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Distinct Patterns of Skeletal Muscle Mitochondria Fusion, Fission and Mitophagy upon Duration of Exercise Training

Yoan Arribat[1], Nicholas T. Broskey[1], Chiara Greggio[1], Marie Boutant[2], Sonia Conde Alonso[1], Sameer S. Kulkarni[2], Sylviane Lagarrigue[1], Elvis A. Carnero[1], Cyril Besson[3], Carles Cantó[2], Francesca Amati[1,4,5]

[1]Aging and Muscle Metabolism Lab, Department of Physiology, University of Lausanne, Lausanne, 1005, Switzerland

[2]Nestlé Institute of Health Sciences, Lausanne,1015, Switzerland

[3]Sport Medicine Unit, University Hospital (CHUV), Lausanne, 1011, Switzerland

[4]Institute of Sports Sciences (ISSUL), University of Lausanne, Lausanne, 1005, Switzerland

[5]Service of Endocrinology, Diabetology and Metabolism, Department of Medicine, University Hospital (CHUV), Lausanne, 1011, Switzerland

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Correspondence:

Francesca Amati, MD-PhD

University of Lausanne

Bugnon 7

1005 Lausanne

Phone +41 21 692 5552

Fax +41 21 692 5505

Email: francesca.amati@unil.ch

Abstract:

Aim: Healthy aging interventions encompass regular exercise to prevent mitochondrial dysfunction, key player in sarcopenia pathogenesis. Mitochondrial biogenesis has been well documented, but mitochondrial remodeling in response to exercise training is poorly understood. Here we investigated fusion, fission and mitophagy before and after an exercise intervention in older adults.

Methods: Skeletal muscle biopsies were collected from 22 healthy sedentary men and women before and after 4 months of supervised training. Eight lifelong trained age- and gender-matched volunteers served as positive controls. Transmission electron microscopy was used to estimate mitochondrial content. Western blotting and qRT-PCR were used to detect changes in specific proteins and transcripts.

Results: After intervention, mitochondrial content increased to levels of controls. While enhancement of fusion was prevalent after intervention, inhibition of fission and increased mitophagy were dominant in controls. Similarly to PARKIN, BCL2L13 content was higher in controls. The observed molecular adaptations paralleled long-term effects of training on physical fitness, exercise efficiency and oxidative capacity.

Conclusions: This study describes distinct patterns of molecular adaptations in human skeletal muscle under chronic exercise training. After 16 weeks of exercise, the pattern was dominated by fusion to increase mitochondrial content to the metabolic demands of exercise. In lifelong exercise, the pattern was dominated by mitophagy synchronized with increased fusion and decreased fission, indicating an increased mitochondrial turnover. In addition to these temporally distinct adaptive mechanisms, this study suggests for the first time a specific role of BCL2L13 in chronic exercise that requires constant maintenance of mitochondrial quality.

Introduction

Mitochondria are key players in muscle dysfunctions that come with aging, such as sarcopenia. In skeletal muscle, mitochondria form a highly dynamic network that adapts to changing energetic stimuli by modifying their reticular nature and their content. Mitochondrial biogenesis, the creation of newly synthesized mitochondria, has been extensively described and studied in humans, particularly in response to exercise.[1, 2] We have recently shown that sedentary older adults were able to increase mitochondrial volume density (MitoVD), electron transport chain proteins and oxidative capacity to levels of chronically trained age-matched controls[3] through an important drive of biogenesis. We also observed limited availability of space between muscle fibers.[4] Little is known with regards to mitochondrial turnover in chronic exercise, which would clarify not only the observation of the limited spatial availability but also the continuous need to maintain mitochondrial quality for the energetic demand of chronic exercise.

Mitochondrial remodeling can be conceptualized as a balancing act combining the generation of mitochondria with their dynamic modifications in the network (called fusion/fission), and subsequent clearance through macroautophagy (referred to as mitophagy).[5, 6] Fusion facilitates ATP synthesis, prevents mitochondrial DNA loss and allows mingling of the organelles.[7] Fusion is mediated by two GTPases, Mitofusin 1 and 2 (MFN1 and MFN2) which ensure the connection of outer membranes.[8] Concomitantly, Optic Atrophy 1 (OPA1), a dynamin related protein, allows fusion of the inner membrane.[9] Fission allows proper distribution of mitochondria, participates in the removal of damaged mitochondria and facilitates apoptosis. Recruitment of the cytoplasmic proteins dynamin-related protein1 (DRP1), mitochondrial fission 1 (FIS1) and mitochondrial fission factor (MFF) trigger fission processes.[10, 11] The most studied mitochondrial degradation pathway, triggered by mitochondrial depolarization, involves the accumulation of PTEN induced kinase 1 (PINK1) in the outer membrane of damaged mitochondria.[12] PINK1 promotes the phosphorylation of its different targets, which in turn recruit PARKIN E3 ligase from the cytosol to the mitochondrial membrane. PARKIN mediates the ubiquitination of its different substrates among which are the voltage dependent anion channel 1 (VDAC1)[13], MFN 1 and 2[14, 15], and DRP1.[16] These ubiquitinated targets bind P62 and the downstream autophagophore machinery[17], via the microtubule-associated protein 1 light chain 3 (LC3), thus linking mitophagy specific cascades with global autophagy. In hypoxia, BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) is another inducer of PARKIN dependent mitophagy, but BNIP3 can also ensure direct interaction with LC3 to load targeted mitochondria into autophagosomes.[18, 19]

Evidence of a PARKIN-independent mitophagy has recently been identified in mammalian cultured cells with B-cell lymphoma 2-Like Protein 13 (BCL2L13) in the mitochondrial membrane[20], serving as a mammalian homolog of the autophagy related protein Atg32 in yeast[21]. BCL2L13, through the direct interaction with LC3, allows engulfment of mitochondria into autophagosomes in PARKIN deficient cells[20]. The role of BCL2L13 pathway in skeletal muscle has yet to be explored.

While numerous human interventional studies have confirmed the effect of exercise training on key regulators of mitochondria biogenesis in multiple populations[22-24], few have explored this effect in fusion and fission[25-27], with contradictory results[6], and none have looked at the effect of an exercise intervention on human muscle mitophagy. The primary aim of this study was to investigate in older adults, markers of mitophagy, fusion and fission using the exact same exercise intervention that increased mitochondrial content and efficiency in human skeletal muscle[3], focusing on internal validity and using a comprehensive picture of mitochondrial biology from the molecular level to the whole-body level. Our main hypothesis was that mitochondrial turnover is necessary to maintain efficient mitochondria as well as to overcome the limited space within human skeletal muscle with chronic exercise. Our secondary aim was to quantify BCL2L13 in the muscle of our volunteers before and after the exercise intervention.

