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Title: Skeletal muscle mitochondria in the elderly: effects of physical fitness and exercise training

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ABSTRACT

Context: Sarcopenia is thought to be associated with mitochondrial (M) loss. It is unclear whether the decrease in M content is consequent to aging *per se* or to decreased physical activity.

Objectives: To examine the influence of fitness on M content and function, and to assess whether exercise could improve M function in older adults.

Design and subjects: Three distinct studies were conducted: 1) a cross-sectional observation comparing M content and fitness in a large heterogeneous cohort of older adults; 2) a case-control study comparing chronically endurance-trained older adults (A) and sedentary (S) subjects matched for age and gender; 3) a 4-month exercise intervention in S.

Setting: University-based clinical research center

Outcomes: M volume density (Mv) was assessed by electron microscopy from *vastus lateralis* biopsies, electron transport chain proteins (ETC) by western blotting, mRNAs for transcription factors involved in M biogenesis by qRT-PCR and *in-vivo* oxidative capacity (ATPmax) by ^{31}P-MR spectroscopy. Peak oxygen uptake (VO_2peak) was measured by GXT.

Results: VO_2peak was strongly correlated with Mv in eighty 60-80 yo adults. Comparison of A vs. S revealed differences in Mv, ATPmax and some ETC complexes. Finally, exercise intervention confirmed that S are able to recover Mv, ATPmax and specific transcription factors.

Conclusions: These data suggest that 1) aging *per se* is not the primary culprit leading to M dysfunction, 2) an aerobic exercise program, even at an older age, can ameliorate the loss in skeletal muscle M content and may prevent aging muscle comorbidities and 3) the improvement of M function is all about content.

KEYWORDS

Mitochondria volume density, Electron transport chain complexes, *PGC-1a, TFAM, NRF-1, NRF-2,* endurance exercise intervention, Phosphorus magnetic resonance spectroscopy, *in vivo* oxidative capacity

INTRODUCTION

Mitochondrial dysfunction and reduced oxidative capacity in skeletal muscle have been linked to the pathogenesis of sarcopenia, aging disabilities and frailty (1). Sedentary lifestyle, an escalating epidemic in western societies, is associated with loss of mitochondrial content and function (2, 3). Increased mitochondrial content in response to exercise training was first reported by Holloszy in 1967 (4). Since then, exercise training has been shown to be an effective strategy to improve muscle oxidative capacity (5, 6).

Aerobic exercise training up-regulates mitochondrial genes (7). Adaptations of skeletal muscle to exercise (8) include upregulation of the master regulator of mitochondrial biogenesis, the peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1α (*PGC-1α*)(9). *PGC-1α* is a transcriptional regulator that induces mitochondrial biogenesis by coactivating a large spectrum of transcription factors, including the nuclear respiratory factors 1 and 2 (*NRF-1, NRF-2*)(10, 11). In turn, *NRF-1* and *2* control the expression of a significant number of the proteins that make up the five respiratory complexes (12, 13) and modulate the expression of the mitochondrial transcription factor A (TFAM), which regulates mitochondrial DNA replication (13, 14). Several studies to date indicate that, in addition to *PGC-1α,* aerobic exercise also up-regulates *TFAM* and *NRF1* in humans (2, 15, 16).

Aging is associated with a loss of mitochondrial content (17, 18) and function (18-20) in muscle. However, studies comparing younger to older subjects are conflicting (21, 22). Indeed, it is not clear whether the decrease in mitochondrial content is associated with aging *per se* or with the decreased physical activity that comes with aging. Given the important role of aerobic exercise in up-regulating genes and transcription factors controlling mitochondrial content and function, it remains to be seen whether aerobic exercise training could play a protective role for mitochondria in aging and if training can help older individuals recover mitochondrial content. Therefore, this study had two main objectives: first to examine the relationship between mitochondrial content and physical fitness in older men and women focusing on external validity with a broad population in terms of physical fitness and body composition. Secondly, focusing on internal validity using a comprehensive picture of mitochondrial biology from the molecular level (mRNA transcripts, protein expression), the organelle level (mitochondrial density), the whole muscle level (in vivo organelle capacity), and whole-body level (VO_2peak), combining invasive

and non-invasive techniques, to assess whether exercise could improve mitochondrial content and function in older adults through up-regulation of mitochondrial master regulators.

RESEARCH DESIGN AND METHODS

Study design

Three distinct studies were conducted. Study 1 is a cross-sectional study comparing baseline levels of mitochondrial content and physical fitness in a heterogeneous cohort of older adults across a spectrum of fitness levels. Study 2 is a case-control study comparing chronically endurance trained older adults and sedentary subjects matched for age and gender. Study 3 is an interventional study comprising a four-month exercise intervention in sedentary older adults.

Study 1 was partially conducted at the University of Pittsburgh and finished at the University of Lausanne as the last author was in the process of changing institutions. All tests were conducted exactly under the same conditions and the analyses were conducted exactly the same way. Studies 2 and 3 were conducted at the University of Lausanne. The institutional review boards of both sites approved all studies and all subjects provided written informed consent.

