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Lifelong physical exercise delays age-associated skeletal muscle decline

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ABSTRACT

Aging is usually accompanied by a significant reduction in muscle mass and force. To determine the relative contribution of inactivity and aging per-se to this decay, we compared muscle function and structure in a) male subjects belonging to a group of well-trained seniors (average of 70 years) who exercised regularly in their previous 30 years; b) age-matched healthy sedentary seniors; c) to active young men (average of 27 years). The results collected show that relative to their sedentary cohorts, muscle from senior sportsmen have: 1) greater maximal isometric force and function; 2) better preserved fiber morphology and ultrastructure of intracellular organelles involved in Ca^{2+} handling and ATP production; 3) preserved muscle fibers size resulting from fiber rescue by re-innervation; and 4) lowered expression of genes related to autophagy and ROS detoxification. All together our results indicate that: a) skeletal muscle of senior sportsmen is actually more similar to that of adults than to that of age-matched sedentaries; b) signaling pathways controlling muscle mass and metabolism are differently modulated in senior sportsmen to guarantee maintenance of skeletal muscle structure, function, bioenergetic characteristics and phenotype. Thus, regular physical activity is a good strategy to attenuate age-related general decay of muscle structure and function. ClinicalTrials.gov: NCT01679977

INTRODUCTION

Aging is a multifactorial process influenced by genetic factors, nutrition, and lifestyle (1, 2). One of the most striking effects of aging on humans is a reduction in muscle mass, known as sarcopenia, which occurs to different degrees in all individuals and results in reduced functional capacities (strength and endurance). Contributing factors include a severe decrease in both myofiber size and number (3) and a decrease in the number of motor neurons innervating muscle fibers (4,5). Age-related reduction of muscle strength/endurance, though, has also been attributed to factors other than simple reduction of muscle mass, (6,7). Reduced amount of Ca^{2+} ions available to sustain muscle contraction (8) and impaired ATP production (9) are definitely important factors to taken into account to explain reduced specific force and resistance to fatigue of skeletal muscle (6). Miss-function of excitation-contraction (EC) coupling, the mechanism linking the action potential into Ca^{2+} release from the sarcoplasmic reticulum (SR), may be the result of an age-related decrease in the number of calcium release units (CRUs) (10). Impairment in ATP production may depend on mitochondrial dysfunction (9,11) and possibly reduced number and miss-placement, as mitochondria-CRUs cross-talk seems to be crucial for efficient ATP production (12-14).

In addition, as maintenance of muscle mass can be also regulated by anabolic (e.g., insulin-like growth factor-1 [IGF-1]) and catabolic (e.g., Atrogin-1, MuRF-1) factors, and by energy supply (15-18), sarcopenia may be also the result of deregulation of this fundamental molecular pathways. Moreover, the emerging field of microRNA (miRNA or miR) biology has begun to uncover roles for these regulatory molecules in skeletal muscle development and disease processes.

One of the crucial issues that would need to be addressed is the relative contribution of aging itself and of inactivity (i.e. sedentary lifestyle) to the dramatic changes that we just described. For example we know that muscle disuse can induce a rapid down-regulation of PGC-1α transcript, the master gene for mitochondrial biogenesis (19). Additionally, oxidative stress - also elevated in disused muscle (20) - may play a role in age-related deterioration of intracellular organelles (contractile elements, sarcotubular membranes, mitochondria, etc.) and related cellular functions (21,22).

Several studies have shown that regular exercise may extend life expectancy and reduce morbidity in aging (23-26). Exercise regulates one of the most important anti-ageing system that is the autophagy pathway. Indeed, autophagy is important for clearance of damaged organelles and proteins allowing rejuvenation of cellular components. In this study, we aimed to define the impact of regular physical activity on the age-related changes that occur in muscle by comparing muscle function and structure in a group of lifelong-trained senior men to that of two other groups of men: i) age-matched healthy sedentary seniors, and ii) young active subjects. Our hypothesis is that lifelong physical activity may counteract age-related decline of muscle functional output and muscle fiber ultrastructure, also activating specific signaling pathways associated with muscle homeostasis, metabolism, and oxidative stress.

METHODS

Study Subjects. Subjects were male volunteers who received detailed information about the study and gave informed consent (demographic details in Table 1). Approval from the ethical committees of the City of Vienna and the Comenius University in Bratislava was obtained at the study outset. Three groups of subjects were enrolled: a) young subjects: 19-33 years of age (n = 5), physically active for 3, but no more than 5, times a week; b) healthy sedentary seniors: 65-74 years of age (n=9), performing only routine daily activities; and c) senior sportsmen: 65-79 years of age (n=15), who routinely practiced (lifelong) sport activities usually more than three times a week (Table 2). All subjects were healthy and declared not to have any specific physical/disease issues. For detailed inclusion and exclusion criteria: ClinicalTrials.gov. NCT01679977. All of the senior sportsmen (group c) declared to have a lifelong (30 years) history of high level training.

Force measurements. An isometric measurement using a force chair (Wise Technologies, Lubljana, Slovenia) was performed to assess the maximal isometric torque (MIT) of the left and right knee extensors (18).

Functional Tests. Functional tests were designed and applied to all groups:

1) 10m-walking test. Subjects walked 10 meter at their preferred speed and then again at a very fast pace (27); - *2) short physical performance battery* (SPPB). Lower extremity function was evaluated using tests of gait speed (2.4 meters), standing balance and the time which the subject needed to rise from a chair five consecutive times as quickly as possible with the arms folded across their chest (28); 3) *static and dynamic body sway tests*. Subjects maintained three positions during quiet stance on a force plate (*static body sway)* (29-31) or stood on a force plate with their hands placed on their hips, knees fully extended and their gaze directed forward at a display which provided feedback on the center of pressure displacement *(dynamic body sway)* (29); and 4) *TUGT*. Subjects stood up from a standard chair, walked a distance of 3 meters as fast as possible, turned, walked back to the chair and sat again (32). **Statistical analyses.** Force measurements and functional tests were analyzed for normal distribution using the Kolmogorov-Smirnov-Test and a one-way analysis of variance (one-way ANOVA) (post-hoc analyses: Tukey-HSD, Tamhane-T2) test was used to evaluate group differences using a SPSS Statistics software package, version 17.1.

Muscle biopsies. Needle muscle biopsies were harvested through a small skin incision (6 mm) from the right and left *Vastus Lateralis* muscles of each patient (33). Resulting specimens were fixed for either light or Electron Microscopy (10,34,35).

Light Microscopy and quantitative histological analyses. Serial cryosections (8 μm) from muscle biopsies were mounted on polysine™ glass slides, air-dried and stained either with Hematoxylin and Eosin (H&E) or conventional techniques for myofibrillar ATPases to evaluate muscle fiber type. For ATPase stains, slow-type fibers are dark while fast-type fibers are lightly stained following preincubation at pH 4.35. Fiber type grouping is identified on the basis that one myofiber is completely surrounded by fibers of the same phenotype. Morphometric analyses were performed on stained cryosections using Scion Image for Windows version Beta 4.0.2 (2000 Scion Corporation) (34,36).