Results

Subjects' characteristics

Twenty-two sedentary men and women participated in this exercise intervention study. Subjects' characteristics are presented in table 1. Three subjects dropped out from intervention due to a flare-up of knee osteoarthritis, time constraint, and the desire to fast during the intervention (lent), which was incompatible with our guidelines of not dieting while in intervention. Among the 19 finishers, adherence to supervised exercise was on average of 2.9 (± 0.2 SD) sessions/week and 145 (± 33 SD) minutes/week in the targeted heart rate range. Each supervised session included cycling on a stationary bicycle (52.2±1.1 % of the session) and walking and/or running on a treadmill (44.0±1.4 % of the session). In order not to wait for equipment, subjects were allowed to row if the treadmills or stationary bikes were not immediately available (3.7±1.4 % of the session). Non-supervised seasonal activities

were on average 0.5 (± 0.1 SD) sessions/week and consisted of walking outdoors in summer or skiing in winter.

Eight age- and gender-matched regular exercisers were recruited as positive controls. Controls reported an average of 3.1 (± 1.3 SD) sessions/week of their principal exercise mode which were walking, running or cycling. Additional seasonal activities were on average 1.0 (± 1.1 SD) sessions/week and consisted of walking or cycling in the summer and skiing in winter. While two controls started to exercise in their 30s, the rest started in their teens. The mean duration of their lifelong exercise was 41.3 years (± 8.3 SD). Some controls took breaks or reduced their activity for short periods due to family or work demands, but none of these breaks were within the last 10 years.

The use of tri-axial accelerometers/temperature sensors to estimate daily physical activity and steps for 7 days confirmed that physical activity levels were significantly lower in pre-intervention and improved at post-intervention. Furthermore, neither steps nor daily physical activity energy expenditure were significantly different between post-intervention and controls (table 2). This is of importance to ascertain that, when comparing the intervention group to controls, molecular outcomes are not explained by exercise loads or intensities, which would be reflected by a higher physical activity energy expenditure. Further, previously sedentary subjects did not compensate with sedentary behaviors during intervention.

Subjects and controls had similar dietary habits. Although this study was not focused on nutritional status, a three-day food recall was administered to assess habitual diet and control for eventual dietary changes with intervention. No differences were observed between groups at baseline, neither in quantitative (subjects average intake of 2193±145 kcal/day, controls 2391±122 kcal/day) nor in qualitative measures (supplemental figure 1). Amount of food intake did not change with intervention (2253±138 kcal/day post-intervention) nor did the dietary pattern. Indeed the amount and relative proportion of macronutrients (carbohydrates, lipids, proteins and fibers) remained the same before and after intervention. Interestingly, we also did not see any seasonal differences in this age group.

Effects of training on exercise parameters

Physical fitness, exercise efficiency and systemic fat oxidation adaptations significantly increased in post intervention, not reaching the highest levels observed in controls (Fig 1A-D). This pattern was seen for all expression of peak oxygen uptake and gross efficiency (i.e. absolute and relative

units). The same pattern was observed for markers of *in vivo* oxidative capacity measured by phosphocreatine recovery (Fig 1E-F).

Mitochondrial volume density (MitoVD) and electron transport chain complexes expression levels

With intervention, skeletal muscle MitoVD increased on average by 36.4%, reaching similar levels as those observed in controls (Fig 2A). Although subjects doubled their Complex I content post-intervention, it remained significantly different from the controls, which had a 3-fold higher content than pre-intervention (Fig 2C). Complex III and Complex V were lower in the sedentary condition and increased to the levels of the controls in post intervention (Fig 2D-E).

Fusion and fission

While MFN2 content was significantly increased with intervention, it remained lower than in controls who had an approximately 6 times higher amount (Fig 3A). MFN1 did not change with intervention, but was two times higher in controls (Fig 3B). OPA1 increased with intervention; controls had similar levels as sedentary at baseline (Fig 3C). Although the intervention had no effect on the amounts of DRP1 and the phosphorylation of its inhibition site DRP1^{S637}, controls exhibited three times lower levels of DRP1 (Fig 3D) and two times higher DRP1^{S637} phosphorylation (Fig 3E). Transcripts encoding fusion/fission proteins were not significantly changed with intervention or across groups (supplemental figure 2).

Mitophagy and global autophagy

Mitophagy inducers, PINK1 and BNIP3, exhibited similar adaptations, increasing protein content from pre- to post-intervention (Fig 4A-B). While significant differences were observed at baseline with controls, no significant differences were observed between post-intervention and controls (Fig 4A-B). Mitophagy effectors, PARKIN and BCL2L13, did not respond to intervention, but were respectively 3 and 6 times higher in controls (Fig 4D-E). VDAC1 followed a similar pattern (three times higher in controls) (Fig 4C).[17, 28]

Regarding global markers of autophagy, LC3-I and its lipidated form LC3-II were not modified with intervention (Fig 4G). The active form, LC3-II was reduced in controls compared to the sedentary

subjects, but LC3-I was not different. P62 was neither modified with intervention nor different in controls (Fig 4F).

Correlations

Pairwise correlations were explored at baseline by combining sedentary volunteers pre-intervention and controls, to avoid the dependency of pre and post-intervention outcomes. Correlations among physiological and molecular outcomes are presented in table 3. Correlations among fusion, fission and mitophagy markers are presented in supplemental table 1. Correlations among physiological outcomes are presented in supplemental table 2.

Discussion

The primary finding of this study is the observation of distinct patterns of fusion, fission and mitophagy, but with similar MitoVD, in skeletal muscle of previously sedentary volunteers that underwent four months of exercise intervention compared to lifelong trained controls. In subjects, the increased mitochondrial content post-intervention was concomitant to increased biogenesis (described in [3]) and increased fusion. Controls presented high contents of mitophagy markers as well as decreased fission, speaking for an enhanced mitochondrial turnover. For the first time in humans, BCL2L13 has been evidenced as a potential novel mitophagy player in exercise induced skeletal muscle adaptations.

