was assessed in whole-muscle homogenates without information on its nuclear translocation, no direct functional conclusions regarding the activation of

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downstream NRF2 pathways can be drawn. Analysis of key antioxidant enzymes showed a visible upward shift in the level of SOD2 and catalase protein content (Figure 6), but these changes were statistically insignificant. SOD2 is localized to the mitochondrial matrix and plays a key role in mitochondrial antioxidant defense, whereas SOD1 is predominantly localized in the cytosol (70). These antioxidant systems contribute to the maintenance of redox homeostasis and mitochondrial function in skeletal muscle (67,71). Therefore, despite the clear tendency toward increased NRF2 protein content, the lack of significant changes in the antioxidant enzymes does not support a robust induction of antioxidant defense following MIIT. Nevertheless, subtle changes in antioxidant-related processes cannot be excluded, especially in view of our previous studies showing a significant positive impact of 20 weeks endurance training on SOD2 in young untrained men (72).

Impact of training workload on mitochondrial function

The results of our study appear to be relevant in the context of the ongoing debate regarding recommended training load (training volume and intensity) potent to increase mitochondrial oxidative capacity and health status in humans (22,35,73,74). Our study clearly demonstrates that MIIT (being far less stressful than HIIT or SIT) (49) is able to markedly enhance skeletal muscle homogenate $\dot{V}O_{2max}$, by enrichment of skeletal muscle mitochondria with OXPHOS complexes and shifting the LT towards higher power output in young, healthy, untrained men. Our study, therefore, provides new evidence that training programs involving significantly lower intensity than HIIT and SIT also hold great potential for improving muscle mitochondrial function and endurance performance in humans. This seems significant in light of the emphasis placed in recent years on the effectiveness of HIIT and SIT. Indeed, it has been reported that work-matched HIIT was more effective than moderate-intensity continuous training (MICT) in terms

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of their impact on skeletal muscle mitochondrial respiration (74,75). Namely, Daussin et al. (75) reported that the HIIT resulted in a greater increase in pulmonary $\dot{V}O_{2max}$ than the MICT (amounting to 15 *vs* 9%, respectively), and the skeletal muscle mitochondrial oxidative capacity in their study was only increased after the HIIT (by ~33%). Similarly, a greater effect of the HIIT than MICT workload matched training on the maximal citrate synthase activity and mitochondrial respiration was reported by MacInnis et al. (74). That evidence undisputedly speaks in favor of the HIIT and SIT as the more time-efficient forms of training than the traditional MICT.

However, it is worth noticing that the very recent study by Botella et al. (76), who studied the effect of eight weeks of MICT vs SIT in young healthy individuals reviled, that the MICT was more effective in terms of enhancement of muscle mitochondrial biogenesis and exercise performance than the SIT. Namely the authors found that only the MICT resulted in significant increases in the muscle protein content of the five OXPHOS complexes, mitochondrial volume density and citrate synthase activity (see Figure 5B, therein), whereas SIT was characterized by a functional increase in mitochondrial respiratory chain function. Furthermore, the authors reported that the MICT was more effective in improving endurance exercise capacity (W_{max}, 4 km and 20 km cycling times) and the SIT did not increase the pulmonary $\dot{V}O_{2max}$ to a greater extent compared to moderate-intensity continuous exercise (MICT) (see, Supplemental Figure 1A, Supplemental Digital Content, https://links.lww.com/MSS/D518). Therefore, the results of their study underlines the importance of the moderate intensity training for enhancement of mitochondrial biogenesis (Figure 5B, therein). These findings in general are in accordance with our results showing a strong impact of MIIT on enhancement of endurance exercise performance and skeletal muscle homogenate $\dot{V}O_{2max}$.The observed in our study increase in the oxidative

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capacity of muscle mitochondria and the shift in the LT, the key determinant of endurance capacity in humans, towards higher power outputs, indicate the potential of the MIIT for improving endurance and health status in humans. It is also worth noting that the positive effect of MIIT on muscle mitochondrial function and exercise tolerance, observed in our study is consistent with recently published studies emphasizing the importance of low-intensity training loads in training programs for amateur runners and endurance athletes (77-79).