Statistical analyses. The **differences** in mean myofiber diameter and percentage of fast and slow myofiber type between groups were analyzed using the two-tailed Student's *t* test (Microsoft ® Office Excel ® 2007, Microsoft Corporation).

Transmission Electron Microscopy (EM) and quantitative analyses of CRUs and mitochondria. Biopsy specimens fixed for EM (in 3.5% glutaraldehyde in 0.1 M CaCaCo buffer, pH 7.4, RT) were rinsed in 0.1 M CaCaCo buffer and post-fixed for 1h in 2% osmium tetroxide. The specimens were prepared, and analyzed (10,12,35). CRU and mitochondrial number/area (and their position relative to the sarcomeres) was determined from electron micrographs of non-overlapping regions randomly collected from longitudinal sections. In each specimen, 6 to 10 fibers were analyzed and in each fiber 6 to 10 micrographs were collected at 14,000X magnification. In each EM image, we determined: a) the number of triads and their morphology (discriminating between triads and dyads) and orientation (transversal vs. longitudinal) (Table 5); and b) number of mitochondria as well as their positioning with respect to the I and A bands, and to triads (Table 6). For additional detail on quantitative analysis see (12). The relative volume occupied by mitochondria (Table 6, column A) was determined using well-established stereology point-counting techniques (37,38) in EM micrographs taken at 14.000X of magnification. **Statistical analyses.** The results presented in Tables 5 and 6, are shown either as: 1) average (± SD) number of CRUs/100μm^2 (Table 5, column A), mitochondria/100μm^2 (Table 6, columns B and C), or mito-CRU couplets/100μm^2 (Table 6, column D) of sectional area; here, statistical significance was determined using a Student's *t* test (Microcal Origin® 6.0 Microcal Software, Inc.), and **differences** were considered statistically significant at $p<0.01$; or 2) percentage (Table 5, columns B and C; Table 6, column A); here we used a chi-squared test to evaluate statistical significance (Microsoft ® Office Excel ® 2007, Microsoft Corporation), **differences** were considered statistically significant at $p < 0.01$.

miRNA and gene expression analyses. Total RNA was extracted from muscle using tissue lyser (QIAGEN) in TriReagentTM (SIGMA) and small RNAs were purified using a PureLink miRNA Isolation Kit (Invitrogen). The miRNA fraction was reverse-transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies); the other RNA fraction was reverse-transcribed using a QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative PCR was performed on an ABI PRISM 7500 SDS (Applied Biosystems, USA), using premade 6-

carboxyfluorescein (FAM)-labeled TaqMan assays for GAPDH, IGF-1 Ea, IGF-1 Eb, IGF-1 Ec, IGF-1 pan, (Applied Biosystems, USA), and for Atrogin1, MuRF1, Bnip3, p62, Nrf2, PGC1a, YY1 and SREBP1 (39). FAM-labeled TaqMan MicroRNA Assays for miR-1, miR-133a, miR-206 and U6 snRNA (Applied Biosystems, USA) were performed. Quantitative RT-PCR sample values were normalized to the expression of GAPDH mRNA or U6 snRNA. Relative levels for each gene and miRNA was calculated using the 2-DDCt method (40) and reported as mean fold change in gene expression. **Statistical analyses.** Statistical analysis was performed with GraphPad Prism v5.0 software; groups were compared by Mann-Whitney Rank Sum test. One-way ANOVAs were performed followed by the Bonferroni post hoc test.

RESULTS

Force measurements and functional tests. MIT was measured as a marker of muscle strength and was significantly higher in the young group compared to all other groups (Table 3); however, the force generated by healthy sedentary seniors was significantly lower compared to both young men and senior sportsmen (Table 3). Similarly, senior sportsmen had **higher** functional capacity compared to sedentary peers (TUGT and chair rise tests, Table 3).

Histology and muscle morphometry. Muscle biopsies from seniors showed well-packed myofibers without inflammatory cell infiltrates, major degenerative changes or evidence of acute or chronic damage (Figure 1, A-C). In muscle from healthy sedentary seniors, some small angulated (denervated) myofibers were detected (Figure 1, B). The mean myofiber diameter was significantly higher in young subjects compared to healthy seniors (Table 4) and importantly was significantly higher in senior sportsmen compared to sedentary seniors for both fast and slow fiber types (Table 4). Interestingly, while the percentages of slow and fast type fibers in young subjects and sedentary seniors were comparable, a significantly **higher** percentage of slow type fibers was observed in senior sportsmen.

Among the total number of myofibers analyzed in young active males, 0.3% have a mean myofiber diameter < 30 μm (Table 4) which has been shown to be typical for denervated myofibers (34,41,42). This percentage increased significantly to 4.0% in healthy sedentary seniors, but only by a significant

2% in senior sportsmen (Table 4). Furthermore, in muscle from senior sportsmen, numerous fiber type groupings were detected (Figure 1, F, encircled), and less frequently observed in age-matched sedentary seniors.

Ultrastructure of muscle fibers and quantitative analyses of CRUs and mitochondria. Qualitative EM reveals that the ultrastructure of fibers from senior sportsmen appears better preserved than that of healthy sedentary seniors (Figure 2). For example, mitochondria, which should be positioned at the I band in proximity of the Z-line (Figure 2A and C, white and black arrows), are often misplaced (Figure 2A, empty arrows) in samples from sedentary seniors. Also, structure/position of EC coupling apparatus is abnormal with CRUs often being miss-oriented with respect to the longitudinal axis of the fiber (Figure 2B, arrows, enlarged inset). In some confined areas of fibers from sedentary seniors, decay in internal organization is more evident with possible accumulation of mitochondria and glycogen granules (Figure 2A, stars). In fibers from seniors sportsmen, though, the overall ultrastructure is strikingly better preserved: mitochondria often form pairs (Figure 2C, white arrows) on both sides of Z-lines (Figure 2C, black arrows points at Z lines); CRUs present as classic triads (Figure 2D, arrows), appear better oriented, and are frequently associated to a mitochondrion (Figure 2D, enlarged inset).

We quantitatively analyzed frequency and positioning of CRUs and mitochondria inside individual muscle fibers. Inactive aging results in a drastic reduction in the number of CRUs (Table 5, A) and in the volume and number of mitochondria (Table 6, A and B) compared to young individuals. Also, structure, position and orientation of these two organelles is challenged by aging, as shown by increase of: a) dyad number, i.e., incomplete CRUs (Table 5, B); b) miss-oriented longitudinal junctions (Table 5, C); and c) miss-positioned mitochondria at the A band (Table 6, C). The combined effect is that the frequency of mitochondria-CRU pairs is **3-fold lower with aging** (Table 6, D). In fibers from senior sportsmen, we did not find significant improvement in the EC coupling apparatus (Table 5), even if the frequency of longitudinal CRUs is reduced in exercising individuals suggesting a slightly improved orientation of triads (Table 5, column C). Effect of exercise on the mitochondrial population appears far more striking: both number and volume of mitochondria in senior sportsmen are as high as in active

young subjects (Table 6, columns A and B) with marked preservation of organelle positioning (i.e., fewer A band mitochondria; Table 6, column C). The maintenance of mitochondrial population (Table 6) results in a striking restoration of the frequency of mitochondria-CRU pairs (Table 6, column D), a parameter which is important for overall muscle performance as it could directly influence ATP production (13,43,44).