Subjects

Volunteers between 60 and 80 years of age in good general health and stable weight were recruited for the studies. Active smokers and participants with abnormal thyroid, liver or kidney function, anemia, taking anticoagulation agents or medication known to affect skeletal muscle homeostasis (such as glucocorticoids or insulin sensitizers) were excluded. All subjects underwent a standard 75g oral glucose tolerance test to rule out diabetes. For study 2 (case-control) and 3 (interventional), volunteers were considered physically active or sedentary based on their self-declared levels of physical activity. Physically active volunteers (named here “active”) were engaging in 3 or more structured aerobic exercise sessions per week for more than one year. “Sedentary” individuals were defined as those participating in a structured exercise session no more than one day per week.

Exercise intervention (study 3)

The exercise training was a 16-week, supervised, moderate-intensity aerobic protocol. Sedentary subjects were asked to engage in at least three supervised sessions in the gym. Each session was

progressively increased from 30 to 60 minutes. Moderate intensity was defined as 75% of the subjects' heart rate (HR). Exercise prescription was individualized based on the subject's peak HR achieved during the baseline VO_2peak test and adapted at midpoint of the intervention with a submaximal ergometer test as described in details in Dubé et al (23). HR monitors (Polar Electro Oy, Kempele, Finland) and exercise logs were used to monitor intensity. Subjects could bike, walk, run or row within their HR target range with at least 80% of the training as walking or biking. Frequency, duration and volume of exercise were recorded and computed as described elsewhere (23). During the training regimen subjects were instructed to follow their typical food intake and not to undertake dietary changes while engaged in the study.

Clinical outcome measures

Height was measured using a wall-mounted stadiometer and **weight** using a calibrated medical digital scale (Seca, Hamburg, Germany). **Lean body mass (LBM)** was determined by dual-energy X-ray absorptiometry (DiscoveryA, Hologic Inc., Bedford, MA). **Physical fitness** was determined by peak oxygen consumption (VO_2peak) using a graded exercise test on an electronically braked cycle ergometer (Lode B.V., Groningen, The Netherlands). HR, blood pressure and ECG were recorded before, during and after the exercise test. VO_2 was computed via indirect calorimetry (Metalyzer3B, Cortex GmbH, Leipzig, Germany). The protocol was adapted from previously used protocols well suited for older volunteers of various degrees of fitness or fatness (24). Briefly, after an initial warm-up consisting in 2 minutes of no-load pedaling, the graded exercise test began at 25W for women or 50W for men for the first 2 minutes and was then increased 25-50W thereafter until volitional exhaustion or if one of the American College of Sports Medicine established criteria for maximal testing had been reached.

Ex-vivo skeletal muscle outcome measures

Percutaneous muscle biopsies were obtained in the fasted state from the *vastus lateralis* under local anesthesia (buffered lidocaine) as previously described (24). Controlled conditions included no exercise for 48-hours, a standardized dinner followed by an overnight fast prior to the biopsy. After trimming of visible adipose tissue with a dissecting microscope (MZ6; Leica Microsystems, Wetzlar, Germany), one portion of the specimen (~5mg) was fixed for transmission electron microscopy and two portions (~30mg each) were flash-frozen in liquid nitrogen and stored at -80°C for western blotting and RT-PCR. Analyses were performed in a blind manner. **Transmission electron microscopy (TEM)**

(study 1, 2 and 3): TEM was used to measure mitochondrial volume density (MitoVd) as a marker of mitochondrial content. A recent validation and detailed description of this stereological method has been described elsewhere (25). **Protein expression (study 2 and 3):** Frozen tissue was homogenized in 200µl of ice-cold lysis buffer containing 50mM Tris-HCl, pH7.5, 150mM NaCl, 1%(v/v)Nonidet P-40, 1mM EDTA and freshly added protease inhibitor cocktail tablet (Roche Diagnostics International, Rotkreuz, Switzerland), using a motor-driven Eppendorf homogenizer. Homogenates were then rotated for 30minutes at 4°C before centrifugation at 15,000rpm for 10min at 4°C. The pellet was discarded, and the supernatant was collected and stored at –80°C until used. Protein was measured by the BCA method (Pierce, ThermoFisher Scientific Inc., Rockford, IL). Western blotting was performed as previously described (26). Protein band intensity was measured by ImageJ and the target protein levels were normalized over the corresponding α-tubulin loading controls for each subject. All antibodies for mitochondrial complex subunits have been purchased from Mitosciences (Abcam, Cambridge, UK). The list of antibodies can be found in supplemental Table 1. **Gene expression analysis (study 2 and 3):** Total mRNA preparations, cDNA synthesis and RT-qPCR were performed as described previously (26). Primers are described in supplemental Table 2. Target mRNA levels were normalized over the geometric mean of b-Actin and CyclophilinB, that were selected as housekeeping genes after having checked their expression stability (27). Relative mRNA expression levels were calculated with the ΔΔCt method, where we used the mean of the ΔCts from 5 sedentary subjects as ΔCt calibrator.