CONCLUSIONS

We demonstrated that moderate-intensity interval training (MIIT): (i) induces changes in skeletal muscle at the mitochondrial level, namely improving maximal muscle mitochondrial respiration, which is likely driven by both an increase in mitochondrial mass and the enrichment of the inner mitochondrial membrane in OXPHOS complexes; (ii) does not increase AMPK activity in skeletal muscles at rest and has no negative effect on detectable changes in insulin signaling pathways; (iii) markedly shifts the lactate threshold towards higher power outputs and moderately increases pulmonary maximal oxygen uptake ($\dot{V}O_{2max}$). The results of our study therefore indicate that MIIT has great potential for improving mitochondrial function and exercise capacity in young, untrained men. Consequently, whilst acknowledging all the benefits of SIT or HIIT, we recommend that MIIT should not be overlooked when planning physical activity, particularly for individuals with low physical fitness.

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FIGURE LEGENDS

Figure 1. Schematic illustration of the study outline.

Figure 2. The training workload. The percentage of the total training time performed at a given range of heart rate (HR) (**panel A**) and the percentage of the maximal HR (%HR_{max}) in function of total training time (% total training time) (**panel B**).

Figure 3. The effect of 7-week cycling endurance training on the power output during the incremental maximal exercise protocol. Power output measured at the lactate threshold (PO at LT) (**panel A**) and at the power output at which the maximal pulmonary oxygen uptake ($\dot{V}O_{2max}$) was reached (PO at $\dot{V}O_{2max}$) - in the final stage of the maximal incremental exercise protocol, determined before and after 7-week cycling endurance training (**panel B**). Data are presented as box-and-whisker plots with individual data points (n = 11). In box-and-whisker plots, boxes indicate the interquartile range, the line represents the median, plus shows the mean, and whiskers denote the minimum and maximum values. The nonparametric Wilcoxon sign-rank test two-sided exact *P*-values are shown.

Figure 4. The effect of 7-week cycling endurance training on pulmonary maximal oxygen uptake and mitochondrial rate of maximal oxygen consumption. Absolute pulmonary maximal oxygen uptake ($\dot{V}O_{2max}$) (**panel A**), relative $\dot{V}O_{2max}$ (**panel B**), skeletal muscle homogenate $\dot{V}O_{2max}$ in muscle biopsies (vastus lateralis) (**panel C**), measured before and after 7-week cycling endurance training. A maximal respiration rate of oxygen consumption is expressed as 100% (Before) = 6.27 nmol O_2/mg of homogenate's prot./min). Data are presented as box-and-whisker plots with individual data points (n = 11). In box-and-whisker plots, boxes indicate the interquartile range, the line represents the median, plus shows the mean, and

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whiskers denote the minimum and maximum values. The nonparametric Wilcoxon sign-rank test two-sided exact *P*-values are shown.

Figure 5. Mitochondrial mass and the levels of OXPHOS complexes in the muscle biopsies of volunteers. **Panel A** shows a representative western blot picture of mitochondrial mass marker protein TOM20, PGC1□ and the mitochondrial oxidative phosphorylation (OXPHOS) system proteins: ATP5A, a subunit of complex V; MTCO1, a subunit of complex IV; UQCRC2, a subunit of complex III; SDHB, a subunit of complex II; and NDUFB8, a subunit of complex I of 3 among 11 vastus lateralis muscle biopsies collected before (B, Pre) and after (A, After) the training intervention in healthy individuals. **Panel B** shows total protein staining of the membrane presented on panel A. **Panel C** shows quantification of TOM20 level; **Panel D** shows quantification of PGC1□ level; **Panel E** shows the level of OXPHOS subunits normalized to the total protein; **Panel F** shows the level of OXPHOS subunits normalized to the TOM20; For panels C-F n=11, two-sided Wilcoxon signed-rank test was used. Data are presented as box-and-whisker plots with individual data points. In box-and-whisker plots boxes indicate the interquartile range, the line represents the median, plus shows the mean, and whiskers denote the minimum and maximum values.