Expression of relevant genes associated with muscle homeostasis. The maintenance of muscle mass is controlled by two parallel processes: cell and protein turnover, reflecting the balance between protein synthesis and degradation. Protein turnover is regulated by a highly conserved pathway composed of IGF-1 and a cascade of intracellular effectors that mediate its effects. In humans, three mRNA variants (known as IGF-1Ea, IGF-1Eb, and IGF-1Ec) with alternatively spliced-end have been identified (45). Real time PCR analysis did not revealed significant modulations in any of the IGF-1 isoforms in human muscle biopsies at different ages or with different physical activity status. In contrast, it should be noted that the expression of the IGF-1Eb isoform showed a trend toward an **higher** level in the muscle of seniors compared to young subjects (Figure 3A). The exact mechanisms of IGF-1Eb signalling are currently unknown. However, it can be activated, as compensatory mechanism, in response to exercise and damage to guarantee muscle homeostasis.

We also analyzed the expression of the muscle specific atrophy-related ubiquitin ligases Atrogin-1 and MuRF1, and, similarly to IGF-1 transcripts, we did not observe any significant modulation among the different subjects (Figure 3B).

MicroRNAs are an increasingly important class of small non-coding RNAs that regulate gene expression post-transcriptionally and different miRs have been characterized to be selectively expressed by muscle tissue (46). In senior sportsmen there was a significant down-regulation of miR-1, compared to healthy sedentary subjects, and with a transcription level similar to that observed in the muscle of young subjects (Figure 3C).

In contrast, miR-133a expression showed a trend toward, not significant, an increase in the muscle of senior sportsmen compared to young and healthy sedentary subjects. Of note, in the muscle of senior

sportsmen we observed a significant up-regulation in miR-206 gene expression compared to young and healthy sedentary seniors (Figure 3C). miR-206 has been shown to play a specific role in the early events of regeneration by repressing Pax7 activity, thus allowing progression of the differentiation program (47).

Mitophagy, mitochondrial function, and ROS detoxification. An alternative system that play a key role in the turnover of muscle protein and that is activated in several catabolic processes leading to muscle atrophy and wasting is the autophagy‑lysosome pathways. We monitored the expression of the critical enzymes involved in autophagy–lysosome and reactive oxygen species (ROS) detoxification. All evaluated autophagy-related genes were significantly up-regulated in sedentary senior subjects when compared to age-matched seniors sportsmen and young subjects (Figure 4A). In senior sportsmen the expression levels of Bnip3 and p62 are not as high as in sedentary seniors, being closer to that of the young subjects. We also monitored the expression of master genes involved in ROS detoxification and mitochondrial function: Nrf2 and PGC-1α (48,49) (Figure 4B). The transcription factor Nrf2 was strongly induced in healthy sedentary subjects when compared to either young men (Figure 4B). Senior sportsmen showed a significant induction of Nrf2 that, however, was significantly **lower** than in healthy sedentary seniors. PGC-1α expression was upregulated in healthy sedentary and senior sportsmen (Figure 4B) when compared to the young .

To get further insight into mitochondrial function and metabolic response, we investigated expression YY1 and SREBP1, two transcription factors crucial for lipid homeostasis and protein synthesis (50) (Figure 4C). YY1 was significantly up-regulated in senior groups relative to the young men (Figure 4C). SREPB1 expression was significantly up-regulated in healthy sedentary seniors (Figure 4C). Interestingly, relative to healthy sedentary subjects, senior sportsmen have significantly less SRBP1 induction.

DISCUSSION

Muscle tissue changes with increasing age and often these changes result in a decline of muscle mass and performance. In the present study, we show that senior sportsmen have a high retention of both

myofiber size and function when compared to sedentary peers. The better functional output of senior sportsmen in comparison to age-matched healthy sedentary seniors may be related to the larger average size of both fast and slow type myofibers (Table 4). The observed **higher** percentage of slow type fibers in senior sportsmen relative to age-matched healthy sedentary seniors can be ascribed to the amount of endurance exercise that these subjects have performed on a lifelong basis, thereby augmenting oxidative muscle metabolism (51). It is important to note that fiber type groupings (i.e., reinnervation events) were predominantly detected in senior sportsmen, together with a smaller proportion of severely atrophic myofibers (diameter < 30 μm) compared to healthy sedentary seniors. These findings indicate that denervation atrophy is counteracted by reinnervation in lifelong exercising seniors, as demonstrated in our previous study on a smaller group of subjects (37).

The improvements in functional output noted in senior sportsmen could be also related to the better preserved ultrastructure of skeletal fibers Indeed, aging is associated with a **significant lower number** of CRUs, the structures responsible for transduction of the action potential into Ca^{2+} release from internal stores (Table 5), (SR), and of the organelles deputed to ATP production, i.e. the mitochondria (Table 6). EM structural data presented here indicate that the general organization of the metabolic apparatus is far better preserved in subjects who exercise regularly than in sedentary individuals (Figure 2, Tables 5 and 6): frequency of mitochondria is higher in athletic than in sedentary seniors with mitochondria being even more positively affected than EC coupling apparatus, with parameters similar to those of healthy young subjects (Table 6). However, the most significant result stemming from our EM quantitative studies is the frequency of CRU-mitochondria pairs, which is three times **higher** in senior sportsmen than in sedentary individuals (Table 6, D), a combined result of the **higher frequency** and improved positioning of both organelles. Recent work indicates that CRUs and mitochondria are functionally coupled, as entry of Ca^{2+} into the mitochondrial matrix is able to stimulate the respiratory chain and up-regulate ATP production when muscle is active (42-44). Some of us have also recently shown that mitochondria and CRUs are specifically linked to one another by small strands, or tethers (12). This mitochondria-CRU tethering seems to be crucial for bi-directional cross-talk between the two

organelles (14,44). As correct association between CRUs and mitochondria may be crucial for efficient ATP production, the findings presented in Table 6 D (together with **higher** myofiber size) is likely a key element explaining the significant improvement in muscle performance/endurance of lifelong exercising seniors.