In-vivo skeletal muscle outcome measures (study 2 and 3)

The rate of post-exercise phosphocreatine (PCr) recovery reflects oxidative ATP synthesis rate and was shown to be correlated with in vitro measurements of oxidative capacity (28). PCr Recovery experiments were performed on a 3T MR-system (VERIO, Siemens, Erlangen, Germany) in supine position. A double-tuned 31P/1H surface coil (RAPID Biomedical, Rimpar, Germany) was placed on the center of the *quadriceps* muscle and spectra were collected with an adiabatic excitation pulse. One fully relaxed spectrum was obtained on resting muscle with a repetition time (TR) of 20s and 4averages. For the PCr recovery spectra, the TR was 2s with 2scans per spectrum, resulting in a time resolution of 4s before, during and for 9minutes after dynamic knee extensions against a rubber band. Contraction frequency was 1extension per second (acoustic cues). The resistance of the rubber band was adapted to

each subject's strength, which got determined beforehand by maximal isokinetic torque of the knee extensors. Default exercise duration was 28s. If the relative decrease of PCr was outside the target of 20 to 40%, exercise duration was changed to 22s, 36s, or 44s; otherwise it was unchanged for a 2nd repetition. Since pH did not decrease below 6.8 in any experiment and recovery rates of experiment 1 and 2 were not significantly different from each other ($p=0.92$), results are shown as average of the 2 experiments. For the post-processing, spectra were analyzed with jMRUI (29) using AMARES for quantitation. The recovery of PCr was fitted to the formula $PCr(t)=PCr_0+\Delta PCr(1-e^{-k\cdot t})$; with PCr_0=PCr intensity at the beginning of recovery; ΔPCr=exercise-induced decrease of the PCr signal. pH was calculated from the chemical shift between inorganic phosphate and PCr. The oxidative phosphorylation capacity (ATPmax) was computed as previously suggested (20) as the product of the recovery rate k and the resting PCr content obtained from the resting spectrum and assuming a constant ATP concentration of 8.2mM.

Statistical Procedures

Data are presented as means±SEM. For study 1 (cross-sectional), the relationship between variables was explored using linear regression. For studies 2 (case-control) and 3 (interventional), data was first explored using nonparametric statistical tests appropriate for small sample sizes including the Wilcoxon Rank-Sum Test (between-group comparison study 2) and the Wilcoxon signed rank test (pre-post comparison, study 3). After assessing normality, parametric tests were performed. These included independent t-tests for study 2 and paired t-tests for study 3. P-values reported in the results are two-tails and from parametric tests unless otherwise specified. Correlations were performed with Spearman correlation coefficient. Significance level was set at 0.05. Statistical analyses were performed using JMP version9 (SAS, Cary, NC), SPSS version20 (IBM, Amonk, NY) and Prism version6c (GaphPad, San Diego, CA) for Macintosh.

RESULTS

Mitochondria content correlates with exercise capacity in older adults (study 1: cross-sectional study)

A total of 80 subjects, 33 men and 47 women, were included in this study. The cohort was heterogeneous with wide ranges of MitoVd, VO_2peak, BMI and body fatness (Table 1). A strong relationship was observed between MitoVd and VO_2peak (Figure 1). This relationship was similar when VO_2peak was normalized by LBM or body weight. These data show that skeletal muscle mitochondrial content is positively associated with peak oxygen uptake in the elderly.

Case-control comparison between age-matched sedentary and chronically trained older volunteers (study 2)

In an attempt to evaluate the effects of chronic exercise on mitochondrial content and function, we compared 60-80 years old active to age and gender matched sedentary adults. Subjects' characteristics are presented in Table 2.

The active exhibited significantly higher MitoVd (+48.9%) compared to sedentary peers (Figure 2A). At the protein level, differences between groups could be observed for the electron transport chain (ETC) complexes (C) I, IV and V, which were significantly higher in the active subjects (Figure 2B-C). Complexes IV and V were positively correlated with MitoVd (rho=0.52 and 0.70, respectively; $p<0.05$). Complexes I, IV and V were positively correlated with VO_2peak/LBM (rho=0.56, 0.76 and 0.55, respectively; $p\leq0.03$). Complexes IV and V were negatively correlated with fat mass ($rho\leq-0.59, p\leq0.01$) and percent body fat ($rho\leq-0.52, p\leq0.05$). No significant differences were detected in the expression levels of genes involved in mitochondrial biogenesis (i.e.*PGC-1α*, *PGC-1β*, *NRF-1*, *NRF-2* and *TFAM*; Figure 2D), despite a clear tendency for *PGC-1α* to have a higher expression in the active group.

In vivo oxidative phosphorylation capacity (reflected in the recovery time constant and ATPmax) was greater in the active than in the sedentary volunteers (+22.0% for k and +21.2% for ATPmax, Table 2). MitoVd and ATPmax were positively correlated (rho=0.74, $p<0.0001$); the same was observed for MitoVd and k (rho=0.61,p=0.002). When taking the ratios rate constant k/MitoVd or ATPmax/MitoVd as a marker of mitochondrial function per volume, there was no difference between groups (Table 2). This suggests that the increase in ATPmax is due to a higher mitochondrial number or content, but not to intrinsic changes per mitochondria.

Exercise intervention in previously sedentary older subjects (study 3)

To investigate the capacity of skeletal muscle from untrained elderly individuals to respond to aerobic training, the sedentary subjects followed a 16-week training program (endurance exercise intervention) with a post-intervention evaluation. Two subjects were excluded from the final data analyses: one man initiated a calorie restriction diet during intervention and had substantial weight loss; the second was a woman who received steroid treatment for acute rheumatoid disease during intervention. Out of the 12 finishers, muscle specimen data was obtained in 10 subjects (6 males, 4 females).