Figure 6. Training-induced changes in proteins involved in mitochondrial adaptation. Representing immunoblots **(Panel A)** with quantification **(Panel B)** of TFAM, NRF2 SOD1, SOD2 and catalase levels in vastus lateralis muscle biopsies collected before (B) and after (A) the training intervention in healthy individuals. Protein levels were normalized to total protein determined by Ponceau S staining. Data are presented as box-and-whisker plots with individual data points (n=10, for NRF2 and TFAM, n=7 for SOD1, SOD2 and catalase; two-sided Wilcoxon signed-rank test). In box-and-whisker plots boxes indicate the interquartile range, the

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line represents the median, plus shows the mean, and whiskers denote the minimum and maximum values.

Figure 7. Training-induced changes in proteins involved in skeletal muscle metabolism. Representing immunoblots **(Panel A)** with quantification **(Panel B)** of indicated proteins in vastus lateralis muscle biopsies collected before (B) and after (A) the training intervention in healthy individuals. Protein levels were normalized to total protein determined by Ponceau S staining. Data are presented as box-and-whisker plots with individual data points (n=10, for: pAMPK, AMPK, or n=7, for: pAKT, AKT, pFOXO1, FOXO1, pPKD, PKD, SCD1; two-sided Wilcoxon signed-rank test). In box-and-whisker plots boxes indicate the interquartile range, the line represents the median, plus shows the mean, and whiskers denote the minimum and maximum values.

SUPPLEMENTAL DIGITAL CONTENT

SDC 1: Supplemental Digital Content

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Table 1. Basal anthropometric characteristics, glucose homeostasis, and cardiorespiratory parameters were obtained at exhaustion during the incremental exercise test performed before and after the training program in the studied group (n = 11, except for glucose homeostasis, where n = 10).

	Before Training		After Training		*P*-value
	median (IQR)	min-max	median (IQR)	min-max	
Anthropometric data					
BM, kg	77.1 (63.9-87.2)	48.7-114.4	75.7 (65.7-88.0)	51.3-115.9	0.10
Fat mass, % BM	14.8 (9.5-17.5)	1.9-27.5	14.5 (9.2-17.2)	3.6-27.5	0.98
BMI, $kg \cdot m^{-2}$	22.8 (19.7-25.9)	15.7-32.7	22.5 (20.2-25.9)	16.8-33.0	**0.04**
Glucose homeostasis					
Glucose, $mmol \cdot L^{-1}$	4.89 (4.82-5.05)	4.43-5.27	4.87 (4.82-5.22)	4.69-5.28	0.55
HbA1c, %	5.05 (4.80-5.30)	4.20-5.50	5.00 (4.80-5.10)	4.50-5.30	0.28
Insulin, $\mu U \cdot mL^{-1}$	7.82 (5.83-11.70)	4.19-13.50	9.51 (8.31-12.50)	7.24-15.50	0.11
HOMA-IR	1.68 (1.26-2.74)	0.89-2.95	2.02 (1.80-2.93)	1.55-3.61	0.11
Cardiorespiratory variables					
HR_{max}, $bt \cdot min^{-1}$	184 (175-186)	172-192	179 (171-187)	160-193	0.28
$\dot{V}_{Emax}$, $L \cdot min^{-1}$	91.5 (71.5-104.4)	56.9-108.7	98.3 (70.2-105.2)	54.4-122.4	0.41
$\dot{V}CO_{2max}$, $L \cdot min^{-1}$	3.16 (2.61-3.42)	2.31-3.93	3.31 (2.89-3.81)	2.70-4.11	0.054
RER_{max}	1.21 (1.11-1.26)	1.06-1.36	1.18 (1.09-1.25)	1.05-1.36	0.68