Increases in MitoVD with endurance training are due to a combination of mechanisms

The observation that four months of training increases mitochondrial content in previously sedentary subjects to lifelong exerciser's levels confirms our previous study[3] and is in line with other studies, in older or younger sedentary subjects, that used different markers of mitochondrial content, such as citrate synthase or succinate dehydrogenase.[25] These results were often explained by increases in mitochondria biogenesis, particularly with elevated levels of PGC1α and TFAM in response to training.[3, 25] The effect of chronic exercise on mitochondrial biogenesis has been extensively explored in cross-sectional studies encompassing elite young up to octogenarian athletes[29, 30] or exercise interventions in multiple populations.[23, 24] Recently Meinild Lundby et al. pointed to the fact that exercise training increases MitoVD by enlargement of existing mitochondria and not solely *de novo* biogenesis.[31]

Importantly, while we focused only on intramyofibrillar mitochondria MitoVD, the authors reported increases in both subsarcolemmal and intramyofibrillar mitochondria MitoVD, showing that the faction of intramyofibrillar mitochondria population contributed more to the augmented MitoVD with training than the subsarcolemmal population.[31] The novelty of our study lies on the identification of distinct adaptive mechanisms of mitophagy, fusion and fission, within the continuous drive for mitochondrial biogenesis formerly described. [3]

Mitochondrial dynamics

Our pre-/post-intervention data point to the fact that four months of exercise training in previously sedentary subjects stimulate fusion, through the response of MFN2 and OPA1. Fission was unchanged with stable levels of DRP1 and DRP1^{S637} phosphorylation. This is in accord with previous aerobic exercise training interventions in humans. Konopka et al. [25] showed that 12 weeks of training increased MFN1 and 2 in human muscle. Meinild Lundby et al. [31] pointed to an increase of ~50% of MFN2 in support to their observation of mitochondrial volume expansion of existing mitochondria. Wyckelsma et al. [32] found increases in MFN2 protein by 16% following 3 months of high intensity interval training in younger but not in older subjects which had lower content of MFN2 post-training in glycolytic fibers compared to oxidative fibers. Fealy et al. [26] presented unchanged levels of DRP1 after 12 weeks of exercise training, but their results exhibited decreased DRP1^{S616} phosphorylation suggesting decreased fission. Konopka et al. [25] observed increased levels of FIS1 but did not measure DRP1. Perry et al. [27] presented increases in FIS1 and DRP1 in human muscle after few high intensity exercise sessions. We chose deliberately not to compare our results to rodent studies, acute bout studies or mRNA modifications due to the confirmed time-course observations showing that mRNA responses to exercise sessions are pulsatile and transient.[33, 34]

Muscle from controls presented distinct content of fusion/fission markers with higher levels of MNF1 and MFN2, but lower levels of OPA1 compared to post-intervention. The later may in part be discussed with the fact that OPA1 may have a double role: in fusion and possibly as an inhibitor of mitophagy. Indeed, Liao et al.[35] have recently shown that patients lacking OPA1 have increased mitophagy. Comparing older sedentary to older sportsmen, Tezze et al. [36] observed higher protein content of MFN1 and OPA1, but not MFN2. Tarpey et al. [37] observed higher MFN2 in endurance trained subjects but not MFN1. In our control group, DRP1 content was reduced in conjunction to an increase of

$DRP1^{S637}$ phosphorylation, speaking to a decrease of mitochondrial fragmentation. Tezze et al. [36] and Tarpey et al. [37] show increased fission in their endurance-trained subjects. It is difficult to explain such discrepancies across studies, these could be due to the fact that normalization was performed differently in the three labs (complex II vs. total protein content of the lane vs. α-TUBULIN), or to different inclusion criteria (for one study sedentary subjects had osteoarthritis, different age ranges, only males vs. balanced gender ratio), or to different control conditions as biopsies were taken in different nutritional status or with a different timing from the last exercise session (no restriction vs. 36h vs. 72h).

Mitophagy and global autophagy

In previously sedentary subjects, markers of mitophagy induction (PINK1 and BNIP3) were increased post-intervention but mitophagy effectors (PARKIN and BCL2L13) were not changed. Markers of autophagy such as P62, LC3-I, LC3-II, or their ratio were not changed with intervention. Thus, our results show that global autophagy and global mitophagy are not modified with four months of training in human muscle. Mejías-Peña et al.[38] have shown increased autophagic fluxes in human peripheral blood cells before and after 8 weeks of exercise. Studies exist in conditions of bed rest [39] or critical care [40], but to our knowledge no previous exercise training intervention has evidenced autophagic outcomes in human skeletal muscle of older adults in general good health.

The cross-sectional comparison with controls suggests that long-term exercise mobilizes both initiators and effectors of mitophagy. While PINK1 and BNIP3 were significantly higher in controls compared to baseline sedentary, but were not different from post-intervention levels, PARKIN and BCL2L13 were higher in controls when compared to sedentary before or after intervention. Our observations are consistent with Drummond et al. [41] who presented lower levels of PARKIN and BNIP3 in inactive frail older women, and with Tarpey et al. [37] who suggest that endurance trained individuals exhibit heightened mitophagy activity. The observed results on mitophagy markers are robust and coherent across studies.

Global analysis of autophagy revealed no modification of P62, while the lipidated form of LC3 appeared to be decreased in controls. This could be explained by the fact that mitophagy is not only specific but also a relatively minute part of global autophagy signaling [42, 43], thus mitophagic adaptations may not be evidenced by markers of global autophagy.

It is important to acknowledge that we investigated dynamic processes with static markers as often in similar clinical studies. [40] Ideally mitophagy and autophagy should be evaluated with dynamic studies *in vitro* or *in vivo*. This being impossible in human muscle specimens, we assessed mitophagy using key and specific actors in conjunction with mitochondrial content. We extensively examined the specimen with electron microscopy but it was impossible morphologically to decipher what is degradation, fusion or fission. Autophagosomes, representing the final (and transient) step of mitophagy, were so rarely visualized that quantification was not conceivable. Thus, even if indirect and static, we believe that we used the best available approach to measure fusion, fission and mitophagy in human biopsy specimens as other clinical and translational researchers. [36, 37, 40] Secondly, due to the limited amount of biopsy material and to logistics, we were not able to use fresh tissue or isolate mitochondria. This would have allowed us, for example to assess respirometry or the translation of PARKIN by separating membranes from cytosol.