Subjects' characteristics and effect of the intervention on clinical outcomes are presented in Table 3. On average, subjects exercised 3.1±0.1 sessions/week, with an average of 55±1.9 minutes per session. Based on their recorded HR, exercise intensity was of 8.5±0.6 kcal/min, thus achieving the goal of a moderate endurance exercise program corresponding to an average of 5.2±0.4 kcal/kg of body weight expanded per session. The exercise intervention promoted modest, but significant, changes in body weight and BMI (both-2.2%). Body composition changed with improvements in LBM (+1.5%), and marked decrease in FM (-6.6%) and percent body fat (-5.9%). Overall fitness was remarkably improved by the exercise program, with a change of +13.9% in absolute VO_2peak, corresponding to +12.5% relative VO_2peak/LBM.

MitoVd increased by 50.7% with training (Figure 3A). Furthermore, the levels of complex III, IV and V were significantly increased post-intervention, accompanied by a strong tendency for complex I towards up-regulation (+29.1%) (Figure 3B-C). In line with previous reports (30), we also observed a significant increase in *PGC1a* and *TFAM* expression levels following the 4-months of exercise intervention (Figure 3D). The changes in the expression levels of *PGC1a* and *NRF2* were significantly correlated to the increase in *TFAM* expression (rho=0.86 and rho=0.76 respectively,$p \leq 0.03$). The above observations indicate that exercise training increases MitoVd and VO_2peak in older sedentary subject, probably by up-regulating key orchestrators of the mitochondrial biogenesis program.

ATPmax improved by 22.5% (Table 3). ATPmax/MitoVd was not significantly changed with intervention (Table 3). This, again, highlights that the increase in ATPmax is due to enhanced mitochondrial content, not to intrinsic changes in mitochondrial function.

DISCUSSION

It is well established that mitochondrial dysfunction and reduced oxidative capacity are associated with insulin resistance and type 2 diabetes. Aging is similarly associated with a loss of mitochondrial content and function (17-20), which might contribute to the development of age-related insulin resistance and physiological decline. While the positive relationship between mitochondrial content and physical fitness has been acknowledged in younger populations (31, 32), the relationship in older populations has yet to be recognized. Furthermore, it is not clear whether the mitochondrial function decline during aging is a direct consequence of the aging process *per se* or secondary to the sedentary lifestyle that is more prevalent in the aging population (33). Finally, it is also not clear if the possible mitochondrial defects in the aged population are due to a defective ability to trigger mitochondrial biogenesis programs.

Herein we demonstrate that physical fitness is exquisitely correlated with mitochondrial density in skeletal muscle in older adults (60-80 years old). Similarly, using a comprehensive picture of mitochondrial biology from the molecular level (mRNA transcripts, protein expression), the organelle level (mitochondrial density), the whole muscle level (in vivo organelle capacity), and whole-body level (VO_2peak), we demonstrate that the mitochondrial content and function of aged individuals can be largely enhanced by an endurance exercise program. As a whole, our results indicate that ageing per se does not impede mitochondrial biogenesis in response to exercise, and that the decreases in mitochondrial function observed in elder adults are likely due to decreased physical activity.

To our knowledge, this is the largest cohort used to date to evaluate this relationship with a direct measure of skeletal muscle mitochondrial content. Thus, while the overall mitochondrial content is known to decrease with age, its positive relationship with whole body oxygen uptake persists. Further confirming this, in the case-control comparison between older sedentary and age-matched active adults, the active exhibited higher levels of fitness with greater mitochondrial volume density. While our study uses direct measures of mitochondrial content and objective measures of physical fitness, our results are consistent with previous reports in smaller cohorts of both young and old individuals or using indirect markers of mitochondrial content (24, 34, 35).

Interestingly, sedentary older individuals submitted to an exercise intervention displayed large improvements in MitoVd. Actually, post-intervention MitoVd values were similar to levels observed in

304 the active group (sedentary post intervention vs. active p>0.05). This, again, clearly indicates that aged
305 individuals do not have any acquired problem to enhance mitochondrial biogenesis. Although one of the
306 limitations of our study is the lack of comparison with a younger cohort and the fact that other authors
307 suggested that chronic exercise is not able to completely restore mitochondrial content in older subjects
308 (2), it is important to note that post-intervention MitoVd are in the range of younger cohorts (36) or
309 previously published chronically trained older subjects (24).
310 To further solidify the information from MitoVd, we also evaluated mitochondrial function in our
311 patients by means of *in vivo* oxidative capacity. Our endurance trained active subjects displayed greater *in*
312 *vivo* oxidative capacity, as determined by the rate of PCr recovery and ATPmax, compared to age-
313 matched sedentary subjects. However, sedentary subjects improved their *in vivo* oxidative capacity by
314 ~22% after training. Importantly, neither active, nor sedentary subjects pre or post intervention, displayed
315 changes in the ratio of ATPmax/MitoVd. This suggests that the increase in the ability to replenish ATP is
316 not primarily due to mitochondrial intrinsic changes in oxidative function but rather to the higher
317 mitochondrial content. Remarkably, a recent paper by Conley *et al*. (37) (based on a previous study from
318 the same group (20, 38) showed an increase in the ratio of ATPmax (23%) but no significant increase in
319 mitochondrial volume (8.8%) after 6 months of endurance training in older men and women. Thus, their
320 reported ratio of ATPmax/MitoVd, which the authors termed "energy coupling", was increased. However,
321 a large difference in comparing our study is that their intervention (one-legged press exercise described in
322 Jubrias et al. (38)) only improved VO_2max by ~5%. Further initiatives will be required to evaluate how
323 different exercise protocols mitigate the enhancement of ATP synthesis by increasing the intrinsic
324 respiratory coupling or by inducing mitochondrial biogenesis. Another important difference with the work
325 of Conley et al. is that their sedentary subjects were less fit than ours to start with (average VO_2peak 1.7
326 vs 2.0l/min with equivalent body weight); this could mean that a certain minimal activity is needed to
327 keep up the "energy coupling", but this again would point to the effect of exercise and lifestyle, and not to
328 aging per se.
329 This higher mitochondrial content in chronically trained individuals was concurrent to an increase
330 in electron transport chain complexes content, particularly in complexes I, IV, and V. Similar differences
331 in complex IV levels were observed between sedentary knee osteoarthritic older patients and age-matched