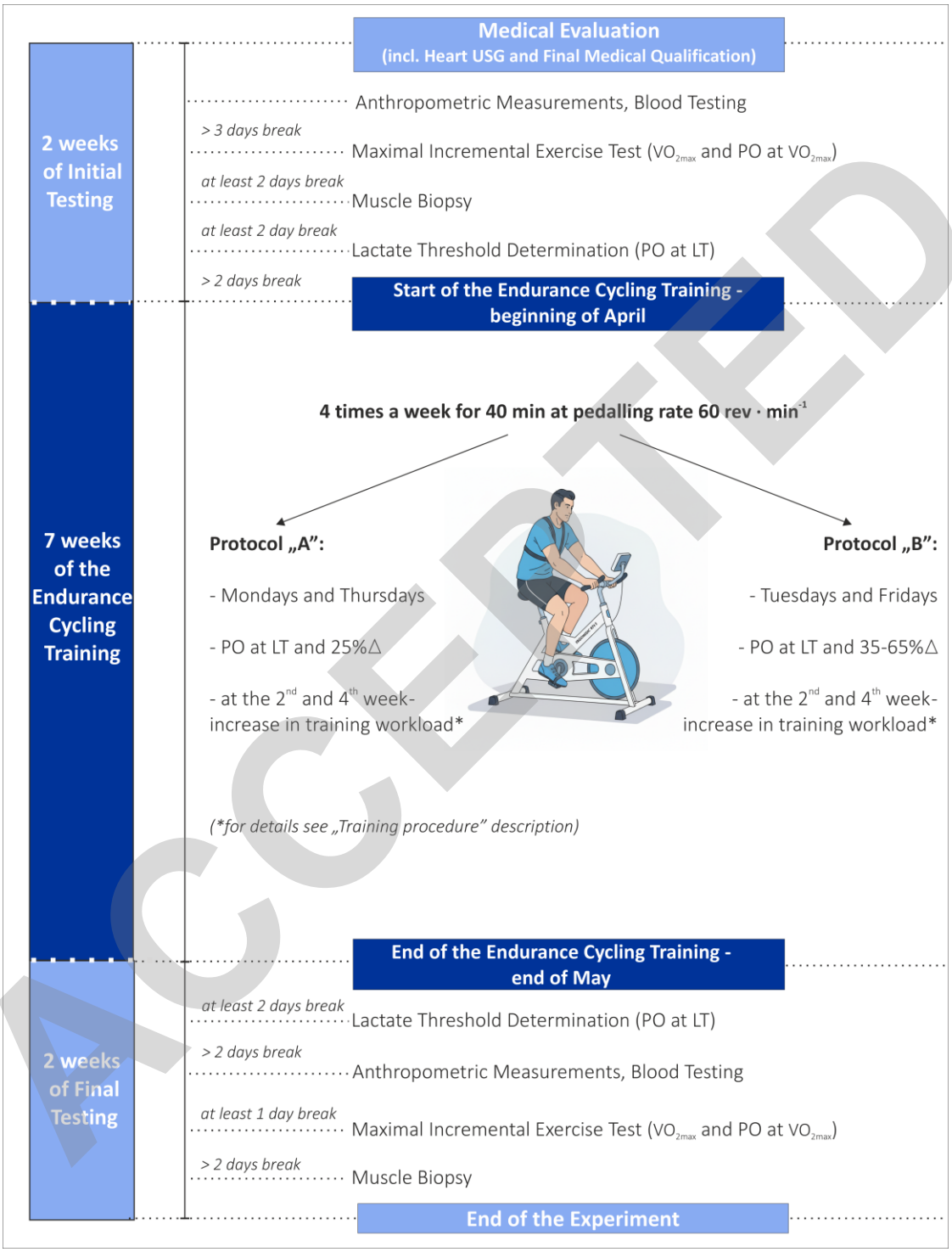
Data are presented as median, interquartile range (IQR: Q1-Q3), and minimum-maximum. The non-parametric matched-pairs Wilcoxon sign-rank test and exact, two-sided *P*-values are shown.

Abbreviations: BM, body mass; BMI, body mass index; HbA1c, glycohemoglobin; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; HR_{max}, heart rate at exhaustion; RER_{max}, respiratory exchange ratio at exhaustion; $\dot{V}_{Emax}$, minute ventilation at exhaustion; $\dot{V}CO_{2max}$, carbon dioxide production at exhaustion.

