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

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54

55 **ABSTRACT**

56

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

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

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

65 *Setting:* University-based clinical research center

66 *Outcomes:* M volume density (Mv) was assessed by electron microscopy from *vastus lateralis* biopsies,
67 electron transport chain proteins (ETC) by western blotting, mRNAs for transcription factors involved in
68 M biogenesis by qRT-PCR and *in-vivo* oxidative capacity (ATPmax) by ³¹P-MR spectroscopy. Peak
69 oxygen uptake (VO₂peak) was measured by GXT.

70 *Results:* VO₂peak was strongly correlated with Mv in eighty 60-80 yo adults. Comparison of A vs. S
71 revealed differences in Mv, ATPmax and some ETC complexes. Finally, exercise intervention confirmed
72 that S are able to recover Mv, ATPmax and specific transcription factors.

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

76

77 **KEYWORDS**

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

80

81 INTRODUCTION

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

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

97 Aging is associated with a loss of mitochondrial content (17, 18) and function (18-20) in muscle.
98 However, studies comparing younger to older subjects are conflicting (21, 22). Indeed, it is not clear
99 whether the decrease in mitochondrial content is associated with aging *per se* or with the decreased
100 physical activity that comes with aging. Given the important role of aerobic exercise in up-regulating
101 genes and transcription factors controlling mitochondrial content and function, it remains to be seen
102 whether aerobic exercise training could play a protective role for mitochondria in aging and if training can
103 help older individuals recover mitochondrial content. Therefore, this study had two main objectives: first
104 to examine the relationship between mitochondrial content and physical fitness in older men and women
105 focusing on external validity with a broad population in terms of physical fitness and body composition.
106 Secondly, focusing on internal validity using a comprehensive picture of mitochondrial biology from the
107 molecular level (mRNA transcripts, protein expression), the organelle level (mitochondrial density), the
108 whole muscle level (in vivo organelle capacity), and whole-body level (VO₂peak), combining invasive

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

111

112 **RESEARCH DESIGN AND METHODS**

113 *Study design*

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

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

124 *Subjects*

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

134 *Exercise intervention (study 3)*

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

137 progressively increased from 30 to 60 minutes. Moderate intensity was defined as 75% of the subjects'
138 heart rate (HR). Exercise prescription was individualized based on the subject's peak HR achieved during
139 the baseline VO₂peak test and adapted at midpoint of the intervention with a submaximal ergometer test
140 as described in details in Dubé et al (23). HR monitors (Polar Electro Oy, Kempele, Finland) and exercise
141 logs were used to monitor intensity. Subjects could bike, walk, run or row within their HR target range
142 with at least 80% of the training as walking or biking. Frequency, duration and volume of exercise were
143 recorded and computed as described elsewhere (23). During the training regimen subjects were instructed
144 to follow their typical food intake and not to undertake dietary changes while engaged in the study.

145 *Clinical outcome measures*

146 **Height** was measured using a wall-mounted stadiometer and **weight** using a calibrated medical
147 digital scale (Seca, Hamburg, Germany). **Lean body mass (LBM)** was determined by dual-energy X-ray
148 absorptiometry (DiscoveryA, Hologic Inc., Bedford, MA). **Physical fitness** was determined by peak
149 oxygen consumption (VO₂peak) using a graded exercise test on an electronically braked cycle ergometer
150 (Lode B.V., Groningen, The Netherlands). HR, blood pressure and ECG were recorded before, during and
151 after the exercise test. VO₂ was computed via indirect calorimetry (Metalyzer3B, Cortex GmbH, Leipzig,
152 Germany). The protocol was adapted from previously used protocols well suited for older volunteers of
153 various degrees of fitness or fatness (24). Briefly, after an initial warm-up consisting in 2 minutes of no-
154 load pedaling, the graded exercise test began at 25W for women or 50W for men for the first 2 minutes
155 and was then increased 25-50W thereafter until volitional exhaustion or if one of the American College of
156 Sports Medicine established criteria for maximal testing had been reached.