active controls (39). Interestingly, in our cohort, sedentary older adults have lower MitoVd than physically active; yet exhibit no differences in complexes II and III concentrations. Similarly, a recent study (40) of young healthy volunteers showed no relationship between MitoVd and the content of complexes I and IV, but a strong correlation with complexes II, III and V. In light of these cumulative data, we propose that MitoVd appears to provide a better representation of mitochondrial content than individual or relative abundance of ETC complexes. It must be kept in mind that analyzing mitochondrial content or function through the evaluation of mitochondrial complexes subunit abundance or by in vitro single complex activities, may be misleading. This overlooks possible additional layers of regulation such as supercomplex assemblies or post-translational modifications, which can heavily affect ETC complexes function without necessarily changing their global content.

Consistent with the increase in mitochondrial content induced by our exercise intervention, transcriptional regulators of mitochondrial biogenesis were markedly upregulated. We observed significant increases in the gene expression of both *PGC-1a* and *TFAM* following the 16-week training, but not in *NRF-1* and *NRF-2*. Prior reports demonstrate that protein expression levels of *PGC1a*, *TFAM* and *NRF1* are increased following 10-weeks of endurance training (2). For *PGC1a*, the magnitude observed in our study (~50%) was similar to the one observed in a 16-week (30) intervention in both younger and older subjects, as well as the one observed for *PGC1a* protein content in a recent 12-week intervention (41). Therefore, exercise can stimulate mitochondrial biogenesis in aged populations and increase this way global respiratory capacity.

This work is not without limitations. First, the common thread between the three parts of this work was the relationship between physical fitness and mitochondrial content/function in older adults. Further studies are needed to address other controversial debates, such as the relationship between mitochondrial function and insulin sensitivity or with the genesis and development of sarcopenia in aged patients. Secondly, we did not explore gender differences, which are thought to influence mitochondrial ATP production (42). Indeed, our measurements of *in vivo* mitochondrial function (study 2 and 3) were performed in a relative small number of volunteers not permitting further stratifications. Lastly, we did not compare our older adults cohorts to a control group of young individuals. Thus, we cannot rule out

that exercise training in a younger population would have enhanced effects in mitochondrial content and function compared to the changes we observed in our 60 to 80 years old population of interest.

In summary, our work, using in-vivo and ex-vivo methodologies thus allowing a comprehensive model of mitochondrial biology, demonstrates (A) that physical fitness is tightly linked to mitochondrial content in a broad and heterogeneous population of older individuals, (B) that aging *per se* is not the primary culprit leading to mitochondrial dysfunction, as (C) aged individuals largely enhance mitochondrial function in response to exercise training. Therefore, the lower oxidative capacity observed in old individuals is likely due to a higher tendency towards a sedentary lifestyle and lower energy demand, as mitochondrial biogenesis programs can be efficiently activated upon stimulation. Accordingly, commencing an aerobic exercise program, even at an older age, can help ameliorate the loss in skeletal muscle mitochondrial content and may prevent muscle aging comorbidities such as sarcopenia and insulin resistance.

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AUTHOR CONTRIBUTIONS

N.T. Broskey collected data, trained all subjects and wrote the manuscript. C. Greggio coordinated volunteers, collected and analyzed data, wrote the manuscript. A. Boss performed MRS, analyzed data and wrote the manuscript. M. Boutant performed RT-qPCR and western blot analysis, analyzed data and reviewed the manuscript. A. Dwyer collected data and edited the manuscript. L. Schlueter reviewed stress tests and edited the manuscript. D. Hans reviewed and edited the manuscript. G. Gremion supervised exercise tests and edited the manuscript. R. Kreis supervised MRS, analyzed data and edited the manuscript. C. Boesch supervised MRS and edited the manuscript. C. Canto analyzed data and wrote the manuscript. F. Amati principal investigator, instigated the project, performed biopsies, analyzed data and wrote the manuscript. All authors have read and agree to the manuscript.

DISCLOSURE SUMMARY

MB and CC are employees of the Nestlé Institute of Health Sciences SA. The work we describe in this manuscript does not have any commercial connection to the work they do at Nestlé. All authors have nothing to disclose.