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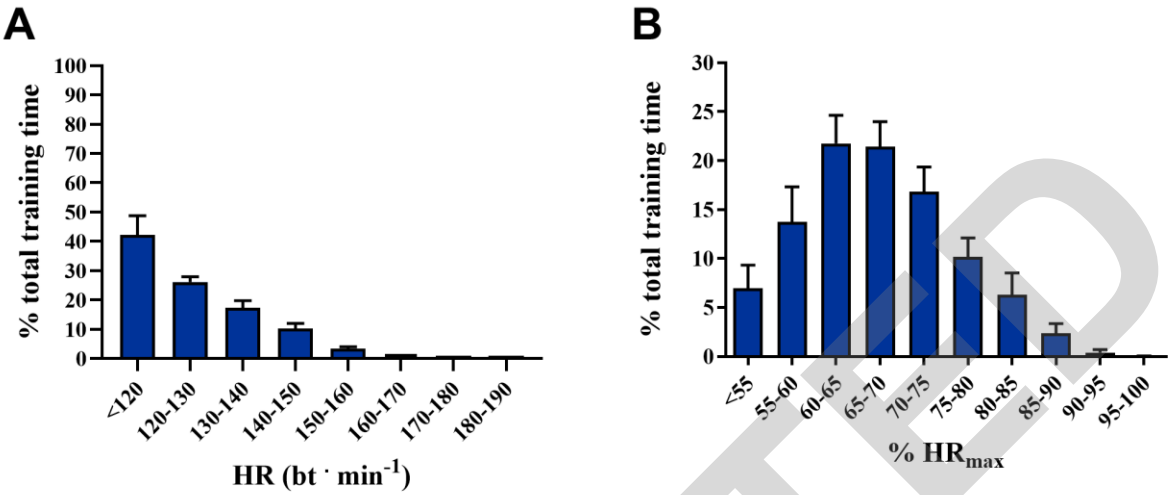
Figure 1



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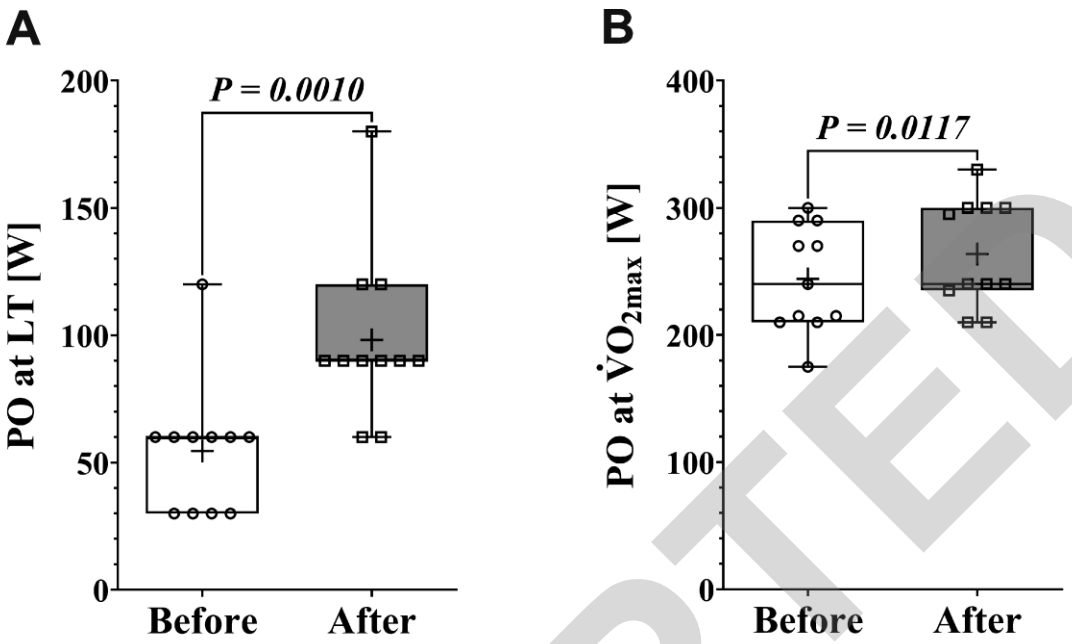
Figure 2