157 *Ex-vivo skeletal muscle outcome measures*

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

165 **(study 1, 2 and 3):** TEM was used to measure mitochondrial volume density (MitoVd) as a marker of
166 mitochondrial content. A recent validation and detailed description of this stereological method has been
167 described elsewhere (25). **Protein expression (study 2 and 3):** Frozen tissue was homogenized in 200 μ l
168 of ice-cold lysis buffer containing 50mM Tris-HCl, pH7.5, 150mM NaCl, 1%(v/v)Nonidet P-40, 1mM
169 EDTA and freshly added protease inhibitor cocktail tablet (Roche Diagnostics International, Rotkreuz,
170 Switzerland), using a motor-driven Eppendorf homogenizer. Homogenates were then rotated for
171 30minutes at 4°C before centrifugation at 15,000rpm for 10min at 4°C. The pellet was discarded, and the
172 supernatant was collected and stored at -80°C until used. Protein was measured by the BCA method
173 (Pierce, ThermoFisher Scientific Inc., Rockford, IL). Western blotting was performed as previously
174 described (26). Protein band intensity was measured by ImageJ and the target protein levels were
175 normalized over the corresponding α -tubulin loading controls for each subject. All antibodies for
176 mitochondrial complex subunits have been purchased from Mitosciences (Abcam, Cambridge, UK). The
177 list of antibodies can be found in supplemental Table 1. **Gene expression analysis (study 2 and 3):** Total
178 mRNA preparations, cDNA synthesis and RT-qPCR were performed as described previously (26).
179 Primers are described in supplemental Table 2. Target mRNA levels were normalized over the geometric
180 mean of b-Actin and CyclophilinB, that were selected as housekeeping genes after having checked their
181 expression stability (27). Relative mRNA expression levels were calculated with the $\Delta\Delta$ Ct method, where
182 we used the mean of the Δ Cts from 5 sedentary subjects as Δ Ct calibrator.

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

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

193 each subject's strength, which got determined beforehand by maximal isokinetic torque of the knee
194 extensors. Default exercise duration was 28s. If the relative decrease of PCr was outside the target of 20
195 to 40%, exercise duration was changed to 22s, 36s, or 44s; otherwise it was unchanged for a 2nd
196 repetition. Since pH did not decrease below 6.8 in any experiment and recovery rates of experiment 1 and
197 2 were not significantly different from each other ($p=0.92$), results are shown as average of the 2
198 experiments. For the post-processing, spectra were analyzed with jMRUI (29) using AMARES for
199 quantitation. The recovery of PCr was fitted to the formula $PCr(t)=PCr_0+\Delta PCr(1-e^{-k \cdot t})$; with $PCr_0=PCr$
200 intensity at the beginning of recovery; ΔPCr =exercise-induced decrease of the PCr signal. pH was
201 calculated from the chemical shift between inorganic phosphate and PCr. The oxidative phosphorylation
202 capacity (ATPmax) was computed as previously suggested (20) as the product of the recovery rate k and
203 the resting PCr content obtained from the resting spectrum and assuming a constant ATP concentration of
204 8.2mM.

205

206 ***Statistical Procedures***

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

217

218 **RESULTS**

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

220 A total of 80 subjects, 33 men and 47 women, were included in this study. The cohort was
221 heterogeneous with wide ranges of MitoVd, VO₂peak, BMI and body fatness (Table 1). A strong
222 relationship was observed between MitoVd and VO₂peak (Figure 1). This relationship was similar when
223 VO₂peak was normalized by LBM or body weight. These data show that skeletal muscle mitochondrial
224 content is positively associated with peak oxygen uptake in the elderly.