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529 **TABLES**

530 **Table 1: Study 1, subjects' characteristics**

	Mean	±	SEM	Minimum	Maximum
Age	66.6	±	0.5	60	79
Body weight (kg)	79.3	±	1.5	55.4	106.6
BMI	28.1	±	0.5	19.9	37.3
Body fat (%)	35.0	±	1.4	7.7	51.8
LBM (kg)	47.9	±	1.01	31.3	71.0
VO_2peak (l/min)	1.91	±	0.08	0.87	4.05
VO_2peak/BW (ml/min/kg)	25.2	±	1.3	11.0	59.1
VO_2peak/LBM (ml/min/kg)	39.2	±	1.2	21.9	66.4
MitoVd (%)	3.78	±	0.21	1.09	10.02

531 BW = Body Weight; LBM = Lean Body Mass; MitoVd = Mitochondria volume density.

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534

535 **Table 2: Study 2, subjects' characteristics and *in vivo* skeletal muscle oxidative capacity (PCr**

536 **recovery)**

Subjects characteristics	Active	Sedentary	*p-value**
N	14	14	
Gender (M/F)	7/7	8/6	
Age (years)	67.4 ± 1.2	65.6 ± 0.7	0.21
Body weight (kg)	59.6 ± 2.2	83.9 ± 4.7	*<0.0001*
BMI (kg/m^2)	21.5 ± 0.5	27.8 ± 1.3	*<0.0001*
LBM (kg)	45.7 ± 2.2	54.7 ± 3.1	*0.03*
FM (kg)	12.0 ± 0.7	27.9 ± 3.0	*<0.0001*
Body fat (%)	20.2 ± 1.2	32.3 ± 2.5	*0.0003*
VO_2 peak (l/min)	2.16 ± 0.15	2.06 ± 0.14	0.64
VO_2 peak/LBM (ml/min/kg)	46.1 ± 2.02	37.7 ± 1.8	*0.005*
PCr recovery			
N	12	14	
k (1/min)	2.33 ± 0.11	1.91 ± 0.11	*0.009*
ATPmax (mmol/l/s)	1.37 ± 0.07	1.13 ± 0.05	*0.01*
pH end exercise	7.11 ± 0.01	7.12 ± 0.01	0.39
pH min	6.94 ± 0.01	6.96 ± 0.01	0.16
Decrease in PCr (%)	28.3 ± 1.9	32.1 ± 1.5	0.13
k/MitoVd (1/min/%)	0.35 ± 0.03	0.41 ± 0.03	0.16
ATPmax/MitoVd (mmol/l/s/%)	0.21 ± 0.04	0.24 ± 0.01	0.13

537

538 Data are means±SEM. LBM = Lean Body Mass, FM = Fat Mass; k = PCr recovery rate constant;

539 ATPmax = maximal rate of ATP regeneration. * 2-tailed independent t-test.

540

Table 3: Study 3, subjects' characteristics and *in vivo* skeletal muscle oxidative capacity (PCr recovery) before and after a 4-months endurance training intervention

Subjects characteristics	Sedentary Pre	Sedentary Post	*p-value**
N	12	12	
Gender (M/F)	7/5		
Body weight (kg)	83.3 ± 5.4	81.5 ± 5.1	*0.04*
BMI (kg/m^2)	27.5 ± 1.3	26.9 ± 1.3	*0.04*
LBM (kg)	54.6 ± 3.7	55.4± 3.5	*0.04*
FM (kg)	27.4 ± 3.0	25.6 ± 2.9	*0.0008*
Body fat (%)	32.0 ± 2.6	30.1 ± 2.6	*0.0005*
VO_2 peak (l/min)	2.01 ± 0.16	2.29 ± 0.17	*0.006*
VO_2 peak/LBM (ml/min/kg)	36.97 ± 1.92	41.60 ± 2.03	*0.004*
PCr recovery			
N	12	12	
k (1/min)	1.88 ± 0.12	2.41 ± 0.13	*0.0009*
ATPmax (mmol/l/s)	1.11 ± 0.06	1.36 ± 0.06	*0.006*
pH end exercise	7.13 ± 0.01	7.12 ± 0.01	0.30
pH min	6.96 ± 0.01	6.96 ± 0.02	0.35
Decrease in PCr (%)	31.4 ± 1.4	30.5 ± 2.0	0.72
k/MitoVd (1/min/%) (N=10)	0.42 ± 0.04	0.35 ± 0.04	0.22
ATPmax/MitoVd (mmol/l/s/%) (N=10)	0.24 ± 0.02	0.20 ± 0.02	0.13

Data are means±SEM. LBM = Lean Body Mass, FM = Fat Mass; k = PCr recovery rate constant; ATPmax = maximal rate of ATP regeneration. * 2-tailed paired t-test.

548 **FIGURE LEGENDS**

549

550 **Figure 1: Study 1 (N=80).** Linear relationship between physical fitness (VO_2peak) and mitochondrial
551 volume density (MitoVd).