Our results on atrophy-related gene expression are consistent with a recent report of MYOAGE (45). In both groups of seniors, the ubiquitin ligases Atrogin-1 and MuRF-1 are not up-regulated in either healthy sedentary subjects or senior sportsmen when compared to young subjects. Conversely, in both studies the autophagy genes are strongly induced in healthy sedentary elderly, suggesting that autophagy is part of the mechanism(s) required for maintenance of muscle homeostasis (52-54). Interestingly, results from senior sportsmen show that exercise maintains some aspect of autophagy, for instance the gene Bnip3, which is involved in mitophagy, is not induced in senior sportsmen suggesting that the mitochondrial network is well functioning. Indeed, ultrastructural analyses confirmed that mitochondria are well preserved in lifelong exercised people.

In the present study, we did not observe significant **differences** in the expression of hypertrophy-related gene IGF-1 isoforms in either sedentary or senior sportsmen; however, we did reveal a downregulation of miR-1 and an upregulation of miR-206 and miR-133a gene expression in senior sportsmen. This down-regulation of miR-1 expression is in line with the evidence indicating that miR-1 is downregulated after 7 days of functional overload in mice and that this downregulation was accompanied by a 45% increase in plantaris muscle weight (55). Moreover, it has been demonstrated that miR-1 is up-regulated in stimuli-mediated atrophy (56).

Overall, a downregulation of miRNA-1 and the up-regulation of miR-206, in the senior sportsmen may represent another level of control on transcripts important for muscle homeostasis and satellite cell function.

The **differences** in genes related to autophagy and/or selective mitophagy in healthy sedentary seniors in comparison to senior sportsmen could mirror a tendency of clear damaged organelles and proteins that would inevitably contribute to ROS production and weakness. It is well known that exercise

maintains/preserves mitochondrial function preventing ROS release. This may explain why Nrf2 is less induced in athletic than in sedentary seniors.

Recent data concerning the transcription factors YY1 and SREBP (which play a critical role in glucose and lipid homeostasis) are also in favor of this hypothesis. Indeed YY1 interacts with PGC-1α via mTOR and controls expression of many genes of the insulin/IGF-1-Akt pathway, including IGF-1, Insulin receptor substrate 1 and 2, Akt1 and Akt2 (57). Inactivation of YY1 in muscle causes abnormalities of mitochondrial morphology and oxidative function associated with exercise intolerance (58). Therefore, the upregulation of YY1 during aging might compensate for both mitochondrial dysfunction and insulin resistance. SREBP1 upregulation may support the metabolic changes favoring the use of lipids rather than glucose as energy sources for ATP production. However, because SREBP1 has recently been found to be a negative regulator of protein synthesis in muscle (50), its induction in healthy sedentary subjects may also account for **lower** protein synthesis in these subjects, while its **lower** level of induction in senior sportsmen may serve to maintain protein synthesis. Therefore, exercise would be expected to preserve glucose homeostasis, insulin sensitivity and protein synthesis, thereby, reducing age-related SREBP induction.

The results of our study strongly highlight the importance of lifelong recreational sport activity in delaying progression of the age-related changes within skeletal muscle which negatively affect either the "quantity" or the "quality" of the muscle. Our findings further suggest that regular skeletal muscle contractility, voluntary or supported by a specifically designed neuromuscular electrical stimulator (56) may represent a good therapy to attenuate or reverse the decline of skeletal myofiber size, strength, and power associated with the ultra-structural abnormalities observed during aging. In this regard, a specific well directed program of training could improve body balance, muscle structure and contractile properties in elderly subjects, which in turn are likely to improve quality of life and reduce risk of falling.

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All authors declare no conflict of interest.

References

1. Schneider EL, Guralnik JM The aging of America. Impact on health care costs. JAMA. 1990;263:2335-2340.
2. Roubenoff R Sarcopenia and its implications for the elderly. Eur J Clin Nutr. 2000;54Suppl 3:S40-47.
3. Faulkner JA, Larkin LM, Cafin DR, Brooks SV. Age-related changes in the structure and function of skeletal muscles. Clin Exp Pharmacol Physiol 2007;34:1091-1096.
4. Deschenes MR, Mackenzie AR, Eason MK, Harris MB. Remodeling of the neuromuscular junction precedes sarcopenia related alterations in myofibers. Exp Gerontol 2010;45:389-393.
5. Clark DJ, Fielding RA. Neuromuscular contributions to age-related weakness. J Gerontol A Biol Sci Med Sci. 2012; 67: 41-7.
6. Manini TM, Clark BC. Dynapenia and aging: an update. J Gerontol A Biol Sci Med Sci. 2012; 67: 28-40.
7. Goodpaster BH, Park SW, Harris TB, Kritchevsky SB, et al. The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. J Gerontol A Biol Sci Med Sci. 2006; 61: 1059-64.
8. Delbono O, O'Rourke KS, Ettinger WH. Excitation-calcium release uncoupling in aged single human skeletal muscle fibers. J Membr Biol. 1995; 148: 211-22.
9. Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A. 1994; 91: 10771-8.
10. Boncompagni S, d'Amelio L, Fulle S, Fanò G, Protasi F. Progressive disorganization of the excitation-contraction coupling apparatus in aging human skeletal muscle as revealed by electron microscopy: a possible role in the decline of muscle performance. J Gerontol A Biol Sci Med Sci. 2006;61:995-1008.
11. Trounce I, Byrne E, Marzuki S. Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. Lancet 1989;1:637-639.

12. Boncompagni S, Rossi AE, Micaroni M, et al. Mitochondria are linked to calcium stores in striated muscle by developmentally regulated tethering structures. Mol Biol Cell. 2009;20:1058-1067.
13. Rossi AE, Boncompagni S, Wei L, Protasi F, Dirksen RT. Differential impact of mitochondrial positioning on mitochondrial Ca(2+) uptake and Ca(2+) spark suppression in skeletal muscle. Am J Physiol Cell Physiol. 2011;301:C1128-1139.
14. Rossi AE, Boncompagni S, Dirksen RT. Sarcoplasmic reticulum-mitochondrial symbiosis: bidirectional signaling in skeletal muscle. Exerc Sport Sci Rev. 2009;37:29-35.
15. Musarò A, McCullagh K, Paul A, et al. Localized IGF-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nature Genetics 2001;27:195-200.
16. Musarò A, Giacinti C, Dobrowolny G, Pelosi L., Rosenthal N. The role of IGF-I on muscle wasting: a therapeutic approach. Basic Appl Myol 2004;14:29-33.
17. Pelosi L, Giacinti C, Nardis C, et al. Local expression of IGF-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines. FASEB J. 2007; 21:1393-1402.
18. Kern H, Pelosi L, Coletto L, et al. Atrophy/hypertrophy cell signaling in muscles of young athletes trained with vibrational-proprioceptive stimulation. Neurol Res. 2011;33:998-1009.
19. Sandri M, Lin J, Handschin C, et al. PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. PNAS USA 2006;103:16260-16265.
20. Pellegrino MA, Desaphy JF, Brocca L, Pierno S, Camerino DC, Bottinelli R. Redox homeostasis, oxidative stress and disuse muscle atrophy. J Physiol. 2011;589:2147-60.
21. Fulle S, Protasi F, Di Tano G, et al. The contribution of reactive oxygen species to sarcopenia and muscle aging. Exp Gerontol. 2004;39:17-12.
22. Staunton L, O'Connell K, Ohlendieck K. Proteomic Profiling of Mitochondrial Enzymes during Skeletal Muscle Aging. J Aging Res. 2011;908035.