225

226 *Case-control comparison between age-matched sedentary and chronically trained older volunteers* 227 *(study 2)*

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

231 The active exhibited significantly higher MitoVd (+48.9%) compared to sedentary peers (Figure
232 2A). At the protein level, differences between groups could be observed for the electron transport chain
233 (ETC) complexes (C) I, IV and V, which were significantly higher in the active subjects (Figure 2B-C).
234 Complexes IV and V were positively correlated with MitoVd ($\rho=0.52$ and 0.70 , respectively; $p<0.05$).
235 Complexes I, IV and V were positively correlated with VO₂peak/LBM ($\rho=0.56$, 0.76 and 0.55 ,
236 respectively; $p\leq 0.03$). Complexes IV and V were negatively correlated with fat mass ($\rho\leq -0.59$, $p\leq 0.01$)
237 and percent body fat ($\rho\leq -0.52$, $p\leq 0.05$). No significant differences were detected in the expression
238 levels of genes involved in mitochondrial biogenesis (i.e. *PGC-1 α* , *PGC-1 β* , *NRF-1*, *NRF-2* and *TFAM*;
239 Figure 2D), despite a clear tendency for *PGC-1 α* to have a higher expression in the active group.

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

247

248 ***Exercise intervention in previously sedentary older subjects (study 3)***

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

255 Subjects' characteristics and effect of the intervention on clinical outcomes are presented in Table
256 3. On average, subjects exercised 3.1 ± 0.1 sessions/week, with an average of 55 ± 1.9 minutes per session.
257 Based on their recorded HR, exercise intensity was of 8.5 ± 0.6 kcal/min, thus achieving the goal of a
258 moderate endurance exercise program corresponding to an average of 5.2 ± 0.4 kcal/kg of body weight
259 expanded per session. The exercise intervention promoted modest, but significant, changes in body
260 weight and BMI (both -2.2%). Body composition changed with improvements in LBM (+1.5%), and
261 marked decrease in FM (-6.6%) and percent body fat (-5.9%). Overall fitness was remarkably improved
262 by the exercise program, with a change of +13.9% in absolute VO_2 peak, corresponding to +12.5%
263 relative VO_2 peak/LBM.

264 MitoVd increased by 50.7% with training (Figure 3A). Furthermore, the levels of complex III, IV
265 and V were significantly increased post-intervention, accompanied by a strong tendency for complex I
266 towards up-regulation (+29.1%) (Figure 3B-C). In line with previous reports (30), we also observed a
267 significant increase in *PGC1a* and *TFAM* expression levels following the 4-months of exercise
268 intervention (Figure 3D). The changes in the expression levels of *PGC1a* and *NRF2* were significantly
269 correlated to the increase in *TFAM* expression ($\rho=0.86$ and $\rho=0.76$ respectively, $p \leq 0.03$). The above
270 observations indicate that exercise training increases MitoVd and VO_2 peak in older sedentary subject,
271 probably by up-regulating key orchestrators of the mitochondrial biogenesis program.

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

275

276 **DISCUSSION**

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

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

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

302 Interestingly, sedentary older individuals submitted to an exercise intervention displayed large
303 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_2 max 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

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

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

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

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

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

371

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385 Diabetes annual scientific meeting in September 2013.

386

387 **AUTHOR CONTRIBUTIONS**

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

397

398 **DISCLOSURE SUMMARY**

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

402

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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 ₂ peak (l/min)	1.91	±	0.08	0.87	4.05
VO ₂ peak/BW (ml/min/kg)	25.2	±	1.3	11.0	59.1
VO ₂ peak/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	<i>p-value*</i>
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

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

Subjects characteristics	Sedentary Pre	Sedentary Post	<i>p-value*</i>
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

543

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

545 ATPmax = maximal rate of ATP regeneration. * 2-tailed paired t-test.

546

547

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). *, $p\leq 0.02$; **, $p=0.0001$. **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