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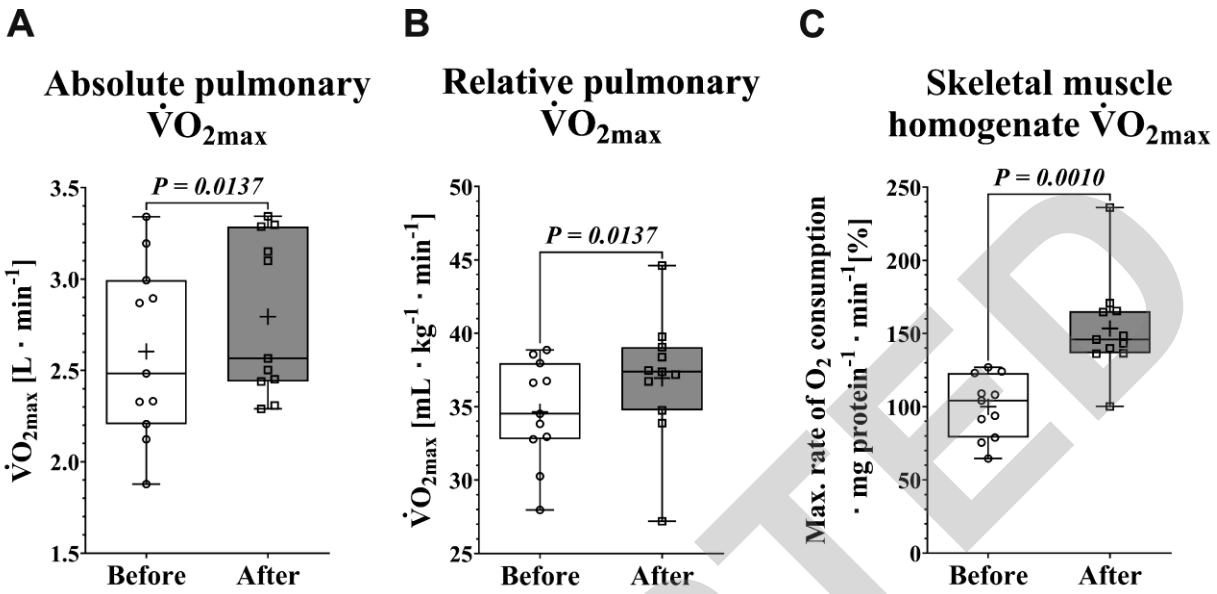
Figure 3