23. Toraman A, Yildirim NU. The falling risk and physical fitness in older people. Arch Gerontol Geriatr. 2010;51:222-226.
24. Hubert HB, Bloch DA, Oehlert JW, Fries JF. Lifestyle habits and compression of morbidity. J Gerontol A Biol Sci Med Sci. 2002;57:M347-351.
25. Crane JD, Macneil LG, Tarnopolsky MA. Long-term aerobic exercise is associated with greater muscle strength throughout the life span. J Gerontol A Biol Sci Med Sci. 2013; 68: 631-8.
26. Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Nilwik R, van Loon LJ. Elderly men and women benefit equally from prolonged resistance-type exercise training. J Gerontol A Biol Sci Med Sci. 2013; 68: 769-79.
27. Sarabon N, Loefler S, Fruhmann H, Burggraf S, Kern H. Reduction and technical simplification of testing protocol for walking based on repeatability analyses: An Interreg IVa pilot study. European Journal of Translational Myology-Based and Applied Myology 2010;1:181-186.
28. Guralnik JM, Simonsick EM, Ferrucci L, et al. A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission. J Gerontol. 1994 ;49:M85-94.
29. Sarabon N, Rosker J, Loefler S, Kern H. Sensitivity of body sway parameters during quiet standing to manipulation of support surface size. J Sport Science Medicine 2010;9:431-438.
30. Sarabon N, Loefler S, Cvecka J, Sedliak M, Kern H. Elderly – Strength for Balance. European Journal Translational Myology - Basic Applied Myology 2013;23:85-89.
31. Bogle Thorbahn LD, Newton RA. Use of the Berg Balance Test to predict falls in elderly persons. Phys Ther. 1996;76:576-585.
32. Podsiadlo D, Richardson S, The timed "Up & Go": a test of basic functional mobility for frail elderly persons. J Am Geriatr Soc. 1991;39:142-148.
33. Kern H, Boncompagni S, Rossini K, et al. Long-term denervation in humans causes degeneration of both contractile and excitation-contraction coupling apparatus that can be

reversed by functional electrical stimulation (FES). A role for myofiber regeneration? J Neuropath Exp Neurol 2004;63:919–931.

34. Kern H, Carraro U, Adami N, et al. Home-based functional electrical stimulation rescues permanently denervated muscles in paraplegic patients with complete lower motor neuron lesion Neurorehabil Neural Repair. 2010;24:709-721.
35. Boncompagni S, Kern H, Rossini K, et al. Structural differentiation of skeletal muscle fibers in the absence of innervation in humans. PNAS USA. 2007;104:19339-19344.
36. Rossini K, Zanin ME, Podhorska-Okolow M, Carraro U. To stage and quantify regenerative myogenesis in human long-term permanent denervated muscle. Basic Appl Myol 2002;12:277-286.
37. Loud AV, Barany WC, Pack BA. Quantitative evaluation of cytoplasmatic structures in electron micrographs. Lab Invest. 1965;14:996-1008.
38. Mobley BA, Eisenberg BR. Sizes of components in frog skeletal muscle measured by methods of stereology. J Gen Physiol. 1975;66:31-45.
39. Brocca L, Cannavino J, Coletto L, Biolo G, Sandri M, Bottinelli R, Pellegrino MA. The time course of the adaptations of human muscle proteome to bed rest and the underlying mechanisms. J Physiol. 2012;590:5211-5230.
40. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-408.
41. Kern H, Hofer C, Mödlin M, et al. Stable muscle atrophy in long-term paraplegics with complete upper motor neuron lesion from 3- to 20-year SCI. Spinal Cord 2008;46:293-304.
42. Mosole S, Carraro U, Kern H, et al. Long term high-level exercise promotes muscle reinnervation with age. 2013; *In press.*
43. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol. 2004;287:C817-833.

44. Shkryl VM, Shirokova N. Transfer and tunneling of Ca^{2+} from sarcoplasmic reticulum to mitochondria in skeletal muscle. JBC 2006;281:1547-1554.
45. Sandri M, Barberi L, Bijlsma AY, et al. Signalling pathways regulating muscle mass in ageing skeletal muscle. The role of the IGF1-Akt-mTOR-FoxO pathway. Biogerontology. 2013; 14:303-23.
46. van Rooij E, Liu N, Olson EN. MicroRNAs flex their muscles. Trends Genet. 2008;24:159-66.
47. Cacchiarelli D, Martone J, Girardi E, et al. MicroRNAs involved in molecular circuitries relevant for the Duchenne muscular dystrophy pathogenesis are controlled by the dystrophin/nNOS pathway. Cell Metab. 2010;12:341-351.
48. Romanello V, Sandri M. Mitochondrial biogenesis and fragmentation as regulators of muscle protein degradation. Curr Hypertens Rep. 2010;12:433-439.
49. St-Pierre J, Drori S, Uldry M, et al. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell. 2006;127:397-408.
50. Dessalle K, Euthine V, Chanon S, et al. SREBP-1 transcription factors regulate skeletal muscle cell size by controlling protein synthesis through myogenic regulatory factors. PLoS One. 2012;7:e50878.
51. Coggan AR, Spina RJ, Rogers MA, et al. Histochemical and enzymatic characteristics of skeletal muscle in master athletes. J Appl Physiol 1990;68:1896-1901.
52. Mammucari C, Milan G, Romanello V, et al. FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab. 2007;6:458-471.
53. Sandri M, Sandri C, Gilbert A, et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell. 2004;117:399-412.
54. Sandri M. Autophagy in skeletal muscle. FEBS Lett. 2010;584:1411-1416.
55. McCarthy JJ, Esser KA. Counterpoint: Satellite cell addition is not obligatory for skeletal muscle hypertrophy. J Appl Physiol (1985). 2007;103:1100-2; discussion 1102-3.

56. Kukreti H, Amuthavalli K, Harikumar A, et al. Muscle-specific microRNA1 (miR1) targets heat shock protein 70 (HSP70) during dexamethasone-mediated atrophy. J Biol Chem. 2013;288:6663-78.
57. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature. 2007;450:736-740.
58. Blättler SM, Verdeguer F, Liesa M, et al. Defective mitochondrial morphology and bioenergetic function in mice lacking the transcription factor Yin Yang 1 in skeletal muscle. Mol Cell Biol. 2012;32:3333-3346.
59. Krenn M, Haller M, Bijak M, et al. Safe neuromuscular electrical stimulator designed for the elderly. Artif Organs. 2011;35:253-256.

Table 1. Study subject demography.