BCL2L13 may be a novel alternative mitophagy pathway implicated in metabolic adaptations

This study highlights for the first time in human muscle a possible role of BCL2L13 as a potential novel non-canonical mitophagic pathway alternative to the canonical PARKIN cascade.[20] Indeed, analyses of Parkinson's disease patients and PARKIN null models [44] revealed the unnecessary role of PARKIN in muscle. Our observations and correlations suggest that exercise may preferentially stimulate the BCL2L13 cascade. This could also explain the fact that fission markers were strongly decreased in controls, implying the existence of a specific form of mitophagy independent from mitochondria fragmentation. DRP1 and its phosphorylated form were associated in opposite directions with BCL2L13. PINK1 and BNIP3, the two mitophagic inducers, were positively correlated with BCL2L13 but not with PARKIN. This is accord with previous observations of crosstalk between BNIP3 and other BCL2 family members [45], and suggests that the BCL2L13 pathway may connect to BNIP3. PARKIN is known to interact with MNF2 to initiate mitophagy, this was confirmed in our cohort by a strong positive relationship between these two proteins. BCL2L13 was also strongly positively associated with MFN2, which suggests a concomitant activation of both BCL2L13 and PARKIN cascades. PARKIN and BCL2L13 were positively correlated, which reinforces previous observations of the relationships between PARKIN and the BCL2 family.[46] BCL2L13 was positively related with mitochondrial content and with

physiological markers of mitochondrial and muscular efficiency, but was negatively related with obesity and body fatness. Although these observational and correlational results need to be interpreted with caution, they cast doubt on BCL2L13 as a novel alternative mitophagy pathway calling for confirmatory and mechanistic studies that are beyond the scope of this project and would require the use of cellular or animal models. We speculate that BCL2L13 could be an important mitophagic effector in conditions where there is a high demand for increased mitochondrial function, which could be achieved by higher turnover and preserved mitochondrial quality.

Physiological relevance

This study was geared at exploring the adaptation to chronic exercise in previously sedentary subjects and not the response to an acute bout of exercise. As 4-months training can already be interpreted as a chronic response, the chosen control group was lifelong exercisers. The cross-sectional comparison between post-intervention and controls revealed different patterns of mitochondrial adaptation within chronic exercise. Based on previous cellular studies showing that efficient mitophagy precedes and drives biogenesis [47, 48], on a recent animal knock-out study showing that mitophagy is required for basal mitochondrial function as well as for the accumulation of normally functioning mitochondria as a result of training adaptations [49], and on the fact that controls had similar MitoVD than subjects at post-intervention, our results suggest a renewal of the mitochondrial pool in controls. Although a hierarchical program of mitochondrial adaptation cannot be evidenced with our study design, including the limitation that exercise history was self-reported for the lifelong group, the observation of temporally distinct patterns upon duration of exercise training is relevant. Interestingly, this temporally distinct pattern of mitochondrial adaptation paralleled patterns observed for the physiological parameters. Our observation questions whether higher mitochondrial turnover impacts higher mitochondrial quality in human skeletal muscle. Indeed, the observed changes in the physiological parameters cannot be explained solely by central adaptations or learning effects.

Conclusion

In summary, this study investigated the impact of chronic exercise on muscle mitophagy, fusion and fission, in healthy humans. Our results evidence temporally distinct levels of adaptations in human skeletal muscle when engaged in regular exercise. While biogenesis and fusion were elicited within few

months of training in previously sedentary adults; we observed a different pattern of adaptations driven by mitophagy with lifelong training. These two distinct patterns of molecular adaptations paralleled long-term effects of training on physiological adaptations such as physical fitness, exercise efficiency and oxidative capacity. These findings suggest that four months of training are sufficient to adapt mitochondria to the energetic demands imposed by a new routine of regular exercise without evidence of eliciting mitophagy. At the opposite, mitochondrial turnover may be necessary to maintain mitochondrial quality in lifelong training thus including a balancing act of increased biogenesis and fusion, decreased fission and a higher rate of mitophagy. In addition to the integrative view, a new molecular key player is highlighted, inviting further investigation. Our results suggest the role of BCL2L13 as putative candidate in the specific condition of a high mitochondrial quality demand, which may be very different from other mitophagic triggers such as hypoxia or stress.

Subjects and Methods

Subjects, study design and ethical approval

In a pre-/post-intervention design, sedentary healthy men and women volunteers were recruited in a 4-month supervised endurance exercise intervention. To be included volunteers needed to be between 60 and 80 years old, in good health and weight stable. Sedentary subjects were required to exercise ≤1 time per week of <20 minutes. Endurance trained age- and gender-matched subjects were recruited as positive controls. These were required to engage in regular structured aerobic exercise sessions (≥3 per week) of either running or cycling for ≥5 years. Habitual physical activity was self-reported and discussed during the screening medical interview, including exercise mode, frequency, and training years. Active smoking, diabetes, and pharmacological treatments known to affect metabolism were among the exclusion criteria. The institutional review boards of the Canton of Vaud approved the study protocol and all subjects provided written informed consent.

Exercise intervention

The exercise training consisted of a 16-week supervised (3 sessions/week), moderate-intensity aerobic protocol described in details elsewhere.[3] Frequency, duration, and volume of exercise were

tightly monitored. Volunteers were requested to follow their typical diet during the study and were weighed once a week to ascertain compliance. Adherence to the exercise program was computed as presented in details elsewhere.[50]

Body composition

Height was measured using a wall-mounted stadiometer and weight using a calibrated medical digital scale (SecaGmBh, Hamburg, Germany). Lean body mass (LBM) and fat mass were measured by dual-emission X-ray absorptiometry (Lunar Prodigy; GE Healthcare, Milwaukee, MI).

Exercise testing

Indirect calorimetry (Metalyzer3B; Cortex, Leipzig, Germany) was used to measure $\dot{V}O_2$ and $\dot{V}CO_2$ in two physiological conditions: during a maximal graded exercise test and during a one-hour steady state bout of exercise. Physical fitness was determined by $\dot{V}O_2$peak using a validated protocol suited for older volunteers of various degrees of fitness.[3, 51] Gross exercise efficiency and systemic rate of fat oxidation were measured during the submaximal exercise test as previously described.[52, 53]

Accelerometry

Total daily physical activity and energy expenditure were measured with a tri-axial accelerometer/temperature sensor (SenseWear Armband, BodyMedia, Pittsburgh, PA).[54] All but two subjects wore the armband for 7 consecutive days at baseline and during the last week of intervention. Analyses were performed on all days where the monitors were worn >90% of the day as presented elsewhere. [55]

Dietary patterns

Total energy, carbohydrate, fat and protein intakes were computed from three-day food record questionnaires. Subjects were requested to write down everything they ate on one weekend and two weekdays. On the following day, food records were validated during an interview with the nutritionist. All analyses were performed by the same nutritionist with Prodi Swiss 5.8 software (Nutri-Science Gmbh, Stuttgart, Germany).