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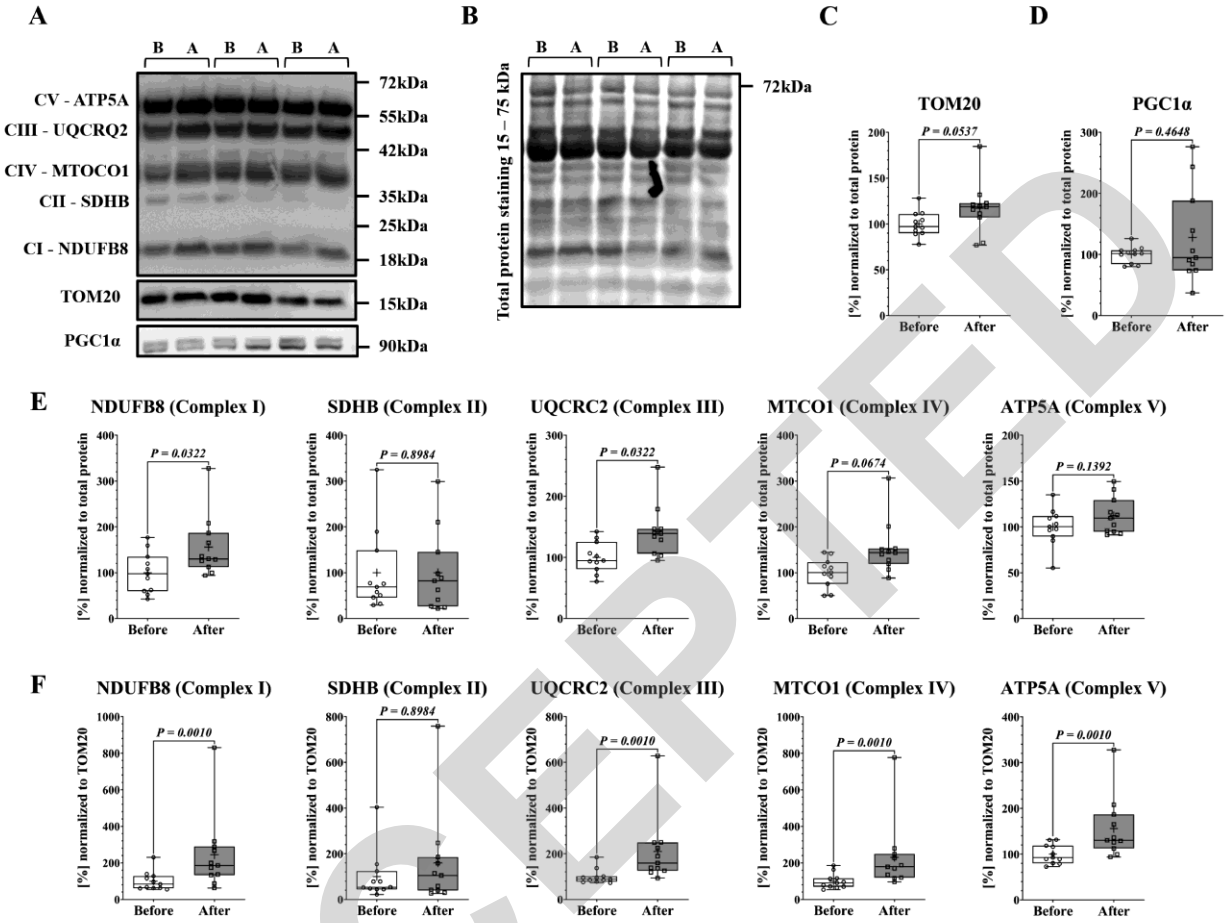
Figure 4



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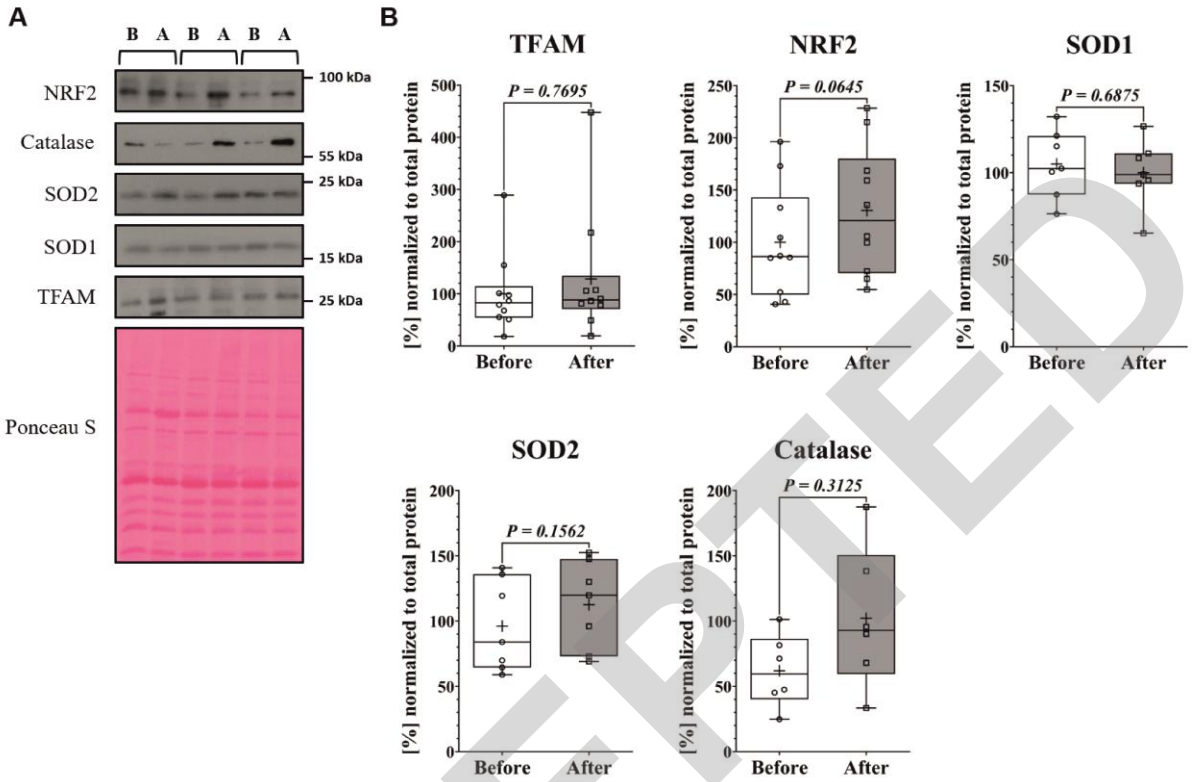
Figure 5