		Seniors	
	Young (n=5)	**Healthy sedentary (n=9)**	**Sportsmen (n=15)**
Age (years)	27.3 ± 4.2 $$^^	71.4 ± 3.0 **	70.2 ± 4.0 **
Weight (kg)	73.8 ± 5.9	84.9 ± 10.08	81.7 ± 8.8
Height (cm)	174.6 ± 4.0	177.3 ± 8.0	176.0 ± 4.9
BMI (kg/m^2)	24.2 ± 2.0	26.9 ± 2.0	26.3 ± 1.9

Values are given as mean ± SD; BMI= body mass index. **p<0.01 vs. young; ^^p<0.05 vs. Sportsmen; $$p<0.01 vs. sedentary;

Table 2. Weekly amount of training detailed by type and duration in senior sportsmen at biopsy.

	Type of Training/week			Total amount of training/week		
Subject	**Force (%)**	**Endurance (%)**	**Game sports (%)**	**Duration of session (hrs)**	**Session (no.)**	**Duration (hrs)**
1	100	0	0	1.5	3	4.5
2	89	11	0	3.0	5	9
3	100	0	0	3.0	3	9
4	65	35	0	2.3	6	6.9
5	0	100	0	2.0	3	6
6	0	25	75	4.0	8	16
7	17	33	50	5.0	7	12
8	12.5	87.5	0	3.0	9	16
9	10	80	10	5.5	5	10
10	0	100	0	2.0	4	8
11	14	86	0	4.0	3	7
12	14	86	0	3.0	8	14
13	0	100	0	1.5	4	6
14	58	18	24	5.5	13	24.5
15	0	100	0	2.0	6	12
mean±SD	**32.0±38.8**	**57.4±40.3**	**10.6±22.6**	**3.2±1.4**	**5.8±2.8**	**10.7±5.2**

Table 3. Maximal isometric torque normalized to body mass and functional tests.

		Seniors	
	Young	**Healthy sedentary**	**Sportsmen**
Maximal Isometric Torque (Nm/kg)	3.2±0.6$^{\wedge\wedge\$\$}$	1.7±0.3$^{**\wedge}$	2.2±0.3$^{**\$\$}$
10-m test (normal, m/s)	1.6±0.2	1.4±0.1	1.5±0.3
10-m test (quick, m/s)	2.5±0.1$^{\$\$}$	1.9±0.2$^{**\wedge\wedge}$	2.3±0.4$^{\$}$
SPPB (total score)	12.0±0.0$^{\$}$	10.9±0.9*	11.9±0.6
SPPB (5x chair rise, s)	5.3±0.7$^{\$\$}$	11.9±2.1$^{**\wedge\wedge}$	6.3±1.3$^{\$\$}$
TUGT (s)	4.0±0.2$^{\$\$}$	6.6±1.3$^{**\wedge}$	4.7±1.1$^{\$}$

Values represent mean±SD; TUGT= timed-up-and-go-test; SPPB= short physical performance battery; n.d.=not determined. Statistical significance: *p<0.05 vs. young; **p<0.01 vs. young; $^{\wedge}$p<0.01 vs. Sportsmen; $^{\wedge\wedge}$p<0.05 vs. Sportsmen; $^{\$}$p<0.05 vs. sedentary; $^{\$\$}$p<0.01 vs. sedentary; Nm=Newton-meter; m/s = meters per second.

Table 4. Morphometric analyses of muscle biopsies. The mean myofiber diameter of muscle biopsies from young men is significantly higher than in all other groups, as well as in senior sportsmen when compared to sedentary seniors. No major **differences** in fiber type distribution were observed in healthy seniors compared to young subjects while a significant **higher** percentage of slow type fiber was detected in muscle biopsies from senior sportsmen.

	Mean myofiber diameter (μm ±SD)			**Myofiberdiameter < 30 μm (%)**
	All fibers	**Slow-type** (%)	**Fast-type** (%)	
Young subjects	73.4 ± 19.3^	68.8 ± 21.8 (50)^	76.8 ± 22.9 (50)^	0.3^
Healthy sedentary seniors	56.2 ± 17.9#	54.8 ± 18.0 (54)#	51.0 ± 16.8 (46)#	4.0#
Senior sportsmen	61.2 ± 17.1*	61.8 ± 15.8* (69)*	59.5 ± 18.2* (31)*	2.0*

Statistical significance: ^ $p < 0,01$ vs senior sportsmen; # $p < 0.01$ vs young sportsmen; * $p < 0.01$ vs healthy sedentary seniors.

Table 5. Quantitative analysis of CRUs. Aging causes a drastic reduction in CRU number (A) and an increase in the number of dyads (B) and of longitudinal junctions (C). In fibers from senior sportsmen, we did not find significant improvement in the EC coupling apparatus (A and B), even if the frequency of longitudinal CRUs is reduced in exercising individuals suggesting a slightly improved orientation of triads (C).

	A	**B**	**C**
	CRUs /100 μm^2	**Dyads (%)**	**Longitudinal CRUs (%)**
Young subjects[$]	32.5±13.4	8.0	0.5
Healthy sedentary seniors[#]	20.3±10.0	10.9	21.9
Senior sportsmen[§]	21.6±10.8*	13.8	10.6*

Values are given as mean ± SD;

[$] Young subjects: n = 50 fibers total; 10 micrographs/fiber;

[#] Healthy sedentary seniors: n = 52 fibers total; 6-10 micrographs/fiber;

[§] Senior sportsmen: n = 72 fibers total (6 for each sample); 6 micrographs/fiber.

* Statistical significance: $p < 0.01$

Table 6. Quantitative analysis of mitochondria: exercise preserves the association between mitochondria and CRUs. Aging causes a dramatic decrease in mitochondrial volume and number (A and B) and a miss-placement of these organelles at the A-band (C). In senior sportsmen, both mitochondrial number and volume are as high as in young subjects (A and B) with an improvement in organelle positioning (i.e., lower frequency of A band mitochondria, C). Maintenance of mitochondrial population results in a striking restoration of the frequency of mitochondria-CRU pair(D).

	A	B	**C**	**D**
	Mito V/V, % of total	**no. of Mito /100 μm²**	**no. of Mito at A band/100 μm²**	**no. of Mito-CRU pairs/100μm²**
Young subjects[$]	5.3±2.9	50.0±20.1	1.8 (4.3%)	13.0±9.1
Healthy sedentary seniors[#]	3.4±1.7	37.1±18.3	7.8 (22.2%)	5.9±5.5
Senior sportsmen[§]	6.3±3.0*	52.0±21.3*	3.2 (6.6%)*	11.1±8.3*

Values are given as mean ± SD;