^{31}Phosphorus magnetic resonance spectroscopy

The mono-exponential rate constant (k) of phosphocreatine (PCr) recovery after dynamic knee extensions performed inside a 3T clinical magnetic resonance scanner (VERIO, Siemens, Erlangen, Germany) was determined from ^{31}Phosphorus magnetic resonance spectra obtained from the quadriceps muscle. *In vivo* phosphorylation capacity (ATPmax) was computed by multiplying k with the PCr concentration estimated from a separately obtained resting spectrum as suggested by Conley et al.[56] Details of acquisition and computations have been described previously. [3, 53]

Skeletal muscle biopsies

Percutaneous muscle biopsies from the *vastus lateralis* were obtained after an overnight fast in tightly controlled conditions as described previously.[3] Subjects were requested not to exercise in the last 72 hours in order to avoid acute effects of the last training bout. Samples were trimmed of all visible adipose tissue with a dissecting microscope (MZ6; Leica Microsystems, Wetzlar, Germany). One portion of the specimen (~5 mg) was fixed for electron microscopy and the remaining specimen was flash frozen in liquid nitrogen and stored at −80°C for protein and mRNA analyses.

Mitochondrial volume density

Transmission electron microscopy was used to measure MitoVD as a marker of mitochondrial content. In brief, samples were fixed, embedded, and cut longitudinally in ultra-thin sections of 60nm. Twenty micrographs were taken per subject at 33,000x magnification and using a plugin known to avoid sampling bias. MitoVD was obtained using the point-counting technique with a superimposed grid of 500x500nm. Upon placing the grid, the number of points (defined as two intersecting grid lines) that touched the mitochondria, were tallied and divided by the total number of points on the grid. This process was repeated for the other 19 images and averaged together to receive the volume density percentage for each grid.[53] A detailed description of this stereological method has been described elsewhere [4] and was validated in human muscle samples.[57]

Muscle lysate and western blotting

Protein content was measured as described in details in [3]. Briefly, frozen specimens were homogenized in ice-cold lysis buffer. Proteins were measured by the BCA method (Pierce,

ThermoFisher Scientific Inc., Rockford, IL). 30µg of each sample were loaded in 10 or 12% SDS-Page gel. Transfer was performed on methanol-activated PVDF membrane (Bio-Rad Laboratories, Hercules, CA) in tris-glycine-20% ethanol buffer. After blocking, primary antibodies were incubated overnight. The list of antibodies and their specific dilutions are detailed in supplemental table 3. HRP-linked secondary antibodies were used for 2 hours at RT. For consecutive primary antibody presentations, stripping was performed using a previously published protocol.[58] Autoradiography films were used for detection (Blue Devil, Genesee Scientific, San Diego, CA). Specific bands were quantified by densitometry using the software ImageJ (National Institutes of Health, Bethesda, MD) as described in [3]. All samples of the entire cohort were loaded on the same day. All gels and membranes were processed at the exact same time with the same solutions. Protein levels were normalized over the corresponding α-TUBULIN loading control for each sample. Further normalization with one specific human sample loaded on each gel was performed to control for gel-to-gel variability. Raw data including uncropped western blots and ponceau images are available in the supplemental section. Protein abbreviations are reported according to the *Genetic Nomenclature Guide* proposed by *Trends in genetics.* [59] All reagents used in this study are available upon request.

Gene expression analyses

Total mRNA preparations, cDNA synthesis, and quantitative RT-PCR were performed as described previously.[3] Primers used were as follows: *MFN2* (For: 5'- ATGCATCCCCACTTAAGCAC-3' , Rev : 5'-CCAGAGGGCAGAACTTTGTC-3'); *MFF* : (For : 5'-ACTGAAGGCATTAGTCAGCGA-3', Rev : 5'-TCCTGCTACAACAATCCTCTCC-3') ; *OPA1* : (For : 5'-CGACTTTGCCGAGGATAGCTT-3', Rev : 5'-CGTTGTGAACACACTGCTCTTG-3') ; *DRP1* : (For : 5'GTCGCAGAACCCTAGCTGTA-3' , Rev : 5'-ACCAGTAGCATTTCTAATGGC-3') ; *β-ACTIN :* (For: 5′-TCGTGCGTGACATTAAGGAG-3′; Rev: 5′-GTCAGGCAGCTCGTAGCTCT-3′).

Briefly, RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was generated using Superscript II enzyme (Invitrogen, Carlsbad, CA) and quantitative real-time PCR was performed as described previously[60] using acidic ribosomal protein (ARP) to normalize the expression. Target mRNA levels were normalized over the geometric mean of β-ACTIN, which was selected as housekeeping genes after having checked its expression stability. Relative mRNA

expression levels were calculated with the ΔΔ cycle threshold (Ct) method. Gene abbreviations are reported according to the *Genetic Nomenclature Guide*[59] described above.

Statistical procedures

Unless specified otherwise, all values presented in the text are mean±SEM. After checking normality and equality of variance, two-tailed independent t-tests were performed to examine group differences between sedentary pre-intervention and controls, or between post-intervention and controls. If the equality of variance assumption was not met, comparisons between groups were performed with the Welch corrected t-test. If the normality assumption was not met, comparisons between groups were performed with the nonparametric median test. For pre- and post-intervention comparisons, two tailed paired t-tests were used. Pairwise correlations were performed with the Pearson product moment correlation coefficient. Statistical analyses were performed using JMP version 11.2.0 (SAS Institute, Cary, NC). Graphs were done using Prism version 6 (GaphPad Inc, La Jolla, CA).

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Author contributions

Conceptualization, Y.A. and F.A.; Methodology, Y.A. and F.A.; Formal analysis, Y.A. and F.A.; Investigation, Y.A., N.T.B., C.G., M.B., S.C.A., S.S.K., Cy.B. and E.A.C.; Resources, F.A. and C.C.; Writing – original draft, Y.A. and F.A., Writing – review & editing, N.T.B., C.G., S.S.K., S.L., Y.A. and F.A.; Visualization, Y.A.; Supervision, F.A. and C.C.; Project administration, F.A.; Funding Acquisition, F.A.