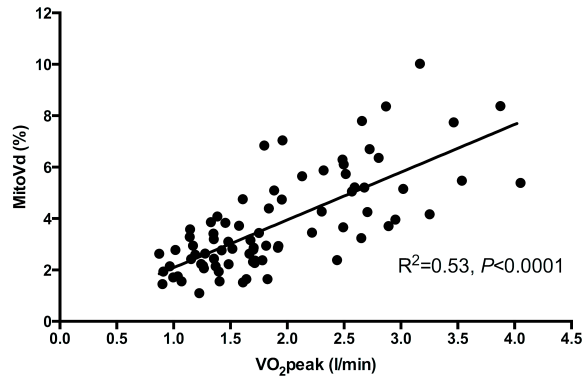


Figure 2

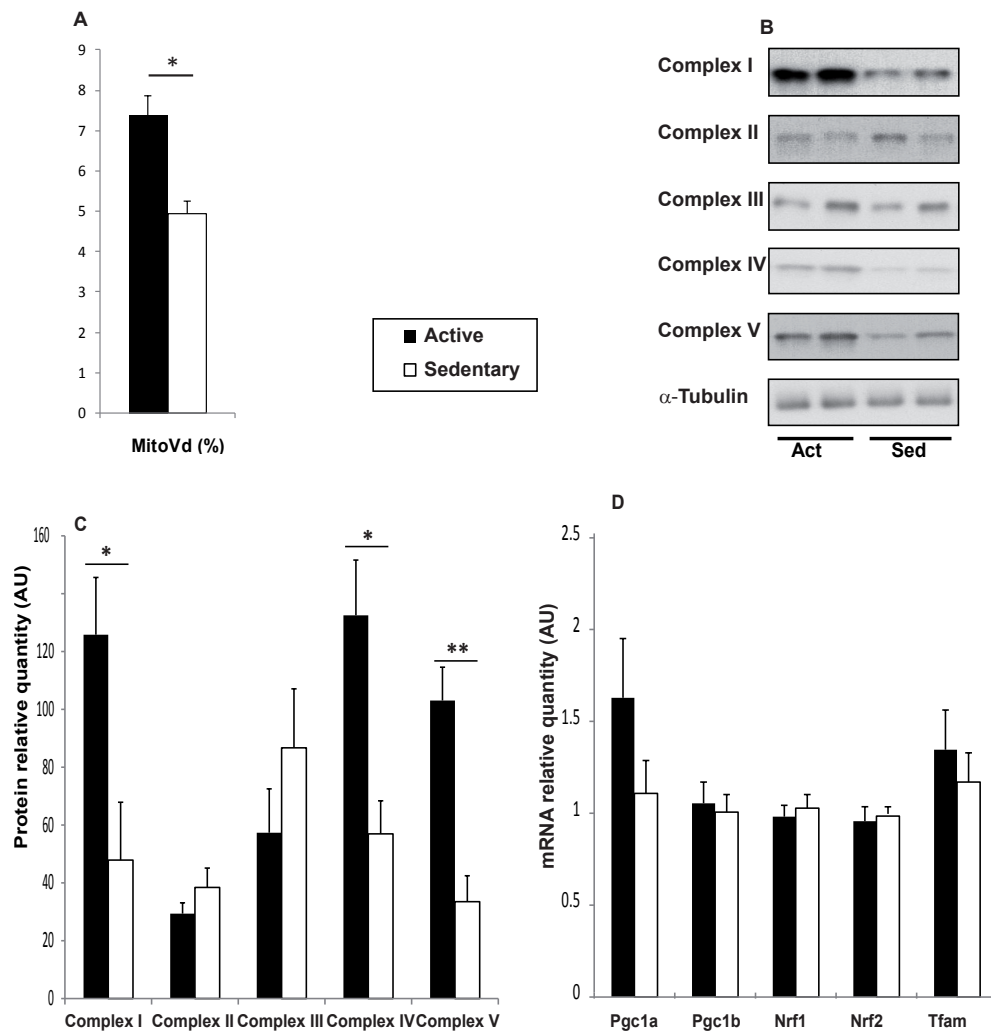
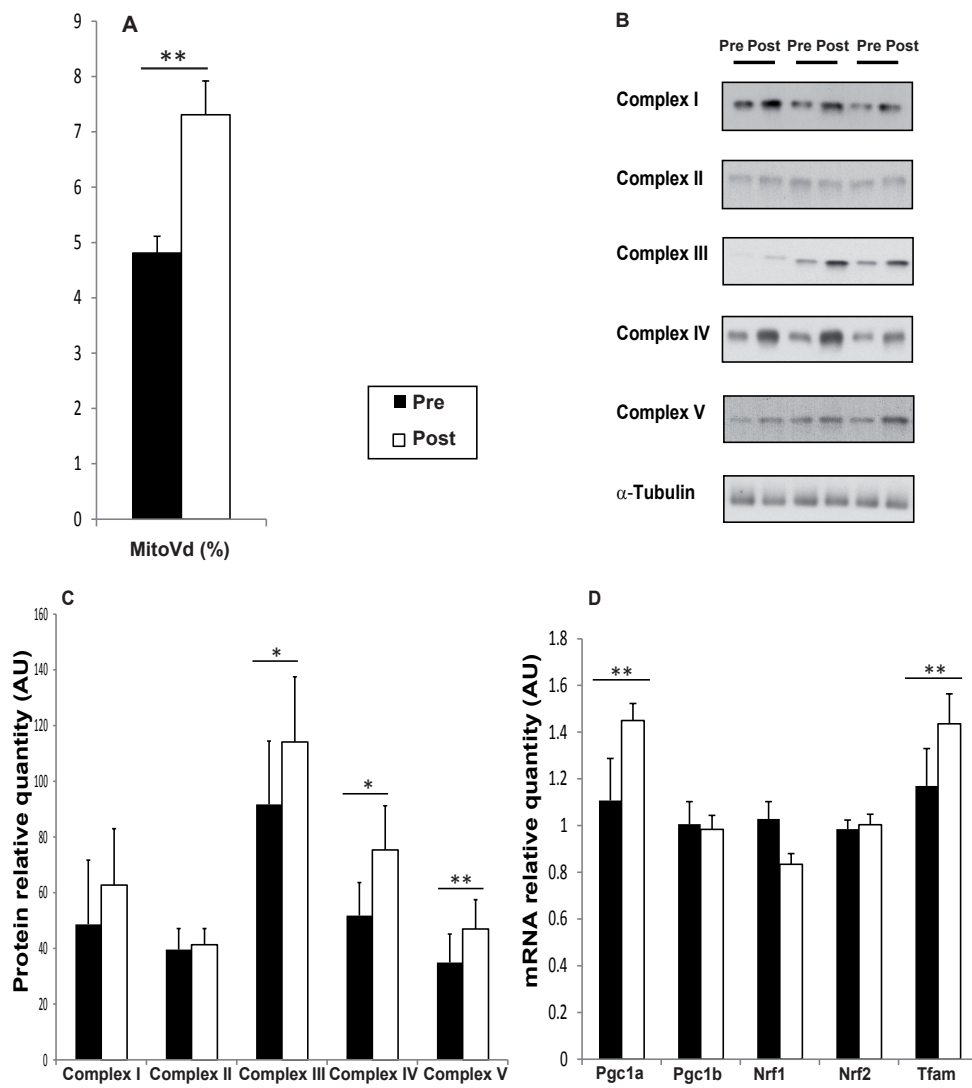


Figure 3



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'-CTTCCCCGATGAGAACTTCAAAC-3' R : 5'-CACCTCCATGCCCTCTAGAACTTT-3'
PGC1a	F : 5'-TCTGAGTCTGTATGGAGTGACAT-3' R : 5'-CCAAGTCGTTACATCTAGTTCA-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'-AAACCACCAATGCAGGACTT-3'
TFAM	F : 5'-GCACCGGCTGTGGAAGTCGAC-3' R : 5'-CAGGAAGTCCCTCCAACGCTGG-3'