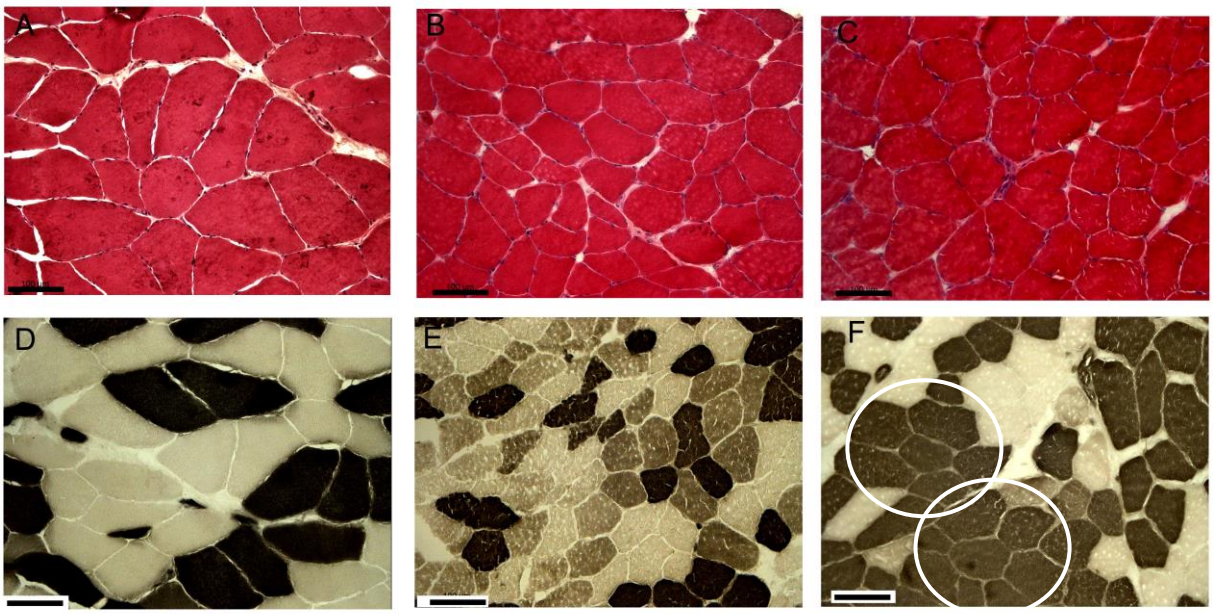
[$] Young subjects: n = 50 fibers total; 10 micrographs/fiber;

[#] Healthy sedentary seniors: n = 52 fibers total; 6-10 micrographs/fiber;

[§] Senior sportsmen: n = 72 fibers total (6 for each sample); 6 micrographs/fiber.

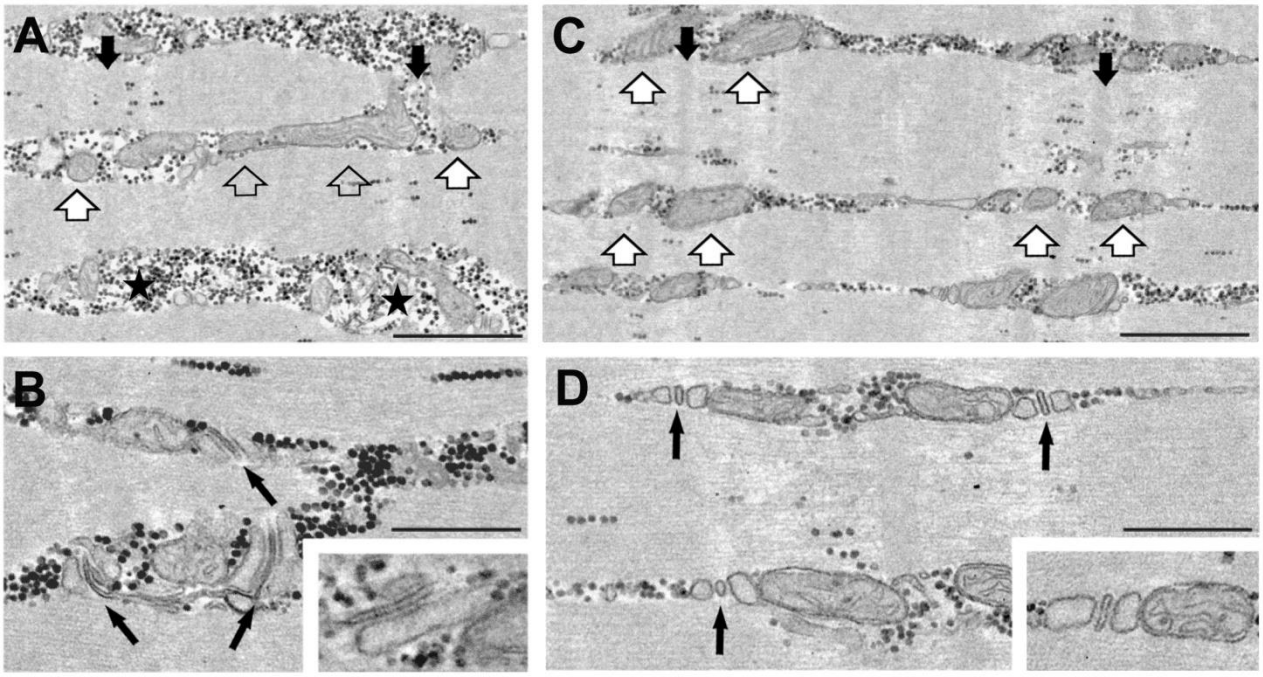
* Statistical significance: $p < 0.01$

Figure 1. Exercise rescues age related fiber atrophy.