Conflict of interest

All authors report no conflicts of interest in this work. M.B., S.S.K., and C.C. are employees of the Nestlé

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TABLES

Table 1, subject's characteristics

	Sedentary Pre-intervention n=22	Post-intervention n=19	Controls n=8
Age (years)	66.2±3.8		65.6±6.7
Gender (m/f)	11/11	10/9	4/4
BMI (kg/m^2)	27.1±4.9$^\#$	26.7±4.7*$^\#$	22.7±1.4
FM (kg)	24.5±9.1$^{\#\#}$	23.5±9.5**$^{\#\#}$	12.6±2.4
BF (%)	30.8±8.2$^{\#\#}$	29.2±9.2**$^\#$	19.8±4.4
LBM (kg)	51.6±11.8	53.7±12.3*	49.2±5.7
V̇O$_2$peak (l/min)	2.01±0.58$^\#$	2.28±0.64**	2.49±0.43
V̇O$_2$peak/BW (ml/min/kg)	26.3±6.6$^{\#\#}$	29.4±7.5**$^{\#\#}$	38.8±4.8

Notes: Data are mean±SD. * Significant effect of intervention (two tailed paired t-test), # Significantly different from controls (two tailed independent t-test). 1 symbol=P<0.05, 2 symbols= P<0.001.

Abbreviations: FM, fat mass; BF, body fatness; LBM, lean body mass; V̇O$_2$peak, peak oxygen uptake; BW, body weight.

Table 2, Daily physical activity, steps and energy expenditure

	Sedentary Pre-intervention	Post-intervention	Controls
Wearing Time (days)	6.53±0.29	5.88±0.30	6.00±0.38
Wearing Time (hours/day)	23.66±0.04	23.53±0.06*	23.66±0.04
Steps (steps/day)	5843.9±650.6##	8208.6±864.6**	10630.9±1397.5
TDPA (min/day)	325.2±20.1#	370.7±22.8*	431.0±41.2
TDPA EE (kcal/day)	1130.5±73.4#	1356.5±116.6**	1481.1±182.7
TDPA NetEE (kcal/day)	832.3±59.7#	1020.2±98.8**	1109.2±149.7
MVPA (min/day)	94.3±16.9##	131.6±28.7*	185.5±26.3
MVPA EE (kcal/day)	449.6±76.2#	645.4±139.4*	860.2±158.1
MVPA NetEE (kcal/day)	364.2±62.4#	529.5±115.5*	698.1±132.3
NEPA (min/day)	325.2±20.1	350.8±21.4	-

Notes: Data are mean±SEM. * Significant effect of intervention (two tailed paired t-test), # Significantly different from controls (two tailed independent t-test). 1 symbol=$P<0.05$, 2 symbols=$P<0.01$.

Abbreviations: TDPA,total daily physical activity computed by the sum of activities ≥1.5 METs; EE, energy expenditure; Net, Net values after subtraction of resting EE; MVPA, moderate and vigorous physical activity computed by the sum of activities ≥3.0 METs; NEPA, non exercise physical activity.

Table 3, Pairwise correlations between markers of mitophagy, fusion and fission, and physiological outcomes

Variables	MFN2	OPA1	DRP1	DRP1 S367	PARKIN	PINK1	BNIP3	BCL2 L13	VDAC1	LC3 II	LC3 I	P62
MitoVD (%)	0.11	0.03	**-0.45**	**0.60**	0.17	0.11	-0.05	*0.37*	**0.43**	-0.25	0.03	0.18
k (1/s)	**0.48**	0.05	*-0.38*	0.28	*0.35*	-0.11	-0.12	**0.41**	*0.31*	-0.29	-0.11	0.17
ATPmax (mmol/L/sec)	**0.54**	-0.07	**-0.53**	0.22	*0.37*	-0.11	-0.08	**0.40**	0.28	-0.27	-0.18	0.11
V̇O$_2$peak (L/min)	**0.46**	-0.10	-0.14	0.14	**0.47**	-0.05	-0.22	0.22	0.26	-0.27	-0.24	-0.06
V̇O$_2$peak/BW (ml/min/kg)	**0.56**	-0.06	-0.23	**0.40**	**0.45**	0.17	0.09	**0.62**	**0.54**	-0.11	0.01	-0.03
GE/Avg leg mass (%/kg)	0.32	-0.05	-0.06	*0.37*	*0.35*	0.18	0.25	**0.70**	**0.58**	-0.07	0.15	0.01
Fat ox/LBM (μmol/min/kg)	0.24	0.30	-0.27	**0.45**	0.11	0.28	0.27	0.18	0.15	0.11	0.22	0.23
Fat mass (kg)	-0.33	0.00	0.13	-0.28	-0.22	-0.12	-0.17	**-0.63**	**-0.53**	-0.05	-0.18	0.02
Body fat (%)	**-0.40**	0.05	0.17	-0.21	**-0.41**	0.03	0.07	**-0.56**	**-0.52**	0.08	-0.02	0.09
BMI (kg/m^2)	-0.18	0.07	0.06	-0.25	-0.02	-0.13	-0.29	**-0.50**	*-0.36*	-0.05	-0.21	0.06

Notes: Values are Pearson coefficient r. In **bold red**, significant correlations with P<0.05. In *italic orange* P=0.05-0.09.

Abbreviations: MitoVD, mitochondrial volume density; k, PCr recovery rate; V̇O2peak/BW, Peak oxygen uptake relative to body weight; Fat Ox/LBM, Fat oxidation during a steady state submaximal exercise bout relative to lean body mass; GE/Avg leg mass, Gross exercise efficiency during a steady state submaximal exercise bout relative to leg mass.

FIGURE LEGENDS

Figure 1, Effects of exercise training on physical fitness and other physiological outcomes. (**A**) Absolute peak oxygen uptake ($\dot{V}O_2$peak), (**B**) Peak oxygen uptake relative to body weight (BW), (**C**) Fat oxidation during a steady state submaximal exercise bout relative to lean body mass (LBM), (**D**) Gross exercise efficiency during a steady state submaximal exercise bout (GE) relative to average leg mass, (**E**) Posphocreatine recovery rate (k) and (**F**) Oxidative phosphorylation capacity (ATPmax). Bars are means, error bars are SEM. Pre=sedentary subjects at baseline, Post=post-intervention, Con= control group. * Significant effect of intervention (two tailed paired t-test), # Significantly different from Con (two tailed independent t-test). 1 symbol=P<0.05, 2 symbols=P<0.01, 3 symbols=P<0.001, §=0.05.