552

553 **Figure 2: Study 2, skeletal muscle comparison between age-matched older** active **and sedentary**
554 **subjects. A**. Mitochondrial volume density (active N=13 and sedentary N=12). *, p=0.0003. B Western
555 Blots from representative subjects belonging either to the active (Act) or sedentary (Sed) group. **C.**
556 Electron transport chain complex relative abundance (active N=7; sedentary N=8; the values normalized
557 over the corresponding α-tubulin levels are shown). *, p≤0.02; **, p=0.0001. **D**. Relative mRNA
558 abundance (active N=7 and sedentary N=9). For all panels, Error Bar = SEM; black bar = active; white
559 bar = sedentary.

560

561 **Figure 3: Study 3, skeletal muscle of older sedentary adults before and after 4-month endurance**
562 **training intervention. A.** Mitochondrial volume density (N=10). **B**. Paired Western Blots on ETC
563 complexes from representative subjects before and after intervention. **C.** Electron transport chain complex
564 relative abundance (N=7; the values normalized over the corresponding α-tubulin levels are shown). **D.**
565 Gene expression profiles (N=8). For all panels, error Bar = SEM; black bar = pre-intervention; white bar
566 = post-intervention; *, p=0.03 (1-tail); **, p<0.05.

567

568

Figure 1

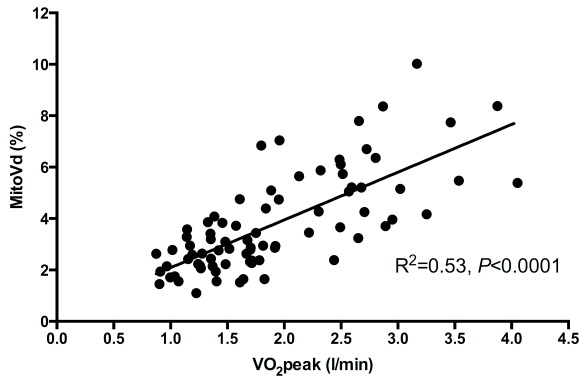


Figure 2

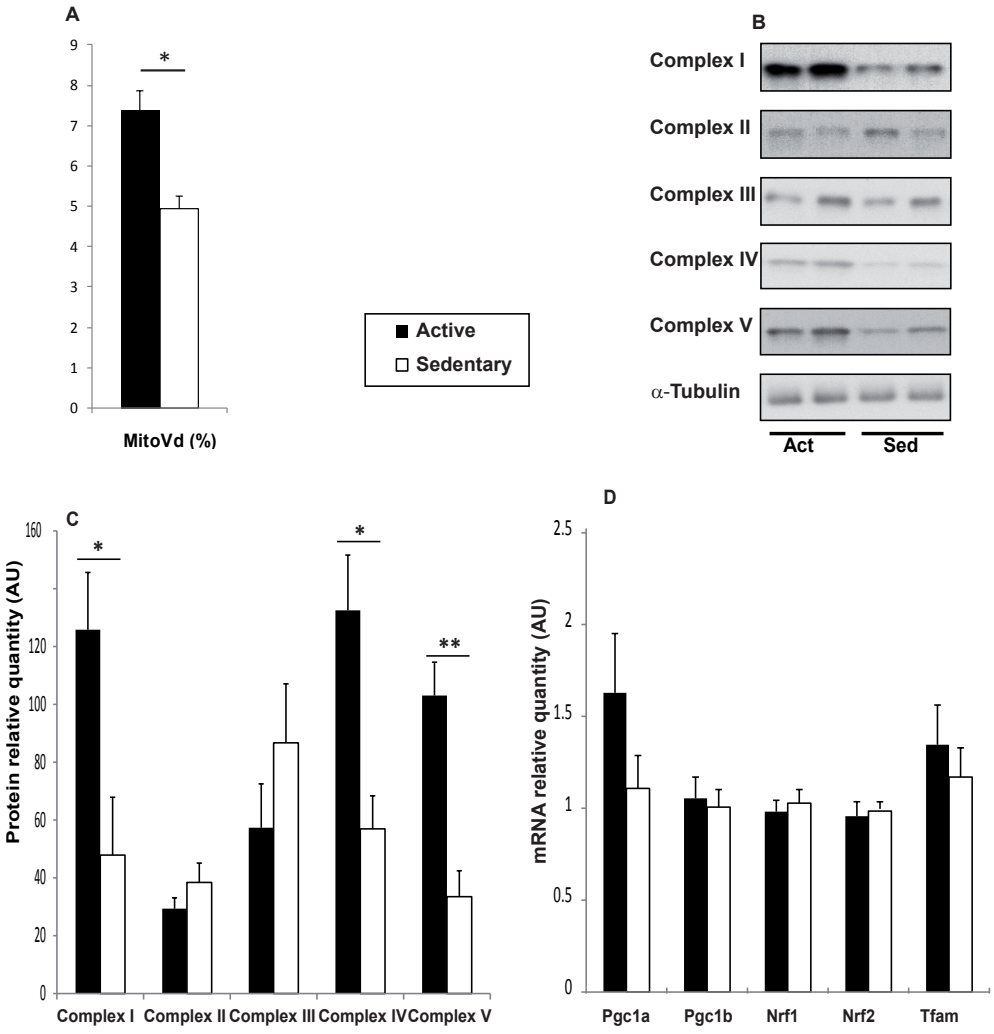
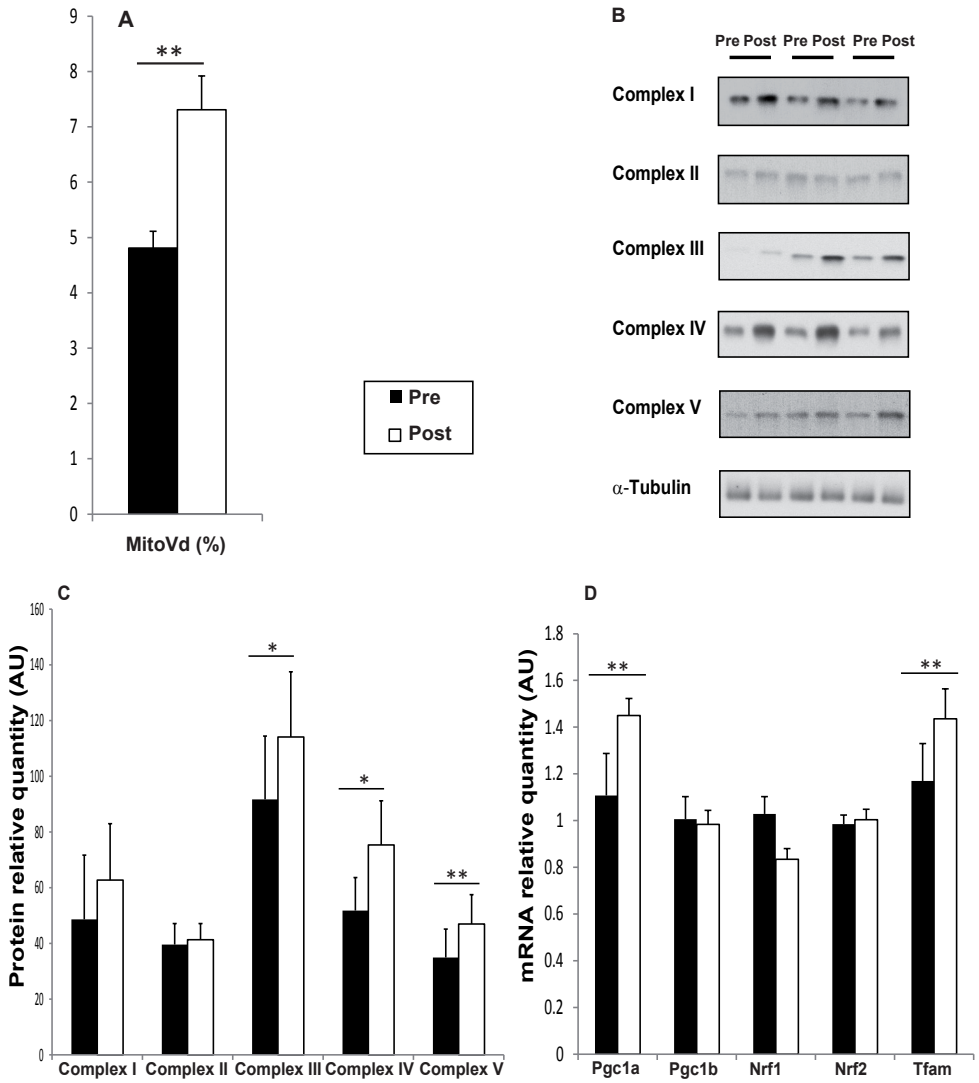