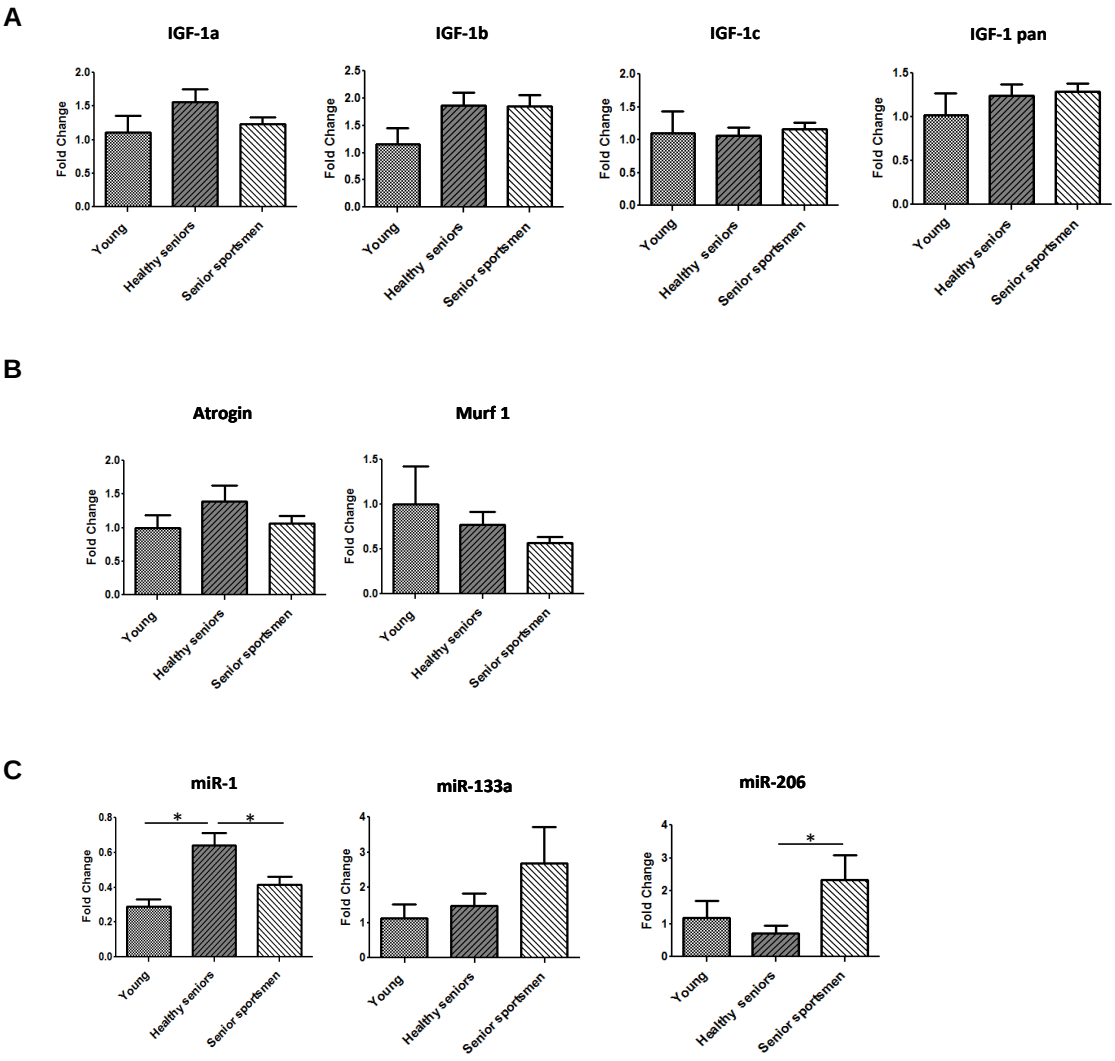
Hematoxylin and Eosin stains (A-C) of muscle sections from young men (A), healthy sedentary seniors (B) and senior sportsmen (C) show well-packed myofibers without evidence of major degenerative **differences**. Fiber type distribution (D,E,F; ATPase pH 4.35) in young subjects (D) and healthy sedentary seniors (E) is similar, while in senior sportsmen slow type (dark) myofibers significantly increase with accompanying fiber type grouping (encircled). Bars=100μm.

Figure 2. Exercise improves cellular ultrastructure.



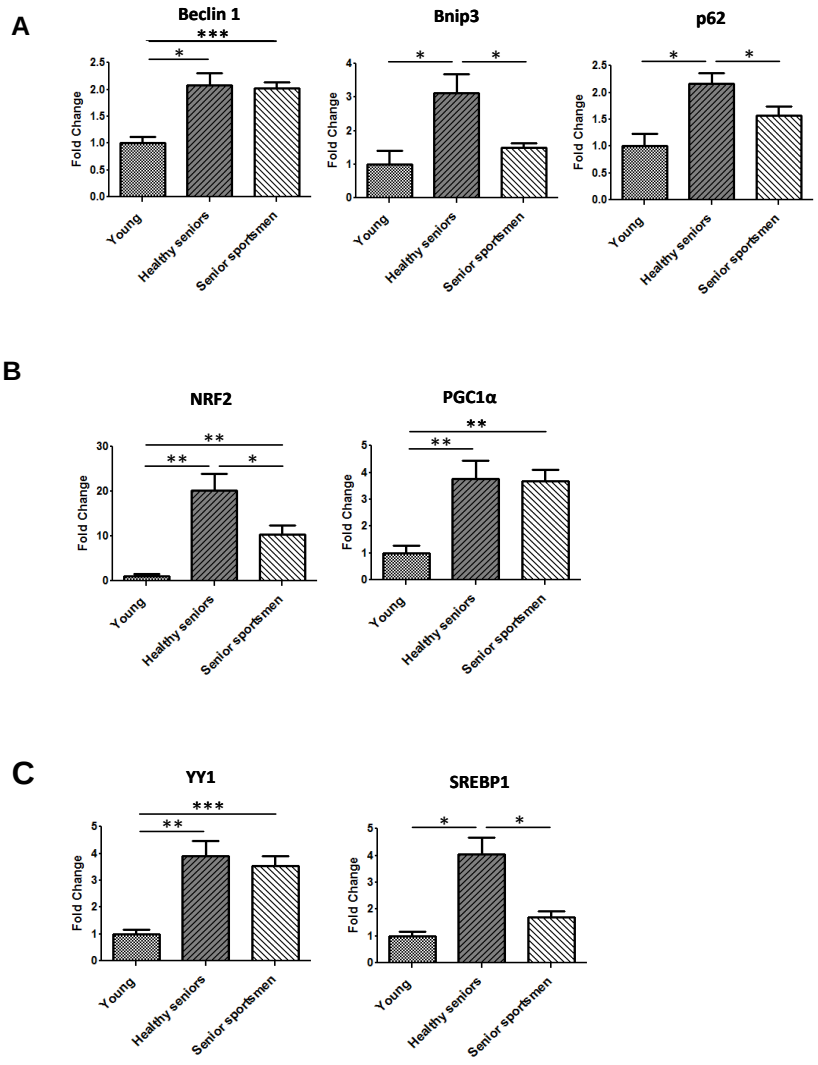
Muscle fibers of healthy sedentary subjects (A-B) exhibit poor internal organization, having mitochondria often localized to the A band (A, empty arrows) instead of the I band (panel A, white arrows) in close proximity to the Z-line (A, black arrows). Decay of internal organization is evident (A, stars) and CRUs are often miss-oriented and abnormally structured (B, arrows and inset). In fibers from senior sportsmen (C-D), internal organelle structure and positioning are strikingly better preserved: mitochondria more often form pairs (C, white arrows) on both sides of Z-lines (C, black arrows); CRUs have the classic triad structure (D, arrows) and are frequently associated with mitochondria (D, inset). Bars A and C=1μm; B and D=0.5μm.

Figure 3. Expression of genes and miRNA controlling muscle mass.



Real time PCR analysis for the expression of IGF-1 isoforms (A), atrophy related genes (B) and miRNA (C) in human muscle biopsies from young, healthy sedentary senior and senior sportsmen subjects. Statistical significance: *p≤0.05; **p≤0.01; ***p≤0.001.

Figure 4. Expression of genes controlling autophagy, oxidative stress and muscle metabolism.



Expression of genes related to: (A) autophagy, (B) regulation of ROS detoxification and mitochondria biogenesis and (C) modulation of lipid homeostasis and protein synthesis. Beclin1, NRF2, PGC-1a and YY1 genes are significantly upregulated in senior sportsmen relative to young men. Statistical significance: *p≤0.05; **p≤0.01; ***p≤0.001.