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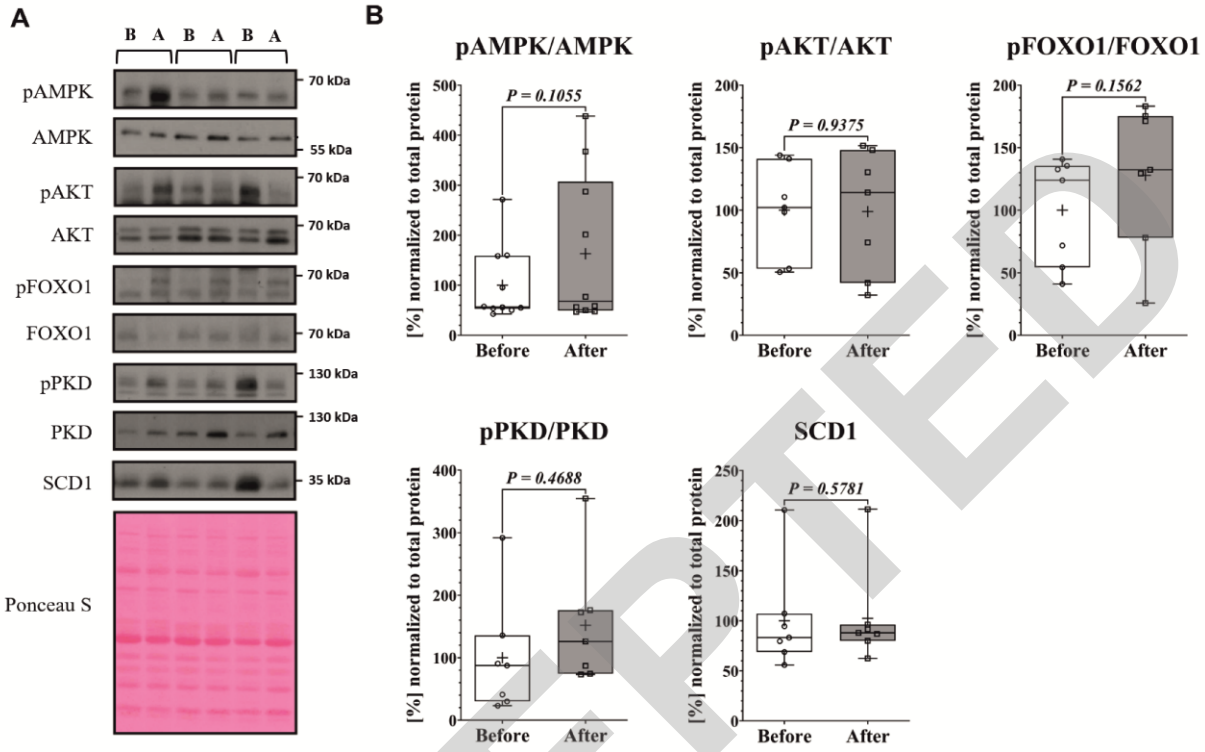
Figure 6



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Figure 7



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