Figure 2, Effect of exercise training on markers of mitochondrial content. (**A**) Mitochondrial volume density (MitoVD), (**B**) Representative muscle micrographs, magnification 33,000x, scale bar is 500nm, (**C**) Complex I content, (**D**) Complex III content and (**E**) Complex V content. Protein contents are normalized by α-TUBULIN (TUB). Representative western blots bands have been isolated within the same gel. Bars are means, error bars are SEM. Pre=sedentary subjects at baseline, Post=post-intervention, Con= control group. *Significant effect of intervention (two tailed paired t-test), # Significantly different from Con (two tailed independent t-test). 1 symbol=P<0.05, 2 symbols=P<0.01, 3 symbols=P<0.001. Arrows represent mitochondria in one intramyofibrillar space.

Figure 3, Effects of exercise training on markers of mitochondrial fusion and fission. Quantification of (**A**) MFN2, (**B**) MFN1, (**C**) OPA1, (**D**) DRP1 and (**E**) phosphorylated DRP1^{S637}. All normalized by α-TUBULIN (TUB). Representative western blots bands have been isolated within the same gel. Bars are means, error bars are SEM. Pre=sedentary subjects at baseline, Post=post-intervention, Con= control group. * Significant effect of intervention (two tailed paired t-test), # Significantly different from Con (two tailed independent t-test). 1 symbol=P<0.05, 2 symbols=P<0.01, 3 symbols=P<0.001.

Figure 4, Effects of exercise training on markers of mitophagy and autophagy.
Quantification of (**A**) PINK1, (**B**) BNIP3, (**C**) VDAC1, (**D**) PARKIN, (**E**) BCL2L13, (**F**) P62, (**G**)
LC3 I and II. All normalized by α-TUBULIN (TUB). Representative western blots bands have
been isolated within the same gel. Bars are means, error bars are SEM. Pre=sedentary
subjects at baseline, Post=post-intervention, Con= control group. * Significant effect of
intervention (two tailed paired t-test), # Significantly different from Con (two tailed
independent t-test). 1 symbol=P<0.05, 3 symbols=P<0.001, §=0.05.

Figure 1, Effects of exercise training on physical fitness and other physiological outcomes.
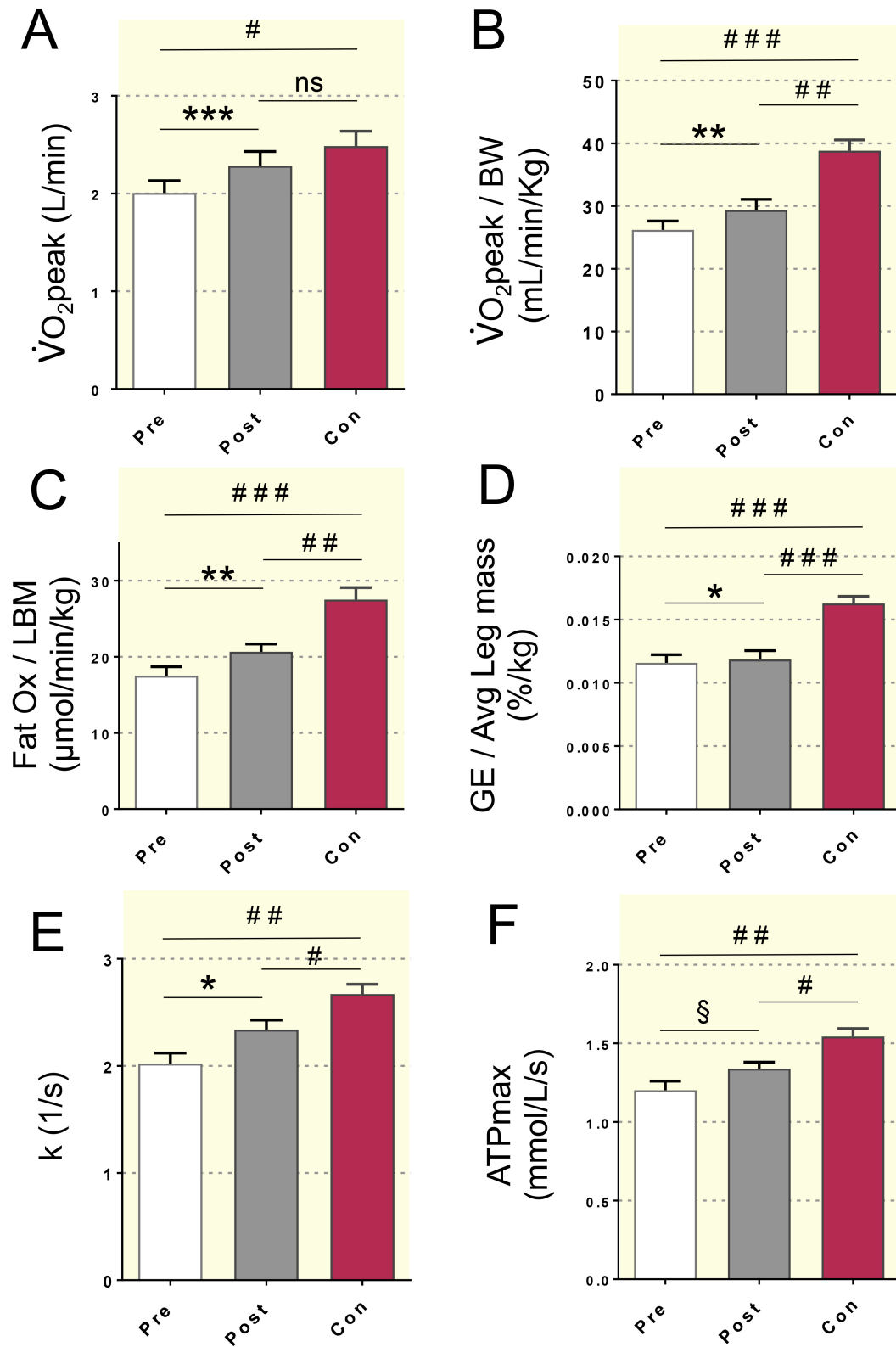
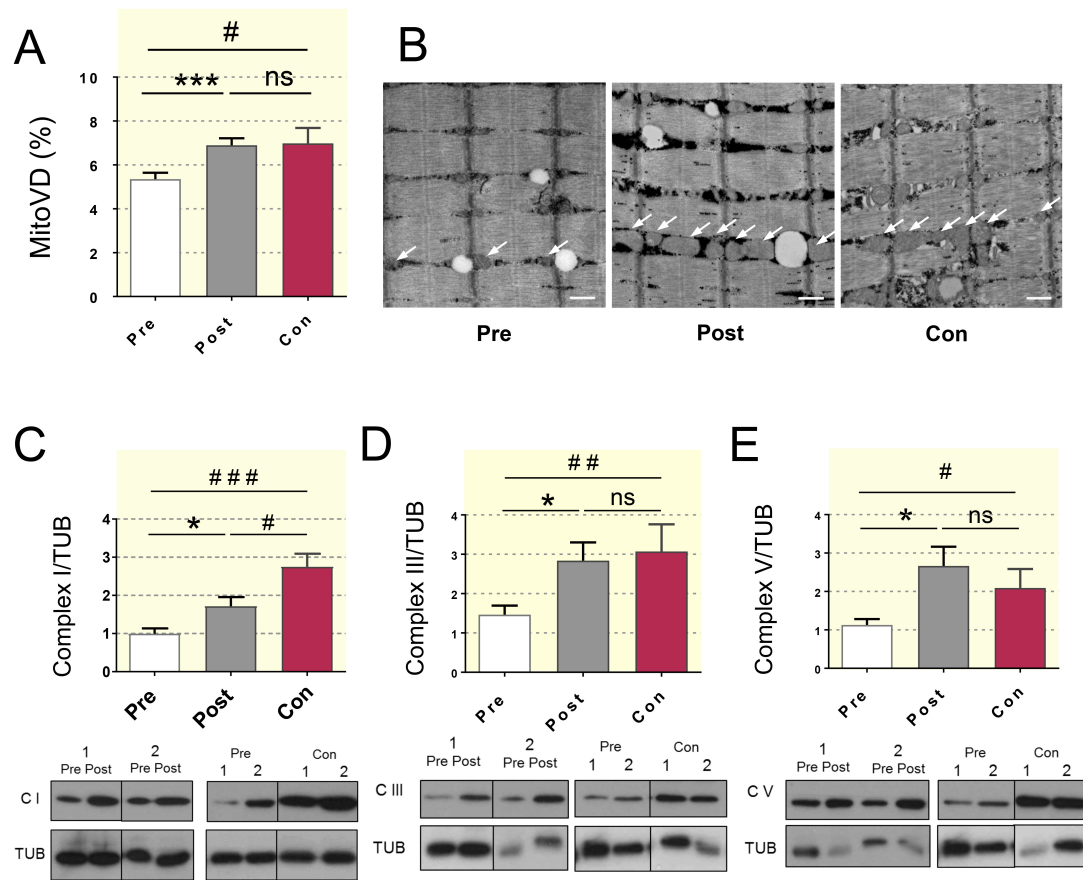**Figure 2, Effect of exercise training on markers of mitochondrial content.**