Figure 3


A
**
MitoVd (%)
Pre
Post
B
Pre Post Pre Post Pre Post
Complex I
Complex II
Complex III
Complex IV
Complex V
α-Tubulin
C
Protein relative quantity (AU)
Complex I
Complex II
Complex III
Complex IV
Complex V
D
mRNA relative quantity (AU)
Pgc1a
Pgc1b
Nrf1
Nrf2
Tfam

Supplemental material

Supplemental table 1: Western-blotting antibodies

Antibody anti-	Brand	Number
α-Tubulin	Sigma-Aldrich	T9026
Complex I NDUFA9	Mitosciences	ab14713
Complex II SDHA	Abcam	ab14715
Complex III UQCRC1	Abcam	ab14705
Complex IV MTCO1	Abcam	ab14748
Complex V ATP5A	Abcam	ab109865

Supplemental table 2: qRT-PCR primers

PCR Primers Gene	Sequence
b-Actin	F : 5'-TCGTGCGTGACATTAAGGAG-3' R : 5'-GTCAGGCAGCTCGTAGCTCT-3'
cyclophilin B	F : 5'-CTTCCCCGATGAGAACTTCAAACT-3' R : 5'-CACCTCCATGCCCTCTAGAACTTT-3'
PGC1a	F : 5'-TCTGAGTCTGTATGGAGTGACAT-3' R : 5'-CCAAGTCGTTCACATCTAGTTCA-3'
PGC1b	F : 5'-GCGAGAAGTACGGCTTCATCA-3' R : 5'-AGCGCCCTTTGTCAAAGAGA-3'
NRF1	F : 5'-GGTGCAGCACCTTTGGAGAA-3' R : 5'-CCAGAGCAGACTCCAGGTCTTC-3'
NRF2	F : 5'-CAAGAACGCCTTGGGATACC-3' R : 5'-AAACCACCCAATGCAGGACTT-3'
TFAM	F : 5'-GCACCGGCTGTGGAAGTCGAC-3' R : 5'-CAGGAAGTTCCCTCCAACGCTGG-3'