Figure 3, Effects of exercise training on markers of mitochondrial fusion and fission.
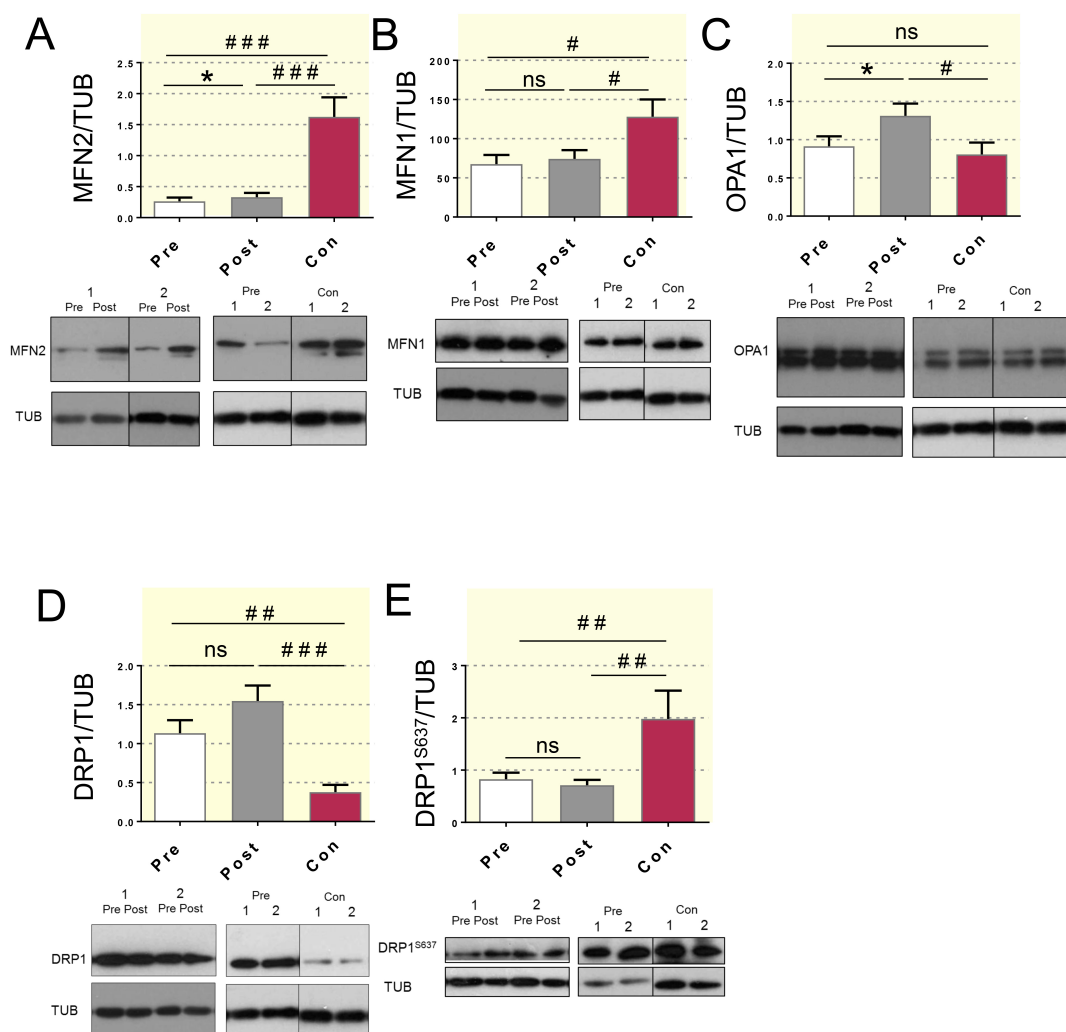
Figure 4, Effects of exercise training on markers of mitophagy and autophagy.